british

TOMATO GROWERS' ASSOCIATION

FINAL REPORT

To:

AHDB Horticulture

Stoneleigh Park, Kenilworth

Warwickshire, CV8 2LT

Tomato: Phase 3 of an investigation into poor pollination performance by the native bumblebee, *Bombus terrestris audax*

16 March 2022

Rob Jacobson Consultancy Science into Practice

WARWICK
THE UNIVERSITY OF WARWICK

Project title:	Tomato: Phase 3 of an investigation into poor pollination performance by the native bumblebee, *Bombus terrestris audax*
Project number:	PE 031b
Project leader:	Mr Philip Pearson (TGA Technical Committee Chairman) British Tomato Growers' Association, Pollards Nursery, Lake Lane, Barnham, West Sussex, PO22 0AD
Report:	Final report, March 2022
Previous report:	Annual report, February 2021
Key staff:	Dr Rob Jacobson, RJC Ltd Dr David Chandler, Warwick University Mrs Gillian Prince, Warwick University Dr Ken Cockshull, Research Fellow, Warwick University
Location of project:	RJC Ltd, Bramham, West Yorkshire Warwick University, Wellesbourne, Warwickshire R & L Holt Ltd, Springhill Nurseries Ltd, Fladbury, Worcs.
Industry Representative:	Dr Philip Morley (TGA Technical Officer) British Tomato Growers' Association, Pollards Nursery, Lake Lane, Barnham, West Sussex, PO22 0AD
Date project commenced:	1 January 2019
Date project to be completed:	31 December 2021

DISCLAIMER

While the Agriculture and Horticulture Development Board seeks to ensure that the information contained within this document is accurate at the time of printing, no warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Dr Robert Jacobson

Director

RJC Ltd, 5 Milnthorpe Garth, Bramham, West Yorkshire, LS23 6TH

Signature ..... Date .....

Dr David Chandler,

Associate Professor

School of Life Sciences, Warwick University, Wellesbourne Campus, CV35 9EF

Signature ..... Date .....

Report authorised by:

Mr Philip Pearson

Chairman of the TGA Technical Committee

British Tomato Growers' Association, Pollards Nursery, Barnham, West Sussex, PO22 0AD

Signature ..... Date .....

Mr Paul Faulkner

TGA Treasurer

British Tomato Growers' Association, Pollards Nursery, Barnham, West Sussex, PO22 0AD

Signature ..... Date .....

CONTENTS

Grower Section

Science Section

GROWER SUMMARY

Headline

- *Bombus terrestris audax* colonies do not perform well in UK tomato crops and this has serious financial implications for growers.
- New evidence will be presented to policy makers with a request to revise the regulations introduced in 2015.
- High air temperatures by day affected production of pollen by tomato flowers in three cultivars. This could become more relevant as air temperatures rise due to climate change.

Background

British tomato growers had successfully pollinated their crops with two non-native species of bumblebees (*B. terrestris terrestris* [Btt] and *B. terrestris dalmatinus* [Btd]) for over 27 years when Natural England (NE) withdrew permission for their use in unscreened glasshouses. As a consequence, growers had to switch to the British native sub-species, *B. terrestris audax* [Bta]. The aim of this project is to understand why fruit set in commercially-important varieties of UK tomato has been problematic since growers made that switch to Bta.

British tomato growers are keen to use bumblebees sourced in the British Isles if this can be done without significant economic loss and they have instigated several studies since 2015 in an attempt to improve the situation. Key findings of particular importance to the present project are highlighted here. The full results of those studies can be found in AHDB's series of PE 031b reports and Knowledge Library Pages (Add Link).

The first such study was an AHDB-funded independent review of the scientific literature relevant to the effects of releasing non-native sub-species of bumblebees as pollinators in commercial crops. The resulting peer-reviewed paper (Add Link) concluded that there was insufficient reliable and consistent evidence to support claims that the use of Btt/Btd was harmful to wild populations of *B. terrestris* in the UK. Furthermore, the review reported the genetic structure of wild populations of *B. terrestris* in the UK to be complex with significant differences between populations from different parts of the British Isles. It also highlighted known hybridisation among European 'sub-species'.

In 2017, the Tomato Growers' Association's Technical Committee (TGA TC) organised an in-depth survey of UK tomato growers to gather more precise information about the use of Bta up to that time. Growers representing 98% of the UK production area participated in the survey. In summary, most growers believed Bta to be less vigorous than the non-natives and more likely to fail to provide adequate pollination should any influencing factor be sub-optimal. Modern small-fruiting tomato cultivars (*eg* cv Piccolo) were most likely to suffer significant issues with fruit set, especially during hot weather, but the physiological reason was unknown. The survey was repeated in 2019. It identified a marginal improvement in growers' perception of the performance of Bta, which could have been due to improved breeding stock and/or improved in-crop management of Bta colonies. Nonetheless, growers still considered the performance of Bta to be substantially inferior to Btt/Btd.

There followed a series of short practical studies which made significant progress in subjects related to both the bumblebees and the plants. First, the team discovered that most Bta colonies went into decline soon after placement in tomato crops, which was in stark contrast to previous experience with Btt/Btd colonies. Second, a study of traffic from Bta hives strongly indicated that there was considerably less flight activity in glasshouse tomato crops than outdoors. However, more detailed work with Bta at that stage was confounded by large variations in the obvious morphology (most notably size) of adult bees and the performance of commercial colonies. The combined evidence from these studies and the literature review began to raise questions about the current classification of *B. terrestris* sub-species and the distinction between 'native' and 'non-native' *B. terrestris*. Molecular genetic studies were then instigated which focussed upon different haplotypes (*i.e.* genetic groups each with its own DNA sequence) rather than sub-species based on geographical origin.

In addition to the bumblebee work, preliminary studies investigated flower development and pollen production in cv Piccolo. This revealed that each flower was usually open on two successive days, although it usually released most of its pollen on the first day with peak pollen release usually occurring between 12:30h and 13:30h. This coincided with the time of peak bumblebee flight activity. The anthers of each cv Piccolo flower had the potential to produce many more pollen grains than were required to fertilise all the ovules in the same flower's ovary. In our experiment, the anther of each flower could produce at least 20,000 pollen grains while the fruit contained fewer than 120 seeds. At that stage, it was not known whether all the grains were viable or whether they would all be released by the actions of bumblebees. Methods of assessing pollen viability reported in the scientific literature did not prompt germination of pollen from modern tomato cultivars so new techniques had to be developed.

Work in 2020 was severely disrupted by restrictions imposed to prevent the spread of Tomato Brown Rugose Fruit Virus (ToBRFV) and the 'lockdown' of research facilities due to Covid-19. All large-scale trials were aborted but the team were able to complete some small-scale laboratory experiments which focused on developing and refining research techniques to be applied in subsequent studies. The present project extension began in January 2021 with studies divided into the following four subject areas.

Summary

Effect of temperature on tomato pollen production and viability

Earlier work had shown that cv Piccolo flowers produced more pollen grains in September than in August which may be due to differences in average temperature. Published data suggested that high temperature had its greatest effect between about 13 days and 7 days before anthesis with the most sensitive period occurring about 9 days before anthesis. The latter being when the pollen mother cells are forming pollen grains. Other factors that might contribute to the setting problems of cv Piccolo could be that this cultivar produces fewer pollen grains than other cultivars when the air temperature rises. In addition, it is possible that not all of the pollen grains that land on the stigma are able to germinate and allow the pollen nucleus to pass along the style to the ovary where the fertilisation of ovules occurs.

Two experiments were done in sequence, each in two fan-ventilated air conditioned glasshouses at Warwick Crop Centre. In each experiment, there were two temperature regimes. The main treatment (HDT) was a high day temperature of 32^{O}C maintained for 12 hours per day followed by a night temperature of 18^{O}C. This was compared to a control with day temperature maintained at 20^{O}C. This was an extreme temperature difference but considered necessary as a 'proof of concept'. If proven, then intermediate temperature regimes could be investigated later. Within each regime, responses in pollen production / viability were recorded in cv Piccolo and compared to cv Duelle (a baby plum tomato) and cv Milandro (a classic round tomato). All flowers were self-pollinated manually, using an 'electric bee', except on the day of, and the day before, sampling for assessment of pollen quantity / quality. The timings of these assessments were carefully planned to provide data from flowers that reached anthesis at critical times in relation to the HDT. Trusses on some plants of each cultivar from each treatment were grown on to produce fruits for seed counts.

The data from the start of the experiments, when flowers had not been exposed to the HDT, showed that all three cultivars produced many more viable pollen grains than were required to fertilise all the ovules in the ovaries of their flowers. No differences were recorded between the HDT and control temperature regimes at that stage. Similarly, there was little or no effect on flowers that reached anthesis on day 21. However, flowers that reached anthesis on day 9 showed a significant reduction in the numbers of seeds per fruit. This effect was amplified in the results from the flowers that reached anthesis on day 15, which showed a dramatic reduction in the number of seeds per fruit leading to the abscission of fruits in all three cultivars. The flowers sampled on day 9 and day 15 had both experienced the HDT between 12 and 7 days before anthesis. This result was consistent with previously published literature which stated that high day temperature could affect fruit set if it occurred 13 to 7 days before anthesis. The effect of the HDT on seed count appeared to be due to an effect on pollen production rather than an effect on pollen viability, or the combined pollen viability index, as pollen viability was relatively high whenever samples were taken.

Genetic structure of populations of B. terrestris.

The decision by NE in 2015 to severely restrict the use of Btt/Btd in the UK was driven by concerns about gynes (sexually reproducing males and females, which are produced at the end of the colony's normal lifespan) 'escaping' from the glasshouse and mating with native British bees to produce a genetic hybrid. However, this rested on the assumption that the *B. terrestris* populations that occur in Great Britain were genetically isolated from the *B. terrestris* bees that occupied mainland Europe.

The project management team felt it was important that we develop in-house molecular genetics methods for *B. terrestris*, with the longer-term aim of providing new data on the population structure of *B. terrestris* that would help decision making and policy development by NE. Studies were initiated to provide DNA sequence data for the *B. terrestris* mitochondrial cytochrome oxidase I (COI) gene, including the identification of nucleotide polymorphisms that can differentiate between different haplotypes of *B. terrestris.*

Our data has shown that there is greater sequence variation in COI than previously reported. While these are preliminary findings, they do suggest that bee 'sub-species' originating from Britain (labelled as Bta) and those from mainland Europe (Btt) do not consist of separate, 'pure' genetic entities, but rather as populations with some haplotypes in common.
This would support the idea that there is already some natural genetic mixing / interactions between populations in Britain and mainland Europe.

Remote monitoring of B. terrestris colonies.

Preliminary studies had indicated that the honeybee remote monitoring system (RMS) produced by 'Arnia Hive Monitors' could be adapted and recalibrated for use with the much smaller commercial bumblebee colonies which would have benefits as an experimental tool and for use in commercial crops. Arnia monitors were set up in a controlled environment room and data sent to Arnia for fine adjustment before the equipment was tested in a commercial tomato crop. However, we were unable to obtain consistent and reliable results from bumblebee colonies within this project.

During 2021, an additional type of bumblebee monitor, produced by Agrolabs, became available and was trialled in a commercial crop. The results with this equipment were more promising and we concluded that this system did have potential to provide high quality, useful data for monitoring bumblebee traffic. However, it would be useful to build the system with temperature and humidity probes so that environmental conditions within hives could be monitored to look for evidence of heat stress effects.

Effect of high temperature on within-hive activity of B. terrestris

Bombus terrestris, collectively thermoregulate their nests in response to cold and heat in order to maintain a relatively constant temperature for brood rearing. In hot conditions, they cool the nest by fanning, which helps maintain a stable temperature for brood rearing. In the wild, the nest temperature is kept within a narrow range independent of the ambient air temperature. For example, brood temperatures measured within a wild *B. terrestris* nest ranged from 31.3 to 33.4 °C despite ambient air temperatures varying from 13.2 to 34.4°C. At extreme ambient conditions, bumblebees spend more time on thermoregulation and less time on other activities such as brood maintenance and foraging. One possible explanation for the poor pollination performance observed with Bta in tomato crops during hot weather is that they are more likely to switch to thermoregulation activities within the hive.

As a first step, a thorough literature search was completed to ensure that we had all the published information on this subject and that information is summarised within the main report. We then ran a set of pilot studies and thereby developed a novel method of observing the effects of elevated temperatures on the behaviour of individual bumblebees. This method was subsequently used to compare the temperature responses of Bta and Btt/Btd.

Our conclusion at this stage is that poor pollination observed with Bta bees is unlikely to be caused by a marked difference in the thermal biology of Bta versus Btt for either (i) the temperature threshold for the fanning response and (ii) the upper lethal temperature for adult bees. We do have some evidence of differences in the proportion of bees that engage in fanning between Bta and Btt, but we would need to perform the same experiment on replicate hives to confirm this.

