



# Mines over matter: Effects of foliar particulate matter on the herbivorous insect, *Helicoverpa armigera*

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## Abstract

Coalmines, which are major contributors of particulate matter in the form of coal dust, are expanding globally into rural environments. However, ecological effects on organisms interacting with coal-dusted foliage in mining landscapes are unknown. We tested how the behaviour, development and survival of a polyphageous insect herbivore, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is affected by consuming tomato leaves *Lycopersicon esculentum* laden with coal dust. We tested (a) feeding site establishment preference of neonates, (b) feeding behaviour and leaf consumption of late-instar larvae, (c) survival of neonates and (d) survival and development of late-instar larvae. We found that coal dust consumption increased the mortality of late-instar larvae but did not influence their development. Despite long-term implications for survival, late-instar larvae did not adjust their feeding behaviour or the amount of leaf material consumed in response to foliar coal dust. Contrastingly, when neonate *H. armigera* were given a choice, they avoided establishing themselves on the coal-dusted adaxial surface of leaves. Neonate mortality was 99% within 7 days, with no effect of coal dust. Our study provides the first data on the impact of coal dust on an insect herbivore. This has implications for ecological interactions in landscapes adjoining coalmines.

## KEYWORDS

air pollution, behaviour, coal dust, herbivore, Lepidoptera, survival

## 1 | INTRODUCTION

Particulate matter is a pervasive air pollutant of growing global concern. These airborne solid and liquid suspensions (<100  $\mu\text{m}$ ) are damaging to both human respiratory (Lim et al., 2013; Samoli et al., 2008; WHO, 2013) and plant (Farmer, 1993; Hirano, Kiyota, & Aiga, 1995; Naidoo & Chirkoot, 2004; Sharifi, Gibson, & Rundel, 1997) health. Defined by size rather than composition, the particulate matter of considerable health concern is the finer  $\text{PM}_{10}$  (<10  $\mu\text{m}$  aerodynamic diameter) and  $\text{PM}_{2.5}$  (<2.5  $\mu\text{m}$  aerodynamic diameter) size fractions. Significant anthropogenic sources of particulate matter include combustion, vehicular transport, agriculture and coal mining (Doley, 2006).

Open-cut coalmines are major contributors of particulate matter in the form of coal dust through processes of blasting, overburden removal, quarrying, handling-plants, exposed pits and transportation (Doley, 2006; Ghose, 2007; Huertas, Huertas, & Solis, 2012). With 1,424 GW of new and planned coal capacity worldwide, coalmines are expanding globally into regions of remnant forest and agricultural use (Shearer, Ghio, Myllyvirta, Yu, & Nace, 2016). The annual processing of 3 million tonnes of ore at an open-cut mine can equate to a daily production of 10 tonnes of particulate pollution (New South Wales State Pollution Control Commission, 1983). Yet the ecological effects of coal dust beyond plant biochemistry and photosynthetic performance remain unexplored (Farmer, 1993; Grantz, Garner, & Johnson, 2003; Naidoo & Chirkoot, 2004). As organisms

such as herbivores are drivers of vegetation community composition in natural and cultivated ecosystems, a limited understanding of their responses to particulate matter, and specifically coal dust, may have broader ecological and agricultural implications. There is a need to understand the effects of coal dust on the behaviour, development and survival of animals that interact with vegetation.

Insect–plant interactions are moderated by a range of environmental stressors (Pincebourde et al., 2017). Whereas abundant research documents the mixed effects of O<sub>3</sub>, NO<sub>x</sub>, and SO<sub>2</sub> air pollutants on the fitness and performance of herbivorous insects, relatively little is understood about the effects of particulate matter pollution (Bell, 2000; Grantz et al., 2003). Historically, inert particles made from fossilized silicon dioxide, Diatomaceous Earth (DE), were employed for their abrasive and hydrocarbon absorption properties which cause insect cuticle damage and desiccation (Hockenyos, 1933; Korunić, 2013). Foliar coal dust may have similar properties that influence herbivorous insect behaviour, including feeding patterns, consumption and establishment on a host plant, as well as their development and survival.

Coal dust deposited on leaf surfaces may influence the feeding behaviour of herbivores directly by acting as a structural barrier to accessing edible leaf material (Massey, Ennos, & Hartley, 2006). The chemical composition of coal dust varies with local geography; however, silicates, carbonates and trace heavy metals are common constituents (Huertas et al., 2012; Merefield et al., 1995). The high silicate content of coal dust may create an abrasive layer that reduces the palatability and digestibility of the leaf, deterring larvae from feeding (Korth et al., 2006; Massey & Hartley, 2009; Massey et al., 2006; Park, Doege, Nakata, & Korth, 2009). The toxicity of heavy metals in coal dust may also act as antifeedants, deterring herbivores (Boyd, 2007; Mogren & Trumble, 2010).

As fine particulates can reduce photosynthetic productivity (Hirano et al., 1995; Naidoo & Chirkoot, 2004; Sharifi et al., 1997) and change foliar chemistry (Farmer, 1993), the nutritional quality, palatability and digestibility of leaves may also be affected by cumulative coal dust deposition. Indirect responses to foliar coal dust by insect herbivores may include deterrence, altered feeding patterns or compensation for lower nutrient quality by increasing leaf consumption (Lee, Raubenheimer, & Simpson, 2004; Raubenheimer & Simpson, 1993).

