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Title:
Expanding Floral Multimodality

Floral Temperature and Floral Humidity

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctor of Philosophy in the Faculty of Life Sciences

School of Biological Sciences

MAY 2019

Word Count: 62632

Abstract

Flowering plants produce floral displays that both attract pollinators and allow them to learn flower identity. How well these displays do this can be critically important to pollinator and plant success, and thus is also important to the evolution of both mutualists. Floral displays are multimodal, they produce many complex floral signals through different sensory modes (such as visual, olfactory and tactile) simultaneously. Why floral displays are multimodal is not fully understood. This is due to the majority of research focusing upon pollinator responses to single signalling modalities, but also due to the majority of research focusing upon scent and visual signals. This has a consequence that we do not yet know the extent of floral multimodality. While scent and visual modalities are obviously of great importance, in order to fully understand the reasons behind the evolution of floral multimodality we must gain a better understanding of other signalling modalities. In this thesis I investigate two floral signalling modalities further, floral temperature and floral humidity. Through investigation of the traits floral displays produce I show that the temperature across floral displays differs, flowers show temperature patterns. Additionally, I show that elevated floral humidity about the flower is not limited to the single specialist species on which it has been recorded previously. Using captive bumblebees and established behavioural techniques I show that bumblebees can respond to and learn floral temperature pattern and floral humidity differences. This shows floral displays to be more multimodal than previously thought, with floral temperature showing a greater level of complexity and floral humidity having potential to be more widely used. In addition to this I investigate further how floral temperature may function within a multimodal display by testing its capacity to perform roles other than floral recognition which have previously been observed in visual patterns.

This thesis is dedicated to my dad

Nick Harrap

Who never got to see it finished

All that I have
is yours
and yours
and yours

Acknowledgements

I would firstly like to thank my supervisors Heather Whitney, Sean Rands and Natalie Hempel de Ibarra. I would like to say thank you for this opportunity and for your support throughout these last four years.

I am also thankful to the NERC GW4+ doctoral training partnership for providing funding for this PhD project and the means by which I could attend the many training opportunities required to do this work.

I would like to thank the various bee-lab members (Karin Kjernsmo, Dave Lawson, Rich Pearce, Nadia Khuzayim, & Cara Doyle), as well as the members of the Whitney plant science group (Tom Swift, Matt Jacobs, Jay Phrathep, Ulrike Bauer, Heath O'Brien, Taryn Fletcher, Nathan Masters & Allanna Kelly), both past and present. In particular Henry Knowles for his help with robots. I would also like to thank the National Botanic Gardens of Wales and its research team (Natasha de Vere, Laura Jones, Zara Riches, Tim Foster, Alice Hope, Lucy Bidgood, Andrew Lucas, Rhiannon Dowling & Louisa Smith) for letting me visit and work at the gardens and carry out fieldwork there. I'd also like to thank Nicholas Wray and the University of Bristol Botanic Garden for letting me use plant materials. I also need to thank Paul Chappell and the rest of the Bristol University Cross Faculty Workshop for their help building things.

Additionally, I would like to thank all my friends at the LSB, I'm afraid I'd run out of space on the page I name you all. I'd also like to thank my friends at the AHF and associated clubs (likewise not listing everybody). You probably had no idea just how much, but you all helped.

Lastly, I would like to thank my family: Dave, Jonny and my mum.

Authors Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE:.....

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Published Material

Work within this thesis has produced two published papers and one manuscript submitted for review at time of thesis submission. Thus, sections of the thesis will thus have high similarity of content. As stated in the author's declaration (page v) none of these publications have been submitted for any other academic reward. These publications are listed here and at the start of the relevant chapters.

Chapter 2 was published in Royal Society Open Science in December 2018 (Harrap et al., 2018). The full citation for this paper is as follows:

> *Harrap, M. J. M., Hempel De Ibarra, N. & Whitney, H. M., Rands, S. A. 2018. Reporting of thermography parameters in biology: a systematic review of thermal imaging literature. Royal Society Open Science, 5, 181281*
>
> *DOI: 10.1098/rsos.181281*
>
> *URL: http://rsos.royalsocietypublishing.org/content/5/12/181281*

Chapter 3 was published in eLife in December 2017 (Harrap et al., 2017). The full citation for this paper is as follows:

> *Harrap, M. J. M., Rands, S. A., Hempel De Ibarra, N. & Whitney, H. M. 2017. The diversity of floral temperature patterns, and their use by pollinators. eLife, 6, e31262.*
>
> *DOI: 10.7554/eLife.31262*
>
> *URL: https://elifesciences.org/articles/31262*

I am the first author on both these publications. Contributions made by me and by my supervisors, who make up all the remaining authors, are the same as would have been for a normal thesis chapter - with the following minor assistance described on page xv. Within these chapters the papers have been reformatted and parts rewritten or reordered as appropriate to better suit the format of the thesis.

Chapter 7 was adapted from the version presented in this thesis to form a paper published in the Journal of Comparative Physiology A. in early 2019 (Harrap et al., 2019). This paper is currently available online only. The full citation for this paper is as follows:

> *Harrap, M. J. M., Lawson, D. A., Whitney, H. M. & Rands, S. A. 2019, Cross-modal transfer in visual and non-visual cues in bumblebees. Journal of Comparative Physiology A.*
>
> *URL: https://doi.org/10.1007/s00359-019-01320-w*

This publication was adapted from the version presented in this thesis, with additional material added by collaborator Dr David Lawson. I remain first author on this manuscript. The version presented within this thesis represents my own work, with normal contributions by supervisors, and is a version of this work prior to Dr Lawson's contribution.

Declaration of Assistance

The following assistance in the work contained within this thesis was provided:

Chapter 2: Non-English language publications (12/1219 papers) that could not be excluded based on English language materials were processed with the aid of the following native language speakers: Gerardo Arias, Ulrike Bauer, Olivia Davis, Kate Maia, Zoe Nemec Venza, Burak Urgancioğlu, and Gilda Varliero.

Chapter 3: 'Large artificial flowers' were constructed by the Bristol University Cross Faculty Workshop, following my designs. Assistance was provided in species identification by Alanna Kelley (University of Bristol, horticulturalist), Nick Wray (University of Bristol, Botanic Gardens), Natasha de Vere, and Laura Jones (National Botanic Gardens of Wales).

Chapter 4: Robot arm programming of humidity transects was done by Henry Knowles, following a humidity transect design of my devising (adapted from similar transects in von Arx et al. 2012). Initial robot arm operation, but not flower preparation and collection (that pertaining to approximately 20% of species sampled) was carried out by Henry Knowles, the rest by myself. Phylogenies and the associated 'Influences on floral humidity' analysis were constructed, carried out and written up by my supervisor Sean Rands (sections 4.3.6 and 4.4.4). All other analysis was carried out by myself.

Chapter 5: As with chapter 4, robot arm programming of humidity transects, here used to measure artificial flower humidity, was done by Henry Knowles following a humidity transect design of my devising.

Chapter 6: Video footage collected in this chapter (15%,14 bees out of 96) and spectrometer readings (figure 6.2) were collected by MSci student Ed Straw under my supervision.

All other experimental design, data collection, data processing, data analysis and writing was carried out by me with the normal contributions from my supervisors.

Chapter 1: Introduction

Pollination, the transfer of pollen between male and female parts of flowers in angiosperms, normally has to be carried out through a pollen vector. These pollen vectors can be abiotic, non-living, such as wind, water and rain (Cox, 1991; Ackerman, 2000; Aguiar et al., 2012). However most flowering plants depend on pollen transport by animal pollinators (Ollerton et al., 2011), biotic pollen vectors. Pollinators are often insects such as beetles (Gottsberger, 1977; Dieringer et al., 1999), flies (Johnson and Midgley, 1997; Johnson and Dafni, 1998), bees (Heinrich, 1979a), butterflies (Reddi and Bai, 1984; Aguiar et al., 2012) and moths (Eisikowitch and Galil, 1971; Oliveira et al., 2004). Vertebrate pollination also occurs, primarily by bats (Fleming et al., 2009) and birds (Cruden, 1972; Stiles, 1978). For biotic pollination to occur flowers must ensure that they are visited by a suitable pollinator. This pollinator must then have pollen deposited upon its body, visit a compatible flower (normally) of the same species and deposit that pollen on that flower's stigma (Rademaker et al., 1997; Larson and Barrett, 2000). To encourage visitation, flowering plants often produce floral rewards that are sought out by foraging pollinators. In collecting or consuming these rewards from different flowers, pollinators transport pollen between flowers, providing a pollination service. The most common floral rewards are the pollen itself or nectar secreted from floral nectaries, but others such as resins (Armbruster, 1984) and scent chemicals, exist also (Simpson and Neff, 1981).

While the floral rewards plants provide are sought by pollinators, they are rarely alone sufficient to ensure pollinator flower visits. Flowers exist in complex natural environments and need to be located by their pollinators (Kilkenny and Galloway, 2008; Tichy and Kallina, 2014). Furthermore, flowers are competing with other flowers for the attention of their pollinators (Heinrich, 1979b; Chittka et al., 1997; Chittka et al., 1999). For this reason, biotically pollinated flowering plants produce complex floral displays to signal pollinators (Raguso, 2004; Leonard et al., 2012). These floral displays act as "*sensory billboards*" (Raguso, 2004) signalling flower location and identity through various floral signalling pathways. These displays attract pollinators and allow pollinators to locate flowers within the environment (Spaethe et al., 2001;

Chittka and Spaethe, 2007). Furthermore, many pollinators show an ability to learn which flowers are most rewarding (Thomson et al., 1982; Duffield et al., 1993; Carter, 2004). This allows pollinators to recognise these more rewarding flowers and adjust their foraging behaviour accordingly (Heinrich, 1979b). These floral displays have a critical influence on pollinator behaviour, pollen transport and the foraging success of pollinators. Floral signalling is thus of critical importance to understanding the fitness and evolution of both plant and pollinator.

Floral displays are multimodal, a single display simultaneously produces signals through many different sensory pathways that can be detected by pollinators (Leonard et al., 2012). Furthermore within these different signalling modalities flowers show a high degree of complexity, such as patterning of signals (Hempel de Ibarra et al., 2001; Hempel de Ibarra et al., 2002; Leonard et al., 2012; Clarke et al., 2013; Lawson et al., 2018). Perhaps the best-known and best understood of floral signalling modalities are visual signals. Many pollinators differ in their visual systems compared to humans. For example many insects and birds see human-invisible ultraviolet light, while bees are less sensitive to human-red wavelengths (Vorobyev et al., 2000; Davies et al., 2013). Furthermore, insects have compound eyes have a fixed focal distance, leading to 'mosaic vision' with lower resolution and effectively short sightedness (Kirschfeld, 1976; Chittka and Raine, 2006; Hempel de Ibarra and Menzel, 2014). Consequently flowers have reduced visual range and appear quite different to many pollinators than to humans (Vorobyev et al., 2000; Hempel de Ibarra and Menzel, 2014). Nevertheless, there are many visual components of floral displays that pollinators respond to. These include: contrast with background (Spaethe et al., 2001; Hempel de Ibarra et al., 2001; Hempel de Ibarra and Vorobyev, 2009), colour (Dyer and Chittka, 2004c; Dyer and Chittka, 2004d; Dyer et al., 2008), patterning in these aspects (Johnson and Dafni, 1998; Hempel de Ibarra et al., 2002; Hempel de Ibarra and Vorobyev, 2009; Whitney et al., 2013), and flower shape (Rodríguez et al., 2004; Krishna and Keasar, 2018). Visual properties of flowers can be altered by structural elements of the flower surface, such as gloss and iridescence, which can

themselves show patterns (Whitney et al., 2009c; Glover and Whitney, 2010; Whitney et al., 2012; Moyroud et al., 2017). Additionally recent evidence has shown bumblebees can detect and learn the patterns of light polarization reflected off flowers (Foster et al., 2014). Floral scent or odour signals are also well studied, being reported in insect and mammal pollination systems (Wright and Schiestl, 2009), but feature less in bird pollination (Knudsen et al., 2004). Pollinators can detect several aspects of scent signals such as variation in the amounts of scent chemicals produced (Gervasi and Schiestl, 2017) or what olfactory chemicals are produced (Cunningham et al., 2004; Riffell et al., 2008; Filella et al., 2011; Farré-Armengol et al., 2015; Junker and Parachnowitsch, 2015). Additionally, floral scent emissions can show structured shapes or patterns within the flower (Kessler and Baldwin, 2006; Howell and Alarcón, 2007; Balao et al., 2011), which recent evidence suggests bees can learn (Lawson et al., 2017a; Lawson et al., 2018).

While floral displays are multimodal, producing signals in many modalities in addition to scent and visual modalities, the majority of this work is based upon scent and visual modalities (Chittka and Raine, 2006; Hegland and Totland, 2005; Wright and Schiestl, 2009; Leonard et al., 2012; Junker and Parachnowitsch, 2015). Other modalities are comparably overlooked. The next most widely studied floral signalling modality is probably tactile or texture signals. These are often created by the shape and arrangement of petal epidermis cells (Kevan and Lane, 1985; Whitney et al., 2009a; Papiorek et al., 2014). Several pollinator groups, such as moths and bees have been demonstrated to detect and learn these tactile differences between flowers (Kevan and Lane, 1985; Goyret and Raguso, 2006; Whitney et al., 2009a). Other modalities are studied even less. Recent evidence has shown that moths can respond to floral humidity (von Arx et al., 2012) and CO_2 emissions (Thom et al., 2004; Guerenstien et al., 2004; Goyret, 2008) from flowers of *Oenothera caespitosa* and *Datura* flowers respectively. Floral temperature can vary between flowers (Totland, 1996; Seymour et al., 2009b; Dietrich and Körner, 2014; Shrestha et al., 2018). Floral temperature can function as a signal to pollinators, with at least bees showing the ability to learn to associate hotter or

colder flowers with rewards (Whitney et al., 2008; Hammer et al., 2009). Floral electrostatic signals and electrostatic patterns can also be generated by flowers (Clarke et al., 2013). These electrostatic signals can be detected by, at least, bee pollinators (Clarke et al., 2013; Greggers et al., 2013) through mechanical manipulation of the bee's hairs by floral electric fields (Sutton et al., 2016). Bat pollinated flowers also possess unique structures that allow these flowers to reflect echolocation sound emissions. This creates an acoustic signature that indicates flower location to echolocating bat pollinators (von Helversen and von Helversen, 1999). Bats can use differences in this acoustic signatures to learn younger and more rewarding flowers (von Helversen and von Helversen, 2003). These modalities are studied less for several reasons. Many require specialist techniques and equipment to monitor, such as: electron microscopy for tactile signals (Whitney et al., 2009a); infra-red thermometers or cameras for temperature signals (Rejšková et al., 2010); and non-contact voltmeter devices for detection of electrostatic signals (Clarke et al., 2013). Many of these signalling modalities are also recent discoveries (Whitney et al., 2008; von Arx et al., 2012; Clarke et al., 2013), possibly the result of the novel equipment needed to detect them, so have not yet been explored to the extent of scent and visual signals. While scent and visual modalities undoubtedly have an important signalling function to pollinators and are of great importance for plant pollinator interactions (Bradshaw and Schemske, 2003; Chittka and Raine, 2006; Wright et al., 2009b; Schiestl and Johnson, 2013; Junker and Parachnowitsch, 2015), we risk overlooking a large part of floral signalling if we focus only on such modalities. If we are to gain a holistic understanding of how pollinators respond to and interact with natural, and therefore highly multimodal, floral displays we must consider the influence of these additional floral signals on pollinator behaviour (Leonard et al., 2012; Junker and Parachnowitsch, 2015). This will help shed light on the foraging decisions pollinators make and the plant pollinator interactions observed in nature (Galen and Newport, 1988; Engel and Irwin, 2003; Waelti et al., 2007; Hopkins and Rausher, 2012). Furthermore it will further our understanding of plant evolution, particularly the reasons behind evolution of multimodal floral displays (Leonard et al., 2012).

1.1 Floral signalling, pollinator behaviour and plant-pollinator fitness

Floral displays are of critical importance to both plants and pollinators. To maximise foraging success, pollinators need to maximise rewards acquired, with minimum costs to time and energy spent (Heinrich, 1979a; Burns, 2005; Charlton and Houston, 2010). Flowers can vary in the amount of floral rewards they provide (Raine and Chittka, 2007). Thus pollinators benefit from being able to easily detect and locate flowers and quickly learn the most rewarding display at a given time (Raine and Chittka, 2008; Charlton and Houston, 2010). This means the nature of floral displays encountered by a pollinator, in terms of flower detectability and how easily more and less rewarding flowers can be distinguished, influences pollinator foraging success. Plant reproductive success is maximized by ensuring high pollen export to conspecifics, male success (Harder, 1990; Harder and Wilson, 1994), and high pollen receipt from conspecifics, female success (Larson and Barrett, 2000; Knight et al., 2005; Morales and Traveset, 2008). Pollinators are the main vector of pollen transport in biotically pollinated plants. So, behaviour of pollinators, especially in terms of how they respond to floral displays and the signals that make up these displays, will have a major influence and plant fitness (Galen and Kevan, 1983; McCall and Primack, 1992; Johnson and Nilsson, 1999; Bradshaw and Schemske, 2003; Schiestl and Johnson, 2013; Wilson et al., 2017). Patterns of pollen transport and outcrossing are often linked to patterns of pollinator foraging behaviour and floral signalling (Galen and Newport, 1988; Engel and Irwin, 2003; Waelti et al., 2007; Hopkins and Rausher, 2012). Thus, floral evolution should favour displays that encourage pollinators to visit flowers and efficiently transport pollen between conspecifics (Wright and Schiestl, 2009; Leonard et al., 2012; Schiestl and Johnson, 2013). This includes floral signalling traits that influence pollinator visitation to the flower, but also traits such as morphological fit to the pollinator that can improve transfer efficiency (Hegland and Totland, 2005; Krishna and Keasar, 2018). However, there is a mismatch in plant and pollinator goals. Pollinators do not 'need' to pollinate if they obtain rewards from flowers. Pollinators will feed from flowers without providing a pollination service, a behaviour known as 'floral larceny' (Inouye, 1980). Floral

larcenists are flower visitors that remove floral rewards but provide little or no pollination services, thus they can have several effects on pollen transport of plants they visit (Hargreaves et al., 2009; Goulson et al., 2013; Solís-Montero et al., 2015). Likewise, floral signals may evolve to manipulate pollinators, encouraging (from the pollinators perspective) less optimal foraging decisions that may be better for pollen transport (Chittka et al., 1999; Schiestl, 2005). There are several strategies to maximise pollen transport. The best strategy of floral signalling for a given species can depend on the community the plant is found within (Schiestl and Johnson, 2013). In this section, I will discuss the various manners in which pollinators respond to floral displays and how this has important consequences on the fitness and evolution of both plant and pollinator.

Flowers that are harder for pollinators to detect, those with less salient floral display signals, take longer to find (Spaethe et al., 2001; Dyer and Chittka, 2004b; Hegland and Totland, 2005; Chittka and Spaethe, 2007; Dyer et al., 2008) or are not visited at all (Borges et al., 2003). If pollinators struggle to find food, foraging success will be low (Raine and Chittka, 2008). A low frequency of flower visits by pollinators will compromise pollen export and lead to pollen limitation, a lack of pollen receipt (Larson and Barrett, 2000; Engel and Irwin, 2003; Ashman et al., 2004; Farré-Armengol et al., 2016). Plants may possess a self-compatibility mechanisms and automatic self-pollination mechanisms which can limit the costs of low visitation (Larson and Barrett, 2000; Aguiar et al., 2012), but self pollination can have separate costs to fitness (Harder and Wilson, 1994; Husband and Schemske, 1995; Husband and Schemske, 1997). For this reason, floral displays appear to be adapted to stand out from the background (Naug and Arathi, 2007). Most floral signals represent to some extent a ‘contrast’ with the background. This is perhaps clearest in visual signals. Bees use their brightness sensitive L-receptor, sensitive predominantly to wavelengths in the human green region, to detect flowers visually at longer distances and chromatic contrast can be used at shorter distances (Spaethe et al., 2001; Hempel de Ibarra et al., 2001; Hempel de Ibarra et al., 2002; Hempel de Ibarra et al., 2015). A greater contrast in terms of both colour and the L-receptor

contrast with the background will lead to faster and longer range discovery by bees (Hempel de Ibarra et al., 2001; Spaethe et al., 2001; Hempel de Ibarra et al., 2002). The colouring of many bee-pollinated flowers have consequently evolved to aid detection in this way (Hempel de Ibarra and Vorobyev, 2009).

Flowers benefit from signals that help them stand out and be attractive to their pollinators. Many attractive signals are those that the pollinators have an innate preference for. Pollinator innate preferences reflect which signals pollinators will favour prior to any learning (Raine and Chittka, 2007; Schiestl and Johnson, 2013). These can result to pre-existing sensory biases of the pollinator. In such instances plants are producing signals to which pollinators have a pre-existing adaption to detect and respond to. This is well documented in colour signals where insect visual systems are adapted to respond to dark-radial patterns associated with insect nest burrows (Biesmeijer et al., 2005). Insect visual systems are evolved to detect such patterns more clearly, especially in terms of the insect L-receptors (Hempel de Ibarra et al., 2001). This more salient colour pattern arrangement is widespread in bee pollinated flowers (Hempel de Ibarra and Vorobyev, 2009; Hempel de Ibarra and Menzel, 2014), likely as a result of its benefits for flower detection and pollinator preferences (Biesmeijer et al., 2005). Similarly, many floral scent compounds have high similarity to insect signalling pheromones, to which pollinators are adapted to detect and innately attracted to (Borg-Karlson, 1990; Schiestl et al., 1999; Schiestl, 2010; Stökl et al., 2011). Other preferences of pollinators may be related to environmental preferences of insects. Such as insect preference for elevated floral temperatures (Seymour and Schultze-Motel, 1997; Seymour et al., 2003; Dyer et al., 2006). Producing signals that align with pollinator preferences will increase the likelihood of these flowers being visited (Galen and Kevan, 1983; Naug and Arathi, 2007; Shi et al., 2008; Stökl et al., 2011; Filella et al., 2011). This may be particularly beneficial for less rewarding flowers as they may gain visitation by naïve bees (those without flower experience) before they learn of other more rewarding species in the environment (Gumbert, 2000; Naug and Arathi, 2007; Goyret et al., 2008b).

This is believed to have resulted in the numerous instances of floral displays converging on signals preferred by or more salient to their associated pollinators (Fenster et al., 2004; Fleming et al., 2009; Hempel de Ibarra and Vorobyev, 2009). Similarly the differential sensitivities and preferences of different pollinator groups is thought to be important in creating isolation mechanisms between different floral displays, driving speciation in angiosperms (Bradshaw and Schemske, 2003; Harder and Johnson, 2009; van der Niet and Johnson, 2012; Gervasi and Schiestl, 2017). While it may seem plants exploit these preferences, innate preferences can be adaptive to the pollinator if they align with the most rewarding flower species, as they will encourage pollinators to visit rewarding flowers without having to acquire experience. Raine and Chittka (2007) demonstrated that bumblebee colonies whose colour preferences aligned with the most rewarding flower species in their environment had greater foraging success. This suggests that pollinator preferences are under selection to align with the most rewarding flower species. Such pollinator preferences can be quickly altered as pollinators learn aspects of floral displays altering foraging to favour signals associated with the most rewarding flowers (Heinrich, 1979b; Gumbert, 2000; Cunningham et al., 2004; Goyret et al., 2008b; Riffell and Alarcón, 2013; Russell et al., 2016). Many pollinators can very quickly learn to favour flowers against their innate preferences, if such displays are more rewarding. This is shown by bumblebee learning to favour visits to cold flowers when naïve bumblebees prefer hot flowers (Whitney et al., 2008). However, signals that align with pollinator preferences can still influence experienced pollinator foraging decisions when choosing to switch foraging species (Gumbert, 2000; Lynn et al., 2005; Riffell et al., 2008).

By learning the floral display pollinators are able to focus visitation on the most rewarding food sources available at a given time. This can reduce the loss of energy and time wasted visiting less rewarding species, maximizing foraging success (Raine and Chittka, 2008). When floral signals are very similar between different flowers, and less easily distinguished, pollinators are more likely to make ‘mistake visits’ to less rewarding species (Dyer and Chittka, 2004c; Dyer and Chittka, 2004d; Peter and Johnson, 2008) and take more

time to make foraging choices (Dyer and Chittka, 2004c; Skorupski et al., 2006; Chittka and Spaethe, 2007; Wright et al., 2009a). Increased time taken and mistake visits will reduce a pollinators' foraging efficiency (Raine and Chittka, 2008; Charlton and Houston, 2010), the cost of mistake visits varying depending on the differences in reward levels and handling (Lynn et al., 2005; Chittka and Raine, 2006). So, pollinators benefit from foraging on easily distinguishable floral displays, where the most rewarding species can be easily identified. When foraging on similar displays pollinators appear to forage in such a way that they reduce uncertainty and the chances of mistake visits. This has been described in terms of 'peak shift' behaviours where, following conditioning to rewarding and unrewarding stimuli (colour), bumblebees favour novel stimuli that are shifted from the rewarding stimulus in a direction away from the unrewarding stimulus (Lynn et al., 2005; Leonard et al., 2011a). This response is the result of generalisation responses to positive and negative stimuli and foraging decisions to avoid stimuli that bees are uncertain are rewarding. This may lead to pollinators favouring the members of a rewarding species that are most distinctive from other displays or a completely separate, but easily identifiable, species. This further demonstrates the importance of flower learning to understanding pollinator foraging and how plants with distinctive floral displays can be at an advantage in terms of pollination as a result of pollinator learning. This shifting to new distinguishable forage can occur when the distinguishable novel forage is less rewarding if costs or frequency of mistakes are sufficiently high (Lynn et al., 2005). Consequentially, pollinator learning may drive evolution of more district floral displays (Belsare et al., 2009).

As pollinators learn a rewarding display their behaviour begins to align better with that plant's goals (Chittka et al., 1999; Wright and Schiestl, 2009; Schiestl and Johnson, 2013). As a flower's display is learnt to be rewarding, it will receive greater visitation rates, with benefits to pollen transport (Heinrich, 1979b; Engel and Irwin, 2003). Additionally, as pollinators learn floral displays, they show increased flower constancy (Wilson and Stine, 1996; Chittka et al., 1999; Gegear and Laverty, 2005; de Jager et al., 2011), a temporary focus on visiting similar

flower displays without switching to another display. This behaviour has been noted to be particularly influenced by learned associations with particular signals such as colouring (Wilson and Stine, 1996; Gegear and Laverty, 2005; de Jager et al., 2011), leading pollinators such as bees, to focus on flowers similar to their experience over other options, even more rewarding options (Wilson and Stine, 1996; Chittka et al., 1999). These behaviour changes with learning increases the likelihood that next flowers visited are of the same species, improving flower export (Rademaker et al., 1997). Similarly, the previous flower visited by a pollinator is more likely to be a compatible conspecific. This increases the chances of receiving compatible pollen and reducing the chances of receiving incompatible pollen. Receipt of incompatible pollen can block the stigma surface and interfere with compatible pollen receipt (Morales and Traveset, 2008; de Jager et al., 2011). Due to these benefits a rewarding flower species may favour selection for floral signals that make their display easy to learn and distinguish from other displays in their environment (Peter and Johnson, 2008; Wright and Schiestl, 2009; Belsare et al., 2009; Leonard et al., 2011b; Schiestl and Johnson, 2013).

While there are benefits of distinctive floral displays to rewarding species (Belsare et al., 2009), for less rewarding species there may be an advantage to possessing similar displays to rewarding species. Indeed, such deceptive strategies and mimicry are common within flowering plants, particularly orchids (Jersáková et al., 2006). Further extreme examples of deception include flowers mimicking female insects for pollination via pseudo-copulation (Schiestl, 2005; Ellis and Johnson, 2010). Though, deceptive strategies include general mimics which do not appear to mimic a specific target flower, rather possessing signals generally attractive to pollinators (Shi et al., 2008), and mimics that replicate displays or signals of specific more rewarding target species in their environment (Hegland and Totland, 2005; Schiestl and Johnson, 2013). These mimics can be less rewarding than targets or totally unrewarding. It would appear that such mimics exploit pollinator preferences, mistake visits or generalization responses to floral signals of pollinators searching for similar displays, exploiting pollinator action but paying reduced costs of reward production (Johnson et al.,

2003; Schiestl, 2005; Jersáková et al., 2006; Peter and Johnson, 2008). However, the advantage of mimicry appears to be dependent on the density of rewarding and mimic species (Schiestl, 2005). As mimicry or erroneously perceiving the target display as less rewarding can lead to its (and the mimics) abandonment for foraging (Lynn et al., 2005).

Learning can be of further importance as reward levels in a pollinators' foraging environment can change dramatically over time (Pleasants, 1981; Kaeasar et al., 2008). This can be the result of plant daily or seasonal cycles (Wyatt et al., 1992; Langenberger and Davis, 2002; Silva and Dean, 2004), or simply the action of pollinator feeding (Heinrich, 1976; Heinrich, 1979b; Kaeasar et al., 2008). Thus, pollinators are required to constantly learn, update information and adjust visitation alongside these changes. This will mean distinctive displays will help pollinators quickly learn 'newly' more rewarding species alongside these changes (Heinrich, 1979b; Pleasants, 1981).

It is worth mentioning that what flowers are 'most rewarding' to a particular pollinator will vary. Not all floral resources will be available to every pollinator (Harder, 1983; Corbet, 2000; Krishna and Keasar, 2018). Flowers may provide vast quantities of floral rewards but be inaccessible for certain pollinators. This can be due to width or depth of flowers limiting access to pollinators that are too small or have short tongues (Heinrich, 1976; Heinrich, 1979a; Harder, 1983; Graham and Jones, 1996). Conversely, certain pollinators may find feeding on shallower, more generalist, flowers difficult and time consuming as a result of long tongues (Heinrich, 1976; Heinrich, 1979a; Peat et al., 2005). Other aspects like pollinator's ability to learn complex displays (Krishna and Keasar, 2018) and perform mechanical manipulations required to access floral rewards (Heinrich, 1979a), such as buzz pollination (De Luca et al., 2013; De Luca and Vallejo-Marín, 2013), may also limit access to floral rewards by certain pollinator species. These differences in handling can mean what flower gives the most rewards for least effort, in terms of time and energy, may differ between pollinator species (Ranta and Lundberg, 1980; Corbet, 2000). Additionally, as some pollinators can show within species variation in morphology, particularly bumblebees, leading to different optimal flower choices

between individuals of the same species (Heinrich, 1979a; Peat et al., 2005). As such partitioning of pollinator and plant resources, and changes in reward availability with time, means that many species will benefit from distinguishable displays, even if they do not produce the most rewards per flower.

How pollinators respond to floral displays is a critical part of understanding the foraging decisions a pollinator makes (McCall and Primack, 1992; Hegland and Totland, 2005), and thus the pollination service it provides to plants. Understanding pollinator responses to floral displays, which they are attracted to and what they can or cannot distinguish, can be important part in explaining patterns of flower visitation and the interactions that occur between plants and pollinators (Memmott, 1999; Hawkins et al., 2015; de Vere et al., 2017; Lucas et al., 2018). Most flowering plants (Ollerton et al., 2011) and approximately 70% of crops, making up 35% of global production volumes, depend on animal pollinators (Klein et al., 2007). As plant reproductive success often depends on how pollinators respond to floral displays, understanding how pollinator respond to floral signals can have further consequences to our understanding and maintenance of pollinator services within natural and agricultural environments. As pollinator responses to floral displays are critical to explaining plant and pollinator success and fitness pollinator behaviour in response to floral signals has a critical impact on understanding floral evolution and the evolution of angiosperms more generally (Bradshaw and Schemske, 2003; Leonard et al., 2011b; Hopkins and Rausher, 2012). Thus, understanding how pollinators respond to floral displays and the signals that are part of these displays, in addition to furthering our understanding floral evolution, can have useful consequences to our understanding of how ecosystems function.

1.2 Floral Multimodality

The responses of pollinators to floral displays have a high impact on both plant and pollinator fitness and are critical to our understanding of pollinator ecology (as described above). However, most of our understanding of floral signalling is based on responses to single modality differences in displays (Leonard et al., 2012; Junker and Parachnowitsch, 2015). While the multimodality of floral signals has been recognised for a long time (von Frisch, 1914; von Frisch, 1919), the reasons for the evolution floral multimodality, and thus the influence of additional floral signalling modalities, are only just beginning to be explored (Leonard et al., 2011b; Leonard et al., 2012; Leonard and Masek, 2014). Multimodal signalling likely increases metabolic costs (Helsper et al., 1998; Galen, 1999) and floral signals can attract unwanted visitors, such as herbivores and larcenists (Baldwin et al., 1997; Adler and Bronstein, 2004; Kessler and Halitschke, 2009; Solís-Montero et al., 2015). Plants must benefit from producing multimodal signals in some manner, otherwise why incur these costs? Several explanations for signal multimodality in nature exist (Candolin, 2003; Partan and Marler, 2005). These explanations have been categorised into three main categories by Hebets and Papaj (2005), and then applied to floral multimodality by Leonard and colleagues across a series of reviews (Leonard et al., 2011b; Leonard et al., 2012; Leonard and Masek, 2014). These categories are “content-based hypotheses”; “efficacy-based hypotheses”; and “inter-signal interaction hypotheses” (Hebets and Papaj, 2005). In the following sections I shall summarise these groups of hypotheses, adding evidence published since these reviews. In this way I demonstrate the potential importance of additional floral signalling modalities on pollinator responses to the floral display, highlighting the need to consider the full range of signal modalities to a gain a complete understanding of plant pollinator interactions.

1.2.1 *Content-based hypotheses*

Content-based hypotheses relate to 'what' the floral message is (Leonard et al., 2012). These suggests that all the information the plant sends to the pollinator, the 'full floral message', is transmitted incompletely via one modality, so many are needed to accurately convey the full message. Perhaps the most straightforward explanations of floral multimodality is the 'redundant signal hypothesis'. This suggests that a single modality transmits the floral message imperfectly. Consequently, many floral signals, transmitting the same information, are needed to send the complete message (Leonard et al., 2012). Flowers often benefit from being more clearly identified, recognised and learnt (see above), this hypothesis suggests multimodality improves this by allowing the plant to send more complete information. From a floral learning standpoint this can be thought of as multimodality allowing a greater number of ways a flower can differ and make itself more distinctive. This is supported in many instances, although this evidence can be consistent with other explanations. When rewarding and nonrewarding artificial flowers differ in scent as well colour bumblebee foragers have been demonstrated to learn faster (Kunze and Gumbert, 2001) and achieve higher levels of correct choices (Kaczorowski et al., 2012; Katzenberger et al., 2013), than when flowers differ only in colour. Kulahci et al. (2008) found similar results with flowers differing in shape and scent. Likewise, Clarke et al. (2013) found flowers differing in electrostatic patterns as well as colour also were learnt faster than flowers differing in colour alone.

Nityananda and Chittka (2015) found bumblebees can use multimodal signals to carry out complex foraging tasks that are beyond the bee's capacity to do based on a single modality. Bees were tasked with distinguishing rewarding flowers, from nonrewarding distractors and avoiding simulated predator attacks on rewarding flowers. Bees were unable to avoid predation when rewarding flowers and 'predators' both had to be distinguished visually. However, when scent indicated rewarding flowers and predators were indicated by their visual signals, bees could select safe rewarding flowers from those with predators. This demonstrates how multimodality may allow pollinators to carry out difficult foraging tasks by

splitting attention across modalities to different aspects of the display. While in Nityananda and Chittka (2015) bees were tasked with both a reward search and a predation avoidance task, with differences in the display pertaining to the spiders' presence rather than the flower itself, it is possible this separation of attention between modalities may similarly apply to difficult floral recognition tasks.

Content-based hypotheses include the 'multiple messages hypothesis'. This posits that different parts of the full message are transmitted through different modalities. Thus, different modalities have additional or alternative functions. So, to elicit a full response flowers need many signals transmitting different information. The exact function of a given signal may vary with flower species or pollinator. Individual modalities may be used to find flowers, signalling flower location. Others may signal flower identity and are thus used for flower learning. Wright and Schiestl (2009) and others (de Jager et al., 2011; Riffell and Alarcón, 2013) proposed that floral scent's primary function is signalling flower identity, suggesting other signals being more important for locating the flower. This may explain why scent varies greatly between flower species (Wright and Schiestl, 2009; Farré-Armengol et al., 2015) while visual signals have been seen to be more similar, varying more in species under high selective pressure to be distinctive (Gumbert et al., 1999). Some floral signals might inform pollinators about the level of rewards in the flower (Galen and Newport, 1988; von Arx, 2013). Signals associated with rewards directly, which get removed with them, may provide this function. This may include scented or coloured nectar and pollen (Kessler and Baldwin, 2006; Hansen et al., 2007; von Arx, 2013). Similarly, floral humidity is associated with nectar levels in individual *O. caespitosa* flowers and may indicate reward presence directly (von Arx et al., 2012). Signals directly indicating reward status, 'honest signals', would allow pollinators to avoid recently emptied flowers, increasing pollinator foraging efficiency, and the attractiveness of those flowers (von Arx, 2013; Knauer and Schiestl, 2015).

Included within the multiple messages hypothesis is the idea that different signals are signalling at different scales. Signalling longer distances has benefits for detection and

pollinator attraction (Spaethe et al., 2001) but pollinators may also need short range signals to find flower location (Goyret et al., 2008a; Balkenius and Dacke, 2010). Bees have been repeatedly seen to use L-receptor contrast to locate flowers at longer range and coloration for shorter range flower location (Hempel de Ibarra et al., 2001; Hempel de Ibarra and Menzel, 2014; Hempel de Ibarra et al., 2015). Hawkmoth *Manduca sexta* struggle to locate feeders at long and short range depending on whether scent or visual cues were missing (Raguso and Willis, 2002; Balkenius and Dacke, 2010). At long range moths could find the flower's general location when scent cues were present but would overshoot and end up circling if no visual signal was paired with it. Suggesting scent cues are needed to detect the flower at long range but shorter range location required a visual cue (Balkenius and Dacke, 2010). *Manduca sexta* showed similar responses to floral CO_2 emissions, as did *Tetralonia* bees foraging on deceptive orchid *Ophrys heldreichii* to loss of scent and visual cues (Streinzer et al., 2009). Other signals may work as indicators of reward locations within the flower, functioning as 'floral guides' to visitors (also known as 'nectar guides'). Colour (Leonard and Papaj, 2011; Goodale et al., 2014) scent (Lawson et al., 2017b) and tactile patterns (Goyret and Raguso, 2006; Goyret and Kelber, 2011) function as floral guides that lead the pollinator to rewards. Floral guides reduce handling time, making flowers less costly to pollinators (Waser and Price, 1983), and improve pollen transfer (Hansen et al., 2011). Floral guides may thus benefit the plant even if they have no function in terms of actually recognising or locating the flower.

While not directly related to signalling and not touched upon by Leonard and colleagues when considering occurrence of many signalling related traits within a display, some floral traits that can function as signals show additional functions independent of the floral signals sent to the pollinator. A well-documented example of this is floral texture's function in influencing insect grip of petals (Whitney et al., 2009a; Papiorek et al., 2014). Tall conical petal epidermal cells can aid flower grip, while ovoid cells can make it difficult for the petal to be gripped. This can make it advantageous for flowers to evolve conical cells on parts of the flower where legitimate visitation should be promoted, and ovoid cells where larcenist

visitation should be discouraged (Papiorek et al., 2014). Petal micro-texture may also influence the generation of other signas, such as structurally generated visual properties (Whitney et al., 2009c; Glover and Whitney, 2010) or temperature (Whitney et al., 2011). Colouration also helps floral temperature generation (Rejšková et al., 2010). Floral temperature itself can warm pollinators while they feed, minimising costs of feeding, acting as a floral reward (Rands and Whitney, 2008). Such additional functions may make these traits beneficial for the plant, thus may evolve or be maintained in many displays even when they do not aid floral signalling (i.e. they have benefit but may not be a floral signal in all cases).

1.2.2 *Efficacy-based hypotheses*

Efficacy-based hypotheses relate to how the signal is effectivity sent or received. 'Signal detection hypotheses', in a manner similar to redundant signal hypotheses, suggest multiple signals sent at once through different modalities are detected more easily and quickly. This would be beneficial to plant and pollinator as visitation rates would improve (Leonard et al., 2012). The similar 'parallel-processing hypotheses' suggest that, rather than transmission being improved, multimodal signals allow pollinators to process floral messages more quickly along different neural pathways relating to each modality. This may lead to faster foraging decisions or improved learning (Leonard et al., 2012). These hypotheses could explain results seen in Kunze and Gumbert (2001), Kulahci et al. (2008) and Katzenberger et al. (2013). Some efficacy-based hypotheses relate to how different floral signals are degraded or obscured as they are transmitted through the environment. The 'efficacy trade-off' hypothesis suggests that the differing modalities have constrains in how well they transmit through the environment. This suggests some signals may be obscured or degraded at range, while others may be less capable of indicating flower location at close range. Possession of many multimodal signals thus allows the floral message to reach the pollinator at varied scales. This hypothesis relates very closely with parts of the multiple messages hypothesis, potentially explaining why different modalities functioning at different scales may be necessary.

The ‘efficacy-backup hypothesis’ suggests multimodality allows flowers to better cope with disruption of floral signalling within variable natural environments. Efficacy backup suggest different signals are disrupted by different environmental conditions. This does appear to be the case. For example, floral colour signal detection is disrupted by darker conditions as many pollinators have only partial colour constancy (Dyer and Chittka, 2004b; Arnold and Chittka, 2012; Chittka et al., 2014), but scent detection is unaffected by light. Similarly, light winds insufficient to prevent pollinator foraging interfere with scent plumes (Lawson et al., 2017a), and likely similar ‘chemical plumes’ like CO_2 and humidity, but light wind is unlikely to influence visual signalling. Multimodality may allow floral signalling to be robust to variable environmental conditions, as it increases the chances that at least one modality should still be detected and recognised by the pollinator when others are interrupted (Ay et al., 2007; Leonard et al., 2012). This suggests more multimodal flowers are more robust to variable conditions. Efficacy backup within multimodal displays has been observed several times. The decrease in bumblebee ability distinguish coloured flowers in dark conditions is lessened when flowers also have different odour signals (Kaczorowski et al., 2012) or differ in shape (Dyer and Chittka, 2004a). Similarly Lawson et al. (2017a) found the impact wind had on bumblebee flower recognition is less when flowers differed in visual patterns as well as scent patterns, than when they differed in scent patterns alone.

The ‘perceptual variability hypothesis’ suggests additional floral signals allow plants to attract a wider range of pollinators (Leonard et al., 2012). As pollinators have different sensory systems and preferences (Fenster et al., 2004), different pollinators may show different responses to different modalities. Attracting a wider range of visitors can be beneficial if pollinator population sizes or composition are variable over the season or from year to year. In this way the plant would be more robust to changes in the pollinator community. Different kinds of pollinator can show different foraging ranges (Walther-Hellwig and Frankl, 2000; Borges et al., 2003; Greenleaf et al., 2007), and can differ in how long they retain pollen and how likely they are to deposit it on flowers immediately following removal (Castellanos et al.,

2003; Richards et al., 2009). Thus, encouraging attraction of a wider compliment of pollinators may allow plants to reproduce with more distant, and likely less closely related, populations. In this way multimodality may be important for promoting connectivity between plant populations. Scent attraction in *Aphelandra acanthus* is associated with nocturnal bat pollinator attraction. However, this species also receives floral visits from bird pollinators that use yellow colour signals to find flowers (Muchhula et al., 2009). As birds do not respond to scent signals, and nocturnal bats do not use *A. acanthus*' visual signals, it is thought the different modalities function evolved to attract the different pollinators, supplementing visitation (Muchhula et al., 2009). Similarly, removal of UV reflective bracts associated with flowers of *Mussaenda frondosa* reduced birdwing and butterfly visitation but not that of bees or bird visitors, while removal of flowers had the opposite effect (Borges et al., 2003). While bird and bee visitors were poor pollinators in this instance, this result might suggest birdwings and butterflies were using the UV signal associated with bracts to locate flowers, while other visitors used signals associated directly with the flower (Borges et al., 2003).

1.2.3 *Inter-signal interaction hypotheses*

Inter-signal interaction hypotheses suggest additional signals alter the context in which pollinators respond to other signals, or help focus pollinator attention to those other signals, enhancing flower learning and detection (Leonard et al., 2011b; Leonard and Masek, 2014). As many floral signals are encountered in other contexts, such as dark radial patterns of nest burrows (Biesmeijer et al., 2005), and insect pheromones (Borg-Karlson, 1990; Schiestl et al., 1999; Stökl et al., 2011), pollinators may need to be given context through other modalities to recognise these signals as a floral food source. Differences between flower morphotypes of *Gorteria diffusa* in tactile and structural elements of spots on petals, as well as the number of spot's influence whether male bee-flies feed from flowers or attempt to mate with spots (pollination by psedo-copulation) (Johnson and Midgley, 1997; Ellis and Johnson, 2010). A possible explanation for these differences in behavioural response is additional signals

change the context in which bee-flies respond to coloured spots on *G. diffusa*. Hawkmoths have been observed to require both visual and olfactory signals to elicit proboscis extension responses (Raguso and Willis, 2002), even though both signals can attract the moth to flower location. As moths will feed on visible flowers with odour present but not coupled to the flower it seems that scent gives context to which moths respond to colour (Raguso and Willis, 2002). CO_2 signals have also been shown to provide a similar contextual signal to hawkmoths (Goyret, 2008; Goyret et al., 2008a). Pollinators can show other responses that are apparently an innate reaction to the floral display. For example, buzz pollination behaviours of bumblebees upon visiting certain flowers (De Luca and Vallejo-Marín, 2013; Morgan et al., 2016). It is possible these innate responses are triggered by some, currently unknown, context signal.

In a similar way additional cues might help focus attention on differences in others. Lawson et al. (2018) found matching scent and visual patterns were learnt faster than non-matching patterns, suggesting overlapping patterns of different modalities reinforced pattern learning. Kunze and Gumbert (2001) found that bees foraging on flowers that presented scent and colour but differed only in colour were learnt faster than flowers showing the same colour differences but lacking scent. Similarly Leonard et al. (2011a) found scents' presence improved bumblebee colour learning, as seen by reduced uncertainty shown by bees in a peak shift experiment, even when scent did not provide information on flower identity. The presence of scent acting to trigger recall and focus attention of bees to colour differences, improving colour learning, is a consistent explanation of these results. This is supported by findings that scents can trigger recall of rewarding visual signals associated with them. When bees are trained on feeders that differ in scent as well as colour, the addition of the associated scent to the hive can encourage visitation to unscented feeders of the colour associated with that scent (Reinhard et al., 2004). Similarly, bumblebee learning of a single modality scent pattern may lead to bees developing preferences for a matching visual pattern (Lawson et al., 2018). This response showed that bumblebees can show cross-modality pattern learning from

scent to visual patterns. These ‘attention triggering’ signal interactions (Leonard et al., 2011b) between scent and visual modalities are likely the result of neurological links between scent and visual memories and learning in bees (Leonard and Masek, 2014).

The ‘attention consuming hypothesis’ suggests that multimodal learning causes focus on multiple aspects of the display thus outcompetes simpler displays for attention (Leonard et al., 2011b), by attempting to monopolise attention via many modalities and encourage bees to focus on the flower. This is supported by increased constancy bees show to multimodal flowers (Gegear and Laverty, 2005). In this way multimodality may represent an adaptation to encourage flower constancy in pollinators.

It is worth noting that many of these hypotheses of the benefits associated with floral multimodality and causes of its evolution have high overlap. These hypotheses work better as a framework to discuss the many potential benefits of multimodality than as distinct explanations (Leonard et al., 2012). This also consequentially means that the results of several studies can be explained in terms of several related hypothesis (Kunze and Gumbert, 2001; Kulahci et al., 2008; Balkenius and Dacke, 2010). Although sometimes this may be the result of work still being required to prize apart the causes of certain pollinator responses. Furthermore, for the most part these various explanations are non-exclusive of each other; one benefit being the case does not prevent others applying alongside or in other situations. For example, it is possible additional signalling modalities both make flowers easier to learn and detect (redundant signal hypothesis) but also make the plant more robust to weather conditions (efficacy backup), these paired benefits were in fact observed by Kaczorowski et al. (2012).

1.3 Overlooked floral signals

Floral signalling and pollinator responses to floral signals have critical influences on plant and pollinator fitness and evolution, as discussed in the previous sections. Furthermore 'additional' modalities incorporated into the floral display appear to have important influences on how pollinators respond to floral displays, even if the full range of advantages of multimodality are not fully understood (Leonard et al., 2011b; Leonard et al., 2012). However, the main focus has been upon pollinator responses to floral scent and visual signals. Other floral signalling modalities, particularly those more recently discovered, are comparably less studied, especially within studies of floral multimodality (but see Goyret and Kelber, 2001 and Clarke et al., 2013). Such modalities include floral texture, temperature, humidity, CO_2, electrostatic and acoustic signals. We lack information on many of these overlooked modalities. In several instances we lack information on their diversity outside of specific specialist pollination systems. This is perhaps best shown by floral humidity signalling, were responses have only been demonstrated by the hawkmoth pollinator of a single flower species, *Oenothera ceaspitosa*. Thus, it is unclear if other flowers produce signals in these modalities, let alone whether other pollinator groups can respond to them. We lack information on what aspects of these signals pollinators can respond to, such as patterns, or differing intensities. Further research on these, thus far, less studied floral signals will improve our understanding of what aspects of the floral display pollinators can respond to and the foraging decisions pollinators make when visiting flowers. This will also expand our scope of how multimodal flowers are, furthering our understanding of floral evolution. Furthermore, once a good grounding in pollinator responses to these additional modalities is gained, we can begin to incorporate these less studied signals into more direct studies of floral multimodality. As the multimodal displays of real flowers are made up of more than scent and visual signals such studies are required to gain a full understanding of floral multimodality, it's evolution and how pollinators respond to natural flowers.

In this thesis I aim to increase our understanding of floral signalling to pollinators by investigating these overlooked floral signalling modalities. I expand our understanding of how multimodal flowers are, by further investigating floral temperature and floral humidity. I investigate floral patterns in temperature, that have (until now) been overlooked in a floral signalling context (chapter 3), despite being observed in floral thermal imaging studies (Rejšková et al., 2010; Dietrich and Körner, 2014). The diversity of floral humidity is then assessed on a wide range of species to evaluate how common this floral trait is (chapter 4). In addition to evaluating the diversity of these two floral traits, I also investigate whether bumblebees can respond to floral temperature patterns and floral humidity in a flower foraging context, thus evaluating these traits capacity to function as floral signals (chapter 3 and 5). Lastly, I begin to assess floral temperature patterns function within a multimodal display. Here, I assess the extent to which temperature patterns show similar functions demonstrated by visual patterns within multimodal displays, namely floral guide functionality (chapter 6) or cross-modality pattern learning (chapter 7).

Furthermore, in this thesis, I aim to develop new methods and improve upon existing methodologies in biological science. This involves development of novel protocols for sampling floral humidity (chapter 4). Additionally, I assess the quality of reporting of thermal imaging techniques in biological sciences and set up guidelines on how best these devices should be used in the future (chapter 2). In this manner I aim to facilitate future study on these overlooked floral signals.

The remaining chapters of this thesis take the following structure:

- **Chapter 2: Reporting of thermography parameters in biology: a systematic review of thermal imaging literature.** In this chapter the principles of infrared thermography tools, used throughout the thesis, are discussed. Then the quality of reporting of such techniques by biological scientists is assessed using a systematic review.

- **Chapter 3: The diversity of floral temperature patterns, and their use by pollinators.** Here the diversity of floral temperature patterns over a range of flowering plants is explored. Then the capacity of bumblebees to respond to differences in temperature patterns as a foraging signal for flower recognition is investigated.

- **Chapter 4: Measurement of floral humidity.** In this chapter floral humidity production by a range of flower species is surveyed using a novel protocol utilizing robotic tools.

- **Chapter 5: Floral humidity signals: bumblebee detection of floral humidity.** In this chapter the capacity for bumblebees to respond to differences in floral humidity is tested. Here whether bumblebees have innate preferences, and whether they can form learnt associations, based on floral humidity differences between flowers is investigated.

- **Chapter 6: The capacity of temperature patterns to function as floral guides.** In this chapter I investigate whether temperature patterns that are spatially associated with floral rewards may function as a floral guide for bumblebee pollinators. Furthermore, the effect of overlapping temperature and visual patterns on floral guide functionality is explored.

- **Chapter 7: Temperature patterns in multimodal displays: cross-modality pattern learning.** Here the capacity for bumblebees to show cross-modality pattern learning between matching visual and temperature patterns is investigated.

- **Chapter 8: Thesis discussion.** In this final chapter I summarize the findings of the previous chapters and discuss the implications of these findings and avenues for future research.

Chapter 2: Reporting of thermography parameters in biology: a systematic review of thermal imaging literature

The following chapter is adapted from a published paper of which I am first author: Harrap et al. (2018), Open Science Royal Society, 5, 181281. It will thus have high similarity with this publication. As indicated on page xiv, this has not been submitted as part of any other academic award, and contributions of my supervisors (the other authors) was as expected for a normal thesis chapter. Assistance in processing foreign language papers was provided by native speakers listed on page xv.

CHAPTER ABSTRACT

Infrared (IR) thermography, where temperature measurements are made with IR cameras, has proven to be a very useful and widely used tool in biological science. Several thermography parameters are critical to the proper operation of thermal cameras and the accuracy of measurements, and these must usually be provided to the camera. Failure to account for these parameters may lead to less accurate measurements. Furthermore, the failure to provide information of parameter choices in reports may compromise appraisal of accuracy and replicate studies. In this chapter, I investigate how well biologists report thermography parameters. This is done through a systematic review of biological thermography literature that included articles published between years 2007 and 2017. I found that in primary biological thermography papers, which make some kind of quantitative temperature measurement, 48% fail to report values used for emissivity (an object's capacity to emit thermal radiation relative to a black body radiator), which is the minimum level of reporting that should take place. This finding highlights the need for life scientists to take into account and report key parameter information when carrying out thermography in the future.

2.1 Introduction

Temperature is an important biological variable. It is a key influence on living organisms (Gates, 1968; Levitt, 1980; Cossins and Bowler, 1987; Gillooly et al., 2001; Azad et al., 2007; Rands and Whitney, 2008; Seymour et al., 2009a; Zhang et al., 2010), and temperature can also be used as an indicator for metabolic activity (Seymour and Schultze-Motel, 1997; Tattersall et al., 2004; Seymour et al., 2009a; Seymour et al., 2009b), disease, injury and stress (Marazziti et al., 1992; Carere and van Oers, 2004; Ring and Ammer, 2012; Pascual-Alonso et al., 2015; Duncan et al., 2016). Temperature of organisms has been measured using thermocouples (Gale et al., 1970; Brinnel and Cabanac, 1989; Barnes, 1989) or thermistors (Togawa, 1985; Kort et al.), though use of thermographic cameras has increased dramatically in recent years with improvement of the technology (Ring and Ammer, 2012; Tattersall, 2016). Thermographic cameras detect the radiation from all objects hotter the absolute zero, usually in the human invisible 'thermal infrared band', wavelength range of 2 to 14μm. These radiation measurements, along with thermography parameters that are input into the camera, can be used to estimate the temperature of an object. The main thermography parameter is the target object's emissivity, which is its capacity to radiate infrared radiation relative to a blackbody radiator at the same temperature. Other parameters used are information about the environment in which measurements are taken place: infrared reflections, distance between camera and target, environmental temperature and environmental humidity (Usamentiaga et al., 2014; Tattersall, 2016; Vollmer and Möllman, 2017). Thermography has a number of benefits when compared to other temperature measurement methods such as thermocouples (Kastberger and Stachl, 2003; McCafferty, 2007). Firstly, in contrast to thermocouples and thermistors with individual contact points, it is easier with thermal cameras to measure the changes of temperature with high spatial resolution, across a target or simultaneously in several targets (Rejšková et al., 2010; Nääs et al., 2010; Ring and Ammer, 2012; Harrap et al., 2017). Secondly, it responds quickly to changes allowing monitoring of subjects that are moving or might change temperature quickly (Rejšková et al., 2010; Moe et al., 2012). Lastly,

and possibly most importantly to biologists, it is non-contact (Kastberger and Stachl, 2003; Usamentiaga et al., 2014; Tattersall, 2016); this is important because attempting contact measurements with biological subjects may disturb or damage the subject, or in more delicate applications disrupt temperature distributions. Using a non-contact technique also means temperature measurements can be made on more distant targets (Lenthe et al., 2007; Bellvert et al., 2014; Gillette et al., 2015).

Infrared thermography is a valuable tool for biologists and has been widely applied for temperature measurements (Kastberger and Stachl, 2003; McCafferty, 2007; Wisniewski and Glenn, 2008; Ring and Ammer, 2012; Tattersall, 2016; Harrap et al., 2017). However, doubt has been expressed over how well biologists understand and use these tools (Tattersall, 2016). Understanding of how thermal cameras estimate the temperature of objects requires an understanding of the thermography parameters that must be entered into the camera. Here, these parameters are discussed and how they are reported in the biological literature is assessed using a systematic literature review. Correct reporting is important, as it is both vital for ensuring repeatability of a thermographic study, and allows a reader to evaluate the correctness of a reported result. By reviewing how often thermographic parameters are reported, we can evaluate how well life scientists appear to understand thermography. Based on our findings, we will provide advice for biological thermographers, and highlight common mistakes that can be easily avoided in future work.

2.2 Background Information

2.2.1 *Principles of thermography*

All objects of a temperature above absolute zero emit electromagnetic radiation. Increased temperature leads to increased levels of radiation (Stefan, 1879; Boltzmann, 1884). This radiation is usually within the thermal infrared (IR) band, which is invisible to humans and has wavelength ranges between 0.8 – 14μm (Usamentiaga et al., 2014; Tattersall, 2016; Vollmer

and Möllman, 2017). However, once heated to a certain point objects will begin to radiate more in the shorter wavelengths, including in the light spectrum visible to humans. Thermal cameras are equipped with infrared-transmitting optics and arrays of sensors that are sensitive to portions of the thermal IR band (Usamentiaga et al., 2014; Tattersall, 2016; Vollmer and Möllman, 2017). The sensor readings are converted to radiometric units and colour-coded to generate false colour images that allow us to visualise thermal IR radiation that cannot be seen by the human eye. Most commercially available thermal cameras are sensitive to either Mid-wave Infrared (MWIR, 2-5μm) or Long-wave Infrared (LWIR, 8-14μm) (Usamentiaga et al., 2014; Tattersall, 2016; Vollmer and Möllman, 2017). These restrictions of wavelengths cameras are sensitive to are of the wavelengths of expected thermal radiation and those that provide high transmission (see below) through the atmosphere and camera optics (Usamentiaga et al., 2014; Tattersall, 2016; Vollmer and Möllman, 2017).

The thermal radiation emitted by an object (W_{obj}) is dependent on the object's temperature (T_{obj}, measured in K) in accordance with the Stefan-Boltzmann formula (Stefan, 1879; Boltzmann, 1884):

$$W_{obj} = \varepsilon \cdot \sigma \cdot T_{obj}^4 , \qquad (2.1)$$

where σ is the Stefan-Boltzmann constant (circa 5.67 x 10^{-8} W m^{-2} K^{-4}) and ε is the emissivity of the object. Emissivity is the capacity of an object to emit thermal radiation relative to a black body at the same temperature. A black body is a theoretical body which is non-transmissive and non-reflective, in other words completely absorbs any kind of incident electromagnetic radiation. Emissivity is normally represented as a fraction between 0 and 1, and black bodies have an ε of 1.

A thermal camera detects electromagnetic waves in the thermal IR band, and just like a regular human-visible light camera does not distinguish between emitted and reflected radiation. Like human visible light, thermal radiation has to be transmitted through the atmosphere. Furthermore, the atmosphere itself emits thermal infrared radiation

(Usamentiaga et al., 2014; Tattersall, 2016; Vollmer and Möllman, 2017). Thus, when imaging a non-transmissive object through air, the total radiation W_{tot} entering a thermal camera will be the sum of the emitted radiation of the object (W_{obj}), the amount of radiation reflected off the object (W_{ref}), and the amount of radiation emitted by the atmosphere (W_{atm}):

$$W_{tot} = W_{obj} + W_{ref} + W_{atm}\,. \tag{2.2}$$

This means that the radiation-based image viewed through the camera does not necessarily indicate the focal object's temperature, and that some level of calibration of the raw radiation image is needed to account for these additional sources of radiation (Vollmer and Möllman, 2017). This uncalibrated thermal image is known as 'apparent temperature'. W_{obj}, W_{ref} and W_{atm} are each influenced by the transmissivity of the atmosphere between the object and camera, τ_{atm}, and can be calculated by:

$$W_{obj} = \varepsilon \cdot \sigma \cdot \tau_{atm} \cdot (T_{obj})^4\,, \tag{2.3}$$

$$W_{ref} = (1-\varepsilon) \cdot \sigma \cdot \tau_{atm} \cdot (T_{ref})^4\,, \tag{2.4}$$

and

$$W_{atm} = \sigma \cdot (1-\tau_{atm}) \cdot (T_{env})^4\,, \tag{2.5}$$

where T_x refers to the temperature of x (x being the object, the environment or reflections). Note that the emissivity of the atmosphere equals (1-τ_{atm}), as objects can either emit, transmit or reflect radiation (Usamentiaga et al., 2014) and the atmosphere is non-reflective within the thermal IR band. Equations 2.3, 2.4 and 2.5 can be substituted into equation 2.2 to give,

$$W_{tot} = \varepsilon \cdot \sigma \cdot \tau_{atm} \cdot (T_{obj})^4 + (1-\varepsilon) \cdot \sigma \cdot \tau_{atm} \cdot \left(T_{ref}\right)^4 + \sigma \cdot (1-\tau_{atm}) \cdot (T_{env})^4\,, \tag{2.6}$$

which can be reorganised

$$T_{obj} = \sqrt[4]{\frac{W_{tot} - (1-\varepsilon) \cdot \tau_{atm} \cdot \sigma \cdot \left(T_{ref}\right)^4 - (1-\tau_{atm}) \cdot \sigma \cdot (T_{env})^4}{\varepsilon \cdot \tau_{atm} \cdot \sigma}}, \qquad (2.7)$$

to give temperature estimates of the object of interest.

The calculation in equation 2.7 is normally carried out by the camera itself, or related software (*e.g.* FLIR tools, FLIR Systems Inc., 2015) after the image has been captured (Vollmer and Möllman, 2017). Equation 2.7 identifies several parameter inputs required by the camera, or software, to accurately measure temperature of the object. These must be applied to images before measurements of temperature are taken from them, using the camera or related software. However, several of these parameter inputs are dependent on the time of image capture. Thus, although they can be applied to images afterwards, they must be measured at the time of thermograph capture. A checklist summary of the requirements for obtaining the most accurate thermographic temperature measurements and how the required timings influence protocol, is provided in table 2.1. The best quality thermographic measurements require accurate estimates of these parameter inputs in addition to correct use of camera optics in terms of image focus (Usamentiaga et al., 2014; Vollmer and Möllman, 2017).

Table 2.1: *A checklist for accurate thermographic temperature measurements. The six aspects needed for accurate thermographic temperature measurements of are listed, as well as where the timing of such aspects should be considered in experimental protocols. Note that the requirements, although all contributing to maximising accuracy do not influence accuracy equally. This checklist assumes thermography is not being carried out through a thermal IR transmissive window. It is very unlikely that researchers conducting biological thermography would need to use a transmissive window, but if this is the case further considerations must be made (see Vollmer and Möllman, 2017).*

Aspect	**Ideal requirements**	**Timing considerations**
Quality Thermographic Image Capture	A well-focused, unobscured image of target organism or tissues.	Thermograph image focus and content cannot be altered after capture. Image contrast and appearance in terms of temperature scales can be altered and are not important for temperate measurements, although they can aid with obtaining good image focus.
Emissivity (ε) estimate	A measurement of emissivity from the same object being thermographed.	Emissivity can normally be applied to images after capture. It does not necessarily need to be known at the time of image capture but needs to be obtained and applied to an image before measurements are taken from images.
Reflected temperature (T_{ref}) measurement	A measurement of reflected temperature off the thermography target.	Measurement of reflected temperature should be made simultaneously with each thermographic image capture. More practically mirrors require T_{ref} measurements to be made immediately after image capture. Reflected temperature can then normally be applied to the relevant thermograph images after capture.
Environmental temperature (T_{env}) measurements	A measurement of the temperature of the environment where the thermal image was captured.	Should be made simultaneously with image capture. Environmental temperature can then normally be applied to the relevant thermograph images after capture.
Environmental relative humidity (rh) measurements	A measurement of the relative humidity of the environment where the thermal image was captured.	Measurements should be made simultaneously with image capture. Environmental relative humidity can then normally be applied to the relevant thermograph images after capture. This is used by the camera or software to calculate τ_{atm}.
Distance between the camera and thermography target (d)	A measure of distance between the camera and thermography target.	This should be either controlled, and therefore known, or measured after image capture. As long as positions are noted, this measurement does not need to occur right away. This is used by the camera or software to calculate τ_{atm}.