It is highly unlikely that temperatures within the glasshouse for tomato production will get high enough to kill adult bees. This is not to say, however, that high temperatures are not detrimental to bees within the glasshouse. Temperatures above 32°C are likely to result in bees staying within the hive to fan rather than foraging and could be detrimental to brood.

Financial Benefits

Benefits to the British Tomato Industry - TGA members initiated this series of projects to reduce financial losses resulting from production deficit, increased labour and excessive hive input caused by the enforced change to Bta for pollination of UK tomato crops. For example, one tomato grower estimated that poor fruit set cost his business £50k / hectare in 2015. An investment appraisal conducted as part of PE 031 demonstrated a potential payback from the cost of the project to be achieved from just one hectare of crop in one growing season. When extrapolated to the whole industry over a 5 year horizon, the potential cost-benefit of phases 1-3 of PE 031 is greater than 1:250. This project has greatly increased the industry's knowledge of the subject and has provided the basis of a case to request NE to reconsider their original decision.

Benefits to the wider scientific / horticultural communities – The project has provided data on flower development, pollen production / viability and bumblebee activity which will benefit not only the tomato sector but the principles and findings can be applied to other sectors growing in similar production systems, such as glasshouse grown soft fruit. The further refinement of remote hive monitoring systems is providing an invaluable research tool for pollinator studies and could also have the potential to be used by the industry as part of an increasingly digitised growing environment (*i.e.* 'Digital Twinning').

Benefits to UK population – There is now irrefutable evidence of rising temperatures due to climate change. This project will indirectly contribute to our general knowledge by indicating how those changes are likely to impact on native pollinators in outdoor habitats.

Action Points

- At the start of this project, it was not clear whether poor fruit set, which resulted when Bta was used to pollinate tomato crops during hot weather, was due to the effect of temperature on plants, bumblebees or an interaction between the two. The findings have greatly improved our understanding and provided evidence to present to policy makers:
- Regarding the plants:
    - The timing of peak pollen release coincides with peak Bta flight activity.
    - A high temperature event reduces pollen production in flowers that are forming pollen grains at that time. This results in poor fruit set from those flowers 7-13 days after the event regardless of the activity of bumblebees. Further studies are required to determine the precise temperature thresholds for different types / cultivars of tomato.
    - These results do not explain the difference in performance between Bta and Btt/Btd.
- Regarding the bumblebees:
    - Bta colonies go into decline soon after placement in tomato crops regardless of environmental conditions.
    - Bta flight activity is less in tomato crops than outdoors.
    - There is no evidence to date to show a marked difference between Bta and Btt/Btd in thermoregulation activities within the hive that would be more detrimental to colony development or to the upper lethal temperature for adult bees.
    - The combined results to date suggest that the tomato plant does not provide a suitable food source for the sustenance of Bta colonies.
- Molecular studies suggest that there is already natural genetic mixing of *B. terrestris* populations in Britain and mainland Europe which questions current classification.
    - Taxonomists should base classification of *B. terrestris* on genetic structure rather than geographical origin.
    - Policy makers should review their decision to restrict the commercial use of *B. terrestris* based on their geographical origin.
    - A meeting has been requested to present the new evidence to NE and Defra.
- The Agrolabs bumblebee remote monitoring system has potential to provide high quality, useful data for monitoring bumblebee traffic but would benefit from the addition of internal temperature and humidity probes to alert growers to adverse conditions.
- High air temperatures by day were shown to affect the production of pollen by tomato flowers in three widely-grown cultivars. Such a response could be a factor adversely affecting fruit set in some cultivars even under present conditions in summer but will evidently become more relevant as ambient air temperatures rise due to climate change.

SCIENCE SECTION

Introduction

The aim of this project is to understand why fruit set in commercially-important varieties of UK tomato has been problematic ever since it became necessary for growers to pollinate their crops using *Bombus terrestris* breeding stock sourced from wild populations in the British Isles.

The issue

For over 27 years, British tomato growers had successfully pollinated their crops with non-native populations of bumblebees when Natural England (NE) withdrew permission for their use in unscreened glasshouses. Those populations had been classified as the sub-species *B. terrestris* (Btt), from central Europe, and *B. terrestris dalmatinus* (Btd), from south east Europe. NE suggested that these non-native bumblebees could escape from glasshouses and hybridise with native populations (classified as sub-species, *B. terrestris audax* [Bta]) and thereby lead to the local extinction of Bta. Although the classification of Btt, Btd and Bta has since become controversial, we will provide continuity by using those abbreviations in this document to distinguish between the geographical origin of the populations.

As a consequence of the NE decision, in the 2015/16 growing season tomato growers had to switch to *B. terrestris* reared from populations collected from within the British Isles. However, the native bumblebees failed to provide the reliable and maintenance-free pollination experience to which the industry had become accustomed. This should not have been a surprise because at least one of the original producers, Brinkman Bunting Bumblebees BV, had tested *B. terrestris* collected from within the British Isles during the 1980s and dismissed them due to inferior performance both in their rearing facilities and in commercial glasshouses (Griffiths, pers. com., 1990).

Work completed prior to 2021

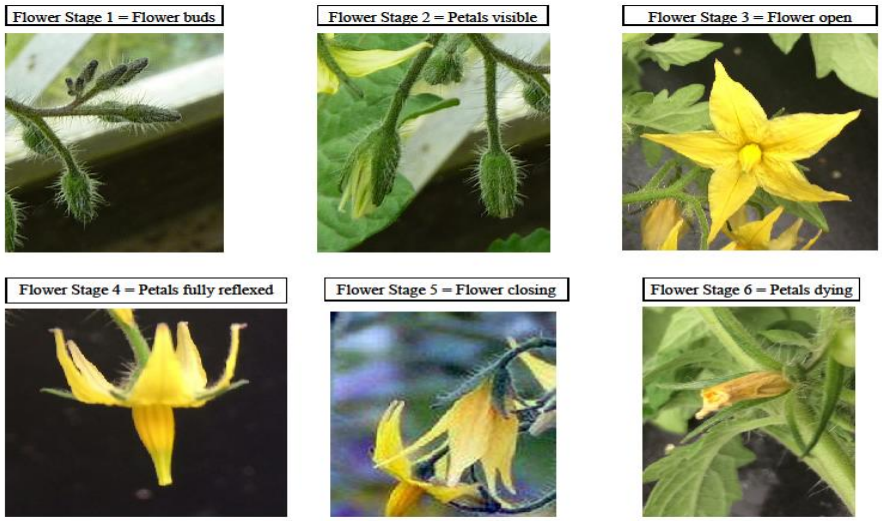
British tomato growers are keen to use bumblebees sourced in the British Isles if this can be done without significant economic loss and they have instigated several studies since 2015 in an attempt to improve the situation. The first was an AHDB-funded (PE 026) independent review of the scientific literature relevant to the effects of releasing non-native sub-species of

bumblebees as pollinators in commercial crops. The resulting peer-reviewed paper concluded that there was insufficient reliable and consistent evidence to support claims that the use of Btt/Btd was harmful to wild populations of *B. terrestris* in the UK (Chandler, *et al.,* 2019). Furthermore, the review reported the genetic structure of wild populations of *B. terrestris* in the UK to be complex with significant differences between populations from different parts of the British Isles. It also highlighted known hybridisation among European 'sub-species'.

In 2017, the Tomato Growers' Association's Technical Committee (TGA TC) organised an in-depth survey of UK tomato growers to gather more precise information about the use of Bta up to that time. Growers representing 98% of the UK production area participated in the survey (Jacobson, 2017). In summary, most growers believed Bta to be less vigorous than the non-natives and more likely to fail to provide adequate pollination should any influencing factor be sub-optimal. Modern small-fruiting tomato cultivars (*eg* cv Piccolo) were most likely to suffer significant issues with fruit set, especially during hot weather, but the physiological reason was unknown. The survey was repeated in 2019 (Jacobson *et al.*, 2020). It identified a marginal improvement in growers' perception of the performance of Bta, which could have been due to improved breeding stock and/or improved in-crop management of Bta colonies. Nonetheless, growers still considered the performance of Bta to be substantially inferior to Btt/Btd.

In 2018, the TGA TC organised a short (6 months) AHDB-funded (PE031) practical project to begin to investigate factors raised by tomato growers in three key subject areas (Jacobson *et al*., 2018*)*. First, the team discovered that most Bta colonies went into decline soon after placement in tomato crops, which was in stark contrast to previous experience with Btt/Btd colonies. Second, a study of traffic from Bta hives strongly indicated that there was considerably less flight activity in glasshouse tomato crops than outdoors. Finally, a preliminary study to investigate flower development and pollen production in cv Piccolo provided a foundation for more detailed experimentation (Figure 1). This work revealed that each flower was usually open on two successive days, although it usually released most of its pollen on the first day with peak pollen release usually occurring between 12:30h and 13:30h. The anthers of each cv Piccolo flower had the potential to produce many more pollen grains than were required to fertilise all the ovules in the same flower's ovary. In our experiment, the anther of each flower could produce at least 20,000 pollen grains while the fruit contained fewer than 120 seeds. However, it was not known at that stage whether all the grains were viable or whether they would all be released by the actions of bumblebees.

Figure 1. The scale of stages of flower development in cv Piccolo



Based on the results of all the above studies, the TGA TC then organised a two year AHDB-funded project (PE031b) to further investigate i) relative performance of Bta and Btt/Btd in commercial crops, ii) effect of high temperature on within-hive activity of both Bta and Btt/Btd, and iii) effect of high temperature on tomato pollen production / viability with emphasis on the cultivars most vulnerable to poor fruit set. This project began in January 2019 and, in the first year, focused on Bta colony development, flight activity and flower visitation as well as aspects of tomato flower / pollen development (Jacobson *et al.,* 2020). At the end of the first year of that project (*i.e.* December 2019), the TGA hosted a review of PE031b which was attended by the TGA TC, WCC team and representatives from AHDB. The work in 2019 had identified the following topics which required further investigation:

Remote monitoring of bumblebee colonies – Evaluation of the prototype Arnia system highlighted some components which required further refinement.

Variation in Bta colonies - Studies in 2019 were confounded by large variations in both the numbers of adult bees in delivered hives and the subsequent development of those colonies. There was also variation in the obvious morphology (most notably size) of adult bees. In parallel to those practical studies, the literature review (PE026) had highlighted variation in the genetic structure of *B. terrestris* populations in the British Isles as well as hybridisation among some sub-species in mainland Europe. The combined evidence raised questions about the current classification of *B. terrestris* sub-species. The review group recommended additional studies which would i) utilise existing molecular techniques to investigate the genetic structure of samples saved from the key populations in our 2019 studies and ii) incorporate such tests into all subsequent studies.

Pollen viability - Several methods of assessing pollen viability reported in historic scientific literature did not prompt germination of pollen from modern tomato cultivars in our 2019

experiments. The review group considered this to be a very important component of the project and recommended that additional studies be done to refine and perfect the techniques prior to continuing with the previously planned work.

Unfortunately, work in 2020 was severely disrupted by restrictions imposed to prevent the spread of Tomato Brown Rugose Fruit Virus (ToBRFV) and the 'lockdown' of research facilities due to Covid-19. All large-scale trials were aborted but the team were able to complete some small-scale laboratory experiments which focused on developing and refining research techniques which could be applied in subsequent studies (Jacobson *et al.,* 2021).

Work planned for this project in 2021

As a consequence of the delays, the project was extended for a further 12 months and the work plan was modified to take into account all findings up to that point. The present project began in January 2021 with the following objectives:

- To determine the effect of temperature on tomato pollen production and viability in modern tomato cultivars.
- To investigate the genetic structure of populations of *B. terrestris* used in the studies.
- To further refine remote monitoring techniques of *B. terrestris* colonies.
- To investigate the effect of high temperature on within-hive activity of *B. terrestris*.

Effect of temperature on tomato pollen production and viability

Our earlier experiments showed that cv Piccolo flowers produced more pollen grains in September than in August which may be due to differences in average temperature because pollen production is reduced at high temperatures. It is generally accepted that high temperatures (c.30^{o}C) will reduce fruit set in most tomato cultivars (*eg*. Picken, 1984; Paupière *et al*., 2017) and that reductions in pollen production and pollen viability contribute to this response. The results of experiments by Sato *et al.* (2002) suggest that high temperature has its greatest effect between about 13 days and 7 days before anthesis with the most sensitive period occurring about nine days before anthesis, when the pollen mother cells are forming pollen grains. Other factors that might contribute to the setting problems of cv Piccolo could be that this cultivar produces fewer pollen grains than other cultivars when the air temperature rises. In addition, it is possible that not all of the pollen grains that land on the stigma are able to germinate and allow the pollen nucleus to pass along the style to the ovary where the fertilisation of ovules occurs.

