Strong impacts of smoke polluted air demonstrated on the flight behaviour of the painted lady butterfly (Vanessa cardui L.)

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Abstract. 1. A major component of biomass burning smoke is fine particulate matter (PM2.5), which has been shown to generate impacts on insect population dynamics and development. However, little is known about its effect on insect flight behaviour, even though this will influence insect dispersal and distribution, and potentially migration and ecosystem services such as pollination.

2. Here we use a tethered flight mill setup to examine the behaviour of adult painted lady butterflies (Vanessa cardui L.) flying in different levels of combustion-generated airborne PM2.5, comparison this to TFM flying under ‘clean air’ conditions.

3. Descriptive statistics and paired sample t-tests indicate that the smoke had a significantly deleterious impact on flight behaviour, with for example total flight distance covered declining by 65% during the first 20 min of flying in the least smoke contaminated air compared to ‘clean air’ control conditions, whilst average speed declined by 54% and flight duration by 32%. A strongly negative and highly significant linear correlation between flight speed and PM2.5 concentration was also observed.

4. This study represents the first time that smoke effects on insect flight behaviour have been experimentally tested, and the longer the butterflies were exposed to the elevated PM2.5 concentrations the more obviously their flight behaviour declined. We conclude that the month(s)-long episodes of air pollution often associated with agricultural burning and deforestation fires in the tropics may well be significantly affecting the behaviour of the flying insects living in those regions and/or who migrate through them.

Key words. smoke pollution, fires, Vanessa cardui L., flight behaviour, tethered flight mill, insect.

Introduction

Landscape fires burn across millions of square km of Earth’s landscapes annually (Giglio et al., 2010), and human-driven fires associated with land clearance and agricultural management are particularly prevalent as a seasonal occurrence across many developing nations of Asia, Africa, and Latin America (Korontzi et al., 2006; Yadav & Devi, 2018). Agricultural residue burning is a seasonal practice in many of these nations for example, burning wheat stubble, rice straw, and other vegetative waste before and/or after harvest (Scholes et al., 1996; Streets et al., 2003; Toledo et al., 2005; Gadde et al., 2009; Mahmud, 2013; Jain et al., 2014; Zhang et al., 2016). Though each of these residue fires maybe individually small (Ranson et al., 2012; Zhang et al., 2017), the very large numbers of fires burning simultaneously can seriously degrade local and regional air quality (Li et al., 2014; Liu et al., 2020) by releasing a complex mixture of gases and aerosols (Li et al., 2007; Gadde et al., 2009; Shi et al., 2014; Zhang et al., 2015). At these times, atmospheric concentrations of fine particulate matter (PM2.5) can sometimes exceed 1 mg m⁻³ in heavily affected areas of China for example (Zhang et al., 2017), and elsewhere in...
Asia extremes even exceeding 3 mg m\(^{-3}\) have been seen during very large fire events associated with large scale land clearance and drought (Wooster et al., 2018). In addition to the significant effects on human health – particularly from the fine particulate matter (Johnston, 2017) – animals are very likely also affected by this air pollution. This includes insects, which have important ecological functions, facilitating plant pollination (Chapman et al., 2010; Ollerton, 2017), seed dispersal (Willson & Traveset, 2000), and soil ventilation (Wardlaw et al., 1998), for example, as well as roles in maintaining important trophic relationships (Belovsky & Slade, 2000). However, whilst a few studies have explored the impact of smoke pollution on insect development (Tan et al., 2018; Wang et al., 2017), there have been no quantitative studies of how it might affect insect flight behaviour. In this context we have designed and performed a series of laboratory experiments to investigate this issue for the first time, focusing on Vanessa cardui, the painted lady butterfly, which is an important indicator species because of their sensitivity to ecosystem conditions (Griffis et al., 2001).

The smoke released from agricultural residue fires and other types of landscape burning includes trace gases such as CO\(_2\), CO, NH\(_3\), CH\(_4\), SO\(_2\), NO\(_x\) (Radojevic, 2003; Ding et al., 2013; Zhang et al., 2015), but it is the fine PM\(_{2.5}\) particles of black carbon (BC) and organic carbon (OC) that pose the most serious risk to air quality (Li et al., 2007; Cao et al., 2008; Zhang et al., 2017). Such fine particles dominate the aerosols present in vegetation fire smoke (Dennis et al., 2002; Zhang et al., 2016; Ni et al., 2017), and when emitted in large quantities by very large and/or long-lived fires (Wooster et al., 2018) or by huge numbers of smaller burns (Zhang et al., 2017), they can dramatically increase the extent and severity of regional haze and smog episodes (Othman et al., 2014; Koplitz et al., 2016; Chen et al., 2017). In humans these fine particles can enter the respiratory system in sufficient numbers to cause serious morbidity and even mortality (Chen & Kan, 2008; Li et al., 2013; Koplitz et al., 2016), with consequential economic impacts on health-care (Othman et al., 2014) and tourism (Anaman & Looi, 2000). However, few studies have examined the consequences of such pollution for other animal species. A few have indicated effects on insect development and thus population dynamics (Ginevan & Lane, 1978; Alstad et al., 1982; Führer, 1985), including for example impairing the development of insect larvae, such as was shown by Tan et al.’s (2018) observations of smoke haze prolonging development time and decreasing pupal weight of Bicyclus anynana (squinting bush brown butterfly). Even the smoke caused the decline of five butterfly species (Lepidoptera: Rhopalocera) in Epping forest (Corke, 1999).