The physical and chemical properties of coal dust may also influence where neonate (newly hatched) insect herbivores choose to establish themselves on a host plant. Although the impact of particulates on where neonates prefer to establish for feeding is yet unstudied, larvae are known to avoid toxic substrates. For example, neonate *Helicoverpa zea* avoid leaves treated with the pesticide *Bacillus thuringiensis* (Ashfaq, Young, & McNew, 2001; Jyoti, Young, Johnson, & McNew, 1996). As neonates disperse before establishing themselves on leaves for feeding, effects of coal dust at this stage may determine the intensity of feeding to different sites of a plant.

The abrasive structural properties and/or toxic trace heavy metal content of coal dust may have a cumulative influence on the development and survival of herbivorous insects. Lepidopteran

larvae experience reduced growth, digestion efficiency and delayed development when consuming abrasive minerals such as silica and calcium oxalate (Clissold, Sanson, Read, & Simpson, 2009; Korth et al., 2006; Massey & Hartley, 2009; Massey et al., 2006; Park et al., 2009). Irreversible mandibular wear is one mechanism proposed for the lower fitness impact of high silica-containing food (Massey & Hartley, 2009). Consumption of the heavy metal selenium reduced the development time and pupal weight of *Spodoptera exigua* (Trumble, Kund, & White, 1998). Similarly, *Pieris rapae* experienced higher mortality and reduced growth when feeding on a nickel-containing diet (Martens & Boyd, 1994). Although heavy metals are present in coal dust as traces, compensatory consumption could increase the ingestion of a toxin to ecological effective concentrations, leading to survival implications in the long term. For example, Velvet bean caterpillars, *Anticarsia gemmatalis*, increased their consumption of a low nutrient, caffeine-containing resource, which led to high ingestion of this alleochemical and overall decreased fitness (Slansky & Wheeler, 1992). It remains unknown how herbivorous insects may respond to the structural and chemical barriers inherent in foliar coal dust.

We tested how the behaviour, development and survival of a polyphagous insect herbivore, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is affected by consuming tomato leaves *Lycopersicon esculentum* laden with coal dust. *Helicoverpa armigera* has been observed on 101 native and crop species across 31 families in Australia alone (Johnson & Zalucki, 2007) and is a globally destructive pest of wide-ranging crop species including tomatoes (Cunningham & Zalucki, 2014; Talekar, Opena, & Hanson, 2006; Tay et al., 2013). Tomato plants were chosen for their high trichome density which makes them readily capable of capturing and retaining coal dust (Leonard, McArthur, & Hochuli, 2016). The ubiquity of *H. armigera* makes it an ideal model species for this study as it likely pervades agro-ecosystems where coalmines are present.

As sensitivity to environmental stressors and optimal host sites differ by insect life history stage (Hochuli, 2001), it is important to assess the impacts of pollutants across multiple immature stages. We thus tested the effects of foliar coal dust on (a) the feeding behaviour and (b) leaf consumption of late-instar larvae as well as (c) feeding site establishment preference of neonates of *H. armigera*. We predicted that late-instar larvae would feed for less time, and take shorter meals less often with longer intermeal rests to process toxins. We hypothesized that deterrence towards coal dust would extend to where neonates choose to establish themselves on the leaf. We further tested the effect of foliar coal dust on (d) survival and development of late-instar larvae as well as (e) survival of neonates. We predicted that *H. armigera* feeding on foliar coal dust would have increased mortality and more delayed development than those feeding on control leaves. Our study provides the first data on the impact of coal dust on an insect herbivore. This is an important step to understand implications for ecological interactions in landscapes adjoining coalmines.

## 2 | MATERIALS AND METHODS

### 2.1 | Preparation of coal dust and plant materials

We sourced coal dust from SGS Energy Minerals, Newcastle Australia. To make coal dust of  $\leq 38 \mu\text{m}$  diameter, unwashed coal from mines in the Hunter Valley, NSW Australia was milled to 0.212 mm using a Raymond Mill (Alan Roberts Products Pty Ltd). It was then sieved to 0.038 m using an Endecotts woven wire sieve (120 mm diameter) in a sieve shaker (Wallaby Mining products Pty Ltd).

Tomatoes, *L. esculentum*, were grown from seed in a greenhouse at  $22 \pm 3^\circ\text{C}$ ,  $59\% \pm 15$  relative humidity in soil mixed with 25% sand (Professional potting mix; Greenlife Pty Ltd). Seedlings were transferred into 20 cm 4.5 L pots after 2 weeks with leaves sprayed once with Seasol (Seasol International Pty Ltd), an organic plant health treatment, to promote growth. Plants were watered daily with an automatic drip watering system that watered directly at the base of the plant to avoid washing coal dust off leaves throughout the experimental period.

After 10 weeks, we partitioned the greenhouse into coal dust and control segments, by installing eight coal dust excluders. Coal dust excluders consisted of clear polyethelene film (200  $\mu\text{m}$  thickness) attached to a wooden frame (1,230 mm  $\times$  1,000 mm) encasing plants within (Appendix Figure 1a). An overlap of 560 mm was sealed with velcro to prevent coal dust from entering or escaping. Treatment excluders included a top flap (500 mm  $\times$  400mm), also sealed with Velcro to facilitate dusting from above. Each 1.8 m<sup>3</sup> coal dust excluder was suspended from the ceiling 1.5 m above the benchtop on which pots were placed. Each tent had an open base to allow for airflow, but walls were tucked underneath pots to minimize dust escaping. Coal dust excluders for coal dust/control treatments were arranged randomly and each contained twenty plants (total = 160 plants).