Genetic structure of populations of *B. terrestris*

The decision by Natural England (NE) in 2015 to severely restrict the use of Btt / Btd in the UK was driven by concerns about gynes (sexually reproducing males and females, which are produced at the end of the hive's normal lifespan) 'escaping' from the glasshouse and mating with native British (*Bta*) bees. This would then create a genetic hybrid. The concern was that hybridisation between bumblebee subspecies that are normally separate could alter the frequencies of natural gene variants and this would affect their evolution and conservation (Balloux & Lugon-Moulin, 2002). However, this rests on the assumption that the *B. terrestris* populations that occur in Great Britain are genetically isolated from the *B. terrestris* bees that occupy mainland Europe.

Historically, *B. terrestris* was separated into different subspecies which occupy different geographical regions and which show some differences in morphology, *e.g.* tail colouration. However, the true genetic relationships between geographic populations are best identified using modern DNA sequencing methods. These molecular genetics studies have shown that the three 'subspecies' of *B. terrestris* that inhabit the contiguous mainland of Europe (Btt, Btd, and *B. t. lusitanicus*) are not separate and are in fact genetically uniform and are best thought of as a single breeding population (Estoup *et al*, 1996). However, there are genetically distinct subspecies on three European islands, namely Sardinia (*B. t. sassaricus*), Corsica (*B. t. xanthopus*) and the Canary Islands (*B. t. canariensis*) (Estoup *et al*., 1996), presumably because these are geographically isolated with little opportunity for genetic mixing with bees from the mainland. The population genetics for Great Britain are much less clear cut. A molecular genetics study by Moreira *et al*. (2015) used the DNA sequences of different genetic markers to investigate the population structure of *B. terrestris* bees sampled in Ireland, Great Britain, and the contiguous European mainland. The markers were of two types: (i) data from the mitochondrial cytochrome oxidase subunit I gene (COI) (which is maternally inherited, and does not undergo recombination through sexual reproduction), and (ii) microsatellite markers, which are short, repeating sequences of DNA that occur across the genome in non-coding regions, and which tend to show greater variation between populations than the mitochondrial gene sequences. The COI gene data showed two different 'haplotypes' (= genetic groups, each with its own DNA sequence), one of which (haplotype A) was common to Ireland and Great Britain, and the other (haplotype B) which was common to all populations in continental Europe but also some populations in Ireland and Britain. The microsatellite data, meanwhile, was used to construct a model of the *B. terrestris* population genetic structure. This grouped bees from Ireland into a single population, while bees from Britain and continental Europe separated into a second cluster. Taken together, the studies

showed that Irish *B. terrestris* populations were genetically separate from populations from Britain and continental Europe, while those from mainland Europe and Britain showed natural genetic mixing and significant interactions (Moreira *et al*., 2015). This has important implications for the conservation policy of *B. terrestris* in Britain and the use of Btt / Btd bees for pollination, as it indicates that natural *B. terrestris* populations in this country are not genetically isolated from those on the European mainland.

The project management team felt it was important that we start to develop in-house molecular genetics methods for *B. terrestris*, with the longer-term aim of providing new data on the population structure of *B. terrestris* that would help decision making and policy development by NE. Studies were initiated to provide DNA sequence data for the *B. terrestris* mitochondrial cytochrome oxidase I (COI) gene, including the identification of nucleotide polymorphisms that can differentiate between different haplotypes of *B. terrestris* as shown by Moreira *et al*. (2015). It is also possible that different genetic primers used for COI amplification and sequencing can give finer resolution of haplotype differences (Manlik *et al*., 2017). Thus, the COI gene sequence is likely to be a useful genetic tool for helping to independently identify *B. terrestris* sub-species.

Refine remote monitoring techniques of *B. terrestris* colonies

Previous work with 'Arnia Hive Monitors' in 2018 had indicated that we could adapt their honeybee remote monitoring system (RMS) to work with our much smaller bumblebee colonies. The RMS system uses an electronic weighing balance combined with temperature and humidity probes and a microphone (https://www.arnia.co/). The system records data which is sent wirelessly to a relay box near the beehive, and the signal is then loaded onto the cloud using the mobile phone network. The data can then be viewed in real time using a dashboard on a smartphone, tablet or personal computer. The weighing balance records changes in hive weight as bees leave the hive to forage and return. The microphone uses an algorithm to decode the buzz signals of bees, and in honeybees it is used to indicate behaviours such as the initiation of foraging, honey processing, fanning etc. within the hive. The aim was that these metrics would also work with bumblebee hives, and we would be able to detect the movement of bees in and out of hives through weight changes, the onset of foraging by a characteristic increase in buzzing within the hive in the morning, and also the sound of individual bees entering and leaving the hive. The RMS not only had the potential to provide continual and more detailed information on Bta activity than labour intensive manual counts but could also provide information on hive environment / health; thus providing a valuable tool for future studies. However initial trials indicated that further refinement and

recalibration of the Arnia system was required. One of the main problems was that the load cells used to record weight did not give consistent readings between different Arnia units, and also that their readings were affected by temperature.

During 2021, an additional type of bumblebee monitor was trialled at Springhill Nursery. The Agrolabs B-control monitor (https://agrolabs.io/) attaches directly to the front of a hive and uses an infra-red beam to directly count the entry and exit of individual bees to the hive. The data is transmitted wirelessly to a relay station within the glasshouse and then to the cloud over the mobile phone network. The bee counter comes with different adaptors which are designed to fit the entrances of different makes of beehive. We found that the attachment to the hive could be a little 'wobbly' and so we used duct tape to strap the counter securely to the hive and prevent it falling off. The counter has an entrance cover which can be controlled remotely - the idea being that hives can be shut remotely at night and the bees kept within the hive if there is pesticide spraying the next day. The Agrolabs bumblebee counter system is available as a commercial product in Europe, USA and Mexico.

Effect of high temperature on within-hive activity of *B. terrestris*

Bumblebees, including *Bombus terrestris*, collectively thermoregulate their nests in response to cold and heat in order to maintain a relatively constant temperature for brood rearing. In hot conditions, they cool the nest by fanning, which helps maintain a stable temperature for brood rearing. In the wild, the nest temperature is kept within a narrow range independent of the ambient air temperature (Vogt, 1986). For example, brood temperatures measured within a wild *B. terrestris* nest ranged from 31.3 to 33.4 °C despite ambient air temperatures varying from 13.2 to 34.4°C (Weidenmuller *et al.,* 2002). At extreme ambient conditions, bumblebees spend more time on thermoregulation and less time on other activities such as brood maintenance (Vogt, 1986) which could be detrimental to the colony. Smaller colonies are reportedly not as good at thermoregulating as larger colonies (Seeley & Heinrich 1981 cited by Weidenmuller *et al*., 2002).

One possible explanation for the poor pollination performance observed with Bta in tomato crops is that periods of high temperature within the glasshouse are detrimental to this particular subspecies. Fruit miss-set tends to occur during periods of very high temperatures in summer. High temperatures would stimulate Bta bees to concentrate on thermoregulating the colony rather than foraging for pollen. This could occur by Bta bees starting to fan at lower temperatures than Btd / Btt subspecies (on the basis that these bees are adapted to warmer summer conditions which are typical of their home range) or by Bta bees showing a greater

propensity to fan at the threshold temperature than Btd / Btt. We might expect this to apply in particular to Btd, which originates from southern Europe. Having said that, *B. terrestris* is known to acclimate to different environments when introduced to new geographical regions (Woodard, 2107) and so may be able to adapt rapidly to summer glasshouse temperatures.

The effects of high temperature are likely to be related to the type of activity that a worker bee is engaged in. Most of the worker bees consistently perform either foraging or house duties, but about a third of workers perform both tasks (Free, 1955). High temperatures could directly inhibit bee flight traffic and pollination activity of foragers, or they might cause house bees to increase their time thermoregulating the brood, which would then reduce their investment in general brood maintenance. A reduction in brood size would be detrimental to crop pollination since the collection of pollen by workers increases with brood size. In the wild, bumblebees nest underground which provides protection against extreme temperatures. Summer temperatures within tomato glasshouses are warmer than the nest temperatures that Bta would normally experience in the wild, where summer soil temperature are on average 20°C (Holland & Bourke, 2015).

Materials and Methods

Effect of temperature on tomato pollen production and viability in modern tomato cultivars.

In the following two experiments, we focussed on the effects of day temperature alone on the grounds that tomato flowers are closed at night and so would not be accessible to bumblebees even if they were flying at night.

Experiment 1

It was proposed that our main glasshouse experiment would investigate the effects of high temperature on pollen production and pollen viability in tomato plants using two of the air-conditioned glasshouse compartments of the Warwick Crop Centre, Wellesbourne. In addition to the cherry tomato, we tested responses of cv Duelle, a baby plum tomato, and cv Milandro, a classic round tomato; neither of which was thought to exhibit problems with mis-setting.

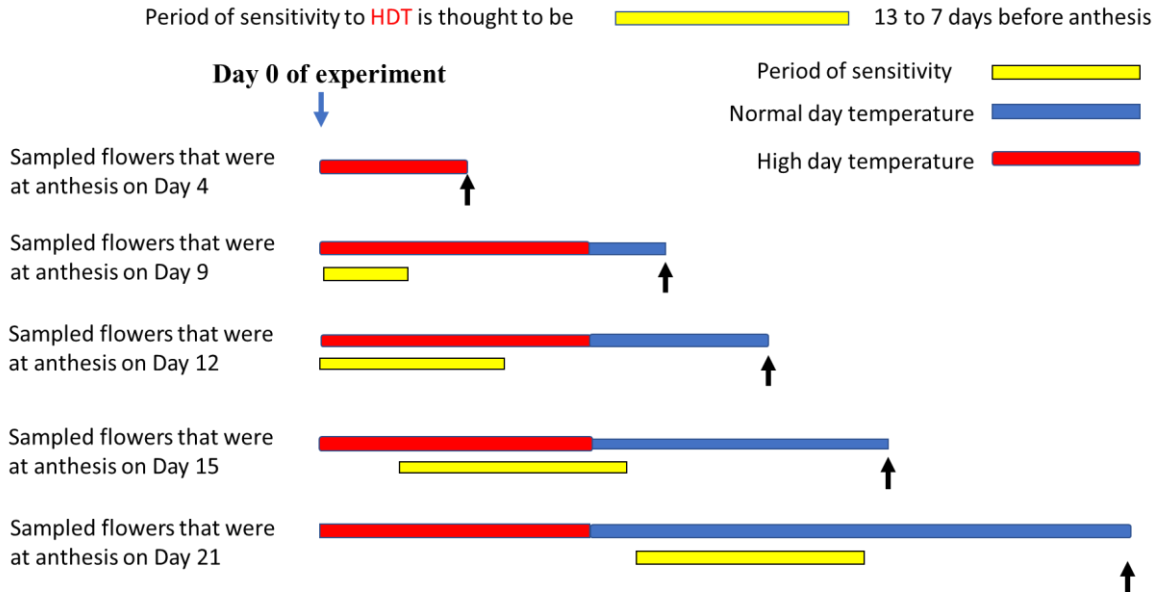
The plants were sown at Delfland Nurseries on 21 February 2021, and then grown in rockwool cubes and raised as double-headed plants. They were delivered to Wellesbourne on 13 April 2021 and were placed in two identical glasshouse compartments, C4 and C5. These compartments are south-facing, fan-ventilated and air conditioned and are each about 18.6m^2 in area. Each compartment contained two rows of six rockwool slabs, each slab was 60cm long and they were placed end to end on the compartment floor. Each slab carried two plants of one cultivar and there were two slabs of each cultivar per row. Each plant had two heads, and so there were 16 heads per cultivar per compartment; *i.e*. a total of 48 heads per compartment, equivalent to a crop density of 2.58 heads per m^2. All flowers were self-pollinated manually, using an 'electric bee', except on the day of, and the day before, sampling for assessment of pollen quantity / quality. The trusses on some plants of each cultivar from each treatment were grown on to produce fruits so that seed numbers per fruit could be counted.

Due to Covid-19 restrictions, the start of the experiment was delayed until 17 May, by which time the plants were all flowering on their fourth truss. On 17 May, all flowers that had reached the fully open stage (our stage 4; petals fully reflexed) on this day, were labelled. Around mid-day, one fully open flower was selected from each branch of five plants of each cultivar to provide a total of 10 flowers per cultivar (day 0 sample). On the following day, the day temperature of C4 was set to 30^{o}C from 08:00 until 20:00 while that of C5 was set to 20^{o}C; the night temperature of both compartments was set to 18^{o}C. Unfortunately, due to a boiler malfunction, it proved impossible to maintain this temperature regime in C4 between 20-24 May and the average day temperature in that compartment during that period was just below 26^{o}C. On 25 May, the day temperature in C4 was restored to 20^{o}C, the same as in the control treatment.