However, the impact of smoke on insect flight behaviour has rarely been considered, despite flight performance largely determining dispersal capacity, which then profoundly influences metapopulation dynamics and ultimately population viability, species persistence, gene flow, and processes of natural selection (Bowler & Benton, 2005; Lester et al., 2007; Berg et al., 2010; Danthnarayana, 1986). Certain of the seasonal biomass burning patterns in Asia (Vadrevu et al., 1986) coincide with the period in which many insects start to migrate (Huang et al., 2012; Jones et al., 2016; Fang et al., 2019), and this is likely to be the case in other continents as well. Increasing our understanding of insect flight performance in smoke-contaminated air may ultimately help elucidate whether the air pollution associated with these fires might affect insect migration, and all the consequent impacts that stem from any such effect.

Butterflies are a predominant insect group present in agricultural lands and are known to be sensitive to environmental changes (Hill et al., 1995; Cleary & Grill, 2004). Cleary & Grill (2004) showed that the richness of Jamides celeno (Common cerulean) has increased over 50% in response to El Niño Southern Oscillation induced fires events as an example and in Indonesia, where vegetation fire smoke contaminated air is an annual occurrence (Wooster et al., 2018), the richness and biodiversity of butterflies are linked to human activities such as forest logging and fires, presumably by directly destroying their habitat (Hill et al., 1995; Cleary & Grill, 2004). The butterflies used herein, therefore, represent a good organism with which to start to explore the impact of smoke pollution on flying insects.

Materials and methods

Study species

Adult (imago) V. cardui were chosen as the experimental subject for this work. V. cardui has a wide global distribution and is found in temperate areas and tropical areas across all continents apart from South America and Antarctica (Ecuador, 1992; Stefanescu et al., 2017; Talavera & Vila, 2017). V. cardui generally maintains a large population through multi successive generations every year (Stefanescu et al., 2013; Talavera & Vila, 2017). V. cardui have excellent flight capacities, reflected in their annual mass migration between Africa and Europe (Talavera et al., 2018; Menchetti et al., 2019; Suchan et al., 2019). Normally, they migrate from Europe to the Afrotropics in autumn and also evidence proved that they had a reverse northwards trans-Saharan migration in Spring (Talavera & Vila, 2017; Talavera et al., 2018). Adults arriving in Britain in May and June are thought to arrive directly from North Africa (Asher et al., 2001; Nesbitt et al., 2009). Massive breeding starts immediately after the arrival of migrants. The offspring appear in the UK in the late summer, such that the population greatly increases at this time (Stefanescu et al., 2013, 2016). The flight capacities of V. cardui depend in part on wing power, with wing size being considered a significant factor on flight performance (Rayner, 1979; Elington, 1984; Steyn et al., 2016). The wingspan of the male is normally 58–70 mm, and that of the female 62–74 mm (Uk butterflies.co.uk, 2020). Adult V. cardui live for 3–4 weeks, often up to 5 (Talavera & Vila, 2017; Stefanescu et al., 2020), providing enough time for experimental treatments such as those detailed here to be completed while the butterflies are in robust health.

To standardise the flight ability of the selected V. cardui subjects as far as possible in the current study, individual differences in V. cardui individuals such as age, wingspan, and body size were minimised by obtaining 20 butterfly pupae of the same age from Gribblybugs LLP, a UK entomological supplier. Pupae were placed in a greenhouse and adults of mixed sexes emerged from the pupae over a one-week period.
Smoke effects on butterfly flight behaviour

Experimental setup

Fig. 1. Experimental setup of (a) the flight chamber and (b) the combustion chamber. The two chambers both contain artificial lighting, a temperature and humidity logger, and four tethered flight mills (TFMs) each of which had a single butterfly subject attached. Different experimental smoke treatments were created in the combustion chamber by burning incense sticks after an initial pre-treatment flight period of 10 min, whilst clean-air ‘control’ conditions were always maintained in the flight chamber. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 2. Tethered flight mill details. (a) An individual TFM (adapted from Jones et al., 2016; Patent: Lim et al., 2013), four of which were used in each chamber as depicted in Fig. 1a,b. (b) Photograph of the TFM configuration used in each chamber, showing four TFMs with a butterfly attached to each. [Colour figure can be viewed at wileyonlinelibrary.com].

Experimental overview

Two test chambers having almost identical ambient environments were used as the location for the experiment, both were located at Rothamsted Research in Southern England and sited within 10 m of one another. The ‘flight chamber’ (Fig. 1a) was always maintained with a neutral ambient ‘clean-air’ environment (PM$_{2.5} < 0.015$ mg m$^{-3}$), whilst the nearby ‘combustion chamber’ (Fig. 1b) had a series of smoke treatments applied by burning unscented incense sticks to increase its PM$_{2.5}$ atmospheric concentration. The butterfly subjects were attached to flight-recording tethered flight mill (TFM) systems (Fig. 2a) in both the flight chamber and the combustion chamber, and in the former always flew in clean air conditions whilst in the latter the flew in clean air conditions for a period prior to the incense stick ignition time, and after this in ‘smoke polluted’ conditions. Three different treatments were used in the combustion chamber, classified as Low, Medium, and High Smoke (LS; MS; HS) based on the number of incense sticks burned simultaneously to create the polluted air conditions (see Table 1). A laser-based particulate matter measurement device (TSI DustTrak II Desktop Aerosol Monitor 8530) was used to record the PM$_{2.5}$ concentration timeseries in the combustion chamber throughout each experiment. The instrument provides PM$_{2.5}$ concentration on the basis of laser backscattering, and is factory calibrated using Arizona road dust. We applied the adjustment factor of 0.61 from McNamara et al. (2011) to deliver concentrations of smoke PM$_{2.5}$ which have a lower density than road dust. Wooster et al. (2018) provides details of a similar