For coal dust treatments, we applied dust weekly for a total of 9 weeks and commenced behavioural/survival assays after 6 weeks of application (Grantz et al., 2003; Leys & McTainsh, 1999). Weekly dust application involved sieving 1.2 g of coal dust per dust excluder over the leaf surfaces from above. We applied coal dust through the access flap of the dust excluders using a metal sieve (9 cm diameter, 75  $\mu\text{m}$  mesh size; Appendix Figure 1b). This amount was chosen to correspond with the maximum monthly allowable dust deposition for a single mine point source of  $4 \text{ g m}^{-2} \text{ month}^{-1}$  (Dec, 2005). The greenhouse fan was switched off when applying coal dust. Control plants were not dusted.

On each experimental day, we randomly picked leaves from the third leaf nodes of all plants in all dust excluders per treatment. Leaves were taken from the third leaf node to control for internode differences in leaf physiological and chemical properties that may affect herbivore consumption and growth (Bhonwong, Stout, Attajarusit, & Tantasawat, 2009). For behavioural assays, we cut leaves into standard leaf discs (4.75 cm<sup>2</sup>) with a vegetable cutter. Whole leaves were fed to caterpillars in survival and development experiments.

The mean amount of coal dust on leaf discs was  $0.3 \pm 0.07 \text{ mg/cm}^2$ . We calculated the mean amount of coal dust on leaf discs by placing a randomly selected subsample of leaves ( $n = 20$ ) with 200 ml of reverse osmosis water in individual glass containers and agitated for 60 s. Washing the leaves in this way is representative of the dust dislodged with precipitation and not that which is retained within the epicuticular wax (Dzierzanowski, Popek, Gawrońska, Sæbø, & Gawroński, 2011). We dried filter papers in a drying oven at  $70^\circ\text{C}$  for 24 hrs before storing them in a desiccating chamber for a further 24 hrs prior to weighing. We weighed predried filter paper with an electronic balance before filtering the dust solution using a 4.5 cm Nalgene filter onto cellulose nitrate filter paper (Sartorius stedim biotech). We dried and desiccated used filter papers by the same method as preweighing to calculate the mass difference as deposited dust. Although particulates were washed from both abaxial and adaxial leaf surfaces, foliar dust deposition was calculated as particulates ( $\mu\text{g}$ )/adaxial leaf area (cm<sup>2</sup>).

### 2.2 | Larval rearing

We obtained neonate and late-instar *H. armigera* larvae from the CSIRO Cotton Research Institute in Narrabri, NSW. Late-instar larvae were reared on CSIRO standard laboratory diet until the 2nd or 3rd instar to avoid high mortality experienced during pilot studies (Singh & Moore, 1985). Larvae were kept in a temperature controlled room maintained at  $23 \pm 2^\circ\text{C}$  with approximately 90% humidity when not observed for behaviour.

### 2.3 | Behaviour and leaf consumption experiments

#### 2.3.1 | Feeding site establishment preference of neonates

We conducted 35 choice tests to determine whether coal dust influences where newly hatched *H. armigera* choose to establish on the leaf for feeding. We randomly placed one control and one coal-dusted leaf disc on opposite edges of a Petri dish (9 cm diameter) lined with moist filter paper ( $n = 35$ ). We introduced 20 *H. armigera* neonates into the centre of each dish and sealed the dish with a lid and tape to prevent escape. We recorded the number of larvae on the abaxial (lower) and adaxial (upper) surfaces of leaf discs after 1, 4 and 24 hrs (Zhang, Wang, Qin, & Guo, 2004).

#### 2.3.2 | Feeding behaviour of late-instar larvae

We conducted 3-hrs feeding trials with 3rd, 4th and 5th instar larvae to determine whether or not coal dust influences the feeding behaviour of *H. armigera*. Larvae were transferred individually into Petri dishes (9 cm diameter) with moist filter paper, sealed with a lid and starved for 12 hrs to standardize for hunger state. Filter paper and frass were removed 1 hr before trials. Directly before observations, either a control or coal-dusted leaf disc was randomly assigned to a larva and placed 1 cm from the head without touching the animal

as this may have influenced feeding behaviour (Low, McArthur, & Hochuli, 2014). Trials were conducted in the laboratory at room temperature  $21 \pm 2^\circ\text{C}$ . All larvae of each instar stage were trialled on a separate day, and each larva was used once. Preweighed larvae were observed for feeding, and nonfeeding behaviours once a minute for 3 hrs continuously. To quantify feeding behaviour, we calculated (a) the total time spent feeding, (b) median meal duration, (c) first meal duration, (d) meal frequency, (e) intermeal frequency and (f) intermeal duration (Shelomi, 2009). Feeding was defined as visible mandibular action or the head touching and vibrating on the leaf. *Helicoverpa armigera* take meals which are comprised of a series of feeding bouts (Shelomi, 2009). We defined a feeding bout as the time a larvae spent feeding, separated by gaps of nonfeeding behaviour shorter than what was determined the "bout criterion" (Blaney, Chapman, & Wilson, 1973; Simpson, 1995). The bout criterion was calculated by plotting the distribution of breaks between feeding as a log survivor function (Simpson, 1995; Slater and Lester, 1982). A meal was defined as a series of feeding bouts separated by breaks greater or equal to the bout criterion (Blaney et al., 1973, Simpson, 1995). Incomplete consecutive feeding or nonfeeding periods after 180 min were removed before meal and intermeal analyses. "Nibbles" were defined as feeding for 1 min or less (Simpson, 1995). Nibbles were analysed separately. We tested 116 larvae in three sets of behavioural trials (3rd instar  $N = 20$ , 4th instar  $N = 20$ , 5th Instar  $N = 18$  for each treatment). As larvae cease feeding before moulting, larvae were left for 48 hrs and checked for the presence of a head capsule or exuviae. As larva does not feed before moulting, any larvae that did not feed in the experiment and moulted within 48 hrs were excluded from analyses.

