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Movement and Survival of *Spodoptera litura* (Lepidoptera: Noctuidae) Neonate Larvae on Cotton Expressing *Bacillus thuringiensis* Proteins

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ABSTRACT

Survival of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) larvae has been reported in transgenic Bollgard 3 (BG3) cotton (*Gossypium hirsutum*) fields in northern Australia, despite high mortality when fed BG3 leaves in laboratory bioassays. Larval movement away from plant material expressing *Bacillus thuringiensis* (Bt) proteins has been proposed as a mechanism enabling lepidopteran larvae to survive in the field. This study examined the movement of *S. litura* neonates on BG3 and cotton expressing no Bt proteins in no-choice and choice leaf disc bioassays, and in whole-plant bioassays under laboratory conditions. In no-choice bioassays, larvae exhibited greater movement when exposed to BG3 leaf discs compared with control cotton expressing no Bt proteins. However, the pattern of movement suggested that larvae could not immediately detect and avoid Bt proteins in the leaf material on which they were feeding. In choice bioassays, when larvae were placed equidistant between BG3 and control cotton leaf discs, approximately 62% of larvae were observed on BG3 leaf discs after 1 h. Thus, their movement away from BG3 cotton appears to be a post-ingestion response. Larval survival was significantly higher in choice bioassays (where larvae placed on BG3 leaf discs could move to control leaf discs) than in no-choice bioassays (where larvae were constrained to BG3 leaf discs). On whole plants, a higher proportion of 1-day old neonate larvae remained near the site of the egg mass on control cotton plants than on BG3 cotton plants, but more larvae disappeared on BG3 cotton plants compared with control plants. These findings suggest that the Bt proteins expressed in BG3 cotton alter the movement behaviour of *S. litura* larvae. In the field, dispersal may increase survival if larvae can move from plant structures expressing lethal levels of Bt proteins to plant structures that express sublethal levels.

1 | Introduction

In Australia, Bollgard 3 (BG3) cotton, which expresses *Bacillus thuringiensis* (Bt) proteins, Cry1Ac, Cry2Ab, and Vip3A, is registered to provide control of key lepidopteran pests, *Helicoverpa armigera* (Hübner) and *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae). However, BG3 cotton has also been shown to cause high mortality in a prominent secondary pest species, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Holman et al. 2025), which is distributed across tropical northern Australia. In laboratory experiments, fewer than 1% of *S. litura* larvae that were fed BG3 leaves survived to adult eclosion (Holman et al. 2025). Despite this, *S. litura* larvae have been intermittently observed surviving in commercial BG3 cotton fields across tropical northern Australia (Holman, personal observation). The extent to which these larvae survive to adult eclosion or contribute to population persistence under field conditions remains unknown;

however, on occasion, larval populations have required control with foliar-applied synthetic insecticides (Holman, personal observation). As large-scale commercial production of Bt cotton in tropical northern Australia has occurred only since 2017, following the commercial release of BG3 cotton (APVMA 2016; Bayer Crop Science 2024), limited information is available on the long-term field performance of BG3 cotton against *S. litura*, including factors influencing larval survival.

Within non-transgenic cotton crops, *S. litura* larvae primarily feed on foliage but have also been observed feeding on reproductive structures, including squares, flowers, and bolls (Michael and Woods 1980). Females typically oviposit egg masses on leaf surfaces, where newly hatched neonates initially feed on the leaf lamina (EPPO 2015), before becoming increasingly mobile and moving within and between plants as they develop (Mitra et al. 2021). Consequently, early larval exposure to Bt proteins is most likely to occur through feeding on vegetative tissues, with subsequent movement to reproductive structures occurring less frequently and typically at older larval instars.

It has been demonstrated that lepidopteran larvae may survive in crops expressing *B. thuringiensis* (Bt) proteins due to behavioural changes, specifically dispersal, movement, and feeding behaviours, that enable them to avoid lethal doses of Bt proteins in the plant material or artificial diet that they ingest (Stapel et al. 1998; Gore et al. 2002; Zhao et al. 2016; Luong et al. 2019; Malaquias et al. 2020). For example, *H. armigera* larvae were more likely to drop off the leaves and squares of Bt cotton compared to non-Bt cotton (Luong et al. 2019). Similarly, Zhao et al. (2016) determined that neonates exhibited higher dispersal and lower establishment on Bt cotton compared with non-Bt cotton. To date, only one study has investigated the effect of Bt proteins on the dispersal and feeding responses of *S. litura* larvae. In a choice bioassay, Singh et al. (2008) reported that after 24 h, more third-instar *S. litura* larvae were found on non-Bt artificial diet compared with artificial diet containing an EC_{95} of formulated Bt (DelfinWGTM, *B. thuringiensis* Berliner subspecies *kurstaki* serotype 3a and 3b). Changes in behaviour that reduce larval exposure to Bt proteins may compromise the efficacy of Bt crops (Head and Greenplate 2012). This could be important when Bt protein expression within a plant is variable (Adamczyk et al. 2001; Kranthi et al. 2005; Knight et al. 2013), as dispersal might result in larvae encountering plant structures expressing sublethal doses, leading to a reduction in Bt crop efficacy (Mallet and Porter 1992; Huang et al. 2011; Head and Greenplate 2012).

This study investigated whether *S. litura* larvae alter their behaviour in response to the Bt proteins expressed in BG3 cotton, and if such behavioural changes influence intra-plant movement, potentially contributing to the survival observed in commercial fields. Specifically, the objectives were to (i) evaluate movement of *S. litura* neonates on BG3 cotton and control cotton that did not express Bt proteins in no-choice and choice leaf disc bioassays, and (ii) determine whether the intra-plant distribution of 1-day old larvae differed between BG3 cotton plants and control cotton plants. It was hypothesised that a higher proportion of *S. litura* neonates would exhibit greater movement and dispersal on BG3 cotton than on control cotton, resulting in greater larval survival.

2 | Materials and Methods

2.1 | Insects

2.1.1 | Laboratory Culture

A laboratory culture was established from wild populations of *S. litura* collected during 2020–2021 in Kununurra, Western Australia (−15.65°, 128.70°) from commercial maize and non-Bt cotton refuge crops. The culture, which was maintained at the Queensland Department of Primary Industries in Toowoomba, Queensland, was supplemented annually with field collected specimens from commercial maize and non-Bt cotton refuge crops from Kununurra to minimise inbreeding.