Pollen was released from each sample flower by shaking its pedicel with an electric vibrator while holding the flower above an Eppendorf tube containing 0.5ml of a liquid medium containing 0.99g H_3BO_3, 0.71g $Ca(NO_3)_2.4H_2O$, 0.197g $MgSO_4.7H_2O$, 0.11g KNO_3, and 1L of H_2O (Jacobson *et al.*, 2021). The pollen was then germinated in this medium for up to 24 hr at 25^{o}C after which aliquots were stained with 10µl of Alexander Dye (10ml 95% ethanol, 1ml malachite green (1% solution in 95% ethanol), 54.5 ml distilled H_2O, 25ml glycerol, 5ml acid fuchsin (1% solution in H_2O), 0.5ml Orange G (1% solution in H_2O) and 4ml of glacial acetic acid (1% solution in H_2O). Samples were placed under a light microscope (magnification x200) to identify germinated pollen grains and non-germinated non-viable pollen grains produced by each flower.

The sampling procedure was repeated on flowers that reached anthesis on days 4, 9 (just after the high temperature treatment ended), 12, 15, and 21. As with the day 0 sample, estimates of pollen numbers and of pollen viability were then made on all samples. In addition, once fruit had been formed, some fruit from each cultivar and treatment were cut open and the numbers of seeds they contained were counted. A diagrammatic representation of the timing of these sample days in relation to the high day temperature treatment and the known sensitivity of tomato to high day temperature is shown in Figure 2.

Figure 2. Times of sampling in relation to the high temperature regime in compartment C4 during experiment 1.



Experiment 2

Given the lower than planned temperature in compartment C4 during experiment 1, it was decided to repeat the experiment using the existing plants. It was agreed that the high day temperature should be raised to 32ºC and to minimise any potential bias, the high day temperature treatment was allocated to C5 while the control treatment was allocated to C4. As the compartments were air conditioned, it was possible to maintain the day temperature in the control compartment at about 20ºC, even in July and August.

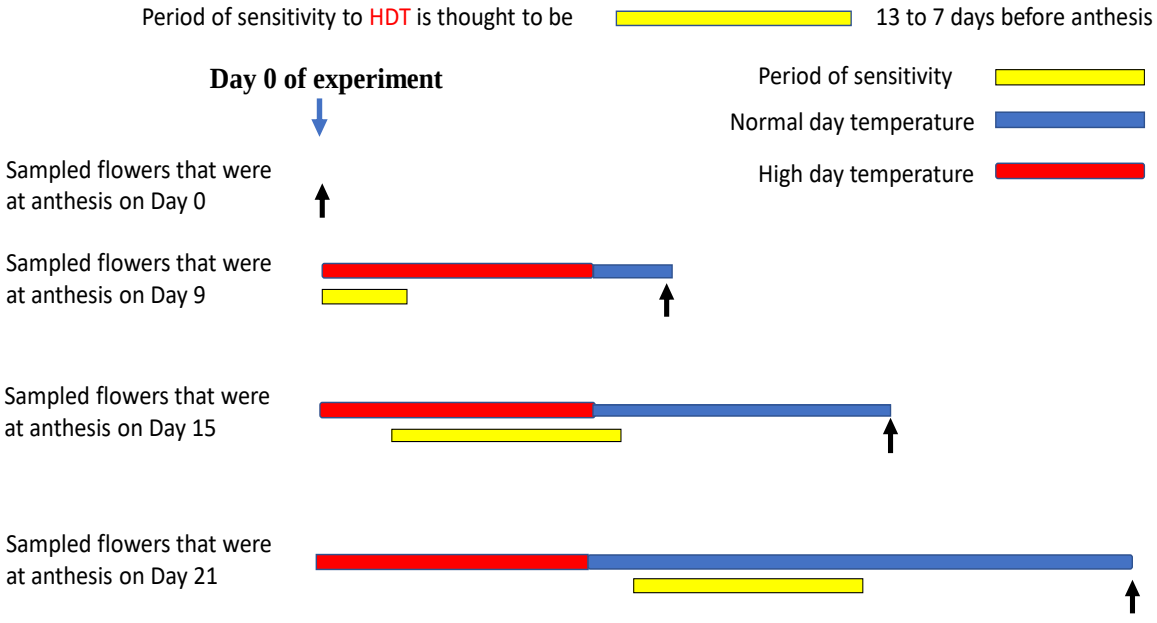
The layout of the plants and the sampling procedures were the same as in experiment 1. The main treatment in C5 was a high day temperature of 32ºC maintained for 12 hours per day followed by a night temperature of 18ºC. This regimen was maintained for seven days before reverting to the temperatures of the control treatment: *i.e*. a day temperature of 20ºC for 12

hours followed by a night temperature of 18ºC. The control treatment was maintained throughout in compartment C4.

The day 0 sample of experiment 2 was taken on 12 July. As with experiment 1, all the flowers that had reached the fully open stage (our stage 4; petals fully reflexed) on this day were labelled. Around mid-day, one fully open flower was selected from each branch of five plants of each cultivar to provide a total of 10 flowers per cultivar. The day temperature of C5 was raised to 32ºC from 08:00 until 20:00 on the following day, while that of C4 was set to 20ºC; the night temperature was 18ºC in both compartments.

While sampling open flowers on day 0 was essential to provide a baseline response, it was not essential to take samples on day 4, and especially not on day 12, provided one was taken on day 15. It was decided, therefore, that samples of open flowers (Figure 1) would be taken on days 0, 9, 15, and 21. As in experiment 1, pollen was released from each flower with an electric vibrator, stained and examined under a light microscope (Jacobson, *et al.,* 2021) to identify the germinated pollen grains, non-germinated but viable pollen grains, and non-germinated non-viable pollen grains. Some plants of each cultivar and each treatment were grown on so that seed numbers per fruit could be counted. A diagrammatic representation of the timing of these sample days in relation to the high day temperature treatment and the known sensitivity of tomato to high day temperature is shown in Figure 3.

Figure 3. Times of sampling in relation to the high temperature regime in compartment C4 during experiment 2.



Genetic structure of populations of B. terrestris used in the studies.

Eighty individual bees, which had been stored at -80ºC from samples of the commercial hives of Bta and Btt studied in 2018 and 2019, were ground to a fine powder in liquid nitrogen using a pestle and mortar. DNA was extracted from approximately one third of the powder using a DNeasy mini kit (Qiagen). Total DNA samples were quantified using a NanoDrop®ND-1000 spectrophotometer (Thermo Scientific).

The *B. terrestris* mitochondrial cytochrome oxidase I (COI) gene was amplified by PCR using the universal primers HC02198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'). This gene has been used previously in population genetics studies between Bta and Btt (Moreira *et al.,* 2015). PCR amplifications were carried out in a final volume of 20 µl containing 10 µl of REDTaq® ReadyMix™ PCR Reaction Mix (Sigma), 0.5 µM of each forward and reverse primer, and 1 µl of DNA template (1/10 dilution). The PCR conditions were as follows: 94°C for 1 min, 35 cycles of 94°C for 45 s, 52°C for 60 s, and 72°C for 45 s, followed by a final extension of 7 min at 72°C. The PCR products were subjected to electrophoresis on a 1.5 % agarose gel. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and then sequenced using the forward and reverse primers (Eurofins-GATC). These sequences were compared, and consensus versions were constructed. A multiple sequence alignment programme (MegAlign, DNASTAR Inc., Madison, USA) was used to compare the sequences with those in the Genebank database.

Remote monitoring of B. terrestris colonies.

Experiments with the Arnia system

During 2021, Arnia monitors were set up in a controlled environment room at a standard temperature and humidity (20°C, 70%RH) and a known weight (Figure 4). All data was sent to Arnia to (i) adjust the settings on the load cells and environmental probes to ensure that units perform consistently and uniformly, (ii) recalibrate the load cells so that weight readings can be adjusted for fluctuations in temperature, and (iii) gains on the microphone modified to exclude external noise.

Following the studies in the controlled environment room, two recalibrated hive scales were placed under two Biobest bumblebee hives at Springhill Nurseries on the 9 June 2021 (Figure 5). Data was collected and interrogated remotely.

Figure 4. Calibration of Arnia monitors in a controlled environment room

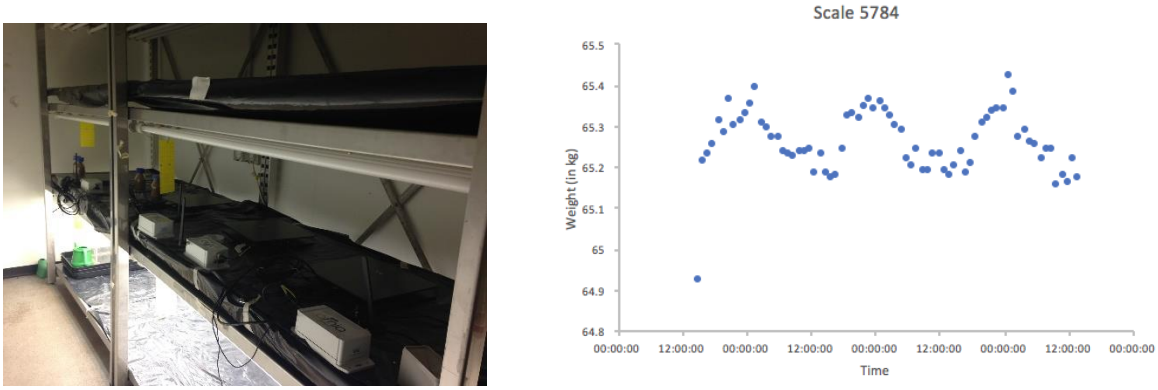


Figure 5. Hives set up with Arnia monitoring equipment attached at Springhill Nurseries on 9 June 2021

In-crop experiments with the Agrolabs system

We worked closely with Agrolabs to get the system up and running at Springhill Nurseries. There were issues at the start with getting a consistent mobile phone signal for the relay; this was solved by changing network providers to one that had the strongest signal in the area, by changing the position / height of the relay on a stanchion within the glasshouse, and by careful adjustment of its aspect so that it was in line of sight with the nearest mobile phone mast. Thus we found that the system was not 'out of the box' ready but we received very good technical support from Agrolabs during the set up and trial process and once the system was in operation it gave us good data. We ran the system to record bee traffic continually and the dashboard provided a summary in terms of bees entering and leaving per hour. It was

possible to reduce the monitoring time which saved on battery life (in our trials the batteries had to be recharged every 3 weeks but with a reduced number of counts the system could go for 2 months between charges). The data relay needed to be plugged into a power socket, which limited where it could be positioned in the glasshouse. The quality of the phone network is dependent on geography, hence it should be easier to set the system up in glasshouses with a better mobile signal. We ran a proof of concept trial over summer 2021 using two different bee counters (Figure 6).

Figure 6. Agrolabs data relay located in glasshouse (left hand picture) and Agrolab monitor attached to a hive (right hand picture).

Effect of high temperature on within-hive activity of B. terrestris.

The aims of this part of the research were (i) to complete a literature review of the thermoregulatory behaviour of bumblebees, (ii) to develop a practical, low-cost method for measuring the behavioural / physiological responses of bumblebees to high temperatures, and (iii) to compare the temperature responses of Bta versus Btd / Btt. The basic idea was to heat bees in a controlled way and determine the temperature at which they start fanning, as well as the upper lethal temperature. The hypothesis was that Bta bees would start to fan at a lower temperature that Btd / Btt bees.

Developing an apparatus to measure the responses of *B. terrestris* to high temperatures

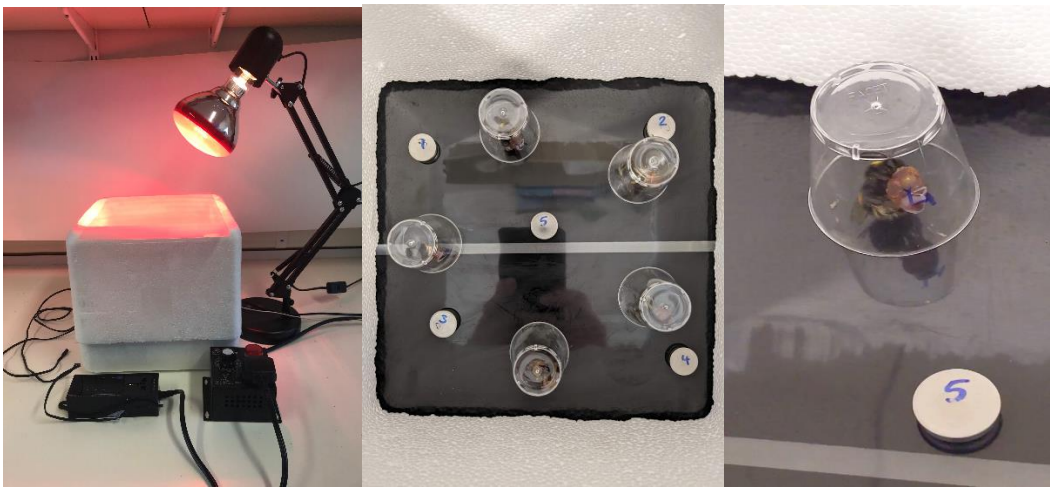
A number of papers have been published that investigate fanning behaviour in *Bombus* (Vogt, 1986; Weidenmuller et al, 2002; Weidenmuller, 2004; Couvillon et al 2010; Duong & Dornhaus, 2012; Holland & Bourke, 2015; Westhus et al 2013). These have generally been done by maintaining bee nests in small, ventilated wooden boxes with plexiglass lids for observation (Vogt, 1986; Weidenmuller et al 2002). The bees are then warmed, and fanning behaviour is measured by recording the numbers of fanning and non-fanning bees. In most cases, temperature is increased gradually: the bees usually respond uniformly, and this gives a threshold temperature at which fanning starts. Often the bees are given access via a plastic tube to a foraging arena containing sugar syrup, with pollen fed directly into the nest (Vogt, 1986; Weidenmuller et al 2002; Weidenmuller, 2004). Fixed age cohorts of bees can be used by marking newly emerged bees, which are paler than older bees (Weidenmuller et al 2002). Different methods have been used to warm the bees. For example Vogt (1986) kept bee colonies in a controlled environment room at a series of constant temperatures, which was used to control the ambient temperature for the whole nest. Others have used an infrared heat lamp with a dimmer switch positioned above the nest (Weidenmuller et al 2002; Weidenmuller 2004; Duong & Dornhaus, 2012). Again this controls the temperature of the whole nest. The challenge here is to be able to increase temperature in a way that is linear over time, as dimmer switches tend to operate in a nonlinear way and this means it can be difficult to obtain consistent, repeatable responses. Similar approaches have been used with honeybees. For example, Cook *et al* (2016) investigated the thermoregulatory fanning response of honeybees (*Apis mellifera*) in which bees were placed in groups (largest group size = 10) in a mesh cage placed on stilts in a glass jar that was placed on top of a Corning laboratory heating plate, which was used to raise the temperature.