### 2.3.3 | Leaf consumption by late-instar larvae

We analysed leaf area eaten from feeding behaviour trials to determine whether foliar coal dust influences leaf consumption by late-instar *H. armigera*. Third and fourth instar larvae were left in the Petri dish with the leaf disc for 24 hrs before removal. Fifth instar larvae were removed from the leaf immediately after trials as they consumed a large proportion of the leaf within the 3-hrs period. Amount eaten was calculated as the leaf area missing from photographs taken after trials against the original known leaf disc area ( $4.75 \text{ cm}^2$ ) using Image-J image analysis software (<http://rsbweb.nih.gov/ij/download.html>).

## 2.4 | Survival and development experiments

### 2.4.1 | Survival of neonates

We placed 400 newly hatched neonates (within 12 hrs) in groups of 10 into 20 clear plastic containers (30 ml) containing either a control or coal-dusted leaf with a mean area of  $4.75 \text{ cm}^2$  ( $n = 20$  replicates per treatment with 10 larvae per replicate). The number of surviving larvae was counted, dead larvae removed, and leaves replaced every second day for 20 days.

### 2.4.2 | Survival and development of late-instar larvae

Three hundred individual *H. armigera* were transferred as 2nd or 3rd instars from laboratory diet to individual (30 ml) clear plastic containers containing either a control or a coal-dusted leaf 5 days after hatching ( $N = 150$ ). Larvae were identified to instar visually, using the presence of the head capsule and exuviae (Johnson & Zalucki, 2007). We assessed development stage and survivorship when replacing leaves every second day until pupation. We compared the number of days from 3rd instars to 4th, 5th and 6th instars, respectively, as measures of development rate. Larvae that were transferred as 2nd instars were excluded from development analyses to standardize for starting instar.

### 2.5 | Statistical analyses

We analysed the difference between control and coal dust treatments on larval feeding behaviour, consumption and development using one-way analysis of variance (ANOVA). Normality and homogeneity of variances were tested using Shapiro-Wilk tests and Welch's tests, respectively. As ANOVA is robust to violation of the assumption of normality (Quinn & Keough, 2002), nonparametric tests were only performed if variances were heteroscedastic.

To test the effect of coal dust on the feeding site establishment preference of neonate *H. armigera* at 1, 4 and 24 hrs, we conducted separate Wilcoxon sign rank tests. Control/coal dust treatment and positioning on the adaxial or abaxial leaf surfaces were the two predictor variables. This test was chosen as data were heteroscedastic. Where significance differences occurred, we conducted post hoc Tukey's HSD tests to determine which predictor variables differed.

For feeding behaviour and consumption analyses, we checked the assumption of no difference in the initial weight of larvae between treatments using one-way ANOVA (Johnson & Zalucki, 2007; Mohammadi, Abad, Rashidi, & Mohammadi, 2010). All larvae that moulted within 48 hrs of behavioural trials fed during the experiment and were included in analyses. As there was no difference in the starting wet weights between treatments for 3rd instars ( $F_{1,38} = 1.047$ ,  $p = 0.313$ ), 4th instars ( $F_{1,38} = 0.190$ ,  $p = 0.666$ ) or 5th instars ( $F_{1,34} = 0.056$ ,  $p = 0.814$ ), wet weights were not included as a covariate for behavioural and consumption analyses. The feeding bout criterion was set at 4 min for 3rd instars and 6 min for 4th and 5th instar larvae.

We tested whether coal dust consumption influenced larval and neonate survival using Cox regression models. We conducted Cox proportional hazards analysis, which measures the probability of surviving to a given time point when survival probability is constant over time, using SPSS software (SPSS, 2013). For late-instar larvae, starting instar was included as a categorical covariate to distinguish differences in survival with starting age. To control for the lack of independence of neonates within the same rearing container, we clustered neonates by group. We reported Wald statistics for Cox proportional hazards analyses to account for multiple covariates.

The level of significance was set at  $\alpha = 0.05$  for all tests. Aside from survival analyses, statistical tests were performed in JMP 11.0.0 (SAS Institute Inc., Cary, NC, USA). Means are reported as  $\pm SE$ .

### 3 | RESULTS

#### 3.1 | Behaviour and leaf consumption experiments

##### 3.1.1 | Feeding site establishment preference of neonates

The feeding site establishment preference of neonate *H. armigera* significantly differed between treatments (on the abaxial and adaxial surfaces of control and coal-dusted leaves) after 1 hr ( $\chi^2 = 31.20$ ,  $df = 3$ ,  $p < 0.0001$ ; Figure 1a), 4 hrs ( $\chi^2 = 50.29$ ,  $df = 3$ ,  $p < 0.0001$ ; Figure 1b) and 24 hrs ( $\chi^2 = 17.45$ ,  $df = 3$ ,  $p < 0.0006$ ; Figure 1c). Post hoc analyses revealed that neonates avoided establishing themselves on the adaxial surface of coal-dusted leaves compared with control leaves for all time periods ( $p < 0.05$  for 1, 4 and 24 hrs, Figure 1). Furthermore, neonates significantly preferred to establish on the abaxial surface of both control and coal-dusted leaves at 1 hr ( $p < 0.05$ ; Figure 1a) and 4 hrs ( $p < 0.05$ ; Figure 1b), but there was no difference after 24 hrs ( $p > 0.05$ ; Figure 1c).