Moths within the colony of *S. litura* were kept in clear plastic 9-L containers (24 × 25 × 15 cm) with a lid and supplied with 10% sucrose solution from a cotton wick in 70-ml plastic specimen jars. A hole was cut in the lid of the container/bucket, and the edges of the lid were used to secure fabric liners (bamboo rayon) as an oviposition substrate. Fabric liners were changed every second day as part of culture maintenance but daily for experimental purposes. Egg masses were cut from the fabric liners and stored in sealed plastic bags until hatching. Upon hatching, neonates were placed in food-grade rectangular clear plastic containers (17.5 × 12 × 6 cm) (Reward Hospitality, Toowoomba) provisioned with a soybean flour-based artificial diet (recipe modified from Teakle et al. 1985; Table S1). When larvae developed to the third instar, individuals were transferred to separate cells in 32-well plastic trays containing diet. Pupae were washed in 1% sodium hypochlorite solution and placed in emergence containers (24 × 25 × 15-cm) until moth emergence. The culture was maintained in an environmentally controlled room (25°C ± 2°C, 12:12-h light:dark cycle, 60% relative humidity).

2.1.2 | Experimental Insects

Egg masses or neonates (< 24 h post-hatching) from the laboratory culture of *S. litura* were used in the whole plant and leaf disc bioassays, respectively, as specified. Egg masses, used in the whole plant bioassays, were laid on fabric liners (bamboo rayon) and collected from the laboratory culture the morning after oviposition. As *S. litura* egg masses are irregularly shaped and multilayered, often containing hundreds of eggs, direct counting is challenging, time-consuming, and destructive. A previous study demonstrated a highly significant regression relationship ($R^2 = 0.96–0.98$) between egg mass weight and egg and larval numbers in a laboratory colony of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Lynch et al. 1983). Consistent with these findings, our method-establishing data, conducted for *S. litura*, also demonstrated a highly significant relationship between egg mass weight and egg number ($R^2 = 0.988$), with an estimated 19.133 eggs per mg of egg mass (experimental details provided with

Figure S1), highlighting its utility as a simplified and effective method for quantifying egg numbers. Each egg mass was cut from the liner and weighed using an analytical balance (A&D HR-250AZ) to estimate the number of eggs in the masses that were used in the whole plant bioassays.

2.2 | Cotton Plants

Sicot 748B3F (Bollgard 3 [BG3], expressing Cry1Ac, Cry2Ab, Vip3A, and CP4 EPSPS) was used in all experiments. In the leaf disc bioassays, Sicot 620 (CC), which lacks Bt proteins and herbicide resistance traits, was used as the control, while Sicot 711RRF (NBT), which expresses only the herbicide resistant trait, CP4 EPSPS, but no Bt proteins, was used as the control in the whole plant bioassays. Previous research has demonstrated glyphosate-tolerant crops have no detrimental effects on non-target organisms including insects (Carpenter 2001; Talyn et al. 2019). All cotton varieties used share a common genetic background and similar growth habit (Stiller, personal communication). All seeds were supplied by Cotton Seed Distributors, Wee Waa, NSW.

Plants were grown in a 2:1:1 mix of potting mix (Searles Premium), sand, and perlite with a slow-release fertiliser (NPK 15.3: 2: 12.6) (Scotts Osmocote). Plants were germinated and grown for 5 weeks in 1.6-L pots in environmentally controlled rooms (29°C ± 2°C, 12:12-h light:dark cycle, 55% relative humidity) before being transferred to a controlled temperature glasshouse (27°C ± 4°C) and natural photoperiod for the remainder of their growth and development. Plants were watered regularly, as required, and no additional fertiliser was applied. Plants were used for experiments during the squaring stage (approximately 45–60 days after sowing), a growth stage during which intermittent larval survival has been observed in commercial BG3 cotton fields, as well as other growth stages (Holman, personal observations).

2.3 | BG3 and CC Cotton Leaf Disc Bioassays

No-choice and choice leaf disc bioassays were conducted to assess the effects of BG3 and CC cotton on the movement and survival of *S. litura* larvae. Leaf discs (15-mm diameter) were cut from fully expanded mid-canopy leaves of BG3 and CC plants and placed in Petri dishes (90-mm diameter) with a 2% water agar base to maintain leaf moisture. The bioassays followed a standardised experimental set up, as described below. Newly hatched neonate larvae from the culture were selected without bias for the experiment.

In no-choice bioassays, a single BG3 or CC leaf disc was placed in the centre of a Petri dish, and a single neonate was placed onto the leaf disc using a fine paint brush. In choice bioassays, one BG3 and CC leaf disc were placed 30-mm apart in the Petri dish. Test larvae were then placed in one of three initial starting positions: (i) on the BG3 leaf disc ('BG3'), (ii) on the CC leaf disc ('CC'), and (iii) equidistant away from both leaf discs ('Choice') [approximately 20-mm from each leaf disc] (Figure S2). Leaf disc placement within the choice bioassays was randomised to minimise any potential light effects on larval orientation. Petri dish lids were sealed with Parafilm to prevent larval escape.

Neonate location (on-leaf [CC or BG3] or off-leaf) was recorded at 1, 4, 8, 24 and 48 h after the experiment began. At each time point the location, survival status, and whether each neonate had escaped or disappeared from the Petri dish was recorded. Each larva was considered a replicate; 70 replicates were conducted per treatment, 10 replicates per treatment on any given day were conducted over a 2-week period in November 2021. No-choice and choice bioassays were conducted simultaneously.

2.4 | Distributions of *S. litura* Neonate Larvae on BG3 and NBT Cotton Plants

To assess the effect of BG3 cotton on *S. litura* neonate distribution, egg masses with a known egg number, as previously described, were used. Egg masses at the 'black-head' stage (developed larvae that are expected to hatch within < 2 h, Kaleka and Kapoor 2024) were affixed to the underside of the main leaf at the sixth node from the terminal (=6th node leaf) of BG3 and NBT cotton plants using non-toxic glue (UHU stic, Bolton Adhesives). Only masses containing 350–650 eggs were used in the experiment. Plants were randomly assigned to individual benches and spaced at least 1.5-m apart along a bench in a controlled temperature glasshouse (27°C ± 4°C). Care was taken to position plants away from vents to minimise the effect of air movement. The hatching time of each egg mass was recorded, and after 24 h, plants were examined and dispersing neonates (i.e., those hanging from the plant by a silk thread) were collected using a fine brush, transferred to a piece of white A4 paper and then counted. The plant was then dissected to determine the locations of neonates across six categories: (i) 6th node leaf (egg mass site), (ii) leaves above the 6th node leaf, (iii) leaves below the 6th node leaf, (iv) squares, (v) dispersing (as previously described), and (vi) unaccounted larvae. The liner was removed from the leaf and examined for unhatched eggs under a microscope (Nikon: SMZ800N).