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Table 1. PM$_{2.5}$ concentration statistics of the three different smoke treatments created in the combustion chamber. Ignition of the incense sticks occurred at time $T_1$, 10 min after the butterflies were placed on the TFMs at time $T_0$. Total time-integrated PM$_{2.5}$ concentration and mean PM$_{2.5}$ concentration are given, both for the period from $T_1$ up to 10 min after incense stick ignition (i.e. $T_1 + 10$ min) and also up to 20 min after ignition ($T_1 + 20$ min).

| Smoke Treatment | Replicates | (T$_1$ + 10 min) Integrated Total (mg m$^{-3}$ s) | Mean (mg m$^{-3}$) | (T$_1$ + 20 min) Integrated Total (mg m$^{-3}$ s) | Mean (mg m$^{-3}$) |
|-----------------|------------|-----------------------------------------------|---------------------|-----------------------------------------------|---------------------|
| Low smoke (LS) | 3          | 54                                            | 0.15                | 208                                            | 0.18                |
| Medium smoke (MS) | 3          | 231                                            | 0.38                | 723                                            | 0.61                |
| High smoke (HS) | 3          | 449                                            | 0.75                | 1544                                           | 1.28                |

Fig. 3. Atmospheric conditions in the flight chamber and combustion chamber, measured across all experiments for (a) air temperature and (b) relative humidity. The higher and lower bars of the plots are the maximum and minimum values respectively, while the rectangle illustrates the first quartile, the median, and the third quartile (bottom to top). The red plus is the mean. [Colour figure can be viewed at wileyonlinelibrary.com].

calibration adjustment for smoke particulates recorded by this sensor. Each experimental treatment involved four butterflies obtained from a total pool of 20 and flown in the combustion chamber, as did the control treatment based in the flight chamber (Fig. 2b). Whilst the PM$_{2.5}$ concentration of the flight chamber was strongly influenced by the smoke produced by the burning incense sticks, the other atmospheric characteristics of the chambers (air temperature and relative humidity) were statistically similar throughout the experiments (Fig. 3). All experiments were conducted under the artificial lights, with the lighting homogenous in the different mills within the two chambers.

Using the setup described above we investigated the following: (i) whether and for how long butterflies keep a constantly stable flight behaviour when flying under ‘clean air’ conditions; (ii) whether smoke causes a significant change in this flight behaviour; and (iii) how any change is affected by increasing smoke PM$_{2.5}$ concentration.

Details of the tethered flight mill technique

The TFM (Fig. 2) is quite widely used to measure insect flight speed and duration across a wide range of insect body sizes and types (Jones et al., 2016; Dällenbach et al., 2018; Minter et al., 2018). A lightweight arm, secured and balanced using a counterweight, is suspended between upper and lower magnets, and an axis between the two magnets allows even an insect with relatively limited flight power to turn the mill successfully when attached to the mill arm. When the insect flies, the mill arm turns in a circular trajectory with a circumference of 50 cm, and a black and white striped disk attached to the axis rotates with the arm. A light detector detects the movements of the banded pattern on the disk, using this to record the flight distance to a precision of 10 cm at a 5-s temporal resolution. In a 5-s period, the V. cardui studied herein covered an averaged flight distance of 300 cm when attached to the TFM system. Four TFMs were used in the flight chamber and four in the combustion chamber, as shown in Fig. 1.

Subject preparation

Several preparatory steps were necessary prior to placing the butterflies on the TFM systems in both chambers. Special pins were constructed to connect the subjects to the TFMs, each made by bending a small length (around 3 cm) of steel wire into a small loop to which the butterflies could be firmly glued (Nesbit
et al., 2009). Because of the fragility of the butterfly wings, all subjects were kept chilled and in a torpid state in refrigeration units prior to this procedure, minimising the risk of damaging their wings and ensuring they were not too active too soon. Each butterfly in its chilled state was placed on a sponge mat and secured with a net and two small weights to avoid it being damaged or escaping during attachment of the pin. The scales on the surface of the butterfly thorax were cleared to make sure the pin could be firmly glued to the butterfly. The pin facilitates weighing, feeding, and minimizes stress to the butterfly during preparation for flight. Before placing the butterflies on the TFM system, they were fed with a mixture of cool water and honey in the ratio of 9:1 by weight by letting them drink from pieces of paper tissue dipped in the liquid. Each butterfly was then mounted on its TFM system at an appropriate angle to ensure normal flight.