2.2.2 *Emissivity*

Object emissivity, ε, alternatively called 'emittance', 'emission', or 'emission coefficient', is a proportion (bound between 0 and 1) that represents the capacity of an object to radiate thermal infrared radiation relative to a black body at the same temperature (Usamentiaga et al., 2014; Tattersall, 2016; Vollmer and Möllman, 2017). An emissivity of 1 treats the target object as a black body. Objects with high emissivity have temperatures that align closely with apparent temperature, while the total radiation entering a thermal camera (W_{tot}) when observing a low emissivity object will be influenced more strongly by reflected IR radiation (equation 2.6).

Emissivity can be measured using several methods, usually involving comparing the radiation from the object with that of a known emissivity of the same temperature (Vollmer and Möllman, 2017). This can be achieved by coating part of the object in something of known emissivity and heating the object evenly. Here a true measurement of the object temperature can be made with the thermal camera, and the emissivity parameter can then be adjusted until matching estimates of temperature are achieved on the uncoated parts of the object (Tattersall et al., 2004; Bulanon et al., 2008; Gallego et al., 2017). Often such coating is difficult on biological subjects, and heating live subjects evenly can be difficult and unethical. Although estimates could be carried out using dead subjects, where suitable and ethically obtainable (Tattersall, 2016; Potts et al., 2018). Alternatively, if the objects' temperature is known through another temperature measurement method, emissivity can be calculated by rearranging equation 2.7 (Idso et al., 1969; Stabentheiner and Schmaranzer, 1987; Bulanon et al., 2008; Lópes et al., 2012).

Inaccurate estimates of emissivity have the largest influence on the accuracy of temperature measurements (Usamentiaga et al., 2014; Tattersall, 2016). As seen in equation 2.7, changing emissivity changes the portion of W_{tot} taken to be from the object itself as opposed to from other sources and can lead to misjudgements in the contribution of reflections to W_{tot} relative to the object radiation. Emissivity has a direct effect on the temperature the

object is estimated to have when emitting a given amount of radiation. Therefore, information on emissivity of the object is key for thermographic measurements.

Emissivity is normally high in biological tissues, approximately 0.9 or higher (e.g. Kastberger and Stachl, 2003; Tattersall, 2016). This has the benefit that the impacts on inaccurate emissivity measurements are reduced when compared to low emissivity objects (see equation 2.7). An inaccurate but still high emissivity value, assuming the targets true emissivity is in fact high, will cause smaller levels of inaccuracy than similar inaccuracy in low emissivity targets (Usamentiaga et al., 2014; Tattersall, 2016). However, such impacts are not removed entirely. Emissivity is primarily influenced by the object's composition, and this can vary across different biological tissues. Emissivity can also be influenced by object properties such as geometry and surface structure (Vollmer and Möllman, 2017). As these can differ across and between different types of biological subjects (Kevan and Lane, 1985; Rubio et al., 1997), it is advised that when appropriate sources for emissivity values are not available, emissivity is measured on the tissues to be thermographed or estimated based on sources on similar tissue.

2.2.3 *Reflected temperature*

Reflected temperature (T_{ref}) is an estimate of the level of background radiation reflected off the thermography target object (Usamentiaga et al., 2014; Tattersall, 2016; Vollmer and Möllman, 2017), and is frequently expressed as a temperature value. Reflected temperature can also be referred to as 'reflected apparent temperature', 'background radiation', 'reflected radiation from ambient sources'. Also, confusingly, simply 'ambient' or 'background temperature' can be used to describe reflected temperature (Kaňa and Vass, 2008; Bowers et al., 2009; Costa et al., 2011; Manier et al., 2015). Such terms for reflected temperature can be easily confused with environmental temperature (T_{env}), and should be discouraged. It should be clearly stated what information is used to estimate reflected and environmental

temperature in calculations. This is especially true as environmental temperature can be used as a reasonable estimate of reflected temperature (Usamentiaga et al., 2014).

There are several ways this value can be estimated alongside thermographic measurements. A mirrored surface (Kaňa and Vass, 2008; Usamentiaga et al., 2014), preferably a multidirectional mirror (Bulanon et al., 2008; Lópes et al., 2012), placed on a plane with the thermography target can be used to measure T_{ref} . Here T_{ref} is taken as the average apparent temperature of the mirror (achieved by setting the camera's emissivity to 1 and distance to 0). Practically speaking this normally involves taking a second thermograph of the target with the mirror placed in frame alongside it in the same plane immediately after measurements are taken. T_{ref} can then be calculated and applied to the initial image (Kaňa and Vass, 2008; Bulanon et al., 2008). Alternatively, the environmental temperature is often a reasonable estimate of reflected temperature (Usamentiaga et al., 2014), as long as no sources of a large amount of light or heat are near the object. Such sources of heat and light may lead to reflected temperature differing form environmental temperature. Efforts can be taken to minimise sources of reflected temperature, such as shielding and repositioning the camera, however an accurate measure of reflected temperature value still has to be entered into the camera, and how reflected temperature was estimated should still be reported. Reflected temperature should be measured simultaneously or immediately following thermographic measurements, as changes in conditions or positioning of objects can alter reflected temperature, as noted in table 2.1.

Inaccurate estimates of reflected temperature can lead to misjudgement of the amount of radiation coming from the target object and other sources. However, biological tissues have a high emissivity, so the contribution of reflected temperature to W_{tot} is usually small (Tattersall, 2016) within biological applications (see equation 2.7). Usually, the best estimate of T_{ref} is achieved by measuring it along with each thermograph using a multidirectional mirror. This can be easier with stationary targets unlikely to move, such as plants. Similarly, in lab conditions multidirectional mirrors can be installed in such a way that T_{ref} measurements are

taken simultaneously with target measurements (as in Lópes et al., 2012). The use of mirrors and constant measurement of reflected temperature can be impractical in some experiments. Biological targets, particularly wild animals can be disturbed by the addition of mirrors or may be too distant or be too fast moving. In such instances, the environmental temperature should be used as an estimate for reflected temperature (Tattersall, 2016).

2.2.4 *Other environmental parameters*

Besides reflected temperature, environmental temperature, T_{env}, and the transmissivity of the atmosphere, τ_{atm}, are also specified in equation 2.7, and require entry into the thermographic camera. Environmental temperature allows the camera to account for the radiation emitted by the air between the camera and the target. Transmissivity of the atmosphere, τ_{atm}, accounts for how well that radiation travels through the air between the camera and target. Transmissivity of the atmosphere is normally estimated by the camera using the distance of the target from the camera, d, and the percentage relative humidity of the environment, rh. Usually both values are entered into the camera which then computes τ_{atm}. Environmental temperature, environmental humidity and camera distance are easily estimated using standard measurement tools. To maximize accuracy these should also be measured simultaneously with thermography measurements, as noted in table 2.1. However, τ_{atm} is typically very close to 1 (Usamentiaga et al., 2014; Vollmer and Möllman, 2017). Consequently, the effects of changes in these parameters are normally very small. In most instances the accuracy in these measurements has little effect on thermography data. Therefore, these parameters are often not measured alongside each thermograph, and an appropriate value is chosen for calculations (Usamentiaga et al., 2014). Such practices have the advantage of saving time with minimal effects on accuracy. The potential exceptions to this are in extreme scenarios such as very hot or humid environments, or where measurements are being taken over a long distance. In such cases these inputs should be measured.

2.3 Impacts of parameter omission

Above we have discussed the thermographic parameters needed to accurately estimate temperature using thermal cameras, and the relative importance of the values chosen for these parameters. Emissivity estimated from the same kinds of tissue can vary (Rubio et al., 1997; Belloni et al., 2015), which means that the chosen emissivity value will have a drastic impact on the accuracy of thermographic measurements. Accuracy of measurement is also affected by the extent to which reflected temperature and other environmental parameters are accounted for (Vollmer and Möllman, 2017): whether they are measured; if so how they are measured; and, if not, what value was assumed for calculations. For this reason, when thermographic temperature measurements are made the values used for emissivity should be included in reports as a minimum standard for accurate reporting, preferably alongside the method by which reflected temperature was accounted for. Assuming that thermography has been carried out correctly, the failure to provide this parameter information represents an incomplete methodology, and potentially misrepresents the accuracy of the thermographic measurements made. This limits the reader's ability to evaluate the choice of parameters, and compromises comparable replicate studies, as experimenters repeating a methodology will need to make an increasing number of assumptions about the methodologies of previous studies. Such assumptions may include: the value of emissivity used in estimates and if or how environmental parameters were monitored and adjusted for. If environmental parameters like T_{ref}, T_{env}, rh and d were not adjusted during the experiment, repeat experimenters will also have to assume the values used for calculations if they are not provided. This need to assume parameter choices will impact on the usefulness of studies where the replication of the described methods is expected. These include standardised monitoring studies such as those screening injury (Ring and Ammer, 2012), disease (Hovinen et al., 2008; Rainwater-Lovett et al., 2009; Johnson et al., 2011), or stress (Marazziti et al., 1992; Carere and van Oers, 2004; Alchanatuis et al., 2010; Kuraoka and Nakamura, 2011; Ballester et al., 2013; Bellvert et al., 2014; Yarnell et al., 2015).

In this chapter I assess the frequency in which key thermography parameters are reported in recent primary biological literature, through a systematic literature review, aiming to evaluate how well thermography is understood and reported by biologists. A lack of inclusion of thermography parameters could be the result of two different scenarios. Firstly, the thermographic camera was used correctly, with parameters adjusted appropriately, but the detail of their adjustment was not provided in the published methodology. Alternatively, the thermographic camera could have been used incorrectly, and consequently parameters are not adjusted or reported. Thus, a lack of information on the thermography parameters, especially emissivity, could indicate that thermography is not well understood by experimenters at some level.

2.4 Methods

2.4.1 *Search criteria*

Our literature search was carried out using the *Web of Science* core collection (Clarivate Analytics), limited to papers published between 2007 and 2017, with the final search taking place on 17th December 2017. This comparably recent search was chosen to allow us to focus our assessments of how biologists are using thermography currently, and to minimise the effects changes in the technology might have on the reporting of methods and applications. The following search terms were used: "[infrared OR infra-red OR infra red] AND [thermograph* OR thermal imag* OR camera]" ('*' denoting derivations of the word, so 'thermal imag*' includes derivations such as 'thermal image' and 'thermal imaging').

The search was then refined further to include only publications in at least one of the following 23 Web of Science Categories: *agriculture dairy animal science*, *agriculture multidisciplinary*, *agronomy*, *behavioural sciences*, *biology*, *biophysics*, *ecology*, *entomology*, *evolutionary biology*, *fisheries*, *forestry*, *horticulture*, *marine freshwater biology*, *ornithology*, *physiology*, *plant sciences*, *psychology*, *psychology applied*, *psychology biological*,

psychology experimental, *psychology multidisciplinary*, *veterinary sciences*, and *zoology*. Full texts of all search results were searched for using University of Bristol library subscriptions and through *Google Scholar*. If publications could still not be found, and the paper could not be excluded based on the information in the abstract provided by *Web of Science* or linked sites alone (see exclusion criteria), the corresponding authors (where contact details provided) were contacted for copies of publications. Any publication that was not obtained through these methods was excluded. A summary of the *Web of Science* search history used in our literature search can be found in Appendix A1.

2.4.2 *Review process*

Search results were examined in a chronological order by myself (both a biological scientist and qualified thermographer - Level 1 thermographer, Infrared training centre, awarded June 2015). Publications were checked for any criteria for exclusion (criteria detailed below), a process which left only primary biological science research papers that reported work utilising infrared thermography in some way. These papers' methodology, how thermographic tools were employed, and the inclusion of thermographic parameters were assessed. Non-English language journals were assessed with aid of a native speaking translator if the journal could not be excluded based on the abstract alone (12 papers in total, translators are listed in the declaration of assistance on page xv). After completion of the full review process all search results were worked through and assessed a second time to ensure confidence and consistency in our assessment.

2.4.3 *Exclusion criteria*

The search criteria used in this systematic review was deliberately broad to allow for the many ways thermal cameras might be described in publications, such as 'thermal camera' and 'infrared camera'. This was done to minimise the chance of accidently excluding papers that

genuinely utilise infrared thermography. This accidental exclusion of relevant papers has been identified as a major issue in systematic reviews (Gerstner et al., 2017). This has the consequence that many publications included in the *Web of Science* search results, were not primary biological science papers that used thermal imaging. The exclusion criteria applied to our search results are summarized in table 2.2.

Only publications which carried out thermography and reported data or images collected by infrared thermography were included in our review, everything else was excluded as 'not thermography'. These excluded works included those using non-thermal infrared technologies, such as triggers and sensors (Séquin Larrucea et al., 2007; Braden et al., 2008; Li et al., 2010; Garrote et al., 2011; Flatters et al., 2014), infrared reflectance cameras (Kaizu and Imou, 2008; White et al., 2009; Sakamoto et al., 2012), hyperspectral cameras (Nagy and Tamás, 2013), and the use of non-thermal infrared devices for night vision (Pierce and Pobprasert, 2007; White et al., 2007; Huckschlag, 2008; Wellman and Downs, 2009). Additionally, publications using 'infrared thermometry' (Graham et al., 2010; Schmidt et al., 2014; Bowman et al., 2016) as opposed to thermography were excluded (although infrared thermometry tools do use the same principles for point measurements). Theoretical studies investigating applications of infrared thermography (Amri et al., 2011; Luna et al., 2012; Absalan et al., 2012; Padra and Salva, 2013), if such studies did not report any thermal imaging measurements, were also excluded.

Table 2.2: *A summary of the exclusion criteria applied to the results of our Web of Science search results. Each criterion for exclusion is given in the order they are applied. For each criterion the publications that are still included, and those that are excluded, when the criteria are applied are summarized. Also summarised here are the papers excluded from our analysis of emissivity reporting after the thermography methods assessment.*

Order	Exclusion Criterion	Included in assessment	Excluded on this criterion
Excluded from thermography methods assessment			
1	Not Thermography	Publications that carry out thermography or images collected by infrared thermography.	Publications that do not utilise thermography in any way. Also excluded are theoretical studies on applications of thermography if studies do not make thermal imaging measurements.
2	Not Biological	Thermography in applied to a biological research application.	Thermography is applied to a non-biological application.
3	Isolated abstract	Publications that are not isolated abstracts from conferences. Conference reports are retained if they have a methods section.	Isolated abstracts from conferences which have no featured section for reporting methods.
4	Retracted article	Publications that have not been retracted by the publishing body at time of last search.	Articles that had been retracted by the publishing body for any reason at time of last search.
5	Review	Article is a primary research paper.	Publication is a secondary research paper reporting or providing commentary on the findings of previous work. (These publications are filed separately for ease of reference).
Excluded from statistical analysis after thermography assessment completed			
6	Quantitative-Qualitative	Paper presents temperature measurements dependent on thermography or data that required thermographic temperature measurements for its calculation. Thus, should report thermography parameter information.	Paper uses thermal imaging in an application that does not involve measuring temperature and is dependent wholly on apparent temperature. Thus, reporting of thermography parameter information is not required to assess accuracy or repeat methods.

This review aims to assess use of infrared cameras in the life sciences area. Thus, if the application of infrared thermography did not appear to be biological, publications were also excluded as ‘not biological’. Such application treated as non-biological included the industrial preparation of baked goods (Deng et al., 2011), materials science (Aoyagi et al., 2014; Mauranen et al., 2015; Antikainen et al., 2015). biomechanical surgery tool maintenance

(Gasiorwski et al., 2011), assessment of building materials in agricultural management (Fiorelli et al., 2012), and canal upkeep (Huang et al., 2009). Biomechanical studies where temperatures of artificial replacements were only monitored outside the body, for example in mechanical stress assessment (Bougherara et al., 2011), and studies where biological tissue mimics were employed instead of real biological targets (Paul et al., 2014; Heussner et al., 2015) were likewise excluded as 'not biological'.

Any isolated abstracts from conferences were excluded, as such summary articles typically do not normally provide detailed information on their methodology. Published conference reports were not excluded if they featured a methods section. Any retracted articles, at time of the search, were also excluded.

Lastly, review articles that either discussed infrared thermography or thermography-dependent results were excluded. Although, for reference review articles were filed separately from other exclusions (see supplementary material of the published version of this chapter *http://rsos.royalsocietypublishing.org/content/5/12/181281.figures-only*).

2.4.4 *Thermography methods assessment*

Included publications were assessed to obtain data on how infrared thermography was used and has been reported. The information extracted from each publication can be found in table 2.3. It was beyond the scope of this review to evaluate in each case how appropriate the parameters used were and how this influenced the value of the thermographic measurements taken within the study. This review process consequently focused on whether primary research papers provided the information needed to make such evaluations of parameter choice or repeat the study without having to assume parameter choice. For emissivity a specific value used in measurements was required. Simply an acknowledgement that emissivity was input was deemed as insufficient as the actual value is needed for appraisal of papers. For environmental thermography parameters (T_{ref}, T_{env}, rh and d), indication that

these were used in calculations was required. The method of T_{ref} measurement was also monitored, and could either be a single quoted value used for the parameter at measurements or a continuous measurement alongside the thermography measurements, as both are acceptable (Usamentiaga et al., 2014). The information listed in table 2.3 could be provided at any point in the paper main text, including within thermograph figures when information was not given in the text. The article main text was the focus of the publication search, and "supplementary" or "supplemental" text was only consulted for this information if the publication explicitly directed us to do so.

Throughout the review we aimed to give authors the benefit of the doubt where possible. If a study indicated at any point in the paper that the environmental factors (T_{env}, rh and d) in the sampling area were known it was assumed they were input into the camera. This could be simply mentioning that these parameters were measured in the thermography sampling area. If the camera was mounted in a fixed position relative to the target it was assumed that distance had been measured and input. As several thermography parameters can be referred to by various names (listed previously), any of these were acceptable. As reflected temperature, T_{ref}, is sometimes referred to as "ambient temperature" (Kaňa and Vass, 2008; Bowers et al., 2009; Costa et al., 2011; Manier et al., 2015), if a study referred to environmental temperature as "ambient temperature" it was assumed that this value was also used for reflected temperature unless stated otherwise. However, a note was made of instances where this assumption was made (table 2.3). For each piece of information noted in table 2.3, page locations within the relevant publication were noted in each publication (using the page numbers on the version accessed).

Table 2.3: *The information extracted from each publication during the thermography methods assessment. Each datapoint, the format of this datapoint, and a description of this datapoint are given.*

Datapoint	Format	Description
Thermography target	Category	The subject for the research involving thermography.
Quantitative temperature values	Boolean Y/N	Whether the paper used thermal imaging for a qualitative or quantitative study. "y" if quantitative, "n" if qualitative.
Emissivity, ε, value given	Boolean Y/N	An indicator of whether the ε value(s) used are given in the publication.
ε value(s)	Value	The ε value(s) used in the study. *'n/a'* if the ε value(s) are not given.
ε value(s) measured or referenced	Category	An indicator of the source for the ε value(s) used. If emissivity was measured by the researchers this is indicated here. If emissivity was taken from an existing source that source is indicated. *'n'* if ε value(s) are given but no indication of measurement or source is given, *'n/a'* if the ε value(s) are not given.
T_{ref} considered	Boolean Y/N	An indicator that the publication accounts for reflected temperature (T_{ref}) in temperature measurements in any way. *'n'* if this information is not given or if the study merely attempted to minimise reflection.
T_{ref} method	Category	How reflected temperature (T_{ref}) was accounted for. If the reflected temperature value is assumed to be ambient this method is listed as 'assumed to be ambient'. *'n'* if publication gives a value for reflected temperature but gives no detail. *'n/a'* if reflected temperature is not accounted for.
T_{env} considered	Boolean Y/N	An indicator of whether the environmental temperature was measured or estimated alongside thermal imaging.
rh considered	Boolean Y/N	An indicator of whether environmental relative humidity was measured or estimated alongside thermal imaging.
d considered	Boolean Y/N	An indicator of whether camera distance was measured or estimated alongside thermal imaging
Camera manufacturer model	Category	The manufacturer and model of the thermal camera(s) used in the publication. *'n'* if this information is not given.

Not all applications of thermal cameras involve measurements of temperature, for example, thermal cameras can be used to spot animals at long distances or in the dark (Betke et al., 2008; Udevitz et al., 2008; Horn et al., 2008; Gillette et al., 2015). In such non-quantitative or 'qualitative' applications, data is dependent only on apparent temperature (Vollmer and Möllman, 2017). Consequently, thermography parameter information is not required to assess accuracy or repeat methods of qualitative studies. It is thus important in our assessment of biological thermography publications to evaluate whether thermal imaging was used in a quantitative manner or not (table 2.3), as this will determine whether failing to report parameters affects study accuracy or repeatability. A publication was determined to be a quantitative study if it presented temperature data dependent on thermal imaging. This thermography-dependent temperature data could be presented graphically, or as quoted temperature values, or as a thermograph with temperature scales. If the paper presented data that required temperature measurements for its calculation, such as Plant Water Stress Index (Alchanatuis et al., 2010; Ballester et al., 2013; Bellvert et al., 2014), such papers were viewed as quantitative. Studies deemed qualitative use infrared thermal imaging but do not measure temperature values.

Each paper was assigned a biological field based on the subject of research in each study. These biological fields are listed in table 2.4. This also allowed assessment of whether certain biological research disciplines are more likely to fail to report infrared thermography parameters when they are required (in quantitative studies). The number of quantitative studies that failed and succeeded in reporting emissivity, the minimum level of parameter reporting for thermographic temperature measurements (see above), was calculated for each biological field. The association between emissivity reporting and biological field was assessed using a chi-squared test using *R* version 3.4.1 (R Development Core Team, 2017). It was deemed acceptable for wholly qualitative studies to not include parameter information (Vollmer and Möllman, 2017); thus, any qualitative publications were not included in this analysis (as described in table 2.2).

Table 2.4: *The biological fields assigned to papers based on the subject of the thermography research. A description of the research subjects of papers in each field is also provided.*

Biological Field	Thermography Research Subjects
Agricultural Animals	Animals used in agricultural practice, such as cows, goats, sheep and pigs
Birds and Poultry	Birds and poultry, includes chickens, turkeys and their eggs
Earth and Soil	Ground, rock or soil when measured within biological studies
Humans/Medical	Humans, including sports science, medical and psychological studies
Insects	Any insects
Mammals	Mammals, excluding humans and agricultural animals
Plants	Any plants, including crop science
Reptiles and Amphibians	Any reptiles and amphibians
Other	Any subject not covered in the above biological fields

2.5 Results

The search yielded a total of 1219 search results. Exclusions accounted for most of this number. 575 publications were excluded in total: 466 ‘not thermography’; 35 ‘not biological’; 36 isolated abstracts; 1 retracted; and 37 that were not obtained by the authors and could not be otherwise excluded. This left 562 primary biological publications which employed infrared thermography and a further 82 reviews featuring infrared thermography. Of these 562 primary publications, 531 (94.48%) were deemed to use quantitative temperature measurements in some way, leaving 31 (5.52%) wholly qualitative studies. The frequency of quantitative and qualitative papers in each biological field is presented in figure 2.1.

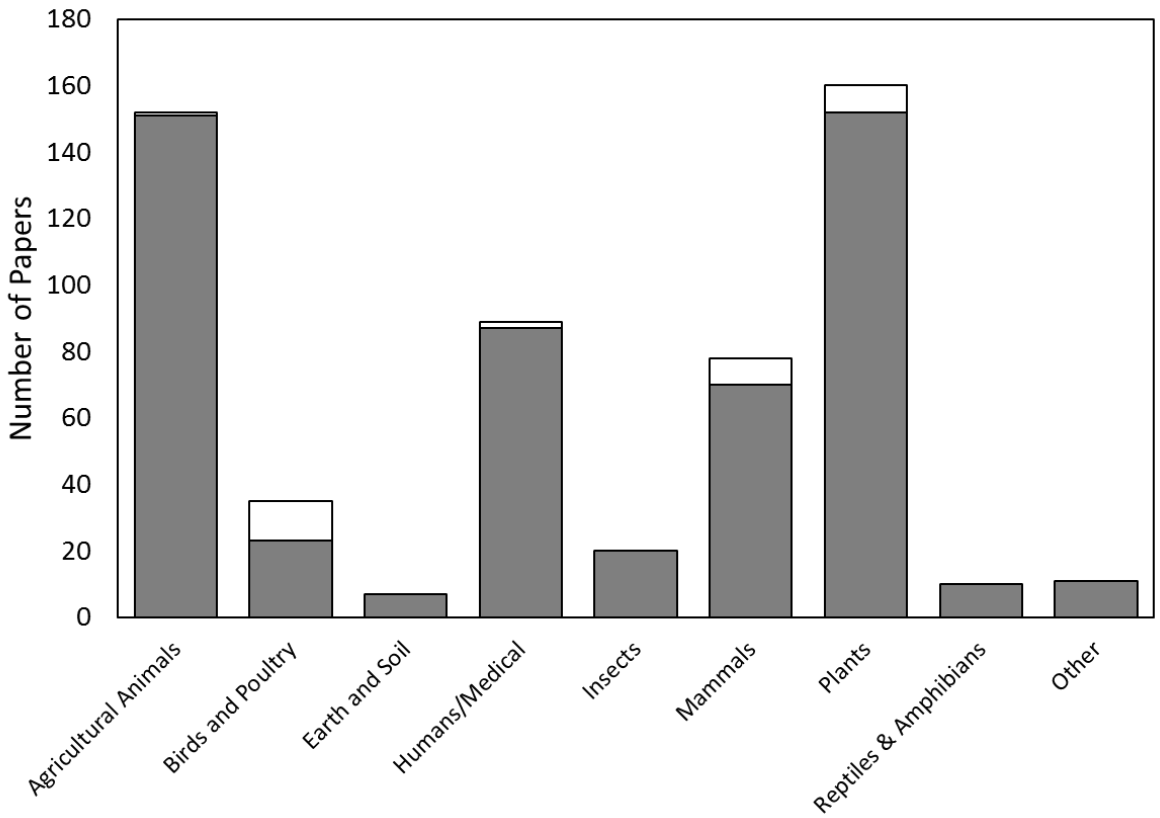


Figure 2.1: *The frequency of thermography papers within each biological field, as categorised in table 2.4. Quantitative and qualitative papers are indicated by shading: quantitative papers, darker grey shading; qualitative papers, clear white.*

Of the 531 quantitative papers, where camera parameter inputs are necessary for accurate temperature measurements, 52.0% of all quantitative studies provided emissivity values (276 publications), and 48.0% of all quantitative studies failed to provide emissivity values (255 publications). Figure 2.2 shows the percentage of quantitative papers in each biological field that report emissivity compared to all quantitative papers. Chi-squared analysis revealed a significant association between the biological field and reporting of emissivity (X_8^2 = 20.235, p = 0.009). This association is largely due papers in the "Birds and Poultry", "Insects" and "Earth and Soil" biological fields reporting emissivity more frequently than expected and

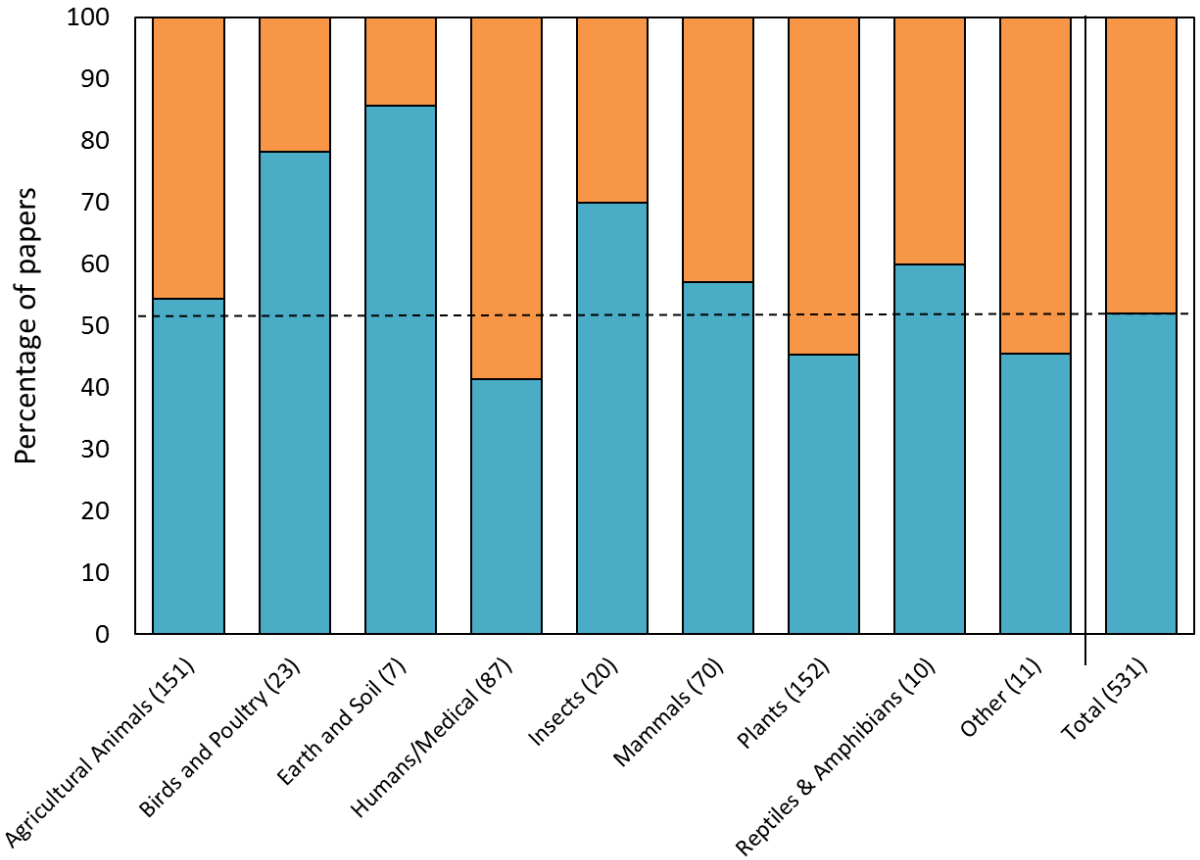


Figure 2.2: *The percentage reporting of emissivity within all quantitative papers (Total) and different biological fields, as categorised in table 2.4. Lower blue bars indicate the percentage of papers that report emissivity, higher orange bars indicate the percentage of papers that fail to report emissivity. The dotted line indicates 52.0%, the percentage of all quantitative papers that report emissivity, allowing comparison of how frequency of reporting differs compared to the overall frequency. Numbers of quantitative papers in each biological field and the total are indicated in brackets.*

the “Plants” and “Humans/Medical” biological fields reporting emissivity less frequently than expected. Table 2.5 gives the frequencies of emissivity reporting across research fields alongside expected frequencies and Pearson residual used in our Chi-squared analysis. Of the 276 papers that provided emissivity values, 45.2% (126 publications) provided a source for that value choice and a further 5.4% (15 publications) measured the value within the study. A summary of emissivity values used in studies measuring similar targets, targets of the same research field, is given in table 2.6.

Table 2.5: *The realised, expected and Pearson residual values of emissivity reporting used in χ^2 analysis of emissivity reporting within each biological field (biological field described in table 2.4). Realised frequency represent the actual observed values of emissivity reporting. Expected frequency represent the frequency of reporting expected if no effect of research field was present, given the size of the groups. Pearson residual values indicate the relative influence of research field on a χ^2 analysis result.*

	Realised Frequency		**Expected Frequency**		**Pearson residuals**	
	Emissivity Value Not Given	Emissivity Value Given	Emissivity Value Not Given	Emissivity Value Given	Emissivity Value Not Given	Emissivity Value Given
Agricultural Animals	69	82	73	78	-0.413	0.397
Birds and Poultry	5	18	11	12	-1.819	1.748
Earth and Soil	1	6	3	4	-1.288	1.238
Humans/Medical	51	36	42	45	1.426	-1.371
Insects	6	14	10	10	-1.163	1.118
Mammals	30	40	34	36	-0.624	0.599
Plants	83	69	73	79	1.171	-1.126
Reptiles and Amphibians	4	6	5	5	-0.366	0.352
Other	6	5	5	6	0.312	-0.300

Table 2.6: *A summary of emissivity values reported by publications monitoring similar biological targets, targets of the same research field and for all studies.*

	Agricultural Animals	Birds and Poultry	Earth and Soil	Humans/ Medical	Insects	Mammals	Plants	Reptiles and Amphibians	Other	**TOTAL**
Mean ε value	0.969	0.956	0.978	0.976	0.964	0.977	0.957	0.970	0.947	**0.967**
Standard deviation in ε	0.018	0.033	0.020	0.007	0.009	0.015	0.038	0.020	0.059	**0.026**
Minimum ε value	0.92	0.86	0.95	0.95	0.95	0.95	0.8	0.95	0.85	**0.8**
Maximum ε value	1	1	1	0.98	0.97	1	1	1	1	**1**

Reflected temperature was only reported in 26.7% (142 publications) of all quantitative papers. Within papers that gave emissivity values, reflected temperature was reported in 41.7% (115 publications) of papers. However, in 52.2% (60 publications) of these papers reflected temperature information was not explicitly given but 'assumed to be ambient'. In papers that failed to give emissivity, reflected temperature was reported in only 10.6% of papers (27 publications).

Environmental parameters associated less directly with infrared thermography tended to be reported more frequently than emissivity and reflected temperature. With environmental temperature, environmental humidity and camera distance being reported in 81.2%, 51.6% and 66.7% of all quantitative papers respectively. Environmental temperature, environmental humidity and camera distance were reported more frequently in papers that gave emissivity values (89.9%, 60.9%, and 80.1% respectively) than those that did not (71.8%, 41.6%, and 52.2% respectively) but this difference between papers that report emissivity and those that did not was less stark than that seen in reflected temperature.

A list of the 1219 papers found in our search categorized into primary papers, reviews and exclusions as well as the data extracted from each paper can be found in the supplementary material of the published version of this chapter (*http://rsos.royalsocietypublishing.org/content/5/12/181281.figures-only*). A summary of frequency of parameter reporting, broken down further by biological field, can be found in table 2.7.

Table 2.7: *A breakdown summary of primary thermography papers in our Web of Science search. Given are frequencies (and percentages where indicated) of publications at each level of our thermography reporting assessment. This is given for all primary thermography publications (total) and broken down by research field (as defined in table 2.4).*

	Agricultural Animals	Birds and Poultry	Earth and Soil	Humans/ Medical	Insects	Mammals	Plants	Reptiles and Amphibians	Other	**TOTAL**
Primary Thermography Papers:										
Qualitative Studies	1	12	0	2	0	8	8	0	0	**31**
Quantitative Studies	151	23	7	87	20	70	152	10	11	**531**
Research Field Total	152	35	7	89	20	78	160	10	11	**562**
Of All Quantitative Studies:										
number that:										
ε value not given	69	5	1	51	6	30	83	4	6	**255**
ε value given	82	18	6	36	14	40	69	6	5	**276**
ε value referenced or measured	25	9	2	19	13	19	47	3	3	**140**
Tref measured	50	13	2	19	5	24	27	2	0	**142**
Tenv measured	119	21	5	73	18	61	119	8	7	**431**
rh measured	90	12	3	43	8	30	83	2	3	**274**
d measured	125	13	5	48	5	51	98	3	6	**354**
Percentage that:										
Failed to give *ε*	46%	22%	14%	59%	30%	43%	55%	40%	55%	**48%**
Gave *ε*	54%	78%	86%	41%	70%	57%	45%	60%	45%	**52%**
Of Quantitative Studies that Gave ε:										
number that:										
Tref measured	34	11	2	17	5	19	25	2	0	**115**
Tref measured but 'Assumed to be ambient'	22	10	1	10	5	7	3	2	0	**60**
Tenv measured	72	18	5	33	12	38	61	5	4	**248**
rh measured	53	10	3	21	6	22	49	2	2	**168**
d measured	72	11	5	27	3	33	63	2	5	**221**
Of Quantitative Studies that Failed to Give ε:										
number that:										
Tref measured	16	2	0	2	0	5	2	0	0	**27**
Tenv measured	47	3	0	40	6	23	58	3	3	**183**
rh measured	37	2	0	22	2	8	34	0	1	**106**
d measured	53	2	0	21	2	18	35	1	1	**133**

2.6 Discussion

Infrared thermography parameters are an important part of making accurate thermography measurements (Kastberger and Stachl, 2003; Usamentiaga et al., 2014; Tattersall, 2016; Vollmer and Möllman, 2017). Failure to include this information represents incomplete reporting on methodologies and can compromise the value and utility of studies that depend on thermography. Furthermore, it can indicate some misunderstanding of parameter importance and the thermal imaging methods used. The systematic review of biological primary research papers presented in this chapter reveals that, of those which carried out some kind of quantitative thermographic measurements, 48% failed to give the emissivity values used. Although this significantly varied between different biological research fields, it is worth noting that a large portion of all fields failed to give any indication of emissivity. Reporting emissivity represents the minimum parameter information that quantitative papers ought to include. Reflected temperature, the other large contributor to accuracy of biological thermographic measurements, was reported less frequently than emissivity, in 26% of all quantitative papers. This value includes those where reporting was unclear but the descriptions suggest that ambient temperature was entered as reflected temperature in calculations. It appears that the true frequency of reflected temperature reporting is likely to be lower. These findings reveal biological literature to be quite poor at reporting basic thermography parameter information used in studies, and suggests that greater effort is needed on the part of authors to report key thermography parameters.

Environmental temperature (T_{env}), relative humidity (rh), and camera distance (d) have little influence on the accuracy of temperature measurements (Usamentiaga et al., 2014; Tattersall, 2016). Nevertheless, reporting of these environmental parameters is found more frequently than explicit statements of values for emissivity and reflected temperature. This tendency for papers to report these less critical parameters seems to be the result of two factors. Firstly, we assumed in our analysis that if these parameters were known they were entered into the camera. Secondly, there is often a biological reason to include monitoring of

these environmental factors independent of their influence on thermography. This is especially true of environmental temperature, a key biological variable (Gates, 1968; Levitt, 1980; Cossins and Bowler, 1987; Gillooly et al., 2001; Azad et al., 2007; Rands and Whitney, 2008; Seymour et al., 2009a; Zhang et al., 2010). This means even without any knowledge of what parameters needed to be entered into the camera, and included in the report, it is likely authors would have monitored and reported these environmental parameters. This explains why many papers that failed to give emissivity and reflected temperature still gave environmental temperature and humidity (table 2.7). This unfortunately suggests the high inclusion frequency of these parameters is not indicative of understanding of thermography.

Without parameter information it is difficult to assess the accuracy of thermographic measurements within papers, or to tell if thermography was carried out correctly or not. A number of studies (10.6%) appear to give information on reflected temperature when emissivity information is not given (Šumbera et al., 2007; Deeming and Pike, 2015; Kleinhenz et al., 2017), or mention that emissivity was input into the camera (Šumbera et al., 2007; Paterson et al., 2010; Johnson et al., 2011; Samara et al., 2014) or even measured (Goff et al., 2017) but provide no information on the value used. These suggest an understanding of thermography and the parameters involved, most likely indicating correct operation of thermal cameras but with incomplete reporting. However, many quantitative studies make use of thermal cameras but make no mention of emissivity or reflected temperature at all (Wright et al., 2008; Holzer et al., 2010; Liang et al., 2010; Kuraoka and Nakamura, 2011; Yarnell et al., 2015). Camera models and sensitivities and the temperature ranges displayed in images are given but not thermography parameters. Camera specifications are useful for assessment of measurement accuracy, and at least the model of camera used should be reported. However, quoted accuracies of the camera only apply when the camera inputs are correct. Likewise the temperature range applied to the image, while influencing the image seen by operators and in reports, does not influence the temperature measurements given (Vollmer and Möllman, 2017). Taken as a whole, the frequent failure to report thermographic parameter information

is likely to be the result of a combination of both scenarios. In both cases, our ability to actually appraise the accuracy and repeatability of these studies is compromised. More worryingly, if no accounting for thermography parameters has been conducted, there is a strong possibility that these papers suffer from larger level of inaccuracy in their measurements. As these two quite different causes of parameter omission cannot be easily distinguished and have quite different effects on the paper's validity and usefulness, it is critical that researchers report parameter information. At the very least, this will then confirm that these settings were taken into account when carrying out thermographic measurements.

We found a significant association between research field and emissivity reporting, although the level of reporting was not high in most research fields (figure 2.2). Research fields with a very large amount of quantitative thermography publications 'Plants' and 'Humans/Medical' tended to report emissivity slightly less often than other fields, while smaller groups like 'Birds and Poultry', 'Earth and Soil' and 'Insects' reported emissivity more often. That said, the largest research field from our review, 'Agricultural Animals', reported emissivity at about the average frequency. It is likely that existing publications, especially those in the same research field, set a precedent for authors, and reviewers, that thermography parameter information does not need to be included in new publications. This may explain the lower frequency of parameter reporting in certain research fields. Such an explanation could be applied more generally to explain the low frequency of parameter reporting throughout biology. It is important that journals ask for this parameter information, at least emissivity, to be included in the future to prevent such a precedent continuing. As the research fields applied to this review are deliberately quite broad, further breakdowns of the research fields would perhaps reveal specific subdivisions more prone to parameter omission than others. However, no field reported emissivity with great frequency, with failure ranging from 20 to 60% of cases across fields. So, tendency to not include parameter information is likely to continue into subdivided fields to some extent.

While our systematic review suggests that an issue exists with thermography parameter reporting in biology, it does not necessarily give a full representation of how well biologists carry out thermography. Successfully reporting parameters such as emissivity does not guarantee thermography was carried out correctly. Other operation issues can still occur when parameter settings are input correctly. Furthermore, it was beyond the scope of our review to evaluate in each instance how applicable the values used for emissivity actually were, and instead our focus was upon whether such appraisals can be done based on the information reported. Consequently, it is possible that the values chosen were still inappropriate and result in inaccurate temperature measurements. However, most often biological tissues have an emissivity of approximately 0.9 (Kastberger and Stachl, 2003; Tattersall, 2016), and this is supported by the values found in the review which range from 0.8 to 1. Although, our review confirms that estimates can vary even within similar applications (table 2.6). In papers where emissivity values are supported by measurements or a source which measures emissivity of the tissues thermographed, we can be more confident in the emissivity values chosen. For this reason, we strongly encourage authors to provide sources for emissivity values chosen. As certain biological targets can be hard to measure emissivity from, particularly when delicate or hard to access, papers providing information on biological tissue emissivity (Idso et al., 1969; Stabentheiner and Schmaranzer, 1987; Rubio et al., 1997; Bulanon et al., 2008; Lópes et al., 2012; Zhang et al., 2016) should be encouraged as they will help biological thermographers make more informed parameter choices and be more precise in their thermography measurements.

Our review treats all quantitative thermography as equally important to studies; we made no evaluation of how critical the temperature measurements were to the paper's findings (outside of assessing if the paper was qualitative and quantitative). It is possible that some papers may use thermography in such a minor way that authors felt parameter detail unnecessary. However, reporting parameter information represents a small addition to the methods. Furthermore, in such instances where the accuracy of measurements is less

important papers should still give the information on parameters, but perhaps need not worry for a precise estimate of emissivity or monitor environmental parameters with every measurement. Our review process did not penalise papers for applying these less accurate approaches if they reported the necessary information, consequently a less precise approach for less critical measurements was acceptable within our review.

Frequently emissivity and other parameter values were provided within a thermograph figure with no mention to it in the main text (Steward et al., 2008; Stokes et al., 2012; Zadeh et al., 2016). Our review process counted this as reporting, as the information was indicative that parameters were adjusted, or at least are known. However, in such instances the value could easily be overlooked if the reader were not experienced with thermography. This is particularly likely when the thermography format is unusual, perhaps due to a less common camera manufacturer. Inclusion of parameters within the article text should be encouraged over inclusion within thermographs.

This chapter has highlighted a common tendency for biologists to omit information on critical thermographic parameters such as emissivity and reflected temperature in published primary literature. This omission suggests a lack of understanding of thermographical methods. More care should be taken to include parameter information in publications. This will improve clarity and confidence in measurements but also to allow the assessment of the limitations of thermography in different types of biological studies. Fortunately, the addition of parameter information represents a small effort which can significantly improve the evaluation of reported research and awareness of the correct use of thermal cameras in biological studies. It is recommended as a minimum that the emissivity values should be given, preferably with sources or measurements supporting the parameter choice. Additionally, the method of assessing reflected temperature should be included as well.

Chapter 3: The diversity of floral temperature patterns, and their use by pollinators

The following chapter is adapted from a published paper of which I am first author: Harrap et al. (2017), eLife, 6, e31262. It will thus have high similarity with this publication. As indicated on page xiv, contributions of my supervisors (the other authors) was as expected for a normal thesis chapter. Assistance in artificial flower construction and species identification was provided as stated on page xv.

CHAPTER ABSTRACT

Pollinating insects utilise various sensory cues to identify and learn rewarding flower species. One such cue is floral temperature, created by captured sunlight or plant thermogenesis. Bumblebees, honeybees and stingless bees can distinguish flowers based on differences in overall temperature between flowers. I report here that floral temperature often differs between different parts of the flower creating a temperature structure or pattern. Temperature patterns are common, with 55% of 118 plant species thermographed, showing within-flower temperature differences greater than the 2°C difference that bees are known to be able to detect. Using differential conditioning techniques, I show that bumblebees can distinguish artificial flowers differing in temperature patterns comparable to those seen in real flowers. Thus, bumblebees are able to perceive the shape of these within-flower temperature patterns. Floral temperature patterns may therefore represent a new floral cue that could assist pollinators in the recognition and learning of rewarding flowers.

3.1 Introduction

Many flowering plants require pollen transport by animals to ensure reproductive success (Ollerton et al., 2011). These pollinating animals are often insects (Kevan and Baker, 1983), such as bees. To encourage pollinator visits flowering plants create floral displays (Raguso, 2004; Leonard et al., 2012) which produce diverse floral cues in different sensory modalities (Kevan and Lane, 1985; Bhagavan and Smith, 1997; Whitney et al., 2009c; Hempel de Ibarra and Vorobyev, 2009; von Arx et al., 2012; Lawson et al., 2017b). These signals allow pollinators to find and locate flowers (Spaethe et al., 2001; Chittka and Spaethe, 2007), and also allow pollinators to learn and recognise them (Heinrich, 1979b; Raine and Chittka, 2008). Bees and other pollinators adjust their foraging behaviour to favour visits to more rewarding species found in their environment (Heinrich, 1979b), avoiding 'mistake visits' to less rewarding flowers in order to enhance their foraging success (Raine and Chittka, 2008). Similarly, a floral display that is easily learnt and distinguished from others in its environment ensures greater visitation to the flower (Galen and Newport, 1988; Lynn et al., 2005) and thus greater reproductive success (Ashman et al., 2004; Bell et al., 2005; Schiestl and Johnson, 2013). Identifiable floral cues are therefore critical to both plant and pollinator.

One flower signal bees can use to recognise flowers is floral temperature (Whitney et al., 2008; Hammer et al., 2009; Norgate et al., 2010). Warming of flowers can occur due to floral thermogenesis (Seymour and Schultze-Motel, 1997; Seymour and Matthews, 2006; Seymour et al., 2009b), but is more frequently the result of captured solar radiation (Totland, 1996; Sapir et al., 2006; Rejšková et al., 2010; Zhang et al., 2010; Atamian et al., 2016) or environmental warming (Shrestha et al., 2018). The absorption of sunlight and heat loss is influenced by floral pigmentation (Kay et al., 1981; Sapir et al., 2006), structure (Rejšková et al., 2010; Whitney et al., 2011) and heliotropism (Totland, 1996; Zhang et al., 2010; Atamian et al., 2016), all of which will contribute to how much a certain flower will heat up in given conditions. This can create differences in temperature between different flower species (Rejšková et al., 2010; Kovac and Stabentheiner, 2011). Using thermal detectors in their

antennae and tarsi (Heran, 1952), bumblebees (Dyer et al., 2006; Whitney et al., 2008), honeybees (Hammer et al., 2009; Kovac and Stabentheiner, 2011) and stingless bees (Norgate et al., 2010) can distinguish flowers based on differences in overall temperature. Greater differences in temperature between flowers appear to be easier for bees to detect (Hammer et al., 2009), although bees have been shown to be able to detect differences in temperature as little as 2^{o}C (Heran, 1952). Floral temperature can also function as a floral reward by keeping pollinators warm while they feed (Herrera, 1995a; Rands and Whitney, 2008). Warmer flowers help insect visitors maintain a body temperature above their minimum threshold for flight (Heinrich, 1979a; Heinrich, 1979c). This allows pollinators to forage and collect nectar in colder conditions (Herrera, 1995a), and avoid the metabolic costs they might incur if they have to warm themselves for flight (Rands and Whitney, 2008). Therefore, floral temperature cues are likely to be salient to insect pollinators.

As well as being sensitive to temperature differences between the flower and its environment (Whitney et al., 2008; Hammer et al., 2009; Norgate et al., 2010; Shrestha et al., 2018), insects should also be sensitive to differences within a floral display. When flowers are observed using infrared thermography (thermal imaging), it is apparent that floral temperature is not necessarily distributed uniformly across the flower surface (Rejšková et al., 2010; Dietrich and Körner, 2014; Ladinig et al., 2015; Atamian et al., 2016; Shrestha et al., 2018). It has not been investigated whether any pollinators can learn to recognise flowers based on which parts of the flower are hotter or colder, which will determine the flower's temperature pattern. Understanding whether pollinators can detect temperature patterns within the flower will improve our understanding of how pollinators interact with flowers, and how floral displays have evolved.

In this chapter, I investigate the capacity of these floral temperature patterns to function as a floral signal. I demonstrate floral temperature patterns are common by taking thermographs the displays of 118 plant species (plus some additional subspecies and horticultural cultivars), that are visited by a range of pollinator groups and show a variety of

flower forms, under good weather conditions. I further ask whether bumblebees, frequently a generalist pollinator group (Heinrich, 1976; Williams, 1989; Goulson et al., 2005), can learn to distinguish rewarding from nonrewarding artificial flowers, based solely on temperature pattern differences comparable to those observed in real flowers.

3.2 Methods

3.2.1 *Sampling of floral temperature patterns*

Thermographs of floral blooms (flowers or flowering heads) were taken in Royal Fort Gardens (51º45'N 2º60'W) and the University of Bristol Botanic Garden (51º47'N 2º63'W) and in the National Botanic Garden of Wales, Carmarthen (51º84'N 4º15'W). 118 species were selected with the aim of sampling flowers visited by a wide range of floral visitor groups and as broad range of floral shapes, colours and phylogeny as possible (assistance with species identification was provided as described on page xv). Cultivars and subspecies were also thermographed where available. Due to thermal camera limitations in minimum area of measurement (I.T.C., 2008; Usamentiaga et al., 2014) very small flowers, when not part of a compound inflorescence, could not be sampled. Thermographs were taken on clear and sunny days, or inside a controlled glasshouse with near UV permeable windows, while in sunlight. Mean ambient temperature during sampling was 14.3ºC (SD 4.7). More detail on the weather conditions is available in appendix A2. All thermographs were taken with a FLIR *E60bx* thermal camera (FLIR systems, Inc., Wilsonville, USA), to a standard acceptable for I.T.C. guidelines (chapter 2, I.T.C., 2008; Usamentiaga et al., 2014). The thermal infrared emissivity was set at 0.98. This value is the estimate for vegetation (Rubio et al., 1997; Lópes et al., 2012) and has been previously used for thermography of floral tissue (Rejšková et al., 2010; Dietrich and Körner, 2014). For the sake of efficiency, reflected temperature was kept at 23ºC for all thermographs, due to the high emissivity of floral tissue this would have a minimal effect on temperature readings (see chapter 2).

All thermographs were viewed and measurements taken using FLIR tools software (FLIR Systems Inc., 2015). Using the point measurement functions, the temperature differences between the hottest and coldest points on the flower were measured and used to calculate the temperature range across each flower. Any additional cultivars and subspecies were counted as the same species as the one they were derived from when calculating temperature pattern occurrence or average within flower temperature difference. In such cases whichever variant showed the lowest within flower temperature difference was used, providing more conservative estimates.

3.2.2 *Bumblebee experiments*

Established bumblebee differential conditioning techniques (Dyer and Chittka, 2004d; Raine and Chittka, 2008; Whitney et al., 2008; Whitney et al., 2009c; Clarke et al., 2013; Lawson et al., 2018; Kjernsmo et al., 2018) were used to investigate whether bumblebees could learn to tell apart flowers based on differences in temperature patterns. Bumblebees are an appropriate choice of pollinator for investigating whether any pollinators can respond to temperature patterns. Many bees are generalist pollinators (Waser et al., 1996; Fenster et al., 2004), and it is known that generalist bees will visit many flower forms and families (Heinrich, 1976; Heinrich, 1979a; Williams, 1989; Fenster et al., 2004; Fontaine et al., 2008; de Vere et al., 2017). Bees also visit flowers which they may not pollinate to carry out larceny (Inouye, 1980; Manning and Snijman, 2002; Castellanos et al., 2004; Fenster et al., 2004). There is great variation in size and tongue length both within and between bumblebee species, with long tongued species tending to be more specialist, and shorter tongued bumblebees (such as *Bombus terrestris*) tending to be more generalist (Heinrich, 1976; Heinrich, 1979a; Williams, 1989; Larson and Barrett, 2000; Goulson et al., 2005; Fontaine et al., 2008; Smith, 2010). Bumblebees also occur all over the globe (Heinrich, 1979a). Thus, bumblebees, both as individual species and as a large functional group, will visit a large portion of the floral species surveyed. Additionally, the temperature sensitivities of bumblebees are understood

better than many other pollinators (Heinrich, 1979a; Dyer et al., 2006; Rands and Whitney, 2008; Whitney et al., 2008) and techniques for bumblebee conditioning experiments used here are well established (Dyer and Chittka, 2004d; Raine and Chittka, 2008), making them ideal for investigating pollinator responses to floral displays.

3.2.3 *Bee colony conditions and flight arena*

Flower naïve bumblebee colonies, *Bombus terrestris audax*, were supplied by Biobest (Westerlo, Belgium) *via* Agralan (Swindon, UK) or Syngenta-Bioline (Clacton-on-Sea, UK). Each bumblebee colony was connected to a wooden flight arena by a transparent plastic tube with gates that could be closed to allow control of bee entry to the arena (figure 3.1a and b). Flight arenas used were 72 x 104 x 30 cm (width x length x height) and had a UV transparent Perspex lid and had six access hatches to allow experimenter access to the arena (figure 3.1a). The floor of this arena was covered with Advance Green Gaffer tape (Stage Electrics, Bristol, UK). The flight arenas were illuminated by a ladder of multiple daylight simulating tube lights (Sylvania Activa 172 Professional 36 W fluorescent tubes, Havells-Sylvania Germany GmbH, Erlangen, Germany) supported from the ceiling 50cm above the arena top. These lights kept bees on a 12 hour day-night cycle. Ambient temperature was maintained at 21ºC and flight arenas were ventilated regularly when access hatches were opened.

Outside of experiments bees were allowed to enter the arena and feed from 30% sucrose solution from PCR racks, gravity feeders or a selection of seven or eight ‘generic’

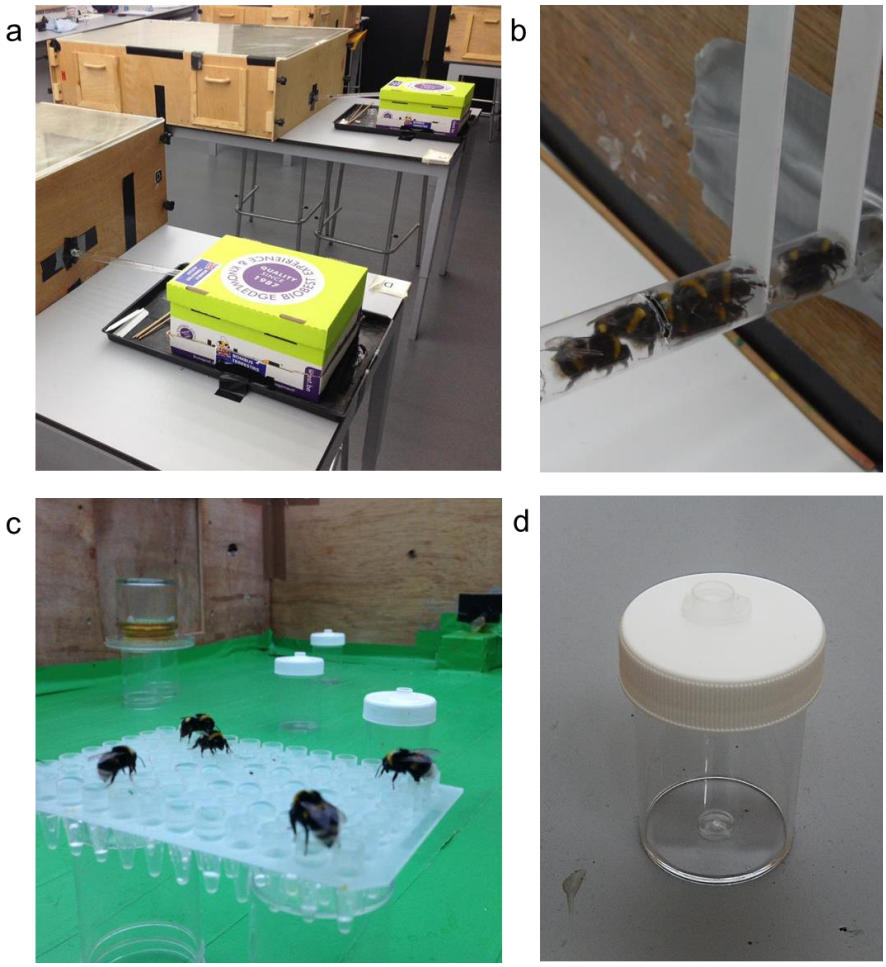


Figure 3.1: *The bee colony and flight arena conditions.* ***a)*** *an exterior view of the flight arenas and the bumblebee colonies (within the green-yellow boxes) attached to them.* ***b)*** *the plastic tubes linking flight arenas and bumblebee colonies. Note in the image how bee entry into the arena is being blocked by gates.* ***c)*** *bumblebee foraging within the flight arena outside of testing.* ***d)*** *a generic artificial flower used outside of testing.*

artificial flowers paced within the arena (figure 3.1). Generic artificial flowers lacked signals bees were to be tested upon, here temperature or temperature patterns, so bees remained naïve to experimental stimuli but were used to feeding from artificial flowers. Normally generic artificial flowers were constructed from specimen jars (Sterilin PS 60ml with white plastic lids, Thermo Fisher Scientific, Newport, UK) with upturned Eppendorf tube lids (Hamburg, Germany) stuck to them to act as a feeding well (figure 3.1d). Pollen was added directly to the colony every Monday, Wednesday and Friday. Individual bees that foraged from PCR racks,

gravity feeders or generic artificial flowers would be marked with non-toxic paint before experiments, this would allow identification of individual forager bees.

3.2.4 *Artificial flowers*

Two separate conditioning experiments, investigating the ability of bumblebees detect and to respond to temperature patterns in a flower foraging context, were carried out. In each of the two experiments bumblebees *B. terrestris* were presented with artificial flowers, either small (40mm in diameter) or large size (85 mm in diameter) depending on experiment (figure 3.2a and 3.2b). The two experiments with different sized flowers allow us to determine the impact of the size of temperature patterns on the identification of rewarding flowers. By using electrical heating elements, we were able to present differing temperature patterns on both sets of artificial flowers. On each flower size, these temperature patterns had two variants in temperature pattern layout and shape, but did not differ in either overall flower temperature, within-flower temperature contrast, or total area heated, to exclude other means by which bees could recognise variants.

Small and large artificial flowers were made from plastic cylinders with an insulated feeding well in the centre of a plastic lid (figure 3.2). Electric heating elements were stuck to the underside of the lid. In the small flowers this heating element was made from resistance wire and a pressure sensitive putty (Blu Tack: Bostik, Paris, France) heat sink. In the large flowers, four 1Ω resistors with a built-in sink were used. In the small flowers these heating elements could be altered in shape to create two different temperature patterns: a ‘circle pattern’ about the edge of the lid, and a ‘bar pattern’ across its centre (figure 3.2e). Altering the arrangement of the resistors in large flowers created two patterns: a ‘cross pattern’, where resistors radiated from the flower’s centre; and another ‘bar pattern’, where resistors were equally spaced across the flower’s length (figure 3.2f).

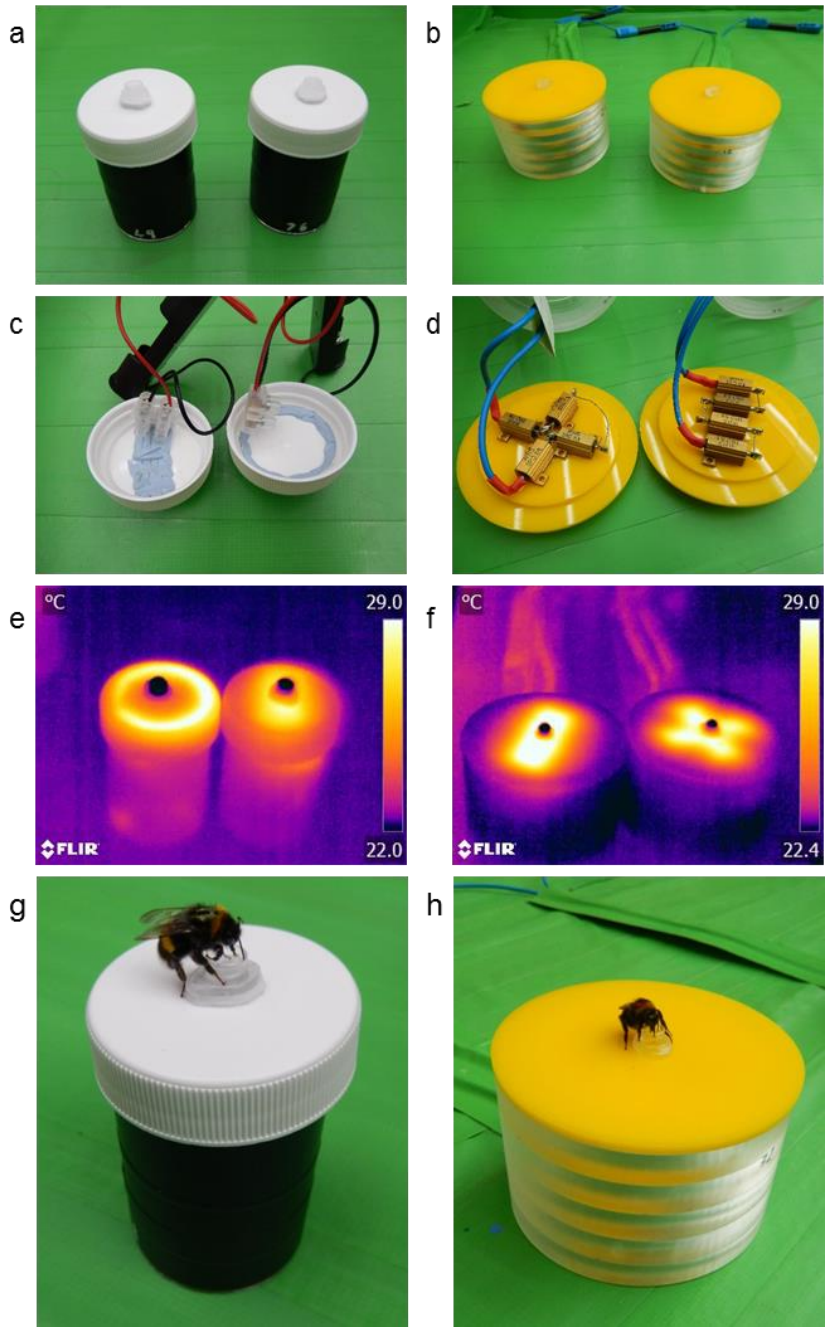


Figure 3.2: *The artificial flowers used in the bumblebee learning experiments.* ***a*** *and* ***b)*** *how both variants of artificial flowers used in the small (****a****) and large (****b****) artificial flower experiments appear.* ***c*** *and* ***d)*** *The heating elements stuck to the underside of the small (****c****) and large (****d****) artificial temperature flowers. When current is applied to these heating elements the areas above them heat up.* ***e*** *and* ***f)*** *thermographs of both artificial flower variants in in the small (****e****) and large (****f****) flower experiments, demonstrating how artificial flowers within each experiment differ in temperature patterns but not visually Colour indicates temperature in ºC as indicated on the scale bar to the right of each panel.* ***g*** *and* ***h)*** *bumblebees feeding from the small (****g****) and large (****h****) artificial flowers.*

Small artificial flowers were built from a specimen jar (Thermo scientific sterilin, PS 60ml, with white plastic lids). An upturned 0.5ml Eppendorf tube lid, insulated by a section of 1mm thick plastic foam, was stuck to the centre of the jar's lid (see figure 3.2a). This Eppendorf tube lid functions as a feeding well to contain sucrose solution or water but, with the plastic foam, also insulates it from heating. A 13cm length of 0.32mm, 17.87 ohms/m kanthal resistance wire was cut and 11cm of this was covered and stuck down to the underside of the feeders lid with blu tack. This left two 1cm 'leads' on each end of this heating element. Two patterns were created by the blu tack (Bostik, Paris, France). The first, a circle about the rim of the jar's lid, placed in such a way that it was still inside the treading of the jars screw. The lipped design of the jar allowed this to be done easily. In the second, where the wire was folded into an M shape along the centre of the jars lid, the blu tack creating a bar shape. Care was taken for the blu tack not to cover more than $3cm^2$ in each temperature pattern. The wire leads were then linked to a single AA battery in a cradle using two cut free sections of a connector block (figure 3.2c). When a 1.2V AA battery was inserted into the cradle, the current begins to heat up the resistance wire, thus causing the blue tack to function as a heat sink, heating up the top of the flower lid creating a circle or bar shaped temperature pattern depending on the shape of the blu tac heat sink (figure 3.2e). As the length of the resistance wire and the battery type was the same in each flower, the amount of heating varied little between flowers (figure 3.2e). As the area covered by the blu tack was also kept the same between patterns, the area heated was also the same between temperature patterns. This battery in the cradle was placed inside the jar and the lid closed over it. Black electrical tape was wrapped about the outside of the jar to conceal the content from bees and prevent the possibility that bees may visually identify the shape of the blue tack heat sink.