The numbers of hatched neonates and unaccounted larvae were estimated as follows:

Hatched neonates = (estimated number of eggs in egg mass)–(number of unhatched eggs)

Unaccounted larvae = (number of hatched neonates)–(recovered neonates)

Each plant with an egg mass was considered a replicate, and there were 15 replicates per treatment. The experiment was conducted from December 2023 to February 2024.

2.5 | Statistical Analysis

All analyses were conducted in GenStat Version 24 (VSN International 2024). Outputs from all analyses are provided in Tables S2–S9. For leaf disc bioassays, behaviour assessments were adapted from the methods described by Wang et al. (2019), and based on two response categories: (i) presence of larvae on leaf discs at 1, 4, 8, 24 and 48 h, recorded as bioassay-specific presence variables (four analyses based on experimental design of bioassay type and/or larval starting position; Table 1) and (ii)

frequency of larvae moving off a leaf disc. Movement off the leaf disc was quantified from observations of larvae present at different locations within the Petri dish.

A generalised linear mixed model (GLMM), with a Poisson distribution, was used to analyse the effect of treatment (BG3 or CC in no-choice bioassays; BG3, CC and choice in choice bioassays) on the frequency of a larva moving off leaf discs, with analyses conducted separately for each bioassay type.

For no-choice bioassays, larval presence on leaf discs over time was analysed using a GLMM with a binomial distribution. Fixed effects included treatment (BG3 or CC), time, and their interaction.

For choice bioassays, larval presence was analysed using GLMMs with a binomial distribution in separate analyses reflecting bioassay design (Table 1). Specifically, presence on the leaf disc on which larvae were initially placed (e.g., CC—for larvae initially placed on CC) and presence on the alternative leaf disc (e.g., BG3—for larvae initially placed on CC) were analysed separately. Fixed effects included larval starting position (BG3 or CC), time, and their interaction. The equidistant 'choice' treatment was analysed separately (Table 1) using a GLMM with a binomial distribution to model larval presence on BG3 leaf discs, with time as a fixed effect.

For all no-choice and choice presence analyses, random effects included the individual larva/petri-dish nested within day, and their interactions with time to account for variation among days, among larvae within days, and for repeated observations of each larva over time. The equidistant 'choice' treatment in the choice bioassays was the exception; this analysis included random effects for variation among days, and individual larvae nested within day only. Post hoc multiple comparisons of model-estimated means were conducted on the transformed (link) scale using a Fisher's protected least significant difference (LSD) test ($p < 0.05$) following binomial GLMM analyses. Larvae that escaped or disappeared from the Petri dish were included in the data at the time point they escaped but were excluded from further analyses, as were those recorded dead at 24 h. Consequently, as time progressed, subsets of replicates were analysed. Figures illustrating the location and survival status of all larvae at key time points in the no-choice and choice bioassays are provided in the Results section.

Larval survival at 48 h was analysed using a GLMM with a binomial distribution to assess the effect of bioassay type (no-choice and choice) and larval placement (BG3, CC, or choice). Random effects included day of the bioassay and individual larva/petri dish nested within day to account for variation among days and among larvae within each day. A post hoc multiple comparisons of model-estimated means were conducted on the transformed (link) scale using a Fisher's protected LSD test ($p < 0.05$). Larvae that escaped or disappeared during the experiment were excluded from the survival analysis.

Due to the variation in the number of hatched neonates across replicates in the whole plant bioassays (range: 355–587), larval counts per location were converted to proportions based on the number of hatched neonates for each replicate. These proportions were analysed using a linear mixed model framework. Location category, cotton variety, and their interaction were fitted as fixed effects, while day of experiment and bench were included as random effects to account for the experimental design. Predictions of the fixed effects were obtained as empirical best linear unbiased estimates (eBLUEs). The linear mixed model was fitted using a linear mixed model function, whereby the variance components were estimated by residual maximum likelihood (REML) (Patterson and Thompson 1971). Fisher's protected LSD test was then used to test for significant differences among the different treatments ($p < 0.05$). Proportions were transformed by square root to meet the assumptions of normality. The transformed proportional means were back-transformed and presented as percentages for reporting.

3 | Results

3.1 | BG3 and CC Cotton No-Choice and Choice Bioassays

3.1.1 | No-Choice Bioassays

In no-choice bioassays, larvae placed on BG3 leaf discs were less likely to remain on the disc, with their presence decreasing over time, whereas larvae placed on CC leaf discs were more likely to stay on the discs across time ($F = 23.6$; df = 4, 444.5; $p < 0.001$; Figure S3, complete statistical outputs presented in Table S2). Larvae were more likely to be recorded on the CC cotton leaf disc than on the BG3 cotton leaf disc at 1, 4, 8, 24, and 48 h,

TABLE 1 | Bioassay type (no-choice or choice bioassays; access to Bollgard 3 (BG3) and/or non-Bt cotton expressing (CC) leaf discs) and treatments based on larval starting positions on BG3 or CC leaf discs, and analysed response variables, with larval presence on leaf discs analysed as four distinct response variables, measured at 1, 4, 8, 24 and 48 h.

Bioassay type	Treatments (starting positions)	Analysed response variable (presence/absence)
No-choice (larvae have access to only BG3 or CC leaf disc)	BG3	Presence on leaf disc
	CC	
Choice (larvae have access to both a BG3 and CC leaf disc; Figure 2)	BG3	Presence on initial (starting) leaf disc
	CC	Presence on alternative leaf disc
	Choice (equidistant between both leaf discs)	Presence on BG3 leaf disc

ranging from 99.8% to 97.6% across the time points ($p < 0.05$; Figure S3). From 1 to 8 h after the experiment began, the percentage of larvae observed on BG3 leaf discs declined from 94% to 87%. By 24 h, only 13% of larvae remained, but this rose to 39% at 48 h.

However, when considering all replicates from the start of the experiment to their final fate at 48 h, fewer larvae remained on BG3 leaf discs, with only 6% alive on the BG3 leaf discs (4 out of 70) and 9% alive but off-leaf (6 out of 70) (Figure 1). In contrast, larvae that were alive in the CC treatment after 48 h were observed on the leaf disc (67 out of 70) (Figure 1). Additionally, larval escape or disappearance was higher in the BG3 treatment at 21% (15 out of 70) compared to 1% (1 out of 70) in the CC treatment (Figure 1).

Larvae placed onto BG3 moved off the leaf discs three times more than larvae placed upon CC in no-choice bioassays ($F = 41.6$; df = 1, 77.1; $p < 0.001$). Over the 48-h period, larvae on BG3 leaf discs moved off the leaf disc an average of 1.5 ± 0.1 times per larva, while those on CC leaf discs moved off the leaf disc an average of 0.5 ± 0.1 times per larva.