Smoke treatments

The unscented incense sticks used to create the smoke within the combustion chamber consisted of a wooden base with incense compounds attached at one end (Jetter et al., 2002). They were considered a suitable medium for generating the smoke for this experiment since they can sustain combustion for around 1 h and the smoke released is similar in composition to that from standard biomass burning, including gases (CO and volatile organic compounds, as well as aldehydes and polycyclic aromatic hydrocarbons (PAHs) (Jetter et al., 2002; Lee & Wang, 2004; Lin et al., 2008; Shi et al., 2014). The incense sticks were positioned close to the ground in one corner of the combustion chamber, well away from the butterflies to avoid any direct increase in their temperature. The three experimental treatments of LS, MS, and HS (Table 1) were generated by burning different quantities of incense sticks simultaneously. The aerosol monitor was placed next to the TFM systems in the combustion chamber to record the airborne PM$_{2.5}$ concentration, and the temporal variation of PM$_{2.5}$ associated with the three different treatments are shown in Fig. 4. There were other substances being created during the smoke treatments, but they were not measured in the experiment. The PM$_{2.5}$ concentration were recorded to represent the smoke conditions during the whole experiment.

Experimental procedure

To conduct the experiments, the available pool of 20 butterflies was divided into five groups of four individuals, and two groups of four in good physical condition were chosen for each run of each experimental treatment. Each treatment was replicated three times to provide more robust statistics, using different butterflies each time. In each case, one group of four butterfly subjects was placed on the TFMs in the combustion chamber and immediately started flying (at time $T_0$), and this ‘experimental group’ flew for a pre-treatment period of 10 min in the clean-air condition before ignition of the incense sticks at $T_1$ and a total treatment period of around 1 h flying in smoke polluted air. Once the incense sticks were burned out after around 1 h, an extractor fan was activated to remove all the smoke in the combustion chamber and the butterflies were remove from the TFM system. Another ‘control group’ set of subjects were placed on the TFMs in the flight chamber and flown in permanently clean-air conditions for the same period, providing a comparison to those flown in the polluted conditions. The collected TFM data of distance flown every 5 s was processed using a script written in MATLAB (version R2019a) to obtain the different flight behaviour variables. These were total flight distance (m), average speed (m s$^{-1}$), maximum speed (m s$^{-1}$), and time spent flying (minutes), each calculated over different durations as detailed below. The means (with SD), median (with Interquartile Range, IQR), minimum and maximum values were also derived.

Because butterflies showed signs of fatigue after flying 30 min in the clean-air environment of the flight chamber (i.e. flew more slowly or even stopped flying completely; see Results section), time periods shorter than this were selected for calculation of the flight variables, such that we could isolate the influence of the smoke from that of fatigue. The two periods were (i) the initial 10 min of flying in the combustion chamber following ignition of the incense sticks ($T_0 + 10$) and (ii) the full 20 min of flying in the combustion chamber following ignition ($T_1 + 20$). Data for the control group flying in the flight chamber were selected for the same periods. We also compared flight data from the combustion chamber taken during the initial 10 min of flying in the clean-air conditions, and in the subsequent polluted conditions post-ignition.

Results

Data were analysed using a variety of statistical techniques, and prior to this the univariate normalities of all data were
confirmed using the Shapiro–Wilk test in the Statistical Package for the Social Sciences (SPSS), version 26, which determined that the data were normally distributed ($P \geq 0.091$). The following suites of analyses were then performed:

**Determining the analytical time period**

To determine the duration of the analysis periods, data from the ‘control group’ were first used to understand the length of time that *V. cardui* typically fly in a consistent way. For this we used data on total flight distance, because average flight speed is dependent on the total distance flown in a certain period, and maximum speed was found to be too random to be statistically representative. Total flight distance flown being 208 m in the 10 min of measurement at an average speed is dependent on the total distance flown in a certain period, and maximum speed was found to be too random to be statistically representative. Total flight distance data from the flight chamber was analysed in 10-min blocks using paired sample *t*-tests across a total of 40 min, so in the periods $T_0$ to $T_0 + 10$, $T_0 + 10$ to $T_0 + 20$, $T_0 + 20$ to $T_0 + 30$, and $T_0 + 30$ to $T_0 + 40$. Total flight distance covered in the different time periods were confirmed as normally distributed ($P > 0.80$) and the null hypothesis ($H_0$) was that no significant difference would be observed between each of the four different flight periods, whilst the alternative ($H_1$) was that a difference would be observed.

Results in Table 2 show that the total flight distance covered in the fourth 10 min period of flying in clean air ($T_0 + 30$ to $T_0 + 40$) was significantly lower than that in first 10 min period ($T_0$ to $T_0 + 10$) ($P = 0.049$, $n = 36$), but that between all other periods the total flight distance covered is statistically similar. This indicates that *V. cardui* can keep consistent flight behaviour for 30 min ($P \geq 0.296$), after which flight behaviour begins to change – presumably due to fatigue. This 30 min threshold was, therefore, used to determine our total experimental duration, meaning that we (i) subsequently compared flight data from the combustion chamber pre-treatment (pre-ignition) period ($T_0 + 10$) to the that from the treatment period ($T_1$ to $T_1 + 10$ and $T_1$ to $T_1 + 20$); and (ii) compared the control group in the flight chamber to that in the combustion chamber with no analysis of data from beyond 30 min of flying time.