Large artificial flowers were built by the University of Bristol cross faculty workshop following my designs (see page xv) using an 8cm yellow cast acrylic disc that was built to slot into an 5cm tall cylindrical stand. Again, an insulated feeding cup was stuck to the centre top of the disk (figure 3.2b). Four 1Ω resistors with a built-in heat sink (1Ω through hole wirewound

resistors, Welwyn Components, Bedlington, UK) were stuck to the underside of the disks with resin. These were arranged in either a cross pattern radiating from the centre of the flower or a bar pattern spaced equally across the underside (figure 3.2d). These resistors were wired in series to two long blue insulated copper wires with connectors. These wire leads were covered by a sleeve made of card and green tape to match the floor of the arena and minimise the distraction to the bees. Eight of these artificial flowers were attached to each other again in series, to a variable power supply (ranging between 1.5V and 15V). During the experiment this allowed 6 artificial flowers to be present in the area and the temperatures of a further two to be monitored outside the area with the IR camera. When the power source was turned on the artificial flower's top heated up above the resistors. This created two patterns of temperature on the flower's top, both hotter in the centre of the flower but differing in shape according to the placement of resistors (figure 3.2f). As each flower had four resistors in series, all flowers heated up at the same rate and the area heated was the same across all the flowers. Varying the voltage allowed control of the heating within all the flowers. The cylindrical stand of the artificial flowers was transparent but clouded and thick, thus bees were unlikely to be able to see though to recognise flowers by the pattern of resistors.

As the Perspex lid of the flight arena was non-transparent to the thermal infrared radiation that the IR camera detects, a method was needed that allowed me, but not bees, to identify the artificial flowers while bees foraged. To allow identification of the temperature pattern in a way humans but not bees would manage, randomly generated even and odd numbers were written on the side of both kinds of artificial flower corresponding with the flowers temperature pattern. These numbers included several digits to allow even and odd digits to occur on all flowers thus bees could not use the presence of the number's shapes to recognise a flower. As jars and cylinder stands could be switched we also were able to change whether even or odd numbers corresponded with rewards (figures 3.2a and b).

The small artificial flowers normally reached a temperature of 33ºC at the heated parts above the heat sink and 25ºC on the parts that were not heated, with temperature differences

approximately 8ºC. These varied slightly between flowers and with time as flowers heated over the experiment but not consistently between temperature patterns in a way that could inform foraging decisions. Large artificial flowers were wired in series to a variable power unit (voltage ranging from 1.5V and 15V). This created a consistent voltage drop across each flower, thus the heating and area heated was the same between patterns. The temperatures of large flowers were monitored during tests using the thermal camera and a pair of flowers outside the arena wired into the same series as those presented to the bees. By varying the voltage temperatures were maintained, at approximately 24ºC in cold parts and 30ºC in hot parts. The temperature difference was maintained between 5ºC and 7ºC. Static electric signals generated by the larger artificial flowers were checked using a non-contact voltmeter and found to be below the 10V charge that bumblebees can detect (Clarke et al., 2013) and thus could not conflate results. As flowers within each experiment had the same heating elements, differing only in the shape, the area heated and the overall temperature of artificial flowers did not differ in a way which could inform bee foraging decisions (figure 3.2).

3.2.5 *Learning experiments*

Before bees began foraging, they were assigned to one of three test groups. This determined which floral temperature pattern provided sucrose solution (rewarding flowers), or water (nonrewarding flowers), in the centre of the flower hidden in the feeding well (figure 3.2g and h). There were three test groups: i) 'Bar rewards' group where the bar temperature pattern was rewarding, and the distractor pattern nonrewarding (cross in large or circle in small flowers); ii) 'Circle/cross rewards' group where reciprocally the circle or cross temperature pattern was rewarding, and the distractor nonrewarding (bar pattern); iii) 'Control' group where heating elements in the flowers were disconnected, and thus neither rewarding or nonrewarding flowers showed temperature patterns. An individual bee only foraged in one test group and were not used in both experiments. 12 bees completed the small flower learning experiment in each test group (36 bees in total from 4 different nests). 18 bees completed the

large artificial flower experiment in the Control and Cross rewards group and 17 bees in the Bar rewards group (53 bees in total from 7 different nests).

Both conditioning experiments began with a learning phase, where bees were presented with a choice of flowers placed randomly about the flight arena floor. Individual bees were let into the foraging arena one at a time and allowed to freely forage on the artificial flowers, and return to their nest. This time between a bee departing the nest to forage and returning was classified as a single foraging bout. During the learning phase feeding wells of the rewarding artificial flowers (as determined by the bee's test group) were filled with 25μl of 30% sucrose solution and the feeding well of nonrewarding artificial flowers with 25μl of water. In small flower tests, sixteen flowers (eight of each temperature pattern) were presented to the bee. In large flower tests, six flowers (three of each temperature pattern) were presented. Typically, bees made contact with the flower top while hovering above it before quitting flight and landing. If a bee landed on the flower it would normally approach the feeding well and extend its proboscis and attempt to feed from the sucrose solution presented in rewarding flowers (figures 3.2g and 3.2h). It could also decide to depart without attempting to feed. As bees detect temperature *via* touch (Heran, 1952), physical contact with the top of the flower was considered a landing, even if the bee did not quit flying. Bees were each observed for 60 flower landings. Bees completed the learning phase in 5.69 ± 1.79 and 8.60 ± 2.63 foraging bouts (mean ± SD) for the small and large flower experiments respectively, making 10.53 ± 6.58 and 6.97 ± 3.96 landings per bout. At each landing we monitored whether the bee fed from the feeder or left without feeding. For small flower experiments the learning phase was followed by a test phase. In the test phase, individual bees that had completed the learning phase were allowed to forage freely as discussed above. Here bees were presented with a fresh set of sixteen small temperature pattern flowers with 25μl of water in feeding wells but presenting the same temperature patterns, or lack of patterns in control group, the bee had experienced in the training phase. Bees were observed for twenty flower landings in this test phase. A test phase was not carried out in the large flower experiment as the large flowers

limited the number that could be sensibly placed within the arena, making them less suitable for a nonrewarding trial.

In small temperature pattern experiments, flowers were not interfered with while the bee was in the flight arena foraging. This was to minimise disturbance of the foraging bees. Once a bee had emptied the feeder of a flower any subsequent returns to that flower during the same bout were not counted. This was done so that a bee's foraging success was not influenced by encounters with empty feeding wells. It is not possible to distinguish whether a bee withholds its probing response because it is correctly responding to a nonrewarding flower (or incorrectly responding to a rewarding flower) or because the feeding well is empty. In large temperature pattern experiments flowers were topped up after the bee departed and moved to a different point in the arena, as the small number of flowers meant bees often had to visit flowers more than once in a bout. Return visits were not counted unless the flower had been moved to a different location and refilled whilst the bee was flying elsewhere in the arena. In both experiments after a bee returned to the colony, the end of a foraging bout, all the artificial flowers were taken out of the arena. Flower feeders were emptied and refilled to prevent differences in reward temperature developing. The flower tops were then wiped down with ethanol removing any scent marks the bees could have left. Thus, flowers were cleaned regularly preventing the bee from using these to recognise rewarding flowers (Stout and Goulson, 2001; Pearce et al., 2017). Temperature patterns were then checked with the thermal camera before placing artificial flowers back in the arena, replacing any flower that ceased to present the temperature pattern due to a fault.

Each flower landing was classed as correct or incorrect, as described in previous bee conditioning studies (Whitney et al., 2008; Whitney et al., 2009c; Clarke et al., 2013). In the learning phase experiments extending their proboscis into the feeding well (probing and/or feeding) on a rewarding flower, or not doing so when landing on a nonrewarding flower, was deemed a correct action. Extending their proboscis into the feeding well on a nonrewarding flower, or not doing so when landing on a rewarding flower, was deemed an incorrect action.

In the test phase all flowers were nonrewarding, therefore scoring flowers as ‘rewarding’ and ‘nonrewarding’ was determined by the reward scheme in the preceding learning phase. So, probing the feeding well of flowers with the temperature pattern that had been rewarding in that bee’s test phase, or not probing after landing on a flower showing the temperature pattern that had been nonrewarding, were correct actions in the test phase. Success rates over the previous 10 visits (starting at visit 10, then 20, 30, *etc*.) in the learning phase and overall success rate in the test phase were calculated for each bee. Comparing foraging success between the control bees and bees that had foraged on flowers with temperature pattern differences allows us to evaluate if temperature patterns aided bumblebee learning.

3.2.6 *Statistical Analysis*

Data were analysed using *R* 3.4.1 (R Development Core Team, 2017). The success rate data from the learning and test phase underwent an arcsine square root transformation in order to account for it being bound between 0 and 1. The arcsine square root of success probability across the whole test phase was compared between the three test groups using analysis of variance. Bee identity was included as a random factor.

Generalised linear model techniques and AIC model simplification was used in our analysis of bumblebee foraging success during the learning phase of our experiments. While differential conditioning data is often analysed by *t*-tests on the first and last 10 visits bees make during learning (Clarke et al., 2013), the model simplification technique used here has the advantage of including all visits made throughout the learning phase in comparisons and allows more specific comparisons of the influences on learning between each test group. For this reason, the following model simplification technique is more appropriate and informative for the learning data collected in this study

Not counting revisits to emptied flowers while scoring foraging success does mean the balance between rewarding and nonrewarding flowers could change as flowers are emptied,

especially during the learning phase, as bees are more likely to empty the wells of rewarding flowers. This effect was minimal in the large flower experiments, as flowers were refilled shortly after bees departed from them. In the small flower experiments, there was a much larger number of flowers in the flight arena and bees seldom visited all of them in a bout. Small flowers were refilled at the end of each bout (here on average every 10.53 visits). So, any changes in the balance of rewarding and unrewarding flowers did not persist for long. Furthermore, bees can carry out correct foraging actions on rewarding and unrewarding flowers showing probing, or not, as described above. Thus, the capacity of bees to forage correctly does not change as flowers are emptied, as long as some flowers still have sucrose or water in their feeding wells. Consequently, the impact of a changing balance of rewarding and nonrewarding flowers on scoring of pollinator foraging success is likely small and short-lived, thus was not included within our analysis.

The following represents our full model before any simplification was applied:

$$y_{nx} = i + (\ln x * l) + T\big(s_t + (\ln x * c_t)\big) + C\big(s_c + (\ln x * c_c)\big) + \big(b_n + (\ln x * r_n)\big). \tag{3.1}$$

Where y_{nx} is the arcsine square root success rate of bee n over the previous 10 visits to the artificial flowers, at x flower visits. x is the number of the visits the bee has made to the artificial flowers, the data for y is calculated in blocks of 10 (10, 20, 30, 40, 50, 60). i is the initial arcsine square root success rate, the intercept, for bees in the bar rewards test group when $x = 0$. Parameter l dictates the change in arcsine square root success rate with increased x in the bar test group, thus l is effectively the learning speed parameter and allows bee's experience to effect success rate. T and C are Boolean parameters which allow the model to alter y depending on which test group the bee is in. C indicates whether the bee is in the control group, where:

$$C = \begin{cases} 0, \text{ bee is not in the control group;} \\ 1, \quad \text{bee is in the contol group;} \end{cases}. \tag{3.2}$$

T indicates whether the bee is in the circle rewards or cross rewards test group, depending on the experiment, where:

$$T = \begin{cases} 0, \text{ bee is not in the cross or circle test group;} \\ 1, \quad \text{bee is in the cross or circle test group;} \end{cases} . \tag{3.3}$$

s_c and s_t are the change in initial arcsine square root success rate, relative to i, for bees in the control and circle or cross test groups respectively. c_c and c_t are the change in learning speed, relative to l, for bees in the control and circle or cross test groups respectively. Variation between individual bees was included in our model as a random factor. b_n and r_n represent the change in initial arcsine square root success rate and learning speed, for bee number n. In the model described in equation 3.1 parameters i, l, s_c, s_t, c_c, c_t, b_n and r_n are parameters to be estimated.

Model simplification procedure involved paired comparisons between the standing 'best model', beginning with the full model described in equation 3.1, with a simpler model. Simpler models were constructed from the standing best model but with further parameters removed (effectively forcing the relevant parameters to equal zero) in the order described below. Should the simpler model have a lower AIC or be comparable to the standing best fitting models based on AIC, as laid out by Richards (2008), this simpler model would become the best model for the next comparison. If removal of a parameter led to a significant increase in AIC, again as laid out by Richards (2008), the standing best (more complex) model would remain the best for the next comparison.

Initially the effects of random factors were compared, a model without r_n was compared to the complete model. This allowed testing of whether individual bees differed only in intercepts or intercepts and learning speed (as in the full model). In both experiments r_n had no significant effect on the model, and is thus not included in subsequent models below. Secondly interaction effects were investigated by removing c_c and c_t. This created a model where the shape of the relationship between x and y in all test groups was dictated only by l.

Should the best model, according to AIC, find no significant interaction the effects of the test groups would be investigated by removing T and C creating a model were all test group both showed the same intercepts and learning. Finally, the impact of experience on success was compared by removing the learning parameter l. Should the best fitting interaction model include interaction effects individual models for each test group would be fitted as follows:

$$y_{nx} = i + (\ln x * l) + b_n. \quad (3.4)$$

For each test group, using the model described in equation 3.4, we tested whether bee foraging success changed with the number of visits the bee has made by removing parameter l.

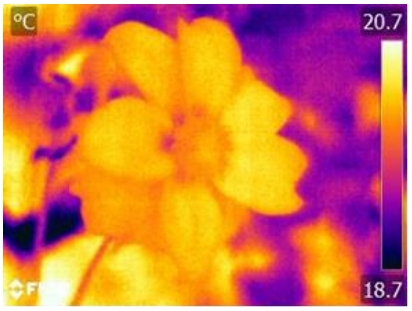
3.3 Results

3.3.1 *Diversity of floral temperature patterns*

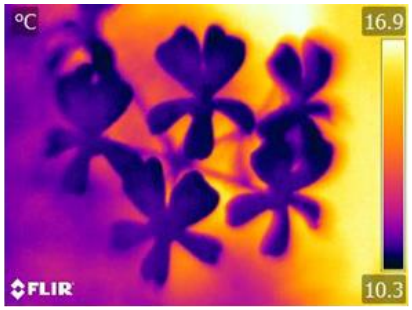
The thermographs of flowers of 118 species in different taxa reveal the variety of temperature patterns of different shapes, sizes and locations that pollinators may encounter. A selection of floral thermographs are provided (figure 3.3), with the human visual photographs collected simultaneously by the camera (figure 3.4). The full library of floral thermographs can be found online (in the Supplementary Data for the published version of this chapter https://datadryad.org/resource/doi:10.5061/dryad.qp244). Appendix A3 lists each species and the within flower temperature differences measured across them. Some species had little to no detectable temperature differences across their surface, for example *Dahlia coccinea* and *Pelargonium echinatum* (figure 3.3). However, most species observed showed some part of the flower that differed in temperature from the rest of the flower, thus displaying a temperature pattern (figure 3.3 and appendix A3). Most often there was a temperature contrast between the flower centre and its periphery, although the extent and shape of contrasting regions varied greatly. In such cases the centre of the flower was often hotter, as in *Bellis perennis* and

Geranium psilostemon, but not always, as with *Papaver rohoeas* or *Hydrangea macrophylla* (figure 3.3). Warming or cooling of a protruding section of the flower, such as 'landing pad' petals of zygomorphic flowers such as *Crinum*, or the reproductive structures of *Papaver* (figure 3.3), also frequently created contrasting regions of temperature. Flowers of all sizes showed temperature patterns, such as the large *Hermerocallis '*autumn red' and small *Bellis perennis* flowers (figure 3.3).

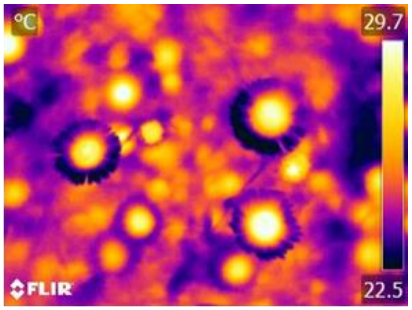
Of the 118 species thermographed, 65 species (55%) showed within-flower temperature differences of at least 2^{o}C (appendix A3). So more than half the species observed show temperature contrasts which at least bees would be able to detect (Heran, 1952). Within these 65 species the average temperature difference was 4.89^{o}C ± 2.28 (mean ±SD). While these findings suggest temperature patterns that can vary greatly between species, we must determine whether pollinators can use such differences in temperature patterns to inform foraging in order to show these differences can be used as floral signals.



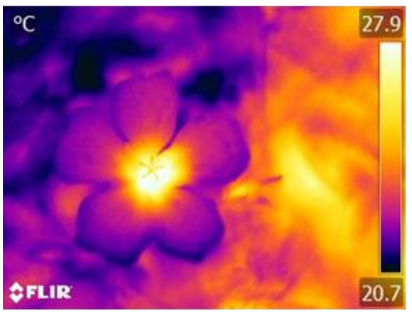
Dahlia coccinea



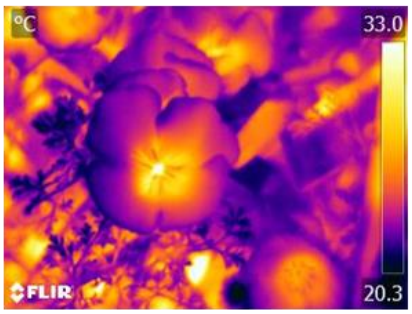
Pelargonium echinatum



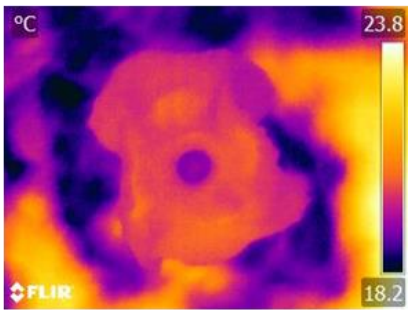
Bellis perennis



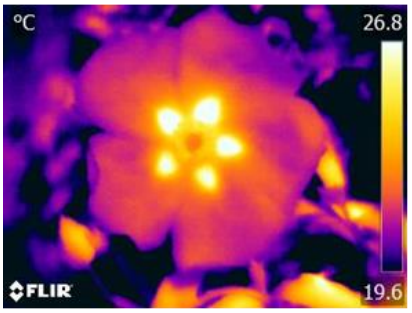
Geranium psilostemon



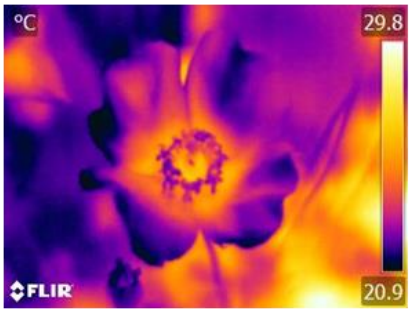
Eschscholzia californica



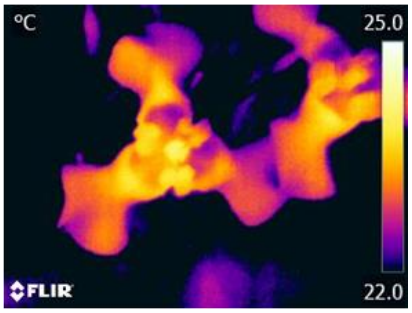
Papaver rhoeas



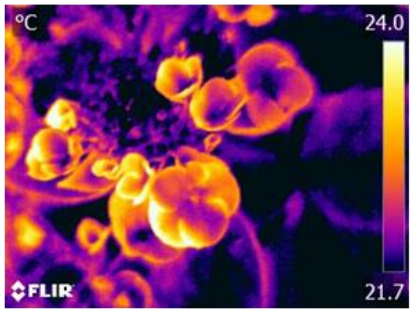
Cistus x *verguinii*



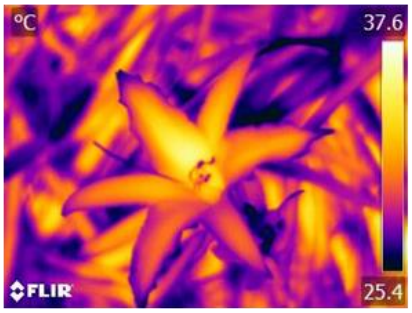
Papaver cambricum



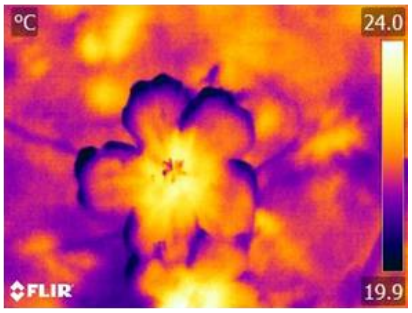
Cypella herbertii



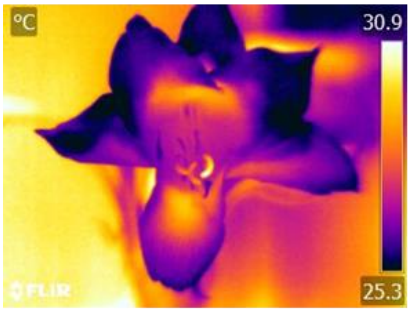
Hydrangea macrophylla



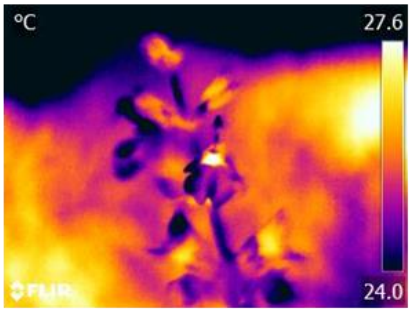
Hemerocallis 'autumn red'



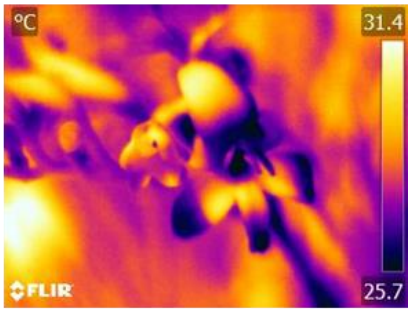
Geranium sylvaticum



Crinum x *powellii*



Scutellaria galericulata



Crocosmia 'Lucifer'

Figure 3.3 [left]: *Floral thermographs demonstrating the range of floral temperature patterns observed. Colour indicates temperature in ºC as indicated on the scale bar to the right of each panel. The flower species is labelled below each thermograph. Human colour images corresponding to each thermograph are presented in figure 3.4. Appendix A3 summarises the temperature differences measured across all 118 species observed, and thermographs of each species can be found in the Supplementary Data for the published version of this chapter* (*https://datadryad.org/resource/doi:10.5061/dryad.qp244*).

Dahlia coccinea

Pelargonium echinatum

Bellis perennis

Geranium psilostemon

Eschscholzia californica

Papaver rhoeas

Cistus x verguinii

Papaver cambricum

Cypella herbertii

Hydrangea macrophylla

Hemerocallis 'autumn red'

Geranium sylvaticum

Crinum x powellii

Scutellaria galericulata

Crocosmia 'Lucifer'

Figure 3.4 [left]: *Human colour images of each flower species shown in Figure 3.3. Species names are at the bottom of each photograph. Note that, when using the FLIR E60bx IR camera, the twinned digital and thermal images are taken at slightly different angles and magnification, so images do not match perfectly. Additionally human colour images are not seen while thermographs are being captured, thus focus, colour saturation and image clarity can differ.*

3.3.2 *Bumblebee experiments*

When foraging on small artificial flowers, bumblebees learnt to identify rewarding flowers when they differed in temperature patterns (figure 3.5) but did not learn in the control group. When models of bumblebee foraging success in the learning phase were compared, the relationship between success and experience varied between test groups (figure 3.5a). Models including individual learning speeds (r_n) where comparable to those that did not (AIC -226.31 *vs*. -223.70 ΔAIC = 2.61, Δdeviance = 1.38, df = 2, $p = 0.501$). Models that allowed test group to have an interacting effect with experience producing a lower AIC (-226.3 *vs*. -216.9, ΔAIC = 9.4) and a better fit (Δdeviance = 13.4, df = 2, $p < 0.01$) than models that did not, meaning the relationship between success and experience varied with the test group the bee was in. Bees from the control group foraged randomly throughout the experiment maintaining a 50% success rate, experience having no effect on success (AIC -83.3 *vs.* -83.0 ΔAIC = 0.3; Δdeviance = 1.6, df = 1, $p = 0.201$). When flowers differed in temperature patterns, bees began with a success rate comparable to the control group but improved with experience; this occurred regardless of which temperature pattern corresponded with rewards (Circle rewards: AIC -92.1 *vs*. -68.5, ΔAIC = 23.6; Δdeviance = 25.6, df = 1, $p < 0.001$. Bar rewards: AIC -50.5 *vs*. -28.0 ΔAIC = 22.5; Δdeviance = 24.5, df = 1, $p < 0.001$). When the conditioned preference was tested in nonrewarding tests, bees in the bar and circle reward groups made more correct visits than the control group ($F_{2,33} = 23.8$, $p < 0.001$, figure 3.5b). These results demonstrate that bumblebees can learn and alter foraging decisions based on differences in temperature patterns.

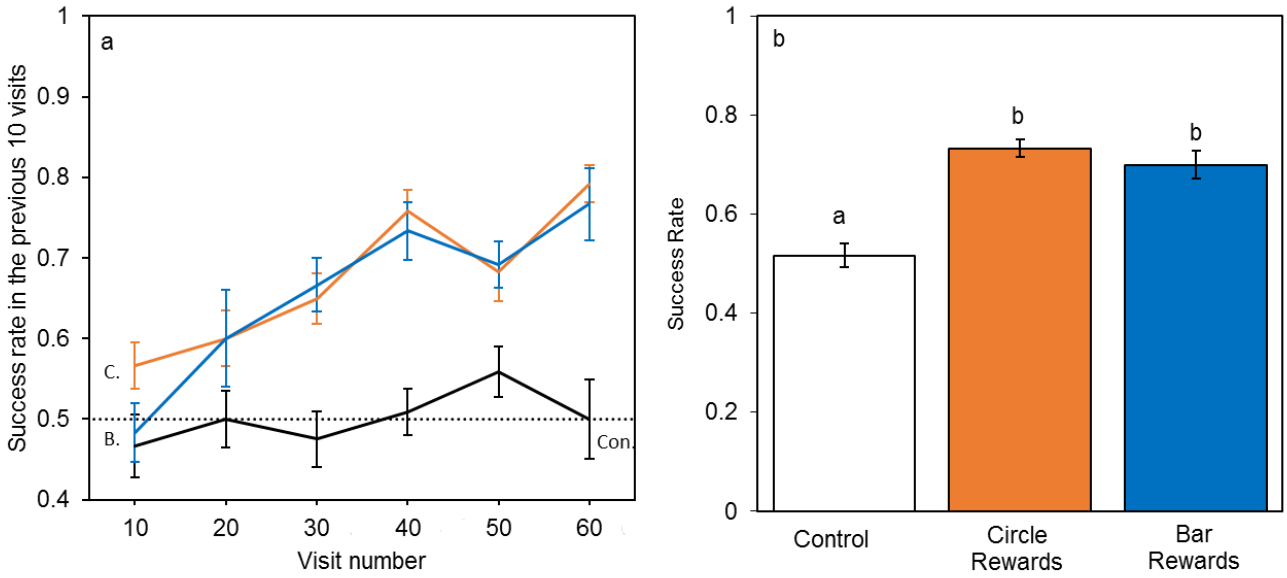


Figure 3.5: *Bumblebee learning within our small artificial flower experiment.* ***a)*** *the relationship between bees' foraging success and experience of the small artificial flowers (flower visits made) during the learning phase. The dotted line indicates the 50% success level. Solid lines indicate the mean foraging success of bees in the previous 10 visits. Error bars represent ± SEM. Colour and label of solid lines and error bars correspond with test group: black, the control group, labelled "Con."; orange, Circle rewards group, labelled "C."; blue, Bar reward group labelled "B.".* ***b)*** *mean foraging success ± SEM of bees in different test groups during the nonrewarding test phase. Letters above bars denote groups as defined by post hoc Tukey's tests where $p < 0.05$.*

Bumblebees also showed the ability to perceive temperature patterns in large-sized flowers (figure 3.6), although test groups showed similar shaped relationships between success and experience. Models that included individual learning speeds for bees were comparable to those that did not (AIC -290.88 *vs*. -290.30 ΔAIC=0.58, Δdeviance = 3.42, df = 2, $p = 0.181$). Models including an interaction between test groups and those that did not, were comparable in terms of AIC (Richards, 2008) (AIC -290.88 *vs*. -287.72 ΔAIC = 3.16), but were a better fit (Δdeviance = 7.16, df = 2, $p = 0.028$). Nevertheless, which test group bees were in still had a significant effect on the level of success achieved (AIC -287.72 *vs*. -266.71 ΔAIC = 21.01, Δdeviance = 25.01, df = 2, $p < 0.01$), with Bar and Cross reward groups achieving a greater level of success than the control. Experience had a significant effect across test groups

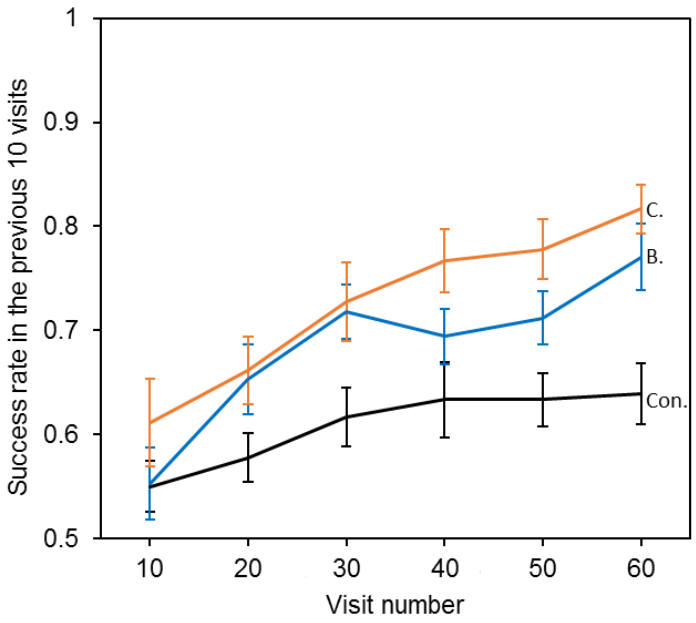


Figure 3.6: *The relationship between bumblebees' foraging success and experience of the large artificial flowers (flower visits). Solid lines indicate the mean foraging success of bees in the previous 10 visits. Error bars represent ± SEM. Colour and label of solid lines and bars correspond with test group: black, the Control group, labelled "Con."; orange, Cross rewards group (rewarding cross pattern), labelled "C."; blue, Bar reward group (rewarding bar pattern), labelled "B.".*

(AIC -287.72 *vs*. -233.06 ΔAIC = 54.66, Δdeviance = 56.66 df = 1, $p < 0.01$). Thus, the presence of temperature patterns improved bumblebee foraging success, indicating their ability to use these larger temperature patterns to distinguish flowers.

The increase in success rate in the control group in the large flower experiment (unlike in the previous small flower experiment) can be explained by the experimental set-up leading to spatial preferences within the arena that developed during training. Three rewarding and three nonrewarding flowers were present in the arena during the large flower experiment due to space constraints, and there was a reduced ability for random re-arrangement of each flower due to wiring constraints. Bees have a great capacity for spatial learning (Burns and Thomson, 2005; Robert et al., 2016), and therefore the control group may have learnt to identify within each foraging bout which regions of the arena contained more rewarding

flowers. However, even with this spatial learning, the presence of temperature patterns improved bumblebee foraging success on the large artificial flowers.

3.4 Discussion

The results of both of the conditioning experiments showed that temperature pattern differences improved the ability of bumblebees to distinguish between rewarding and nonrewarding artificial flowers (figures 3.5 and 3.6). This suggests that floral temperature patterns can function as a floral signal. The main variation observed in floral temperature patterns were between flowers with hot centres and cold edges and *vice versa* (see figure 3.3, appendix A3), and bees foraging on the small artificial flowers were observed to be able to distinguish similar differences (figure 3.5). Furthermore, bees foraging on large artificial flowers could distinguish between two differently-shaped patterns where the centre of the flower was hotter (figure 3.6), demonstrating that bumblebees can detect more detailed aspects of temperature signals. Artificial flowers showed within-flower temperature differences similar to that of real flowers (figure 3.2, appendix A3). Real flowers can show a greater degree of variety in the temperature differences than those used in our experiments, which represented flowers showing the clearest temperature patterns (appendix A3). However, bees have been shown to have a high sensitivity to differences in temperature (Heran, 1952; Dyer et al., 2006) and are therefore likely to detect the lower within flower temperature differences as well as the higher temperature differences. The use of floral temperature might not be limited to bumblebees, since other pollinating insects have been observed to detect and respond temperature differences between different flowers (Sapir et al., 2006; Kleineidam et al., 2007; Hammer et al., 2009; Kovac and Stabentheiner, 2011), and therefore may also be able to use temperature patterns as foraging signals. Furthermore, it did not appear that temperature patterns were limited to flowers associated with bumblebees. Temperature patterns appear to be a floral phenomenon, rather than a ‘bee flower’ phenomenon. Several

of the Asteraceae (which are known to be visited by a variety of insects including bumblebees, Goulson et al., 2005), as well as primarily bee pollinated flowers such as *Eschscholzia californica* (Smith, 2010), were among those that produced the most contrasting temperature patterns (figure 3.3, appendix A3). However, other plants attracting similar pollinators were also observed to produce little temperature contrast across their surface. Additionally, some plants associated with moths and hummingbirds, such as *Crinium* and *Crocosmia* (Manning and Snijman, 2002; Goldblatt and Manning, 2006), were also observed to produce contrasting temperature patterns (figure 3.3. and appendix A3).

Demonstrating that floral temperature patterns could present a floral signal raises the question as to how they might be generated, and there are several potential mechanisms. Different flower species differed in which structures generated temperatures patterns (figure 3.3). Some patterns are created by hotter or colder parts of the petals, and others by hotter or colder reproductive structures. The variation in shape and contrast of temperature patterns between different plants derived from the same species (*i.e.* cultivars, subspecies) suggest that small changes in floral characters can influence temperature patterns. This is perhaps most evident in the various *Cistus, Gazania* and *Knautia* flowers thermographed (appendix A3). Floral morphology appears to influence temperature pattern generation, as structures in a position more likely to capture light tended to be warmer (e.g. the exposed petals in the landing pad of *Crinum*). Structures that were more densely packed, and might retain heat better were often warmer (such as the florets of composite inflorescences). Likewise, colour differences in the visible spectrum often appeared to occur alongside temperature differences (figure 3.3 and 3.4). Such observations are in agreement with our understanding of the influence of solar radiation (Totland, 1996; Sapir et al., 2006; Rejšková et al., 2010; Kovac and Stabentheiner, 2011) and floral structure (Miller, 1986; Davis et al., 2008) on floral temperature. Additional potential influences on temperature include floral metabolism (Seymour and Schultze-Motel, 1997; Seymour and Matthews, 2006), active heat loss by transpiration (Gates, 1968; Tsukaguchi et al., 2003) and petal epidermal cell shape effects

(Whitney et al., 2011). Further study of how these influences differ across the floral surface will help us gain a greater understanding of floral temperature pattern generation.

Ecological factors might influence a flower's capability to generate temperature patterns pollinators can detect. The amount of sunlight captured limits floral warming in non-thermogenic plants (Totland, 1996; Rejšková et al., 2010; Zhang et al., 2010). While Rejšková et al. (2010) found that artificially shaded *Bellis perennis* flowers maintained temperature patterns, overall temperature of the flower and temperature contrast between regions decreased, and shaded *Anemone nemorosa* cooled to even temperature across the flower. Pollinators may only be able to use temperature pattern signals during sunny weather and when flowers grow in open non-shady environments. Understanding how floral temperature patterns change with environmental conditions, and the sensitivity of pollinators to changing temperature patterns (including how small a contrast in temperature that pollinators are able to detect), will reveal the level of influence that environmental factors have on temperature patterns. It may be that flowers that grow in less sunny climates and in shadier habitats may not be under strong selection to produce complex thermal cues such as temperature patterns. Plants in these conditions may seldomly generate temperature patterns (Rejšková et al., 2010), and pollinators may not be able to detect or respond to these patterns. Several of the flower species that produced the greatest contrasts in temperature within the flower are associated with hot and dry climates (*e.g*. *Osteospermum* and *Dimorphotheca* species) or with more open environments (*e.g*. *Geranium psilostemon* and *Eschscholzia californica*, figure 3.3 and appendix A3), even though all sampling took place in similar conditions. This may reflect such plants experiencing greater selection to produce thermal cues.

Flowers are multimodal displays - they produce many different kinds of floral signal simultaneously (Raguso, 2004; Leonard et al., 2012), despite pollinators often being able to distinguish flowers based on a single signal (Bhagavan and Smith, 1997; Dyer and Chittka, 2004d; Clarke et al., 2013). The benefits of this multimodality are only just starting to be understood (Leonard et al., 2012; Kaczorowski et al., 2012; Leonard and Masek, 2014;

Lawson et al., 2017b; Lawson et al., 2017a). 'Novel' signalling modalities, such as floral electrostatic fields, have been found to be equally beneficial in foraging maintaining accuracy (Clarke et al., 2013). The discovery of another floral signal that bumblebees can use to recognise flowers, temperature patterns, encourages further investigation into this apparent redundancy in floral signalling and the potential benefits multimodal signalling confers. The frequent overlap of temperature patterns with structural and visual elements of the floral display perhaps makes them ideal for investigation of how floral signals interact within multimodal displays.

Thermal imaging of floral temperature reveals that flowers show a diversity of temperature patterns. It is known that bees can distinguish differences in temperature between flowers (Whitney et al., 2008) and using temperature as a reward (Rands and Whitney, 2008), and we have shown here that bumblebees can use these floral temperature patterns as a signal to recognise flowers and make informed foraging choices based upon them. This ability does not seem to be influenced by the size of the flower and its floral temperature pattern. Thus, floral temperature patterns may be added to the growing number of floral signals (Raguso, 2004; Leonard et al., 2012) that pollinators, at least bumblebees, may be able to utilise to identify more rewarding flowers in their environment.

Chapter 4: Measurement of floral humidity

Data presented in this chapter was obtained with the assistance of a collaborator Dr Henry Knowles, as described on page xv. Some of the analysis presented in this chapter, that contained in sections 4.3.6 and 4.4.4, were carried out by my supervisor Dr Sean Rands, also described on page xv.

CHAPTER ABSTRACT

Due to the combined effects of liquid nectar evaporation and floral transpiration the area of space immediately around the floral display is likely to have increased humidity relative to the environment around it. This floral humidity production could act as a cue for pollinators. However, with a few exceptions, relatively little is known about patterns of floral humidity in flowering plants. Here, I present a survey of 42 radially-symmetrical species (representing 21 widely-spread families), measured using a novel robot arm technique that allowed us to take transects of humidity across and above the floral surface. 30 of the species surveyed presented levels of humidity exceeding a control comparable to background humidity levels, demonstrating that floral humidity is produced by a wide range of species. The structure of floral humidity differed across species, but tended to be highest near the centre of the flower, and decreased logarithmically with increasing distance above the flower. Repeated transects revealed that in many flower species, floral humidity declined over time. These results suggest that there is a greater level of complexity and diversity to floral humidity patterns than previously known.

4.1 Introduction

Flowers can simultaneously produce many signals that can be detected by a pollinator using differing sensory modes. It is not well understood why a flower's display should be multimodal. Explanations (chapter 1; Hebets and Papaj, 2005; Leonard et al, 2012), many of which are not excusive of each other, suggest that additional floral signalling modalities might: improve detection and recognition of the flower (Kulahci et al., 2008; Kunze and Gumbert, 2001); improve robustness to interference from environmental conditions (Johnstone, 1996; Lawson et al., 2017a; Dyer and Chittka, 2004a; Kaczorowski et al., 2012); or attract different kinds of pollinator (Muchhula et al., 2009). It is possible that additional signals within a floral display convey different information to the pollinator, the 'multiple messages hypothesis' (Møller and Pomiankowski, 1993; Johnstone, 1996; Leonard et al., 2012). As part of a multiple-message display, some floral cues might provide information on the flower's reward status (Leonard et al., 2012; von Arx, 2013), with specific signals potentially indicating the type (Kong et al., 2017), presence (von Arx et al., 2012), quality (Knauer and Schiestl, 2015) and quantity of rewards (Hansen et al., 2006) present in a flower, or indicating how recently flowers have been visited to pollinators (Clarke et al., 2013). When flowers produce signals that provide information about individual flowers' reward status, this is known as 'honest signalling' (Hansen et al., 2007; von Arx, 2013; Knauer and Schiestl, 2015). An individual foraging on an honest signalling flower species can avoid wasteful visits to emptied or unrewarding flowers. Because a visitor's foraging efficiency will be increased when visiting species that produce honest signals (Raine and Chittka, 2008; Wright and Schiestl, 2009), this suggests that honest signallers should be preferred by their visitors, and receive greater pollinator visitation and other benefits to their reproductive success (chapter 1; Hansen et al., 2007; von Arx et al., 2012).

Floral humidity has been suggested to be an honest signal (von Arx et al., 2012; von Arx, 2013). Floral humidity is characterised by an area of increased humidity relative to background levels about the flower headspace (the area of empty space about the floral

display). von Arx et al. (2012) demonstrated that evening primrose *Oenothera caespitosa* flowers create an area of elevated humidity at the hovering distance of their hawkmoth pollinator, *Hyles lineata*. This humidity increase was approximately 4% higher than the background ambient level. This difference from the background was highest directly above the flower's corolla tube and decreased with increased distance from the flower. The hawkmoth was demonstrated to show a preference for more humid artificial flowers generating similar humidity levels to the primrose over those producing none, indicating floral humidity can be detected by *H. lineata* and influence foraging decisions (von Arx et al., 2012). Removal of nectar from primrose flowers, or plugging the nectar tube, reduced floral humidity greatly but not completely (von Arx et al., 2012). This suggests that floral humidity is generated by a combination of evaporation of liquid floral nectar rewards and transpirational water loss from flowers. Nectar removal induced changes to the humidity signals produced by the primrose, indicating that floral humidity can potentially function as an honest signal directly linked to the presence of nectar rewards. Further stationary probe monitoring showed that humidity signals produced by the primrose began at flower opening and dropped off with time, and so could also function as an indicator, honest signal, of flower age (von Arx et al., 2012; von Arx, 2013).

It is likely that other flower species produce floral humidity, particularly because liquid nectar is abundant in angiosperms (Percival, 1960; Brandenburg et al., 2009) and transpiration is a ubiquitous plant process (Morgan, 1984; Jarvis and McNaughton, 1986; Gates, 1968; Azad et al., 2007; Liang et al., 2010). Relative humidity has been reported to increase with proximity to *Digitalis*, *Echium* and *Helleborus* flowers (Corbet et al., 1979a; Corbet et al., 1979b). Likewise, flowers preferred by flies in Indian alpine environments have higher humidity levels about them than those that flies visit less (Nordström et al., 2017). However, such surveys did not always consider or monitor background humidity levels (Nordström et al., 2017) or characterise floral humidity structure as done in von Arx et al. (2012). It remains unclear to what extent any flower species other than *O. caespitosa* produce floral humidity.

As floral humidity is the result of a combination of transpirational water loss and nectar evaporation (von Arx et al., 2012; von Arx, 2013), floral characteristics are likely to play a role in a flower's ability to generate humidity. Larger flowers may allow greater volumes of nectar to accumulate. Nectar volume present in the flower will influence how much nectar evaporation can actually occur (von Arx et al., 2012). Nectar volume can differ between species as can patterns of nectar secretion (Biernaskie and Carter, 2002; Carlson and Harms, 2006; Kaeasar et al., 2008; Mačukanović-Jocić et al., 2004) and reabsorption (Langenberger and Davis, 2002). Some flower species differ in the sugar concentration (Corbet, 2003; Brandenburg et al., 2009), types of sugars (Percival, 1960; Corbet et al., 1979b) and the amount of secondary metabolites that are secreted in to the nectar (Baker, 1977; Kessler and Baldwin, 2006; Richardson et al., 2015). These influence the viscosity and concentration of dissolved material which influences evaporation (Corbet et al., 1979b). As evaporation takes place at the exposed surface of liquids, factors that influence the exposure of liquid nectar to the environment will influence the extent of nectar evaporation (Corbet, 2003). In this way the flower structure can play a part in nectar evaporation. Exposed nectaries and open floral architecture allow increased nectar evaporation (Corbet et al., 1979a; Plowright, 1987; Corbet, 2003). Deep, narrow corollas and nectaries capable of closing can limit evaporation for the same reasons (Plowright, 1987; Corbet, 2003). However, open floral architecture may not be sufficiently shielded (von Arx et al., 2012), preventing humidity from accumulating, while deep corollas create and enclosed space that may allow areas of high humidity to accumulate (Corbet et al., 1979b; von Arx et al., 2012; von Arx, 2013). Transpirational water loss can also be influenced by floral characteristics. Petals often have more permeable cuticles than leaves, allowing greater water loss (von Arx et al., 2012; Buschhaus et al., 2014). This can be due to differences in the chemical composition of the cuticle (Corbet et al., 1979b; Schreiber and Riederer, 1996; Goodwin et al., 2003; Guo et al., 2017) and cuticle thickness (Hajibagheri et al., 1983). Many flower species have floral stomata, although at a lower density to leaves (Inamdar, 1968; Shah and Gopal, 1971; Azad et al., 2007; von Arx et al., 2012; Davis et al., 2005; Hew et al., 1980). Stomata have a major influence on transpiration water loss from plant

tissues (Jarvis and McNaughton, 1986; Azad et al., 2007). Floral stomata vary in their gaseous exchange and transpiration activity between species. In some flowers floral stomata carry out a similar levels of transpiration to leaf stomata (von Arx et al., 2012; Huang et al., 2018), while in others floral stomata have been observed to be non-functioning (Hew et al., 1980; van Doorn, 1997). In orchid *Maxillaria anceps* floral stomata have been observed to be nectar secreting (Davis et al., 2005). Floral stomata have also been observed to open in sequence with flower opening (Azad et al., 2007; Effmert et al., 2005). The presence, density, location, functionality and opening patterns of floral stomata are therefore likely to influence floral humidity generation. As several floral characters can potentially contribute to floral humidity generation (von Arx et al., 2012) species may differ in their capacity to generate floral humidity. Varying levels of floral humidity between species may mean that humidity can be used by pollinators to distinguish flower species, in addition to it giving information about the location or presence of rewards.

Many floral traits have recently been revealed to have structural elements such as patterns, which are detectable to floral visitors but difficult for humans to identify (chapter 3; Clarke et al., 2013; Harrap et al., 2017; Lawson et al., 2018). While the greatest humidity within the floral headspace of *O. caespitosa* was found above the flower corolla tube (von Arx et al., 2012), differences in flower characteristics may influence, in addition to humidity intensity, the structure of floral humidity between species. Such structural changes to floral humidity may include where the greatest humidity is found about the flower's headspace, or how much of the floral headspace is at elevated humidity. Differences in the location and orientation of nectaries may alter where evaporated vapour accumulates about the flower. Differences in permeability across the petal cuticle, as well as the location and density of petal stomata (Azad et al., 2007; Huang et al., 2018) will similarly alter where transpirational water loss occurs. These contributions of transpiration and nectar evaporation to a species' floral humidity may alter shape of humidity structures and how much humidity differs across the flower. This potential for floral humidity structure to differ across species has a further consequence when

sampling floral humidity. A stationary probe can detect a difference in floral humidity compared to the background (Nordström et al., 2017). However, such measurement methods carry a risk of underestimating the humidity generated by the flower (if they are measuring in the wrong location) and fail to evaluate any structural differences in the floral humidity. Furthermore, humidity appears to decline with distance from the flower (Corbet et al., 1979a; Corbet et al., 1979b; von Arx et al., 2012), and a stationary probe does not provide any information on the distance floral humidity might reach. Multidimensional transects of the flower headspace, similar to those used by von Arx et al. (2012), allow evaluation of how much species differ in floral humidity structure and allow further consideration of how this humidity changes with distance from the flower.

Understanding the capacity of flowers, other than *O. caespitosa*, to produce floral humidity will increase our knowledge of how multimodal flowers actually are. Furthermore, understanding in what ways that different flower species' floral humidity can potentially vary in intensity and structure may reveal potential ways in which floral humidity might be utilised by flower visitors. Understanding which flower species produce floral humidity will increase our knowledge of the mechanisms behind floral humidity generation. In this chapter, we analyse the humidity in the headspace above 42 species of flowers (including resampling *O. caespitosa*). This is done using a humidity probe supported by a robotic arm carrying out a similar transect method to that described by von Arx et al. (2012). Using these measurements, we evaluate the structure and intensity of floral humidity from these species.

4.2 Methods

4.2.1 *Robot sampling setup*

All floral humidity measurements were taken within a 6.11 × 3.67m room within the Bristol University Life Sciences Building (51º45'N 2º60'W). A 3.72 × 3.67m section of this room, hereafter referred to as the 'sampling zone', was separated off within a 2m high wall of 10mm thick clear polycarbonate and a 6-axis articulated *Staubli* RX 160 robot arm (Pfäffikon, Switzerland) was mounted in the centre of this space. A scale floorplan for this room is given in figure 4.1. The mean background temperature and background humidity of the room during sampling was 23.01ºC (SD=1.38) and 49.1% (SD=12.3) respectively. Relative humidity is a percentage measure relative to the amount of water vapour in the air needed for saturation. Consequently the amount of water vapour indicated by a relative humidity value varies dependent on aspects like air temperature (Tichy and Kallina, 2014), for example an increase in air temperature of 10ºC approximately doubles the amount of water vapour indicated by the same relative humidity value. However, here, the consistent lab temperature means that the relative humidity values indicate a similar amount of water vapour across all measurements.

All humidity measurements were taken using DHT-22 humidity probes (Aosong Electronics Co., Ltd., Huangpu, China) attached to an *Arduino UNO* microcontroller (Adafruit Industries, New York, USA), with the *Arduino* transmitting the measurements via USB to a PC outside the sampling zone (behind the polycarbonate screen), where all measurements were stored. Two probes were used in measurements: a 'background probe', which measured the ambient background humidity levels, and a 'focal probe', which measured the humidity about the headspace of the object being sampled. The focal probe was mounted on the robot as detailed in figure 4.2.

A 75 × 90 × 74cm (width × length × height) wooden table was placed in the sampling zone. A rack for 24cm^3 horticultural tubes was fixed along one length of the table (figure 4.1),

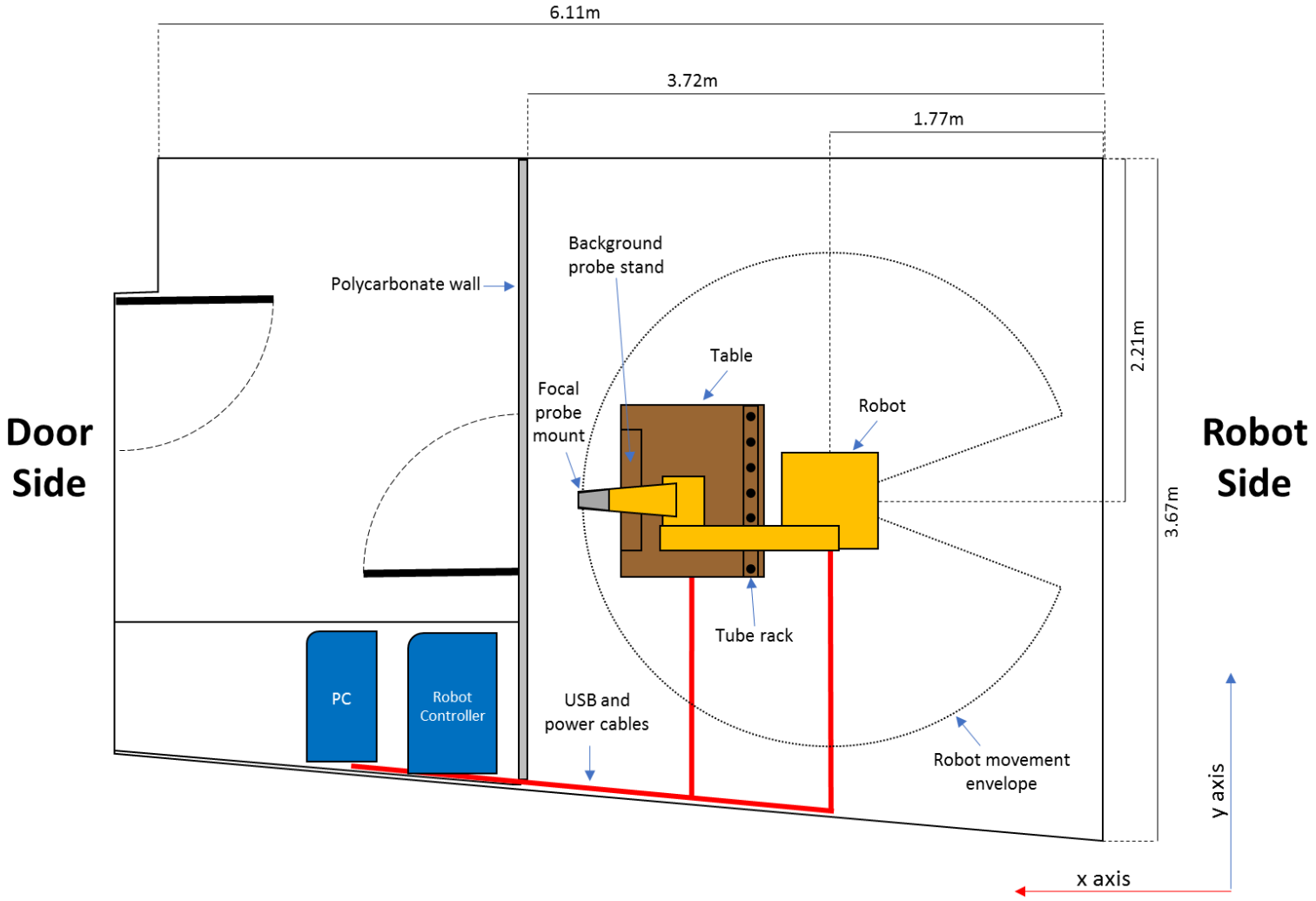


Figure 4.1: *A scale floorplan of the lab where humidity signal sampling took place, adapted from building and arm installation blueprints. The location of elements related to humidity signal sampling are indicated. The 'door' and 'robot' sides of the room as well as the robot's coordination of x and y axis are indicated, image is looking down the z axis.*

where horticultural tubes containing flowers could be placed. A 17cm high mount was attached on the other side of the table for the background probe, with its microcontroller sitting on the table itself. The background probe was between 44.5 and 54.5cm from any hole in the rack (figure 4.1 and 4.2).

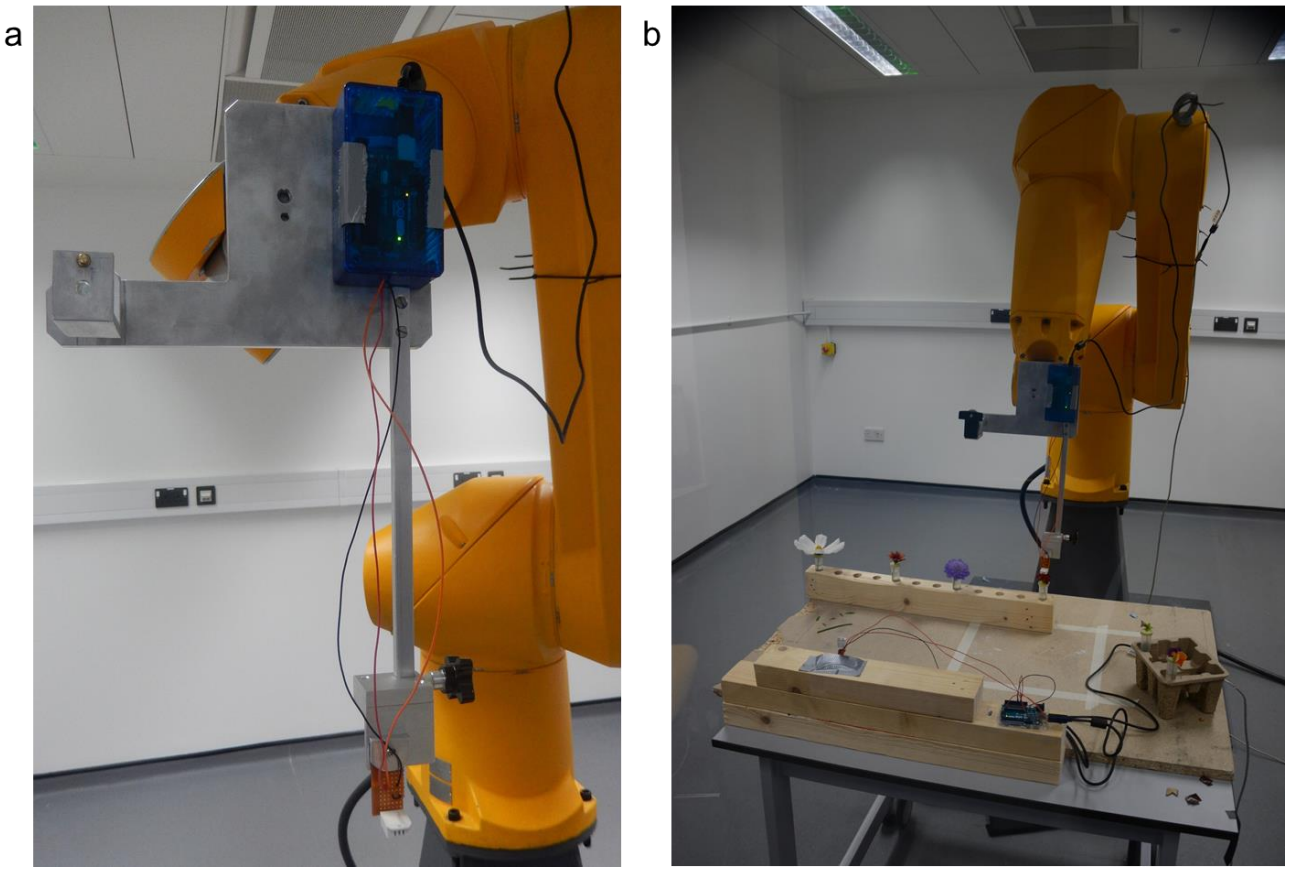


Figure 4.2: *The 6-axis robot arm used for floral humidity sampling.* ***a)*** *the humidity sensing tool mounted onto the robot arm. An adapter mount (a modified Manfrotto 625 adapter, Leicestershire, UK) is attached to the robot flange (the tool mounting surface at the end of the arm) and a purpose-built sensor tool fitted. This tool consists of a metal plate attached to the adapter mount with a 30cm long steel bar running parallel to the flange screwed onto it. The DHT-22 humidity sensing probe can be seen at the bottom of the panel attached to the end of the 30cm steel bar, the probe's microcontroller at the top of the panel on the metal plate mounting. The position the arm is seen in here is the 'safe' position the robot returns to throughout sampling.* ***b)*** *The arm during transect central point teaching. Note flower positions and location of the other background probe on the table in front of the arm.*

The two microcomputers registering the values from the focal and background probes were at a distance of 34.5 and 32cm from their respective probes. They did not heat up significantly or possess any elements that might generate turbulence, whilst all elements relating to the control of the robot arm and PC to which humidity probe data was sent, were set up behind the polycarbonate screen (figure 4.1). This means that humidity probes were unlikely to be affected by either turbulence or heat generated by the computers within the room. The 30cm bar of the measurement tool (see figure 4.2) meant that the probe was held far from the moving parts of the robot arm. Additionally, the arm moved slowly during sampling (at a maximum of 3% of nominal speed; estimated to be below 200mms^{-1}) while sampling and paused before samples to allow any disturbances to settle (see below). Consequently, the arm was also unlikely to generate large amounts of turbulence or interfere with any floral humidity.

4.2.2 *Preparation of flowers*

Humidity in the headspace of cut flowers in horticulture tubes would be sampled. Cutting of flowers meant floral organs could isolated from other sources of humidity on the plant. Additionally, cutting allowed orientation of flowers to be controlled during sampling (figure 4.2b). Flowers were either collected from University of Bristol gardens (Royal Fort Garden, the School of Chemistry gardens and Woodland Road gardens, all within 51^{o}45'N 2^{o}60'W), the University of Bristol Botanical Garden (51^{o}47'N 2^{o}63'W), or grown within the Bristol Life Sciences Building glasshouse (51^{o}45'N 2^{o}60'W). Flowers would be cut on the stem so no leaf remained on the cutting. Sepals of flowers remained on the cutting however. The stems of cut flowers were stuck through a hole in the cap of a water-filled 24cm^3 plastic horticulture tube, filled to ensure the flower's stem had access to the water, immediately after cutting. Flowers would be cut from the plant outside or in the glasshouse (depending where the plant was growing) then taken into the lab in these tubes. If flowers were collected from outside, sampling would only take place if flowers had no standing water on them *in situ* from condensation or

rain. Only flowers that were fully open, did not appear to show signs of age or damage and appeared healthy were cut.

42 species were sampled in total. Table 4.1 provides information on each flower species sampled and its growth conditions, along with the number flower replicates. Six individual flower replicates were achieved in 39 species. Normally individual flower replicates were also from different plants, however when insufficient plant material was available (10 species, indicated in Table 4.1), individual flowers were collected from plants sampled previously. In most species, flowers were the flower unit sampled, but in species where the inflorescence unit was comparable to a single flower, for example the compound inflorescences of Asteraceae (Baude et al., 2016; Hicks et al., 2016), the whole inflorescence was the unit sampled. As these inflorescences are small we were able to be sample them in exactly the same way as true flowers. Either three or four flowers were sampled each day. The species of flowers sampled each day was mixed. Most often on a single day four individuals of different species would be sampled. However, this varied dependent on available flowers of each species, and shorter flowering periods of some species meant they were prioritised on some days. The full list of dates of individual flower sampling, ordered by species, are found in appendix A6. Once flowers were taken into the lab, they were placed in the rack on the table in the sampling zone, and spaced at least 15.5cm apart from one another. Flowers were orientated so that they faced vertically upwards: when needed, support was provided to a flower using mouldable putty (*blu tack*: Bostik, Paris, France) stuck to the tube lid.