3.1.2 | Choice Bioassays

Larvae that were initially placed on BG3 leaf discs were less likely to be recorded remaining on BG3 leaf discs, with their presence decreasing over time, while larvae that were initially placed on CC leaf discs also moved off CC leaf discs before returning ($F = 25.4$; df = 4, 594.8; $p < 0.001$; Figure S4A, complete statistical outputs presented in Table S3). The percentage of larvae placed and remaining on BG3 leaf discs decreased significantly over 48 h, declining to 86% at 1 h and to 72% at 8 h (Figure S4A). The number of larvae present on BG3 leaf discs continued to decline, with approximately 18% remaining at 24 h and 12% at 48 h. For larvae placed on CC leaf discs, a proportion moved from the leaf discs initially, with 67% of larvae observed on CC leaf discs at 1 h; however, this increased to 77% at 8 h. The number of larvae that were present on CC leaf discs continued to increase until 48 h, by which time 90% of larvae were present on CC leaf discs.

Larvae that were initially placed on BG3 leaf discs were more likely to move off those leaf discs and onto the alternative [CC] leaf disc over time, whereas larvae that were initially placed on CC leaf discs were less likely to move off those leaf discs and onto the alternative [BG3] leaf disc ($F = 25.6$; df = 4, 399.8; $p < 0.001$; Figure S4B, complete statistical outputs presented in Table S4). For larvae initially placed on BG3 leaf discs, the percentage of larvae subsequently observed on CC leaf discs increased over time, from 6% at 1 h to 53% at 48 h (Figure S4B). Of the larvae that were initially placed on CC leaf discs, 29% and 24% were observed on BG3 leaf discs after 1 and 4 h, respectively. However, this percentage declined to 2% by 48 h.

In the choice bioassays where larvae were placed equidistant away from BG3 and CC leaf discs ('choice treatment'), the percentage of larvae recorded on BG3 leaf discs also declined over time ($F = 25.8$; df = 4, 255.5; $p < 0.001$; Figure S5, complete statistical outputs presented in Table S5). At 1 h, 62% of larvae were observed on BG3 leaf discs, but at 24 and 48 h, only 4% and 3% were observed on BG3 leaf discs, respectively.

When considering all replicates from the start of the experiment to their final fate at 48 h, fewer larvae remained on the

FIGURE 1 | The percentage of *Spodoptera litura* larvae recorded in different locations (leaf, off-leaf, and escaped) and survival status (dead, alive) in Bollgard 3 (BG3) and non-Bt expressing cotton (CC) leaf disc no-choice bioassays over time.

BG3 leaf discs compared to the CC leaf discs (Figure 2). Of the larvae that were placed on the BG3 leaf disc, only 1% (1 out of 70) were located alive on BG3, while 41% (29 out of 70) were alive on the CC leaf disc and 6% (4 out of 70) had escaped the Petri dish after 48 h (Figure 2). For larvae that were placed on the CC leaf disc, 76% of larvae (53 out of 70) were alive on CC, while only 1% (1 out of 70) was found alive on BG3. Like the BG3 starting treatment, 6% of all larvae placed on the CC leaf disc (4 out of 70) also escaped or disappeared from the Petri dish after 48 h. In the choice treatment, 41% of larvae (29 out of 70) were found alive on CC, compared with 6% of larvae (5 out of 70) located alive on BG3 after 48 h, while 26% of larvae (18 out of 70) escaped or disappeared from the Petri dish (Figure 2).

As in the no-choice bioassays, larvae that were initially placed on BG3 leaf discs moved off leaf discs more frequently compared with larvae placed on CC leaf discs ($F = 4.45$; df = 2, 141.2; $p = 0.013$). Larvae initially placed on BG3 leaf discs moved off the leaf disc an average of 1.7 ± 0.1 times per larva while larvae that were initially placed on CC leaf discs moved off the leaf disc an average of 1.2 ± 0.1 times per larva. There was no difference in the number of movements recorded from larvae that were placed between leaf discs, at 1.4 ± 0.1, compared with larvae placed on either CC or BG3 leaf discs.

3.1.3 | Survival of Larvae in No-Choice and Choice Bioassays

There was a significant interaction between bioassay type (larvae have access to only one leaf disc [BG3 or CC 'no-choice bioassays'] or access both leaf discs [BG3 and CC 'choice bioassays']) and initial larval placement on the survival of larvae at 48 h ($F = 29.8$; df = 1, 240.6; $p < 0.001$; Figure 3, complete statistical outputs presented in Table S6). Survival was higher when larvae were placed on CC leaf discs compared with BG3 in no-choice bioassays ($p < 0.05$). In choice bioassays, larvae placed on CC leaf discs had higher survival than those placed on BG3 or equidistant between both leaf discs ($p < 0.05$). Larvae placed on BG3 leaf discs in choice bioassays had higher survival compared with those on BG3 in the no-choice bioassays ($p < 0.05$).

3.2 | Distribution of *S. litura* Neonates on BG3 and NBT Cotton Plants

The mean number of neonates that hatched per egg mass was 497 ± 69 and 457 ± 85 on BG3 and NBT cotton, respectively. The recovery of larvae showed location-specific differences between BG3 and NBT cotton ($F = 51.4$; df = 5; 120.7; $p < 0.001$; Figure 4, complete statistical outputs presented in Table S7). For BG3 cotton, the proportion of larvae unaccounted for was 53%, compared to only 14% of larvae on NBT cotton ($p < 0.05$; Figure 4). The second most likely outcome for BG3 cotton was larvae remaining on the 6th node leaf, at 20%. However, this was much lower than the 78% recorded on NBT cotton ($p < 0.05$; Figure 4), where remaining on the 6th node leaf was the primary outcome. Although representing smaller percentages, significantly more larvae were found on the upper leaves, lower leaves, or dispersing on BG3 cotton compared with NBT cotton ($p < 0.05$; Figure 4). No difference was observed in the proportion of larvae recovered from reproductive structures between BG3 or NBT cotton (Figure 4).

FIGURE 2 | The percentage of *Spodoptera litura* larvae recorded at different locations (on a leaf disc [non-Bt expressing (CC)/Bollgard 3 (BG3)], off-leaf discs, or escaped) and survival status (alive/dead) in choice bioassays when larvae initially started on the BG3 or CC leaf disc, or placed equidistant between both leaf discs ('choice') in choice bioassays.