##### 3.1.2 | Feeding behaviour of late-instar larvae

For 3rd, 4th and 5th instar larvae, there was no effect of coal dust on total feeding time (3rd instar:  $F_{1,38} = 0.47$ ,  $p = 0.498$ ; 4th instar  $F_{1,38} = 0.10$ ,  $p = 0.759$ ; 5th instar  $F_{1,34} = 0.25$ ,  $p = 0.622$ ), meal frequency (3rd instar:  $F_{1,30} = 0.004$ ,  $p = 0.950$ ; 4th instar:  $F_{1,33} = 1.06$ ,  $p = 0.311$ ; 5th instar:  $F_{1,31} = 0.80$ ,  $p = 0.378$ ), median meal duration (3rd instar:  $F_{1,30} = 1.26$ ,  $p = 0.271$ . 4th instar:  $F_{1,32} = 0.19$ ,  $p = 0.667$ , 5th instar:  $F_{1,33} = 0.13$ ,  $p = 0.724$ ), first meal duration (3rd instar:  $F_{1,30} = 0.004$ ,  $p = 0.951$ , 4th instar:  $F_{1,33} = 0.58$ ,  $p = 0.453$ ; 5th instar:  $F_{1,31} = 2.53$ ,  $p = 0.122$ ), the number of nibbles taken (3rd instar:  $F_{1,30} = 0.08$ ,  $p = 0.774$ ; 4th instar:  $F_{1,33} = 1.84$ ,  $p = 0.184$ ; 5th instar:  $F_{1,31} = 0.16$ ,  $p = 0.690$ ), intermeal frequency (3rd instar:  $F_{1,29} = 0.035$ ,  $p = 0.853$ ; 4th instar:  $F_{1,32} = 0.11$ ,  $p = 0.741$ ; 5th instar:  $F_{1,28} = 0.52$ ,  $p = 0.476$ ), or median intermeal duration (3rd instar:  $F_{1,29} = 0.002$ ,  $p = 0.965$ ; 4th instar:  $F_{1,32} = 3.36$ ,  $p = 0.076$ ; 5th instar:  $F_{1,28} = 0.63$ ,  $p = 0.434$ ; Table 1).

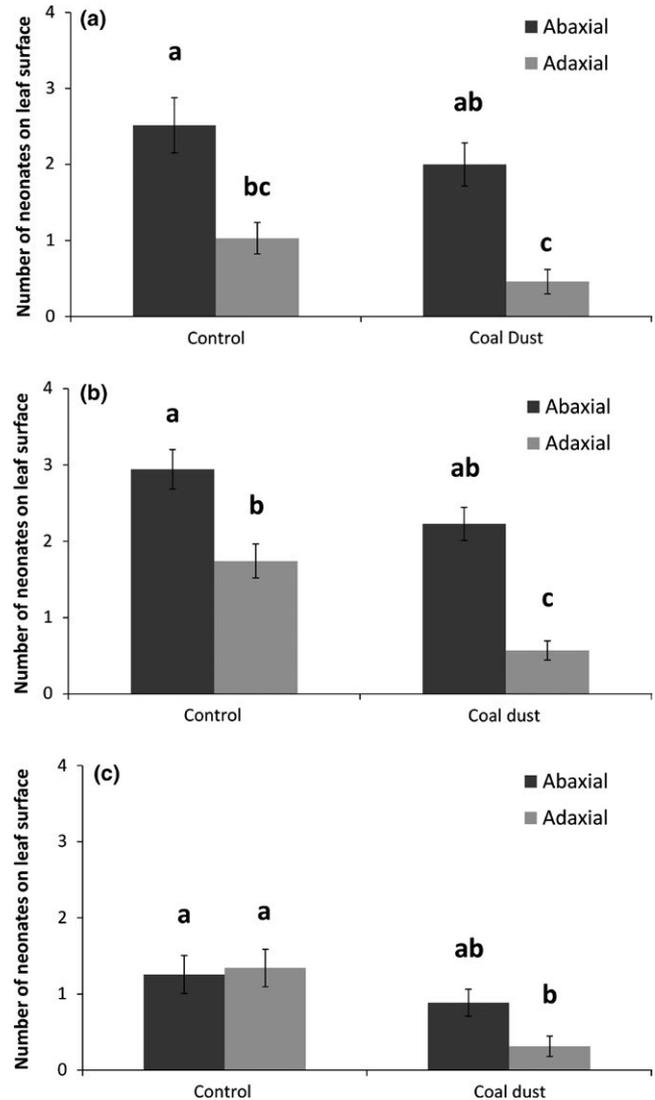
##### 3.1.3 | Leaf consumption by late-instar larvae

There was no difference in the leaf area eaten (% original) for 3rd instar ( $F_{1,38} = 3.66$ ,  $p = 0.063$ ), 4th instar ( $F_{1,38} = 3.84$ ,  $p = 0.057$ ) or 5th instar larvae ( $F_{1,34} = 0.76$ ,  $p = 0.389$ ).