Table 4.1: *The plant species sampled with our floral humidity headspace methods. Species are sorted by order and family, as listed on (The Plant List, 2013). The level of the floral unit sampled is indicated, either a single flower or an inflorescence. Growth conditions of plants used for sampling are indicated as follows Bristol University Glasshouse (Glasshouse), Bristol University Gardens (Gardens), Bristol University Botanic Gardens (Botanics) as well as the number of flowers sampled (n). Number in brackets after n value indicates the number of plants these flowers was collected from when it differs from the number of flowers (no bracket value indicates all flowers were collected from different plants).*

Species	Order	Family	Floral Unit	*n*	Growth Conditions	Growth Location
Allium ursinum	Asparagales	Amaryllidaceae	Flower	4	Outside	Gardens
Tulbaghia violacea	Asparagales	Amaryllidaceae	Flower	6	Outside	Botanics
Achillea millefolium	Asterales	Asteraceae	Umbel Inflorescence	6	Outside	Gardens
Bellis perennis	Asterales	Asteraceae	Compound Inflorescence	6	Outside	Gardens
Centaurea segetum	Asterales	Asteraceae	Compound Inflorescence	6	Outside	Gardens
Centaurea montanus	Asterales	Asteraceae	Compound Inflorescence	6	Outside	Gardens
Coreopsis sp.	Asterales	Asteraceae	Compound Inflorescence	6 (2)	Inside	Glasshouse
Cosmos parviflorus	Asterales	Asteraceae	Compound Inflorescence	6 (2)	Inside	Glasshouse
Leucanthemum vulgare	Asterales	Asteraceae	Compound Inflorescence	5	Outside	Gardens
Osteospermum sp.	Asterales	Asteraceae	Compound Inflorescence	6 (2)	Inside	Glasshouse
Rudbeckia hirta	Asterales	Asteraceae	Compound Inflorescence	6 (3)	Outside	Gardens
Taraxacum officinale	Asterales	Asteraceae	Compound Inflorescence	6	Outside	Gardens
Xerochrysum bracteatum	Asterales	Asteraceae	Compound Inflorescence	6 (3)	Inside	Glasshouse
Campanula sp.	Asterales	Campanulaceae	Flower	6 (2)	Inside	Glasshouse
Nepenthes sp.	Caryophyllales	Nepenthaceae	Flower	6 (4)	Inside	Glasshouse
Scabiosa sp.	Dipsacales	Caprioliaceae	Compound Inflorescence	6 (2)	Inside	Glasshouse
Trifolium pratense	Fabaceae	Leguminosae	Umbel Inflorescence	6	Outside	Gardens
Vinca herbacea	Gentianales	Apocynaceae	Flower	6	Outside	Gardens
Geranium 'Roxanne'	Geraniales	Geraniaceae	Flower	6	Outside	Gardens
Geranium robertianum	Geraniales	Geraniaceae	Flower	6	Outside	Gardens
Geranium sanguinemum	Geraniales	Geraniaceae	Flower	6	Outside	Gardens

Species	Order	Family	Floral Unit	*n*	Growth Conditions	Growth Location
Lavandula angustifolia	Lamiales	Lamiaceae	Racemose Inflorescence	6	Outside	Gardens
Lantana sp.	Lamiales	Verbenaceae	Umbel Inflorescence	6 (4)	Inside	Glasshouse
Lilium sp.	Liliales	Liliaceae	Flower	6	Inside	Glasshouse
Euphorbia milii	Malpighiales	Euphorbiaceae	Compound Inflorescence	6 (3)	Inside	Glasshouse
Linum grandiflorum	Malpighiales	Linaceae	Flower	6	Outside	Gardens
Linum usitatissimum	Malpighiales	Linaceae	Flower	6	Outside	Botanics
Cistus 'greyswood pink'	Malvales	Cistaceae	Flower	6	Outside	Gardens
Abutilon × *milleri hort.*	Malvales	Malvaceae	Flower	6	Outside	Botanics
Epilobium hirsutum	Myrtales	Onagraceae	Flower	6	Outside	Gardens
Fuchsia sp.	Myrtales	Onagraceae	Flower	6	Outside	Gardens
Oenothera caespitosa	Myrtales	Onagraceae	Flower	6	Inside	Glasshouse
Eschscholzia californica	Ranunculales	Papaveraceae	Flower	6	Outside	Gardens
Papaver cambricum	Ranunculales	Papaveraceae	Flower	6	Outside	Gardens
Papaver rhoeas	Ranunculales	Papaveraceae	Flower	6	Outside	Gardens
Clematis chinensis	Ranunculales	Ranunculaceae	Flower	4	Outside	Botanics
Ranunculus acris	Ranunculales	Ranunculaceae	Flower	6	Outside	Gardens
Ranunculus lingua	Ranunculales	Ranunculaceae	Flower	6	Outside	Gardens
*Potentilla*sp.	Rosales	Rosaceae	Flower	6	Outside	Gardens
Calystegia silvatica	Solanales	Convolvulaceae	Flower	6	Outside	Gardens
Convolvulus sabatius	Solanales	Convolvulaceae	Flower	6	Outside	Botanics
Nicotiana tabacum	Solanales	Solanaceae	Flower	6	Inside	Glasshouse

4.2.3 *Control groups*

The flowers sampled under the robot are were placed within a water filled horticultural tube. The lid of this tube had a 3mm diameter hole in it but was otherwise sealed. Although this hole was largely blocked by the flower, it is possible that evaporation of the water within the tube may create an extraneous humidity difference. Furthermore, humidity across rooms is rarely completely even, due to how air mixes within a room (Schellenberg, 2002; Lake et al., 2003). Thus, some level of difference in humidity between the focal and background probes' readings may have been detected, simply because they are in different positions. Lastly, the *blu tack* applied to the flowers may also create humidity extraneous to the flower as it dries. Consequently, controls were implemented to assess the extent of differences in humidity that may occur between the focal and background probe due to influences extraneous to the flower. Controls sampled included combinations of empty or water filled tubes, with or without their lid, and with or without *blu tack* covering the top of the tube lid, listed in table 4.2. Six individual tubes set up for each control were sampled under the robot, except the *EmptyLid* control where seven individual tubes were sampled.

Table 4.2: *The controls used to support our survey and check for humidity differences extraneous to the flower.*

Control Designation	Description
Empty	The empty horticultural tube without lid
EmptyLid	The empty horticultural tube with its lid
EmptyLidBlue	The empty horticultural tube with its lid and *blu tack* covering the top of its lid
Water	Water filled horticultural tube without lid
WaterLid	Water filled horticultural tube with its lid
WaterLidBlue	Water filled horticultural tube with its lid and *blu tack* covering the top of its lid

4.2.4 *Humidity transect: robot preparation*

Humidity transects commenced within a maximum of an hour after flowers were picked. The mean start of sampling across all sampling days (taken as the time humidity measurements began rather than robot activation) was 11:01h, this ranged between 9:10h and 14:00h. A full list of the times of the first measurements taken on each individual control and flower are given found in appendix 5 and 6 respectively. This will mean plants will typically begin transects in a late-morning or noon state in terms of plant daily cycles such as transpiration, stomatal opening, nectar secretion or floral metabolism.

All humidity transects were carried out autonomously by the robot. On each day, once flowers were ready to be sampled the focal probe was manoeuvred using manual controls to a 'transect central point' above each flower – the point above the centre of the flower, 5mm higher than the flower's highest point. The transect central points for all the current flowers, along with a point less than 2.5cm of the background probe, were stored in the robot's memory. Once stored, the robot was able to return the probe to each flower's transect centre, or points relative to it, and a full series of humidity transects could be conducted. This manual teaching and input of transect central points had to be carried out each day as flowers differed in size and shape. Controls were sampled under the robot in exactly the same manner as flowers.

4.2.5 *Humidity transect method: robot motion*

The robot began each sampling cycle by starting in a 'safe' position 50cm above the table (figure 4.2a). In the robot co-ordinate system, x axis movements are horizontal, moving towards and away from the robot (negative and positive respectively); z axis movements are up and down (positive and negative respectively). The axes of the robot relative to the room are indicated in figure 4.1. The order in which individual flowers were sampled on each day would be selected via a randomization algorithm within the arm's software.

Once activated, and flower order selected, the arm would begin sampling on a flower. From the 'safe' position the arm would move to the flower, stopping at a point 30mm closer to the arm then that flowers transect central point, -30mm offset in the x axis relative to the transect central point. Once at this position, the robot would begin transects of the floral headspace. The first a transect was conducted horizontally, the x axis transect, the second conducted vertically, the z axis transect. These paired horizontal and vertical transects through the flower's headspace are an adaptation of the similar transects carried out by von Arx et al. (2012). The x axis transects involved measurement humidity at sampling points across the front of the flower. The x axis sampling points started and ended at 30mm on either side of the transect central point (x axis offset -30 and 30, relative to the transect central point) and were separated by 5mm intervals (13 points in total). Once the x axis was finished, the robot would commence the z axis transect. The z axis transects sampling points started 5mm above the transect central point and moved vertically upwards for 30mm in 5mm steps (6 points in total). The transects' spatial layouts are described in Figure 4.3. At each sampling point along both transect the arm would stop the probe and take humidity measurements as described

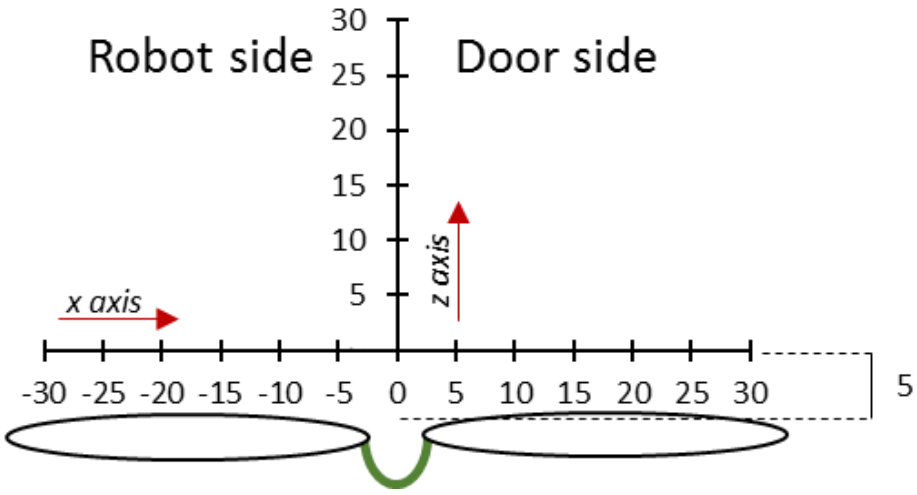


Figure 4.3: *The spatial layout of the humidity headspace sampled above the flower in our transects. The flower is viewed in cross section sideways on. All offset measurements are given in millimetres. Each measurement point is marked along the transect with a dash and the distance along that transect relative to the transect central point (where X=0 and Z=0).*

below, measurements at each sampling point taking 230 seconds. Movements within the transects (e.g. from x axis offset point 20 to 25, of from the end of the x axis and start of the z axis transects) were made directly. As with all movements during sampling, the arm moved at 3% of nominal speed (estimated to be below 200mms^{-1}). A third y axis transect was deemed unnecessary as all flowers and inflorescences sampled showed high radial symmetry.

Following completion of both transects on a flower the robot would return to the 'safe' position 50cm above the table, then move the focal probe to the point 2.5cm away from the background probe. Here it would carry out a 'probe-control' measurement, as described below. This probe control measurement would also take 230 seconds. Following completion of a probe control measurement the robot would move the focal probe back to the 'safe' position. From here it would begin sampling of the next flower in its sequence, carrying out the same sequence of transects followed by a probe-control measurement as described above. The movement via the 'safe' position when moving to and from probe control measurements was to avoid the robot moving the probe through and potentially interfering with floral headspaces. The robot would then continue this sequence of transects and probe-control measurements for each flower in turn.

Four replicate samples of all the flower headspaces (x and z axis transects, with probe-control measurements, for each flower) were carried out back to back. The initial randomized order chosen by the robot was maintained across all the replicate samples taken on the same day. This meant that any sampling point on the same flower would be measured in the following replicate sample within 231 and 308 mins on days where 3 and 4 flowers were being sampled respectively. Following completion of the probe calibration on the final flower in the sequence on the last replicate sample the robot would return to the 'safe' position and power down.

4.2.6 *Humidity transect method: humidity measurement at sampling points*

At each sampling point along transects (the points indicated in figure 4.3) the probe was held stationary for 230 seconds. The first 30 seconds were a non-sampled settling time, to mitigate for any disturbance in the humidity profile, before 200 seconds of sampling, the measurement period, where the DHT-22 probe sampled approximately 100 relative humidity measurements. These measurements were used as the 'uncorrected relative humidity' values of that sampling point. Whilst these measurements were taken, the background probe simultaneously recorded the background humidity.

4.2.7 *Humidity transect method: probe control measurements*

Probes can vary slightly in their estimations of the same levels of humidity (±5% according to the manufacturer's specifications). Thus, the probe-control measurements were necessary to calculate how much the probes differed in estimates and reduce this source of inaccuracy. Upon moving the focal probe next to the background probe, the focal probe waited for 30 seconds before measuring humidity for 200 seconds, and the background probe made simultaneous measurements. Assuming that both probes are measuring a point of the same humidity during the probe-control measurements we could later compute linear regression parameters, using the MATLAB '*regression*' function (MathWorks®, 2012), to predict one probe's measurements from the other when in this position. This could then later be used to adjust the 'uncorrected focal relative humidity' ($f_{uncorrected}$) values for other points using

$$f_{corrected} = W \cdot f_{uncorrected} + M \qquad (4.1)$$

where W and M are respectively the slope and intercept parameters obtained from regressing the focal probe measurements against the background probes measurements for the time period of the probe-control measurements. This focal probe correction (equation 4.1), would be calculated and applied across each replicate sample of the flowers sampled on the same

day (*i.e.* one set of x and z axis transects on every flower sampled each day). This was done to account for any possibility that the difference between probes may change over time.

4.2.8 *Flower physiological correlates*

The presence of petal stomata was surveyed in 14 flower species. Petals were removed from fresh unsampled flowers, and a mould was made of the upper petal surface using dental wax (*Elite HD+ A-silicone Impression Material*, Bada Polesine, Italy). A cast was made from this mould using clear nail polish, and was mounted on a microscope slide using tape (*Scotch Crystal tape*, St Paul, USA). Mounted casts were surveyed from petal base to tip using a light microscope at ×100 magnification, and the presence of stomata was recorded. A species' presence of stomata was expressed as the percentage of petals surveyed that showed the presence of any stomata.

Flower size, horizontal span in mm, was measured on fresh flowers using a tape measure. This was done on fresh flowers to avoid disturbance of any floral humidity before sampling or effects of flowers drying post cutting and sampling afterwards. In instances where additional flowers were not available flower span was taken from measurements of the same species published elsewhere. Flower or inflorescence structure, where appropriate to each species, was classified based on species descriptions from Stace (2010) for flower structure, and the inflorescence classification system of Troll (1969) and Endress (2010).

4.3 Statistical analysis

All statistical analyses were carried out in *R* 3.4.1 (R Development Core Team, 2017). The differences between corrected focal humidity measurements ($f_{corrected}$) and the simultaneous background measurements ($f_{background}$) taken throughout the transects can be expressed as a relative humidity where

$$\Delta RH = f_{corrected} - f_{background}. \quad (4.2)$$

Thus, where $\Delta RH > 0$ an increase in relative humidity is detected by the focal probe.

4.3.1 *Evaluation of robot measurements*

Relative humidity at a given measurement period (*i.e*. the *c.*100 measurements at each sampling point on the *x* and *z* transect, on each replicate transect, for each flower) may not be stable over the approximately 100 measurements made at each measurement point. This may be because some level of turbulence is generated while the probe is being moved into place remains after the end of the 30 second wait period. It is also possible the humidity generated from the flower itself shows low stability over this 200 second measurement time. Understanding how stable humidity is at a single measurement period will be critical to evaluation of the accuracy of the robot's measurements. It also has implications on how we analyse humidity transect data and will inform best practice in future application of this robotic humidity measurement protocol.

To assess the repeatability of the measurements of the difference between focal and simultaneous background humidity, ΔRH, the residual variation taken at the same sampling point along our transects was calculated (Nakagawa and Schielzeth, 2010). This was done using the *rpt* function (with 100 bootstrap model repeats) within *rptR* 0.9.21 (Stoffel et al., 2017) using each set of *c*.100 measurements taken during the measurement period at each sampling point as the replication level of interest. The repeatability of measurements within

each measurement period from all transects, across all species and controls were assessed together.

The turbulence generated by the movement of the probe may cause humidity to have not settled by the end of the 30 second settling time. This may cause a change in humidity measurements at the focal probe between the start of a measurement period, where remaining turbulence would be greatest, and the end, where it would be most stable. It is possible this difference may differ dependent upon the humidity of a measurement point: if little water is present in the air, disruption of that area may affect humidity estimates less than more humid air. The directionality (positive or negative) of a change in humidity may also differ depending on how the air mixes as a result of turbulence and the humidity air being mixed in. Thus, to assess the effect of remaining turbulence, the change across a measurement period, regardless of directionality, should be assessed and how humid the disturbed area is should also be accounted for. The mean of focal humidity ($f_{uncorrected}$) measurements taken during the first 20 seconds and the last 20 seconds at each measurement period, f_{focal}^{first} and f_{focal}^{last} respectively, were calculated. The non-directional change in focal humidity between the beginning and end of each measurement period can then be calculated as

$$f_{focal}^{change} = \sqrt{\left(f_{focal}^{first} - f_{focal}^{last}\right)^2}. \quad (4.3)$$

f_{focal}^{change} is thus an indicator how much humidity differs between the start and end of a measurement period. The mean focal humidity across the whole of each measurement period (200 seconds, *c.* 100 measurements), f_{focal}^{mean}, was also calculated. A linear regression model describing how the f_{focal}^{change} value of a measurement period changes with f_{focal}^{mean} was fitted to this data. The extent of f_{focal}^{change}, and how this is influenced by f_{focal}^{mean} could then be evaluated by testing the significance of this model's parameters.

4.3.2 *Assessment of floral humidity*

Within each measurement period (the *c.*100 measurements made at each point on the flower transects) the average difference between the focal and background probe (ΔRH, equation 4.2) value was calculated and then used for analyses of humidity structure. For each flower species (and control), models were fitted to the x and z axis data. A quadratic model was fitted to the x axis transect, and a logarithmic model fitted to the z axis. These models described the humidity structure across transects and allowed these to vary with subsequent replicate transects. This full model was then compared against simpler models using AIC to select the best fitting humidity structure model. Throughout all models, flower identity was included as a random factor influencing the intercept, or amount of humidity produced.

4.3.3 *Statistical models*

The full model fitted to the x axis transect of a given species or control is

$$\Delta RH_{xnt} = I_x + i_{xt} + (A_x + a_x)X + (B_x + b_x)X^2 + v_{xn}\ , \qquad (4.4)$$

where X refers to the x axis offset of the sampling point relative to the transect central point (where $X = 0$), figure 4.3. The value of t represents the replicate transect number: 1 being the first transect; 2, 3 and 4 being the second, third and fourth transects of the flower respectively. ΔRH_{xnt} is the mean ΔRH for the measurement period at sampling point X, on transect replicate t, on flower n. A_x and B_x are parameters that describe the positioning and slope of the x axis humidity profile in the initial transect. Parameter I_x is the model intercept in the initial transect, and I_x is modified by v_{xn}, which represents the change in model intercept, and consequently the intensity of humidity generated, by individual flower (or control tube) *n* on the x axis transect. A_x, B_x and I_x are also modified by parameters a_x, b_x and i_x, which represent the change in the offset, slope and intercept (A_x, B_x and I_x) respectively between first and latter replicate transects. The values of A_x, B_x and I_x within each transect are modified as follows:

$$a_x = F(g_{2x}) + S(g_{3x}) + E(g_{4x}) , \tag{4.5}$$

$$b_x = F(c_{2x}) + S(c_{3x}) + E(c_{4x}) , \tag{4.6}$$

$$i_x = F(r_{2x}) + S(r_{3x}) + E(r_{4x}) . \tag{4.7}$$

Where g_{tx}, c_{tx}, r_{tx} are the values of α_x, b_x and i_x during transect t, when

$$F = \begin{cases} 0, t \neq 2 \\ 1, t = 2 \end{cases}, \tag{4.8}$$

$$S = \begin{cases} 0, t \neq 3 \\ 1, t = 3 \end{cases}, \tag{4.9}$$

$$E = \begin{cases} 0, t \neq 4 \\ 1, t = 4 \end{cases}. \tag{4.10}$$

Consequently g_{tx}, c_{tx}, r_{tx} are the changes applied to in A_x, B_x and I_x in transect t. Here parameters I_x, A_x, B_x, v_{xn} and all iterations of g_{tx}, c_{tx} and r_{tx} are parameters to be estimated.

The full model applied to the z axis transect is as follows:

$$\Delta RH_{znt} = I_z + i_z + (B_z + b_z)\ln Z + v_{zn} . \tag{4.11}$$

Here parameter Z refers to the sampling point's z axis offset relative to the transect central point. All other parameters (I_z, i_z, B_z, b_z and v_{zn}) function in the same manner described for respective parameters in the x axis model. Here parameters I_z, B_z, v_{zn} and all iterations of c_{tz} and r_{tz} are parameters to be estimated.

4.3.4 *Model selection process*

The full models for the x and z transects described in equations 4.4 and 4.11, as well as simpler versions of these models, were fitted to the x and z transect data of each species and each control group. Simpler models were achieved by removing certain parameters from the full models, done by forcing these parameters to have values of zero. Eleven x axis models and five z axis models were compared for each species. These models are summarised in tables 4.3 and 4.4 for the x and z axis models respectively. These models were then compared using AIC to select the best models for the x and z transects of each flower species following the guidelines within Richards (2008).

Table 4.3: *The models fitted to each species' and control group's x axis transect humidity data. Model name, the omitted parameters from the full model and a model description is given.*

Model	Omitted parameters	Model description
m0	$A_x,\ B_x,\ r_{tx},\ c_{tx}, g_{tx}$	Flat linear model with no influence of replicate transects.
m1	$B_x,\ r_{tx},\ c_{tx}, g_{tx}$	Linear model with no influence of replicate transects.
m2	$A_x,\ r_{tx},\ c_{tx}, g_{tx}$	Quadratic model with no influence of replicate transects.
m3	$r_{tx},\ c_{tx}, g_{tx}$	Quadratic model with an x axis offset with no influence of replicate transects.
m4	$A_x,\ B_x,\ c_{tx}, g_{tx}$	Flat linear model with differing intercepts between replicate transects.
m5	$B_x,\ c_{tx}, g_{tx}$	Linear model with differing intercepts between replicate transects.
m6	$A_x,\ c_{tx}, g_{tx}$	Quadratic model with differing intercepts between replicate transects.
m7	c_{tx}, g_{tx}	Quadratic model with an x axis offset and differing intercepts between replicate transects.
m8	$B_x,\ c_{tx}$	Linear model with interacting effects of replicate transects.
m9	A_x, g_{tx}	Quadratic model with interacting effects of replicate transects.
m10	none	The full model: Quadratic model with an x axis offset and interacting effects of replicate transects.

Table 4.4: *The models fitted to each species' and control group z axis transect humidity data. Model name, the omitted parameters from the full model and a model description is given.*

Model	Omitted parameters	Model description
z0	B_z, r_{tz}, c_{tz}	Flat linear model with no influence of replicate transects.
z1	r_{tz}, c_{tz}	Logarithmic model with no influence of replicate transects.
z2	B_z, c_{tz}	Flat linear model with differing intercepts between replicate transects.
z3	c_{tz}	Logarithmic model with differing intercepts between replicate transects.
z4	none	Logarithmic model with interacting effects of replicate transects.

4.3.5 *Summary value calculation*

To evaluate the intensity of humidity generated by each flower species and by controls ΔRH_x^{max}, the peak mean humidity difference across the x transect as predicted by the best fitting model, was calculated for each flower species. This was done by calculating X_t^{max}, the x axis offset (parameter X) value that the best fitting model predicted to give the largest difference in humidity on transect t, for each species. ΔRH_x^{max} and X_t^{max} were calculated using the parameter values estimated by the best fitting model for each species' x axis model. If the best fitting x axis model was z0 or z2, any value of X could be used for X_t^{max}, but 0 was used. In species where the best fitting model showed a linear relationship, X_t^{max} would be either 30 or -30 (depending on whether $A_x + a_x$ came to a positive or negative value respectively). In species where the x axis showed a quadratic relationship in the best fitting model, X_t^{max} would be the X value of the vertex of the quadratic curve described by equation 4.4 (as all species favouring quadratic models showed negative values for $B_x + b_x$). In quadratic curves the vertex is also the plane of symmetry (Strang, 1991). Due to this a formula for X_t^{max} of a species showing a quadratic best fitting x axis model can be derived as follows. Equation 4.4 can be reorganised to

$$\Delta RH_{xnt} = I_x + i_x + v_{xn} + X\left((A_x + a_x) + \left((B_x + b_x)X\right)\right) \quad (4.12)$$

From equation 4.12 two values of X spaced equally either side of the vertex, two points that mirror each other in the curves' symmetry, can be identified, where $\Delta RH_{xnt} = I_x + i_x + v_{xn}$. These are when

$$X = 0, \quad (4.13)$$

and

$$X = -\frac{(A_x + a_x)}{(B_x + b_x)}. \quad (4.14)$$

As the curve is symmetrical, the x coordinates of the vertex for the curve (described by equations 4.4 and 4.12) is halfway between these points. Consequently, X_t^{max} of a species showing a quadratic x axis relationship can be calculated using

$$X_t^{max} = -\frac{(A_x + a_x)}{2(B_x + b_x)} \quad (4.15)$$

and the parameter values estimated by the best fitting model for each species' x axis model. Equation 4.15 is an adaption of the standard formula for the x axis coordinates of a quadratic vertex (Strang, 1991; Stapel, 2014), with the corresponding parameters form the model described in equation 4.4 substituted in. This equation can also be derived by calculus (instead of the solution based on graphical properties presented here) to give the same answer. This is shown on standard quadratic formulae in Stapel (2014).

Once X_t^{max} is calculated ΔRH_x^{max} of a species could then be estimated by inserting X_t^{max} into equation 4.4 and using the parameter estimates of the best fitting model, with v_{xn} was set to zero. In instances where replicate transects had an influence on ΔRH_x, the X_t^{max} and ΔRH_x^{max} would be calculated for each replicate transect and the largest ΔRH_x^{max} value used. In instances where multiple comparable models best fitting these summary values would be calculated with whichever of these comparable best models had the lowest AIC.

4.3.6 *Influences on floral humidity*

Phylogenies were constructed within *R* 3.4.1 (R Development Core Team, 2017) using the megaphylogeny and *S.Phylomaker* algorithm described by (Qian and Jin, 2016). A number of the species studied were either absent from the megaphylogeny or only identified to the genus level, but at least congeneric sister species was present allowing a degree of accuracy in placement. The data for the horticultural cultivar *Geranium* 'Roxanne' was not considered in these analyses as it was not possible to place this cultivar within the genus. The default algorithm given by Qian and Jin (2016) constructs phylogenies based on the position sister species using three separate rulesets. We used a tree generated by their second scenario, where an absent species is placed randomly within its genus, but trees from the other scenarios suggested were also considered, and gave identical results.

How ΔRH_x^{max} was affected by flower span, floral type (taken as either a single flower or an inflorescence), and whether plants had grown in field conditions or in a greenhouse was tested using a phylogenetically-controlled generalised least squares regression (Grafen, 1989; Martins and Hansen, 1997; Symonds and Blomberg, 2014) fit using a maximum likelihood model and run within *nlme* 3.1-137 (Pinheiro et al., 2018), assuming a correlation matrix based on either Brownian Motion (BM) or an Ornstein Uhlenbeck (OU) process with an estimate of α generated with ape 5.1 (Paradis et al., 2004), with floral type and growth environment coded as binary dummy variables. ΔRH_x^{max} was log-transformed so that test assumptions were met (Mundry, 2014). Models with OU correlation were compared to those with BM correlation using likelihood tests and AICs, and the full models that best explained the data were then compared to a corresponding null model where ΔRH_x^{max} was fit to the grand mean.

4.4 Results

4.4.1 *Evaluation of robot measurements*

The robot arm showed high repeatability over each set of approximately 100 measurements made in the same measurement period (R = 0.971, SE < 0.001, 95% CI = [0.971, 0.972], p < 0.001). This means that within each measurement period the majority are about the mean value. This confirms the robot's consistency of measurements and validates our decision to use mean values for each measurement periods for our analyses of humidity structure, as measurements within each period are largely similar.

Analysis of f_{focal}^{change} values found focal humidity measurements from the start of measurement periods differed by a small amount from those taken at the end (table 4.5). This suggests there may be small amounts of turbulence from robot movement remaining after the 30 second waiting time. However, this change across measurement periods was small (effect sizes in table 4.5). Furthermore, the high repeatability across measurement periods suggests humidity was settled across the majority of measurement periods. Humidity measurement accuracy might be improved by a longer waiting time or further reduced robot speed, however such changes in the protocol are likely have very slight effects on humidity measurements.

Table 4.5: *Summary of the parameter effects of the linear regression model fitted to the mean focal humidity measurements taken during the first and last 20 seconds of each measurement period. Given here are: the predicted Model values, parameter standard errors and confidence intervals. Significance of each parameter is evaluated based on a t test of each parameter individually (in all cases* df = *21582).*

Parameter	**Estimate**	**Standard Error**	**Confidence Intervals**		t_{21582}	p
			2.5%	**97.5%**		
Model Intercept	0.091	0.0056	0.0796	0.1016	16.12	<0.01
f_{focal}^{mean} (%)	0.001	0.0001	0.0006	0.0011	7.61	<0.01

4.4.2 *Extraneous humidity*

The best fitting models, X_t^{max} and ΔRH_x^{max} values for the controls can be found in table 4.6, the parameter values for best fitting x and z axis models and humidity structure for controls are given in appendix A4 and AIC tables for model selection are found at appendix A5. The transects of the control groups confirm that the arm detects a humidity source if present, for example water filled tubes (*Water, WaterLid)*, but detects little difference when sources of humidity are absent, as in empty tubes (*Empty, EmptyLid*).

Table 4.6: *The best fitting models, X_t^{max} and ΔRH_x^{max} values for the controls. Subscript letters following best fitting models indicate shape of that model: F, flat models; L, linear models; and Q, quadratic models (note quadratic models were not fitted to the z axis). Subscript values next to x_t^{max} values indicate replicate effects: the number itself referring to the value of t, which replicate transect, at which ΔRH_x^{max} was found; no subscript values indicates replicate transects have no effect on humidity*

Control	Best fitting model for x axis	z axis	X_t^{max}	ΔRH_x^{max} (%)
Empty	$m7_Q$	$z1_L$	-5.01_3	0.06
EmptyLid	$m5_L$	$z2_F$	-30_3	0.10
EmptyLidBlue	$m4_F$	$z2_F$	0_1	0.14
Water	$m2_Q$	$z1_L$	0	1.17
WaterLid	$m2_Q$	$z0_F$	0	0.46
WaterLidBlue	$m5_L$, $m6_Q$	$z2_F$	-30_3	0.09

When the horticulture tubes had no water in them we see little change in humidity between focal and background probes, hence the low ΔRH_x^{max} values of the *Empty* and *EmptyLid* controls, table 4.6. This suggests that very little of the observed humidity differences are the result of humidity varying within the room due to air mixing. The slight differences between *Empty* and *EmptyLid* controls are likely to be due to influences on changes in airflow about the tubes due to the addition of the tube's lid. The control *EmptyLidBlue* shows a slight increase compared to the *EmptyLid* control, suggesting *blu tack* gives off a small amount of water as it dries out.

The addition of water to the controls increases humidity, as seen by the higher ΔRH_x^{max} values of the *Water* and *WaterLid* controls compared to the *Empty* and *EmptyLid* controls

(table 4.6). This shows that there is potentially a small amount of humidity generated by the water filled horticulture tubes flowers were placed within. The addition of the lid to a water filled tube decreases the humidity generated, by limiting the escape of water vapour to the hole in the tube's lid. This resulted in the lower ΔRH_x^{max} of the *WaterLid* control compared to *Water* (table 4.6). Humidity intensity decreases between controls *WaterLid* and *WaterLidBlue* (table 4.6). This is likely because, despite *blu tack* giving off water vapour, the *blu tack* also further obscures the hole in the lid of the horticultural tube, limiting escape to water vapour from the tube in the same way as the lid. It seems that at most humidity extraneous to the flowers from the horticulture tube will be similar to that of the *WaterLid* control (ΔRH_x^{max}=0.46%). However, as flowers also obscure the hole in the horticultural tube lid, this extraneous humidity may be lessened in the same manner as in *WaterlidBlue* controls.

4.4.3 *Floral humidity*

The transects of flower headspace found flower species to vary in the humidity levels they produce. Table 4.7 provides for all 42 species sampled: the best fitting models of x and z axis transects, physiological correlates, and values for X_t^{max} and ΔRH_x^{max}. The ΔRH_x^{max} values for each species alongside those of the controls are shown in figure 4.4. Values for estimated parameters of our best fitting models can be found in appendix A4 and AIC tables for each species in appendix A6. In some species the floral humidity detected was lower than or comparable to extraneous humidity expected from the horticultural tube (where ΔRH_x^{max}= 0.46%) and it was difficult to confirm the floral unit is the source of the humidity (figure 4.4). However, 30 species produced floral humidity at an intensity that exceeded expected from sources extraneous to the flower (the *WaterLid* control). 13 species produced floral humidity of a greater intensity than that detected from an unobscured water source, the *Water* control (ΔRH_x^{max} >1.17%), the largest ΔRH_x^{max} produced by any control.

Table 4.7: *The results of our humidity survey, summarized by ΔRH_x^{max}, alongside physiological correlates. Species are ordered by ascending rank order of ΔRH_x^{max}. Bracketed values after the mean span and Stomata occurrence values are the number of flowers and petals sampled for span or stomatal presence. Where span was taken from a different source this is indicated by a letter where: a, Clapham et al. (1981); b, Cartalano and Kruetr (2010); c, Huxley et al. (1999); and d, Plants Database (2018). Subscript letters following best fitting models indicate shape of that model: F, flat models; L, linear models; and Q, quadratic models (note quadratic models were not fitted to the z axis). Subscript values next to x_t^{max} values indicate replicate effects: the number itself referring to the value of t, which replicate transect, at which ΔRH_x^{max} was found; (z) indicates replicate effects only in the z axis model; no subscript values indicates replicate transects have no effect on humidity.*

Rank	Species	Structure	mean span mm (n/source)	Stomata (n)	Best fitting model for x axis	Best fitting model for z axis	X_t^{max}	ΔRH_x^{max}
1	*Fuchsia* sp.	Funnel	33.7 (7)		$m0_F$	$z2_F$	$0_{(Z)}$	0.05
2	*Nicotiana tabacum*	Funnel	23.6 (5)		$m2_Q$	$z2_F$	$0_{(Z)}$	0.10
3	*Vinca herbacea*	Bell	48.6 (12)	0% (5)	$m7_Q$	$z0_F$	-2.49_2	0.24
4	*Allium ursinum*	Unfused	20 (a)		$m2_Q$	$z2_F$	$0_{(Z)}$	0.24
5	*Nepenthes* sp.	Unfused	80 (b)		$m2_Q$	$z2_F$	$0_{(Z)}$	0.26
6	*Papaver rhoeas*	Unfused	56.4 (9)	100% (5)	$m3_Q$	$z2_F$	$-4.35_{(Z)}$	0.29
7	*Euphorbia milii*	Compound Inflorescence	12 (c)		$m6_Q$	$z2_F$	0_3	0.29
8	*Centaurea montanus*	Compound Inflorescence	53.4 (11)		$m7_Q$	$z2_F$	-1.88_2	0.31
9	*Abutilon* × *milleri* hort.	Funnel	30.8 (6)	0% (4)	$m6_Q$	$z2_F$	0_1	0.32
10	*Campanula* sp.	Bell	20.7 (3)		$m2_Q$	$z2_F$	$0_{(Z)}$	0.36
11	*Linum grandiflorum*	Unfused	38 (3)	0% (2)	$m10_Q$	$z3_L$	7.22_2	0.36
12	*Geranium robertianum*	Unfused	15.4 (11)		$m2_Q$	$z2_F$	$0_{(Z)}$	0.41
13	*Tulbaghia violacea*	Funnel	29 (9)		$m9_Q$	$z2_F$	0_1	0.52
14	*Papaver cambricum*	Unfused	48.3 (8)	100% (5)	$m2_Q$	$z1_L$	0	0.58
15	*Bellis perennis*	Compound Inflorescence	18.1 (14)	0% (3)	$m2_Q$	$z3_L$	$0_{(Z)}$	0.58
16	*Epilobium hirsutum*	Funnel	15.1 (7)		$m2_Q$	$z2_F$	$0_{(Z)}$	0.59
17	*Trifolium pratense*	Umbel Inflorescence	20.1 (9)		$m9_Q$	$z2_F$	0_4	0.61
18	*Lilium* sp.	Funnel	93 (5)		$m8_L$	$z2_F$	-30_4	0.65
19	*Clematis chinensis*	Unfused	50 (d)		$m7_Q$	$z2_F$	-2.91_1	0.66
20	*Cistus* 'greyswood pink'	Unfused	43.6 (11)		$m2_Q$	$z3_L$	$0_{(Z)}$	0.66
21	*Cosmos parviflorus*	Compound Inflorescence	92.5 (6)		$m7_Q$	$z1_L$	2.99_2	0.67
22	*Geranium* 'Roxanne'	Unfused	42.6 (8)	0% (4)	$m7_Q$	$z3_L$	-1.66_3	0.67

Rank	Species	Structure	mean span mm (n/source)	Stomata (n)	Best fitting model for x axis	Best fitting model for z axis	X_t^{max}	ΔRH_x^{max}
23	*Potentilla* sp.	Unfused	29 (12)		$m7_Q$	$z1_L$	1.28_1	0.70
24	*Coreopsis* sp.	Compound Inflorescence	46.8 (4)	0% (2)	$m3_Q$	$z3_L$	$0.94_{(Z)}$	0.71
25	*Lavandula angustifolia*	Racemose Inflorescence	18.6 (10)		$m6_Q$	$z2_F$	0_4	0.72
26	*Geranium sanguinemum*	Unfused	41 (12)		$m9_Q$	$z3_L$	0_2	0.79
27	*Linum usitatissimum*	Unfused	16.7 (6)		$m9_Q$	$z2_F$	0_2	0.80
28	*Convolvulus sabatius*	Funnel	32.8 (12)	0% (4)	$m9_Q$	$z3_L$	0_1	0.87
29	*Centaurea segetum*	Compound Inflorescence	36.4 (14)		$m6_Q$	$z0_F$	0_1	1.10
30	*Osteospermum* sp.	Compound Inflorescence	51.9 (10)	20% (5)	$m10_Q$	$z4_L$	5.49_3	1.20
31	*Rudbeckia hirta*	Compound Inflorescence	56.3 (10)		$m7_Q$	$z1_L$	2.97_2	1.25
32	*Scabiosa* sp.	Compound Inflorescence	39.6 (5)		$m3_Q$	$z1_L$	1.61	1.36
33	*Lantana* sp.	Umpel Infloresence	42.3 (11)		$m2_Q$	$z1_L$	0	1.47
34	*Achillea millefolium*	Umbel Infloresence	33.3 (9)		$m3_Q$	$z1_L$	1.69	1.73
35	*Leucanthemum vulgare*	Compound Inflorescence	47.2 (6)		$m7_Q$	$z4_L$	2.17_1	1.79
36	*Oenothera caespitosa*	Funnel	54.2 (5)		$m10_Q$	$z1_L$	2.54_1	1.79
37	*Ranunculus lingua*	Unfused	35.7 (7)		$m7_Q$	$z3_L$	2.61_2	3.16
38	*Eschscholzia californica*	Unfused	48.8 (9)	80% (5)	$m7_Q$	$z4_L$	21.49_4	3.24
39	*Taraxacum* agg.	Compound Inflorescence	39.3 (9)	0% (4)	$m9_Q$	$z4_L$	0_4	3.35
40	*Ranunculus acris*	Unfused	24 (19)	0% (3)	$m9_Q$	$z3_L$	0_2	3.41
41	*Xerochrysum bracteatum*	Compound Inflorescence	48.4 (9)	0% (3)	$m3_Q$	$z1_L$	1.41	3.67
42	*Calystegia silvatica*	Funnel	61.8 (12)		$m8_L$	$z1_L$	30_3	3.71

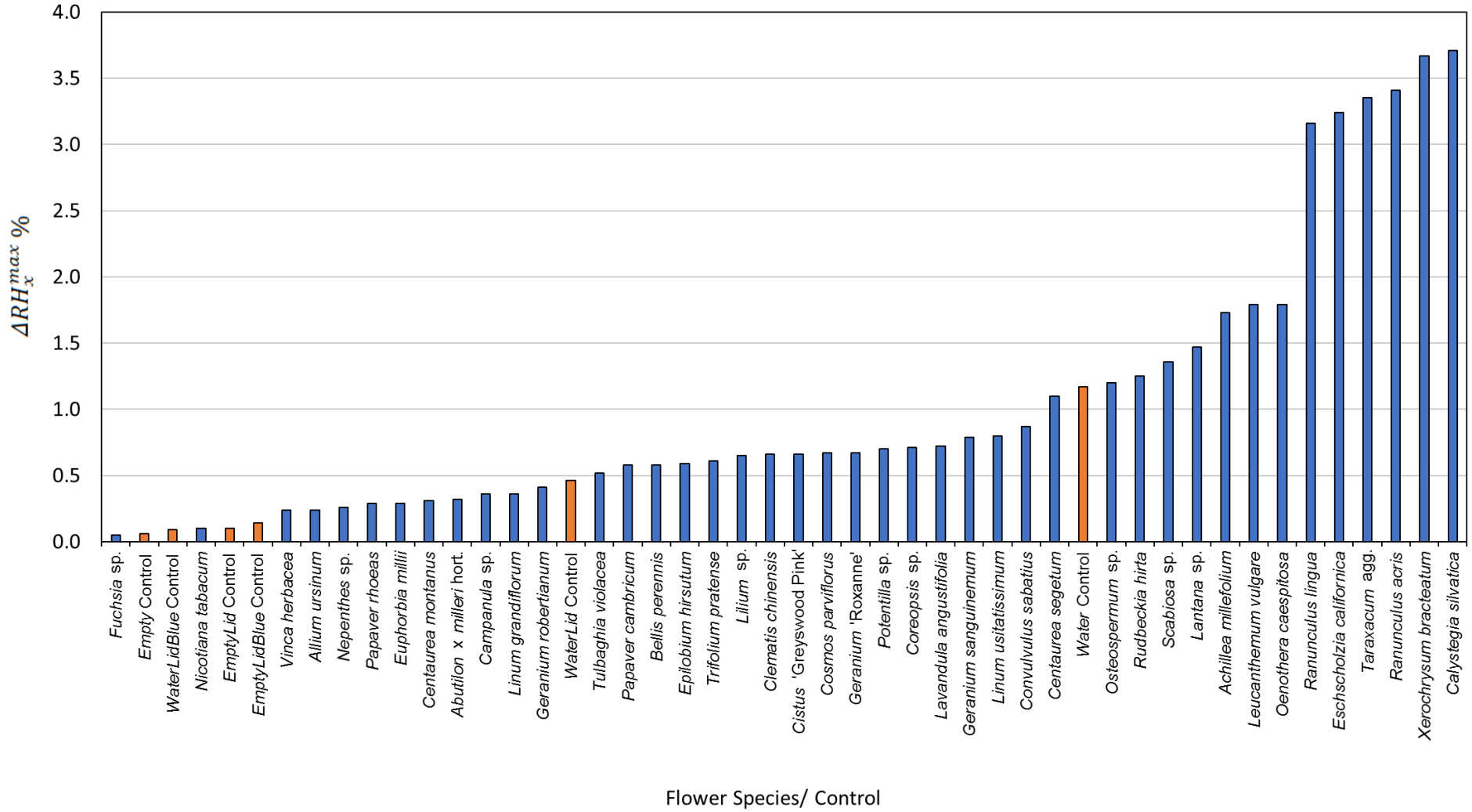


Figure 4.4: *The peak mean humidity differences between species across the x transect as predicted by the best fitting models, the* ΔRH_x^{max} *values, of each flower species (blue bars) and control (orange bars). Species and controls are presented together in rank order.*

Typically, the humidity structure detected in the x axis transect showed a quadratic relationship between x axis offset and ΔRH_x. Of the 42 species studied, only three (*Fuchsia* sp., *Lilium* sp., and *Calystegia silvatica*) had non-quadratic best fitting models, as shown in table 4.7, all other best fitting models included parameter B_x in some way. The x axis humidity structures of six species are given in figure 4.5. The point where humidity intensity was greatest, X_t^{max}, was the transect central point ($X = 0$) for 22 species, table 4.7. In species where X_t^{max} did not equal 0, X_t^{max} was normally only slightly offset, with all but five species showing X_t^{max} to be less than 5mm offset from the transect central point, table 4.7.

Z axis humidity profiles were less consistent in structure. The Z axis structures of six species are given in figure 4.6. In 25 species, the best fitting z axis models showed a logarithmic relationship with humidity declining with increased distance from the flower (increased z axis offset). In most instances humidity reaching background levels by the end of the 30cm transect. Although 17 species favoured a flat z axis model where humidity remained level across the transect (table 4.7) as seen in figure 4.6a and b.

37 species favoured models with changes in humidity with replicate transects in at least one axis, 19 of which favoured changes with replicate transects in both axis. Most changes with replicate transects were changes in floral humidity intensity (non-interacting effects determined by r), but in 13 species (ten in x axis only, two in z axis only, one in both) showed some changes in structure of floral humidity with replicate transects (interacting effects determined by g and c). In most species the humidity decreased in the later replicate transects (figure 4.5 and 4.6), but in several species, there was an increase in the humidity signal after the first transect, as seen in *Ranunculus acris* (figure 4.5f and 4.6f). In most species (all except seven) the largest humidity signal was detected in the first or second replicate transect, table 4.7.

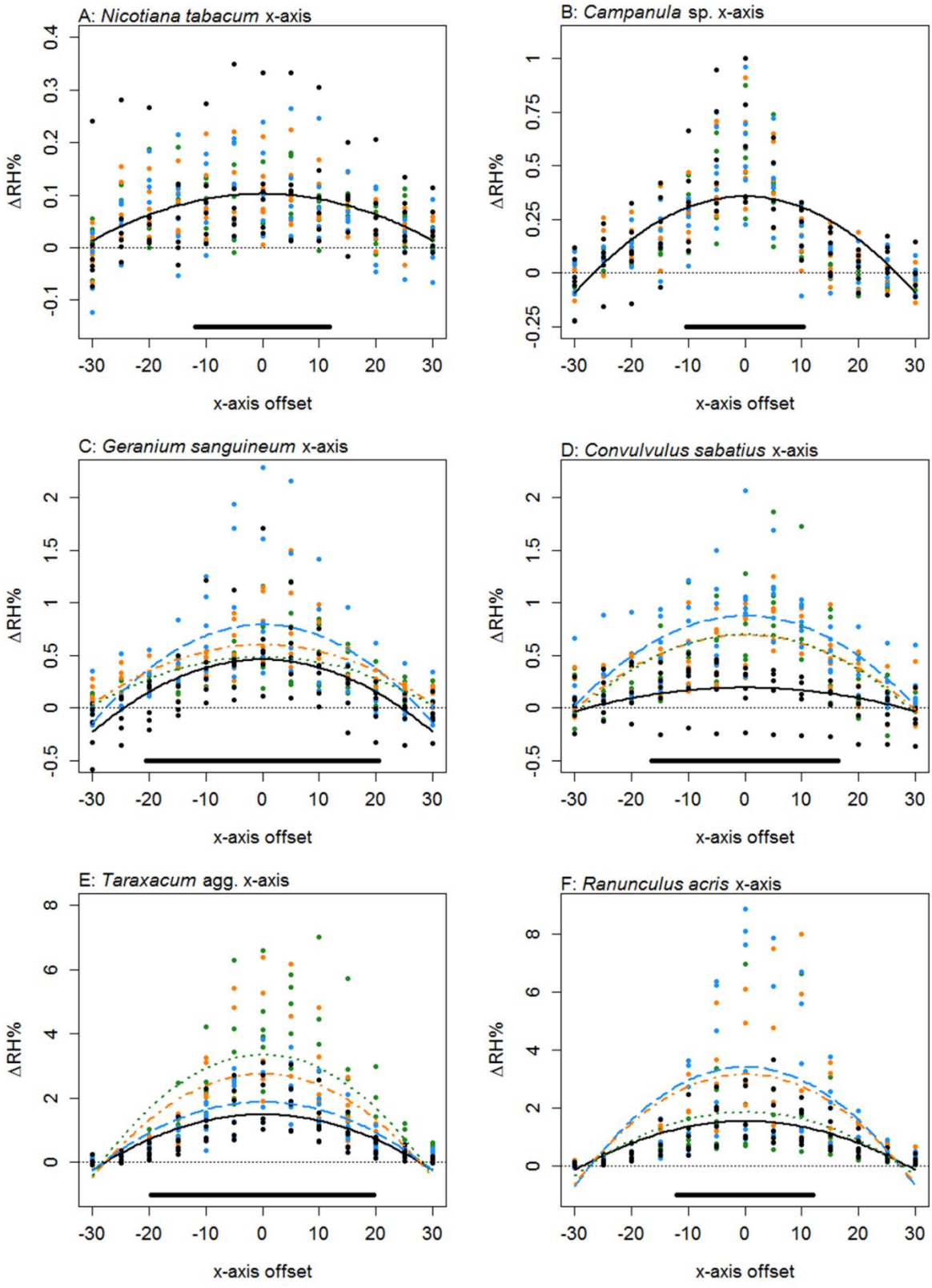

A: Nicotiana tabacum x-axis
B: Campanula sp. x-axis
C: Geranium sanguineum x-axis
D: Convulvulus sabatius x-axis
E: Taraxacum agg. x-axis
F: Ranunculus acris x-axis
ΔRH%
x-axis offset

Figure 4.5 [left]: *The difference in humidity relative to the backgrounds (ΔRH) for the x axis transects of Nicotiana tabacum (**A**), Campanula sp. (**B**), Geranium sanguineum (**C**), Convulvulus sabatius (**D**), Taraxacum agg. (**E**) and Ranunculus acris (**F**). All axis offsets are relative to the transect central point and in millimetres. The thin dotted line indicates a 0% change in humidity (the background level). Bold lines indicate the mean change in humidity as predicted by the best fitting model for that flower. Colour and dashing of bold lines and points indicate the replicate transect: solid black, first transect; long-dash blue, second transect; dash-dot orange, third transect; dotted green, fourth transect. The solid bar above the x axis indicates the mean flower span for that species relative to the x axis.*

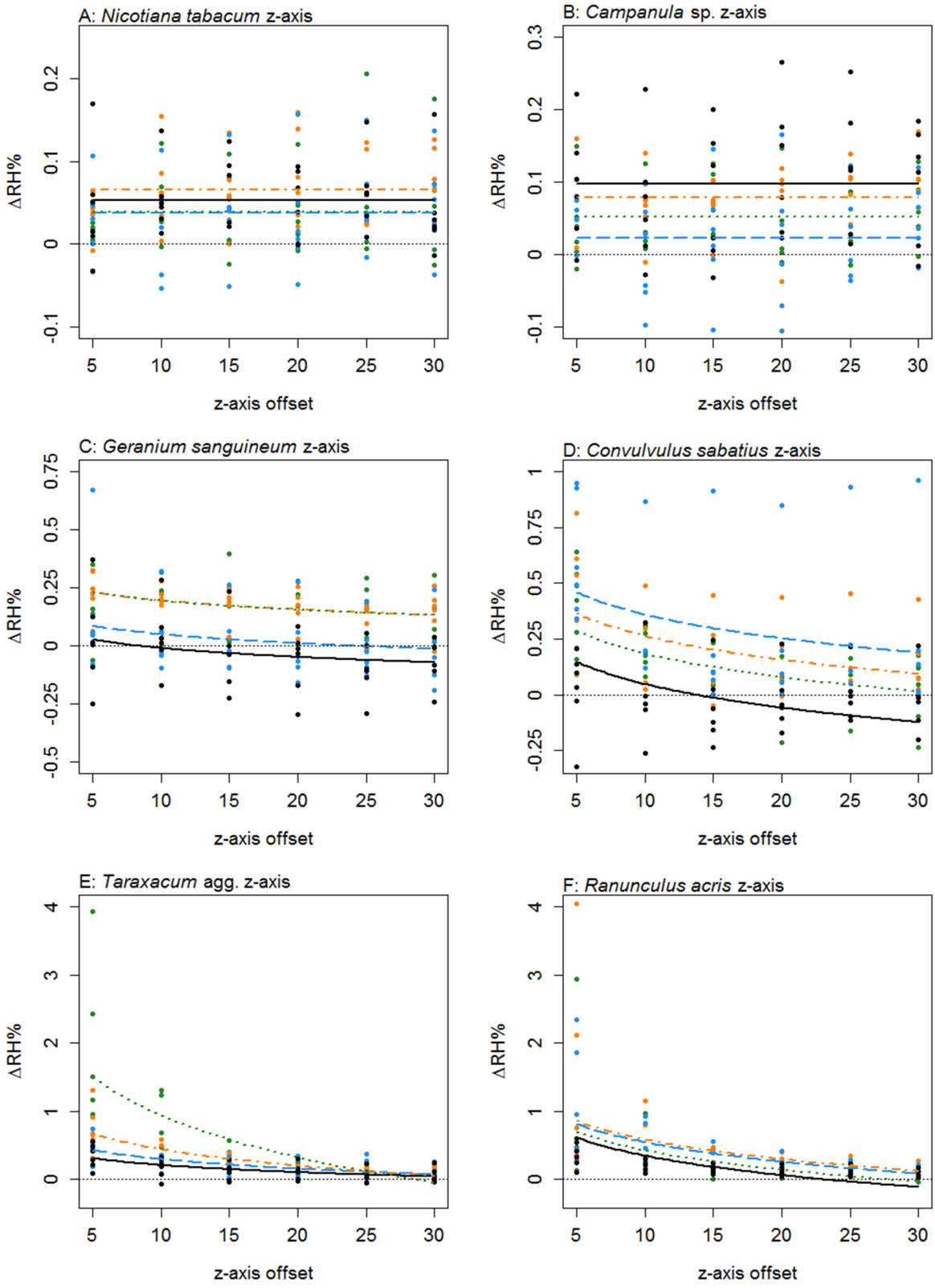

A: Nicotiana tabacum z-axis
B: Campanula sp. z-axis
C: Geranium sanguineum z-axis
D: Convulvulus sabatius z-axis
E: Taraxacum agg. z-axis
F: Ranunculus acris z-axis
ΔRH%
z-axis offset

Figure 4.6 [left]: *The difference in humidity relative to the backgrounds (ΔRH) for the z axis transects of Nicotiana tabacum (**A**), Campanula sp. (**B**), Geranium sanguineum (**C**), Convulvulus sabatius (**D**), Taraxacum agg. (**E**) and Ranunculus acris (**F**). All axis offsets are relative to the transect central point and in millimetres. The thin dotted line indicates a 0% change in humidity- the background level. Bold lines indicate the mean change in humidity as predicted by the best fitting model for that flower. Colour and dashing of bold lines and points indicate the replicate transect: solid black, first transect; long-dash blue, second transect; dash-dot orange, third transect; dotted green, fourth transect*.

4.4.4 *Influences on floral humidity*

The fitted full model with OU correlation (estimated α = 31) explained the data better than the model with BM correlation (AIC = 119.0 vs 173.7 respectively, ΔAIC=54.7, χ_1^2 = 56.64, p < 0.001). The model parameter effects are summarised in table 4.8. Explanatory variables had no significant effects on ΔRH_x^{max} within the OU full model, leaving the model intercept as the only significant effect. Comparing the OU full model to a null model with an OU correlation confirmed that there was no measurable effect of the explanatory variables on humidity (AIC = 119.05 full vs 119.50 null, ΔAIC = 0.45, χ_3^2 = 6.45, p = 0.092).

Table 4.8: *Summary of the parameter effects of the OU model. Given here are: the predicted Model values and standard errors, on log transformed ΔRH_x^{max} values for each species and the back-transformed effect of these aspects and confidence intervals on ΔRH_x^{max} according to the model. Significance of each parameter is evaluated based on a t test of each parameter individually (in all cases* df = *37).*

Parameter	Model value	Standard error	Back-transformed effect size (RH%)	Back-transformed Confidence Intervals		t_{37}	p
				2.5%	97.5%		
Model Intercept	-1.23	0.48	0.29	0.11	0.74	-2.60	0.01
Grown Outside	0.28	0.34	0.09	0.67	2.58	0.81	0.42
Inflorescence	0.55	0.31	0.21	0.95	3.17	1.79	0.08
Span (mm)	0.01	0.01	$\cdot e^{0.01(span)}$	0.98	1.03	1.61	0.12

4.5 Discussion

The transects of floral headspace presented here find many species across a wide range of the angiosperms produce floral humidity (table 4.7). While the controls demonstrate that humidity extraneous to the flower was produced by the horticultural tubes that flowers were placed within (table 4.6), many species (30 of 42) produced humidity of an intensity that exceeded the humidity produced by the lid capped horticultural tube alone, the *WaterLid* control (figure 4.4). This indicates that the flower was the source of at least some floral humidity. Cutting of flowers can separate flowers from hormonal control and lead interference with water uptake (van Doorn, 1997). However, problems with water uptake take time to develop (Lü et al., 2011) and experiments with cut flowers show they function normally in terms of transpiration, showing normal daily cycles (Lü et al., 2011; Fanourakis et al., 2012; Huang et al., 2018). Thus, within the timescales of our sampling (less than a day) flowers were likely functioning normally in terms of transpiration and water uptake for at least the earlier transects. Species whose flowers were picked from the outside showed no significant difference in capacity to produce humidity than those that grew indoors (table 4.8). This suggests the floral humidity detected is unlikely to be an artefact of flower treatment or nectar being allowed to accumulate to unnatural levels due to lack of exposure to floral visitors. These results support the hypothesis of previous researchers that floral humidity is not limited to *O. caespitosa* and is more prevalent than previously thought (Corbet et al., 1979a; Corbet et al., 1979b; von Arx, 2013).

Different flower species showed a wide range of floral humidity intensities (table 4.7, figure 4.4), ranging from apparently background levels, or comparable to humidity extraneous to the flower, to those producing much more with ΔRH_x^{max} reaching as high as 3.71% in *Calystegia sylvatica*. The intensity of floral signals did not appear to be determined by flower size (span) or form (single flower or inflorescence) when phylogeny was taken into account (table 4.8). This finding indicates that more specific characteristics of a species determine its capacity to produce floral humidity. The most likely candidates for such characters relate to

floral nectar and petal permeability. Nectar-related traits may determine variation in floral humidity between species, and could include the volume of nectar within these species (von Arx et al., 2012; von Arx, 2013), the surface area of liquid presented (Corbet et al., 1979a; Corbet et al., 1979b), or nectar composition (Corbet, 2003). It was not possible for us to measure nectar volume in species before sampling without also disrupting any humidity produced, and information on the liquid volume of nectar standing crop, and its variation between species is not available, most information being based on dry sugar mass (Baude et al., 2016; Hicks et al., 2016). Many aspects can influence transpiration differences between species (Buschhaus et al., 2014; Guo et al., 2017; Corbet et al., 1979b; Schreiber and Riederer, 1996; Hajibagheri et al., 1983). While stomata appeared absent among many of the highest humidity producers (table 4.7), transpirational water loss can occur directly through the petal cuticle (Buschhaus et al., 2014; Guo et al., 2017). Thus, differing humidity intensity between species may relate to petal permeability. Lastly, structural aspects beyond the general form, flower verses small inflorescence, may influence floral humidity generation by creating enclosed spaces and buffer zones allowing humidity to accumulate (Corbet et al., 1979a; Corbet et al., 1979b; Corbet, 2003). Understanding how these traits, and variation in these traits, relate to floral humidity production may start to explain this variation in signal intensity observed in the transects presented here. Now that several species have been identified to produce floral humidity, further investigation of how these differ from species that do not produce humidity can be carried out. This will also help reveal the extent to which floral humidity relates to the nectar levels or presence in species other than *O. caespitosa*. This will have consequence on how humidity may be used as an honest signal of reward presence by floral visitors.

The structure of floral humidity patterns was reasonably consistent, usually showing a quadratic x axis and decreasing z axis relationship (table 4.7). Such humidity structures are similar to that observed by von Arx et al. (2012). There were species that differed from this structure however, indicated in table 4.7. This was particularly the case in the z axis where

humidity was found frequently to be level across the 30mm transect. This often appeared to be due to a lack of humidity generated across the z axis, resulting in humidity differences remaining constant at a low level. Many of the flowers favouring flat humidity models in the z axis produce lower intensity floral humidity (table 4.7). The lack of humidity generated *Fuchsia* could also explain why it showed a flat humidity structure in the x axis. Lack of humidity generated was not always the cause of an atypical humidity structure, *Calystegia sylvatica* produced the highest measured floral humidity intensity and showed a linear x axis structure. *C. sylvatica* was among the largest flowers sampled, with a flower span slightly exceeding the x axis transect width (table 4.7). Consequently, it is probable only the zenith of a quadratic structure was sampled in the transect. Had the transect carried on away from the flower as it does in most other species, the ΔRH_x will probably have dropped off. Even when species showed a similar shaped humidity structure species still differed in broadness and shape of these humidity structures (differences in B_x and b_x shown in appendix A4) demonstrated in figure 4.5 and 4.6. X_t^{max} tends to be near the flower centre, about where nectaries tend to be within radially symmetrical flowers. This supports the association between nectar and floral humidity (von Arx et al., 2012). Differences in humidity structure shape and the location of X_t^{max} may be due variation in positioning and orientation of nectaries, or nectar producing florets within inflorescences. Alternatively, transpiration may differ across the flower surface due to location of petal stomata and differences in cuticle thickness and composition. Responses to between species differences in structural arrangement or patterns of floral signals have been observed in visual (Goodale et al., 2014; Hempel de Ibarra et al., 2015), olfactory (Lawson et al., 2017b; Lawson et al., 2018), electrostatic (Clarke et al., 2013) and thermal (chapter 3; Harrap et al., 2017) flower signals. It may be of interest to investigate further variation in humidity structure shown by flowers as well as whether pollinators that use floral humidity, hawkmoths (von Arx et al., 2012), can respond to the variation in humidity structure independent of its intensity.

Floral humidity was often shown to vary with replicate transects. The increases in humidity in latter transects may represent floral humidity taking time to establish once flowers are moved into the sampling area. The subsequent decreases in humidity we observed in latter transects are likely to represent flowers aging, and potentially drying out (van Doorn, 1997; Lü et al., 2011; von Arx et al., 2012). Alternatively, floral humidity may be responding to changes in transpiration or nectar secretion and reabsorption in the flower that correspond to time of day (Burquez and Corbet, 1991; Langenberger and Davis, 2002), which occur even when flowers are cut (Lü et al., 2011; Fanourakis et al., 2012; Huang et al., 2018). Many flowers also show daily cycles of flower opening and closing (van Doorn and van Meeteren, 2003; van Doorn and van Meeteren, 2014). Closing of flowers leads to changes in flower shape and exposure of nectar that might likewise change humidity production. In tulips flower opening and closure is associated with floral stomata opening (Azad et al., 2007), if such responses occur in other species this may explain changes in humidity production. All but one of the species that showed no influence of replicate transects were species sampled at the inflorescence level (*Scabiosa* sp*., Lantana* sp*., Achillea millefolium, Xerochrysum bracteatum*). This floral humidity stability over time may be the result of such blooms having many florets that open gradually over the day, potentially maintaining the signal where single flowers might wilt or dry out. Through the replication of x and z axis transects over the day, we show here that changes in floral humidity with flower age are not limited to signal intensity, as several species (13 of 42) showed changes humidity structure between replicate transects. In this way our repeated transects support the idea proposed by von Arx et al. (2012), that floral humidity intensity may act as an indicator, or honest signal, of flower age in some species. More direct assessment of how floral humidity production relates to the plant daily rhythms and plant age may help assess the cause of these humidity changes with time.

Some variation in species floral humidity intensities may be due to the way we conducted transects. As the transect central point needed to clear the highest point of the flower, in species with protruding structures may lead to the transect being carried out at increased

distance from the flower. As floral humidity declines with distance from the flower (figure 4.6; Corbet et al. 1979a; Corbet et al., 1979b), this may mean we under estimate the humidity generated by certain flower species. This is particularly true in *Lilium* which has very tall anthers and stigmas. More detailed mapping of flower structure may reveal some species do in fact produce a larger humidity signal. However, most of the flowers sampled had flatter surfaces, where this effect would be small.