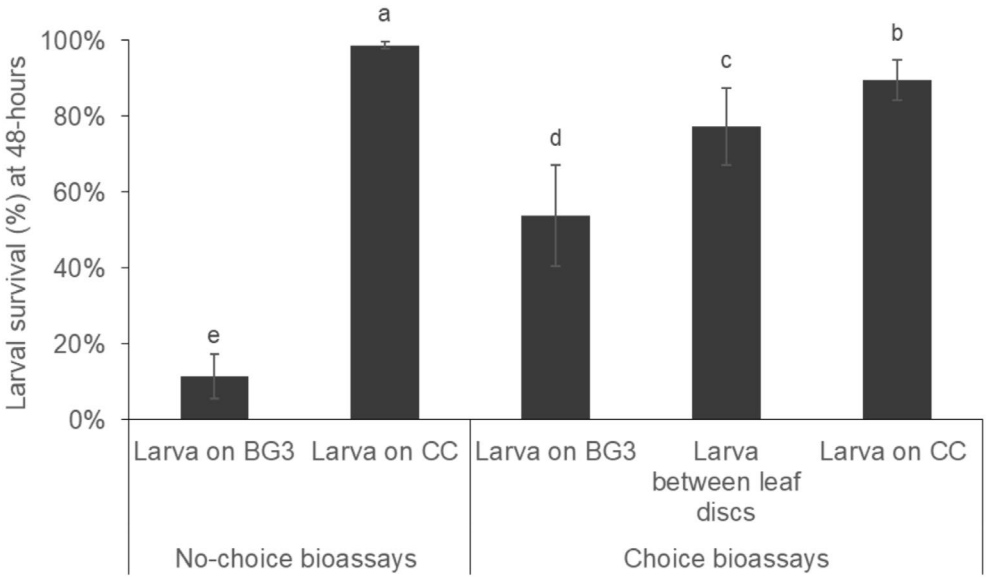

FIGURE 3 | *Spodoptera litura* neonate larvae (mean ± SE) survival rate at 48 h when starting on Bollgard 3 (BG3) or non-Bt expressing cotton (CC) leaf discs in no-choice and choice bioassays, or equidistantly between the leaf discs (=choice) in choice bioassays. Means sharing the same letter are not significantly different (LSD, $p > 0.05$).

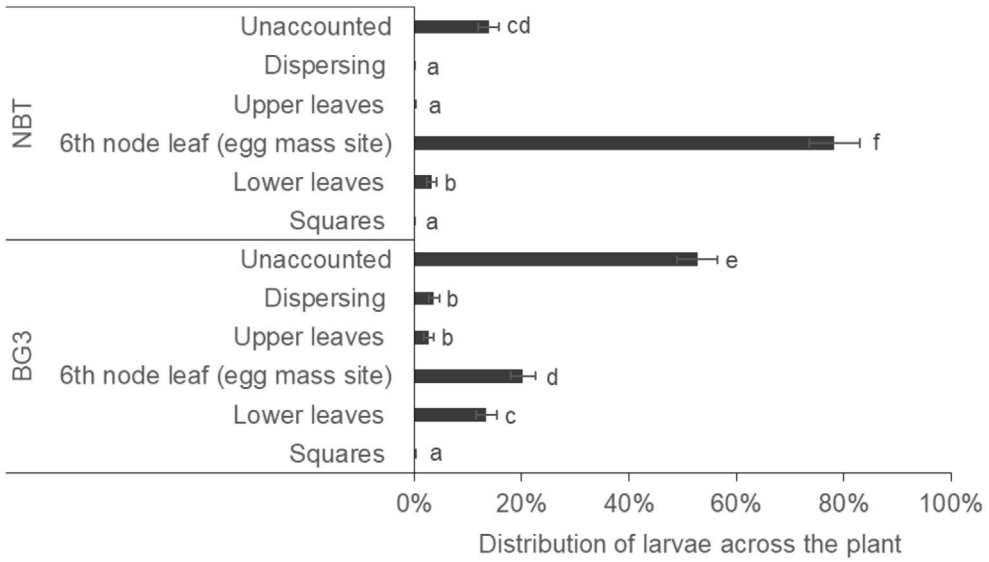

FIGURE 4 | *Spodoptera litura* larvae (mean ± SE) recorded at different locations across Bollgard 3 (BG3) and non-Bt (NBT) cotton plants approximately 24 h after hatching. Means sharing the same letter are not significantly different (LSD, $p > 0.05$).

4 | Discussion

Spodoptera litura neonate larvae are mobile; however, movement is greater when neonate larvae are on BG3 cotton compared with cotton that does not express Bt proteins (viz. NBT/CC) (Figures 1 and 2), potentially influencing greater distributions of larvae both within and between plants (Figure 4). Leaf disc bioassays demonstrated neonate larvae were more likely to move off BG3 leaf discs than CC leaf discs in both no-choice and choice experiments, and movement increased over time (Figures 1 and 2, Figures S3–S5). This is consistent with previous studies indicating that Bt protein exposure increases the likelihood of lepidopteran larvae abandoning feeding sites (Luong et al. 2018; Luong et al. 2019). *Helicoverpa armigera* larvae also exhibit this behaviour, with Luong et al. (2018) reporting the larval distribution within Petri dishes containing non-Bt and Bt leaf discs changed after 12 h, with more larvae leaving Bt leaf discs and/or moving to non-Bt cotton leaf discs.

Although larvae eventually dispersed from BG3 leaf discs, bioassays suggest larvae did not immediately differentiate between plant material with or without Bt proteins (Figure S5). In choice bioassays, larvae placed equidistant away from BG3 and CC leaf discs did not show a strong initial preference towards either leaf disc, with approximately 62% of larvae on BG3 leaf discs at 1 h (Figure S5). However, over time, dispersal from BG3 leaf discs increased, with few larvae remaining by 24 and 48 h (Figures S4A and S5), suggesting that larval dispersal away from BG3 proteins

is likely driven by a post-ingestion response. Similarly, Zhang et al. (2004) found no differences in the location of *H. armigera* larvae between the leaf discs of non-Bt and Bt cotton, expressing Cry1Ac, in choice bioassays at 3 h, but by 24 h, a higher proportion of larvae were observed on non-Bt leaf discs. Interestingly, in our study, movement also occurred in the absence of direct Bt exposure. At 1 h, approximately 67% of larvae initially placed on CC leaf discs remained on the leaf discs in choice bioassays (Figure S4A), while in no-choice bioassays, 98% of larvae remained on CC leaf discs (Figure S3). Odors influence lepidopteran larval behaviour, sometimes inducing movement towards or away from host plant volatiles (Castrejon et al. 2006; Becher and Guerin 2009), which may explain increased larval movement in choice bioassays due to the additional plant material emitting volatiles within the Petri dish. Alternatively, larval movement may represent exploratory behaviour prior to feeding, which would allow larvae in a choice bioassay to encounter another leaf, whereas in a no-choice bioassay they are more likely to return to the same feeding site due to the absence of alternative resources.