**Comparisons of flying in the pre-treatment and treatment conditions**

The first determination of whether subject flight behaviour changed in response to smoke exposure was made by comparing the total flight distance covered by the experimental group flown for 10 min in the combustion chamber pre- and post-treatment. Specifically, for each different smoke treatment (LS, MS, and HS) we compared the flight variables for the pre-treatment period ($T_0$ to $T_0 + 10$ min) prior to incense stick ignition to those recorded for the same butterflies in the period ($T_1$, $T_1 + 10$ min) immediately after ignition. Paired sample *t*-tests were used and the null hypothesis ($H_0$) was that no significant difference would be observed between these two flight periods, whilst the alternative hypothesis ($H_1$) was that a difference would be observed.

Descriptive data of each of the flight behaviour variables for the pre-treatment and post-treatment periods are shown in Table 3. During the pre-treatment period, the subjects showed similar flight behaviour characteristics, with average distance flown being 208 m in the 10 min of measurement at an average speed of 0.34 m s$^{-1}$. However, flight behaviour changed in the 10 min that they flew in the smoke conditions, and some butterflies stopped flying on occasion, especially in the MS treatments (highlighted in Table 3). Total flight distance covered was 25% less in the 10 min of the MS smoke treatment than in the subsequent treatment period, and average speed 26% lower. Also, the maximum speed in the MS condition was lower than that in the pre-treatment period by 43%, though the average flight duration was similar. Paired sample *t*-tests comparing total flight distance in both pre-treatment and treatment periods identified significant differences between the two flight periods for the MS treatments ($P = 0.024$, $n = 12$); while no significant difference was found for either the LS treatment ($P = 0.936$, $n = 12$) nor the HS treatment ($P = 0.832$, $n = 12$).

**Flight comparisons between control and treatment conditions**

To further determine whether flight behaviour during smoke exposure was different to that seen under control conditions,
Table 3. Descriptive statistics of flight behaviour variables (total flight distance, average speed, maximum speed, and flight duration) for the pre-treatment flight period in the combustion chamber (T₀ to T₀ + 10 min; Vanessa cardui L. was put on TFM at T₀) and in the post-treatment period (T₁ to T₁ + 10 min; Ignition of the incense sticks occurred at T₁). Data shown here only come from subjects flown in the combustion chamber, with each flown in the pre-treatment condition for 10 min prior to ignition of the incense stick(s).

Table 4. Descriptive statistics of flight behaviour variables (total flight distance, average speed, maximum speed, and flight duration) between the pre-treatment and treatment conditions, the disadvantage was that flight periods since each subject had to be fed, rested and moved between the two chambers. However, the advantage was that flight periods of 20 min under clean air and polluted air conditions could be compared, and were twice as long as the 10 min possible with the flights conducted in the pre-treatment and treatment conditions (Table 3). The period up to 20 min after incense stick ignition

**Total flight distance covered** was compared between the treatment flight periods of the experimental group (T₃ to T₃ + 20 min) flying in the polluted air of the combustion chamber and the same butterflies flying in the clean air of the flight chamber.

Compared to the prior comparison of the butterflies flying in the pre-treatment and treatment conditions, the disadvantage here was that the control run and experimental run with the same butterfly could not be conducted immediately after each other (so the ambient temperature and humidity may have differed) since each subject had to be fed, rested and moved between the two chambers. However, the advantage was that flight periods of 20 min under clean air and polluted air conditions could be compared, and were twice as long as the 10 min possible with the flights conducted in the pre-treatment and treatment conditions (Table 3). The period up to 20 min after incense stick ignition also allowed the mean PM₃₅ concentrations to increase to higher values compared to just the first 10 min (Table 1).

The analysis was again performed using paired sample t-tests, and the null hypothesis (H₀) was that no significant difference would be observed between the two, whilst the alternative hypothesis (H₁) was that a difference would be observed.

Results (Table 4) show that the control group flew an average total flight distance of 833 m in the 20 min period, while in smoke treatments the average was far lower at 335 m. Total flight distance covered in the three smoke treatments (LS, MS, and HS) decreased by 65%, 58%, and 56%, respectively compared to that in the control treatments performed using the same butterflies, whilst the average speed declined by 54%, 58%, and 56%, respectively. Maximum speed was also lower than that in control treatments, by 43%, 42%, and 40%, respectively.
Fig. 5. Exemplar data from four butterflies flown under (a) control conditions in the flight chamber and (b) under the medium smoke (MS) treatment in the combustion chamber. Mean flight speeds from all four subjects are shown, and in (a) the flights under control conditions (T₀ to T₀ +30 min) in the flight chamber show no discernible trend, as do those in (b) collected under the pre-treatment conditions (T₀ to T₀ +10 min) in the combustion chamber. However also in (b) after the smoke treatment is applied 10 min into the flight time (via ignition of the incense sticks) the flight speed shows a steady reduction. Also shown in (b) is the mean PM₂.₅ atmospheric concentration recorded in the flight chamber by the TSI DustTrak II Desktop Aerosol Monitor. [Colour figure can be viewed at wileyonlinelibrary.com].