In an alternative system, Westhus *et al* (2013) placed worker bees individually in circular, ventilated Plexiglas arenas. Groups of 8 arenas were held on a water filled aluminium block that connected to a programmable circulating water bath. The block had 8 pins, each of which protruded up into the centre of a test arena. Prior to experiments, each pin was covered in a thin layer of wax collected from the colony. This was then overlaid with a small amount of wax that covered larvae. The pin acted as a 'brood dummy' that was designed to elicit brood fanning behaviour in bees. The temperature of the block and the brood dummy pin was controlled using the water bath. A layer of insulation sat between the aluminium block and the arena, and the brood dummy pin protruded through it. With this set up, the air temperature remained constant and only the temperature of the brood dummy - and its wax covering - was changed. This is a sophisticated system and it reduces the variability documented with other

experimental set ups, for example by controlling for social feedbacks from nest mates. However, it is expensive: a programmable water bath costs >£10k which was outside this project's budget.

We ran a set of pilot studies and investigated different systems in order to develop a practical, affordable method for observing the effects of elevated temperatures on bumblebee behaviour. We decided to use a method in which bees were kept and monitored individually during temperature elevation. This would avoid complicating effects associated with social feedback if bees were kept in groups, when fanning by one bee induces the same behaviour in other bees. The challenge was to have a reliable method for raising the temperature in a controlled way. We investigated a range of methods including water baths, thermostat controlled heating mats used for keeping pet reptiles, and infra-red heat lamps. The only method that gave a repeatable linear increase in temperature over time was by using a 250W variable infra-red heat lamp controlled through a digital voltmeter (Figure 7). We initially used a digital voltmeter used for temperature management of vivaria used for keeping pet reptiles but subsequently had one designed and built for us by a local electronics company.

Figure 7. Equipment used to assess fanning behaviour of bumblebees



Individual adult bumblebees were collected from commercial bumblebee hives, placed in Falcon tubes and maintained in the laboratory at room temperature until assessed. Bees were then placed individually under plastic 'shot glass' cups contained within an insulated box of expanded polystyrene and a clear plastic lid. Each cup contained a pea-sized ball of bumblebee wax which served as a 'brood dummy' to help instigate fanning behaviour. The bees were acclimatized at 25°C for 10 minutes prior to the experiment. Heat was provided using a Philips BR125 Industrial Heat Infra Red incandescent reflector lamp (IR 250w E27

230-250v, dimmable) placed 30 cm above the bees and powered through mains electricity (240V ac) via the digital voltmeter. The temperature was increased by 1°C every minute and the experiment terminated at 50°C. Temperature was monitored using a digital thermometer but was recorded using 'ibutton' temperature data loggers placed next to the bee cups (Figure 8). The bees were observed continually throughout the experimental period and the temperature at which the bees began fanning and the temperature at which the bees died was recorded. The weight of each individual bee was also recorded.

Preliminary experiments were done using cohorts of 5 Bta to get some starter data. These showed that the temperature threshold for Bta fanning was between 33 and 37°C with 62% exhibiting fanning behaviour (Figure 9). As temperatures increased above 34°C, bees began to die with 56% dying by 40°C. There was no correlation between fanning temperature and bee weight.

Figure 8. Linear increase of temperature over time obtained using the IR heat lamp controlled through a digital voltmeter

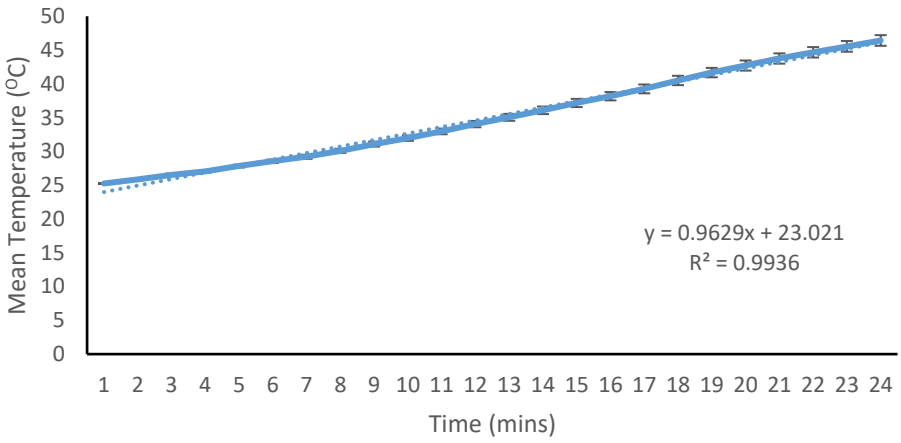
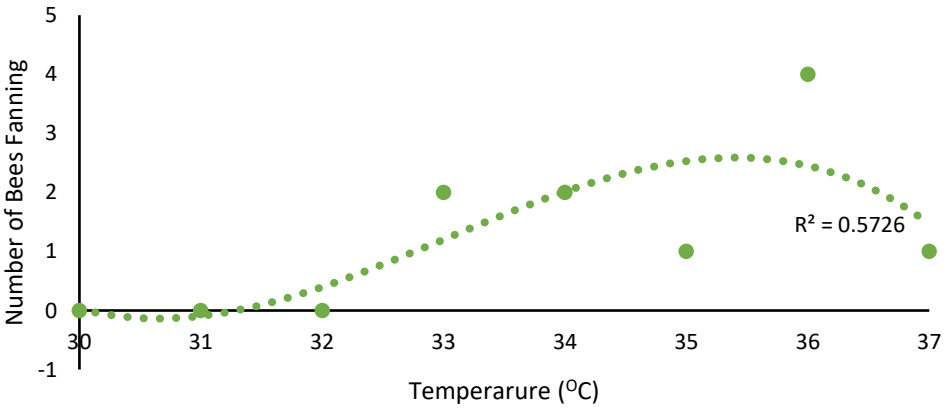


Figure 9. Example results from preliminary study showing total number of Bta bees fanning versus temperature.



Quantifying the response of individual bumblebees to elevated temperatures

Once the system had been finalised, an experiment was done to compare the response to temperature of adult bees from commercial Bta and Btt hives. One hive of each bee subspecies was compared. The hives were the same age and comparisons of the subspecies were done on the same day, with alternating batches of Bta and Btt bees. The experiment was done as three batches of 10 bees of each subspecies (n = 30). Bees were observed throughout the experimental period. We recorded the time at which individual bees first started fanning, as well as the time at which they died. Fanning was defined as having occurred once a bee had fanned its wings for a minimum of 10 seconds. Death was defined as the point at which bees became totally inactive, with no movement in any part of the body. We then used data from the ibuttons to draw a calibration graph of temperature versus time, and used this to determine the temperatures for fanning and death. Weights of individual bees were also recorded.

Results and Discussion

Effect of temperature on tomato pollen production and viability in modern tomato cultivars.

Experiment 1

The seed counts (Table 1) showed no effects of high day temperature on the number of seeds per fruit and indicated that the flowers formed enough viable pollen to fertilise all the ovules present, even in the high day temperature treatment. Fruits of cv Milandro usually contained significantly more seeds than those of either cv Piccolo or cv Duelle,

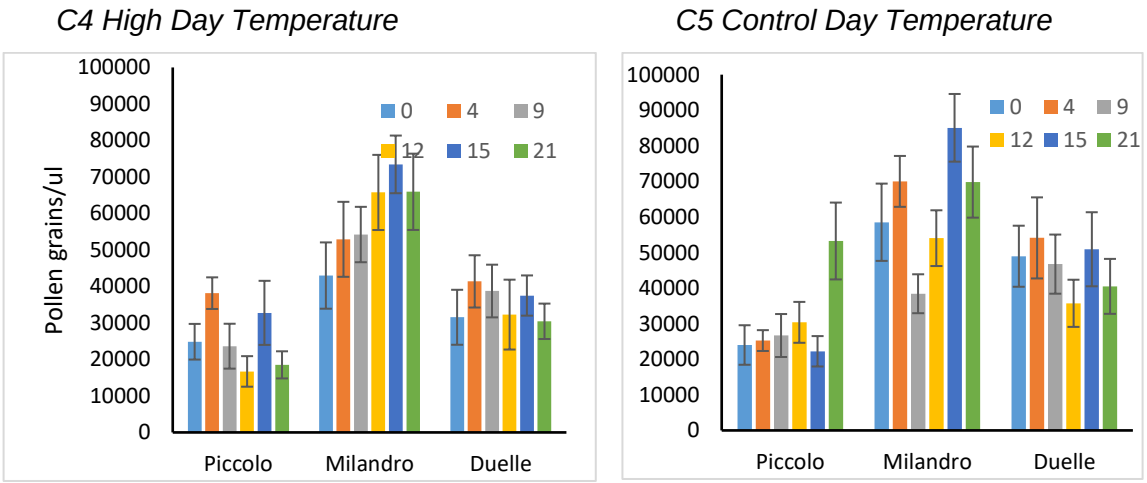
Pollen counts from all three cultivars, in both treatments on each of the six sample dates are shown in Figure 10. There was considerable variation but little consistency. The one possible treatment effect seemed to be that pollen production by flowers of cv Piccolo in the high day temperature treatment was reduced in flowers that reached anthesis on day 12. According to Figure 1, we might expect that samples from flowers that reached anthesis on day 12 would have spent much of the sensitive period (*i.e* 13 to 7 days before anthesis) at the high day temperature. On the other hand, so would flowers that reached anthesis on day 15. Oddly, flowers of cv Piccolo that reached anthesis on day 21 in the control treatment produced more pollen than flowers from any other treatment or sample date.

Table 1. Seed counts in all three cultivars, in both treatments on each of the six sample dates in Experiment 1.

	Day	0	4	9	12	15	21	Average	SEM
C4 HDT	Piccolo	83	82	86	88	86	75	83.3	1.89
	Milandro	127	134	146	139	132	122	133.3	3.48
	Duelle	79	74	84	78	85	69	78.2	2.47
C5 Control	Piccolo	93	85	73	81	84	54	78.3	5.53
	Milandro	141	104	134	71	126	136	118.7	10.92
	Duelle	66	82	81	86	76	74	77.5	2.90

There were no effects of high day temperature on pollen number in cv Duelle while pollen production by the control treatment of cv Milandro was very variable. Indeed, the data appeared to show that were significant differences in pollen production by cv Milandro and cv Duelle on different sample days in the control treatment which did not receive a high day temperature.