The different movement responses of larvae, depending on the leaf that they were placed on in the leaf disc bioassays, were similarly reflected in the contrasting larval distributions on BG3 and NBT cotton plants at 24 h (Figure 4). For BG3 cotton plants, larvae were more likely to be unaccounted for (53% of larvae), whilst on NBT cotton plants, larvae were more likely to remain at the site of the egg mass (78% of larvae), located on the 6th node from the terminal (Figure 4). Similarly, Gore et al. (2002) reported distinct differences in larval dispersal and distribution patterns for *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in Bt cotton, expressing Cry1Ac, compared with non-Bt cotton. At 24 h, *H. zea* larvae were more likely to remain on the terminals of non-Bt cotton, while larvae were more likely to be located within the flowers and bolls of Bt cotton (Gore et al. 2002). Although Gore et al. (2002) used flowering cotton plants compared with the squaring cotton plants in this experiment, they observed a higher percentage of larvae within the squares (and other reproductive structures) of both cotton types. Whereas, in our study, few larvae were observed within the squares of BG3 and NBT cotton (Figure 4). This may be attributed to plant age, as older plants often develop additional reproductive structures that larvae could access, as well as differences between lepidopteran species, larval age, and previous feeding status of larvae. For example, Gore et al. (2002) used 2-day old larvae which had fed on artificial diet, whereas we used neonate larvae. Neonate larvae spend a greater portion of time waving and resting (Johnson and Zalucki 2007), whereas slightly older larvae may have been more mobile, which may influence patterns of movement on Bt cotton plants.

The dispersal behaviour and movement of neonate *S. litura* larvae on BG3 cotton plants may have important implications for pest management. Although *S. litura* larvae are highly mobile and move within and between plants in non-transgenic crops, particularly as older instars (Mitra et al. 2021), increased movement of neonate larvae in response to BG3 cotton (Figures 1, 2, 4) may enhance survival outcomes in BG3 crops. This is particularly notable as early instar lepidopteran larvae are generally more susceptible to Bt proteins than later instars (Farhan et al. 2019), making early exposure critical to the effective use of Bt crops. Bt cotton can express variable levels of Bt protein in different plant structures (Greenplate 1999; Gore et al. 2001; Kranthi et al. 2005). Consequently, if larvae can move from plant structures expressing lethal doses of Bt protein to those expressing sublethal levels of or no Bt protein, survival is likely to be higher. Low larval survival was recorded after 48 h when *S. litura* larvae were on BG3 leaf discs in no-choice bioassays (Figure 3). However, survival increased in choice bioassays when larvae, initially placed on BG3 leaf discs, could move from BG3 leaf discs onto CC leaf discs. Larvae showed higher survival when first placed onto CC leaf discs in choice bioassays (Figure 3). Whether this is due to lack of movement from the leaf discs or whether initial feeding on leaf material expressing no Bt proteins contributes to increased survival if larvae then feed on BG3 material is unclear. Likewise, Luong et al. (2018) also found that *H. armigera* larvae had higher survival rates when released onto non-Bt diet compared with larvae released on Bt diet in choice bioassays.

However, in the field, increased dispersal on BG3 cotton plants could also be disadvantageous and lead to higher neonate mortality. Zalucki et al. (2002) determined that neonate mortality in lepidopteran species is generally high but variable, with a large proportion of mortality and disappearance identified in the early larval instars, attributed to unknown factors. When neonates disperse from an unsuitable feeding site, this may or may not result in death. Whilst larvae that disperse may locate more suitable feeding sites, increased movement may also increase their exposure to the impacts of weather as well as predators and entomopathogens, increasing their mortality risk (Zalucki et al. 2002).

In conclusion, our findings partially support the hypothesis that *S. litura* neonates exhibit greater movement on BG3 cotton compared with cotton without Bt proteins, resulting in greater larval survival. While larvae exhibited increased movement when exposed to BG3 cotton, survival only improved when larvae could move to leaf discs without Bt proteins. Further research is needed to determine whether increased larval movement and dispersal would likely increase survival in commercial BG3 cotton fields, where ongoing exposure to BG3 proteins is likely. Specifically, it is essential to determine if a lethal dose of Bt protein is expressed across all BG3 plant structures that *S. litura* larvae may be exposed to and feed upon.

Author Contributions

Sharna Holman: conceptualization [lead], data curation [lead], formal analysis [lead], investigation [lead], methodology [lead], writing – original draft [lead], writing – review and editing [equal]. **Paul Grundy:** conceptualization [equal], funding acquisition [lead], methodology [equal], writing – review and editing [equal]. **Helen Spafford:** conceptualization [equal], methodology [equal], writing – review and editing [equal]. **Michael J. Furlong:** conceptualization [equal], methodology [equal], writing – review and editing [equal].

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Ethics Statement

This study involved laboratory-reared insects only and did not include human participants or vertebrate animals. Ethical approval, informed consent, and collection permits were therefore not required.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the broader findings of this study are available from the corresponding author upon reasonable request.

References

Adamczyk, J. J., Jr., D. D. Hardee, L. C. Adams, and D. V. Sumerford. 2001. "Correlating Differences in Larval Survival and Development of Bollworm (Lepidoptera: Noctuidae) and Fall Armyworm (Lepidoptera: Noctuidae) to Differential Expression of Cry1A(c) δ-Endotoxin in Various Plant Parts Among Commercial Cultivars of Transgenic *Bacillus thuringiensis* Cotton." *Journal of Economic Entomology* 94, no. 1: 284–290. https://doi.org/10.1603/0022-0493-94.1.284.

APVMA. 2016. "Public Release Summary on the Evaluation of the New Vip3A in the Production Bollgard III." https://apvma.gov.au/sites/default/files/publication/19591-prs-bollgardiii_0.pdf.

Bayer Crop Science. 2024. "Bollgard 3 Resistance Management Plan (RMP) for Northern Australia."

Becher, P. G., and P. M. Guerin. 2009. "Oriented Responses of Grapevine Moth Larvae *Lobesia botrana* to Volatiles From Host Plants and an Artificial Diet on a Locomotion Compensator." *Journal of Insect Physiology* 55, no. 4: 384–393. https://doi.org/10.1016/j.jinsphys.2009.01.006.