Previously reported *O. caespitosa* humidity signals had an average peak x axis humidity signal on the of approximately 4%. Here the comparable ΔRH_x^{max} value of *O. caespitosa* was lower at 1.79%. Temperature and humidity conditions here (23 ºC and 47%) and in von Arx et al. (2012) were similar (23.1ºC and 49.1%), so it is unlikely this difference is due to the influence on conditions on relative humidity readings (Tichy and Kallina, 2014). Reduced humidity from *O. caespitosa* may be due to it being a nocturnal plant surveyed in daytime. All the flowers we used were fresh, as we would have expected the signal to drop off to zero as flowers aged as observed by von Arx et al. (2012). Alternatively, the differences in measured floral humidity intensity may be the result of advantages of the transect method used here, using the robot arm, compared to mechanically-operated screws controlling probe movement in von Arx et al. (2012). Firstly, the robot, due to its ability to autonomously move at slow speeds, allowed transects to be carried out in a larger room, as opposed to a sealed 46cm^3 box. Here a more natural humidity equilibrium could be reached. This would allow floral transpiration and nectar evaporation to occur in a manner more similar to how it would in a natural open environment. Secondly, the arm was capable of more complex motion, allowing the arm to carry out the calibration step throughout the experiment, reducing variation as a result of differences between in probes. Finally, the robot was able to pause during transects. This allowed waiting time for floral humidity to stabilise after disruption from probe motion before humidity measurements, resulting in the high measurement repeatability and small turbulence effects seen here (table 4.5). In this way several sources of inaccuracy are

removed by use of the robot, potentially explaining the differences in *O. caespitosa* floral humidity.

This survey of floral humidity across different plant species has revealed floral humidity can be detected in many species. This wider occurrence of floral humidity expands our knowledge of floral multimodality, as floral humidity can be used by hawkmoth visitors. Our transects showed floral humidity intensity and humidity structure to vary between species, although the reasons behind this variation remain unclear. Repeated transects revealed that in many flower species humidity declined over time, suggesting humidity may in some way indicate flower age. In this way our transects reveal floral humidity to show a greater level of diversity and complexity than previously reported.

Chapter 5: Floral humidity signals: bumblebee detection of floral humidity

Data pertaining to artificial flower humidity structures were obtained with the assistance of a collaborator Dr Henry Knowles, as stated on page xv.

CHAPTER ABSTRACT

Floral humidity, an area of elevated humidity proximal to the flower, has been shown to occur frequently in different flower species. Previously, hawkmoth pollinators of evening primroses have been demonstrated to show a preference for flowers with elevated floral humidity. This preference is believed to aid hawkmoths in locating rewarding flowers. However, floral humidity's use by pollinators other than hawkmoths in this primrose pollination system is unclear. Here I investigate how bumblebees respond to the differences in floral humidity. Using captive bumblebees and artificial flowers that produce floral humidity intensities comparable to those detected on real flowers, I demonstrate that bumblebees also show a preference for flowers producing elevated floral humidity levels. Furthermore, in an additional experiment bumblebees demonstrate the ability to learn to distinguish rewarding artificial flowers from nonrewarding flowers, when these flowers differ in floral humidity production. This learning of flower identity based on floral humidity differences occurred regardless of whether it was the rewarding or nonrewarding flowers that were associated with elevated humidity. These findings demonstrate that between flower differences in floral humidity can be perceived by bumblebees and may influence their flower preferences as well as flower recognition and learning. Thus, floral humidity may be a more widely utilised floral signal and may have a more complex influence on pollinator behaviour than previously thought.

5.1 Introduction

Floral humidity, an area of elevated humidity within the headspace of the flower, has been demonstrated to occur in several flower species (chapter 3; Corbet et al., 1979a; von Arx et al., 2012; Nordström et al., 2017). Floral humidity appears to be created by a combination of nectar evaporation and floral transpiration (Corbet et al., 1979a; Azad et al., 2007; von Arx et al., 2012) although the contribution of these two influences may vary between species (chapter 4). Transects of the flower headspace of 42 species found 30 produce floral humidity of an intensity greater than expected from any conflating humidity sources extraneous to the flower (like the tubes that flowers were placed within) (chapter 4). Higher intensity floral humidity (based on the average peak difference from the background: ΔRH_x^{max}) does not appear to be limited to species visited by a particular group of pollinators (chapter 4). Species with high floral humidity intensity include: moth pollinated evening primrose *Oenothera caespitosa* (von Arx et al., 2012); bee pollinated flowers like *Eschscholzia californica* (Smith, 2010), *Convolvulus sabatius* and *Calystegia sylvatica* (Baker, 1961; Ushimaru and Kikuzawa, 1999); several flower species that practice generalist pollination strategy, attracting mainly bees and flies, such as *Ranunculus acris*, *R. legria* (Steinbach and Gottsberger, 1994), and several Asteraceae compound inflorescences (Goulson et al., 2005). Elevated humidity has also been associated with fly-pollinated flowers (Nordström et al., 2017).

While floral humidity appears to occur widely (chapter 4), the capacity for floral visitors to respond to floral humidity in a flower signalling context is poorly understood, having been only demonstrated in a single species, *Hyles lineatala,* the hawkmoth pollinator of *O. caespitosa* (von Arx et al., 2012; von Arx, 2013). *H. lineatala* was demonstrated to show a preference to artificial flowers producing floral humidity comparable to that produced by *O. caespitosa*. Furthermore, floral humidity was demonstrated to have the capacity to function as an 'honest signal' of nectar presence in *O. caespitosa*. Honest signals correspond with the level of floral rewards, indicating temporary rewardlessness to pollinators (due to for example recent visitation by a pollinator), allowing pollinators to avoid wasteful visits to unrewarding

flowers, increasing pollinator efficiency and preference to honest signallers (von Arx, 2013; Knauer and Schiestl, 2015). Although it is not clear how much floral humidity functions as an indicator of nectar rewards in other flower species, floral humidity may represent an overlooked floral signal, with important signalling functions within multiple messaging multimodal floral displays (von Arx, 2013). It is possible that the floral humidity could be used as a signal in limited circumstances, exclusively by moth pollinators. If so, floral humidity produced by generalist flowers may serve to attract moth visits, increasing the suite of pollinators visiting the flower as suggested by the 'perceptual variability' hypothesis for floral multimodality (Leonard et al., 2012). However, several flower species that produce humidity are rarely visited by moths, *E. californica* (Smith, 2010) and *R. acris* (Steinbach and Gottsberger, 1994), or are unsuitable for moth visitation, as in the case of bindweeds *C. sabatius* and *C. sylvatica* (Baker, 1961; Ushimaru and Kikuzawa, 1999). The floral humidity of such flower species is unlikely to be a signal for moths, and so it is possible the floral humidity may act as a signal for non-hawkmoth pollinators. It is therefore important that we understand the capacity of pollinators other than hawkmoths to respond to floral humidity in a foraging context.

Investigation of the capacity of pollinators other than *H. lineatala* to respond to floral humidity as a floral signal is limited (von Arx, 2013). Elevated relative humidity proximal to flowers was associated, along with other signals, with flowers flies had been observed to visit more in Indian alpine environments (Nordström et al., 2017), but no tests on whether flies actually could or were responding to floral humidity were carried out. No test of bee responses to floral humidity, similar to responses to other floral signals (Whitney et al., 2008; Clarke et al., 2013), have been carried out. However, it is known that many insects possess the capacity to perceive and respond to humidity in other contexts, such as responding to environmental humidity (Enjin, 2017). The presence of hygrosensative (humidity detecting) antennal sensilla, the ceolocapitular sensilla (Yokohari et al., 1982; Yokohari, 1983), is well reported and appear to be in common across insects (Altner et al., 1983; Altner and Loftus, 1985). These sensilla

have been reported in: beetles (Havukkala and Kennedy, 1984); cockroaches (Tichy and Kallina, 2010); dragon flies (Redora et al., 2008); moths (Steinbrecht and Müller, 1991; Altner et al., 1983); stick insects (Tichy and Kallina, 2010); and several other insect groups (Altner et al., 1983; Altner and Loftus, 1985). Such sensilla are also found in both flies (Liu et al., 2007) and in bees (Yokohari et al., 1982; Yokohari, 1983; Tichy and Kallina, 2014; Ahmed et al., 2015) but are more common and show a wider distribution across the antenna in *Bombus* bumblebees (Fialho et al., 2014). This may allow bumblebees to show higher humidity sensitivity (Fialho et al., 2014). These hygrosensative sensilla allow insects to detect to humidity (Enjin, 2017). The exact mechanism by which these ceolocapitular sensilla detect humidity is uncertain (Enjin, 2017). Although it appears these sensilla contain humidity sensitive cells whose shrinking and swelling, due to a change in water content, alters nerve signals to the insect's brain (Tichy and Kallina, 2010). Insects always possess two types of humidity sensitive cells within ceolocapitular sensilla: dry cells, which respond to a lack of humidity; and moist cells, that respond to humidity's presence (Yokohari et al., 1982; Yokohari, 1983). These cells have overlapping sensitivities and signal antagonistically, in so much as changes humidity lead to corresponding increases and decreases in signalling between cell types. In addition to signalling based on the humidity at a given instant, moist and dry cells signal with at a greater frequency in response to the rate of humidity changes (Tichy, 2003; Tichy and Kallina, 2010; Tichy and Kallina, 2014). In this way insects can detect the humidity at a given time but also the rate and direction of humidity changes, getting drier or moister. In *Apis mellifera*, within gradual cycles between 15% and 45% relative humidity a 1 impulse per second change can be elicited in the moist cells by a relative humidity increase of +5% or a rate of humidity change of +2% relative humidity per second. A 1 impulse per second change can be elicited in the dry cells a decrease of -5% relative humidity or a rate of humidity change of -1.2% relative humidity per second (Tichy and Kallina, 2014). In larger cycles the sensitivity to humidity was reported to be higher (Tichy and Kallina, 2014). Such humidity changes are similar to those produced by flowers (chapter 4).

While insect responses to floral humidity have only been demonstrated by *H. lineataIa* visiting *O. caespitosa* (von Arx et al., 2012), insect responses to environmental humidity are well reported (Havukkala and Kennedy, 1984; McCall and Primack, 1992; Kwon and Saeed, 2003; Peat and Goulson, 2005; Enjin, 2017). Honeybees respond to humidity levels within the nest, regulating humidity to different levels in different parts of the nest (Human et al., 2006; Nicolson, 2009). Elevated humidity triggers nest ventilation behaviours in bees like fanning nest structures, and low humidity encouraging behaviours to increase nest humidity by evaporation of nectar water or water collection (Human et al., 2006; Ellis et al., 2008; Abou-Shaara et al., 2017). In a foraging context, but not a flower foraging context, biting flies and mosquitoes are thought to respond to humidity given off by their host organisms, among other signals (Smart and Brown, 1956; Olanga et al., 2010; Chappuis et al., 2013). Furthermore, following presentation of sugar water droplets that touch their antenna, restrained honeybees have been seen to show a proboscis extension response to droplets of water placed near, but not touching the antenna (Kuwabara, 1957; Mercer and Menzel, 1982; Blenau and Erber, 1998). Such responses are likely in response to the water vapour, humidity, given off by the droplet, suggesting bees can be conditioned based on humidity to some degree (Kuwabara, 1957; Mercer and Menzel, 1982; Blenau and Erber, 1998). These responses, along with the presence of hygrosensitive sensilla in many pollinating insects means that pollinator groups other than hawkmoths, particularly social bees, possess the necessary sensory apparatus to detect humidity and can respond to humidity in non-flower signalling contexts. However, it remains to be seen whether these other pollinators, such as bees, can detect and use floral humidity as a flower signal and incorporate it into foraging decisions as shown by *H. lineataIa*.

As *H. lineataIa* showed a preference for floral humidity (von Arx et al., 2012), this might be expected from other pollinator groups, such as bees, should they be able to detect floral humidity. Many adult pollinating insects only feed on liquid nectar rewards (Heinrich, 1979a; Krenn et al., 2005), so may possess an association between evaporation of water, humidity and food (Mercer and Menzel, 1982; Blenau and Erber, 1998). Optimum relative humidities

for bee brood rearing tend to be at elevated humidity (Yoon et al., 2002; Human et al., 2006; Al-Ghamdi et al., 2014; Abou-Shaara et al., 2017). Furthermore, foraging bees have difficulties maintaining water balance in lower humidity environments (Atmowidjojo et al., 1997; Abou-Shaara et al., 2012; Kühsel et al., 2016; Abou-Shaara et al., 2017). Environmental preferences similar to these have been shown to carry over to influence floral trait preferences with regards to environmental temperature preferences and floral temperature signal preferences (Dyer et al., 2006; Norgate et al., 2010). Consequently, it is likely bees will also show a preference for floral humidity if they can perceive it.

Different flower species produce a variety of floral humidity intensities, the amount of humidity produced, this is demonstrated in table 4.7 and figure 4.4. Floral humidity intensity was summarised in chapter 4 by the largest mean relative humidity difference between flower and background across an x transect, as predicted by the best fitting model of humidity structure, ΔRH_x^{max}. Intensities ranged from: those that apparently produced no humidity, like flowers of *Papaver rhoeas* and *Campanula* sp.; to *C. sylvatica* flowers where ΔRH_x^{max} equalled 3.71% higher than the background levels. *O. caespitosa* flowers have been demonstrated to produce a 4% increase in relative humidity compared to background levels based on similar transects by von Arx et al. (2012), but chapter 4 found this to be of a lower intensity. These differing levels of floral humidity between flower species or, at least, the presence and absence of floral humidity between species, might be able to be used as an indicator for flower identity. Pollinators have been observed to be able to learn differences between flowers in various floral traits to distinguish more rewarding flowers from less rewarding flowers, such as: colour (Streinzer et al., 2009), scent (Daly et al., 2001; Galen and Newport, 1988), floral temperature (Dyer et al., 2006; Whitney et al., 2008), floral texture (Kevan and Lane, 1985), electrostatic properties (Clarke et al., 2013) and patterning of these signals (Whitney et al., 2009a; Harrap et al., 2017; Lawson et al., 2018; Hempel de Ibarra et al., 2015). Learning allows foraging decisions that can go against initial preferences of naïve pollinators. In this way learning based on floral humidity differences between flowers is different from showing preferences.

Understanding if floral humidity can be used for floral learning broadens our understanding of the foraging decisions pollinators might make when visiting flowers and the scope by which floral humidity might be used as a floral signal to pollinators.

In this chapter the capacity of a non-hawkmoth pollinator to detect and respond to artificial flowers, producing floral humidity, comparable to that detected from real flowers surveyed in chapter 4, is investigated. This is done using bumblebees, *Bombus terrestris audax*. In addition to tests of bumblebee floral humidity preference, differential conditioning experiments in response to floral humidity were carried out to investigate their capacity to learn differences in floral humidity and use floral humidity as a flower species recognition signal.

5.2 Methods

Responses to floral humidity were tested in lab conditions using captive bumblebees, *Bombus terrestris audax* (Biobest, Westerlo, Belgium via Agralan Swindon, UK). Bumblebees are an appropriate choice of forager to test responses to floral humidity as they visit a wide range of species including many found to produce floral humidity in chapter 4. Bee Lab conditions were again as described in chapter 3. Artificial flowers were constructed that produced either elevated humidity in the proximity of the flower's top, the 'humid' artificial flower variant, or alternatively produced a 'dry' artificial flower variant, where floral humidity would be lower. These artificial flowers were designed to remain dry to the touch (to avoid conflating responses to wet flower surfaces), and to not differ in temperature or other characteristics bees could respond to. Two different sets of artificial flowers were used: 'active humidity flowers' and 'passive humidity flowers', both of which had humid and dry variants.

5.2.1 *Artificial flower design: active humidity flowers*

Active humidity flowers were similar in design to those utilised by von Arx et al. (2012) but modified slightly to better suit bumblebee foragers. These artificial flowers had a flower top with small holes (figure 5.1a) to a chamber below the flower head (figure 5.1b). This chamber was connected by 6mm external, 4mm internal diameter airline tubing (*MARINA blue airline*, Hagen, Mansfield, USA) to a pump assembly outside the foraging arena (figure 5.1d and 5.1e). In humidity-producing flowers, airflow was through water in a bubbler in this pump assembly that elevated humidity of the air that was fed to the flower head. Dry non-humid flowers were also created, where the pump assembly was the same but the bubbler was empty. Thus, airflow at the flower head was the same between flower variants but the air reaching the flower head in dry flowers had not had its humidity increased.

A full schematic of an active artificial flower's pump assembly and its installation in the flight arena is given in figure 5.1e. Airflow from a mechanical fish tank air pump (*MARINA cool 11135*, Hagen, Mansfield, USA) was connected to a bubbler chamber by a 22cm section airline. The last 7cm of this 22cm tube was inserted within the bubbler chamber and the last 2cm of this section of tube was cut away at a 20º angle. This allowed the tube from the pump to be below the water level and allowed surface tension at the end of the pipe to be weaker promoting movement of bubbles through water when the bubbler was full.

This bubbler chamber was made with an airtight 150ml tupperware cylinder (made with either '*Snac-Pacs food tubes*', Wilko, Worksop, UK or '*Snack tubes*', Smash Nude Food Movers, Mitcham, Australia). Two holes were drilled into the lid of this and fitted with rubber grommets to match tube diameters. This chamber would be filled with either 100ml of water (that had been allowed to settle at room temperature overnight) in humid flower variants, or left empty in dry flower variants. This meant that in humidity producing flowers air that had undergone mixing with the water travelled up to the top of the bubbler, while flow of air continued in the same way in dry flowers but without humidity being increased in this air.

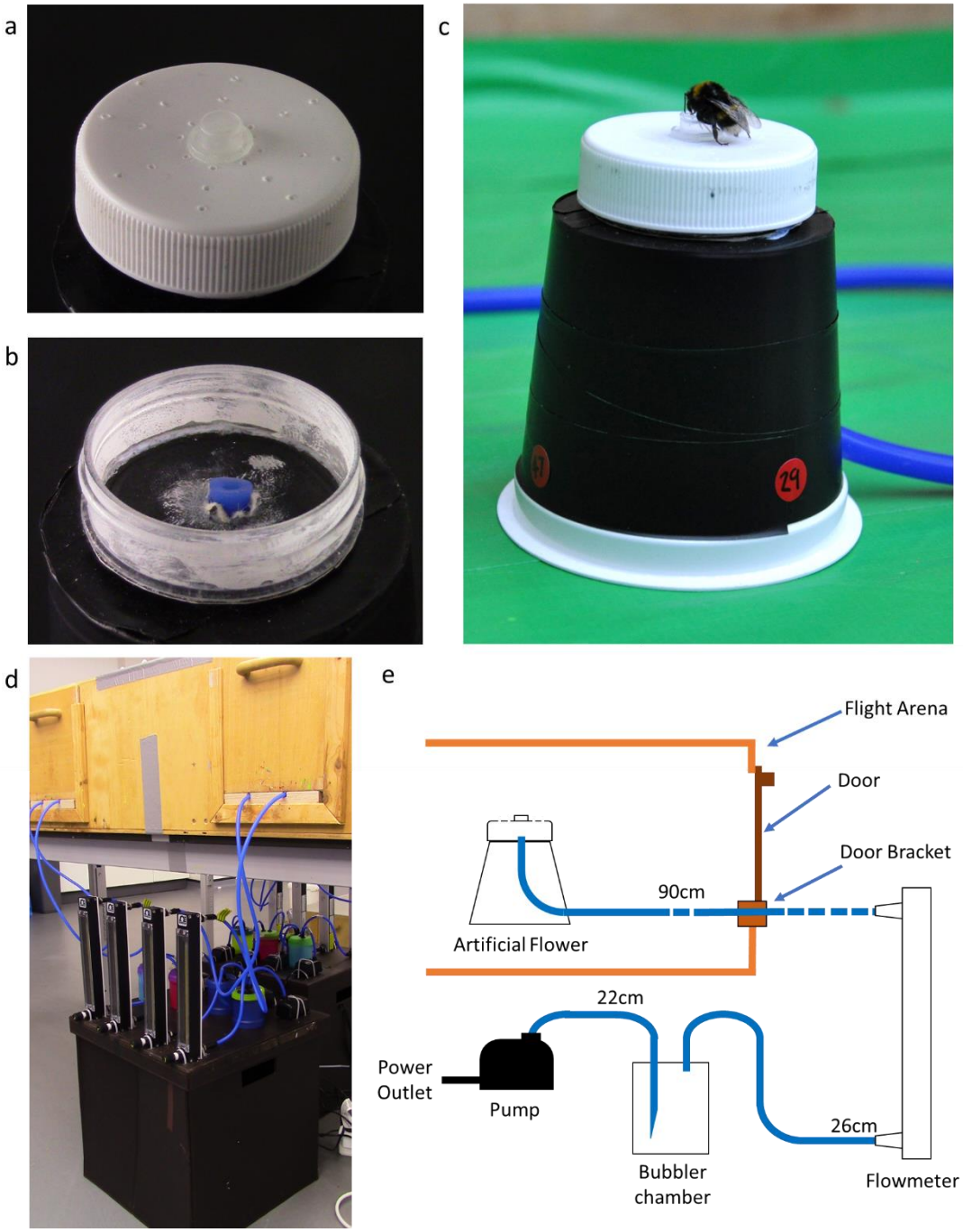


Figure 5.1: *The active humidity artificial flowers used in bumblebee experiments. Panel* ***a****) The artificial flower head. Note the holes on the flower head for air to escape.* ***b****) The flower head with the head unscrewed, allowing the chamber under and pipe entry point to be seen.* ***c****) Bumblebee feeding from active humidity flower as they appear in the flight arena.* ***d****) The pump-bubbler-rotameter assembly installed below the flight arena. Note the rubber tubes entering the arena through brackets below the doors.* ***e****) A diagrammatical representation of each artificial flower and its pump mechanism and how it installs through the flight arena through a door bracket. Rubber tubes are represented by blue lines connecting components, the lengths of tubes are given above each tube.*

A 26cm section of airline tubing was then connected to a rotameter (*Omega FL-3802C*, *Omega engineering*, Manchester, UK). Only enough tubing of this section to clear the grommet was inserted into the bubbler chamber (3mm). This meant that this tube was always above the water level and collected humid air (and dryer air) collecting at the top of the bubbler. These rotameters regulate airflow using a screw to obstruct airflow. Airflow was set at 2.69ml s^{-1}, controlling the flow of humid or dry air to the artificial flower head. The rotameter output was linked to a 90cm long section of tubing that entered the flight arena through holes in a wooden bracket installed on the doorways of the foraging arenas. This 90cm tube would link to the artificial flower itself. Eight active humidity flowers were used at any one time. Four would enter the arena from either side through two door brackets (figure 5.1d).

A 25mm diameter hole would be cut into the bottom of a plastic cup (Dart C71-130, Huntingdon, UK), and a 6mm diameter hole was punched 3mm from the top on one side, just above the lip on the top. This cup was upturned, and all but the lip was covered with black electrical tape (figure 5.1c). This functioned as the flower stand, holding the artificial flowers upright. The 90cm tube from the rotameter was fed into the 3mm hole and up through the 25mm hole. This base was then weighted using modelling clay allowing it to stand in place against any elastic tension created by bending the tube.

The head of the artificial flower was made from a specimen jar (Thermo scientific sterilin, PS 60ml, with white plastic lids), where the top 1cm of the jar (containing the screw lid thread) was cut away. A 0.5ml Eppendorf tube lid (Hamburg, Germany) was upturned and stuck down in the centre of the jar top, to function as the feeding well containing sucrose solution or water. 24 holes were made in the jar lid using a thumbtack pin. These 24 holes would be in lines of 3, each line being at a 45^{o} angle from the next, the first hole at the base of the feeding well, the others separated by 5mm (figure 5.1a). The screw thread would be stuck to thick card using super glue (Precision super glue, Loctite, Hemel Hempstead, UK). Once dry, the joint of this card and the screw thread was covered in glue in order to ensure as good a seal as possible. A 6mm diameter hole was then punched through the centre of this

card base, and the last 3mm of the 90cm tube leading to the rotameter was inserted through it (figure 5.1b) and secured with electrical tape. The flower lid was then screwed tight and the tubing pulled taut so that the flower head would rest on the stand. A small amount of *blue tac* was stuck to the underside of the flower head, to hold it in place against the stand. Artificial flowers thus appeared to bees as the jar lid on top of a trapezoid base (figure 5.1c).

To aid identification of individual flowers by experimenters, in a way bees would not be able to identify, red sticky dots were stuck about the base of the flower stand, and two-digit numbers written on these with black permanent marker (figure 5.1c). These numbers were odd on half the flowers entering the foraging arena on each side, even on the other side (*i.e*. two of each side's four flowers were even, two odd). The black on red colours of these numbers would be hard for bumblebees to make out given their visual systems (Davies et al., 2013). Additionally, these numbers were two digit, this allowed the initial digit to be even number in odd number stickers and *vice versa*. This meant bees were unlikely to recognise flower based on the number shapes (if they can be seen at all) as even and odd digits were present on all flowers. As the bubblers that contained water could be changed, whether even or odd numbered flowers corresponded with humid or dry flower variants could be changed between experiment days.

5.2.2 *Artificial flower design: passive humidity flowers*

Passive humidity flowers created humidity by evaporation of water from components internal to the flower through a permeable lid. In dry, non-humid artificial flower variants, construction was identical but without water being added to the flowers internal components.

Passive artificial flowers were built from a specimen jar (Thermo scientific sterilin, PS 60ml, with white plastic lids). The bodies of the jars were covered with black electrical tape in the same way described in the 'small artificial flowers' in chapter 3, to prevent bees visually identifying the artificial flowers by contents. Flowers were numbered with randomly generated

numbers as described in chapter 3 to allow visual identification of humid or non-humid variant flowers by human experimenters (figure 5.2).

A 35 mm circular hole was cut into the centre of each jar's lid, and the edges were smoothed to remove any excess plastic. This hole removed most of the flat top of the jar but maintained the screw threading assembly of the jar lid (figure 5.2b and 5.2c). The top surfaces of the artificial flowers were made with a sheet of fine gauze material (made from cut out segments of TERESIA curtains, IKEA, Leiden, Netherlands) stretched over the jar aperture and screwed into place using the cut away lid screw assembly. Any excess gauze visible below the screw lid on all flower types was cut away. This created a gauze top to the artificial flower slightly lower than the plastic rim of the artificial flower (figure 5.2d). This gauze surface was firm enough for the bee to walk upon, would help obscure jar contents, and was permeable to the evaporation produced by internal components of artificial flowers (see below). An upturned 0.5ml eppendorf tube lid was painted black and placed in the centre of the gauze indentation, functioning as a feeding well during experiments. This lid was not stuck down and could be moved by the bees while feeding, however it was too heavy for the bees to easily lift and the plastic rim of the artificial flower prevented bees upturning the lid or pushing it off the artificial flower (figure 5.2e).

Three discs of 1cm thick sponge were placed inside the specimen jars within each artificial flower. These discs (cut from cellulose sponge wipes, Co-op, Manchester, UK) were 40 mm diameter, the width of the specimen jar, (figure 5.2a). The top (visible) sponges were all identical green. For humid artificial flower these discs were wetted prior to experiments, and at the midpoint of conditioning experiments, as per the protocol laid out in the following section. The evaporation from this wet sponge increased the relative humidity above these artificial flowers. Dry artificial flowers did not have any water added to the sponges.

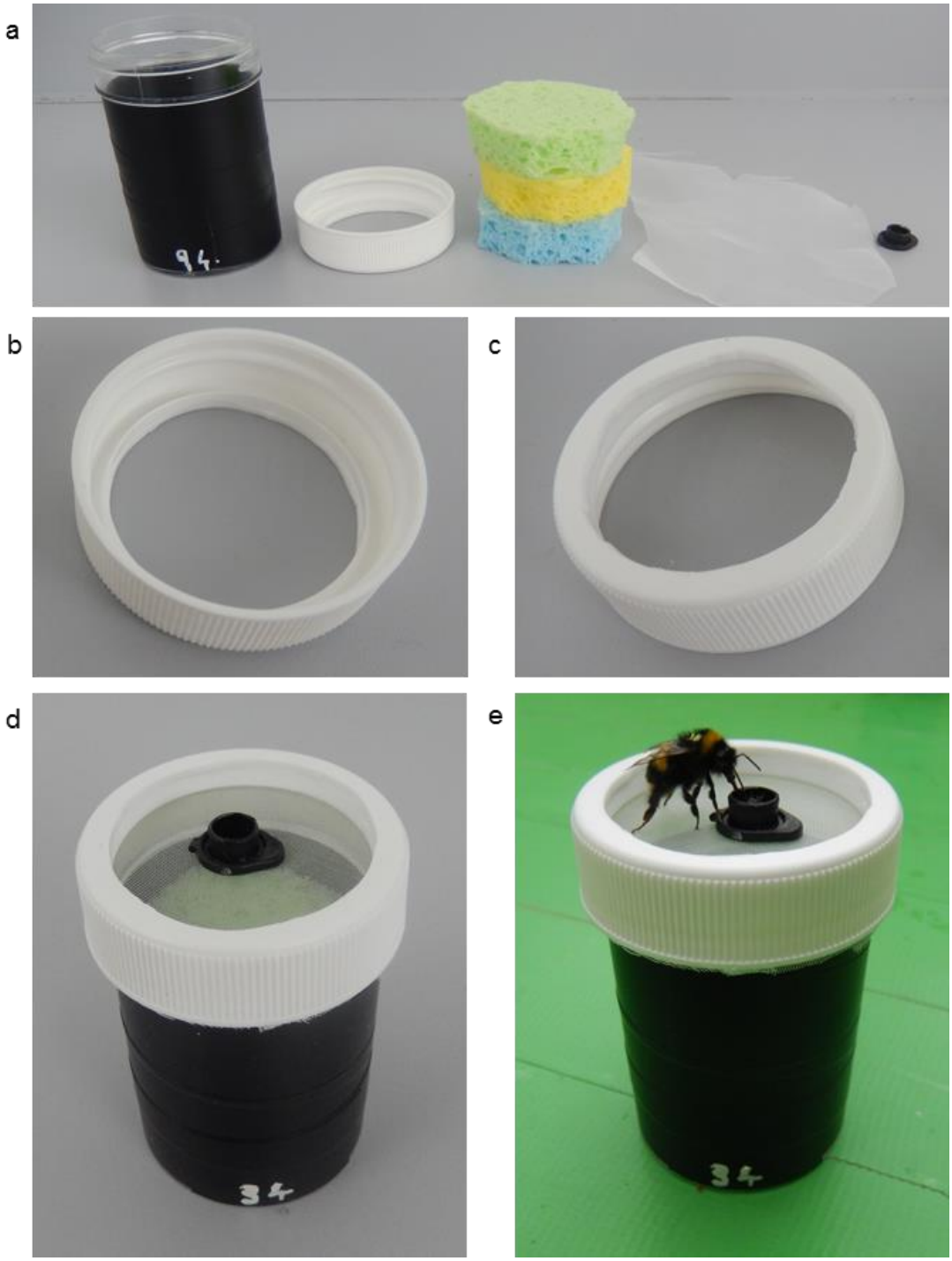


Figure 5.2: *The passive humidity artificial flowers used in bumblebee experiments.* ***a****) The artificial flower components, from left to right: the specimen jar; the specimen jar lid; three sponge discs; gauze fabric; and an Eppendorf tube lid.* ***b****) The specimen jar lid showing the cut away section, leaving the screw assembly, and the lip, from below.* ***c****) The same, but from above.* ***d****) The assembled artificial flower.* ***e****) Bumblebee feeding from the artificial flowers.*

Each batch of 24 sponge discs were stored in a sealed bag following being cut from sheets until needed. As each flower needed 3 discs and 8 flowers were presented to the bee during trials, all the discs used in one day to be from the same sponge batch and stored in the same way. All sponge discs were discarded after a day of use.

5.2.3 *Artificial flower setup*

Before preference experiments using active artificial flowers, the pump assembly for four artificial flowers would be placed under the flight arena table on both the right and left sides of the arena (as shown for one side in figure 5.1d). This allows artificial flowers to be placed in the arena through door brackets placed in the doors on that side. On each side two of the artificial flowers would have odd numbering, two even numbering (making eight flowers in total, four odd, four even). The bubbler chambers of either odd or even numbered flowers would be filled with 100ml of water, the other dry, as described above. In order to ensure a good seal on the Tupperware and the input and output for the rotameter and grommet seals for the bubbler all these seals would be strengthened with electrical tape. The airflow on all rotameters was then set to a 2.69ml s^{-1} using the rotameter screw seal.

Passive artificial flowers would be prepared as follows before each bee's trial in both preference and learning experiments. Sponge discs for dry artificial flower variants were inserted as they were from the bag into the specimen jar before the gauze and flower lid were screwed in. Sponge discs for humid artificial flower variants were submerged until sodden in a pitcher of water, that had been allowed to settle at the lab's temperature overnight, before insertion into the specimen jar and screwing down of gauze and lid. To avoid conflating indicators of which flowers contained wet sponge this was done so that the gauze remained dry. If the gauze got wet at any part of the experiment it was removed and replaced with a fresh dry sheet. Once sponge discs were inserted and tops screwed on, the humidity produced by humid flowers were checked using a handheld hygrometer (Maplin Electronics, UK). If the

relative humidity 5mm above the artificial flowers did not read at least 2% higher than the ambient humidity of the lab using this hygrometer, sponge discs would be removed and re-soaked. As humid flowers contained water, inserting of sponge and evaporation may cause a drop in temperature, artificial flower temperature was checked before trials began using a thermal camera (FLIR systems, Inc., Wilsonville, USA). During all thermal imaging emissivity parameter value used were 0.95 (I.T.C., 2008) and lab reflected temperature was measured using a tin foil mirror (chapter 2) to have a consistent value of 20ºC. As the water used for sponge wetting had been allowed to settle at room temperature, humid flower variants and dry flowers rarely differed in temperature enough for bees to detect (where detectability is presumed to occur if the temperature difference is more than 2ºC, Heran, 1952). However, if the humid flowers differed in temperature from the dry flowers by more than 1ºC, whichever flower variant was warmer would be cooled by placing them on a tray inside a refrigerator at 5ºC until the temperature difference between flower variants was below 1ºC. If both these humidity and temperature requirements were met flowers were ready to be presented to bees and experiments could start. During our learning experiments passive artificial flowers were also be re-prepared, as described above, at the end of the foraging bout when the bee crossed the halfway point in terms of visit number (35 visits or more). A foraging bout constituting the time between a bee leaving the nest to forage in the flight arena and exiting the arena to return to the nest.

5.2.4 *Artificial flower cleaning and maintenance*

Both artificial flower types were cleaned regularly throughout the experiments to prevent any conflating scent marks left behind be bee visits (Stout and Goulson, 2001; Pearce et al., 2017). Cleaning occurred at the end of each foraging bout.

When active artificial flowers were cleaned, all flowers were removed from the arena and the tops were wiped with ethanol, with care taken to not apply liquid over the holes. The

tubing prevented flowers from being moved to completely different locations during tests, due to tubes being linked to door brackets, so following cleaning the door bracket holes each artificial flower entered the arena by were changed on each side (*i.e*. a single flower would now enter the arena from a different hole on the same side of the arena). As tupperware seals, the tightness of the rotameter screw, rotameter input or output seals and grommet seals can weaken under the pressure system of the pump assembly, rotameter airflow was checked after any flower cleaning and rotameter adjusted. Where necessary other seals were repaired to maintain a 2.69ml s^{-1} airflow rate.

When passive artificial flowers were cleaned there was a risk that the fabric top of the flower retained scent better than plastic parts. Furthermore, returning a passive artificial flower to the arena with an ethanol wetted top may conflate the humidity differences between flowers under consideration in foraging tests. Consequently, when flowers were removed from the arena for cleaning, the lids and gauze were removed. The plastic parts of the lip were wiped down with ethanol, and a fresh sheet of unused gauze was screwed down onto the flower top with the clean lid. Excess gauze outside of the screw assembly would be cut away as before (figure 5.2d). This cleaning and replacement of fabric prevented scent marks that might aid reward discrimination from accumulating on the flower tops and allowed artificial flowers to remain consistently dry to the touch of the bees between foraging bouts.

5.2.5 *Evaluation of artificial floral humidity*

Both humidity-producing and dry variants of both artificial flower types, eight of each active artificial flower variant and twelve of each passive artificial flower variant, were sampled under the robot arm transect method described in chapter 4 to evaluate the artificial floral humidity they produce. This was done with a 25μl droplet of water in their feeding well, as bees would normally encounter flowers with water or sucrose solution present in the feeding well (see below). The pump assembly of active artificial flowers was set up under the table within the

sampling area (see figure 4.1). Flowers were otherwise set up as described above. Best fitting models for x and z axis humidity transects were found as described in chapter 4 and summary values X_t^{max} (the point in the x axis transect where humidity difference is greatest) and ΔRH_x^{max} (the greatest mean humidity difference generated) according to the best fitting models was calculated as described in chapter 4. The best fitting models, X_t^{max} and ΔRH_x^{max} values are given in table 5.1. Humidity structures for the active humidity flower transects are given in figure 5.3, and for the passive humidity flowers in figure 5.4. AIC tables for model selection and values for best fitting models are found in appendix A7 and A4 respectively.

Table 5.1: *The best fitting models, X_t^{max} and ΔRH_x^{max} values for the both humidity variants of both humidity artificial flowers. Subscript letters following best fitting models indicate shape of that model: F, flat models; L, linear models; and Q, quadratic models (note quadratic models were not fitted to the z axis). Subscript values next to x_t^{max} values indicate replicate effects: the number itself referring to the value of t, which replicate transect, at which ΔRH_x^{max} was found; no subscript values indicates replicate transects have no effect on humidity*

Artificial Flower Type	Humidity variant	Best fitting model for the		X_t^{max}	ΔRH_x^{max}
		x axis	z axis		
Active Humidity	Humid	$m3_Q$	$z1_L$	2.19	3.08
	Dry	$m10_Q$	$z4_L$	-0.12_1	0.92
Passive Humidity	Humid	$m9_Q$	$z3_L$	0_1	3.49
	Dry	$m10_Q$	$z4_L$	-0.61_1	2.13

Both dry artificial flower variants produced a small amount of humidity, table 5.1. This humidity was from evaporation of the water in the feeding well. This is supported by its proximity to the flower's centre and the diminishing humidity in replicate transects as this water evaporates (figures 5.3 and 5.4). The humidity produced by the feeding well was likely to be lower in dry active humidity flowers than the dry passive flowers due to the effect of the airflow in active flowers dispersing water vapour.

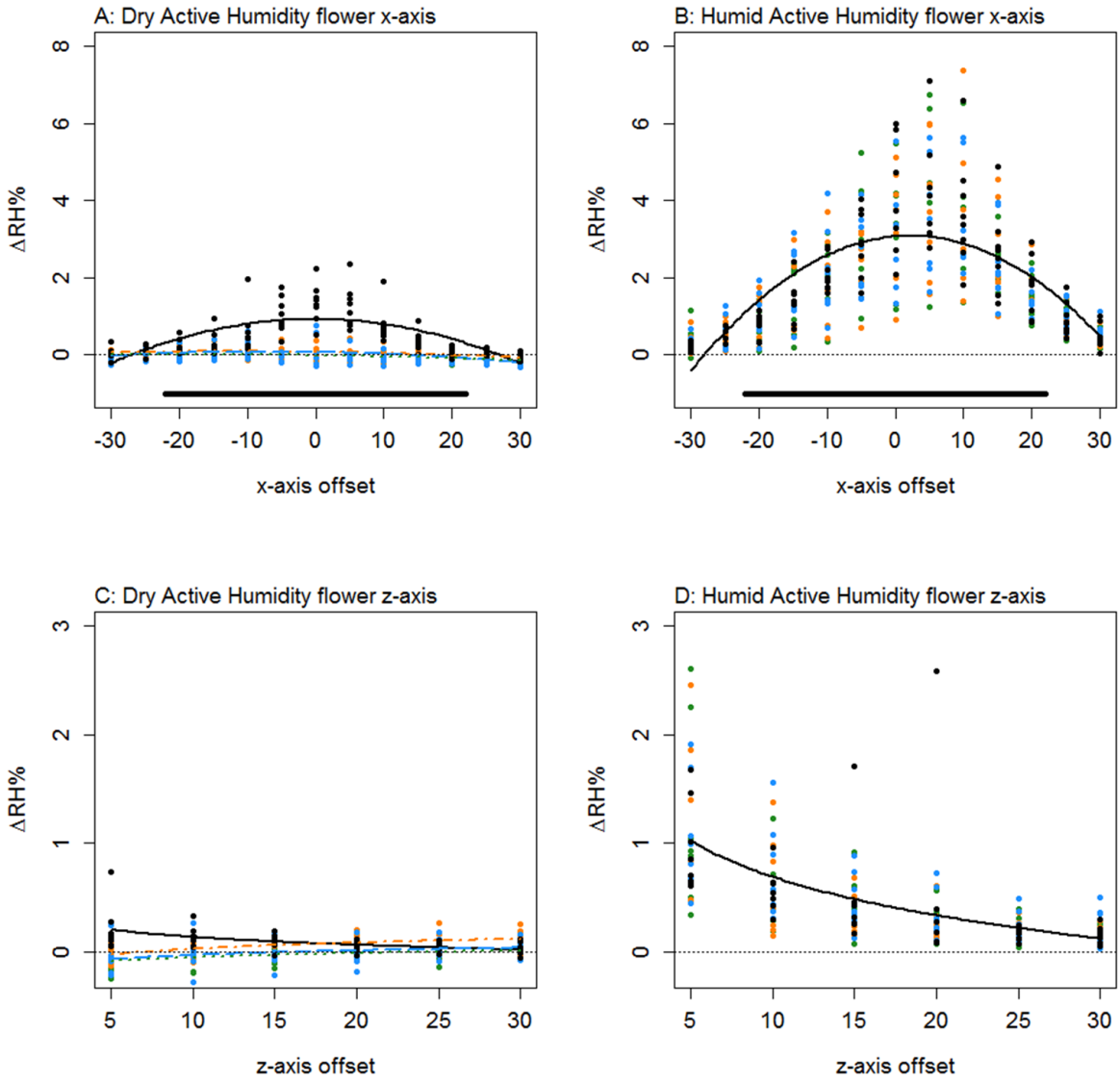


Figure 5.3: *The difference in humidity relative to the background (ΔRH) for transects of active humidity flowers. x and z axis transects are given for: dry, non-humid, flower variants in **A** and **C** respectively; and for the humid flower variants in **B** and **D** respectively. All axis offsets are relative to the transect central point and in millimetres. The thin dotted line indicates a 0% change in humidity- the background level. Bold lines indicate the mean change in humidity as predicted by the best fitting model for that flower. Colour and dashing of bold lines and points indicate the replicate transect: solid black, first transect; long-dash blue, second transect; dash-dot orange, third transect; dotted green, fourth transect. The solid bar above the x axis transects indicate the diameter of the flower top (44mm) relative to the x axis.*

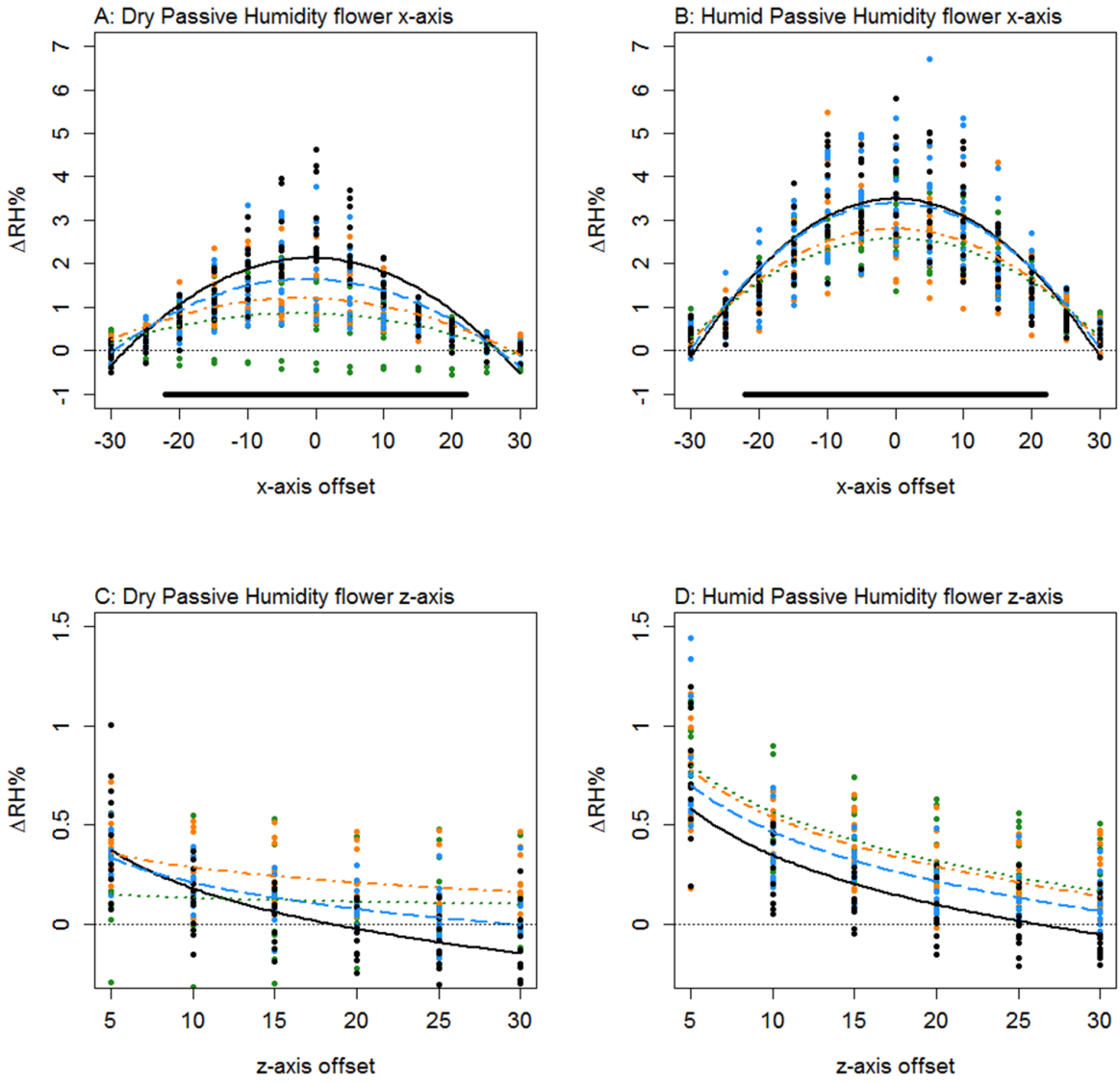


Figure 5.4: *The difference in humidity relative to the background (ΔRH) for transects of passive humidity flowers. x and z axis transects are given for: dry, non-humid, flower variants in* ***A*** *and* ***C*** *respectively; and for the humid flower variants in* ***B*** *and* ***D*** *respectively. All axis offsets are relative to the transect central point and in millimetres. The thin dotted line indicates a 0% change in humidity- the background level. Bold lines indicate the mean change in humidity as predicted by the best fitting model for that flower. Colour and dashing of bold lines and points indicate the replicate transect: solid black, first transect; long-dash blue, second transect; dash-dot orange, third transect; dotted green, fourth transect. The solid bar above the x axis transects indicate the diameter of the flower top (44mm) relative to the x axis.*

While a convenient way to confirm sufficient wetting of the internal sponge components had been achieved, the handheld hygrometer was not a particularly accurate measure of passive artificial flower floral humidity. Thus, the 2% difference on the hygrometer used as a measure of humidity generated was smaller than the average differences detected under the robot arm. Humidity produced by both active and passive flower humid flower variants was similar in intensity to floral humidity produced by flower species with ΔRH_x^{max} values greater than 3%, such as *Eschscholzia californica* (3.24%), *Taraxacum* agg. (3.35%), or *Ranunculus acris* (3.41%) (chapter 4). Structurally, the humidity produced was similar to that of most flower species with a quadratic x axis structure coming to a peak about the transect central point and a declining z axis relationship. The differences in relative humidity intensity between humid and dry flower variants was 2.16% in active humidity flowers and 1.36% in passive humidity flowers. Such differences are similar to those observed between different flower species, and between different flower species and the background humidity levels (figure 4.4, table 4.7), meaning that the floral humidity levels on which bee responses are tested in our experiments were within bounds of differences bees might experience when foraging on natural flowers.

The humidity produced by active humidity flowers remained largely stable throughout the sampling period (figure 5.3). The only change being a drop in humidity of the dry flower variant after the initial transect (figure 5.3a). This humidity in the dry flower variant was produced by the evaporation of the water in the feeding well during the initial transect. As dry active artificial flowers were regularly refilled throughout bee experiments after being emptied (see below), it is likely that the humidity differences bees are exposed to are maintained at levels shown in the initial transect. So, the mean difference in humidity intensity (in terms of ΔRH_x^{max}) between dry and humid active flower variants remained at ~2.16% during experiments. The passive humidity flowers were less stable, with the floral humidity regularly dropping with replicate transects in the dry flower variant (figure 5.4a) and dropping in the second and third replicate transects in the humid flower variant (figure 5.4b). This was due to the drying out of components and again evaporation of water from the feeding well. However,

the passive artificial flowers were refilled and, where appropriate, re-wetted during bee trials. The dry variant, being refilled with water or sucrose solution is likely to maintain humidity differences similar to the first replicate, or at worst second replicate transect. The passive humid variant flowers show stable average humidity intensities for the first and second transect replicates, this means the initial peak in humidity lasts for at least ten hours (see chapter 4) before drying out affects humidity intensity. Preference and learning trials rarely took this long, so it is unlikely that the humidity would drop much below the initial intensities in the time allowed between re-wetting (the bout at 35 visits). Thus, while the difference between dry and humid flower variants of the passive artificial flowers is probably less stable during test then active artificial flowers, the difference in average humidity intensity (in terms of ΔRH_x^{max}) should remain at ~1.36%.

5.2.6 *Bee trials*

Two kind of experiments were carried out on captive bumblebees. Firstly, preference experiments, like those used by Lehrer et al. (1995), Dyer et al. (2006) and von Arx et al. (2012) were carried out using both artificial flower types. Secondly, differential conditioning techniques, like those implemented in chapter 3, Dyer and Chittka (2004d), Clarke et al. (2013) and Lawson et al. (2018), were carried out with passive artificial flowers only. This was due to the limits on how much and how quickly active artificial flowers could be moved about the arena due to their piping. Individual bees were not reused between experiments: an individual bee would only take part in one experiment (preference or conditioning) as part of a single test group on a single type of artificial flower (active or passive).

5.2.7 *Preference experiments*

In preference experiments, individual bees would have their preference tested on either active or passive humidity flowers. Two different bumblebee nests were used in the passive flower tests. Bees used in the active flower tests came from four different nests, which included the two nests used in the passive flower tests. During preference tests, bees were presented with eight artificial flowers of the kind assigned to them, placed randomly about the foraging arena floor. Four of these were the humid flower variant, and the other four were the dry flower variant. All artificial flowers would be rewarding, containing a 25μl droplet of 30% sucrose solution within their feeding wells.

Individual bees were released into the arena alone, and bees were allowed to forage freely on these artificial flowers, and were free to return to the nest at all times. Whether bees made contact with the top of artificial flowers (which was recorded as a landing behaviour) was monitored, and whether the bee extended its proboscis into the feeding well or left without doing so at each landing. Following feeding on a flower the flower was refilled and moved. In the passive humidity flowers this would involve taking the flower out of the arena and placing it back in in a different position. In active humidity flowers the ability to move the flowers was limited by their pipes and the flowers' current arena entry points. Consequently, active artificial flowers would not be taken out of the arena but would still be moved to a different point. If a bee fed from a flower and revisited it before it was able to be moved, then these revisits were not counted. When the bee returned to the nest all the flowers were removed from the arena and cleaned as described above.

This carried on until the bee had made at least 20 flower landings. This was normally achieved in 4.38 ± 0.44 (mean ± SEM) foraging bouts with an average of 5.06 ± 0.41 visits per bout in with the active humidity flowers and 3 ± 0.34 bouts with an average of 7.40 ± 0.67 visits per bout in the passive humidity flowers. 16 bees completed the preference experiment on each type of artificial flower (32 bees in total).

The rate at which bees made a positive response to floral humidity (the 'humidity response rate') over the 20 flower landings was calculated for each bee. A positive response was classed as either landing on a humid flower and extending the proboscis into the feeding well or landing on a dry flower and leaving without extending the proboscis into the feeding well. This humidity response rate underwent the arcsine square root transformation, to minimise influences of response rates being bound between 0 and 1. A Wilcoxon signed-rank test was used to test whether the median value of the transformed humidity response rate differed from that expected from random choice (a 0.5 humidity response rate, 0.79 once arcsine square-root transformed) in *R* 3.4.1 (R Development Core Team, 2017).

Temperature differences between humid or dry flower variants might occur as a result of evaporative water loss or action of mechanical components within artificial flowers. As bees can respond to floral temperature showing preferences and learning (Dyer et al., 2006; Whitney et al., 2008), differences above bee's sensitivities to temperature, a temperature difference greater than 2ºC (Heran, 1952), may conflate bee responses. For this reason, flower temperature differences of artificial flowers were monitored alongside the preference experiment, using a thermal camera (FLIR E60bx, FLIR systems, Inc., Wilsonville, USA) to see if the flower develop a temperature difference bees could respond to. This was done at the start of foraging or after flower cleaning at the end of foraging bouts, by randomly selecting one humid and one non-humid artificial flower and measuring the temperature of the flower top. Emissivity parameter value used was 0.95, an accepted value for plastics (I.T.C., 2008), and reflected temperature used was a consistent value of 20ºC.

5.2.8 *Learning experiments*

In learning experiments only the passive artificial flowers were used. Bees were assigned to one of three test groups. These test groups were: *i*) 'Humid rewards' group, where the rewarding flowers were humid flower variants (wet sponges), and the nonrewarding distractor flowers, dry flower variants (dry sponges); *ii*) 'Dry rewards' group, where the rewarding flowers were dry flower variants, the distractors produced humid flower variants; *iii*) 'Control' group, where neither rewarding or nonrewarding distractors produced humidity (i.e. all the sponges were dry), meaning that flowers only differed in their rewards. Rewarding flowers had a 25μl droplet of 30% sucrose solution within their feeding wells and nonrewarding flowers contained a 25μl droplet of water. Four different bumblebee nests were used in this experiment, none of these nests were used in preference experiments.

Individual bees were allowed into the arena alone and were presented with eight artificial flowers placed randomly about the flight arena: four rewarding and four nonrewarding with humidity production by these flowers assigned as per the bee's test group. Bees were allowed to forage freely on these artificial flowers, again allowed to return to the nest as required. Whether bees made contact with the top of artificial flowers, classed as landing, was monitored. Whether a bee extended its proboscis into the feeding well or left without doing so at each landing was also monitored, as described in chapter 3. Bees were observed for 70 flower visits. Bees achieved 70 visits in, on average, 5.13 ± 0.31 foraging bouts (mean ± SEM) with 13.78 ± 0.56 landings in each bout. 15 bees completed this learning trial in each test group (45 bees in total).

Following a feeding on a flower, that flower was removed from the arena. The feeding well of the removed flower was refilled with sucrose or water as appropriate and placed back in the arena at a different location. If a bee fed from a flower and revisited it before it was able to be moved, then these revisits were not counted. When the bee returned to the nest, all the flowers were removed from the arena and cleaned as described above.

Flower visits were determined as correct or incorrect and success rate over the previous ten visits at ten visit intervals (10 visits, 20, 30... *etc*.) was calculated as described in chapter 3. Generalised linear models were fitted to this data and the AIC model simplification techniques, as described in chapter 3, were used to analyse the effects of experience on the flowers (number of visits made) and test group had on bumblebee foraging success. Models used for analysis, the model simplification process and selection of the 'best model' of pollinator learning were identical to that presented in chapter 3 (equation 3.1 representing the full model before any model simplification), with the following exceptions: i and l now refer to the intercept and learning speed of bees in the control group; s_c and s_t now refer to the change in intercept of bees in the dry rewards and humid rewards test groups; c_c and c_t now refer to the change in learning speed in the dry rewards and humid rewards test groups; C now indicates the bee is in the dry rewards group where

$$C = \begin{cases} 0 & \text{bee is not in the dry rewards group,} \\ 1 & \text{bee is in the dry rewards group,} \end{cases} \tag{5.1}$$

and T indicates the bee is in the Humid Rewards group where

$$T = \begin{cases} 0 & \text{bee is not in the humid rewards group,} \\ 1 & \text{bee is in the humid rewards group.} \end{cases} \tag{5.2}$$

5.3 Results

5.3.1 *Artificial flower temperature*

Artificial flower temperature differences, as measured during the preference experiments, were small and probably not sufficient to elicit a meaningful response from bees. In passive humidity flowers, dry variants were on average 0.31 ± 0.03ºC (mean ± SEM) hotter than humid variants throughout the experiment. In active artificial flowers, humid and dry variants differed less in temperature. Dry active humidity flowers were 0.03 ± 0.03ºC colder than humid flowers. Measured differences between dry and humid flower variants (dry flower variant temperature minus humid flower variant temperature) ranged from -0.2 to 0.9ºC in passive humidity flowers and -0.5 to 0.5ºC in active artificial flowers. These differences in temperature are small, and below the threshold of temperature detection by bumblebees (Heran, 1952) and are unlikely to elicit a response by bumblebees.

5.3.2 *Bee trials*

The preference experiments revealed that bumblebees show a slight preference for flowers artificially producing floral humidity (figure 5.5). The median humidity response rates differed from that expected from random foraging, for both the passive humidity trial (Wilcoxon Test, $W = 109$ $n = 16$, $p = 0.006$) and the active humidity trial (Wilcoxon Test, $W = 119$, $n = 16$, $p = 0.0008$). In both instances median bee humidity response rates were greater than 0.5, it being 0.55 (ranging from 0.4 to 0.7) for bees presented with passive humidity flowers (figure 5.5a), and 0.6 (ranging from 0.45 to 0.85) for bees presented with active humidity flowers (figure 5.5b).

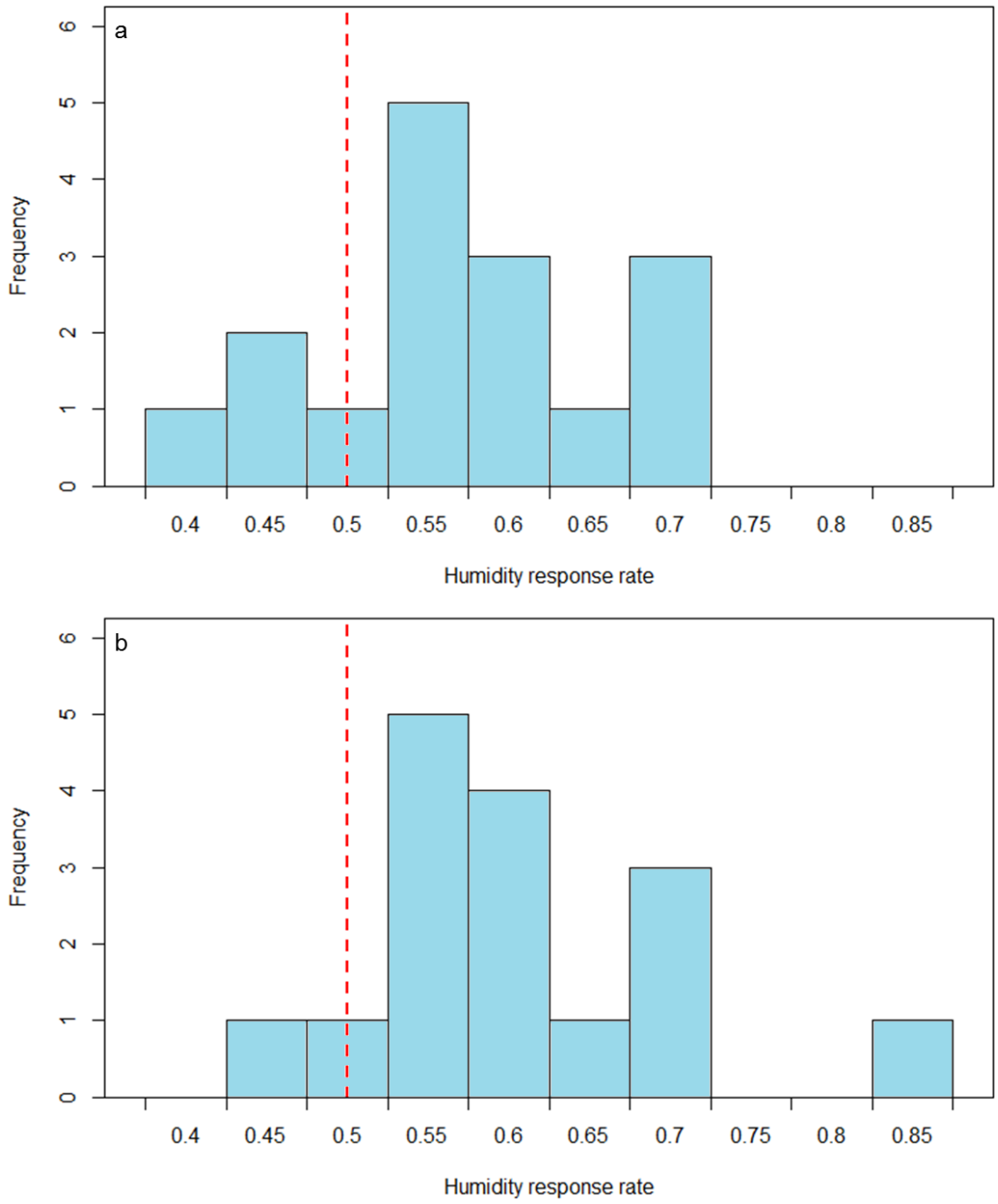


Figure 5.5: *Histograms showing the responses of bumblebees to humidity producing passive (**a**) and active (**b**) humidity flowers in the preference experiments. The number of bees achieving each humidity response rate (bars) is given as well as the expected humidity response rate for randomly foraging bees (dashed vertical red line).*

The learning experiment revealed that bumblebees to be able to detect artificial floral humidity differences between flowers and distinguish rewarding from nonrewarding flowers using them. This appeared to be the case regardless of whether floral humidity corresponded to a reward or not (figure 5.6). Models that allowed individual bees to have different intercepts and different learning speeds (random slopes and intercepts) were not better fits (AIC -298.3 *vs*. -301.3, ΔAIC = 3, Δdeviance = 1.01, df = 2, $p = 0.602$) than models that only had individual variation in intercepts. Models that allowed experience (number of flower visits made) to have a different effect on foraging success dependent on the test group bees were in (interacting effects) had a lower AIC (AIC -301.29 *vs*. -288.16, ΔAIC = 13.13) and a significantly better fit (Δdeviance = 17.13, df = 2, $p < 0.001$) than models that forced experience to have the same effect across test groups. Bees in the control group began the experiment with a success rate of 0.5, as one would expect from random forging, and improved only slightly. Consequently, models of the control group that allowed success to change with experience, learning, were comparable to those that allowed no change in success in terms of AIC (AIC -124.45 *vs*. -121.65, ΔAIC = 2.8) but did have a better fit (Δdeviance = 4.79, df = 1, $p = 0.029$). In test groups that differed in floral humidity between rewarding and nonrewarding flowers, bees began with success rates similar to that achieved by the control group, but success improved as bees made more flower visits. Thus, models that allowed success rate to change with experience had lower AIC and better fit regardless of whether bees were trained to associate humid flowers with rewards (Humid rewards: AIC -100.0 *vs*. -79.7, ΔAIC = 20.24; Δdeviance = 22.24, df = 1, $p < 0.001$) or not (Dry rewards: AIC -74.8 *vs*. -36.7, ΔAIC = 38.16; Δdeviance = 40.16, df = 1, $p < 0.001$) (figure 5.6).