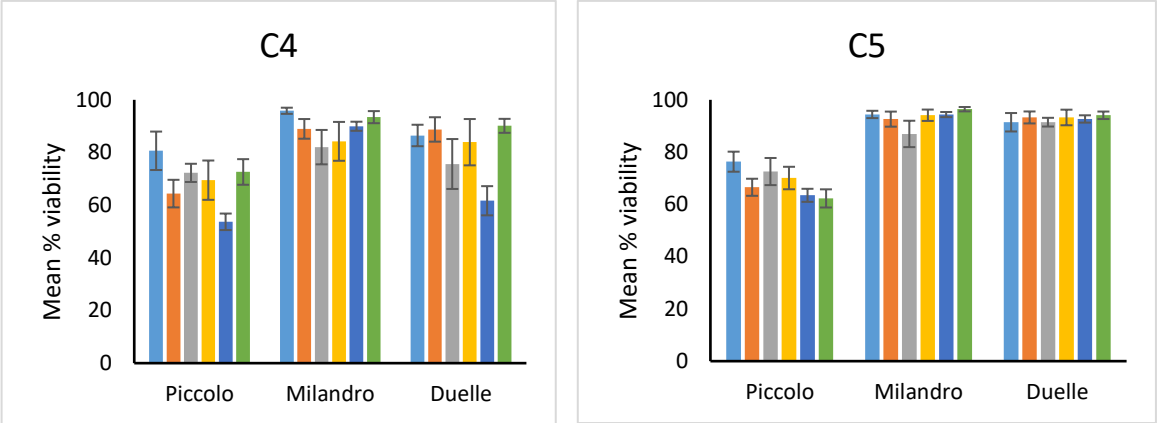
Figure 10. Pollen counts from all three cultivars, in both treatments on each of the six sample dates in Experiment 1.



Pollen viability in all three cultivars, in both treatments on each of the six sample dates are shown in Figure 11. Pollen viability was depressed a little in flowers of cv Piccolo and cv Duelle that reached anthesis on day 15 and were developing, therefore, through almost the whole of the period of sensitivity to high day temperature. In general, pollen viability was reasonably high, being more than 60% in cv Piccolo, and more than 80% in the other two cultivars.

Figure 11. Pollen viability in all three cultivars, in both treatments on each of the six sample dates in Experiment 1.

C4 High Day Temperature *C5 Control Day Temperature*



Experiment 2

The average day/night temperatures in compartments C4 and C5 between 1 July and 12 July were 20.5/18.4ºC for C4 and 20.7/18.5ºC for C5. Once the seven-day period of high day temperatures began on 13 July, the average day/night temperatures over that period were 32.0/21.0ºC for C5 and 20.9/18.6ºC for C4. Over the remainder of July, the average day/night temperatures were 21.0/18.7ºC for C5 and 20.7/18.5ºC for C4. The control of temperature was evidently superior to Experiment 1 and the difference in day temperature between C5 and C4 was more than 11ºC.

The main effect of the high day temperature on flowers of cv Piccolo, cv Duelle, and cv Milandro was that most of those that reached anthesis on day 15 either failed to set fruits or set very small fruits (Figure 12). According to Figure 3, these flowers were exposed to the high day temperature over almost the whole of the period when pollen formation was believed to be at its greatest sensitivity to temperature.

Seed counts in all three cultivars, in both treatments on each of the four sample dates are shown in Table 2. The flowers of all three cultivars that reached anthesis on day 9 also formed fruits and these too had fewer seeds in them but more than from those flowers that reached anthesis on day 15. The day 9 flowers had been exposed to two days of high day temperatures from 9 to 7 days before anthesis which was the earlier part of the temperature-sensitive period. The samples from flowers that reached anthesis on day 21 showed that if

the high day temperature occurred before the temperature-sensitive period occurred, it had no impact on the seed counts.

Figure 12. Examples of trusses from all three cultivars in both treatments towards the end of experiment 2.

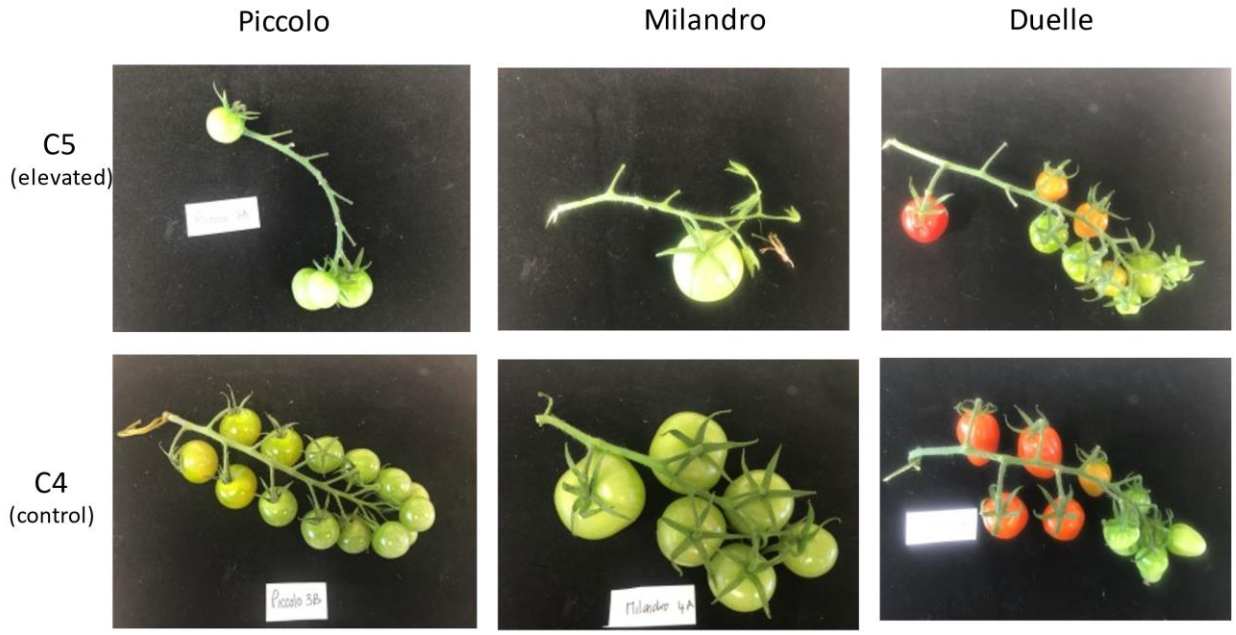


Table 2. Seed counts in all three cultivars, in both treatments on each of the four sample dates in Experiment 2.

	Day	0	9	15	21	Average	SEM
C4 Control	Piccolo	87	81	71	73	78	3.02
	Milandro	111	122	117	111	115	2.17
	Duelle	85	79	73	66	76	3.32
C5 HDT	Piccolo	75	22	0	75	43	15.52
	Milandro	126	49	7	128	78	24.36
	Duelle	74	1	1	67	36	16.42

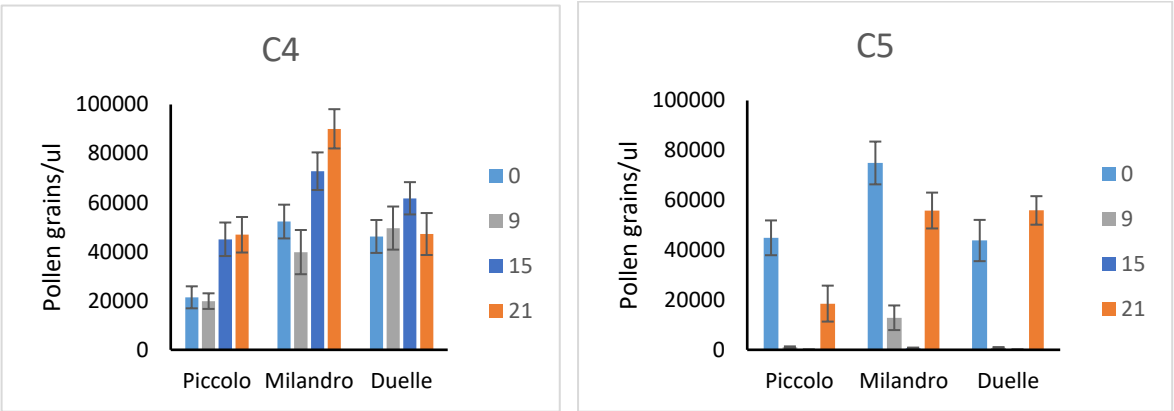
Pollen counts in all three cultivars, in both treatments on each of the four sample dates are shown in Figure 13. Although the pollen counts in the control treatment (C4) varied between 20,000 and 50,000 in cv Piccolo according to the day of the sample (Figure 13), the flowers of cv Piccolo under the high day temperature treatment produced almost no pollen when sampled on reaching anthesis on day 15 and produced only an average of about 1,000 grains per flower for those that reached anthesis on day 9. Cv Duelle produced a very similar picture.

On the other hand, the flowers of cv Milandro produced many more pollen grains (between 50,000 and 70,000) when sampled on day 0 but this number dropped to fewer than 700 grains for flowers reaching anthesis on day 15 after receiving the high day temperature treatment. As with the other cultivars, the flowers reaching anthesis on day 9 were less badly affected as they received the high day temperature treatment earlier in the temperature-sensitive period. The flowers that received the high day temperature treatment and afterwards, reached anthesis on day 9 formed fewer than 13,000 grains.

The samples from flowers that reached anthesis on day 21 showed, as with the seed counts, that if the period of high day temperature was encountered before the temperature-sensitive period occurred, it had no impact on the pollen count.

Figure 13. Pollen counts from all three cultivars, in both treatments on each of the four sample dates in Experiment 2.

C4 Control Day Temperature *C5 High Day Temperature*



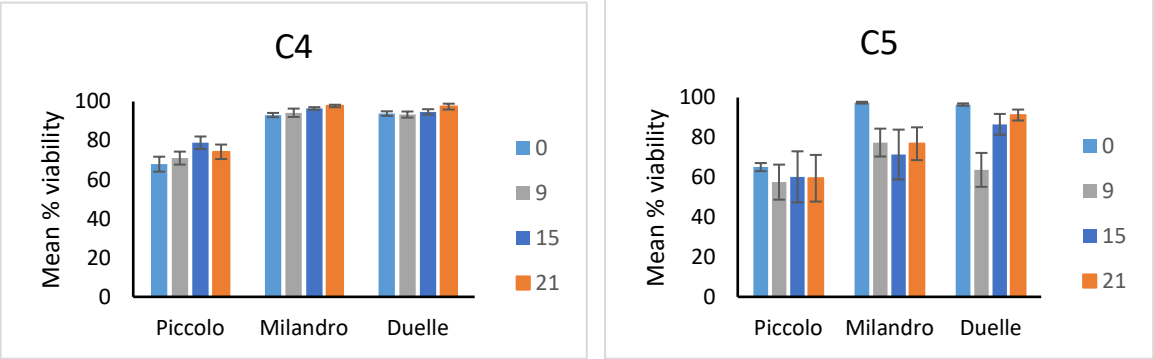
Pollen viability in all three cultivars, in both treatments on each of the four sample dates are shown in Figure 14.

Evidently, pollen viability was depressed a little by the high day temperature treatment, but the effect was not large. The viability of cv Piccolo pollen was generally below 60% in plants receiving the high day temperature but above 60% in the control. With cv Duelle, pollen viability was nearly 90% in the control but dropped around 60% if flowers reaching anthesis on day 9 had received the high day temperature treatment but was well above 80% in most other samples. With cv Milandro, the viability of pollen in flowers that reached anthesis after receiving a high day temperature treatment was below 80% but well above 90% in flowers

that reached anthesis having experienced only the control environment. In general, it seemed that the effect of the high day temperature on pollen viability was not exerted on a specific stage in pollen development but could influence pollen viability at any stage of flower development.

Figure 14. Pollen viability in all three cultivars, in both treatments on each of the four sample dates in Experiment 2.

C4 Control Day Temperature *C5 High Day Temperature*



Genetic structure of populations of B. terrestris used in the studies.

The quantity of DNA extracted from an individual bee ranged from 7.4 to 38.4 ng/µl. The majority of the samples produced a PCR product of ca. 600 base pairs (Figure 15).

Studies by Moreira *et al*. (2015) identified three positions on the mitochondrial cytochrome oxidase I (COI) gene sequence in which Bta and Btt had different nucleotides. Their data also separated out Bta into two haplotypes, termed Bta A and Bta B, which differed at two of these three polymorphic sites. Figure 16 is a diagrammatic representation of these polymorphic sites. Bta and Btt are sequences taken from the Genbank gene sequence database. BtA is haplotype A, reported to occur in Britain & Ireland and BtB is haplotype B, reported in mainland Europe & England, both from Moreira *et al*. (2015) and deposited as reference sequences in Genbank. Bta B is a hybrid sequence that shares features of Bta Btt. Our sequence data, taken from Bta bees sold commercially in Britain, identified that both of the Bta A and Bta B haplotypes were present. We also identified at least six other positions on the gene that were polymorphic. These will need to be checked in future studies using both biological and technical replicates to eliminate sequencing errors. In addition, we observed

nucleotide insertions or deletions in 12 of the bee samples, including single base pair insertions / deletions but also four cases of insertions of up to six bases. Again these will need to be checked in repeated experiments to eliminate sequencing errors. However, at the moment it indicates greater variation in Bta diversity than previously reported.

Figure 15. Alignment of bee sequences.