Carpenter, J. E. 2001. "Case Studies in Benefits and Risks of Agricultural Biotechnology: Roundup Ready Soybeans and Bt Field Corn." https://www.ncfap.org/documents/benefitsandrisks.pdf.

Castrejon, F., A. Virgen, and J. C. Rojas. 2006. "Influence of Chemical Cues From Host Plants on the Behavior of Neonate *Estigmene acrea* Larvae (Lepidoptera: Arctiidae)." *Environmental Entomology* 35, no. 3: 700–707. https://doi.org/10.1603/0046-225x-35.3.700.

EPPO. 2015. "PM 7/124 (1) *Spodoptera littoralis*, *Spodoptera litura*, *Spodoptera frugiperda*, *Spodoptera eridania*." *EPPO Bulletin* 45, no. 3: 410–444. https://doi.org/10.1111/epp.12258.

Farhan, Y., J. L. Smith, and A. W. Schaafsma. 2019. "Susceptibility of Different Instars of *Striacosta albicosta* (Lepidoptera: Noctuidae) to Vip3A, a *Bacillus thuringiensis* (Bacillaceae: Bacillales) Protein." *Journal of Economic Entomology* 112, no. 5: 2335–2344. https://doi.org/10.1093/jee/toz118.

Gore, J., B. Leonard, G. Church, B. R. Leonard, G. E. Church, and D. R. Cook. 2002. "Behavior of Bollworm (Lepidoptera: Noctuidae) Larvae on Genetically Engineered Cotton." *Journal of Economic Entomology* 95, no. 4: 763–769. https://doi.org/10.1603/0022-0493-95.4.763.

Gore, J., B. R. Leonard, and J. J. Adamczyk. 2001. "Bollworm (Lepidoptera: Noctuidae) Survival on 'Bollgard' and 'Bollgard II' Cotton Flower Bud and Flower Components." *Journal of Economic Entomology* 94, no. 6: 1445–1451. https://doi.org/10.1603/0022-0493-94.6.1445.

Greenplate, J. T. 1999. "Quantification of *Bacillus thuringiensis* Insect Control Protein Cry1Ac Over Time in Bollgard Cotton Fruit and Terminals." *Journal of Economic Entomology* 92, no. 6: 1377–1383. https://doi.org/10.1093/jee/92.6.1377.

Head, G. P., and J. Greenplate. 2012. "The Design and Implementation of Insect Resistance Management Programs for Bt Crops." *GM Crops & Food* 3, no. 3: 144–153.

Holman, S., P. Grundy, H. Spafford, and M. Furlong. 2025. "Lethal and Sublethal Effects of Cotton Expressing Single and Pyramided Proteins of *Bacillus thuringiensis* (Bt) on *Helicoverpa armigera* (Lepidoptera: Noctuidae), *Spodoptera litura* (Lepidoptera: Noctuidae), and *Spodoptera frugiperda* (Lepidoptera: Noctuidae)." *Journal of Economic Entomology* 118: toaf089.

Huang, F., D. A. Andow, and L. L. Buschman. 2011. "Success of the High-Dose/Refuge Resistance Management Strategy After 15 Years of Bt Crop Use in North America." *Entomologia Experimentalis et Applicata* 140, no. 1: 1–16. https://doi.org/10.1111/j.1570-7458.2011.01138.x.

Johnson, M., and M. Zalucki. 2007. "Feeding and Foraging Behaviour of a Generalist Caterpillar: Are Third Instars Just Bigger Versions of Firsts?" *Bulletin of Entomological Research* 97, no. 1: 81–88. https://doi.org/10.1017/S0007485307004750.

Kaleka, A. S., and Y. Kapoor. 2024. "Immature Stages and Chaetotaxy of *Spodoptera litura* (F)." *Indian Journal of Entomology* 87: 1–7.

Knight, K., G. Head, and J. Rogers. 2013. "Season-Long Expression of Cry1Ac and Cry2Ab Proteins in Bollgard II Cotton in Australia." *Crop Protection* 44: 50–58. https://doi.org/10.1016/j.cropro.2012.10.014.

Kranthi, K. R., S. Naidu, C. Dhawad, et al. 2005. "Temporal and Intra-Plant Variability of Cry1Ac Expression in Bt-Cotton and Its Influence on the Survival of the Cotton Bollworm, *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera)." *Current Science* 25: 291–298.

Luong, T. T., M. P. Zalucki, L. E. Perkins, T. T. A. Luong, and S. J. Downes. 2018. "Feeding Behaviour and Survival of *Bacillus thuringiensis*-Resistant and *Bacillus thuringiensis*-Susceptible Larvae of *Helicoverpa armigera* (Lepidoptera: Noctuidae) Exposed to a Diet With *Bacillus thuringiensis* Toxin." *Austral Entomology* 57, no. 1: 1–8. https://doi.org/10.1111/aen.12265.

Luong, T. T. A., B. W. Cribb, S. J. Downes, L. E. Perkins, and M. P. Zalucki. 2019. "Stay or Move: How Bt-Susceptible *Helicoverpa armigera* Neonates Behave on Bt Cotton Plants." *Entomologia Experimentalis et Applicata* 167, no. 10: 868–879. https://doi.org/10.1111/eea.12837.

Lynch, R., S. Pair, and R. Johnson. 1983. "Fall Armyworm Fecundity: Relationship of Egg Mass Weight to Number of Eggs." *Journal of Georgia Entomological Society* 18, no. 4: 507–513.

Malaquias, J. B., M. A. Caprio, W. A. C. Godoy, C. Omoto, F. S. Ramalho, and J. K. S. Pachú. 2020. "Experimental and Theoretical Landscape Influences on *Spodoptera frugiperda* Movement and Resistance Evolution in Contaminated Refuge Areas of Bt Cotton." *Journal of Pest Science* 93, no. 1: 329–340. https://doi.org/10.1007/s10340-019-01145-1.

Mallet, J., and P. Porter. 1992. "Preventing Insect Adaptation to Insect-Resistant Crops: Are Seed Mixtures or Refugia the Best Strategy?" *Proceedings of the Royal Society of London, Series B: Biological Sciences* 250, no. 1328: 165–169. https://doi.org/10.1098/rspb.1992.0145.

Michael, P., and W. Woods. 1980. *Entomological Review of Cotton Growing in the Ord River Area of Western Australia*. Technical Bulletin: Western Australian Department of Agriculture.

Mitra, S., D. Firake, K. Umesh, et al. 2021. "Polyphagous Caterpillars of *Spodoptera litura* Switch From a Trap Crop to the Main Crop, Improve Fitness, and Shorten Generation Time." *Journal of Pest Science* 94, no. 4: 1091–1103. https://doi.org/10.1007/s10340-021-01351-w.