Relationships between flight speed and PM₂.₅ concentration

The previous sections have already shown flight distance to be clearly influenced by the presence of smoke, so ordinary least squares (OLS) linear regression was used to further investigate relationships between flight speed and PM₂.₅ concentration. Calculations were performed at the 5-s maximum resolution of the flight mill data, with the null hypothesis (H₀) being that no significant relationship would be observed, whilst the alternative hypothesis (H₁) was that a significant relationship would be observed.

The data were divided into 10 groups of increasing PM₂.₅ concentration, and flight speeds corresponding to each group were extracted and displayed using boxplots (Fig. 6a). The first boxplot in both Fig. 6a,b (PM₂.₅ concentration = 0) contains data from both the control runs in the flight chamber and the pre-treatment period (i.e. first 10 min flying) of the experimental groups in the combustion chamber. These PM₂.₅ concentration = 0 data highlight that under clean air conditions, maximum flight speed can reach 1.7 m s⁻¹. The remaining nine boxplots incorporate flight speed data collected under various smoke concentrations in the combustion chamber, and it can be seen that as PM₂.₅ concentration increased the median of the flight speeds first decreased slightly, then increased up to 1.6 mg m⁻³, after which the median flight speeds once again decreases somewhat. Maximum flight speed also continuously decreased as PM₂.₅ concentration increased.

For PM₂.₅ concentrations less than 1.2 mg m⁻³ (first four boxplots of Fig. 6a) the flight speeds have a larger dynamic range and more values in the outliers. However, at concentrations higher than 1.2 mg m⁻³ the boxplots show a smaller dynamic range and no outliers. Even though more flight speed data were obtained at low PM₂.₅ concentrations compared to high PM₂.₅ concentrations, the far narrower range of flight speeds seen as PM₂.₅ concentration increases (Fig. 6a) indicates that flight speed may be impacted by smoke concentration. Figure 6b shows the same data as in Fig. 6a, but excluding the zero flight speed values when the V. cardui subjects stopped flying altogether. These Fig. 6b data, therefore, represent the actual speed when the butterflies were in flight, rather than including the data when they were paused, though the trends are essentially the same as in Fig. 6a which included both types of behaviour.

Figure 7 shows the OLS linear best fit relationships between subject flight speed and airborne PM₂.₅ concentration, and as with Fig. 6 we show the data both with (Fig. 7a) and without (Fig. 7b) the zero (i.e. non-flying) values included. In Fig. 7a the data points trace out an ‘S’ curve, with a PM₂.₅ concentration of...
Smoke effects on butterfly flight behaviour

Fig. 6. Boxplots showing the flight speed of butterflies separated into 10 different PM$_{2.5}$ concentration classes. Data for the lowest PM$_{2.5}$ concentration class come from the pre-treatment period of the experimental group flown in the combustion chamber, and for the other (higher PM$_{2.5}$) classes from all smoke treatments applied in the combustion chamber up to 20 min after ignition of the incense sticks (T$_{1}$ + 20 min). (a) includes all data, whilst (b) excludes that from any period when a butterfly stopped flying completely. The higher and lower bars of the plots are the maximum and minimum values respectively, whilst the rectangle illustrates the first quartile and the third quartile (bottom to top). The median value of flight speed in each group are represented by the blue circle with centre of the box, whereas the red plus is the mean. Beyond these ranges, outliers are plotted as blue circles. [Colour figure can be viewed at wileyonlinelibrary.com].

Fig. 7. Scatterplots of mean flight speed against mean PM$_{2.5}$ concentration (mg m$^{-3}$) calculated (a) from all data as and (b) with the zero value flight speeds removed as was the case with Fig. 6b. Dashed lines represent the OLS linear best-fit to the data, the equation for which is shown along with the coefficient of variation ($r^2$). [Colour figure can be viewed at wileyonlinelibrary.com].

around 2 mg m$^{-3}$ seeming to be the approximate middle point, whilst in Fig. 7b this shape is damped, and the relationship appears more linear. Overall, both Fig. 7a,b (both $P < 0.001$) demonstrate a clear and strongly negative linear correlation between flight speed and PM$_{2.5}$, backing up the interpretation of the data shown in Fig. 6.

Discussion

Smoke from landscape fires is the greatest single source of airborne fine particulate matter (PM$_{2.5}$) in Earth’s atmosphere, and one that is particularly prevalent in many developing nations (Huang et al., 2014). Local atmospheric concentrations of PM$_{2.5}$ can sometimes exceed 1 mg m$^{-3}$ during intense agricultural burning seasons (Zhang et al., 2017), and even higher values have been seen to persist for weeks over some regions affected by extreme landscape fire events (Wooster et al., 2018). Previous studies have demonstrated that exposure to PM$_{2.5}$ can have a significant impact on insects. Tan et al. (2018) demonstrated that a PM$_{2.5}$ concentration of 0.05 mg m$^{-3}$ can restrict butterfly development, whilst Wang et al. (2017) showed that exposure to concentrations of 0.08 mg m$^{-3}$ shortened the lifespan of Drosophila melanogaster from 48 to 20 days. Here for...
the first time we have explored the effect of atmospheric PM$_{2.5}$ on insect flight behaviour, choosing V. cardui butterflies as our test subjects. The experimental treatments detailed herein exposed subjects to different levels of PM$_{2.5}$ smoke pollution from burning incense sticks, and over the first 10 min from the ignition point ($T_1 + 10$ min) concentrations were found to be an average of 0.15 mg m$^{-3}$ in the LS treatment, 0.38 mg m$^{-3}$ in the MS treatment, and 0.75 mg m$^{-3}$ in the HS treatment. In all cases, PM$_{2.5}$ concentrations increased further after the first 10 min of burning, and so for 20 min tests ($T_1 + 20$ min) concentrations were often even higher (averaging 0.18 mg m$^{-3}$ for LS, 0.61 mg m$^{-3}$ for MS and 1.28 mg m$^{-3}$ for HS). These concentrations are well within the levels found in the ambient atmosphere in areas affected by smoke from biomass burning. In Northern India for example, the post-monsoon agricultural burning season in the NW Indian States of Punjab and Haryana, added to local urban sources within Delhi, quite often results in levels of PM$_{2.5}$ frequently exceeding 0.3 mg m$^{-3}$ (Pant et al., 2015).