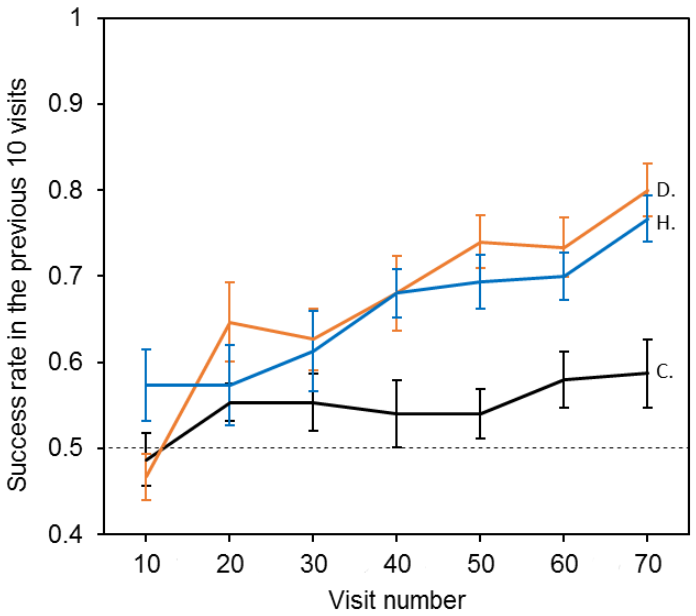


Figure 5.6: *The relationship between bees' foraging success and experience of the humid artificial flowers (flower visits the bee made). Dotted line indicates the 50% success level. Solid lines indicate the mean foraging success of bees in the previous 10 visits. Error bars represent ±SEM. Colour and label of solid lines and error bars correspond with test group: black, the control group, labelled 'C'.; orange, Dry rewards group, labelled 'D.'; blue, Humid reward group labelled 'H'.*

5.4 Discussion

The behavioural experiments presented here show that bumblebees are able to respond to floral humidity, when produced artificially, in a flower foraging context. Bumblebees showed a preference for floral humidity (figure 5.5) and an ability to distinguish rewarding and nonrewarding flowers based on these floral humidity differences (figure 5.6). The artificial flowers used here, both humid and dry variants, produced floral humidity of similar intensities to natural flowers and therefore differed in humidity a similar way (compare table 5.1 with table 4.7 and figures 5.3 and 5.4 with figures 4.5 and 4.6). Consequently, bumblebees should show similar responses to floral humidity produced by natural flowers. Bees may respond to the amount of humidity produced by the flower itself (Yokohari et al., 1982; Yokohari, 1983) and the rate of change in humidity experienced as bees approach or pass flowers (Tichy and

Kallina, 2010; Tichy and Kallina, 2014). Thus, the approach behaviour of bees to humidity-producing flower may have important influences on floral humidity detection. Humidity perception is an important environmental signal for bees (Abou-Shaara et al., 2017), having important influences on nest maintenance (Human et al., 2006; Nicolson, 2009). Our bee experiments show that, bumblebees can additionally use humidity perception to detect floral humidity to recognise and distinguish flowers. Furthermore, rather than being signals detectable by moth pollinators alone, floral humidity can be utilised by bumblebees. In this way the diversity of floral humidity found across flower species (chapter 4; Corbet et al., 1979a; von Arx et al., 2012) represents a floral signal that can be used far more widely than previously thought. This may explain the presence of elevated floral humidity in flowers that receive minimal moth visitation (chapter 4) and expands our knowledge of how many different signalling modalities flowers may be utilising at once to signal pollinators.

When presented with a choice of equal numbers of humidity-producing flowers and dry, non-humid flowers that otherwise didn't differ in a manner bees could detect, bees showed a slight preference for humidity signals (figure 5.5). This preference for floral humidity was verified in both active and passive artificial flowers. Temperature differences between flowers were small but in passive flowers the preferred humid flowers were slightly colder. If bees had been responding to temperature differences, we would expect the reverse of the preferences observed (Dyer et al., 2006), confirming that the bees are responding to humidity differences between the flowers in this experiment. The elevated difference between humid and dry flowers in the active artificial flowers led to a stronger preference, but it remains unclear if bees would show a similar preference between flower species producing lower intensity or showing smaller differences in floral humidity. This preference to humidity producing flowers is similar to that shown by *H. lineataIa* (von Arx et al., 2012) and implied by flower preferences of alpine fly pollinators in India (Nordström et al., 2017). As bees prefer higher intensity floral humidity, flowers may produce humidity to encourage increased bumblebee visitation, especially from

naïve bees. In this way adaptations that might encourage floral humidity production may be favoured.

In addition to showing a preference to floral humidity, bumblebees also demonstrated an ability to learn to distinguish rewarding and nonrewarding flowers based on floral humidity differences. A learning response to floral humidity differences between flowers has not previously been demonstrated in any pollinator group. Other pollinator preferences to floral humidity (von Arx et al., 2012; Nordström et al., 2017) suggests that pollinators other than bumblebees may be able to show similar learning responses. Likewise, other flies (Liu et al., 2007) and bees (Fialho et al., 2014) possess hygrosensitive receptors and show a good flower learning capacity (Mobbs, 1982; Hammer et al., 2009; Wright et al., 2009b) so may be capable of floral humidity detection and learning. However, bumblebees possess an unusually high frequency of hygrosensative receptors (Fialho et al., 2014), and this may be required to perceive and learn floral humidity. Bumblebees showed learning regardless of which flower variant was rewarding, as shown by learning in both the dry rewards and humid rewards test groups (figure 5.6). This is possible due to the antagonistic detection of both presence and absence of humidity *via* dry and moist cells (Yokohari et al., 1982; Yokohari, 1983; Tichy and Kallina, 2014). This learning of humid and dry flowers has important consequences. Humidity transects of several flower species, such as *Vinca herbacea* and *Linum grandiflorum*, showed no floral humidity or less humidity than extraneous humidity sources (figure 4.4). The findings of these learning experiments show that bumblebees can use this the lack of humidity to distinguish these flowers from humidity-producing species. This means an absence, or lower intensity floral humidity, remains a signal that bumblebees can learn. In this way adaptions that limit humidity production may still aid the recognition of flowers from floral humidity-producing conspecifics. Similar adaptation of the floral display that go against naïve bee preferences have been observed previously in non-blue coloured flowers (Gumbert et al., 1999; Dyer and Chittka, 2004d; Lynn et al., 2005) and cold flowers (Whitney et al., 2008). The

ability of bumblebees to learn flowers based on floral humidity intensity differences adds to our understanding of floral display complexity and flower species recognition by pollinators.

Floral humidity has largely been considered in the context of honest signalling. While some links have been demonstrated between floral humidity and floral nectar rewards in some species (Corbet et al., 1979a; von Arx et al., 2012), it is unclear that this is the case in every species (chapter 4). Here the preferences for floral humidity (figure 5.5) suggest that, when foraging on flower species where floral humidity is linked to reward levels, naïve bees should favour visits to the more humid and more rewarding flowers. Similarly, bees can learn floral humidity (figure 5.6), so bees can learn that nectar rewards correspond to the flowers producing humidity when foraging on a honest signalling flower species. This suggests that bees have the capacity to use floral humidity as an honest signal exists. However, more work needs to be carried out to demonstrate the capacity of floral humidity to function as an honest indicator of reward levels or presence in flowers other than *O. caespitosa*. Furthermore, while artificial flowers had visual properties that remained unchanged alongside floral humidity changes, it remains unclear to what extent a change in floral humidity within a more multimodal (but otherwise unchanged) floral display will influence behaviour and foraging choices. Floral humidity preferences are slight here (figure 5.5) despite artificial flowers showing larger humidity differences when compared to naturally occurring floral humidity (table 4.7). Humidity preferences may therefore be overridden by other more salient unchanged signals of other modalities within a multimodal display. The capacity for bees to learn may be critical for use of floral humidity as an honest signal within multimodal displays. It has been shown that learning of one signal can be improved when other signal modalities are present, even when they do not differ with that initial signal (Kunze and Gumbert, 2001; Leonard et al., 2011a). This may be due to a contextualizing of other signals or focusing of the pollinators attention (Leonard et al., 2011b; Raguso, 2004). Thus, in a multimodal display showing an honest floral humidity signal, pollinator attention may be focused by other modalities to humidity differences. That said, the findings of this study suggest that even if humidity does not

frequently function as an honest signal of reward presence it may still have beneficial influences on plant-pollinator interactions. A humidity signal that does not function honestly will still be preferred by naïve bumblebees (figure 5.5), hawkmoths (von Arx et al., 2012) and possibly also flies (Nordström et al., 2017) and, at least, bumblebees will be able to use this signal to identify that flower species. Higher visitation and being identified with clarity will benefit the plant (Larson and Barrett, 2000; Ashman et al., 2004). Likewise, finding and recognising flowers with greater accuracy will improve foraging success of pollinators (Raine and Chittka, 2008). Thus, while the extent of honesty humidity signals show influences how they may be used, it does not govern their potential to be used by flower visitors, at least bumblebees.

Floral humidity has been detected in many flower species (von Arx et al., 2012; Nordström et al., 2017), and shown to differ between species (chapter 4). However, the capacity of pollinators other than *H. lineataIa* to respond to floral humidity as a foraging signal, and the ability of any pollinator to learn floral humidity differences, had not been demonstrated prior to this study. Here, bumblebees are demonstrated to show a preference for and be able to learn to distinguish differing levels of floral humidity. This not only shows floral humidity to be a more widely used floral signal, not limited to use by moths, but also shows it can be used for flower recognition. Floral humidity appears to be able to be used by a wider range of pollinators and has a wider range of potential functions within multimodal floral displays than previously believed.

Chapter 6: The capacity of temperature patterns to function as floral guides

A portion of video data presented in this chapter (15%) and the spectrometer readings presented in figure 6.2 were collected by Msci student Ed Straw under my supervision.

CHAPTER ABSTRACT

Floral guides are signal patterns that lead pollinators to floral rewards, increasing foraging efficiency and pollen transfer. Patterns of several floral signalling modalities, particularly colour patterns, have been identified to be able to function as floral guides. Floral temperature frequently shows patterns that can be used by bumblebees for flower recognition, but whether these temperature patterns can also function as a floral guide has not been explored. Furthermore, how patterns of multiple modalities affect floral guide function has only been investigated in a few modality combinations. We assess how artificial flowers induce guided behaviours in bumblebees when rewards are indicated by unimodal temperature patterns, unimodal colour patterns or multimodal combinations of these. Bees visiting flowers possessing unimodal temperature patterns showed an increased probability of finding rewards and increased learning of reward location, compared to bees visiting flowers without patterns. However, flowers possessing contrasting unimodal colour patterns showed further guide-related behavioural changes in addition to these, such as reduced reward search times and attraction to the rewarding feeder without learning. This shows that temperature patterns alone can function as a floral guide, but their effectiveness is reduced compared to contrasting colour patterns. When rewards were indicated by multimodal patterns of temperature and colour, bees showed limited behavioural changes when responses were compared bees visiting the flower's component patterns. This supports previous evidence, using combinations of other floral signalling modalities, that multimodal patterns do not reinforce floral guide functionality.

6.1 Introduction

Floral displays communicate with flower visitors through various signalling modalities at once. Such modalities include colour, scent, texture, temperature, and electrostatics in addition to patterns of these signals (Raguso, 2004; Leonard et al., 2011b; Leonard et al., 2012). A possible explanation for this multimodality in floral displays is that additional signalling components convey different information to the pollinator: the 'multiple messages hypothesis' (Leonard et al., 2012). These floral messages could include information on flower identify, flower reward type or status (von Arx, 2013). Additional signals might also provide spatial information about the flower. Certain floral signals may be used by pollinators to identify flower location in the environment, but others may indicate the location of rewards within the flower functioning as 'floral guides' – sometimes known as 'nectar guides' (Leonard and Papaj, 2011; Lawson et al., 2017b; Lawson and Rands, 2018). Floral guides are contrasting signal patterns that help lead pollinators to the location of rewards within a flower. The most studied floral guides are colour patterns (Manning, 1956; Dafni and Kevan, 1996; Lunau et al., 2006). These colour guides are found across many diverse floral taxa, and normally appear as radiating lines, speckles or solid blocks of contrasting colouring corresponding to the corolla entrance or nectary location (Manning, 1956; Penny, 1983; Dafni and Kevan, 1996; Hempel de Ibarra and Vorobyev, 2009; Lawson et al., 2017b; Lawson and Rands, 2018). How other modalities function as non-visual floral guides is less well studied. Patterns of scent have been demonstrated to be capable of guiding bumblebees to rewards (Lawson et al., 2017b). Similarly, tactile patterns, particularly radiating floral groves, guide moth proboscis placement (Goyret and Raguso, 2006; Goyret and Kelber, 2011). Other signalling modalities show structured patterns, such as electrostatics and temperature, but the capacity of these floral signalling modalities to function as floral guides has yet to be been demonstrated.

Floral guides lead the pollinator to the rewarding region of the flower (Manning, 1956; Goyret and Raguso, 2006; Lunau et al., 2006; Leonard and Papaj, 2011; Goyret and Kelber, 2011; Hansen et al., 2011; Goodale et al., 2014). When compared to flowers lacking such

patterns, floral guides have been reported to: reduce the time spent searching for rewards on each flower (Leonard and Papaj, 2011; Goyret and Kelber, 2011; Goodale et al., 2014; Lawson et al., 2017b); increase incidence of flower visits where pollinators find floral rewards (Goyret and Raguso, 2006; Goyret and Kelber, 2011; Hansen et al., 2011); reduce the amount of time pollinators spend searching the flower after feeding (Leonard and Papaj, 2011) and reduce the incidence of floral larceny (Leonard et al., 2013). In these ways floral guides increase pollinator foraging efficiency (Heinrich, 1979a; Pelletier and McNeil, 2003; Charlton and Houston, 2010). These improvements in flower handling behaviours, relative to visitors of flowers that don't have guides, appear to be consistent between naïve pollinators and pollinators with experience, in bumblebees at least (Leonard and Papaj, 2011). This means that the initial responses of naïve pollinators are improved and that pollinators can learn the guide and the spatial information it provides to achieve further enhanced responses. By increasing visit efficiency, flowers with guides will be preferred over those that lack them (Waser and Price, 1983). Increasing the total amount of visits, the incidence of successful and legitimate (non-larceny) visits, as well as the speed at which visits take place, will all have beneficial consequences on pollen transport (Hansen et al., 2011; Leonard and Papaj, 2011). Furthermore, guides can help flowers control the direction of pollinator approach and its position while visiting, and this can allow plants to manoeuvre pollinators to a position that is best for pollen transfer (Armbruster et al., 2004; Hansen et al., 2011). Additionally, Leonard and Papaj (2011) found that once a bumblebee has learned to forage on flowers with floral guides, it is less likely to switch to visit flower species that show no guides. This will improve pollinator flower constancy on flowers with guides, further enhancing guides' benefits to pollen transfer (Chittka et al., 1999; Osborne et al., 1999). This also means pollinator responses to floral guides, after experiencing them previously, may be enhanced when encountering novel flowers with similar guides compared to pollinators without such experience.

Floral temperature patterns, where parts of the flower show elevated temperature compared to the rest of the flower, are a common phenomenon (chapter 3; Rejšková et al.,

2010; Dietrich and Körner, 2014; Harrap et al., 2017). Flowers often show radial gradients in temperature from the flower centre, near the nectary or on landing pad structures that protrude from corolla (figure 3.3). Warmer flowers keep pollinator body temperature from dropping while they feed. In this way, floral temperature functions like a secondary floral reward by reducing the foraging costs of pollinators associated with maintaining body temperature (Rands and Whitney, 2008). This is of particular benefit in colder environments (Heinrich, 1972; Heinrich, 1979a; Rands and Whitney, 2008). Consequently, many pollinating insects show a preference for elevated floral temperature (Seymour et al., 2003; Sapir et al., 2006; Dyer et al., 2006; Norgate et al., 2010). A preference for elevated temperature may attract pollinators to the warmer regions of flowers. Therefore, temperature patterns may be able to lead pollinators to floral rewards, when elevated temperature is localized to reward location, and function as a non-visual floral guide. Bumblebees can also learn floral temperature patterns (chapter 3; Harrap et al., 2017), so temperature may allow further improved flower handling with experience, as seen with other guides (Goyret and Kelber, 2011; Leonard and Papaj, 2011). Whether floral temperature patterns can function as a non-visual floral guide has not been demonstrated. However, in carrion mimic dead-horse arum, *Helicodiceros muscivorus*, temperature across the appendix of the flower trap, created naturally or artificially by electrical components, increases the numbers of blowfly pollinators lead into the trap (Angioy et al., 2004). Additionally preferences for patterned signals, like those many insects show for elevated temperature, are important to the function and evolution of floral guides (Lawson and Rands, 2018). Pollinating insect preferences for radial (Lehrer et al., 1995) and darker (Johnson and Dafni, 1998; Hempel de Ibarra et al., 2001) floral colour patterns are well documented, evolving in insects as a result of pre-existing sensory bias for identifying nest-burrows (Biesmeijer et al., 2005). These preferences are thought to cause the guided behaviours pollinators show at flowers with these patterns (Johnson and Dafni, 1998; Biesmeijer et al., 2005; Goodale et al., 2014). Thus, floral temperature shows several aspects that might help it function as a floral guide in addition to other reward and signalling functions.

When considering pollinator responses to individual floral signals, it is important to consider that pollinators visiting natural flowers will interact with a multimodal floral display. Multimodal displays can enhance floral learning beyond that of their unimodal components (Kulahci et al., 2008; Kaczorowski et al., 2012; Leonard and Masek, 2014) or alter pollinator responses to other signals (Goyret et al., 2007; Goyret et al., 2008a). Considering pollinator responses to combinations of signals if they frequently occur together will therefore provide a better understanding of how pollinators actually respond to natural flowers. Floral temperature is often the result of a flower's ability to intercept solar radiation (Totland, 1996; Sapir et al., 2006). Thus, pigmentation of a flower has a strong influence on heating (Sapir et al., 2006; Rejšková et al., 2010) and floral temperature patterns often correspond with dark coloured patterns of flowers (chapter 3; Rejšková et al., 2010; Harrap et al., 2017). Temperature patterns do occur without such dark pigmented colour patterns, as floral temperature can be influenced by other factors like: flower structure (Miller, 1986), epidermal textures (Whitney et al., 2011), floral transpiration (Tsukaguchi et al., 2003) or environmental temperature (Shrestha et al., 2018). However, it appears that, due to this mechanistic association between floral colour and temperature, pollinators visiting natural flowers with coloured guides will often encounter an overlapping temperature pattern simultaneously. It is currently unknown whether a pattern that combines thermal and colour components functions better as a floral guide than colour alone. To understand the guiding ability of natural flowers, we must assess how pollinators respond to combined thermal and visual patterns.

This chapter aims to investigate whether floral temperature patterns function as a non-visual floral guide in a manner similar to tactile (Goyret and Raguso, 2006; Goyret and Kelber, 2011) and scent patterns (Lawson et al., 2017b). Additionally, this chapter considers whether coloured visual guides are enhanced in their function by overlapping heating of the guide, like that seen often in nature. The methods used represent an adaptation of several approaches applied to investigation pollinator responses to floral guides (Goodale et al., 2014; Lawson et al., 2017b). Captive bumblebees are filmed over several visits to artificial flowers where reward

location can be indicated by temperature, colour, or multimodal patterns. From this footage we analyse how various flower-handling metrics, associated with pollinator responses to floral guides, differ between foragers on flowers with different patterns and those without such patterns.

6.2 Methods

6.2.1 *Basic artificial flower construction*

The bottom of a petri dish (100mm x 20mm, *Sarstedt*, Nümbrecht, Germany) was turned upside down and aqua blue sticky back plastic (d-c-fix® adhesive film, Hornschuch group, Weissbach, Germany) was stuck to it, forming the artificial flower's top. Emissivity of the plastic was measured, using a standard emissivity reference (electrical tape, see chapter 2), known to be 0.95. This emissivity value was used throughout all thermography measurements presented in this chapter. Eppendorf tube lids (Multiply-pro cup 0.2mlPP, Sarstedt, Germany) were cut off and a sheet of a 1mm thick white plastic foam was stuck to cover the lids' top. When upturned, these Eppendorf tube lids function as feeding wells for bees. The plastic foam insulates the feeding well form temperature patterns (see below). However, to limit differences between artificial flower variants foam was required on all artificial flowers. Three feeding wells would be constructed for each flower and struck 5mm in from the edge of the flower orientated so that the lids' cap pointed outwards at 120^{o} angles from each other.

Each flower top was supported on a 42mm tall, 85mm diameter card cylinder that fit inside the petri dish. This cylinder was wrapped in black electrical tape to protect it from wear and give rigidity. The petri dish lid functioned as the base of the artificial flower, preventing heating elements (when present) from falling out and detaching themselves, and also allowing the experimenter to carry flowers without touching or interfering with the other parts. Three 3cm by 1cm card rectangles were stuck to the top of the lid of the petri dish (thus the underside of the bottom of the artificial flower), at 120° angles so that 2cm taps extended over the dish's

edge (figure 6.1). A single circular red sticker was stuck to the upwards facing parts of each of the card tabs. Two-digit numbers were drawn on each of these red stickers with a black pen. These numbers were selected so that each lid had two tabs with odd numbers and one with an even number, allowing identification of rewarding and unrewarding feeders by researchers (but not bees) during trials where no visible cue was available as the even numbered tab could be orientated to be next to the rewarding feeder. These numbers all had an even and an odd number in their two digits (*e.g*. 12, 47, 34, 29) to prevent bees identifying feeders by the shapes of the numbers. Black writing on a red background would also be difficult for a bee to see due to their visual insensitivity to human-red colours (Davies et al., 2013).

The bulbs of 3ml plastic pipettes (Pastettes, Alphalaboratories, UK) were cut down to create a plastic hood shaped tunnel, 16mm in length, as used by Pearce et al. (2017) and Lawson et al. (2017b). Three of these bulbs were placed over the feeding wells so that the open end of the tunnel faced into the flower centre (figure 6.1). These were taped down at the beginning of testing each day with a fresh section of clear tape (*Scotch* Easy Tear, St. Pauls, USA). This limited the directions that allowed access to feeding wells, meaning that bees could only approach feeding wells from the central region of the flower. To account for small differences between tunnels, tunnels were randomly assigned to each feeder (across all flowers) at the start of each sampling day.

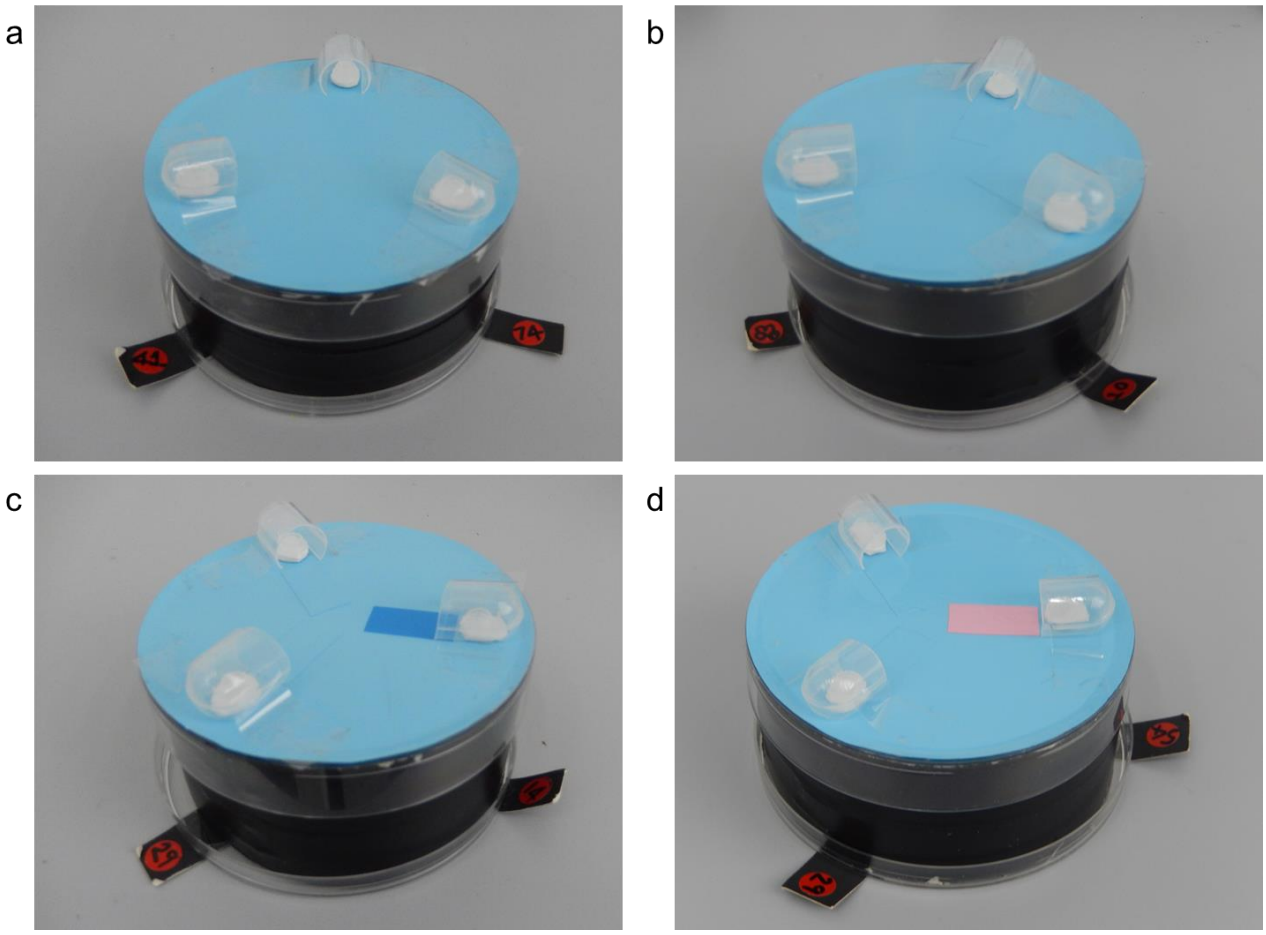


Figure 6.1: *The different appearances of artificial flowers used in our tests, demonstrating each of the colour patterns applied across artificial flower variants.* ***a)*** *a test flower appears with no colour patterns and no control panels, as used in Plain Control, Unimodal Warm and Hot artificial flower variants.* ***b)*** *a test flower with no colour pattern but with control panels, as used in the group 'Panels Control' flower variant. Note the control panels are barely visible.* ***c)*** *a test flower with a blue colour panel, in front of the rewarding feeder, as used in test groups Unimodal and Multimodal Blue flower variants.* ***d)*** *a test flower with a pink colour panel, in front of the rewarding feeder, as used in test groups Unimodal and Multimodal Pink flower variants.*

6.2.2 *Artificial flower patterns*

Colour or temperature patterns were added to the basic construction of the experimental artificial flowers described above. Two types of temperature patterns and two types of visual patterns were used in this experiment.

Colour patterns were created by sticking down a 1cm by 2cm adhesive plastic panel (d-c-fix® adhesive film, Hornschuch group, Weissbach, Germany) in front of feeder tunnels. These panels were placed in front of a feeder to create a solid area of contrasting colour about the reward location. Two types of visual guide panels were used in this experiment: 'Blue panels', which were a darker shade of blue than the flower top (figure 6.1c), and 'Pink panels', which were a pale pink (figure 6.1d). It is possible the very slightly raised platform of the panels may influence bee responses independently of their colouring. So, to help account for such effects, 'Control panels' that were the same shade as the flower top were also used alongside coloured panels and in a separate control testing for such non-colour related effects of panels (figure 6.1b).

The reflectance spectra of the aqua blue plastic and Control, Blue and Pink panels stuck onto aqua blue plastic are presented in figure 6.2. The excitation that these colours would induce on bee colour receptors was calculated for average daylight conditions. The difference between colours to the bee could then be expressed as distance from the aqua blue background in bees' 2D colour hexagon space, their 'hexagon units' as described in Dyer and Chittka (2004a) and Dyer and Chittka (2004c). Control panels showed a distance of 0.01 hexagon units (to 2 d.p.) from background; Pink panels a distance of 0.02 hexagon units from the background, and Blue panels a distance of 0.04 hexagon units. This suggests that to the bees, blue panels show twice as much contrast with the background as Pink panels, Control panels showing even less contrast.

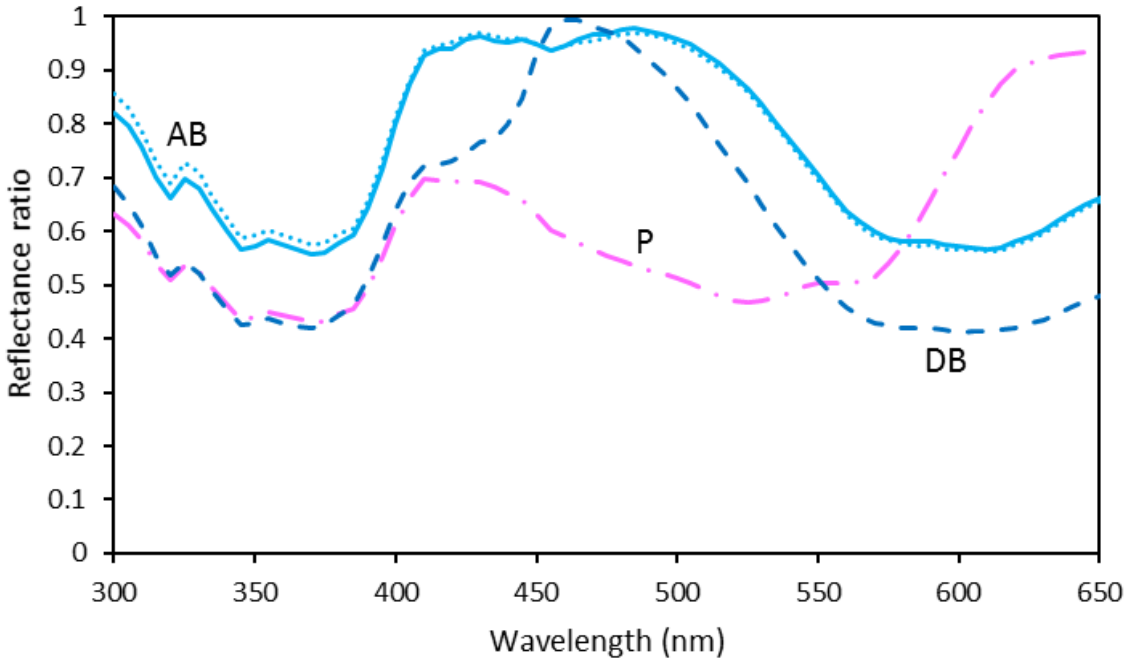


Figure 6.2: *The reflectance spectra of the PVC plastics used in artificial flower construction (measured by Msci student Ed Straw under my supervision). These are measured using an Ocean Optics, Flame-S-UV-VIS miniature spectrometer (Ocean optics, Largo, USA), with a deuterium-halogen tungsten lamp (DH-2000-BAL UV-VIS-NIR, Ocean optics, Largo, USA) as light source and a QR400-7-UV-VIS reflection probe (Ocean optics, Largo, USA). All spectra measurements were taken against a white Spectralon standard (WS-1-SL Ocean optics, Largo, USA). All panels are measured stuck to a sheet of the background plastic. Reflectance is given as a ratio of the Spectralon standard. Colour, labels, and dashing of spectra indicate the plastic colour. Aqua blue lines, labelled AB, indicate the spectra of the aqua blue plastic. The background sheet when dotted, and the Control panel plates when solid. Dark blue dashed line, labelled DB, is the spectra of the dark blue plates. Pink, dash-dot line, labelled P, is the spectra of the pink plates.*

Temperature patterns that corresponded with reward location were generated using heating elements placed on the underside of the flower (as described in chapter 3). Two kinds of temperature patterns were used which will be identified as ‘Hot’ or ‘Warm’ (figure 6.3). The two temperature patterns differed in the level of temperature generated, however both patterns show within flower temperature contrasts comparable to natural flowers (chapter 3; Harrap et al., 2017).

Heating elements for the ‘Hot’ temperature pattern were created by cutting a 2×3cm section of 5mm neoprene foam (Camthorne Industrial Supplies Ltd, Staffordshire, UK) and a further two sheets of 1mm plastic foam were glued to underside of the flower top, just in front of one feeding well. These sheets were stuck so that it faced outwards from the centre of the flower towards a feeder (figure 6.3a). A 13cm length of 0.32mm, 17.87 Ωm^{-1} kanthal resistance wire was bent into an M shape with two 1cm leads on each end. This wire was then covered and stuck down by a pressure sensitive putty (Blu Tack: Bostik, Paris, France) and wire leads attached to a single AA battery cradle with cut free sections of a connector block (Figure 6.3c). When the battery was inserted into the cradle the area above and around the heat sink heated up. This warmed the third of the artificial flower and was hottest in front of the tunnel above the heating elements (Figure 6.3e). Artificial flowers temperature settled at approximately 30°C in front of the ‘Hot’ feeder and 26°C in front of the cooler ones. This resulted in a temperature difference of about 6-5°C across the flower.

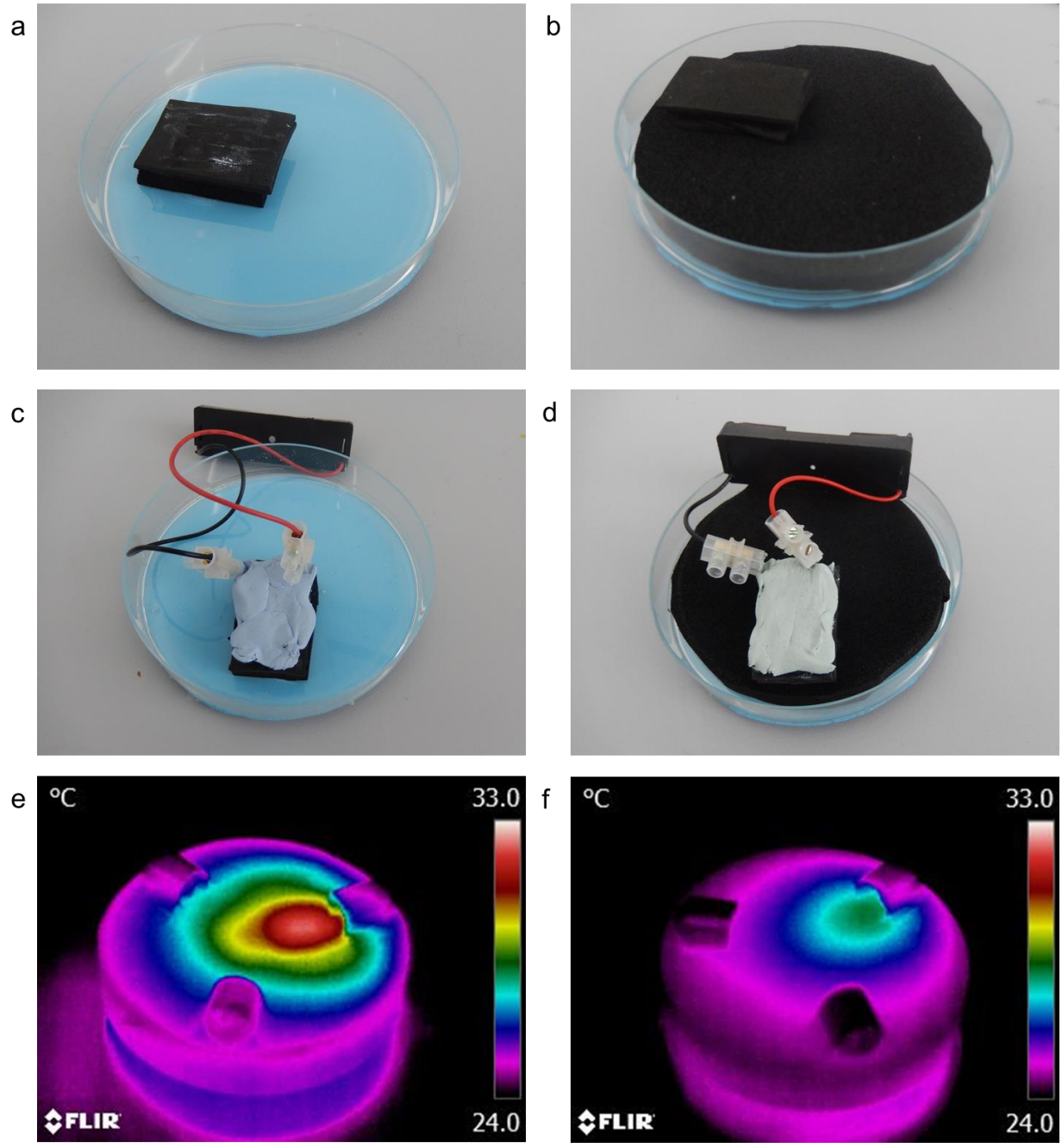


Figure 6.3: *the construction and heating of the temperature patterns in artificial flowers used in our tests.* ***a)*** *An underside view of the heat sinks used to generate a Hot temperature guide.* ***b)*** *An underside view of the heat sinks used to generate a Warm temperature guide.* ***c)*** *Hot guide flowers' undersides with heating elements attached.* ***d)*** *Warm guide flowers' undersides with heating elements attached.* ***e)*** *A thermal image demonstrating the heating that occurs when Hot guide heating elements are turned on.* ***f****) A thermal image demonstrating the heating that occurs when Warm guide heating elements are turned on. The temperature scale in panels* ***e*** *and* ***f*** *is given in the colour scale to the right of each panel in ºC. Note both thermal images have the same temperature scale to allow comparison of the spread and amount of heating each thermal pattern generates.*

‘Warm’ temperature patterns were made in a similar way as ‘Hot’ patterns. Here the whole underside of the flower top was insulated using a disc of 5mm neoprene foam with a 76mm diameter. Four 3×2cm sheets of 1mm neoprene foam were stuck down on the underside of this foam disc, again placed so that it led out from the flower centre towards a tunnel (Figure 6.3b). A 20cm section of resistance wire was bent into an alternating zig-zag shape to fit on the 3x2 cm space, allowing 1cm leads on each end. This was then covered and stuck down with Blu Tack and connected a single AA battery cradle as in the Hot patterns (figure 6.3d). When these flowers heated up, the increased resistance of the wire and insulation meant this heating did not spread as far across the flower surface and was reduced compared to the ‘Hot’ pattern. ‘Warm’ temperature pattern heating elements created a bar of warmer temperature localised to the area in from of the feeder tunnel (figure 6.2f). Artificial flower temperature settled at approximately 28°C in front of the ‘Warm’ feeder and 24°C in front of the cooler ones. This resulted in a temperature difference of approximately 4-5°C across the flowers with the Warm pattern.

Using the guides described above, or combinations of them, eight different variants of experimental artificial flowers were constructed. These included two control, four unimodal, and two multimodal flower variants. These are described in table 6.1.

Table 6.1: *The artificial flower variants, and additionally the experimental test groups, used in experiments. Each flower type is listed and the signals associated with rewarding and nonrewarding feeders are given. Additionally, the number of bees presented with each artificial flower variant during the test phase, alternatively the number in each test group, are given.*

Artificial Flower	Number of bees	Floral patterns
Plain Control	12	Rewarding feeder: No colour or heating Nonrewarding feeders: No colour or heating
Panels Control	12	Rewarding feeder: Control panel; No heating Nonrewarding feeders: Control panel; No heating
Unimodal Pink	12	Rewarding feeder: Pink panel; No heating Nonrewarding feeders: Control panel; No heating
Unimodal Blue	12	Rewarding feeder: Blue panel; No heating Nonrewarding feeders: Control panel; No heating
Unimodal Warm	12	Rewarding feeder: Warm thermal pattern; No colour Nonrewarding feeders: No colour or heating
Unimodal Hot	12	Rewarding feeder: Hot thermal pattern; No colour Nonrewarding feeders: No colour or heating
Multimodal Pink	12	Rewarding feeder: Pink panel; Warm thermal pattern Nonrewarding feeders: Control panel; No heating
Multimodal Blue	12	Rewarding feeder: Blue panel; Warm thermal pattern Nonrewarding feeders: Control panel; No thermal heating

6.2.3 *Bee experiments*

The artificial flowers described in table 6.1 were presented repeatedly to captive bumblebees to investigate bumblebees' guided responses and handling of artificial flowers. Flower naïve bumblebees, *Bombus terrestris audax*, were supplied by Biobest (Westerlo, Belgium) *via* Agralan (Swindon, UK). Unless stated otherwise bee lab conditions and flight arenas in which foraging took place, were as described in chapter 3.

Outside of testing bees were fed sucrose solution daily from PCR racks, gravity feeders and a selection of 'generic' artificial flowers placed within their flight arena. Most generic artificial flowers were constructed from a 44 mm wide specimen jar (Sterilin PS 60ml, with white plastic lids, Thermo Fisher Scientific, Newport UK), or resin disks of a similar size, with single feeding wells stuck to them. At least a week prior to bee trials some of these generic artificial flowers were substituted with flowers to prepare bees for this experiment. These flowers showed one or two of the following: a different size (being either made from a larger specimen jar or a petri dish); multiple feeders; feeders not in the centre of the flower; or tunnels over the feeder (constructed as described above). These 'new' generic artificial flowers allowed bees to get used to feeding from flowers showing aspects of those used in this experiment. However, none of these new generic artificial flowers showed all these aspects together. Furthermore, these new generic artificial flowers never showed feeders at fixed angles about the flower edge, and never showed any visual or temperature patterns, or any colours associated with visual patterns. Additionally, other generic artificial flowers, described in chapter 3, were still present and made up the majority (four out of seven or eight) of artificial flowers presented outside of trials.

Preliminary trails found the experimental artificial flowers were too complicated for bees to learn to use in a single visit. Naïve bumblebees did not land on experimental flowers. Thus, a pre-training phase with 'pre-training artificial flowers' (figure 6.4a) was included before the test itself. A pretraining phase allowed bees to learn how to feed from artificial flowers

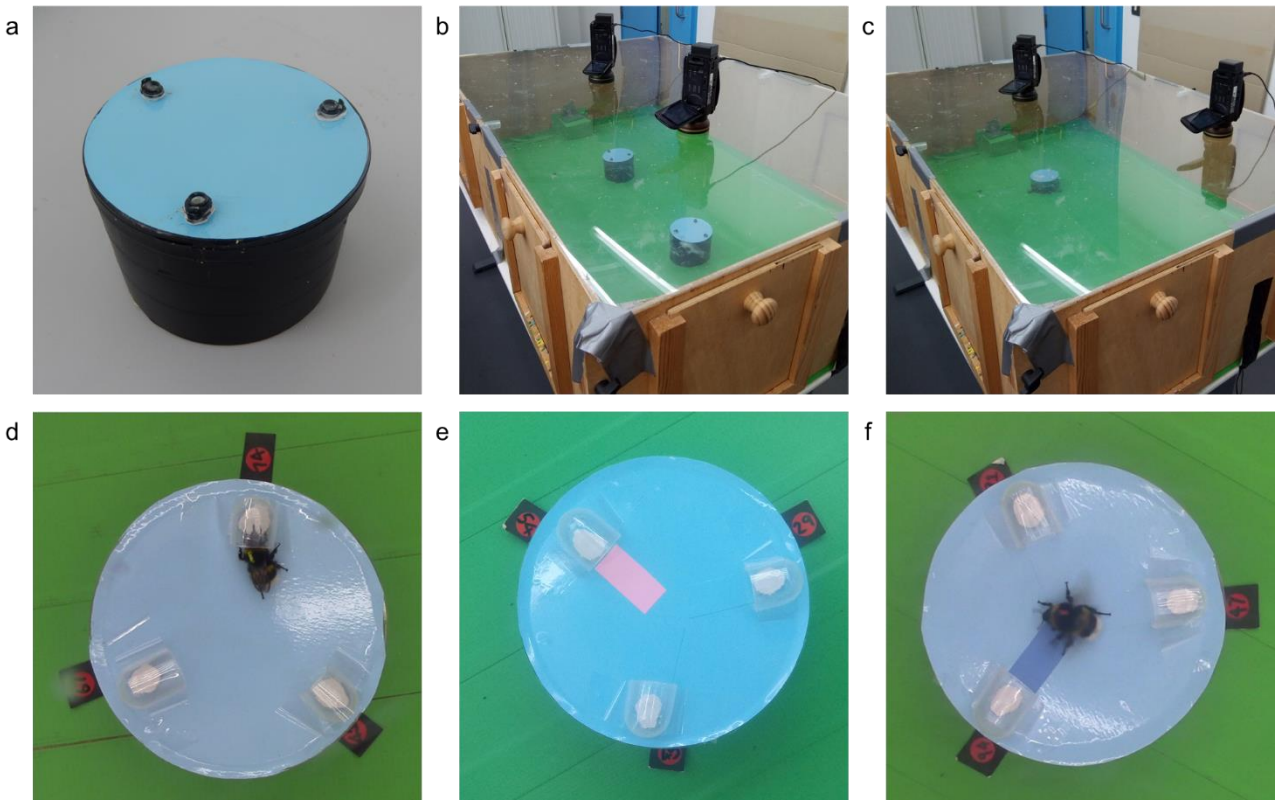


Figure 6.4: *The experimental setup at differing points in the trail process.* ***a)*** *A fully constructed pretraining artificial flower. Note the differences between the visual appearance of the test phase artificial flowers (figure 6.1).* ***b)*** *The area as set up during pretraining. Note that the cameras are still in place.* ***c)*** *The arena at the start of the test phase. Note how the test flower at the start of trials is in a similar location to one of the pretraining flowers.* ***d, e*** *and* ***f)*** *The view from a camera during the test phase for a Unimodal Warm (**d**), Unimodal Pink (**e**) and Unimodal Blue (**f**) test flower, with visiting bees in **d** and **f**. Note how the alignment of the numbered tabs can be used to identify the rewarding feeder on Unimodal Warm flower.*

similar to experimental flowers without gaining direct experience on the experimental flower or patterned signals. The top of these pre-training flowers was a petri dish lid covered with the same aqua blue sticky back plastic as the test flowers. Three feeding wells with foam bottoms were stuck to the top of the artificial flower, as in the test flowers (described above), however here the sides of the Eppendorf tube lids were painted black. This lid was then supported on a 55mm tall card cylinder, wrapped in black electrical tape and taped to the outside of the petri dish lid. Pre-training flowers did not possess tunnels, visual or temperature patterns, a base, or feeder labels.

During pre-training, marked forager bees were released into a foraging arena containing a clean pair of pre-training flowers with a droplet of 30% sucrose solutions in all their feeders. These pre-training flowers were placed in the centre of the foraging arena about 30cm apart from each other in line with the bee's entrance to the arena (figure 6.4b). A camcorder (Legria HF r36; *Canon*, Tokyo, Japan) was placed above each of the pre-training flowers. Each camcorder had a wide-angle lens attachment (XIT pro series 0.43X HD wide-angle lens 52mm, *Xit Group*, Brooklyn, USA), placed facing down to view the artificial flower (figure 6.4b). Though pretraining was not recorded, the camcorder was present in pre-training so bees acclimated to it.

Multiple bees could be released into the flight arena together during pre-training. Bees were allowed to feed freely and return to the nest at will, with feeders being refilled when empty. If a bee completed two foraging bouts feeding on the pre-training flowers (departing and returning to the nest being one bout), it was deemed to have completed the pre-training phase. On a given sampling day, bees that had completed pre-training that day could then be used in the test phase. If a bee completed pre-training but was not used for the test phase that day (for example because other bees took too long to complete testing), it could be used another day but would need to recomplete the pre-training phase. If a bee began the test phase it had to complete it in a single day. Bees that began the test phase were never reused in this experiment, even if they did not complete the test phase.

6.2.4 *Test phase*

Following completion of the pre-training phase, bees were assigned artificial flower variants as described in table 6.1. Individual bees were be presented one variant of experimental artificial flower throughout the test phase. Consequently, the variant of artificial flower that a bee was presented with also describes its experimental test group. Throughout the experiment, temperature patterns were monitored by a FLIR e60bx thermal camera (FLIR

systems, Inc., Wilsonville, USA). Before the bee began a foraging bout the rewarding and unrewarding feeders (described in table 6.1) of 8 experimental flowers were filled with 25µl of 30% sucrose solution or water respectively. Volumes of water and sucrose were measured using an electronic pipette (HandyStep® Electronic, Wartheim, Germany).

During the test phase, bees that had completed the pre-training phase that day were allowed to make successive foraging bouts on their assigned test flowers. Each test bee foraged alone in the arena during the test phase, other bees would not be released into the arena during testing. When a bee began a foraging bout a single artificial flower of the variant assigned to that bee was present in the arena. On the first foraging bout, this first artificial flower was placed in the same position as one of the pre-training flowers had been (figure 6.4c). The bee was allowed to land and forage on the artificial flower. Once a bee had extended its proboscis into any of the test flower's feeding wells (recorded as a 'drinking event'), a fresh artificial flower was placed in the arena at least 15cm away from the bee. If the bee had drunk from any of the feeding wells on a flower, that flower was removed from the arena after the bee had departed.

A visit began when bees first made physical contact with artificial flowers. As artificial flowers were quite large, bees often flew from one part of the flower to another when searching. Thus, classing departure from the flower simply as the moment a bee broke contact with a flower after landing would not be representative of the bee's searching effort and would often result in many aborted landings occurring before the first feeding. For this reason, a bee was classed as departing if it broke contact with any part of the flower, then either flew away from the flower and did not return within 5 seconds, or flew over 30cm away from the flower, or landed on another. These criteria allowed for bees to fly from one part of the flower or hover about the flower after landing without being classed as departing when they were still apparently searching the flower. Additionally, these criteria allowed for bees to climb about and search the lower parts and sides of the flower without being classed as departing.

This process of placing fresh artificial flowers in the arena as bees drank from them, and removing used flowers upon departure, continued until the bee returned to the nestbox on its own accord or had fed from all eight experimental artificial flowers in a single foraging bout. Once a bee had departed from the eighth flower in a bout, the eighth flower was removed and no more flowers were placed in the arena in that bout. The empty arena typically caused the bee to return to the nestbox. Bees reluctant to return to the nestbox were captured in a jar and encouraged to return home.

Artificial flowers were reused in subsequent foraging bouts. Once a bee had completed a foraging bout, all flowers were removed from the flight arena. Any water and sucrose solution left in the feeding wells of flowers visited in the previous bout were emptied using paper towel, and wiped down with ethanol, removing scent marks that may conflate bee decisions (Stout and Goulson, 2001; Pearce et al., 2017). The feeding wells of these flowers were then refilled with sucrose solution and water as appropriate. The cycle of removal and replenishing of water and sucrose between bouts reduced the chance of differences in the temperature of the feeding well contents developing. Flower temperature was checked with the thermal camera and any flower that had overheated or ceased to produce a temperature pattern, due to a fault was replaced. After ethanol washing any thermal signals were allowed to re-settle before re-use, as ethanol evaporation cools flowers.

Before the bee was let back into the arena an artificial flower was placed inside. Bee foraging was then allowed to continue as described above. The first artificial flower placed in the arena in bouts after the initial foraging bout were placed anywhere in the arena, rather than the same positions of pre-training flowers. Individual bees were allowed to carry out successive bouts of foraging until the bee completed the bout where the number of flowers visited across all bouts was 30 or more. At this point the bee was deemed to have completed the test phase.

Occasionally bees would be reluctant to visit the flower in the arena. To encourage the bee, another artificial flower would be placed into the arena. In this scenario if a bee drank

from and departed from either flower, that flower would be removed but a new flower would not be placed inside the arena, as one was already present. Otherwise the experiment carried on as normal. If there were already two artificial flowers within the arena and bees still seemed reluctant to visit an artificial flower, an artificial flower would be moved to a new position. Moving flowers would not be carried out if the bee had already visited a flower but not fed. At any one time there were never more than two artificial flowers in the arena.

Simultaneously to the bee foraging (described above) video cameras were used to record bee flower visits in the test phase (figure 6.4c). This recorded behaviour would be used to collect video data for analysis of flower handling. During the whole test phase the same camcorders as present in the pre-training phase were placed on the Perspex roof of the flight arena, facing down (figure 6.4). Whenever an artificial flower was placed in the arena, either at the start or during a foraging bout, a camera that was not already viewing a flower would be moved into position above it (figure 6.4). This was done immediately after a flower was placed in the arena. When a flower was removed during the foraging bouts, this freed up a camera to be moved to a new position. Viewing the artificial flowers from above meant that the entire flower top and at least two of the numbered tags at the bottom of the flower were visible. This allowed the entire of the bee's foraging activity on each flower to be seen (figure 6.4).

96 bees from 14 colonies completed the test phase, with 12 in each of the 8 test groups (table 6.1). Bee lab trials and video recording was carried out by myself (82 bees) and a second experimenter, an MSci student, Ed Straw under my supervision (14 bees). Bees completed the test phase in 31.59 ± 0.01 visits (mean ± SEM). However, due to camera recording error the visits after focal visit 29 for bee 5 (in Plain Control group) and 84 (in Multimodal Pink group) were not recorded, similarly visit 30 (of 33) of bee 81 (in Multimodal Pink group) was not recorded due to an error in camera placement. This meant the number of filmed visits for each bee ranged from 29 to 35.

6.2.5 *Data processing*

Data on flower handling was collected by reviewing the recordings of a bee's behaviour during visits to experimental artificial flowers. For this processing, aspects of the flower visit and bee behaviours were defined as in table 6.2. Flower handling data was only collected with reference to the 'focal flower visits' as defined in table 6.2. This has the result that visits prior to the focal visit were the bee does not feed, and return visits to the flower after the focal visit (in instances where the flower could not be removed in time) were ignored.

During each focal visit, I recorded whether bees found rewarding feeders on the first, second, or third feeder they drank from (ignoring revisits to drink again from the same feeder), or whether they departed after having failed to feed on the rewarding feeder. On focal flower visits where rewards were successfully found, the time between the start of the focal visit and the start of the first drinking event from the rewarding feeder (as defined by table 6.2) was measured with a stopwatch while replaying video in real time, as done in Lawson et al. (2017b). This time is referred to as the 'reward search time'. While video data was collected by two experimenters, video footage was processed solely by me, giving consistent reaction times, interpretation of definitions of these behaviours and focal visits.

Table 6.2: *The definitions of events and bee behaviours used in assessment of video footage of bee behaviour on experimental artificial flowers.*

Behaviour	Definition
Visit	The time during which the bee is on the flower. Begins with a landing and ends with departure.
Landing	The time at which a bee makes first contact with top flower or the feeder tunnels at the start of a visit.
Departure	The time where a bee leaves a flower. Counted as the point where a bee breaks contact with the flower before it meets any of the following departure criteria: flew away from the flower and did not return within 5 seconds; flew over 30cm away from the flower; or flew away from the flower and landed on another flower.
Focal Visit	The flower visit during which a bee first drinks from any feeding well on the flower. Or the last flower visit a bee makes on flower where feeding wells are never drank from.
Abort Visit	Flower visits where the bee departs before drinking, that occur before the focal visit.
Drinking	When the bee is consuming the contents of a feeding well. Bees were considered to start a drinking event when they extended their proboscis into the well. A bee finishes a drinking event when the bee retracted its proboscis and left the tunnel of the feeding well.

For each bee the proportion of visits where the bee failed to find rewards (failed visits), and the proportion of visits were the rewarding feeder was the first bees chose to drink from (first-feeder visits), was calculated for the previous 10 focal flower visits at 10, 20 and 30 focal visits. In instances were camera errors meant focal visit 30 was not recorded, noted above, the proportions of failed and first-feeder visits at 30 focal visits for these bees were calculated using the previous 9 visits recorded, but were otherwise treated the same. For these proportions, focal visits made after visit 30 were ignored for analyses. All data on reward search times was used in analyses, including recorded focal visits that occurred after 30 visits. These three flower handling metrics identify several aspects of pollinator responses to floral guides. Guides have been previously identified to reduce reward search time (Leonard and Papaj, 2011; Goyret and Kelber, 2011; Lawson et al., 2017b) and the proportion of failed visits made by the pollinator (Goyret et al., 2007; Hansen et al., 2011). Guides have also been identified to indicate reward locations and draw pollinators to them, which would lead to

increased incidence of first-feeder visits (Johnson and Dafni, 1998; Lunau et al., 2006; Goodale et al., 2014) and allow learning of reward location within a flower (Leonard and Papaj, 2011), which would lead to increases the proportion of first-feeder visits and likely other metrics with increased focal visit number.

6.2.6 *Statistical analysis*

All data were analysed using R version 3.4.1 (R Development Core Team, 2017). proportions of failed visits and first-feeder visits underwent the arcsine square root transformation, as this data was bound between 0 and 1. How proportions of failed visits, proportions of first-feeder visits and reward search time were altered by the floral patterns presented to each bee, and how these changed with experience (the number of focal visits) were analysed independently using a generalised linear models and AIC model simplification techniques. These techniques are similar to those applied to learning behaviours in chapters 3 and 5. This involves a sequential process of paired comparisons between a standing 'best model' and a simpler model fitted to the data using AIC. Simpler models were constructed by removal of parameters from the standing best model (forcing parameters to equal zero). If removal of parameters resulted in a significant increase in AIC, based on Richards (2008), the standing best (more complex) model would remain the best for the next comparison. If otherwise, simpler models would become the standing best model for the next comparison. The best fitting model being the one remaining at the end of the sequence of comparisons. Each metric was assessed independently in turn, through the same sequence laid out below.

Before the effect of patterns were analysed, bee responses in the Panels Control and Plain Control flower were compared. This allowed us to assess whether these groups could be treated as equivalent, greatly simplifying later models. The full model, before any simplification applied, used to compare two test groups responses is as follows

$$y_{nx} = i_a + (v * l_a) + G\big(i_b + (v * l_b)\big) + b_n + (v * r_n). \quad (6.1)$$

Here y_{nx} is the bee handling metric in question (the arcsine transformed failure rate, arcsine transformed first-feeder rate or reward search time in seconds) of bee n on focal visit number x, where x is the focal visit number in bee n's sequence of visits. v relates to focal visit x by the following. For reward search time

$$v = \ln x, \tag{6.2}$$

for failure and first feeder rates

$$v = \ln(x + 1 - 10). \tag{6.3}$$

The transformations shown in equations 6.2 and 6.3 allow the model to show a logarithmic relationship. In the case of equation 6.3 the transformation also allows model intercepts to begin at focal visit 10, as is the case with the data. Note that for each bee x has values of 10, 20 or 30 for failure and first-feeder rates, and 1 to at least 29 for reward search time. i_a is the intercept, the initial value of the metric in question, for bees in test group a (a being either of the test groups, initially either Panel Control or Plain Control). l_a represents the change in y_{nx} with experience, increased values of v and thus also x, this indicated bee learning speed. Parameter i_b represents the change in model intercept, relative to i_b, for bees in test group b (b being the other test group in this comparison). Parameter l_b represents the change in learning speed, relative to l_a, for bees in group b. These modifications are applied to bees in test group b by operator G,

$$G = \begin{cases} 0, bee\ is\ in\ test\ group\ a \\ 1, bee\ is\ in\ test\ group\ b \end{cases}. \tag{6.4}$$

Random factors are included in this model with b_n and r_n, that represent the change in model intercept and learning speed of bee n, relative to i_a and l_a. When comparing test groups, the initial standing best model would be as in equation 6.1. This would be compared with a model where l_b was removed, removing different learning speeds between the groups; interaction

between experience and test group. If this had no effect on AIC the new standing best model (without l_b) would be compared to one where differences in intercepts were removed, by removing i_b. A significant effect of either parameter implied the groups differ.

A different model was used to compare bee responses to patterns. This model allowed common effects of patterns, particularly those that make up multimodal patterns, to be assessed across test groups. This accounts for a common response to the same pattern across different test groups. The full model applied to failure rate, first-feeder rate and reward search time before any model simplification is as follows

$$y_{nx} = i + (v \cdot l) + b_n + (v \cdot r_n) + P\left(i_p + \left(v \cdot l_p\right)\right) + B\left(i_b + (v \cdot l_b)\right) + W\left(i_w + (v \cdot l_w)\right) + H\left(i_h + (v \cdot l_h)\right) + P \cdot W\left(i_{pw} + \left(v \cdot l_{pw}\right)\right) + B \cdot W\left(i_{bw} + (v \cdot l_{bw})\right).$$

(6.5)

Equation 6.5 does not include any effects of panels on bee responses (as none were detected in the above test group comparisons). Here parameters y_{nx} and v work as described above. i is the model intercept, the initial value of y_{nx} when $v = 0$, for bees presented with no patterns. l represents the change in y_{nx} relative to i with increased values of v for bees presented with no patterns. The learning speed of bees is thus represented with l. Random factors b_n and r_n work as above but now refer to changes in model intercept and learning speed in bee n relative to i and l. i and l are modified when bees are presented with flowers which have floral patterns. This is controlled through operators P,B, W and H, where

$$P = \begin{cases} 0, & flower\ presented\ to\ bee\ n\ does\ not\ have\ a\ Pink\ colour\ pattern \\ 1, & flower\ presented\ to\ bee\ n\ does\ have\ a\ Pink\ colour\ pattern \end{cases} \quad (6.6)$$

$$B = \begin{cases} 0, & flower\ presented\ to\ bee\ n\ does\ not\ have\ a\ Blue\ colour\ pattern \\ 1, & flower\ presented\ to\ bee\ n\ does\ have\ a\ Blue\ colour\ pattern \end{cases} \quad (6.7)$$

$$W = \begin{cases} 0, flower\ presented\ to\ bee\ n\ does\ not\ have\ a\ Warm\ thermal\ pattern \\ 1, \quad flower\ presented\ to\ bee\ n\ does\ have\ a\ Warm\ thermal\ pattern \end{cases} \quad (6.8)$$

$$H = \begin{cases} 0, flower\ presented\ to\ bee\ n\ does\ not\ have\ a\ Hot\ thermal\ pattern \\ 1, \quad flower\ presented\ to\ bee\ n\ does\ have\ a\ Hot\ thermal\ pattern \end{cases} . \quad (6.9)$$

Pattern induced changes in intercept and learning speed relative to i and l are given by: i_p and l_p when bees are presented with Pink colour patterns; i_b and l_b when bees are presented with Blue colour patterns; i_w and l_w when bees are presented with Warm temperature patterns; and i_h and l_h when bees are presented with Hot temperature patterns. When bees are presented with a combination of patterns as in the multimodal groups the model will apply both patterns' induced changes (see equation 6.5) but will also apply further specific multimodal changes. The parameters i_{pw} and l_{pw} represent the change in model intercept and learning relative to i and l for bees presented with Warm and Pink patterns (*i.e*. those in the Multimodal Pink group). Likewise, parameters i_{bw} and l_{bw} represent the change in model intercept and learning relative to i and l for bees presented with Warm and Blue patterns (i.e. those in the Multimodal Blue group). With these multimodal interaction effects, the model may magnify bee responses to multimodal flowers if positive, alternatively these parameters can reduce or remove the additive benefits of additional patterns if negative. If these values equal zero the model allows the effects of one pattern to be added to the other with no further modification. Note that due to the arcsine transformations applied to proportions these effects will not be truly additive, as they will be reduced as bees approach values of 1 or 0.

The model simplification process applied to the model in equation 6.5 is as follows. The initial standing best model was that as presented in equation 6.5, this would be compared to a model where individual bee learning speeds (r_n) were removed. The standing best model would then be compared to one where parameter l was removed. Testing the effects of l first was necessary as it established what responses control group bees gave. This thus ensured subsequent comparisons were made against bees in the control group, or groups responding the same way as in the control group. Next, the effect of removal of multimodal interaction

effects of bees presented with the Pink Multimodal flowers, removing parameters i_{pw} and l_{pw}. Then the same effects on bees presented with Blue multimodal flowers, removing parameters i_{bw} and l_{bw} was tested. After this each pattern's individual effects would be investigated. Whether each pattern alters the learning speed of bees would be tested by comparing the standing best model to one where that patterns' modifier of l (l_p, l_b, l_w, and l_h respectively) would be removed. The standing pattern after that comparison would be used to investigate whether each pattern alters the initial response, intercept, of bees by comparing the best model to one where that patterns' modifier of i (i_p, i_b, i_w, and i_h respectively) would be removed. These two comparisons would be carried out for each pattern in the following order: Pink, Blue, Warm and Hot. Model intercept i and random individual effect on bee intercepts (b_n) were not assessed and remained in the model. Following this model simplification, if any patterns making them up were found to affected handling (i.e. were in the best fitting model), the response of bees in the Multimodal test groups would be compared with that of bees who were presented with the unimodal components. This would be done using the test group comparison model (equation 6.1) described above.

6.3 Results

6.3.1 *Proportion of failed visits*

Proportions of failed visits by bees across test groups are shown in figure 6.5. Comparisons of the control groups found both groups showed similar relationships with experience and similar initial proportions of failed visits (*Interaction term*: standing best model AIC -5.91, simpler model AIC -6.70, ΔAIC = 0.78, Δdeviance = 1.21, df = 1, $p = 0.270$, *Intercepts*: standing best model AIC -6.70, simpler model AIC -8.16, ΔAIC = 0.78, Δdeviance = 0.53, df = 1, $p = 0.464$).