### 3.2 | Survival and development experiments

#### 3.2.1 | Survival of neonates

There was no effect of coal dust on the survival of neonates ( $\chi^2 = 0.12$ ,  $df = 1$ ,  $p = 0.735$ ). Over 60% of neonates died by the



**FIGURE 1** Mean number of neonates settling on the abaxial and adaxial surface of control and coal-dusted leaves after (a) 1 hr, (b) 4 hrs and (c) 24 hrs. Letters indicate statistical differences between treatments

second day and 99% died within 7 days. All neonates in all treatments died within 20 days of leaf consumption.

#### 3.2.2 | Survival and development of late-instar larvae

Larval survival was significantly reduced in coal dust treatments ( $\chi^2 = 4.81$ ,  $df = 1$ ,  $p = 0.028$ , Figure 2). Larvae transferred as 2nd instars had lower survival than those transferred as 3rd instars ( $\chi^2 = 30.414$ ,  $df = 1$ ,  $p < 0.0001$ ). Most larvae (98.7%) died before pupation, irrespective of treatment.

There was no difference in the development time from 3rd to 4th instars (Mean:  $3.9 \pm 1.2$  days,  $F_{1,49} = 0.35$ ,  $p = 0.559$ ), 5th instars (Mean:  $6.7 \pm 4.4$  days,  $F_{1,49} = 2.23$ ,  $p = 0.142$ ) or 6th instars (Mean:  $16.57 \pm 2.9$  days,  $F_{1,26} = 0.17$ ,  $p = 0.684$ ).

**TABLE 1** Total, mean and median feeding behaviour values for late-instar larvae (3rd, 4th and 5th) in observation experiments lasting 180 min each

	Control	Coal dust
3rd instar		
Total feeding time (min)	37.95	30.15
Meal Frequency	4.47	4.53
Median meal duration (minutes)	9.24	7.27
First meal duration (min)	13.06	12.73
Number of nibbles taken	2.67	2.81
Intermeal frequency	7.63	7.40
Median intermeal duration (min)	9.13	9.03
4th instar		
Total feeding time (min)	32.95	35.30
Meal Frequency	5.22	5.88
Median meal duration (min)	8.39	9.16
First meal duration (min)	17.83	13.29
Number of nibbles taken	1.94	1.35
Intermeal frequency	7.33	7.56
Median intermeal duration (min)	12.97	10.34
5th instar		
Total feeding time (min)	62.83	56.33
Meal Frequency	3.56	3.06
Median meal duration (min)	20.31	18.19
First meal duration (min)	35.44	24.76
Number of nibbles taken	0.81	0.71
Intermeal frequency	3.67	3.20
Median intermeal duration (min)	15.67	20.10

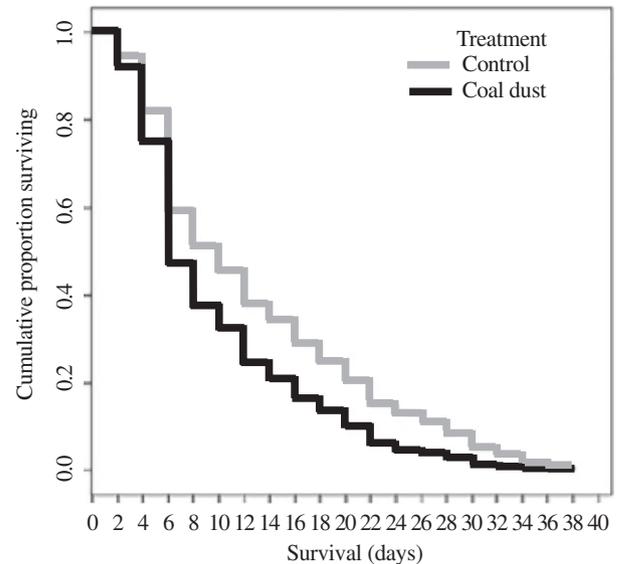
Note. There were no statistical differences between treatments for all feeding behaviour metrics.

## 4 | DISCUSSION

We found that consuming foliar coal dust increased the mortality of late-instar *H. armigera* larvae. We did not detect an effect of foliar coal dust consumption on neonate mortality, nor on larval development between the third and sixth instar. When neonate *H. armigera* were given a choice, they avoided coal dust. Late-instar larvae did not adjust their feeding behaviour or the amount of leaf material consumed in response to foliar coal dust despite long-term implications for survival.

### 4.1 | Increased mortality in late-instar larvae feeding on foliar coal dust

Potential mechanisms for the elevated mortality of late-instar larvae may include the accumulation of trace heavy metals in larvae by cumulative consumption (Sun et al., 2007), desiccation or cuticle



**FIGURE 2** Number of days survived by *Helicoverpa armigera* larvae ( $N = 150$ ). All but one larvae died before pupation

damage as occurs with inert Diatomaceous Earth (Korunić, 2013), or the cost of mandibular wear from consuming abrasive surfaces (Ennis, Mader, Burnside, Bauce, & Despland, 2015; Kyi, Zalucki, & Titmarsh, 1991). High larval mortality in the first 10 days of the experiment was likely due to (a) high background mortality of second instars that are at a more vulnerable life stage and (b) the shock of transferring from undefended and nutritionally balanced laboratory diet to relatively low-quality leaf material and (c) intraspecific competition between neonates grouped in survival tests. Subsequently, the lack of an effect on development to the 6th instar may be because there was insufficient time for toxins to accumulate, or mandibles to wear down.