We used TFMs to fly V. cardui in smoke polluted air, and have shown this to be an effective way of quantifying flight behaviour. It is known that TFMs can limit natural flight behaviour somewhat by potentially hindering wing-flapping (Jones et al., 2016), including in butterflies such as V. cardui which have a 'clap-and-flapping' position of flight (Srygley & Thomas, 2002). The tethered and screened position of the butterfly in the flight mill, along with an absence of appropriate visual cues for take-off and flight, could for example have prolonged flight duration and delayed ‘landing’ (Gatehouse & Hackett, 1980; Jones et al., 2016). For these reasons, in addition to the smoke treatments that the subjects were exposed to during each experimental run, we also flew the same butterflies in clean air on the same TFM system and for the same 30 min time period to provide a control dataset for assessing flight performance effects of the smoke. We also flew each butterfly for an initial 10 min period in clean air prior to application of each smoke treatment, so as to have a pre-treatment performance period for comparison purposes.

Our data show a clear and significantly negative linear correlation between mean flight speed and PM$_{2.5}$ concentration, indicating that the higher the pollution level the lower the mean flight speed becomes (Fig. 7). However, the data in Fig. 7a also suggest a possible change in relationship at around 2 mg m$^{-3}$, and that when concentrations rise above this level the butterflies may react by reducing flight speed, possibly as an attempt to escape the polluted conditions. Higher mean PM$_{2.5}$ concentrations were also reached in each experimental smoke treatment after 20 min of smoke production compared to the first 10 min (Table 1), thus providing more contrast in the control versus treatment comparisons than in the pre-treatment versus treatment comparisons.

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Our results show that the flight variables of speed, duration, and distance were all significantly reduced when flying for 20 min under the smoke contaminated conditions compared to the clean air conditions of the control group, highlighting the deleterious effect that the smoke had on the flight performance. In terms of flight duration, for example, we found this to be shortened by 32% in the LS conditions, 15% in MS and 10% in HS conditions respectively during the first 20 min of flying compared to the control ('clean air') conditions. Some studies have suggested that certain insect species may become disoriented under smoky atmospheric conditions due to changes in the polarisation of sunlight (Hegedüs et al., 2007), however, the distance between the light source and the butterflies in our experiment was only a few meters and the smoke concentrations such that the effect of the particulates on the light was certainly not observable by eye. Thus, whilst in natural conditions smoke can darken the skies and change the polarisation field of incoming sunlight (Johnson et al., 2005; Hegedüs et al., 2007) we do not believe that changes in the light field were responsible for our findings. Rather we think the direct presence of the smoke is what is affecting the butterfly performance. Our data show that as smoke conditions worsened from MS to HS conditions, flight duration actually increased again, becoming closer to that under clean air conditions. One explanation is that the increasingly polluted environment stimulates an escape response, resulting in an increase in flight duration. It is not uncommon for insects to respond to a change in environmental conditions; for example, mosquitoes can use a flutter stroke to double the wingbeat frequency of normal flight, in order to remove drops of water on their wings before taking off (Dickerson & Hu, 2014). Although the butterflies may have increased their flight duration to ‘escape’ the smoke, their flight speed remained significantly lower than under the clean air control conditions. This effect could be related to the fine particulate matter attaching to their wings and causing a decrease in flight speed; in the same way that water can accumulate on mosquito wings and bend them out of shape, eventually preventing flight in foggy conditions (Dickerson & Hu, 2014; Dickerson et al., 2015).

When the 10 min flight data for the pre-treatment ('clean air') conditions were examined and compared to the first 10 min of the subsequent ‘smoke contaminated’ period, the flight distance and flight speed were both reduced, but this was only statistically significant in the MS conditions. The flight duration of butterflies under MS conditions was similar to that under the pre-treatment conditions, but the average flight speed reduced by 26%, resulting in a 25% shorter flight distance. We found, however, that the differences between butterfly flight behaviour pre-treatment and post-treatment, under both the LS and HS treatments, were relatively small compared to that under the MS treatment. They were also less significant than the differences found between the longer control and experimental treatment runs. This suggests that longer periods of smoke exposure are required to produce significant differences in flight behaviour under the lower and higher PM$_{2.5}$ concentrations. It may also be the case that impacts manifest themselves more obviously (or earlier) in the MS conditions, perhaps because whilst the MS conditions are detrimental to flight, the butterflies are still able to fly quite well and are thus, effectively trying to escape the polluted conditions. Higher mean PM$_{2.5}$ concentrations were also reached in each experimental smoke treatment after 20 min of smoke production compared to the first 10 min (Table 1), thus providing more contrast in the control versus treatment comparisons than in the pre-treatment versus treatment comparisons.