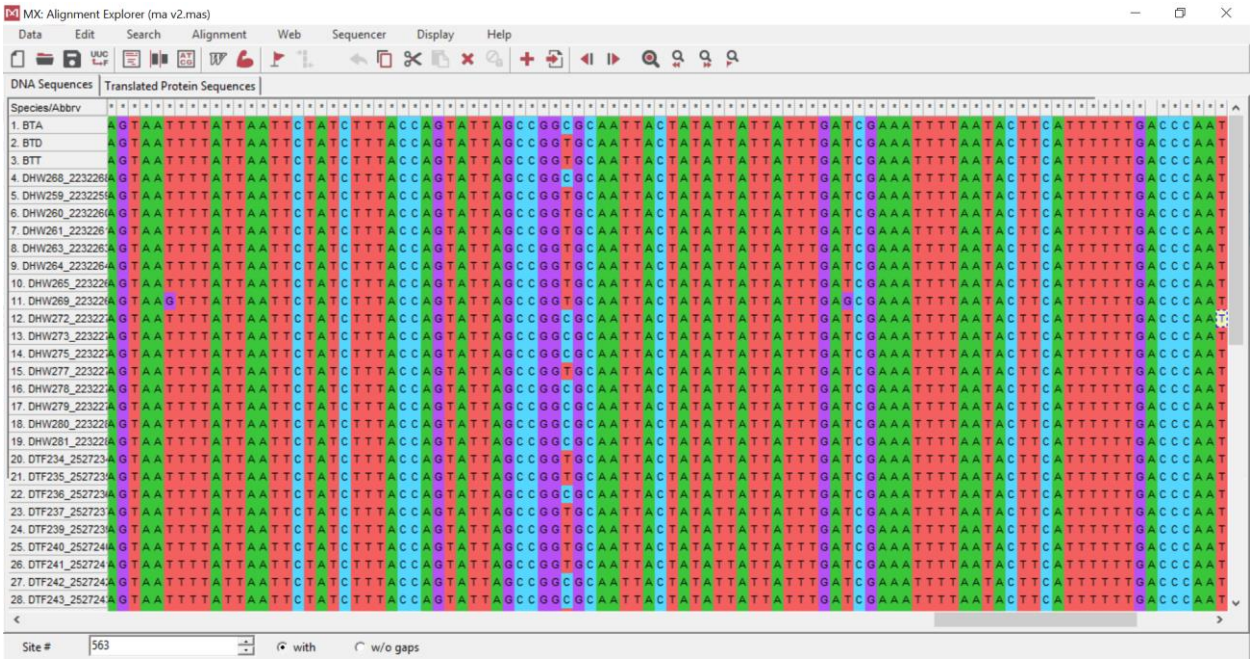


Figure 16. Diagrammatic representation of variation on the mitochondrial cytochrome oxidase I (COI) gene. Bta and Btt are sequences from Genbank database. BtA is haplotype A, reported to occur in Britain & Ireland and Bt B is haplotype B, reported in mainland Europe & England, both from Moreira *et al*. (2015).

Bta: C-----C-----T

Btt: T-----T-----C

Bta A: C-----C -----T

Bta B: T -----T-----T

Remote monitoring of B. terrestris colonies.

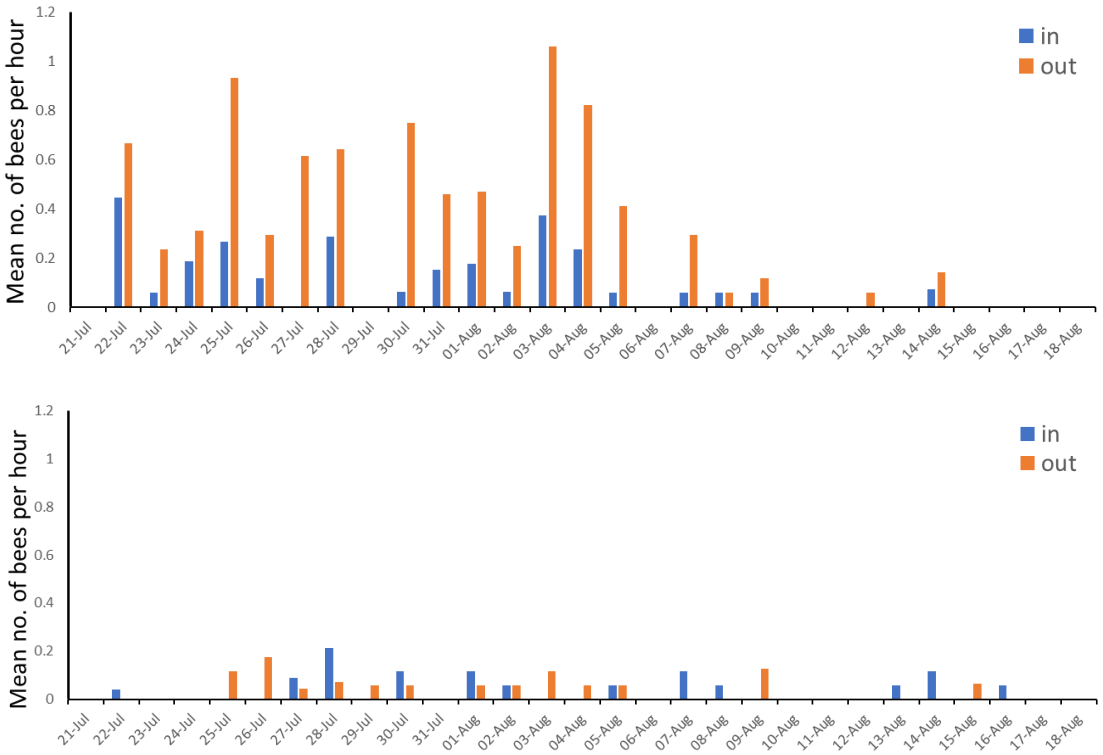
In-crop studies with the Arnia system

Data from the two recalibrated hive scales placed under two Biobest bumblebee hives at Springhill Nurseries on the 9 June 2021 showed that although the weights of the colony could be measured accurately to give a measurement of colony health they were not sensitive enough to determine the activity of individual bees leaving and entering hives. Moreover, the microphone failed to pick up diurnal changes in the sound within the hives compared to a 'dummy' hive that we set up containing no bees.

In-crop studies with the Agrolabs system

Bee activity between the two hives monitored was considerably different (Figure 17), despite being of the same age. This backs up our previous manual bee activity observations which showed that bee hives vary considerably in their activity. Activity in hive 1 hive peaked within the first two weeks of the hive being placed within the glasshouse, again supporting previous observations. There was also evidence from both hives that numbers leaving the hives were generally greater than the numbers entering the hives – thus indicating that bumblebees visited other hives or got lost within the crop. Overall, the amount of flight traffic was low and it confirmed our observations done using manual counts of bee traffic earlier in the project.

Figure 17: Hive activity from two hives measured with the Agrolabs B-control monitor.



*Effect of high temperature on within-hive activity of **B. terrestris**.*

Literature review of the thermoregulatory behaviour of bumblebees

In research on bumblebee thermoregulation with *B. impatiens* done by Vogt (1986), the ambient temperature was controlled by keeping the bee colony in a controlled environment room. Whole bee colonies were maintained at a constant temperature and their behaviour observed, while measurements were taken of brood temperature and oxygen consumption. Wing fanning commenced at 25°C, with 20% of bees engaged in fanning at 32°C and 60% of bees at 35°C, which is a temperature that was considered to be lethal to the brood. Incidence of brood maintenance was highest at 25-30°C and declined at temperatures >30°C. Under conditions of heat stress in the experimental set up, high levels of fanning at ambient temperatures of 35°C and above did not enable the bees to maintain the brood temperature within their preferred range of 28-32°C (Vogt, 1986).

Weidenmuller et al (2002) examined heat effects on *B. terrestris* by holding nests at a constant temperature of 22°C for 15 minutes, then increasing the ambient temperature with a heat lamp every 5 minutes for 45 minutes, and then reducing the temperature every 5 minutes for a further 15 minutes. The highest temperature was 30°C to avoid damaging the brood. The response of small colonies (< 60 bees) was compared to large colonies (> 60 bees). The maximum percentage of workers fanning varied between colonies, with fanning starting to occur from 22-24°C. At a temperature of 28-30°C, the percentage of bees fanning ranged from 8-28% depending on the colony. Larger colonies responded faster to ambient temperature change than smaller colonies. The proportion of workers recruited for fanning did not vary with colony size, so that a larger colony recruited more fanning bees than a smaller colony.

Weidenmuller (2004) studied 4 colonies, each contained in a wooden nest box, with temperature controlled using a heat lamp. The temperature regime was controlled as in Weidenmuller *et al* (2002). Data were collected on individual, numbered bees. Each bee was observed five times. Observations were used to calculate the response threshold (mean temperature at onset of fanning) and duration of fanning. Workers were found to vary in their responsiveness, with c.40% never showing fanning behaviour. Some started to fan at low temperatures while others consistently started to fan at high temperatures. This may indicate that some bees ‘specialise’ in fanning, in that they respond to a lower threshold temperature than others. The average temperature threshold also varied between colonies.

Westhus *et al* (2013) - working in Weidenmuller's lab - used a more advanced set up to that developed by Weidenmuller (2002) (the brood dummy system, see above). Newly delivered bumblebee colonies (each containing 1 queen and up to 26 workers) were maintained as above, with colonies kept at 22°C so that all the workers were naïve to fanning. Individual worker bees were placed in temperature-controlled test arenas, each arena containing a 'brood dummy' comprised of a temperature-controlled metal pin coated in wax that would elicit brood fanning behaviour. Temperatures were varied over the experiment: (i) they were first dropped from 32 to 27°C - this was found to increase the chances of bees performing thermoregulatory brood fanning, and (ii) temperature was then increased at a rate of 0.1, 0.6 or 1.1°C per minute up to a maximum of 48°C. Fanning behaviour was observed over this time. Over all experiments, fanning or brood incubation was observed in 80% of bees, with 48% showing fanning in response to temperature rise. Most fanning occurred on or near the brood dummy. A faster temperature increase led to a lower probability of fanning, with 78% of bees showing fanning at an increase rate of 0.1°C per minute compared to 33% at 1.1°C per minute. The rate of temperature increase also affected the response threshold: the threshold was 43.3°C at an increase rate of 0.1°C per minute compared to 46.6°C at 1.1°C per minute. Colony origin or body mass had no effect on response threshold. Bees that repeatedly performed fanning tests resulted in a decrease in the fanning response threshold temperature. This reduction occurred even after the first trial. In a second study using the same set up, Garrison *et al* (2018) found that bees were less likely to engage in thermoregulatory fanning in a group setting (8 bees per arena) than when tested individually.

In contrast to the above, Duong & Dornhaus (2012) found that the fanning threshold of *B. impatiens* did not decrease with bee age or experience. Workers were selected from commercial colonies, marked, and randomly assigned to treatment. Temperature was increased using a heat lamp for 70 min before being turned off to let the colony cool, with temperatures ranging from 25 to 35.5°C. Marked workers were transferred from their mother colony to a separate nest box for heating experiments, with between 44-100 bee per box. Heating runs were done a total of 4 times for each group in order to see how the fanning temperature threshold varied with experience.

Couvillon *et al* (2010) investigated the effect of nest temperature on foraging flight activity for *B. impatiens*. Commercially supplied nests were insulated in drink coolers to simulate underground nesting, and then connected by plastic tubing to a foraging arena that contained feeders hung from its roof. The whole set up was housed in a controlled environment room which allowed the temperature to be changed from 16 to 36 °C from a set point of 26°C. Bees were individually tagged. The identity of bees foraging was recorded at each temperature,

and bee size was measured at the end of the experiment. The average temperature at which a bee foraged could not be predicted by body size, nor did worker body size correlate with maximum body temperature. Bees of all sizes flew and foraged from 16 to 36 °C. This was an unexpected result because the authors hypothesized that larger bees may have suffered from heat stress at the higher temperatures. In contrast, in a greenhouse experiment with hot pepper in which colony traffic from *B. terrestris* nests was measured and compared against temperature, it was found that colony traffic and foraging activity was highest at 25.7, but at 32.7°C foraging activity had declined by 70% while colony traffic had declined by 48% (Kwon & Saeed, 2003).

Quantifying the response of individual bumblebees to elevated temperatures

Bees that engaged in fanning behaviour typically did so by climbing onto the beeswax 'brood dummy' and fanning their wings, which was usually done in short, repeated bursts of 1-2 seconds each rather than a continuous wing beat. The point of death occurred very rapidly: typically a bee would switch from being fully active (rapid walking and some wing buzzing) to lying on the floor, completely inactive, within 1 second. The temperatures at which fanning began for individual bees ranged from 28.7 to 34.3°C (Bta) and 26.4 to 37.2°C (Btt). There was no difference between Bta and Btt in the average temperature at which fanning began (31.6°C and 31.9°C respectively, Table 3). However, while 58% of the Btt cohort engaged in fanning, only 20% of the Bta cohort fanned. The temperatures at which the bees died ranged from 39.8 to 50.3°C (Bta) and 39.9 to 46.7°C (Btt). There was no observed difference between Bta and Btt in the average temperature at which bees died (43°C). There was no correlation between fanning temperature and bee weight.