High larval mortality is consistent with prior studies on lepidopteran life histories (Massey & Hartley, 2009). It is likely that high background mortality masked any potential effects of coal dust. Neonates can become trapped in the glandular trichomes of tomato leaves, increasing their mortality risk (Kyi et al., 1991; Zalucki, Clarke, & Malcolm, 2002). Considering neonates were in groups of ten within a confined space, cannibalism and intraspecific competition could also have contributed to high mortality (Bentivenha et al., 2016). In a study testing inter and intraspecific competition between Heliiothinae species, 20% of 2nd instar *H. armigera* survived when reared together on maize in confined laboratory tubs compared with 30% when on maize ears in the field with unlimited dispersal potential (Bentivenha et al., 2016). Contrastingly, cannibalism is thought not to be a major issue in *H. armigera* laboratory rearing until they reach the 3rd instar (Kakimoto, Fujisaki, & Miyatake, 2003). We observed and removed the dead bodies of larvae in survival experiments, suggesting that cannibalism was also not a major reason for high fecundities in our experiment. As 99% of neonates died within 1 week of consuming leaf material, it is also possible that toxins had insufficient time to accumulate to toxic doses.

## 4.2 | No changes to feeding behaviour of late-instar larvae despite survival cost

The cost of feeding on coal-laden leaves did not translate to feeding deterrence or compensatory consumption of late-instar larvae. When consuming road dust, an insect herbivore, *Uraba lugens* had lowered daily performance, but compensated by consuming more over time to equalize performance with controls over the instar (Leonard, 2013). It is possible that *H. armigera* may not alter their daily consumption, but would adjust feeding patterns over longer time periods on the same material. It is also possible that late-instar larvae were simply indifferent to the quality of leaf material when making feeding decisions. If given a choice between clean and coal-dusted resources, larvae may have chosen to feed differently.

No differences in the number of nibbles larvae took demonstrate that the progression from tasting to feeding was unchanged by the presence of foliar coal dust. No difference in the duration of the first meal supplements this finding to suggest that larvae made no gustatory distinction between treatments. Furthermore, no difference in meal duration or rests between meals suggests that larvae did not require additional time to process toxins in coal-dusted material (Bernays, Singer, & Rodrigues, 2004; Simpson, 1995). The trace metals present may have been in insufficient concentrations to detect. For example, lepidopteran larvae are only deterred from  $\text{CuSO}_4$  at relatively high concentrations (0.2M for *Mamestra brassicae* and *Mamestra oleracea*, Elbassiouny, 1991). The potential for dose dependent effects of coal dust is an important consideration for future studies.

The high baseline toxicity of secondary metabolites present in solanaceous plants may have masked any effects of coal dust toxicity on feeding behaviour in the short term (Liu, Li, Gong, & Wu, 2004). However, it is possible that an additive or interactive effect of coal dust and noxious plant secondary compounds consumed over time contributed to lower survival in coal dust treatments as well as ubiquitous mortality before pupation across treatments. As we used excised leaves, induced defences may also have reduced leaf quality and increased toxicity more than in experiments on whole plants (Green & Ryan, 1972). Analysing the nutrient, phenolic and tannin content between control and coal leaves would be useful to test whether coal dust altered foliar chemistry. Additionally, assessing the effects of coal dust to herbivorous insects on different host plants or as a constituent of laboratory diet may address the issue of ubiquitous mortality in this experiment.

The disjoint between fitness and optimal feeding decisions is not uncommon with respect to dose-dependent toxins. Velvet bean caterpillars will consume caffeine despite the cost on their survival (Slansky & Wheeler, 1992). Fourth instar larvae of *S. exigua* even preferred plant material containing lethal doses of selenium to controls (Vickerman, Young, & Trumble, 2002). We did not measure ingestion or digestion in this experiment. Determining whether digestion efficiency is affected by coal dust consumption may bridge the gap between the lack of change in consumptive behaviours with the long-term impact on larval survival.

## 4.3 | Neonates avoided coal dust

Neonate *H. armigera* avoided establishing themselves on the adaxial surface of coal-dusted leaves. As leaves were cumulatively deposited with coal dust on the adaxial surface, this demonstrates an avoidance of coal dust. Behavioural avoidance of toxins also occurs in other Lepidopteran larvae. For example, first instar *H. zea* disperse from leaves treated with the pesticide *B. thuringiensis* (Ashfaq et al., 2001; Jyoti et al., 1996). As in our study, *H. zea* avoided the adaxial surfaces sprayed with *B. thuringiensis* pesticide (Ashfaq et al., 2001; Jyoti et al., 1996). Furthermore, the tendency of neonate *H. armigera* to settle on the abaxial leaf surface may also be as a mechanism of concealment from predators to avoid structural defences such as glandular trichomes which concentrate on the adaxial surface (Jyoti, Young, Johnson, & McNew, 1999), and to facilitate ease of penetration to edible material (Simmons, Gurr, McGrath, Martin, & Nicol, 2004). For example, first instar *Helicoverpa virescens* fed exclusively on the lower epidermis without penetrating the upper leaf surface (Parrott, Jenkins, & McCarty, 1983). After 24 hrs, however, the overall effect of leaf surface disappeared and neonates only avoided the abaxial surface of coal-dusted leaves compared with controls. These results suggest that particulate deposition may be a more important predictor of larval settling than positioning on the leaf in coal mining areas. If reproductive parts and leaves accumulate dust differently, this avoidance of coal-dusted sites may influence intraplant dispersal, and consequently plant damage. Similar findings of deterrence at the local scale to pesticides have been used to persuade larval movements to less economically valuable sites of the plant (Parrott et al., 1983). Assessing whether particulate matter accumulates differently to different sites of host plants is needed to determine whether herbivores are likely to avoid certain plant parts in nature. Unlike some lepidopteran larvae which can balloon to different plants (e.g., *Chilo suppressalis*), dispersal of neonate *H. armigera* are largely constrained by where eggs are laid in the field (Dirie, Cohen, & Gould, 2000). Oviposition preferences of adult females are more likely to influence neonate dispersal at greater scales. Further investigation into host plant preference for adult females is required to determine whether coal dust may influence egg-laying behaviours and host plant choice in insect herbivores.