Our data show a clear and significantly negative linear correlation between mean flight speed and PM$_{2.5}$ concentration, indicating that the higher the pollution level the lower the mean flight speed becomes (Fig. 7). However, the data in Fig. 7a also suggest a possible change in relationship at around 2 mg m$^{-3}$, and that when concentrations rise above this level the butterflies may react by reducing flight speed, possibly as an attempt to seek escape or shelter from the polluted conditions. Once PM$_{2.5}$ concentrations reach approximately 3.4 mg m$^{-3}$, the mean flight speed increases again slightly with increasing PM$_{2.5}$ concentration. This may reflect a more ‘panicked’ attempt to move faster to escape the smoke conditions. These potential behavioural explanations are only hypotheses, and remain to be rigorously tested in future work.
It is also possible that beyond fine particulates, combustion gases may also influence the flight behaviour of *V. cardui*. Ginevan *et al.* (1980) found that *Lasiglossum zephrum* (Sweat bee) exposed to a polluted environment with SO₂ levels at 0.14–0.28 ppm for 16–29 days demonstrated reduced flight activity compared to a control population for example. Such poor air quality could cause insects to close their spiracle valves longer, decrease oxygen intake and reduce metabolism (Tan *et al.*, 2018). Toxic compounds may also influence butterfly flight performance by affecting the insects’ body functions. For instance, muscles of *Cecropia* moths are sensitive to CO₂ (and O₂) levels, with changes in concentration causing spiracle closures (Burkett & Schneiderman, 1974). Since the smouldering combustion style of the incense sticks used as the source was constant across the period of burning, CO₂ is released in direct proportion to PM₂.₅ (Zhang *et al.*, 2015), it is possible in our experiments that an increase in CO₂ concentration, and perhaps in some of the other gases compounds released by burning, could have induced longer closure of the insect spiracle valves and contributed to the decreased flight performance seen. This remains to be tested in future experiments taking into account more pollutant species, as does any effect of the particulates on the incoming light field even over the very short optical paths involved, but smoke (as assessed here via PM₂.₅) clearly has a negative impact on *V. cardui* flight behaviour, even at concentration ranges significantly lower than that found ‘naturally’ in some biomass burning affected regions. More research is required to show how the specific behavioural and physiological mechanics of butterfly flight respond to increasing concentrations of different smoke constituents.

The work presented herein is the first to experimentally quantify the impact of smoke pollution on butterfly flight performance, and highlights the deleterious impact on both the flight speed and flight duration of adult butterflies. Although the effect was demonstrated in a controlled experiment where insects were flown on a TFM, and was based on non-natural populations that can sometimes suffer inbreeding, it is an indication of the potentially harmful effect of smoke pollution on flight behaviour in the real world. It is a first step towards understanding the impact of smoke on natural flight performance, and ultimately on issues such as insect migration in regions affected by biomass burning smoke. A reduction in flight speed due to smoke pollution could have a substantial impact on the ability of butterflies to migrate successfully, as slower flying speeds will reduce the distance it is possible to fly in a single flight. This could have serious consequences if the insects must fly over large water bodies in such a flight for example, where a diminished flight capability during polluted conditions might mean insects being unable to make the necessary distance. Since smoke from fires can be released into the boundary layer or lofted high into the atmosphere (e.g. Paugam *et al.*, 2016), particulate matter concentrations can vary widely with altitude, meaning that insects could encounter them at different altitudes or even possibly make adjustments in their behaviour to avoid the highest concentrations. Further studies are required to determine if the fine particulates focused on here are those controlling all the impact on the insects flight behaviour during the polluted conditions, or whether some of the gaseous compounds released by burning might also have a detectable affect.

**Acknowledgements**

We extend thanks to Ceri Watkins and Rebecca Nesbit for training and advice on the TFM and on butterfly handling, James Johnson and Bruce Main for additional assistance with the experiment, and Su Yan for assistance with coding. Aspects of this work were supported by National Capability funding awarded by NERC to the National Centre for Earth Observation (NERC grant no. NE/R016518/1). Certain aspects of this research were supported by funding from the Leverhulme Centre for Wildlife, Environment, and Society through the Leverhulme Trust, grant number RC-2018-023. This work forms part of the outcome of the UKRI projects ST/S003029/1, ST/R00286X/1, and EP/P510804/1. The contribution from K.S.L (Rothamsted) forms part of the Smart Crop Protection (SCP) strategic programme (BBS/OS/CP/000001) funded through the Biotechnology and Biological Sciences Research Council’s Industrial Strategy Challenge Fund. The authors declare that they have no conflict of interest. The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author contributions**

M.J.W. and K.S.L. proposed the project and designed the plan. Y.L. and M.J.G. designed the experimental details. Y.L., M.J.W., and R.A.F. conducted the experiment. Y.L. analysed the data using scripts in part written by Y.L. All authors contributed to writing and revising the manuscript.

**Data availability statement**

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

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Accepted 6 September 2020
First published online 2 October 2020
Associate Editor: Simon Leather