The results of the model selection process for proportions of failed visits across all test groups are summarised in table 6.3. Bees showed a consistent change in proportions of failed visits with experience (figure 6.5, figure 6.6) regardless of the patterns presented to them. Bees presented with Blue or Warm temperature patterns had lower intercepts compared to other bees. Hot or Pink patterns had no significant effect on visit failure. No interaction effects between Blue and Warm patterns, or Pink and Warm patterns were found. This meant that the best fitting model favoured a combined reduction in proportion of failed visits for bees presented with both Warm and Blue patterns (figure 6.6). However, comparisons of test groups found proportions of failed visits of Multimodal Blue test group bees to be comparable to both Unimodal Warm (*Interaction term*: standing best model AIC 9.28, simpler model AIC 7.37, ΔAIC = 1.92, Δdeviance = 0.08, df = 1, $p = 0.771$, *Intercepts*: standing best model AIC 7.37, simpler model AIC 8.71, ΔAIC = 1.35, Δdeviance = 3.35, df = 1, $p = 0.068$) and Unimodal Blue test group bees (*Interaction term*: standing best model AIC 10.8, simpler model AIC 9.91, ΔAIC = 0.87, Δdeviance = 1.13, df = 1, $p = 0.288$, *Intercepts*: standing best model AIC 9.91, simpler model AIC 10.6, ΔAIC = 0.70, Δdeviance = 2.70, df = 1, $p = 0.101$). Similarly, bees in the Multimodal Pink group did not differ from those in the Unimodal Warm (*Interaction term*: standing best model AIC 9.66, simpler model AIC 8.14, ΔAIC = 1.53, Δdeviance = 0.47, df = 1, $p = 0.493$, *Intercepts*: standing best model AIC 8.14, simpler model AIC 7.83, ΔAIC = 0.30, Δdeviance = 1.70, df = 1, $p = 0.193$).

Table 6.3: *The results of model selection for bee proportions of failed visits. Comparisons of standing best models and a simpler version where a focal parameter is removed are given for each effect tested in our model selection process. AIC is given for both models (note how standing best model AIC matches one of the previous model AICs. Also given is an assessment of model fit, Δdeviance=Δdev. A verdict on each comparison is given: Asterisks '∗' indicate parameters where models including them have lower AIC, based on Richards (2008), and are thus included in best model. Note that the AIC criteria of Richards (2008) require a ΔAIC of at least 6 for a more complex models to be favoured. More complex models may still have better fit, but be comparable in terms of AIC. Our model inclusion was based on AIC. The p values of parameters where inclusion significantly improved fit but not AIC sufficiently are labelled with a '†'.*

Tested effect		Standing best model AIC	Simpler model AIC	ΔAIC	Δdev.	df	*p*	Verdict	Value
Model Intercept	i								0.70
Individual learning speeds	r_n	-15.88	-19.27	3.39	0.61	2	0.737		
Background learning in Controls	l	-19.27	-3.88	15.40	1740	1	<0.01	∗	-0.09
Pink Multimodal Interaction Effects	i_{pw} l_{pw}	-19.27	-22.56	3.29	0.71	2	0.701		
Blue Multimodal Interaction Effects	i_{bw} l_{bw}	-22.56	-22.99	0.42	3.57	2	0.167		
Altered learning of Pink Patterns	l_p	-22.99	-24.52	1.53	0.47	1	0.493		
Altered intercepts of Pink Patterns	i_p	-24.52	-25.07	0.55	1.45	1	0.229		
Altered learning of Blue Patterns	l_b	-25.07	-25.04	0.03	2.03	1	0.154		
Altered intercepts of Blue Patterns	i_b	-25.04	0.53	25.57	27.57	1	<0.01	∗	-0.19
Altered learning of Warm Patterns	l_w	-25.04	-26.98	1.94	0.06	1	0.811		
Altered intercepts of Warm Patterns	i_w	-26.98	-5.29	21.69	23.70	1	<0.01	∗	-0.15
Altered learning of Hot Patterns	l_h	-26.98	-27.66	0.68	1.32	1	0.251		
Altered intercepts of Hot Patterns	i_h	-27.66	-23.25	4.42	6.42	1	0.011†		

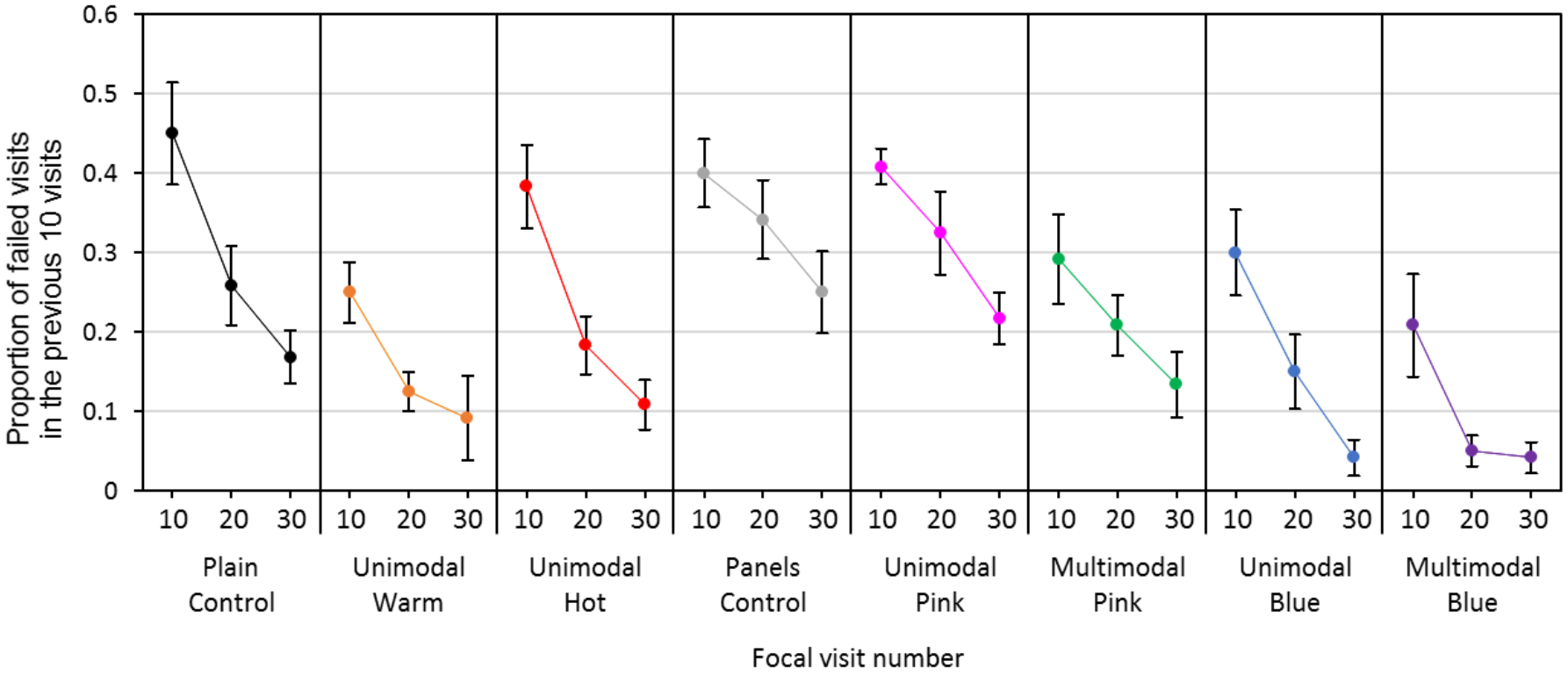


Figure 6.5: *The relationship between incidence of failed visits and experience (focal visit number) across bees that foraged on different artificial flower variants, different test groups. Plotted are mean proportions of failed visits over the previous ten visits at ten, twenty and thirty focal flower visits over each test group. Error bars indicate ± one standard error of the mean.*

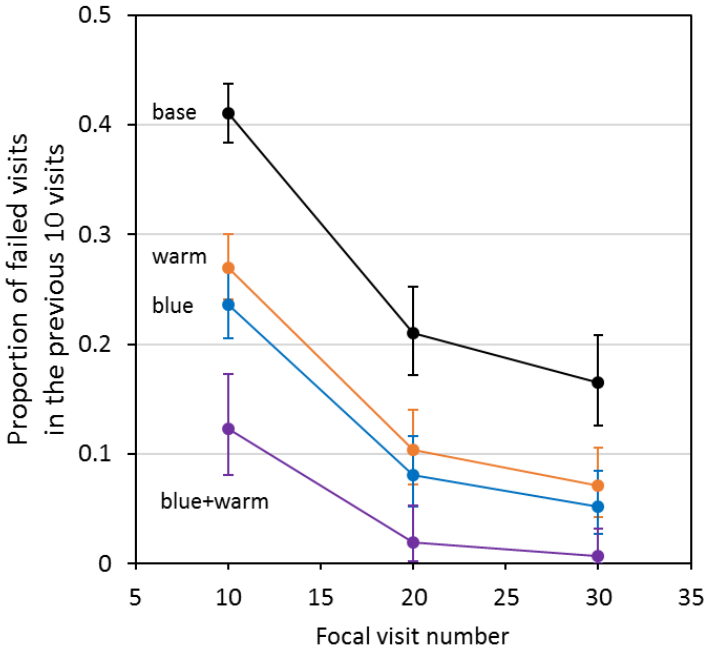


Figure 6.6: *A summary of the best fitting model for proportions of failed visits for bees foraging across all flower test groups, for comparison against figure 6.5. Points plotted are the mean proportions of failed visits for bees presented with different pattern or pattern combinations, as predicted by the best fitting model. Error bars indicate ± one standard error of the model mean estimates. Colours and labels indicate the patterns presented to bees: 'blue+warm' and purple colour, bees presented with both Warm temperature and Blue colour patterns together; 'blue' and blue colour, indicates bees presented with blue colour patterns; 'warm' and orange colour, bees presented with warm temperature patterns; 'base' and black colour, bees not presented with the patterns (control groups) or patterns not indicated by any other line, thus represents bees whose responses were indistinguishable from control bees.*

6.3.2 *Proportion of first-first feeder visits*

Proportions of first-feeder visits of bees across test groups are given in figure 6.7. Comparisons of the control groups found both groups showed similar relationships with experience and similar overall proportions of first-feeder visits (*Interaction term*: separate group model AIC -13.29 *vs.* equivalent group AIC -14.23, ΔAIC = 0.94, Δdeviance = 1.06, df = 1, $p = 0.304$, *Intercepts*: separate group model AIC -14.23 *vs.* equivalent group AIC -16.18, ΔAIC = 1.95, Δdeviance = 0.05, df = 1, $p = 0.827$).

The results of the model selection process for proportions of first-feeder visits across test groups are summarised in table 6.4. The best fitting model found bees presented with Blue patterns showed a higher intercept and improved proportions of first-feeder visits with experience. Bees presented with Hot or Warm temperature patterns increased in proportions of first-feeder visits with experience but showed no altered intercepts (figure 6.7, figure 6.8). Pink patterns had no effect on bees. Bees not presented with Blue, Hot or Warm patterns appeared to maintain a constant proportion of first-feeder visits, comparable with random choice of which feeder to feed from first. No interaction effects between Blue and Warm patterns, or Pink and Warm patterns were found. As Warm patterns had no influence on intercepts this meant bees presented with Blue and Warm patterns had similar intercepts to bees presented with Blue patterns alone, but with experience bees presented with multimodal patterns showed a greater incidence of first-feeder visits. Comparisons between test group proportions of first-feeder visits found models that separated Multimodal blue group from the Unimodal Warm group had comparable AIC but better fit then models that did not (*Interaction term*: separate group model AIC -8.12 *vs.* equivalent group AIC -5.50, ΔAIC = 2.63, Δdeviance = 4.63, df = 1, $p = 0.032$, *Intercepts*: separate group model AIC -5.50 *vs.* equivalent group AIC -0.28, ΔAIC = 5.22, Δdeviance = 7.22, df = 1, $p = 0.007$). Bee first-feeder visits did not differ between the Multimodal blue group from the Unimodal Blue group (*Interaction term*: separate group model AIC -7.76 *vs.* equivalent group AIC -9.37, ΔAIC = 1.62, Δdeviance = 0.38, df = 1, $p = 0.536$, *Intercepts*: separate group model AIC -9.37 *vs.* equivalent group AIC -10.08,

ΔAIC = 0.71, Δdeviance = 1.29, df = 1, p = 0.255). Unimodal Warm group bees did not differ when compared directly with Multimodal Pink bees (*Interaction term*: separate group model AIC -6.03 *vs.* equivalent group AIC -7.89, ΔAIC = 1.86, Δdeviance = 0.14, df = 1, p = 0.706, *Intercepts*: separate group model AIC -7.88 *vs.* equivalent group AIC -9.88, ΔAIC = 2.00, Δdeviance = 0.01, df = 1, p = 0.932).

Table 6.4: *The results of model selection for proportions of first-feeder visits. Comparisons of standing best models and a simpler version were parameters are removed are given in for each effect tested in our model selection process. AIC is given for both models (note how standing best model AIC matches one of the previous model AICs. Also given is an assessment of model fit, Δdeviance=Δdev. A verdict on each comparison is given: Asterisks '∗' indicate parameters where models including them have lower AIC, based on Richards (2008), and are thus included in best model. For fixed effect parameters that are in best model their value in the best model is given, with reference to equation 6.5.*

Tested effect		Standing best model AIC	Simpler model AIC	ΔAIC	Δdev.	df	*p*	Verdict	Value
Model Intercept	i								0.67
Individual learning speeds	r_n	-86.15	-76.64	9.51	13.51	2	<0.01	∗	-
Background learning in Controls	l	-86.15	-88.10	1.95	0.05	1	0.830		
Pink Multimodal Interaction Effects	i_{pw} l_{pw}	-88.10	-91.02	2.91	1.09	2	0.581		
Blue Multimodal Interaction Effects	i_{bw} l_{bw}	-91.02	-93.33	2.31	1.69	2	0.429		
Altered learning of Pink Patterns	l_p	-93.33	-94.55	1.23	0.77	1	0.379		
Altered intercepts of Pink Patterns	i_p	-94.55	-96.32	1.76	0.24	1	0.626		
Altered learning of Blue Patterns	l_b	-96.32	-69.72	26.59	28.59	1	<0.01	∗	0.10
Altered intercepts of Blue Patterns	i_b	-96.32	-88.59	7.72	9.72	1	<0.01	∗	0.14
Altered learning of Warm Patterns	l_w	-96.32	-87.35	8.97	10.97	1	<0.01	∗	0.07
Altered intercepts of Warm Patterns	i_w	-96.32	-97.01	0.70	1.31	1	0.253		
Altered learning of Hot Patterns	l_h	-97.01	-90.07	6.94	8.94	1	<0.01	∗	0.06
Altered intercepts of Hot Patterns	i_h	97.01	97.75	0.74	1.26	1	0.261		

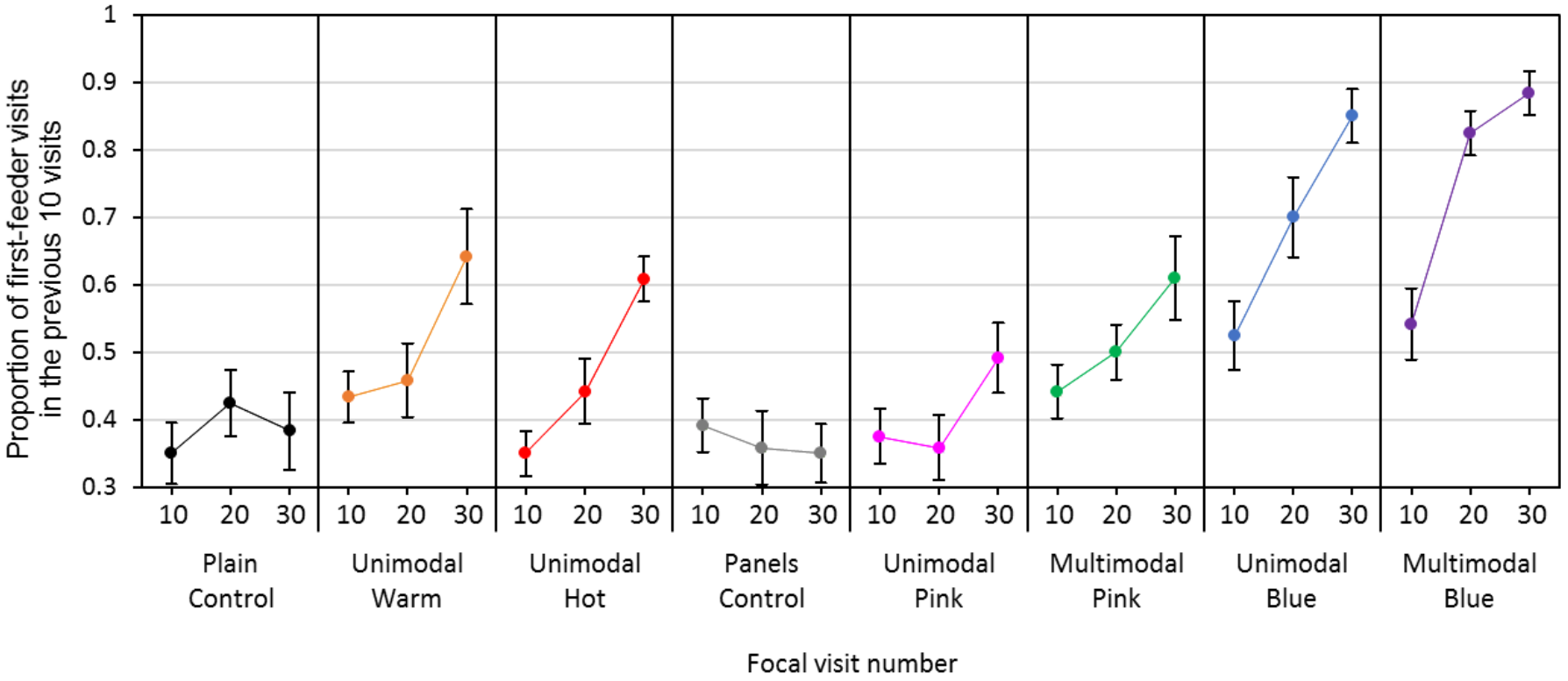


Figure 6.7: *The relationship between incidence of first-feeding visits and experience (focal visit number) across bees that foraged on different artificial flower variants, different test groups. Plotted are mean proportion of first-feeder visits over the previous ten visits at ten, twenty and thirty focal flower visits for each test group. Error bars indicate ± one standard error of the mean.*

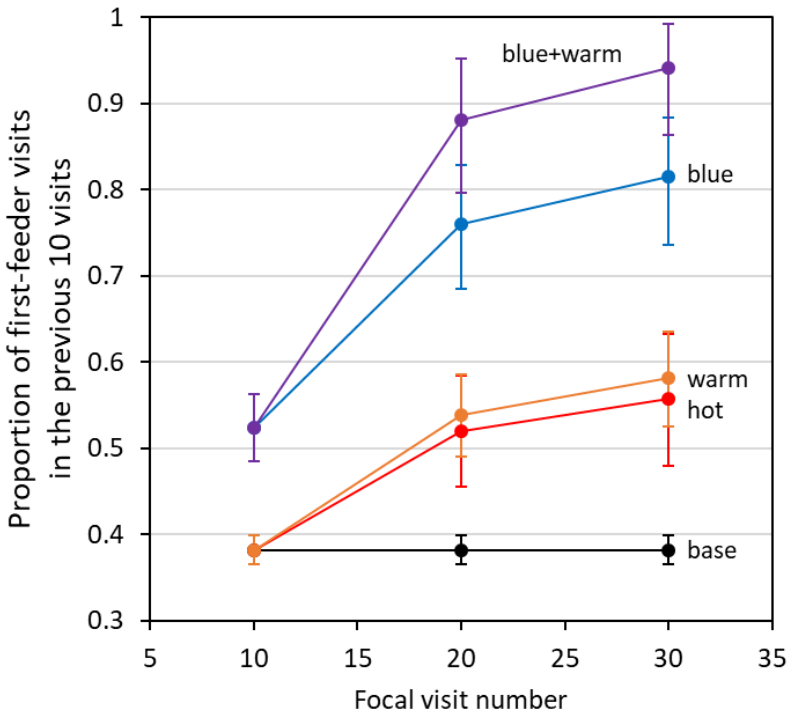


Figure 6.8: *A summary of the best fitting model for proportions of first-feeder visits for bees foraging across all flower test groups, for comparison against figure 6.7. Points plotted are the mean proportions of first-feeder visits for bees presented with different pattern or pattern combinations, as predicted by the best fitting model. Error bars indicate ± one standard error of the model mean estimates. Colours and labels indicate the patterns presented to bees: 'blue+warm' and purple colour, bees presented with both Warm temperature and Blue colour patterns together; 'blue' and blue colour, indicates bees presented with blue colour patterns; 'warm' and orange colour, bees presented with warm temperature patterns; 'hot' and red colour, bees presented with Hot temperature patterns; 'base' and black colour, bees not presented with the patterns (control groups) or patterns not indicated by any other line, thus represents bees whose responses were indistinguishable from control bees.*

6.3.3 *Reward Search Time*

Reward search times across test groups are summarised in figure 6.9. Comparisons of the control groups found both groups showed similar relationships with experience and similar overall first-feeder rates (*Interaction term*: standing best model AIC 3431.5 simpler model AIC 3432.2, ΔAIC = 0.7, Δdeviance = 2.72, df = 1, $p = 0.099$. *Intercepts*: standing best model AIC 3432.2 simpler model AIC AIC 3430.5, ΔAIC = 1.7, Δdeviance = 0.30, df = 1, $p = 0.585$).

The model selection process is summarised in table 6.5. The best fitting model found bees presented with Blue patterns showed reduced reward search times (figure 6.9, figure 6.10). No other effects of patterns or interactions between patterns (multimodal effects) were found. When reward search times of bees in the Multimodal Blue group and Unimodal Blue group were compared they were comparable based on AIC (*Interaction term*: standing best model AIC 3477.6 simpler model AIC 3475.7, ΔAIC = 1.9, Δdeviance = 0.06, df = 1, $p = 0.802$. *Intercepts*: standing best model AIC 3475.7 simpler model AIC AIC 3475.3, ΔAIC = 0.4, Δdeviance = 1.65, df = 1, $p = 0.200$).

Table 6.5: *The results of model selection for bee reward search times rates. Comparisons of standing best models and a simpler version were parameters are removed are given in for each effect tested in our model selection process. AIC is given for both models (note how standing best model AIC matches one of the previous model AICs. Also given is an assessment of model fit, Δdeviance=Δdev. A verdict on each comparison is given: Asterisks '∗' indicate parameters where models including them have lower AIC, based on Richards (2008), and are thus included in best model. Note that the AIC criteria of Richards (2008) require a ΔAIC of at least 6 for a more complex models to be favoured. More complex models may still have better fit, but be comparable in terms of AIC. Our model inclusion was based on AIC. The p values of parameters where inclusion significantly improved fit but not AIC sufficiently are labelled with a '†'.*

Tested effect		Standing best model AIC	Simpler model AIC	ΔAIC	Δdev.	df	*p*	Verdict	Value
Model Intercept	i								9.97
Individual learning speeds	r_n	14241	14291	50	54.06	2	<0.01	∗	-
Background learning in Controls	l	14241	14256	15	16.68	1	<0.01	∗	-1.71
Pink Multimodal Interaction Effects	i_{pw} l_{pw}	14241	14237	4	0.22	2	0.897		
Blue Multimodal Interaction Effects	i_{bw} l_{bw}	14237	14235	2	2.12	2	0.346		
Altered learning of Pink Patterns	l_p	14235	14234	1	1.06	1	0.302		
Altered intercepts of Pink Patterns	i_p	14234	14237	3	4.55	1	0.033†		
Altered learning of Blue Patterns	l_b	14237	14238	1	3.26	1	0.071		
Altered intercepts of Blue Patterns	i_b	14238	14278	40	41.54	1	<0.01	∗	-2.28
Altered learning of Warm Patterns	l_w	14238	14236	2	0.00	1	0.961		
Altered intercepts of Warm Patterns	i_w	14236	14235	1	1.12	1	0.289		
Altered learning of Hot Patterns	l_h	14235	14234	1	0.34	1	0.560		
Altered intercepts of Hot Patterns	i_h	14234	14233	1	1.04	1	0.307		

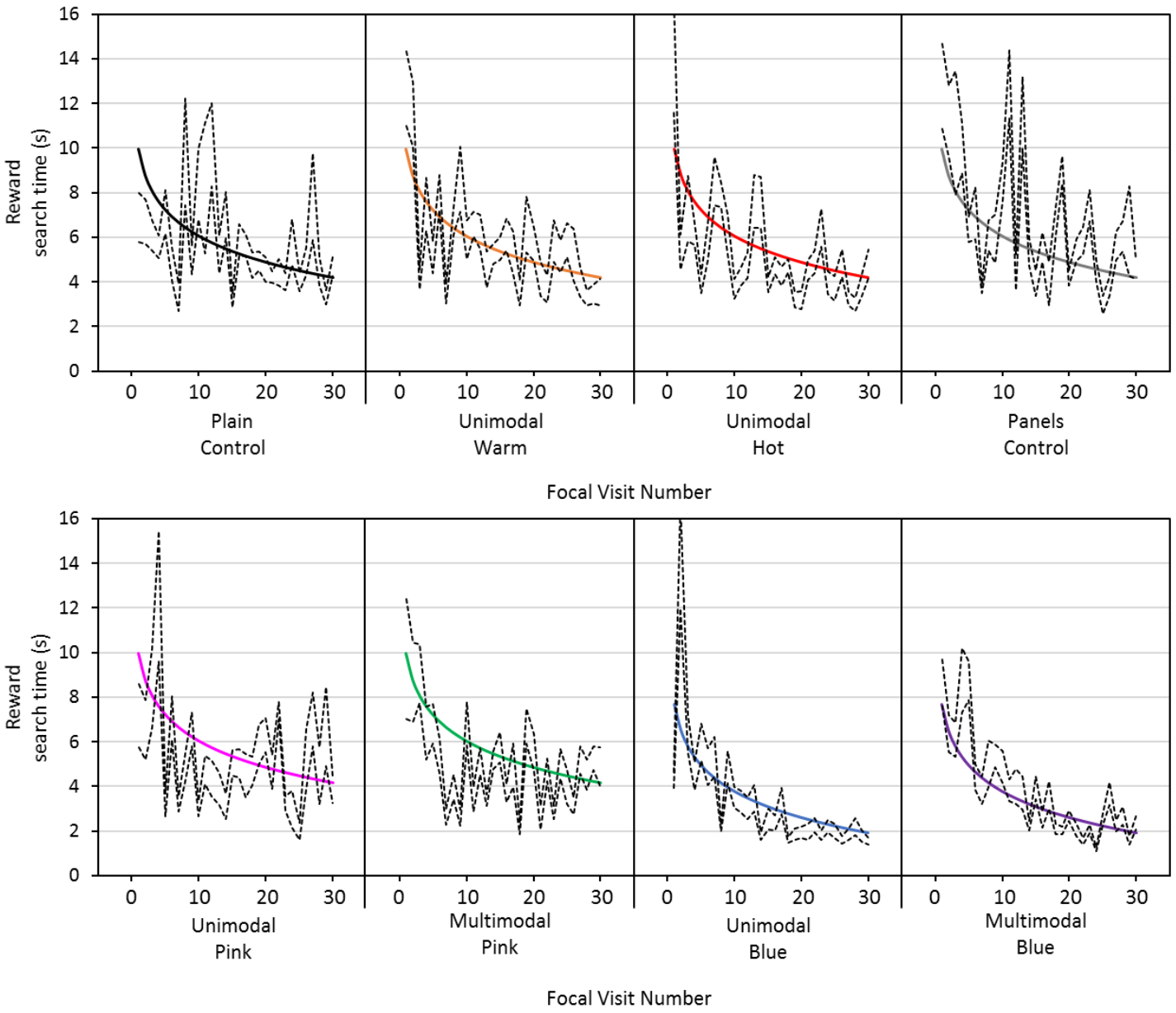


Figure 6.9 [left]: *The relationship between bee reward search time (in seconds) and experience (focal visit number) across bees that foraged on different artificial flower variants, different test groups. Dashed lines indicate the mean reward search time ± one standard error of the mean, calculated at each flower visit. Solid lines indicate mean reward search times of each test group according to the best fitting models.*

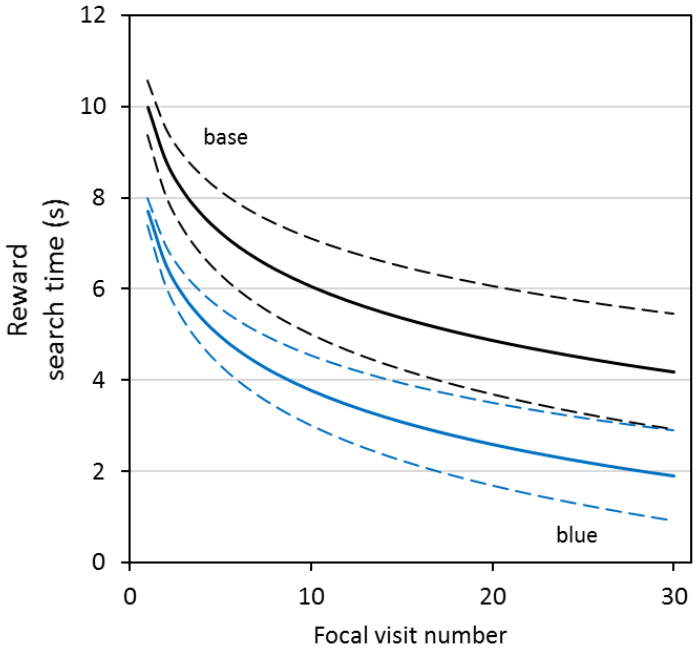


Figure 6.10: *A summary of the best fitting model for reward search times (in seconds) of bees foraging across all flower test groups, for comparison against figure 6.9. Solid lines indicate the mean reward search times for bees presented with different patterns, as predicted by the best fitting model. Dashed lines indicate ± one standard error of the model mean estimates. Colours and labels indicate the patterns presented to bees: 'blue' and blue colour, indicates bees presented with blue colour patterns; 'base' and black colour, bees not presented with the patterns (control groups) or patterns not indicated by any other line, thus represents bees whose responses were indistinguishable from control bees.*

6.4 Discussion

By assessing footage of bumblebee visits to experimental artificial flowers, we find temperature patterns can induce some changes in bee behaviour associated with pollinator responses to floral guides (figure 6.5 and 6.7). Bees visiting flowers presenting 'Warm' temperature patterns showed reduced incidence of visits where they failed to find rewards (failed visits, figure 6.5 and 6.6) and were able to learn the reward location of flowers (figure 6.7 and 6.8), as shown by increases with experience in the proportion of visits where they fed from the rewarding feeder first (first-feeder visits). Hot temperature patterns allowed similar

learning of reward locations but elicited no change in the incidence of failed visits. Pink colour patterns appeared to have no influence on bee foraging responses (figures 6.5 to 6.10). This is likely to be due to Pink flowers showing little contrast with the flower top and are not easily detected by bees. Contrasting colour patterns, blue colour guides, induced changes in bee behaviour in all the handling metrics assessed, as expected for a contrasting colour guide (Lunau et al., 2006; Hansen et al., 2011; Leonard and Papaj, 2011; Goodale et al., 2014). Bees visiting flowers with blue colour patterns had reduced search time for floral rewards (figure 6.9 and 6.10) and reduced proportions of failed visits (figure 6.5 and 6.6). Additionally, bees presented with blue patterns showed increased initial proportions of first-feeder visits (figure 6.9 and 6.10), indicating bees were showing an innate tendency to investigate the patterned area, and were able to learn reward location (figure 6.9 and 6.10). This confirms that a well-functioning floral guide, as in the dark blue patterns, could induce all of the responses associated with guides in bees visiting the experimental artificial flowers. This suggests that the lack of all these guide-associated responses by bees visiting flowers with temperature patterns is not due to the bees being unable to show them in our experiment, perhaps due to the flower design, but due to temperature patterns' reduced functionality as a guide. This suggests that temperature patterns can convey messages of within flower reward location to bumblebees, functioning like floral guides. However, temperature patterns do not elicit all the responses which contrasting colour patterns would, so appear to function less well as guides.

When temperature and colour patterns were combined as multimodal patterns the effects these displays had on bees was less clear. Bees in the Multimodal Pink group, where temperature and pink colour patterns were combined, behaved in a similar manner to bees in the Unimodal Warm group, the addition of a poor-quality colour signal not enhancing responses at all. Best fitting models across test groups suggested bees presented with Multimodal Blue flowers show further reductions in proportions of failed visits (figure 6.5 and 6.6) and enhanced learning of reward location, shown by higher proportions of first-feeder

visits (figure 6.7 and 6.8). However, the addition of temperature to a blue colour guide did not enhance initial proportion of first-feeder visits of bees visiting multimodal flowers (figure 6.7 and 6.8). Similarly, reward search time was not enhanced by the addition of temperature to a blue colour pattern (figure 6.9, figure 6.10). In these latter cases bees responded similarly to bees presented with blue colour patterns alone. Additionally, when test groups were compared directly, bees presented with Multimodal Blue patterns never performed significantly better than bees visiting Unimodal Blue patterned flowers, their best performing unimodal component. Temperature may have some benefit within a multimodal guide in terms of reward location learning and reducing incidence of failed visits. Multimodality has been observed to enhance learning of differences between flowers (Katzenberger et al., 2013; Kulahci et al., 2008), perhaps further in overlapping patterns (Lawson et al., 2018), as our models suggest. However, these effects on reward location learning are small, thus, when the relevant test groups are compared side by side they do not significantly differ. Lawson et al. (2017b), showed found scent and colour patterns did not enhance bee nectar discovery times over unimodal components. Goyret and Kelber (2011) similarly found moths respond to multimodal tactile and colour guides in the same manner as they do to unimodal colour guides. The results presented here support these finding that reward search times are not enhanced in a multimodal guide beyond the ability of the displays component parts (Lawson et al., 2017b), and additionally more generally suggest that multimodal patterns do not greatly enhance other floral guide functions.

Bees showed consistent improvements with experience in reward search time and proportions of failed visits (figures 6.5 to 6.8) even in non-patterned control groups, or in flowers with apparently undetectable Pink patterns. However, these responses were improved further by the presence of certain patterns, and bees required pattern signals to show changes in proportions of first-feeder visits. The decrease in proportions of failed visits with increased experience independent of pattern signal presence, is probably the result of bees learning that rewards are always present in the experimental flowers. Even though bees in control groups

cannot distinguish which feeder is rewarding, their motivation to stay increases with experience (Lefebvre et al., 2007; Taneyhill, 2010). Thus, they become more likely to continue to search after a nonrewarding feeding attempt. The increase in reward search time independent of pattern signal is the result of bees learning how to better access feeders in tunnels. This leads to faster reward search time, as bees in the control group more quickly investigate each tunnel.

The responses of bees to temperature patterns would suggest any guiding benefits temperature pattern might have to natural flowers, and their pollinators, are reduced compared to contrasting colour patterns. That said, plants using floral temperature patterns as guides will ensure more visitors reach the correct position for pollen transfer (Armbruster et al., 2004; Hansen et al., 2011). Additionally, reductions in failed visits and learning the location of rewards would improve bee foraging efficiency, likely in terms of wasted effort. This is particularly true on complex flowers where learning the correct position to find rewards is more beneficial. It also is important to note that any small improvement in foraging efficiency or success in finding rewards does magnify across bee colonies to larger benefits to colony success (Pelletier and McNeil, 2003; Raine and Chittka, 2008; Charlton and Houston, 2010). Furthermore, temperature patterns are common (chapter 3). Bees are unlikely to remain naïve to temperature patterns while foraging in natural environments. As seen in colour guides, learning and guided responses can carry over to novel displays with similar guides (Leonard and Papaj, 2011). This may occur with temperature patterns also. Understanding how previous experience on displays with similar patterns influences bee responses on novel flowers may be important to understanding how temperature patterns, and other floral guides, influence bee behaviour within natural flowers (Lynn et al., 2005; Leonard and Papaj, 2011).

It was predicted, based on bees' preferences for temperature (Dyer et al., 2006; Norgate et al., 2010), that bees would be innately attracted to temperature patterns corresponding with the rewarding feeder and would therefore visit that feeder first, leading to a higher initial proportions of first-feeder visits and reduced reward search times. However,

bees appeared to have to learn the temperature pattern guide to show improved proportions of first-feeder visits using temperature patterns and no temperature related benefit to reward search time was seen. Proportions of failed visits were consistently reduced in flowers with Warm temperature patterns, this suggests that naive bees are more likely to find rewards but are not more likely to correctly choose to approach the correct location first. The decreased proportions of failed visits, but unaffected initial proportions of first-feeder visits or reward search time of naïve bees in response to temperature patterns is probably the result of range of temperature signals. In bees temperature is detected by conduction or touch (Dyer et al., 2006; Whitney et al., 2008). So, unlike with visual signals (Lunau et al., 2006), bees are not informed or attracted to the reward location until they have landed and actually made contact with the temperature pattern during their search. Naïve bees are likely to land and search similarly to control group bees until they actually encounter the temperature patterns and are therefore more likely to erroneously approach and feed from nonrewarding feeders. When presented with Warm temperature patterns, the lower incidence of failed visits of naïve bees indicates they are more likely to feed from the warm feeder once they encounter the pattern (Angioy et al., 2004; Dyer et al., 2006). However, as bees do not detect the temperature pattern until they have searched the flower we see no improvement in incidence of first-feeder visits in naïve bees or in naïve bee reward search times. The lack of improvement in bee incidence of failed visits when presented with Hot temperature patterns may be due to the excess heat discouraging bees. Although bees prefer higher floral temperature, at a point flowers get too hot and will deter bees (Norgate et al., 2010; Shrestha et al., 2018). This point will depend on the environmental conditions, particularly the environmental temperature (Norgate et al., 2010; Shrestha et al., 2018). It is possible the Hot temperature patterns are sufficiently hot to deter naïve bees, hence they are less likely to search the rewarding feeder if they encounter it than bees encountering Warm feeders. Once bees learn the temperature pattern, they reduce errors in first feeder choice, learning not to drink in the feeders they find without the temperature pattern. However, as bees still must physically search the flower top

to find these patterns, we see no improvement in reward search time over control bees, even with experience.

The difference in range of signalling modalities between temperature and visual signals can also explain the differences in bees' responses to temperature and contrasting visual patterns. Contrasting colour patterns can be detected at some range by bees (Manning, 1956; Lehrer et al., 1995; Hempel de Ibarra et al., 2001; Hempel de Ibarra et al., 2002), certainly compared to temperature. Consequently, bees receive the guide and reward location messages from visual patterns prior to landing. Thus, bees are attracted to the rewarding feeder before landing and can land closer to, or immediately precede to, rewarding feeders upon landing (Lunau et al., 2006). This means that all metrics, including reward search time, are improved when flowers have contrasting blue guides, even in naïve bees. Whether other shorter-range patterns show reduced guiding functionality to bees, similar to temperature patterns, has yet to be seen. Tactile guides have only been demonstrated with moth pollinators (Goyret et al., 2007; Goyret and Kelber, 2011), and it is unclear how bees will respond to them. Although, Goyret and Kelber (2011) found tactile guides were ignored by moths in the presence of, longer range, colour guides. Scent patterns, which also work at longer range (Goyret et al., 2007), reduce bee reward search time (Lawson et al., 2017b) appearing to function similarly to how Blue visual guides functioned here. Taken together these results suggest effective range of a signalling modality plays an important role in the extent that modality can change bee foraging responses when functioning as a guide.

A lack of, or very minor, interaction between these different modality guides does not mean possession of floral patterns of both modalities is of no benefit. Floral temperature may still aid flowers by warming pollinators (Rands and Whitney, 2008). If presented alongside the visual pattern, a temperature pattern will, presumably, warm visitors that follow the visual guide, further improving their efficiency of the pollinator, leading to associated benefits to plants. Furthermore, the multiple messages hypothesis is not the only theory explaining multimodality (Leonard et al., 2011b; Leonard et al., 2012). While reward location learning did

not appear to be enhanced significantly, learning of flower identify may be improved. Bees can learn both thermal and visual patterns and use these for flower identification, and so flower recognition may be improved by possessing both (Kulahci et al., 2008; Kaczorowski et al., 2012; Leonard and Masek, 2014). Having both guide signals may be beneficial if one is disrupted by environmental conditions (the efficacy backup hypothesis – Dyer and Chittka, 2004a; Leonard et al., 2012; Kaczorowski et al., 2012; Lawson et al., 2017a). The bee responses to Unimodal Pink flowers suggest temperature patterns can aid the flower as a lesser guide when visual patterns are non-functioning. Colour pattern recognition by insect pollinators is influenced by light conditions (Dyer and Chittka, 2004b; Dyer and Chittka, 2004a), however, so is temperature pattern generation (Rejšková et al., 2010). Thus, it is likely both patterns will be disrupted together. Bees have approximate colour constancy, meaning similar colours get confused in altered light conditions (Kevan et al., 2001; Dyer and Chittka, 2004a; Dyer and Chittka, 2004b; Kaczorowski et al., 2012; Chittka et al., 2014). However, bees in such conditions may still remain sensitive to small differences in temperature (Heran, 1952; Dyer et al., 2006). The extent to which disruption temperature pattern generation occurs alongside a change of light conditions sufficient to disrupt bee colour pattern recognition is not clear. Further investigation of the effects of weather disruption on natural temperature patterns and visual pattern detection is required to evaluate the potential benefit of thermal and colour patterns on efficacy backup.

Bee responses to temperature patterns within the flower reveal that temperature patterns can signal reward location, functioning like a floral guide. Floral temperature patterns appear to perform this role less effectively than contrasting colour guides. This expands the potential functionality of floral temperature and patterns of temperature within a floral display. We also show evidence supporting previous work that multimodal guides do not appear to perform much better as a floral guide than their best functioning components. Based on the findings of this study and existing work it would appear that the effective range of the floral

guide signal has an important effect on both how well different modalities function as guides as well as which guides bees utilise within a multimodal display.

Chapter 7: Temperature patterns in multimodal displays: cross-modality pattern learning

As described in page xv, the following chapter was adapted to form a paper published (online only at time of writing) in the Journal of Comparative Physiology A (Harrap et al., 2019) and will thus have high similarity with this publication. The paper represents an adaptation of the chapter presented in this thesis with additional material from a collaborator, Dr D. A. Lawson. The version presented here represents my own work, with normal contributions from supervisors, prior to adaptations and additions by Dr Lawson.

CHAPTER ABSTRACT

Bumblebees can learn many patterned floral signals of different sensory modalities. It has been recently shown that bumblebees, following conditioning to a pattern of floral scent, will have improved recognition of a matching visual pattern. This suggests that bumblebees can show cross-modality transfer of learning between matching patterns. However, this cross-modality pattern learning has not been demonstrated to occur between matching patterns of other floral sensory modalities. Here I investigate whether a similar cross-modality transfer of pattern learning occurs between floral temperature patterns and dark coloured visual patterns. Bumblebees conditioned to temperature patterns showed no enhanced responses to matching visual patterns compared to bees with no such conditioning. A similar lack of learning transfer was observed for bees conditioned to visual patterns when presented with matching temperature patterns. This suggests cross-modality pattern learning may be limited to specific modality pairings, such as scent and visual patterns.

7.1 Introduction

Many floral displays show differences in intensity, composition and location of floral signalling components across the flower which can be learned by pollinators (Hempel de Ibarra et al., 2015). Where different floral displays differ in these patterns of signals, these can be used as a recognition signal by a visitor. The best understood example of these is visual patterns. Visual patterns include differences in terms of colouring and brightness across the flower. In bee-pollinated flowers, this most frequently involves a darker coloured flower centre (particularly in terms of the green sensitive L-receptor found in bees), and a brighter periphery (Hempel de Ibarra and Vorobyev, 2009). Such colour patterns are more salient to insect pollinators (Johnson and Dafni, 1998; Hempel de Ibarra et al., 2001; Spaethe et al., 2001). Flowers can also create other visual patterns such as iridescence (Whitney et al., 2009b; Whitney et al., 2009c) or polarization patterns (Foster et al., 2014) which bees have been demonstrated to be able to learn. Non-visual patterns include: scent patterns, where different amounts of floral volatiles or different floral volatile chemicals are released across the flower (Bergström et al., 1995; Balao et al., 2011; Lawson et al., 2018); electrostatic patterns, where properties of the flower allow charge to accumulate differentially across the flower surface and between flowers (Clarke et al., 2013); texture patterns, where shape of cells on the flower surface differ (Kevan and Lane, 1985); and temperature patterns; where different parts of the flower differ in how they heat up (chapter 3; Harrap et al., 2017). All these pattern-types have been demonstrated to differ between flower species and to be used by pollinators, particularly bumblebees, for learning flower identity. Additionally, I have demonstrated that flowers show differences in their structure and location of floral humidity signals (chapter 4), which could be another (as yet undemonstrated) pattern that visitors could learn to respond to.

While pollinators can learn isolated patterns of floral signals they will experience such patterns as part of a multimodal floral display in natural environments. If we are to understand how pollinators respond to natural flowers, we must understand how pollinators respond to combinations of floral signals. Additional signals within floral displays may serve different

functions from each other, sending different floral messages. In this way patterns of visual (Leonard and Papaj, 2011; Goodale et al., 2014), scent (Lawson et al., 2017b), texture (Goyret and Raguso, 2006) and temperature (chapter 6), in addition to being used for recognition, have been demonstrated to have a guide function enhancing flower handling. Additional floral signal modalities might make displays more salient or increase the ways in which they can differ improving the pollinator's ability to locate and distinguish flowers (Goyret et al., 2007; Kulahci et al., 2008; Katzenberger et al., 2013). This may be due to different modalities sending the same or different parts of the floral message in parallel or interacting with other signals (Leonard et al., 2011b; Leonard et al., 2012). Pattern signals may allow a further complexity to multimodal floral displays allowing flowers to further differentiate themselves from other flowers, as patterns of signal can be used even when flowers differ in no other aspect (Hempel de Ibarra and Vorobyev, 2009; Clarke et al., 2013; Harrap et al., 2017; Lawson et al., 2018).

When visual and scent patterns spatially overlap on the flower they are learnt faster by bumblebees than patterns that do not match (Lawson et al., 2017b), due to either reinforcement of the pattern signal through multiple modalities, or signal interactions. Additionally, overlapping patterns of different modalities may exploit the learning mechanisms of pollinators to allow consistent responses towards the different modality patterns. Lawson et al. (2018) discovered that bumblebees conditioned to a cross shaped scent pattern showed a preference to unscented flowers presenting matching visual patterns over circular visual patterns. This cross-modality pattern learning is believed to be a result of the links between neural pathways relating to scent and visual perception in bees. Visual and olfactory sensory signals are known to be neurologically linked in the brains of hymenopterans (Leonard and Masek, 2014). Neural pathways from hymenopteran antennal and optic lobes, which carry scent and sight signals respectively, meet at the mushroom body calyx (Gronenberg, 1999; Gronenberg, 2001), where some structures receive information from both pathways (Mobbs, 1982; Gronenberg, 2001; Ehmer and Gronenberg, 2002). This has the consequence that

spatial arrangement of visual patterns may elicit similar stimulation as a matching scent pattern in the bee's brain. Mushroom bodies are strongly associated with memory formation and learning (Menzel, 2001; Davis, 2005). Thus, bees likely process scent and sight memories together (Leonard and Masek, 2014) allowing the cross-modality learning observed between patterns of these signals (Lawson et al., 2018).

Matching scent and visual patterns due to this cross-modality pattern learning and response may improve the efficacy of the floral display and benefit the plant and pollinator. Many floral signals can be interrupted by environmental conditions, however different signal modalities are disrupted by different conditions (Dyer and Chittka, 2004b; Dyer and Chittka, 2004a; Rejšková et al., 2010; Kaczorowski et al., 2012; Lawson et al., 2017a). Cross-modality learning may prime bees for a similar response to matching patterns in the event of disruption of the conditioned pattern by weather conditions (Lawson et al., 2018). With the disruption of one floral signal, recognition of a multimodal display will normally depend on the strength of learning of other undisrupted floral signals (Dyer and Chittka, 2004a; Kaczorowski et al., 2012; Lawson et al., 2017a). When learning transfer occurs between overlapping patterns, some level of learning is maintained when one pattern is disrupted, reducing the impact of this signal disruption. Similarly, overlapping multimodal patterns with differing signalling ranges (like scent and visual patterns) might induce similar stimulations of the bees brain at differing ranges, allowing the conditioned response to be elicited over a longer range than the conditioning stimulus alone may travel (Lawson et al., 2018). This cross-modality transfer of pattern learning has only recently been discovered, likewise the presence of and capacity of pollinators to distinguish many non-visual floral patterns are a recent discoveries (chapters 3 and 4; Clarke et al., 2013; Balao et al., 2011). So, it is currently unknown whether any other overlapping patterned floral signals induce similar cross-modality pattern learning in bumblebees.

Many flower species vary in floral temperature (Dyer et al., 2006; Whitney et al., 2008), which can show varied patterns across the flower surface. Bees have quite high temperature

sensitivity (Heran, 1952) and I demonstrated that these temperature patterns can be learned by bumblebees (chapter 3). Floral temperature is largely dictated by the ability of flowers to intercept solar radiation (Herrera, 1995b; Totland, 1996; Zhang et al., 2010). Consequently, there is a mechanistic link between colour patterns and temperature patterns: dark coloured areas of flowers reach a higher temperature than paler areas, creating temperature patterns that often correspond with colour patterns, (Sapir et al., 2006; Rejšková et al., 2010; Dietrich and Körner, 2014). Although temperature patterns can occur without co-occurring colour patterns (chapter 3), other floral characteristics such as texture and floral architecture can also influence the generation of floral temperature and temperature patterns (Miller, 1986; Davis et al., 2008; Whitney et al., 2011). As there is a frequent association between floral temperature patterns and dark-coloured visual patterns, bumblebees may also show cross-modality pattern learning between these matching visual and temperature patterns when they occur. Floral temperature detection works at short range (Whitney et al., 2008), while visual pattern signals to insects function at comparably longer ranges (Hempel de Ibarra and Vorobyev, 2009; Hempel de Ibarra et al., 2015). Similarly, environmental conditions like lower light intensities may affect temperature signals differently than they might influence colour (Rejšková et al., 2010; Arnold and Chittka, 2012; Chittka et al., 2014). This is similar to floral scents and visual signals, where scent signal can be detected at longer distances than visual signals, and scent can be disrupted by aspects like the wind that do not greatly influence visual signals (Lawson et al., 2017a). Thus, temperature and visual signals are likely to complement each other in the same ways as scent and visual signals. In this way, flowers may gain the same kind of benefits of cross-modality pattern learning between temperature and visual patterns if such transfers are possible then they might gain between scent and visual pattern associations. Investigation of whether temperature and visual pattern learning can transfer between modalities occurs will expand our understanding of how pollinators can interact with multimodal floral displays. This will additionally help establish how often this transfer of learning between matching patterns of different floral signalling modalities actually occurs.

In this chapter we investigate whether bumblebees show cross-modality pattern learning between overlapping dark visual and temperature pattern signals. Bee lab conditioning techniques (Dyer and Chittka, 2004d) as described in chapter 3 and a sequence of nonrewarding test phases, like those used by (Lawson et al., 2018), were employed in this study. Bees are conditioned to associate certain visual or temperature patterns with a reward. Bees are subsequently presented with matching patterns of the other modality and foraging responses are compared to bees that had received no such conditioning prior to exposure to either patterned signal. In this way we can evaluate how conditioning to one modality pattern can alter responses to the other.

7.2 Methods

7.2.1 *Artificial flower design*

Three artificial flower types were used: temperature pattern flowers, visual pattern flowers, and test phase visual pattern flowers. All variants of the visual artificial flowers used in this experiment can be seen in figure 7.1. Temperature pattern flowers can be seen in figure 3.2.Control artificial flowers were also created that were identical to the artificial flowers but with the corresponding patterned signals removed (figure 7.1a and 3.2a).

The temperature pattern artificial flowers used in this experiment are the ‘small artificial flowers’ described in chapter 3. These artificial flowers create a $3cm^2$ temperature pattern in either a ‘circle’ about the edge of the flower’s lid or a ‘bar shape’ across the flowers centre (figure 3.2e). These artificial flowers reach an average temperature of 33ºC over the heated parts and 25ºC over the non-heated parts. As before these artificial flowers could have their batteries removed to create control flowers that presented no temperature pattern (see chapter 3). For full detail on how these artificial flowers are constructed and how they relate to real flowers temperature patterns see chapter 3.

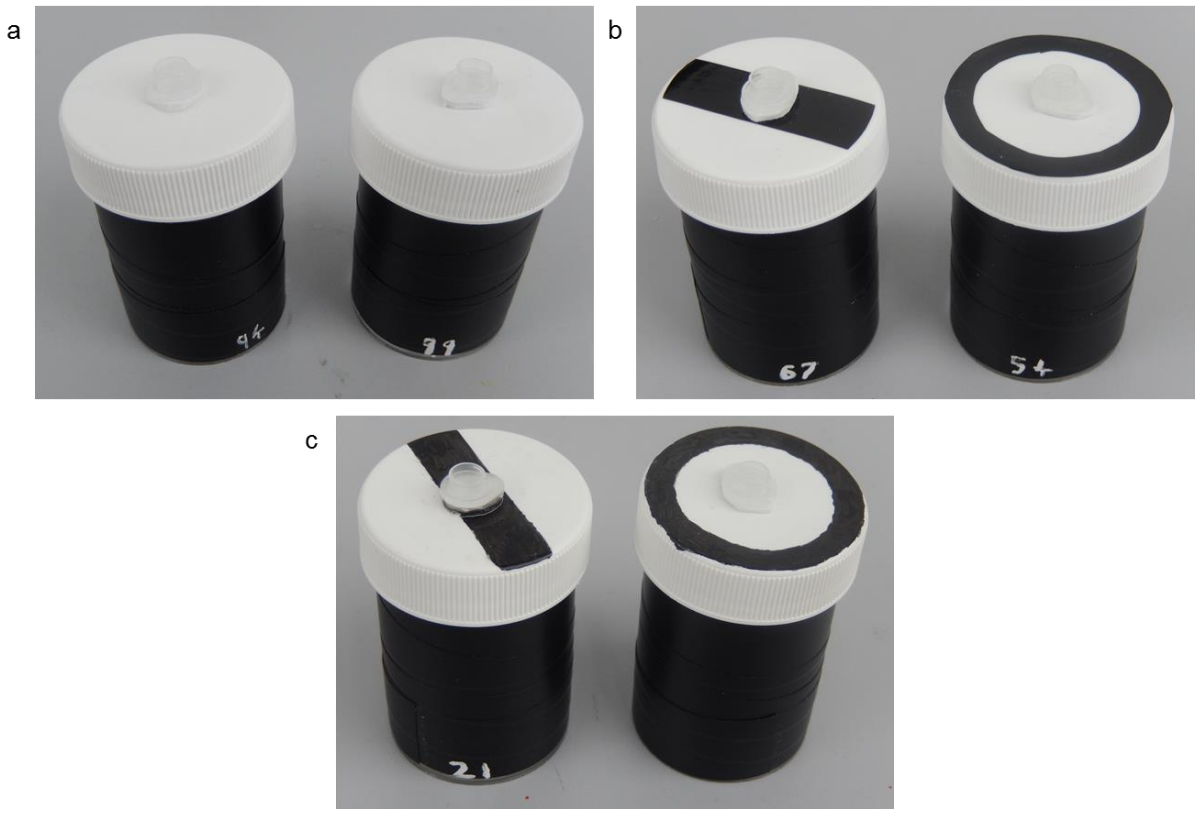


Figure 7.1: *The visual pattern artificial flowers used in our experiments.* ***a)*** *the control artificial flowers that present no visual pattern.* ***b)*** *the visual pattern artificial flowers used in learning experiments.* ***c)*** *the visual pattern artificial flowers used in both test phases.*

Visual pattern artificial flowers were constructed from a specimen jar (Sterilin PS 60ml with white plastic lids, Thermo Fisher Scientific, Newport, UK). The jars themselves were covered with black electrical tape and marked with randomly generated numbers (see chapters 3 and 5 for details). Even though visual patterns would allow distinction of rewarding and unrewarding flowers without these numbers, this was done on all flowers in order to maintain consistency between the different kinds of artificial flowers used across the tests and to allow distinction of rewarding an unrewarding controls. In all flowers an upturned 0.5ml Eppendorf tube lid (Hamburg, Germany), with a 1mm thick section of white plastic foam stuck to its underside was stuck to the centre of the artificial flower's lid. This Eppendorf tube lid would function as a feeding well. The plastic foam, which functions as insulation in the temperature pattern flowers (see chapter 3) was not required to shield the well's content from

heating in the visual pattern flowers but was, again, done to maintain consistency in flower appearance outside of the temperature and visual pattern differences.

Two types of visual patterns were created one set for the learning phase, one for test phases. Learning phase visual patterns were created by sticking a $3cm^2$ section of black adhesive plastic (d-c-fix® adhesive film, Hornschuch group, Weissbach, Germany). These sections of plastic were either in a circle shape about the edge of the flowers lid or a bar shape across the flower's centre (figure 7.1b). These visual patterns corresponded to the same regions heated in the temperature pattern flowers. In the test phase (see below) visual patterns were marked onto the corresponding regions using a black permanent marker but were otherwise constructed in the same way as the learning phase artificial flowers (figure 7.1c). Artificial flowers used for the visual control group were constructed in the same way but had no such markings (figure 7.1a). The dark visual patterns used corresponded to similar darker patterns that correspond with temperature patterns, such as the dark radiations in *Geranium psilostemon* or the dark petal bases of many *Cistus* flowers (such as those shown in figure 3.4).

The test phase flowers had their marks produced with ink, as opposed to plastic, in order to remove any previous learning of or innate response to any tactile element or the raised surface the sticky back plastic. This meant that the tests of learning and responses of bees to test flowers were limited to the visual aspects of the display. As in chapter 3 flowers, learning phase visual pattern and temperature patterns would be cleaned at the end of foraging bouts with ethanol. To clean test phase flowers, I wiped them with a dry cloth. These test phase flowers would be set aside between bees' test phases (during learning phases) for well over the 40 minute lifespan of bee scent marks (Stout and Goulson, 2001); and used only in short (20 visit) unrewarding test phases, which bees rarely required more than a single foraging bout to complete.

7.2.2 *Bee experiments*

Bee lab conditions are described in chapter 3. Flower naïve bumblebees, *Bombus terrestris audax*, were supplied by Biobest (Westerlo, Belgium) via Agralan (Swindon, UK). This study makes use of the same bees conditioned to 'small temperature patterns' in chapter 3, immediately following the nonrewarding test phase described in that chapter (section 3.2.5), and an additional set of bees conditioned to visual patterns. These sets of bees are used to in experiments to investigate 'temperature to visual' and 'visual to temperature' cross-modality pattern learning respectively. Each experiment involved an identical sequence of: learning, nonrewarding test, retraining, and cross-modality learning test phases. The sequence and type of artificial flowers presented to bees within each test group is described for both sets of bees in table 7.1.

Table 7.1: *The sequence of the experiment for all test groups in both sets of bees and the flowers presented to them. Sections in grey shading are described and recorded in chapter 3. Artificial flower types are listed as follows: 'circle TP', Circle temperature pattern; 'bar TP', Bar temperature pattern; 'no TP', Control temperature pattern, off flowers; 'circle VP', Circle learning visual pattern; 'bar VP', Bar learning visual pattern; 'no VP', Control visual pattern; 'circle TVP', Circle test visual pattern; 'bar TVP', Bar test visual pattern. Also provided are the number of bees tested in each test group.*

Bee Set	Test group	Number tested	Learning phase (60 visits)		Nonrewarding test (20 visits)		Retraining (20 visits)		Cross-modality test phase (20 visits)	
Temperature to visual	Circle rewards	12	Rewarding	circle TP	Nonrewarding	circle TP	Rewarding	circle TP	Nonrewarding	circle TVP
			Nonrewarding	bar TP	Nonrewarding	bar TP	Nonrewarding	bar TP	Nonrewarding	bar TVP
Temperature to visual	Bar rewards	12	Rewarding	bar TP	Nonrewarding	bar TP	Rewarding	bar TP	Nonrewarding	bar TVP
			Nonrewarding	circle TP	Nonrewarding	circle TP	Nonrewarding	circle TP	Nonrewarding	circle TVP
Temperature to visual	Control	12	Rewarding	no TP	Nonrewarding	no TP	Rewarding	no TP	Nonrewarding	circle TVP
			Nonrewarding	no TP	Nonrewarding	no TP	Nonrewarding	no TP	Nonrewarding	bar TVP
Visual to temperature	Circle rewards	12	Rewarding	circle VP	Nonrewarding	circle TVP	Rewarding	circle VP	Nonrewarding	circle TP
			Nonrewarding	bar VP	Nonrewarding	bar TVP	Nonrewarding	bar VP	Nonrewarding	bar TP
Visual to temperature	Bar rewards	12	Rewarding	bar VP	Nonrewarding	bar TVP	Rewarding	bar VP	Nonrewarding	bar TP
			Nonrewarding	circle VP	Nonrewarding	circle TVP	Nonrewarding	circle VP	Nonrewarding	circle TP
Visual to temperature	Control	12	Rewarding	no VP	Nonrewarding	no VP	Rewarding	no VP	Nonrewarding	circle TP
			Nonrewarding	no VP	Nonrewarding	no VP	Nonrewarding	no VP	Nonrewarding	bar TP

7.2.3 *Temperature to Visual cross-modality learning*

Immediately following completion of the nonrewarding test phase in the 'small artificial flower' experiment described in chapter 3 section 3.2.5, bees entered a 'retraining phase'. Here bees were allowed back into the arena and presented once again with 16 artificial flowers, presenting the temperature patterns (8 presenting circle and 8 bar temperature patterns) or lack of them for bees in the control group. Feeders of these artificial flowers contained either 25µl of 30% sucrose solution or 25µl of water as corresponding to the test group that bee was in during the learning phase (as described in chapter 3 and table 7.1). Bees were allowed to forage freely, returning to the hive as required, until they completed at least 20 flower visits and returned to the hive. This retraining phase was carried out in order to allow bees to refresh any associations made between flower signals and rewards that may have been damaged by repeated encounters with nonrewarding flowers presenting the same temperature pattern during the previous nonrewarding test phase. Within this retraining phase flowers were cleaned and checked for faults as described in chapter 3.

After completing retraining the bee was allowed to begin the 'cross-modality learning test' (this being the 'second test' phase these bees experienced). Here we investigate how the bees conditioned to temperature patterns, or not in the case of the control group, respond to matching visual patterns. Bees were presented with 16 test visual pattern flowers (eight of each visual pattern) in the flight arena. This was the case even with bees in the control group, as we must establish a baseline of how unconditioned bees respond to these novel pattern signals. Artificial flowers in the cross-modality learning phase were nonrewarding, their feeding wells containing 25µl of water, regardless of the visual pattern they presented. Bees were allowed to forage freely until they completed 20 flower visits. Which flowers they landed on, and whether the bee probed the feeding well, was observed and recorded. As in chapter 3, contact with the flower surface was considered a flower landing even if bees did not quit flight.

During the cross-modality learning test, bee landings were classed on their response to the circle pattern. A 'positive response' was classed as either landing on a circle pattern

flower and probing the feeder, or landing on the bar pattern flower and leaving before feeding on the feeder. Landing on a circle pattern flower and leaving without probing the feeding well or landing on a bar pattern flower and probing the feeding well, was classed as a 'negative response'. For each bee the circle pattern response rate, the proportion of the 20 flower visits that showed a positive response to the circle pattern, was calculated. This circle pattern response rate is similar to the 'humidity response rate' utilised in chapter 5. It allows us to evaluate how bees in the control group, which were not conditioned to a particular pattern, respond when presented with the novel visual signals and adjust our comparisons between test group to account for any innate preferences bees might have. When compared to the control group, if bees showed a higher circle response rate in the circle rewards pattern test group or a reduced circle response rate in the bar rewards test group, this would show a cross-modality learning between visual and temperature patterns.

Circle pattern response rate was bound between 0 and 1, therefore underwent the arcsine square-root transformation for all analyses. The circle pattern response rate in the cross-modality learning phase was compared across different test groups using analysis of variance. Additionally, the correlation between each bee's success rate in the temperature pattern nonrewarding test phase and the circle pattern response rate in the cross-modality test phase was also compared using ANCOVA, including the test group as a categorial variable.

7.2.4 *Visual to Temperature cross-modality learning*

Flower naïve bees underwent an identical learning phase to that described in chapter 3, except that the bees used here were presented with the learning phase visual pattern artificial flowers, instead of temperature pattern flowers. Individual bees were assigned test groups as shown in table 7.1: 'control group', no visual patterns during learning phase; 'bar rewards', rewarding flowers presented a bar visual pattern, nonrewarding flowers a circle pattern; 'circle rewards', rewarding flowers presented a circle visual pattern, nonrewarding distractors a bar visual

pattern. No bee used in the temperature to visual pattern experiment was also used in the visual to temperature pattern experiment.