Patterson, H. D., and R. Thompson. 1971. "Recovery of Inter-Block Information When Block Sizes Are Unequal." *Biometrika* 58, no. 3: 545–554. https://doi.org/10.1093/biomet/58.3.545.

Singh, G., P. J. Rup, and O. Koul. 2008. "Selective Feeding of *Helicoverpa armigera* (Hübner) and *Spodoptera litura* (Fabricius) on Meridic Diet With *Bacillus thuringiensis* Toxins." *Journal of Insect Behavior* 21, no. 5: 407–421.

Stapel, J. O., D. J. Waters, J. R. Ruberson, and W. J. Lewis. 1998. "Development and Behavior of *Spodoptera exigua* (Lepidoptera: Noctuidae) Larvae in Choice Tests With Food Substrates Containing Toxins of *Bacillus thuringiensis*." *Biological Control* 11, no. 1: 29–37. https://doi.org/10.1006/bcon.1997.0576.

Talyn, B., R. Lemon, M. Badoella, et al. 2019. "Roundup, but Not Roundup-Ready Corn, Increases Mortality of *Drosophila melanogaster*." *Toxics* 7, no. 3: 38. https://doi.org/10.3390/toxics7030038.

Teakle, R., J. Jensen, P. Singh, et al. 1985. *Handbook of Insect Rearing*. Vol. 2, 313–322. Elsevier.

VSN International. 2024. "Genstat Reference Manual (Release 24), Part 1 Summary."

Wang, P., M. J. Furlong, T. K. Walsh, and M. P. Zalucki. 2019. "Moving to Keep Fit: Feeding Behavior and Movement of *Helicoverpa armigera* (Lepidoptera: Noctuidae) on Artificial Diet With Different Protein: Carbohydrate Ratios." *Journal of Insect Science* 19, no. 5: 1–9. https://doi.org/10.1093/jisesa/iez098.

Zalucki, M. P., A. R. Clarke, and S. B. Malcolm. 2002. "Ecology and Behavior of First Instar Larval Lepidoptera." *Annual Review of Entomology* 47, no. 1: 361–393. https://doi.org/10.1146/annurev.ento.47.091201.145220.

Zhang, J. H., C. Z. Wang, J. D. Qin, et al. 2004. "Feeding Behaviour of *Helicoverpa armigera* Larvae on Insect-Resistant Transgenic Cotton and Non-Transgenic Cotton." *Journal of Applied Entomology* 128, no. 3: 218–225. https://doi.org/10.1111/j.1439-0418.2004.00841.x.

Zhao, D., M. Zalucki, R. Guo, et al. 2016. "Oviposition and Feeding Avoidance in *Helicoverpa armigera* (Hübner) Against Transgenic Bt Cotton." *Journal of Applied Entomology* 140, no. 9: 715–724. https://doi.org/10.1111/jen.12304.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** A strong positive relationship was observed between egg mass weight and the number of eggs per *S. litura* egg mass. **Figure S2:** Diagrammatic representation of the experiment design for choice bioassays conducted in Petri dishes (90-mm diameter). Three initial starting positions for larvae were used: (1) single neonate placed on the Bollgard 3 (BG3) leaf disc, (2) single neonate placed on non-Bt expressing cotton (CC) leaf disc, and (3) single neonate placed equidistant between both leaf discs (at approximately 20-mm). **Figure S3:** The percentage of *S. litura* neonate larvae (mean ± SE) that were observed on the leaf discs of Bollgard 3 (BG3) and cotton expressing no Bt proteins (CC) in no choice bioassays. Larvae that escaped or disappeared from the Petri dish were included in the data at the time point they escaped but were excluded from further analyses, as were those recorded dead at 24 h. Consequently, as time progressed, subsets of replicates were analysed. Means sharing the same letter are not significantly different (LSD, $p > 0.05$). **Figure S4:** Percentage of S. litura neonate larvae (mean ± SE) observed (A) on the leaf discs where they were initially placed: Bollgard 3 (BG3) (square markers) or cotton expressing no Bt proteins (CC) (triangle markers) and (B) on the alternative leaf disc in the arena, at various observation points in choice bioassays. In both graphs, black lines represent BG3 leaf discs, and grey lines represent CC leaf discs available within the Petri dish. Larvae that escaped or disappeared from the Petri dish were included in the data at the time point they escaped but were excluded from further analyses, as were those recorded dead at 24 h. Consequently, as time progressed, subsets of replicates were analysed. Means sharing the same letter are not significantly different (LSD, $p > 0.05$). **Figure S5:** Percentage of S. litura neonate larvae (mean ± SE) observed on Bollgard 3 (BG3) leaf discs at different observation points in choice bioassays when larvae were placed at an equidistant position from leaf discs of BG3 and cotton expressing no Bt proteins (CC). Larvae that escaped or disappeared from the Petri dish were included in the data at the time point they escaped but were excluded from further analyses, as were those recorded dead at 24 h. Consequently, as time progressed, subsets of replicates were analysed. Means sharing the same letter are not significantly different (LSD, $P > 0.05$). **Table S1:** Ingredients for artificial diet used in maintaining a laboratory Spodoptera litura culture. **Table S2:** Analysis of no-choice bioassays using larval presence on leaf discs of Bollgard 3 (BG3) or non-Bt protein expressing (CC) leaf discs as the response variable. **Table S3:** Analysis of choice bioassays using larval presence on the initial leaf disc as the response variable, with the larval starting position on Bollgard 3 (BG3) or non-Bt protein expressing (CC) leaf discs as the treatment. **Table S4:** Analysis of choice bioassays using larval presence on the alternative leaf disc as the response variable, with the larval starting position on Bollgard 3 (BG3) or non-Bt protein expressing (CC) leaf discs as the treatment. **Table S5:** Analysis of equidistance 'choice' treatment within the choice bioassays using larval presence on the Bollgard (BG3) leaf disc as the response variable. **Table S6:** Analysis of the survival rate of larvae in different bioassay types (no choice or choice bioassays; access to one or two food sources [Bt and/or non-Bt]) and larval starting position on Bollgard 3 (BG3) or non-Bt protein expressing (CC) leaf discs. **Table S7:** Analysis of the proportion of neonates, 24 h after hatching, found distributed across different locations (location categories) on Bollgard 3 and non-Bt expressing cotton (CC) plants. **Table S8:** Analysis of the frequency of larvae off leaf discs in no-choice bioassays. **Table S9:** Analysis of the frequency of larvae off leaf discs in different starting position treatments in choice bioassays.