## 4.4 | Comparing early and late instars

As early and late instars are ecologically distinct, feeding behaviours and consumption patterns may be different in first and late-instar larvae (Hochuli, 2001). For example, lepidopteran *S. exigua* were deterred from feeding on organic selenium compounds as neonates, whereas third instars did not alter their behaviour to this toxic heavy metal (Vickerman & Trumble, 1999). Feeding bout continuation is also more strongly influenced by secondary metabolites and trichome density in younger rather than older instars of *H. zea* (Jyoti et al., 1999). Given that neonates show a distinct feeding site

establishment preference for cleaner leaves when given a choice, assessing whether earlier instars change feeding behaviours in response to coal dust is worthy of investigation.

## 5 | CONCLUSIONS

Insect herbivores experienced mixed behavioural responses to foliar coal dust, and negative fitness outcomes when reared on foliage laden with particulate matter. The decreased survival on a life stage of a herbivorous insect suggests that concurrent species in natural and agro-ecosystems bordered by coal mines may also be at risk. Further studies should include a range of invertebrates with varying feeding strategies as test organisms. As herbivorous insects tend to have faster reproductive rates than their predators and parasitoids, even short-term population decreases could disrupt tritrophic interactions (Agrawal, 2000). If toxin accumulation was indeed a major factor contributing to lowered survival, the potential for bioaccumulation to higher trophic levels could disproportionately affect predators feeding on foliar dust consuming insects (Cheruiyot, Boyd, Coudron, & Cobine, 2013). Understanding how the structural and chemical attributes of coal dust affect insect survival, and by what mechanism, is critical to determining the broader ecological implications of these results.

This study demonstrates that coal dust presents ecological implications of coalmines on natural and cultivated ecosystems.

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## AUTHORS' CONTRIBUTIONS

AMV, TL, RJL and DFH conceived the project. AMV collected the data. AMV, TL and DFH conducted analyses. AMV, TL, RJL and DFH wrote the manuscript. All authors gave final approval for publication.

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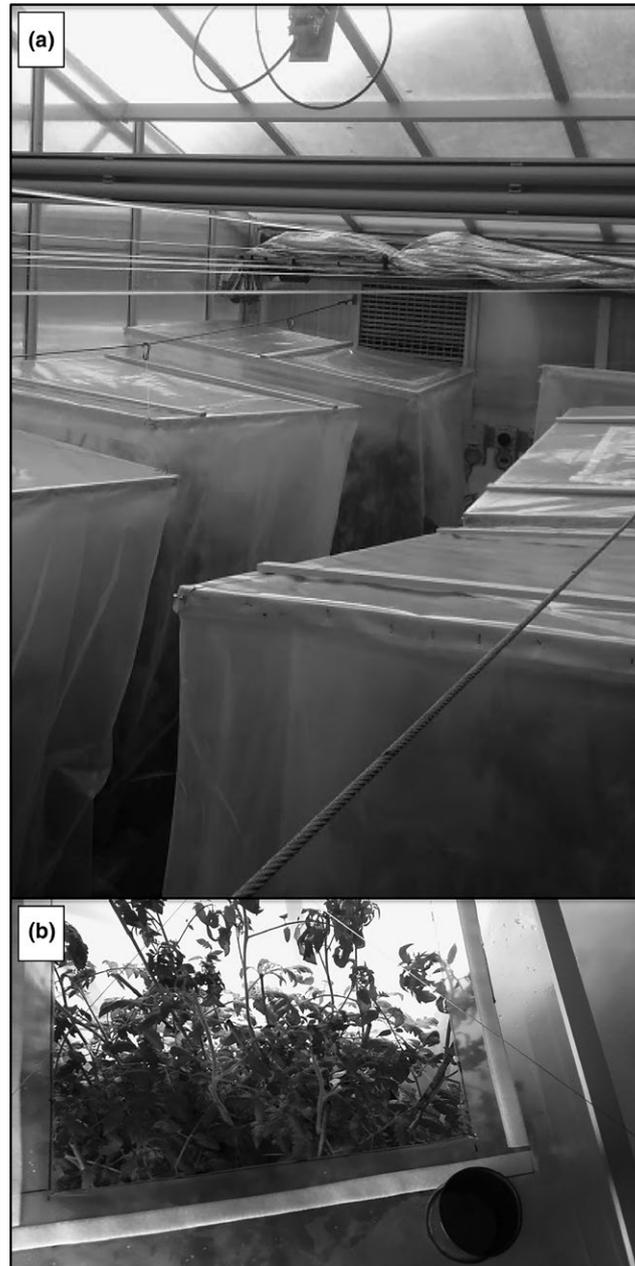
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## APPENDIX 1



**FIGURE A1** Coal dust excluders: (a) View of eight coal-dust excluders arranged in greenhouse from above. Four contained control plants and four were sieved with coal dust weekly. (b) Sealable access flap from which coal dust was sieved to plants. Also, pictures metal sieve (9 cm diameter, 75  $\mu\text{m}$  mesh size) used in coal dusting