Table 3. Observed behavioural responses of Bt audax versus Bt terrestris bees to increasing temperature.

Sub species	% Fanning	Mean (SE) temperature of fanning (°C)	% Dead	Mean (SE) temperature of death (°C)	Mean (SE) weight (g)
Bta	20.00	31.64 (0.400)	83.33	43.02 (0.575)	0.24 (0.012)
Btt	58.62	31.92 (0.586)	89.66	43.15 (0.354)	0.25 (0.008)

Observing the thermal responses of isolated, individual bees in the experiment done here is a simplified system compared to previous studies done on whole bee colonies, in which the brood is heated using an IR lamp or in a heated room and the response of attendant worker

bees is monitored (Vogt, 1986; Weidenmuller *et al*., 2002; Weidenmuller, 2004). Our system used balls of beeswax as 'brood dummies': the benefit is that each dummy receives the same temperature exposure, whereas in a whole colony, the position of individual brood cells in the complex three-dimensional structure of the colony is likely to determine the temperature that each experiences, which will lead to greater experimental variation. This may help explain why our system provided data with low standard errors for both the mean temperature at which bees started fanning, and the mean temperature of bee death. Weidenmuller (2004) reported that the average temperature at which *B. terrestris* bees started to fan was 27.7-28.7°C: this was done in a whole-colony experiment, and is slightly lower than the average fanning temperature observed in our study. Westhus *et al*. (2013) noted that *B. terrestris* assessed individually fanned at higher threshold temperatures than those reported previously in colonies, which may explain our results. The average fanning temperature we observed is identical to the optimum temperature reported for brood rearing for *B. terrestris*, 32°C (Weidenmuller *et al*., 2002). Vogt (1986) reported that the incidence of fanning of *B. impatiens* increased markedly >32°C, reinforcing the idea that bumblebees work to keep the brood at the optimum temperature, and hence this should be the critical metric to consider when using bees in the glasshouse. We found no significant difference in the mean fanning temperature or the mean lethal temperature for Bta and Btt, suggesting that these aspects of their thermal biologies are not linked to their subspecies. There were differences in the percentage of bees that fanned. It is not known if this is linked to subspecies, as it may simply reflect natural variation between colonies - the literature suggests that different colonies can vary considerably in the proportion of bees that engage in fanning (see Weidenmuller *et al*, 2002). The only way to address this would be to repeat the experiment using a representative number of different hives. However, if Bta bees had a lower propensity for fanning, this could have a detrimental effect on the brood when temperatures are high.

Conclusions

Effect of temperature on tomato pollen production and viability in modern tomato cultivars.

Experiment 1

Although the day temperatures varied in both treatments and the high day treatment was only about 6^{o}C higher than the control on average, the results suggest that pollen viability might have been reduced in cv Piccolo and cv Duelle if the high day temperature was given for almost the whole of the sensitive period running up to seven days before anthesis. However,

as even the flowers of cv Piccolo produced more than 12,000 viable pollen grains with some 60% viability it seems that they produced more than enough grains to fertilise all the available ovules and produce fewer than 100 seed.

Experiment 2

- The results clearly showed that when the day temperature was maintained at 32^{o}C throughout the 12 hours of the day period, it dramatically reduced fruit set in some flowers.
- The published literature suggests that high day temperature can affect fruit set if it occurs from 13 to 7 days before anthesis and it was thought to interfere mainly with pollen production.
- Each seed is the result of the fusion of germ cells from one pollen grain and one ovule. Consequently, the data on the number of seeds per fruit, pollen numbers, and pollen viability and the index of pollen viability from the day 0 samples, show that all three cultivars produced many more viable pollen grains than were required to fertilise all the ovules in the ovaries of their flowers.
- The results of experiment 2 showed that flowers that reached anthesis on day 9 and had experienced the HDT treatment 9 and 8 days before anthesis showed a significant reduction in the numbers of seeds per fruit.
- This effect was amplified in the results from the flowers that reached anthesis on day 15 as they showed a dramatic reduction in the number of seeds per fruit leading to the abscission of fruits, especially in cv Piccolo and cv Duelle. Plants in this treatment had experienced the HDT 12 to 7 days before anthesis.
- The HDT treatment had little to no effect upon the seed count and fruit set of flowers that reached anthesis on either day 0 or day 21. These flowers had been exposed to the HDT treatment either later than 7 days before anthesis (the day 0 sample) or before 13 days before anthesis (the day 21 sample).
- The effect of the HDT on seed count appeared to be due to an effect on pollen production rather than an effect on pollen viability, or the combined pollen viability index, as pollen viability was relatively high whenever samples were taken.
- The day temperature treatment of 32^{o}C maintained for 12 hours per day is an extreme environment and is most unlikely to be encountered in the UK at present. However, the question remains as to whether the same or similar temperature given for fewer hours in the day, which can occur in glasshouses in the UK, is likely to have any effect.
- The data strongly suggest that these responses to high day temperatures are likely to become a more serious problem if the present rate of climate change is sustained.

- These results do not preclude the possibility that the problems with fruit set that are encountered in summer in the UK are caused by, or exacerbated by, the bumblebees that are currently used to pollinate tomato crops as those failings happen at temperatures below those tested in these experiments.

Genetic structure of populations of B. terrestris used in the studies.

- Our data shows that there is greater sequence variation in COI than previously reported.
- While these are preliminary findings, they do suggest that bee 'subspecies' originating from Britain (labelled as Bta) and those from mainland Europe (Btt) do not consist of separate, 'pure' genetic entities, but rather as populations with some haplotypes in common.
- This would support the idea of some natural genetic mixing / interactions between populations in Britain and mainland Europe.

Remote monitoring of B. terrestris colonies.

- We were unable to obtain consistent and reliable results with the Arnia system when used with commercial bumblebee hives in this project.
- Our results with the Agrolabs equipment were more promising and we concluded that this system has potential to provide high quality, useful data for monitoring bumblebee traffic. However, it would be useful to build the system with temperature and humidity probes so that environmental conditions within hives could be monitored to look for evidence of heat stress effects.

Effect of high temperature on within-hive activity of B. terrestris.

- Our conclusion at this stage is that poor pollination observed with *Bta* bees is unlikely to be caused by a marked difference in the thermal biology of Bta versus Btt for either (i) the temperature threshold for the fanning response and (ii) the upper lethal temperature for adult bees.
- We do have some evidence of differences in the proportion of bees that engage in fanning between Bta and Btt, but we would need to perform the same experiment on replicate hives to confirm this.
- This is not to say, however, that high temperatures are not detrimental to bees within the glasshouse. Because the mean bee fanning temperature closely matched the optimum

temperature for brood rearing (32°C), this suggests that beehives should not be allowed to get hotter than this if possible. Temperatures above 32°C are likely to result in bees staying within the hive to fan rather than foraging and could be detrimental to brood. These conditions are unlikely to occur to any great extent at present but it would be worth monitoring temperatures within hives to find out.

- However, temperatures within the glasshouse for tomato production will not get high enough to kill adult bees.

Knowledge and Technology Transfer

Jacobson, Chandler, Prince and Cockshull (2021). Drafts of 11 Knowledge Library Pages submitted to AHDB in February 2021. Subsequently revised with final versions submitted to AHDB on 8 September 2021.

Jacobson (2021). Interim report on progress in PE031b presented to TGA Technical Committee meeting. Via internet, 1 June 2021:

Chandler, Jacobson, Prince and Cockshull (2021). Presentation to TGA Technical Committee meeting. Via internet, 7 December 2021

Jacobson (2021). Pollination issues in 2020 and 2021. An exchange of information with the Tomato Study Group via the internet on 25 May 2021.

Chandler, Jacobson and Cockshull (2021). Presentation to Tomato Working Party. 8 March 2021.

Chandler, Jacobson and Cockshull (2021). Presentation to Tomato Study Group. 22 March 2021.

References

Balloux, F. and Lugon-Moulin, N. (2002). The estimation of population differentiation with microsatellite markers. *Molecular Ecology*, **11**, 155-165.

Chandler, D., Cooper, E. and Prince, G. (2019). Are there risks to wild European bumble bees from using commercial stocks of domesticated *Bombus terrestris* for crop pollination? *Journal of Apicultural Research*, DOI: 10.1080/00218839.2019.1637238.

Cook, C.N., Kaspar, R.E., Flaxman, S.M. and& Breed, M.D. (2016). Rapidly changing environment modulates the thermoregulatory fanning response in honeybee groups. *Animal Behavior*, **115**, 237 - 243.

Couvillon, M.J., Fitzpatrick, G. and Dornhaus, A. (2010). Ambient air temperature does not predict whether small of large workers forage in bumble bees (*Bombus impatiens*). Psyche: A Journal of Entomology, 2010, ID 536430, 8 pp. https://doi.org/10.1155/2010/536430

Duong, N. and Dornhaus, A. (2012). Ventilation response thresholds do not change with age or self-reinforcement in workers of the bumble bee *Bombus impatiens*. *Insectes Sociaux*, 59, 25–32

Estoup, A., Solignac, M., Cornuet, J-M., Goudet, J. and Scholl, A. (1996). Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. Molecular Ecology, 5, 19-31.

Free, J.B. (1955). The division of labour within bumblebee Colonies. *Insectes Sociaux*, **2**, 195–212.

Garrison, L.K., Kleineidam, C.J. and Weidenmuller, A. (2018). Behavioral flexibility promotes collective consistency in a social insect. *Nature Scientific Reports*, 8, 15836 DOI:10.1038/s41598-018-33917-7 2

Holland, J.G. and Bourke, A.F.G. (2015). Colony and individual life-history responses to temperature in a social insect pollinator. *Functional Ecology*, **29**, 1209 - 1217.

Jacobson, R.J. (2017). Tomato: An investigation into poor pollination performance by the native bumblebee, *Bombus terrestris audax*. Final report of contract work undertaken for AHDB. December 2017, 27pp.

Jacobson, R.J., Chandler, D., Cockshull, K. and Prince, G. (2018). Tomato: Phase 2 of an investigation into poor pollination performance by the native bumblebee, *Bombus terrestris audax*. Final report of contract work undertaken for AHDB. December 2018, 36pp.

Jacobson, R.J., Chandler, D., Cockshull, K. and Prince, G. (2020). Tomato: Phase 3 of an investigation into poor pollination performance by the native bumblebee, *Bombus terrestris audax*. Annual report of contract work undertaken for AHDB. February, 2020, 33pp.

Jacobson, R.J., Chandler, D., Cockshull, K. and Prince, G. (2021). Tomato: Phase 3 of an investigation into poor pollination performance by the native bumblebee, *Bombus terrestris audax*. Annual report of contract work undertaken for AHDB. January, 2021, 17pp.

Kwon, Y.J. and Saeed, S. (2003). Effect of temperature on the foraging activity on *Bombus terrestris* L. (Hymenoptera: Apidae) on greenhouse hot pepper (*Capsicum annuum* L.). *Applied Entomology & Zoology*, **38**, 275 - 280.

Manlik, O., Schmid-Hempel, R. and Schmid-Hempel P. (2017). Parasite infection of specific host genotypes relates to changes in prevalence in two natural populations of bumblebees. Infection, Genetics and Evolution, **56**, 125-132.

Paupiere, M.J., van Haperen, P., Rieu, I., Visser, R.G.F., Tikunov, Y.M. and Bovy, A.G. (2017). Pollen tolerance to high temperatures in tomato. *Euphytica*, **213**, 130.

Picken, A.J.F. (1984). A review of pollination and fruit-set in the tomato *(Lycopersicon esculentum* Mill.). *HortScience,* **59,** 1-13.

Sato, S., Peet, M.M. and Thomas, J.F. (2002). Determining critical pre- and post-anthesis periods and physiological processes in *Lycopersicon esculentum* Mill. exposed to moderately elevated temperatures. *Journal of Experimental Botany,* **53,** 1187-1195.

Vogt, F.D. (1986) Thermoregulation in bumblebee colonies. I. Thermoregulatory versus brood maintenance behaviours during acute changes in ambient temperature. *Physiological Zoology*, **59**, 55 – 59

Weidenmuller, A., Kleineidam, C. and Tautz, J. (2002). Collective control of nest climate parameters in bumblebee colonies. *Animal Behaviour*, **63**, 1065 - 1071.

Weidenmuller, A. (2004). The control of nest climate in bumblebee (*Bombus terrestris*) colonies; interindividual variability and self reinforcement in fanning response. *Behavioral Ecology*, **15**, 120 - 128.

Westhus, C., Kleineidam, C.J, Roces, F. and Weidenmuller, A. (2013). Behavioral plasticity in the fanning response of bumblebee workers: impact of experience and rate of temperature change. *Animal Behavior*, **85**, 27 - 34

Woodard, S.H. (2017). Bumble bee ecophysiology: integrating the changing environment and the organism. *Current Opinion in Insect Science*, **22**, 101 - 108.