The learning phase of experiments and recording of bee landing and probing behaviours were carried out as described in chapters 3 and 5. Bees then undertook a nonrewarding test phase. Unless stated otherwise all experimental procedures were as in the 'small flower experiments' described in chapter 3. Here bees were presented with nonrewarding test phase visual artificial flowers or control flowers, dependent on test groups. As in the temperature pattern experiment, bees were allowed to forage freely for 20 flower visits and landing and probing behaviour was observed and recorded. Following completion of the test phase, bees were presented with rewarding and nonrewarding learning phase visual artificial flowers or control flowers as determined by test group, and allowed to forage in a retraining phase. Following at least 20 flower visits in the retraining phase, bees were allowed to begin the cross-modality learning test phase upon beginning the next foraging bout. This test phase was carried out as described above except that bees were presented with nonrewarding temperature pattern flowers, instead on nonrewarding visual pattern artificial flowers. Visitation and feeding well probing responses were again recorded during the cross-modality learning test phase.

Success rate in the learning phase and nonrewarding test phase were calculated for bees, as described in chapter 3, based upon whichever visual pattern had been rewarding in that test group. Circle pattern response rate was calculated for the cross-modality test phase, as described above. However, here these described responses to the visual patterns in the learning and nonrewarding test and temperature patterns in the cross-modality learning test. Success rate for both learning and nonrewarding test phases was analysed as described in chapter 3. Circle pattern response rate was analysed as described above.

7.3 Results

Bees conditioned to temperature patterns (details of learning given in chapter 3) did not show any cross-modality pattern learning when presented with matching visual patterns in the cross-modality learning test. No significant differences were detected between the responses of bees in test and control groups to visual patterns (ANOVA, $F_{2,33} = 0.75$, $p = 0.482$, figure 7.2). When presented with the visual patterns bees appeared to forage randomly, achieving response rates of *c*. 50% regardless of whether they were conditioned to bar, circle or no temperature patterns (the control group). Bees in the control group showed a mean circle pattern response rate in the cross-modality learning phase of 49% suggesting bees have no strong preference for either visual pattern. The success a bee achieved when their conditioned stimuli (Bar temperature patterns, Circle temperature patterns or no patterns, dependent on

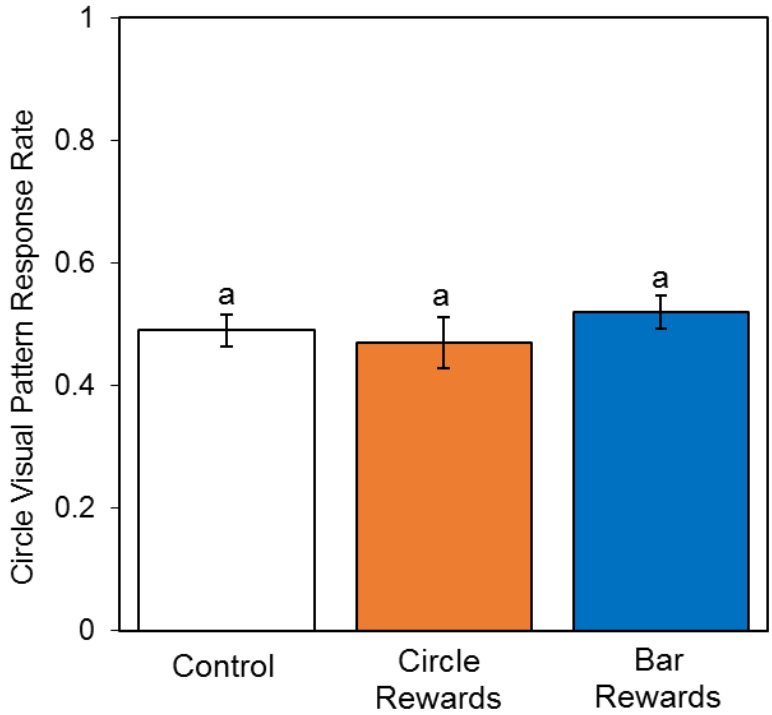


Figure 7.2: *The mean circle pattern response rate ± SEM of bees conditioned in the temperature pattern experiment when presented with matching visual patterns in the cross-modality learning test, ordered by test group. Letters above bars denote groups as defined by post hoc Tukey's test where* $p < 0.05$.

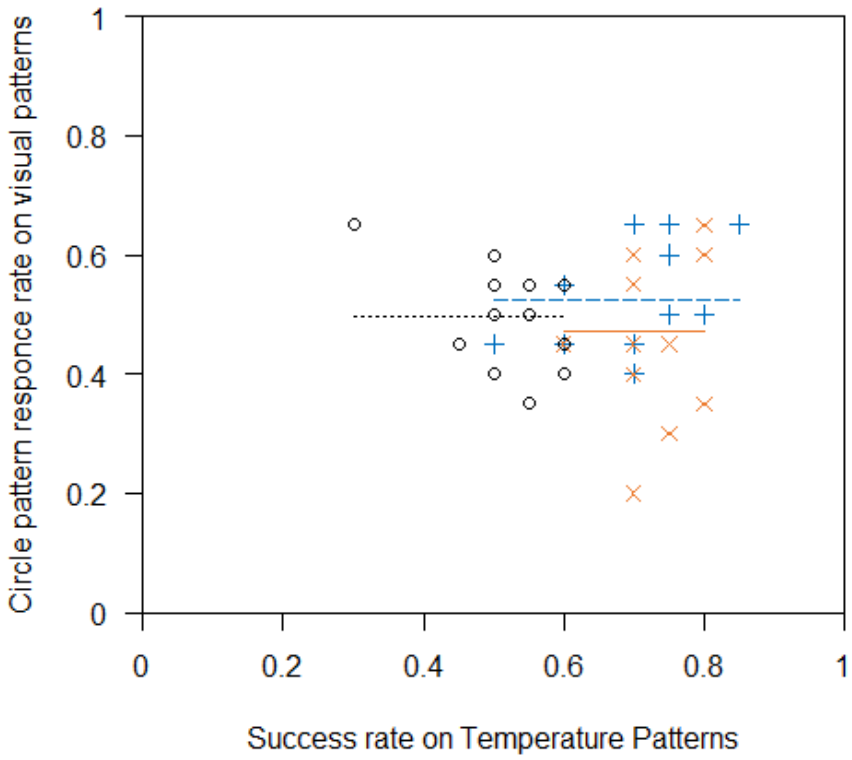


Figure 7.3: *The relationship between success rate of bees in a nonrewarding test of conditioning to temperature patterns and the circle pattern response rate when those bees are presented with matching visual patterns. Lines indicate the average circle pattern response rates for each test group. Shape of points and dashing of lines corresponds with what temperature pattern the bee was conditioned on (their test group), as does colour of both. Black circles, o, and dotted line, control group (no temperature pattern conditioning); orange crosses, x, and solid line, circle rewards (bees conditioned to circle temperature patterns); blue plus signs, +, and dashed line, bar rewards (bees conditioned to bar temperature patterns).*

test group) was tested showed no significant correlation with the circle pattern response rate shown in the visual pattern test, regardless of which test group the bee was in (ANCOVA: *interaction term*, $F_{2,30} = 2.51$, $p = 0.098$; *test group term*, $F_{2,30} = 0.97$, $p = 0.392$; *success in temperature pattern test*, $F_{1,30} = 0.09$, $p = 0.769$, figure 7.3). These findings suggest temperature pattern learning does not inform recognition and learning of matching visual patterns.

When foraging on visual pattern signals in the learning phase, the bumblebees learnt to distinguish flowers when presented with visual pattern cues but not in the control group (figure 7.4a). Models of bumblebee foraging success during the learning phase which allowed individual bees to differ in both their initial success rates (random effects) and learning speed were comparable in terms of AIC (-196.24 *vs*. -192.33, ΔAIC = 3.91) to models which only allowed initial success rates and were not a significantly better fit (Δdeviance = 0.09, df = 2, *p* = 0.955). Models of bumblebee foraging success during the learning phase which allowed bee experience (number of visits made) to have an interacting effect on test group were comparable in terms of AIC (Richards, 2008) and were not a significantly better fit than models that allowed no such effects (AIC -196.24 *vs*. -195.67, ΔAIC = 0.57, Δdeviance = 4.56, df = 2,

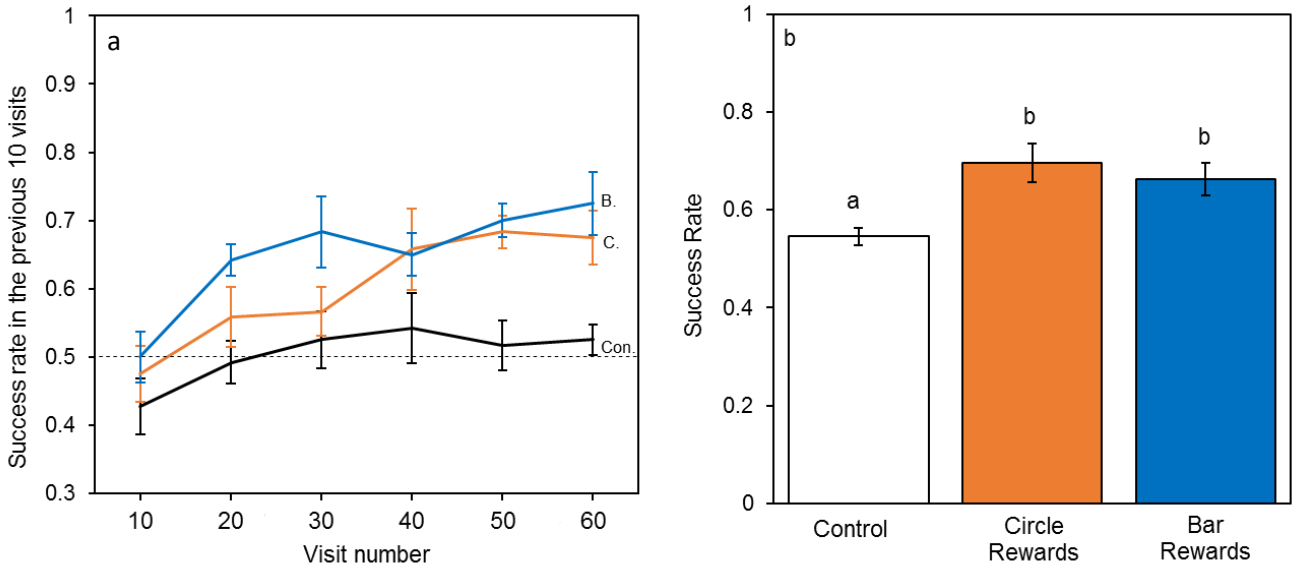


Figure 7.4: *Bee learning when foraging on visual pattern artificial flowers.* ***a****) The relationship between bee foraging success and experience of the artificial flowers (flower visits made) during the learning phase. The dotted line indicates the 50% success level. Solid lines indicate the mean foraging success (± SEM) of bees in the previous ten visits. Colour and label of solid lines and error bars correspond with test group: black, the control group, labelled "Con."; orange, Circle rewards group, labelled "C."; blue, Bar reward group labelled "B.".* ***b****) The mean foraging success (± SEM) of bees in different test groups during the nonrewarding test phase. Letters above bars denote groups as defined by post hoc Tukey's tests where p < 0.05. Twelve bees completed this experiment in each test group (36 bees in total across three nests).*

p = 0.102), meaning that bees showed similar slopes of learning. Models that allowed test groups to vary in success achieved a lower AIC (AIC -195.67 *vs*. -177.43, ΔAIC = 18.24) and a significantly better fit (Δdeviance = 22.24, df = 2, $p < 0.01$) than those that did not, meaning test groups differed in success rate achieved. Experience had a significant effect on bee success independent of test group (AIC -195.67 *vs*. -161.72, ΔAIC = 33.95, Δdeviance = 35.96, df = 1, $p < 0.01$), meaning that bees in all test groups showed improvement in success rate as they made more flower visits, even in the control group. This was largely because control group bees achieved a lower than expected (0.5) success rate early in the learning phase, with success 'improved' to random foraging levels. Meanwhile, circle reward and bar reward test groups began foraging randomly and improved with experience (figure 7.4a). When conditioned visual pattern stimuli were tested, bees presented with visual patterns (the Circle rewards and Bar rewards) achieved a greater success rate than those in the Control group (ANOVA, $F_{2,33} = 6.18$, $p < 0.01$, figure 7.4b).

When bees conditioned to visual patterns were presented with artificial flowers with corresponding temperature patterns, bees did not appear to show a response that was altered by their prior conditioning in the visual modality (figure 7.5). Test group had no effect on the circle pattern response rate (ANOVA, $F_{2,33} = 1.961$, $p = 0.157$). Bees in the control group, which had no prior conditioning to visual patterns showed a mean circle pattern response rate of 61%, suggesting that bees had a preference for the circle temperature pattern over the bar, and this preference is reflected in the similar foraging choices of the other test groups (figure 7.5). The success rate bees achieved in the first test phase had no influence on the later circle response rate (ANCOVA: *interaction term*, $F_{2,30} = 0.59$, $p = 0.561$; *test group term*, $F_{2,30} = 2.01$, $p = 0.152$; *success in visual pattern test*, $F_{1,30} = 0.06$, $p = 0.814$, figure 7.6).

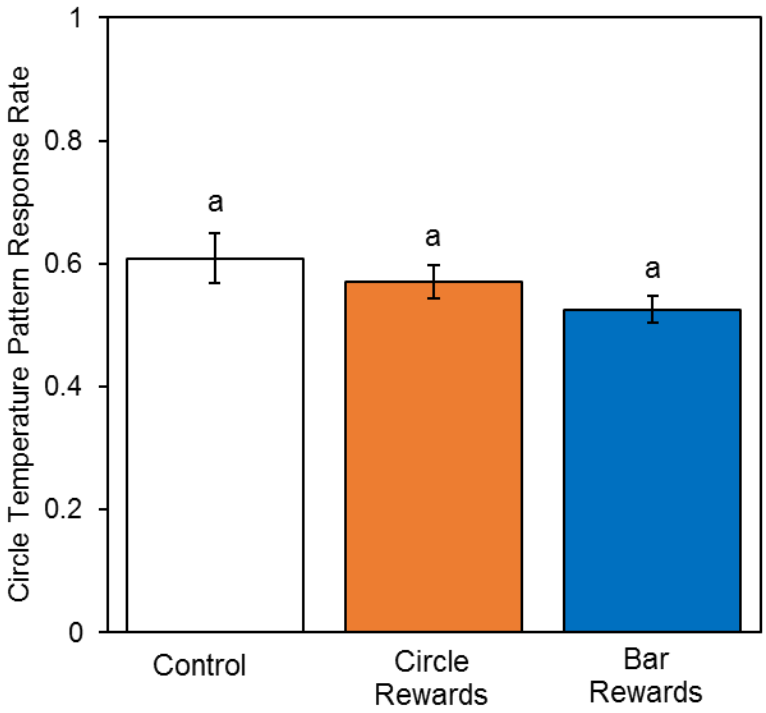


Figure 7.5: *The mean circle pattern response rate (± SEM) of bees conditioned in the visual pattern experiment when presented with matching temperature patterns in the cross-modality learning test, ordered by test group. Letters above bars denote groups as defined by post hoc Tukey's test where $p < 0.05$.*

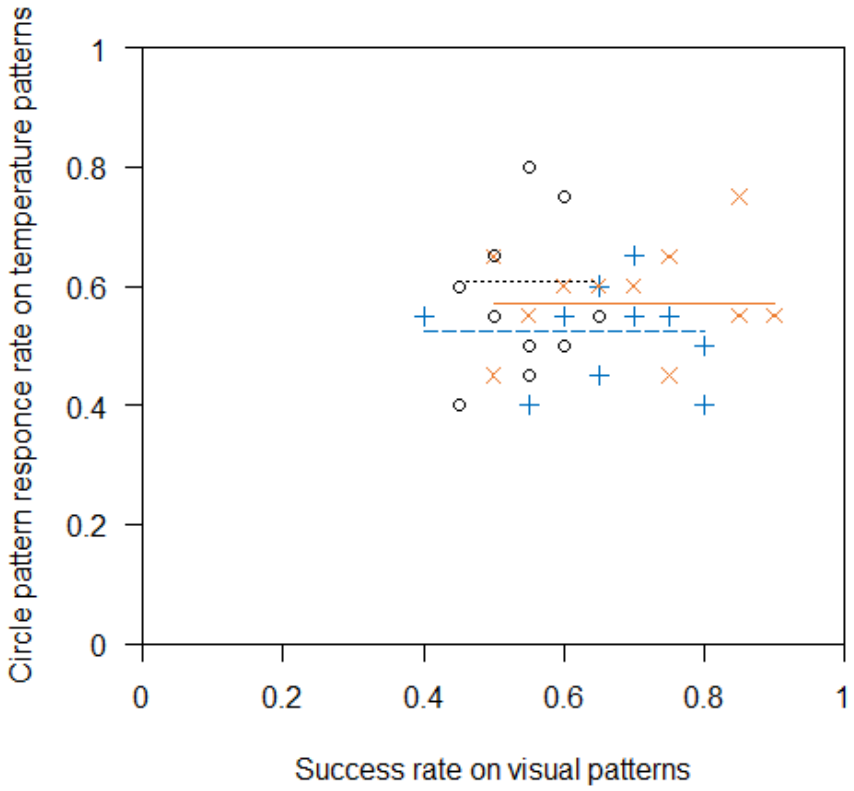


Figure 7.6: *The relationship between success rate of bees in a nonrewarding test of conditioning to visual patterns and the circle pattern response rate when those bees are presented with matching temperature patterns. Lines indicate the average circle pattern response rates for each test group. Shape of points and dashing of lines corresponds with what visual pattern the bee was conditioned on (their test group), as does colour of both. Black circles, o, and dotted line, control group (no temperature pattern conditioning); orange crosses, x, and solid line, circle rewards (bees conditioned to circle temperature patterns); blue plus signs, +, and dashed line, bar rewards (bees conditioned to bar temperature patterns).*

7.4 Discussion

My experiments suggest that cross-modality learning of patterns does not appear to occur in either direction between temperature and visual patterns. Bees conditioned to temperature patterns appeared to forage randomly when presented with matching dark visual patterns (figure 7.2). Likewise, bees conditioned to visual patterns when presented with matching temperature patterns showed a consistent response to their preferred circle temperature pattern (figure 7.5). Bumblebees demonstrated they were capable of learning and recognising the visual and temperature patterns presented to them (figure 3.5 and figure 7.4). Consequently, it is unlikely the lack of cross-modality transfer of pattern learning observed here is the result of bees being unable to detect any of the patterns when presented to them. Such transfers in pattern learning have been seen to occur with bumblebees conditioned to scent patterns when presented with matching visual patterns (Lawson et al., 2018). The results of this study reveal that such cross-modality learning transfer is not universal across all patterned signal modalities and seems to occur between specific patterned signals.

Pattern-learning across different floral signal modalities does not occur between visual and temperature patterns, perhaps because they are detected through more different means. Scent and sight are detected by the bee's antennae and eyes, and have close neurological links (Leonard and Masek, 2014). We do not know if processing of temperature cues is as directly linked to visual processing as scent is. Temperature signals are perceived by touch or conduction, which can occur via the antenna but also across the whole body via their tarsi and other receptors (Heran, 1952; Whitney et al., 2008; Fialho et al., 2014). Consequently, temperature patterns may not elicit similar stimulation and responses to learned visual patterns or vice versa. Thus, cross-modality pattern learning may be limited to modalities that have stronger neurological links. Investigation into other signal modalities' neurological pathways, and the extent of their linkage between them, such as those that have shown scent and visual learning to be linked (Mobbs, 1982; Gronenberg, 2001; Ehmer and Gronenberg, 2002), may identify pairings where such signal transfer might occur. Identification of instances

where cross-modality pattern learning occurs and of the level of neurological links between signalling modalities will help to reveal the underlying cause of these interactions. Although for cross-modality pattern learning to be beneficial to bee foraging success or flower pollination success such modalities need to co-occur and overlap on natural flowers.

Alternatively, this failure to achieve pattern learning between temperature and visual patterns may be a consequence of how bumblebees are interacting with temperature patterns. When a bumblebee lands on a flower with a temperature pattern alone it may not learn the pattern as a whole. It may, for example, learn to associate hot edges of the display with rewards, without 'visualising' the entire circular ring pattern of temperature across the whole flower. Thus, the corresponding visual pattern is not recognised as matching the conditioned temperature pattern and vice versa. However, bees did not require such patrolling of the flower surface to associate a scent pattern with a matching visual pattern and still achieved cue transfer (Lawson et al., 2018). Additionally, bees were able to distinguish temperature patterns of different shapes in a similar location, as in the large artificial flower experiment presented in chapter 3.

The lack of any apparent cross-modality pattern learning responses between visual and temperature patterns does not exclude the possibility that they may show such pattern learning transfer with other overlapping patterns of different modalities. Floral temperature patterns often correspond with other signal modalities. Floral temperature can be influenced by aspects of the flower surface structure, due to texture influencing both heat loss by trapping air (Miller, 1986) and surface area of the flower surface which impacts water loss and light inception (Whitney et al., 2011). Texture signals are detected across the tarsi and antenna of bees (Kevan and Lane, 1985; Whitney et al., 2009a), as is temperature (Heran, 1952; Whitney et al., 2008). Texture and temperature patterns may thus induce a more similar stimulations than temperature and visual patterns. Floral temperature may influence floral volatile emission (Jakobsen and Olsen, 1994; Seymour et al., 2009b), as well as the rates and amount of nectar evaporation (Corbet et al., 1979a; Corbet et al., 1979b) and transpiration (Gates, 1968; Azad

et al., 2007), that contribute to humidity signals (von Arx et al., 2012). Visual patterns can show varied structure that overlaps with many other floral patterns. Floral humidity signals often correspond with the flower centre in a manner similar to many visual signals, chapter 4, although pollinators capacity to distinguish floral humidity structure differences is unknown. Texture-related signals often overlap with visual signals, and this is particularly true where visual patterns are the result of structural aspects of the petal surface, such as floral iridescence (Whitney et al., 2009b; Whitney et al., 2009c; Kjernsmo et al., 2018) or gloss (Glover and Whitney, 2010; Whitney et al., 2012).

Floral temperature and visual signals may not show cross-modality pattern learning, but this does not mean there is necessarily no benefit of possessing both signals. They may interact or reinforce the floral signal in a different manner when they occur together. Many of the proposed hypotheses of why flowers produce multimodal signals are non-exclusive, thus additional modalities may grant different kinds of benefits (Leonard et al., 2012). Visual (Leonard and Papaj, 2011; Goodale et al., 2014) and temperature patterns present at least some ability to function as a nectar guide (chapter 6). Using two modalities at once to recognise and distinguish flower identities has been shown to improve learning speed and accuracy (Goyret et al., 2007; Kulahci et al., 2008; Leonard et al., 2011a; Kaczorowski et al., 2012; Katzenberger et al., 2013). Consequently, multimodal displays with visual and temperature patterns may grant such benefits to salience and recognition. However, the lack of cross-modality learning between temperature and visual patterns has important consequences for our understanding of pollinator interactions with multimodal displays. Much of the evidence of benefits of floral multimodality to pollinators and flowers are based on responses to displays that combine scent and visual signals (Goyret et al., 2007; Kulahci et al., 2008; Katzenberger et al., 2013; Leonard and Masek, 2014). While scent and visual signals are certainly the best understood and likely have the a prominent role in pollinator interactions with floral displays (Leonard and Masek, 2014), it is demonstrated here that benefits found in displays that combine scent and visual signals do not always apply to

combinations of other signal modalities. This highlights the importance to consider pollinator responses to a wide variety of floral signals and more complex multimodal floral displays (with more than two modalities) if we are to properly understand why floral multimodality evolved. That said, several of the benefits of floral multimodal displays have been demonstrated to occur in flowers that combine scent or visual signals with another modality (Dyer and Chittka, 2004a; Dyer et al., 2006; Goyret and Raguso, 2006; Goyret et al., 2008a; Whitney et al., 2009a; Clarke et al., 2013), so it is likely some benefits of multimodal displays are not unique to scent-visual pairings.

In this study, we investigated the capacity of matching temperature and visual patterns to show cross-modality pattern learning transfer like that was described by Lawson et al. (2018) between scent and visual patterns. This has shown that similar pattern learning transfer is not universal among floral modalities. The lack of learning transfer between temperature and visual patterns appears to be due to how bees interact and with temperature patterns or due to differences between the neurological pathways by which temperature, vision and scent learning takes place in bees. These finding demonstrate that the benefits of certain signalling modality combinations do not always apply to all other signalling modality combinations. Despite finding no such cross-modality learning, we have gained important information about how different floral signals might be experienced by bees visiting multimodal displays and how transfer of pattern learning occurs.

Chapter 8: Thesis discussion

8.1 Summary of thesis findings

In this thesis I aimed to expand our understanding of multimodal floral signalling to pollinators. This was done by exploring the largely overlooked floral signalling modalities of floral temperature and floral humidity. Prior to the research presented in this thesis, floral temperature had been demonstrated to function as a floral signal (Dyer et al., 2006) with at least bees being able to use differences in temperature between flowers to distinguish rewarding flowers (Whitney et al., 2008; Hammer et al., 2009). Differences in the arrangement of temperature within flowers, temperature patterns, had been ignored in a flower signalling context, despite being observed in several floral thermal imaging studies (Rejšková et al., 2010; Dietrich and Körner, 2014). Similarly floral humidity had been demonstrated to be produced by *Oenothera caespitosa*, and to be used as a signal by hawkmoth *Hyles lineata* (von Arx et al., 2012; von Arx, 2013), its production by other flower species and it use by other pollinators receiving little research (but see Nordström et al., 2017). Additional floral signalling modalities can have important impacts on pollinator responses to flowers even within multimodal displays (Kunze and Gumbert, 2001; Kaczorowski et al., 2012; Dyer and Chittka, 2004a), and such responses can have great importance to plant and pollinator fitness (Schiestl and Johnson, 2013; Chittka and Raine, 2006; Leonard et al., 2011b). Consequentially, our lack of understanding of these floral signals represents a conspicuous gap in our understanding of the foraging decisions pollinators make, and thus our understanding of plant fitness and evolution. This is most conspicuous in terms of the reasons why flowers evolved multimodal signals, the lack of knowledge of pollinator responses to these individual signals acting as a barrier to incorporating them in multimodal studies of floral displays.

I have demonstrated through the research presented in chapters 3 and 4 that both floral temperature patterns and floral humidity occur widely across several flower species of different lineages. Plants appear to vary in the within flower temperature contrast (appendix

table A3) as well as the shape of temperature patterns (figure 3.3). In terms of floral humidity many flower species show elevated floral humidity levels (table 4.7). I have additionally shown that bumblebees, *Bombus terrestris*, can learn temperature patterns and use these to distinguish rewarding flowers, the first demonstration of a pollinator's ability to do so (chapter 3). This ability to do so seems unaffected by the size of temperature pattern or the flower. Similarly, I have shown bumblebees show a preference for higher floral humidity (figure 5.5), similar to that of hawkmoths (von Arx et al., 2012), but also I demonstrate that bumblebees can use differences in floral humidity intensity between flowers as a signal to recognise rewarding and nonrewarding flowers (Figure 5.6). This represents the first demonstration that floral humidity can be used by a pollinator other than *Hyles lineata*, here by the more generalist and widespread bumblebee. Furthermore, it is the first evidence that floral humidity, as well as a lack of floral humidity, may be learnt by a pollinator to distinguish rewarding flowers. These findings expand our understanding of how multimodal floral displays are. This increases our knowledge of what floral traits pollinators may be responding to. This may help explain pollinator foraging decisions observed in the field (Raine and Chittka, 2007; Peter and Johnson, 2008; Heinrich, 1979b; Riffell and Alarcón, 2013). As additional floral signalling modalities may have several benefits to plant fitness (Leonard et al., 2012; Leonard et al., 2011b) understanding what aspects of the floral display pollinators may respond to is critical to understanding floral evolution. This increases our understanding of what floral traits may be under, or have been subject to, pollinator mediated selection (Schiestl and Johnson, 2013). Furthermore, it reveals other aspects of the floral display pollinators may be responding to, this may help explain foraging patterns observed by pollinators in the field (Galen and Newport, 1988; McCall and Primack, 1992; Hegland et al., 2009).

The discovery of temperature pattern signals and the revelation that humidity signals occur more widely allows such modalities to be incorporated into direct studies of floral multimodality. Such studies are important to our understanding of floral evolution. Within this thesis, I began such investigation of the roles of temperature patterns within multimodal

displays. It was discovered that floral temperature patterns can show some ability to be used as a floral guide for bumblebees (chapter 6) in a manner similar to that demonstrated by visual (Goodale et al., 2014), scent (Lawson et al., 2017b) and tactile patterns (Goyret and Kelber, 2011). However, this function seemed greatly reduced compared to visual patterns (chapter 6). Furthermore, when thermal and contrasting colour patterns were presented together it did not seem that bumblebee flower handing was greatly improved beyond the response to the colour guide (chapter 6). That said our experiments still demonstrate temperature patterns may convey some handling benefit to flowers where visual guides are absent or visual patterns insufficient to elicit a guided response. This suggests temperature patterns may be advantageous in at least some instances as a floral guide, even in flowers where they do not aid flower location or recognition. I additionally show that bumblebees do not show cross-modality pattern learning between temperature and visual patterns (chapter 7). Such cross-modality pattern learning does occur between scent and visual patterns (Lawson et al., 2018). This demonstrates that certain combinations of floral signalling modalities do not convey the same responses and benefits to floral signalling as others. This highlights that is particularly important to include modalities other than just scent and visual in studies of multimodal floral signalling. Although several benefits seen in scent-visual combinations are seen in other modality combinations (Dyer and Chittka, 2004a; Goyret et al., 2008a; Clarke et al., 2013), basing our understanding of multimodality on displays of just scent and visual signals may cause us to gain an inaccurate idea of the benefits and evolution of different signal modalities and floral multimodality as a whole.

8.2 Future research directions

By revealing temperature pattern signals and floral humidity signals to be used by bumblebees, the research presented in this thesis opens new avenues of research into pollinator responses to these floral signals, many of these have been discussed in the relevant chapters (chapters 3, 4 and 5). It is important to further explore the sensitivities of bumblebees,

and other pollinators to variation in these signals. Such studies will determine how widely these signals can be used and the extent to which they can be used to distinguish flowers. It is also important that we investigate the mechanisms by which these signals are generated. This will provide a good grounding for understanding their function within multimodal floral displays. This will also provide more information on what floral characteristics may be subject to selection based on pollinator responses to these signals.

8.2.1 *Floral temperature patterns and floral humidity in multimodal displays*

It is important that floral humidity and temperature pattern signals be considered further in terms of floral multimodality and their functions within multimodal floral displays. We have shown temperature patterns to have some limited guide function (chapter 6), and that it does not appear to be the case that cross-modality pattern learning leads to temperature pattern evolution within multimodal displays (chapter 7). However various other benefits of additional floral signals have been proposed and demonstrated with other signals (Leonard et al., 2011b; Leonard et al., 2012; Leonard and Masek, 2014) and these are yet to be explored in terms of floral temperature pattern or humidity signals. Floral humidity has the potential to function as an honest signal of nectar rewards (von Arx et al., 2012; von Arx, 2013). My findings do show this is potentially possible (chapter 5). However, further investigation into the mechanisms by which floral humidity is generated in flowers, particularly the influences of nectar removal on floral humidity, are needed to evaluate how honest an indicator of nectar presence humidity is in species other than *O. caespitosa*. Additionally, studies of the sensitivities of pollinators to any associated change in floral humidity caused by nectar removal will be required. We do find some evidence that flower age may be indicated by floral humidity, however, a more direct study controlling for flower age would be required to be sure of this potential function. As bumblebees can learn floral humidity signals if it does not function honestly, it might still be used for flower recognition. It may be particularly worthwhile to investigate how these signals influence flower detection and learning when combined with other signalling modalities. This

would involve replicating similar studies methodologies (Clarke et al., 2013; Kunze and Gumbert, 2001) with temperature pattern or floral humidity signals in a manner not unlike the studies presented in chapter 6 (Goodale et al., 2014; Lawson et al., 2017b) and 7 (Lawson et al., 2018). As humidity may be associated with liquid rewards, it may be function as a context signal for nectar feeding pollinators, like scent (Kunze and Gumbert, 2001; Raguso and Willis, 2002) and CO_2 (Goyret et al., 2008a; Goyret, 2008) have been demonstrate to. Thus, this research opens further ways in which floral multimodality can be investigated.

A further avenue for research of temperature and humidity signals is investigation of their signalling with environmental variability. Temperature pattern generation seems to depend on influences of light conditions and environmental temperature (Rejšková et al., 2010; Dietrich and Körner, 2014; Shrestha et al., 2018). In this way exploring weather effects on floral temperature patterns is necessary to investigate the mechanisms generating these signals (discussed in chapter 3). Floral humidity is likely directly influenced by wind, like scent (Lawson et al., 2017a). However, it is also probably influenced indirectly by environmental conditions that allow nectar evaporation (Corbet et al., 1979a; Corbet et al., 1979b) and transpiration (Gates, 1968; Azad et al., 2007). Thus, environmental temperature and water availability likely play a key role in floral humidity signalling. In addition to furthering our mechanistic understanding of these signals, such research would also allow evaluation of how floral signalling is affected by environmental variability and change (Shrestha et al., 2018). Additionally understanding how signals are disrupted by variable conditions is necessary to investigate whether these or other modality signals may function as a backup for floral signalling within variable conditions, the efficacy-backup hypothesis (Lawson et al., 2017a; Dyer and Chittka, 2004a; Kaczorowski et al., 2012). Thus, understanding disruption of these signals by environmental conditions may improve our understanding of the mechanisms of these signals' generation and environmental effects on pollination, but also may aid our understanding of floral evolution and multimodality.

8.2.2 *Signal interactions between floral humidity and floral temperature*

Of particular interest may be investigation of how these two modalities, floral humidity and floral temperature patterns interact. Floral temperature and temperature patterns were not monitored alongside humidity transects (chapter 4). However, temperature is an important component in transpiration and evaporation (Gates, 1968; Azad et al., 2007). So, the flower's temperature is likely to influence humidity signal generation. Furthermore, which parts of the flower are hotter may also moderate the structure of humidity signals, promoting evaporation or transpiration from a certain part of the flower. Conversely, evaporation and transpiration may cause temperature loss at certain parts of the flower, contributing to the generation of temperature patterns. Thus, these two signals may be linked in some way. That said bees can detect both signals independently of each other (chapters 3 and 5). Consequentially, exploring how these different floral traits influence each other, as well as pollinators' responses to these different modalities presented together may reveal some interesting interactions that have not yet been explored. These effects may be further complicated by the differing environmental influences on these signals. It has shown very hot flowers may deter bees (Norgate et al., 2010; Shrestha et al., 2018). Flowers have shown some capacity to prevent overheating (Shrestha et al., 2018), this possibly involves transpiration. This may mean overheated flowers may have increased humidity generation. Elevated humidity is also preferred by bees, thus may compensate for the bee's response to overheating.

8.2.3 *Signal characteristics and their role in multimodal displays*

More generally our research into floral temperature's potential uses within multimodal floral displays identified several aspects of a floral signalling modality that may influence its functionality within a multimodal display. The range at which a signal is received appeared to influence how well a signal functions as a floral guide (chapter 6). Temperature patterns functioning less well as guides compared to longer range visual patterns. In previous studies

scent patterns, a long range signal, have shown functionality more similar to visual guides (Lawson et al., 2017b). Goyret and Kelber (2011) similarly found visual guides took priority over tactile signals, another short-range signal. In a similar manner cross-modality pattern learning appears to be influenced by either, how the pollinator interacts with the signal, which itself may again be a product of the signal range, or the neurological links between signals (chapter 7). Many different signalling modalities differ in their effective range (Hempel de Ibarra and Menzel, 2014; Dyer et al., 2006; Goyret et al., 2008a; Balkenius and Dacke, 2010). It is possible that the range signal modality influences what functions it can show within multimodal displays and therefore the benefits it confers to the plant. It may be valuable to understanding the evolution of floral multimodality to explore what effects different ranged modalities have on pollinator responses, and whether we see common responses depending on signal range. Additionally, what impacts combinations of modalities of different effective ranges have on responses and learning of multimodal displays. The findings of chapter 7 also justify further investigation of whether other modalities show neurological links, and how this influences inter-signal interactions and the effect of combination of these modalities within floral displays.

8.2.4 *Other overlooked floral signal modalities*

The findings in this thesis that a largely overlooked floral signal such as floral humidity may be used more widely encourages further research into other signals that have been largely overlooked. It seems unlikely the acoustic signals associated with bats are used by insects, insects not emitting sound similar to echolocation and having comparably limited hearing (Dreller and Kirchner, 1995; Yack, 2004; Göpfert and Hennig, 2016). However, floral carbon dioxide emissions may, in a similar vain to humidity, be more widespread than previously demonstrated (Guerenstien et al., 2004; Guerenstien and Hildebrand, 2008). CO_2 emissions from *Datura* flowers have been shown to function as a long-range signal for moths (Guerenstien et al., 2004; Thom et al., 2004; Goyret et al., 2008a). However, the ability of other flowers to produce similar CO_2 signals is unknown. That said respiration produces CO_2

and metabolic activity in the flower, particularly nectar production, may lead to a CO_2 plume similar to in *Datura* (Hew et al., 1978; Guerenstien et al., 2004). Furthermore, insects other than moths, including bees and flies, are regularly demonstrated to respond to environmental CO_2 levels (Stange and Diesendorf, 1973; Jones, 2013). Honeybees have even been demonstrated to be capable of conditioning to ambient CO_2 levels (Lacher, 1967) and has been observed feeding from *Datura* flowers (Guerenstien and Hildebrand, 2008). Thus, monitoring of a wider range of flower species for above ambient CO_2 emissions and associated study of pollinator responses, like research carried out here on floral humidity (chapter 4 and 5), may prove useful in similarly expanding the scope of floral multimodality.

8.3 Methodological developments within the thesis

A secondary goal of this thesis was to improve existing and develop new methodologies and technologies that might aid study of these overlooked signalling modalities. In terms of this goal, I have highlighted several common mistakes within the literature in the reporting of thermography-based biology research (chapter 2). I also set out guidelines that will hopefully increase the accuracy and value of research using thermographic tools in the future. Such guidelines should make application of thermographic tools to study floral temperature easier. In terms of floral humidity monitoring, I have developed a novel method for monitoring floral humidity with several advantages over methods applied to monitoring this signal previously (chapter 4). This method of transecting humidity probes supported by a robot arm has a further advantage of being programmable. Thus, this tool can be applied to other humidity monitoring tasks, many of which I have discussed previously (chapter 4, chapter 5 and above). Additionally, our humidity transects revealed that in most instances the humidity at the approximate flower centre is a good estimate of the intensity of floral humidity (table 4.7), at least in the radial flower species sampled in chapter 4. This means a fixed probe set up to monitor humidity at a point above the flower centre (with appropriate background humidity monitoring) should be a suitable estimate of floral humidity intensity, although not providing

information on structure of humidity. This might be well applied to monitoring humidity intensity in the field, or when such robotic tools are not available. Thus, the research in this thesis will hopefully aid other researchers in incorporating floral humidity and temperature into their research.

8.4 Thesis conclusion

Floral multimodality, its evolution and the benefits it conveys to plants, remains a major focus of pollination biology research (Leonard et al., 2011b; Leonard et al., 2012; Kaczorowski et al., 2012; Leonard and Masek, 2014; Nordström et al., 2017; Lawson et al., 2018). While potential benefits of multimodality are beginning to be understood, as floral displays are made up of many signalling modalities we must incorporate a wide range of floral signalling modalities into future research if we are to gain a full understanding of multimodality's effects in plant pollinator signalling. In this thesis, by investigating floral temperature patterns and floral humidity, I demonstrate that there are more aspects of the floral display that pollinators can respond to than previously known. Identification of these additional signal modalities gives us a more complete idea of what aspects of the floral display pollinators may be responding to. I also provide guidelines and new methods by which thermal and humidity signals could be investigated, facilitating incorporation of these modalities into future research. Incorporating these overlooked modalities and others into future research, alongside scent and visual signals, will allow us to gain a better understanding of how pollinators respond to natural, and therefore multimodal, floral displays. Consideration of many floral signalling modalities may reveal differences and similarities between floral displays not apparent in one modality alone. Understanding pollinator responses to multimodal displays, and the differences between floral displays across modalities, may be important in explaining differences in pollinator responses to different displays. In this way a multimodal approach may help explain the interactions between plants and pollinators and the patterns of pollen transport we observe in nature. Understanding the full range of floral display traits that pollinators may respond to, and their

influence pollinator behaviour and plant fitness when presented together, will allow a greater understanding of the drivers of floral evolution. Likewise, understanding the range of signals presented by flowers, on how this aids pollinator foraging success, may help explain the evolution of pollinator floral preferences and sensitivities. Thus, the findings presented within this thesis, by expanding the scope of floral multimodality and the tools by which largely overlooked modalities can be studied, improves our understanding of floral signalling but can also be applied to aid a more multimodal approach to future pollination ecology and evolution research.

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Appendix

A1: A summary of the search used in our systematic literature review of *Web of Science*

The full literature search presented in chapter 2, was made up of several searches. Each search made is identified by the order in wich the searches were made (#1, #2 etc.). Settings applied for various *Web of Science* criteria are given within each search. Throughout all searches the WOS criteria Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH and ESCI Timespan=2007-2017

#1: TOPIC=(INFRARED)

#2: TOPIC=(INFRA-RED)

#3: TOPIC=("INFRA RED")

#4: TOPIC=(THERMOGRAPH*)

#5: TOPIC=("THERMAL IMAG*")

#6: TOPIC=(CAMERA*)

#7: #3 or #2 or #1

#8: #6 or #5 or #4

#9: #8 and #7

#10: #9 Refined by publication years: (2007 OR 2008 OR 2009 OR 2010 OR 2011 OR 2012 OR 2013 OR 2014 OR 2015 OR 2016 OR 2017 OR 2018)

#11: #10 Refined by: *Web of Science* categories: (AGRICULTURE DAIRY ANIMAL SCIENCE or AGRICULTURE MULTIDISCIPLINARY or AGRONOMY or BEHAVIORAL SCIENCES or BIOLOGY or BIOPHYSICS or ECOLOGY or ENTOMOLOGY or EVOLUTIONARY BIOLOGY or FISHERIES or FORESTRY or HORTICULTURE or MARINE FRESHWATER BIOLOGY or ORNITHOLOGY or PHYSIOLOGY or PLANT SCIENCES or PSYCHOLOGY or PSYCHOLOGY APPLIED or PSYCHOLOGY BIOLOGICAL or PSYCHOLOGY EXPERIMENTAL or PSYCHOLOGY MULTIDISCIPLINARY or VETERINARY SCIENCES or ZOOLOGY)

A2: Weather data pertaining to thermography carried out in chapter 3

Appendix table 10.1: *Hourly weather data is provided for each hour thermographs presented in chapter 3 were collected. All weather data was obtained from the nearest Met Office weather station: for Bristol survey days, Filton weather station (51°31'15.6"N 2°34'33.6"W); for Botanic Garden of Wales survey days, Aberporth weather station (52°07'48.0"N 4°32'20.4"W).*

Date	Hour	Thermograph Sampling Location	Met Office Weather station location	Hourly Temperature (°C)	Hourly Relative Humidity (%)	Hourly Total Cloud Cover (oktas)	Hourly Pressure at Mean Sea Level (hPa)	Hourly Rainfall Total (mm)
04/06/2013	13	Bristol	Filton	19.7	n/a	0	1026	0.0
06/05/2014	9	Bristol	Filton	11.9	79.5	7	1007	0.0
25/06/2014	10	Bristol	Filton	16.9	51.2	7	1019	0.0
25/06/2014	11	Bristol	Filton	18.4	48.3	1	1019	0.0
25/06/2014	12	Bristol	Filton	18.6	47.8	0	1018	0.0
25/06/2014	13	Bristol	Filton	19.9	41.9	0	1018	0.0
25/06/2014	14	Bristol	Filton	19.8	41.7	1	1018	0.0
25/06/2014	15	Bristol	Filton	20.7	40.9	2	1017	0.0
26/06/2014	10	Bristol	Filton	18.0	50.3	4	1015	0.0
26/06/2014	11	Bristol	Filton	18.6	45.4	1	1014	0.0
26/06/2014	12	Bristol	Filton	20.2	44.0	1	1014	0.0
26/06/2014	13	Bristol	Filton	20.8	42.7	0	1014	0.0
26/06/2014	14	Bristol	Filton	19.8	46.5	5	1013	0.0
26/06/2014	15	Bristol	Filton	19.0	49.4	8	1013	0.0
09/02/2015	15	Bristol	Filton	7.2	68.0	0	1035	0.0
18/02/2015	14	Bristol	Filton	10.1	63.9	2	1035	0.0
18/02/2015	15	Bristol	Filton	10.0	63.9	7	1035	0.0
24/03/2015	10	Bristol	Filton	7.4	67.3	7	1012	Trace
24/03/2015	11	Bristol	Filton	7.3	58.9	5	1011	0.0
24/03/2015	15	Bristol	Filton	7.9	66.5	6	1010	0.0
26/03/2015	11	Bristol	Filton	10.6	71.2	8	1003	0.0
26/03/2015	15	Bristol	Filton	10.4	57.7	6	1008	0.0
23/04/2016	12	Botanic Garden of Wales	Aberporth	8.3	68.2	0	1024	0.0
26/04/2016	12	Botanic Garden of Wales	Aberporth	6.6	70.4	7	1013	0.0
26/04/2016	13	Botanic Garden of Wales	Aberporth	6.8	69.2	6	1013	0.0
26/04/2016	14	Botanic Garden of Wales	Aberporth	7.1	72.4	3	1013	0.0

Date	Hour	Thermograph Sampling Location	Met Office Weather station location	Hourly Temperature (°C)	Hourly Relative Humidity (%)	Hourly Total Cloud Cover (oktas)	Hourly Pressure at Mean Sea Level (hPa)	Hourly Rainfall Total (mm)
06/05/2016	11	Botanic Garden of Wales	Aberporth	12.0	79.5	7	1011	0.0
06/05/2016	12	Botanic Garden of Wales	Aberporth	13.1	58.4	8	1010	Trace
08/05/2016	10	Botanic Garden of Wales	Aberporth	18.1	71.9	7	1004	0.0
14/05/2016	12	Botanic Garden of Wales	Aberporth	11.8	68.7	0	1022	0.0
04/06/2016	11	Botanic Garden of Wales	Aberporth	15.7	71.6	6	1018	0.0
08/06/2016	16	Bristol	Filton	23.3	63.0	2	1023	0.0
23/06/2016	10	Botanic Garden of Wales	Aberporth	14.7	71.8	4	1018	0.0
01/07/2016	11	Botanic Garden of Wales	Aberporth	13.3	70.5	8	1007	0.0
01/07/2016	12	Botanic Garden of Wales	Aberporth	15.1	63.1	5	1007	0.0
01/07/2016	15	Botanic Garden of Wales	Aberporth	14.7	68.9	6	1007	0.0
03/07/2016	9	Botanic Garden of Wales	Aberporth	12.8	90.5	0	1018	0.0
05/07/2016	15	Botanic Garden of Wales	Aberporth	15.1	63.9	0	1022	0.0
06/07/2016	12	Botanic Garden of Wales	Aberporth	16.7	68.6	8	1023	0.0
13/07/2016	12	Botanic Garden of Wales	Aberporth	14.1	71.2	2	1022	0.0
14/07/2016	12	Botanic Garden of Wales	Aberporth	14.3	75.5	3	1027	0.0
14/07/2016	13	Botanic Garden of Wales	Aberporth	14.5	75.7	2	1028	0.0
			Mean	**14.3**	**62.4**	**3.9**	**1016.8**	**0.0**
			SD	**4.7**	**12.4**	**3.0**	**8.0**	**0**

Appendix table 10.2: *Daily weather data for days where thermographic sampling described in chapter 3 took place. All weather data was obtained from the nearest Met Office weather station: for Bristol survey days, Filton weather station; for Garden of Wales survey days Saron weather station (52°01'N 4°37'W) for daily temperature and rainfall data, Aberporth weather station for all other data.*

Date	Thermograph Sampling Location	Met Office Weather station location	Daily Maximum Temperature (0900-0900) (^{O}C)	Daily Minimum Temperature (0900-0900) (^{O}C)	Daily Mean Temperature (0900-0900) (^{O}C)	Daily Total Rainfall (0900-0900)(mm)	Daily Mean Windspeed (0100-2400) (kn)	Daily Maximum Gust (0100 - 2400) (kn)	Daily Total Sunshine(0100-2400) (hrs)
04/06/2013	Bristol	Filton	20.8	8.4	14.6	0.0	10	23	14.9
06/05/2014	Bristol	Filton	16.1	9.5	12.8	1.8	10	21	6.1
25/06/2014	Bristol	Filton	21.5	12.4	17.0	0.0	7	15	10.0
26/06/2014	Bristol	Filton	21.2	13.8	17.5	3.2	7	21	1.8
09/02/2015	Bristol	Filton	7.8	-4.2	1.8	0.2	2	9	8.7
18/02/2015	Bristol	Filton	10.7	2.7	6.7	Trace	10	21	6.3
24/03/2015	Bristol	Filton	8.9	4.0	6.5	1.8	5	20	4.2
26/03/2015	Bristol	Filton	12.1	3.6	7.9	0.0	11	31	4.6
23/04/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	12.2	2.4	7.3	0.0	9	21	10.1
26/04/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	9.1	1.4	5.3	4.4	23	39	5.1
04/05/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	14.6	5.3	10.0	0.0	12	24	11.0
06/05/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	19.9	4.7	12.3	0.1	3	9	1.1
08/05/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	24.1	13.0	18.6	0.0	14	33	6.9
14/05/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	15.0	8.8	11.9	0.0	6	13	11.4
08/06/2016	Bristol	Filton	24.1	14.8	19.5	Trace	4	11	3.6
23/06/2016	Bristol	Filton	20.0	15.4	17.7	Trace	7	20	0.5
01/07/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	16.0	11.7	13.9	3.3	18	34	5.3
03/07/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	18.7	9.2	14.0	Trace	8	17	15.1
05/07/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	16.3	11.0	13.7	0.0	13	28	9.7
06/07/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	17.7	6.2	12.0	1.2	10	21	1.6
13/07/2016	Botanic Garden of Wales, Carmarthen	Saron/Aberporth	16.8	9.3	13.1	3.4	12	22	7.7
		Mean	**16.4**	**7.8**	**12.1**	**1.1**	**9.6**	**21.6**	**6.9**
		SD	**4.8**	**5.0**	**4.7**	**1.5**	**4.8**	**7.9**	**4.1**

A3: A summary of temperature patterns presented by all 118 species

Appendix table 10.3: *A summary of the temperature patterns observed on each of the 118 species thermographed, and the additional 18 cultivars and subspecies, in chapter 3. Species are ordered taxonomically. The temperature at the hottest and coldest region of the flower and the difference in temperature between these points is also given. Plants derived from the same species where counted together for occurrence or average temperature difference calculations. Plants not used in the calculations are marked with a '*' next to their Δ temp. value. The individual thermographs used for measurements of each species can be found in the Supplementary Data for the published version of this chapter https://datadryad.org/resource/doi:10.5061/dryad.qp244.*

Plant species	Order	Family	Floral Symmetry (Radial/Bilateral)	Time	Date	Location	Hot region temp. °c	Cold region temp. °c	Δ temp. °c
Anthurium scherzerianum	Alismarales	Araceae	Bilateral	14:37	26/06/2014	Bristol	24.8	23.3	1.5
Zantedeschia aethiopica	Alismarales	Araceae	Bilateral	14:58	26/06/2014	Bristol	20.9	19.9	1
Astrantia major	Apiales	Apiaceae	Radial	11:22	26/06/2014	Bristol	19.2	18.7	0.5
Allium cristophii	Asparagales	Amaryllidaceae	Radial	13:16	04/06/2013	Bristol	32.2	29.5	2.7
Crinum × powellii	Asparagales	Amaryllidaceae	Bilateral	11:59	25/06/2014	Bristol	30.9	25	5.9
Galanthus nivalis	Asparagales	Amaryllidaceae	Radial	15:10	18/02/2015	Bristol	9.6	8.2	1.4
Narcissus hispanicus	Asparagales	Amaryllidaceae	Radial	15:13	18/02/2015	Bristol	10.5	9.4	1.1
Narcissus pseudonarcissus	Asparagales	Amaryllidaceae	Radial	10:45	24/03/2015	Bristol	12.8	8.1	4.7
Tulbaghia violacea	Asparagales	Amaryllidaceae	Radial	11:39	26/06/2014	Bristol	22.7	21.1	1.6
Hyacinthoides non-scripta	Asparagales	Asparagaceae	Radial	12:48	26/04/2016	Wales	12.4	10.3	2.1
Paradisea lusitanica	Asparagales	Asparagaceae	Radial	11:36	26/06/2014	Bristol	21.8	20.6	1.2
Crocosmia 'Lucifer'	Asparagales	Iridaceae	Bilateral	13:45	25/06/2014	Bristol	30.7	24.9	5.8
Crocus vernus	Asparagales	Iridaceae	Radial	14:46	18/02/2015	Bristol	13.9	11.7	2.2*
Crocus × stellaris	Asparagales	Iridaceae	Radial	14:44	18/02/2015	Bristol	12.3	11.2	1.1
Cypella herbertii	Asparagales	Iridaceae	Radial	14:32	26/06/2014	Bristol	24	22.3	1.7
Dietes bicolor	Asparagales	Iridaceae	Radial	14:46	26/06/2014	Bristol	21.9	21.7	0.2
Iris sibirica	Asparagales	Iridaceae	Bilateral	15:28	09/02/2015	Bristol	6.3	6	0.3

Plant species	Order	Family	Floral Symmetry (Radial/Bilateral)	Time	Date	Location	Hot region temp. °c	Cold region temp. °c	Δ temp. °c
Iris unguicularis	Asparagales	Iridaceae	Bilateral	13:25	04/06/2013	Bristol	26	21.3	4.7
Hemerocallis 'Autumn Red'	Asparagales	Xanthorrhoeaceae	Radial	13:47	25/06/2014	Bristol	35.5	26.8	8.7*
Hemerocallis sp.	Asparagales	Xanthorrhoeaceae	Radial	15:54	26/03/2015	Bristol	11.1	10.8	0.3
Pasithea caerulea	Asparagales	Xanthorrhoeaceae	Radial	12:39	23/04/2016	Wales	21	20.1	0.9
Arctotis acaulis	Asterales	Asteraceae	Radial	14:36	26/06/2014	Bristol	24.7	22.9	1.8
Bellis perennis	Asterales	Asteraceae	Radial	13:41	25/06/2014	Bristol	29.3	22.3	7
Brachyscome iberidifolia	Asterales	Asteraceae	Radial	11:37	01/07/2016	Wales	27.2	22.4	4.8
Coreopsis verticillata	Asterales	Asteraceae	Radial	13:47	25/06/2014	Bristol	32.8	23.3	9.5
Dahlia coccinea	Asterales	Asteraceae	Radial	14:47	26/06/2014	Bristol	20.3	19.6	0.7
Dimorphotheca pluvialis	Asterales	Asteraceae	Radial	12:00	23/04/2016	Wales	26	19.6	6.4
Dimorphotheca sinuata	Asterales	Asteraceae	Radial	13:31	04/06/2013	Bristol	38.7	30.1	8.6
Dimorphotheca sp.	Asterales	Asteraceae	Radial	12:00	23/04/2016	Wales	26.8	19.9	6.9
Felicia amelloides	Asterales	Asteraceae	Radial	15:29	09/02/2015	Bristol	10.1	7.6	2.5
Gazania 'Daybreak Bright Orange'	Asterales	Asteraceae	Radial	15:45	01/07/2016	Wales	26.1	21.8	4.3*
Gazania rigens	Asterales	Asteraceae	Radial	13:31	04/06/2013	Bristol	39.7	30.2	9.5*
Gazania rigens 'Cookie'	Asterales	Asteraceae	Radial	12:55	01/07/2016	Wales	27.3	23.3	4
Helichrysum sp.	Asterales	Asteraceae	Radial	12:20	23/04/2016	Wales	24.3	19.8	4.5
Leucanthemum vulgare	Asterales	Asteraceae	Radial	11:24	04/06/2016	Wales	25.6	19.4	6.2
Matricaria chamomilla	Asterales	Asteraceae	Radial	14:59	25/06/2014	Bristol	25	19.9	5.1
Osteospermum jucundum	Asterales	Asteraceae	Radial	13:18	04/06/2013	Bristol	33.8	22	11.8
Rhodanthe chlorocephala	Asterales	Asteraceae	Radial	12:22	23/04/2016	Wales	26.6	20.6	6
Taraxacum officinale	Asterales	Asteraceae	Radial	14:36	04/05/2016	Wales	27	17.2	9.8
Xerochrysum bracteatum	Asterales	Asteraceae	Radial	12:09	23/04/2016	Wales	22	17.3	4.7

Plant species	Order	Family	Floral Symmetry (Radial/Bilateral)	Time	Date	Location	Hot region temp. °c	Cold region temp. °c	Δ temp. °c
Xerochrysum bracteatum 'Florabella Pink'	Asterales	Asteraceae	Radial	12:21	23/04/2016	Wales	27.8	22	5.8*
Zinnia peruviana 'Red Spider'	Asterales	Asteraceae	Radial	15:02	26/06/2014	Bristol	20.6	20.2	0.4
Campanula persicifolia	Asterales	Campanulaceae	Radial	15:12	26/06/2014	Bristol	19.2	18.7	0.5
Campanula portenschlagiana	Asterales	Campanulaceae	Radial	12:25	25/06/2014	Bristol	26.4	22.2	4.2
Campanula poscharskyana	Asterales	Campanulaceae	Radial	14:19	18/02/2015	Bristol	9.4	8.9	0.5
Borago officinalis	Boraginales	Boraginaceae	Radial	14:57	25/06/2014	Bristol	23.9	22.3	1.6
Brunnera macrophylla	Boraginales	Boraginaceae	Radial	11:45	06/05/2016	Wales	23.5	21.4	2.1
Arabis alpina	Brassicales	Brassicaceae	Radial	11:52	06/05/2016	Wales	24.3	23	1.3
Aubrieta deltoidea	Brassicales	Brassicaceae	Radial	11:40	24/03/2015	Bristol	12.5	11.3	1.2
Silene coronaria	Caryophyllales	Caryophyllaceae	Radial	15:19	25/06/2014	Bristol	24.7	21.9	2.8
Hydrangea macrophylla	Cornales	Hydrangeaceae	Radial	13:38	25/06/2014	Bristol	24.2	22	2.2
Hypericum calycinum	Cornales	Hypericaceae	Radial	15:20	25/06/2014	Bristol	28.8	24.5	4.3
Begonia coccinea	Cucurbitales	Begoniaceae	Bilateral	14:39	26/06/2014	Bristol	24.2	23.5	0.7
Knautia macedonica	Disacales	Caprifoliaceae	Radial	15:11	26/06/2014	Bristol	31.8	22.6	9.2*
Knautia macedonica 'Red Knight'	Disacales	Caprifoliaceae	Radial	13:51	25/06/2014	Bristol	19.8	19.4	0.4
Impatiens tinctoria	Ericales	Balsaminaceae	Bilateral	15:08	26/06/2014	Bristol	18.6	16.4	2.2
Azalea sp.	Ericales	Ericaceae	Bilateral	12:01	06/05/2016	Wales	21.1	20	1.1
Rhododendron carolinianum 'P.J. Mezitt'	Ericales	Ericaceae	Radial	10:37	08/05/2016	Wales	27.2	23.5	3.7
Rhododendron sp. (white)	Ericales	Ericaceae	Radial	10:39	08/05/2016	Wales	26	24	2
Polemonium foliosissimum	Ericales	Polemoniaceae	Radial	11:24	26/06/2014	Bristol	19	18.5	0.5
Primula vulgaris (mauve form)	Ericales	Primulaceae	Radial	14:16	18/02/2015	Bristol	17.8	13.2	4.6

Plant species	Order	Family	Floral Symmetry (Radial/Bilateral)	Time	Date	Location	Hot region temp. °c	Cold region temp. °c	Δ temp. °c
Primula vulgaris (white form)	Ericales	Primulaceae	Radial	10:14	08/05/2016	Wales	32.6	27.1	5.5*
Camellia fraterna	Ericales	Theaceae	Radial	11:48	06/05/2016	Wales	21.6	20.2	1.4
Lotus corniculatus	Fabales	Fabaceae	Bilateral	12:30	14/05/2016	Wales	18.7	16.4	2.3
Mimosa pudica	Fabales	Fabaceae	Radial	14:42	26/06/2014	Bristol	26.6	26	0.6
Piptanthus napalensis	Fabales	Fabaceae	Bilateral	13:11	04/06/2013	Bristol	26	21.3	4.7
Vinca herbacea	Gentianales	Apocynaceae	Radial	11:51	25/06/2014	Bristol	28.2	23.7	4.5
Vinca minor	Gentianales	Apocynaceae	Radial	11:42	06/05/2016	Wales	28.8	25.1	3.7
Geranium albanum	Geraniales	Geraniaceae	Radial	11:15	26/03/2015	Bristol	19.1	19	0.1
Geranium californicum	Geraniales	Geraniaceae	Radial	12:25	23/04/2016	Wales	21.5	19.4	2.1
Geranium pratense	Geraniales	Geraniaceae	Radial	13:04	14/07/2016	Wales	25.1	20.7	4.4
Geranium procurrens	Geraniales	Geraniaceae	Radial	11:29	26/06/2014	Bristol	21.6	18.6	3
Geranium psilostemon	Geraniales	Geraniaceae	Radial	12:39	14/07/2016	Wales	29.3	22.3	7
Geranium pyrenaicum	Geraniales	Geraniaceae	Radial	14:32	25/06/2014	Bristol	25.3	23.9	1.4
Geranium sylvaticum	Geraniales	Geraniaceae	Radial	12:26	13/07/2016	Wales	23.4	20.2	3.2
Pelargonium cucullatum	Geraniales	Geraniaceae	Bilateral	15:53	26/03/2015	Bristol	11.1	10.7	0.4
Pelargonium echinatum	Geraniales	Geraniaceae	Bilateral	15:54	26/03/2015	Bristol	11	10.5	0.5
Pelargonium quercifolium	Geraniales	Geraniaceae	Bilateral	14:31	26/06/2014	Bristol	19.7	19.5	0.2
Acanthus hungaricus	Lamiales	Acanthaceae	Bilateral	11:50	26/06/2014	Bristol	24.1	19.5	4.6
Nepeta 'Six Hills Giant'	Lamiales	Lamiaceae	Bilateral	15:01	25/06/2014	Bristol	23.8	23	0.8
Phlomis fruticosa	Lamiales	Lamiaceae	Bilateral	15:10	25/06/2014	Bristol	27.1	26.6	0.5
Salvia forsskaolii	Lamiales	Lamiaceae	Bilateral	11:38	26/06/2014	Bristol	20.9	20.9	0
Scutellaria galericulata	Lamiales	Lamiaceae	Bilateral	15:08	25/06/2014	Bristol	28	23.8	4.2
Jasminum officinale	Lamiales	Oleaceae	Radial	14:46	25/06/2014	Bristol	28.4	25.1	3.3

Plant species	Order	Family	Floral Symmetry (Radial/Bilateral)	Time	Date	Location	Hot region temp. ◦c	Cold region temp. ◦c	Δ temp. ◦c
Mimulus aurantiacus	Lamiales	Phrymaceae	Bilateral	11:45	26/06/2014	Bristol	24.1	23.2	0.9
Tulipa 'Daydream'	Liliales	Liliaceae	Radial	12:57	23/04/2016	Wales	19.9	14.6	5.3*
Tulipa 'Golden Appledoorn'	Liliales	Liliaceae	Radial	12:54	23/04/2016	Wales	19.1	13.9	5.2*
Tulipa hageri 'Red Cup'	Liliales	Liliaceae	Radial	12:57	23/04/2016	Wales	20.5	14.4	6.1
Tulipa 'Honky Tonk'	Liliales	Liliaceae	Radial	12:07	06/05/2016	Wales	25.7	22.1	3.6*
Tulipa kaufmanniana	Liliales	Liliaceae	Radial	12:59	23/04/2016	Wales	17.4	13.1	4.3
Tulipa tarda	Liliales	Liliaceae	Radial	12:07	06/05/2016	Wales	23.8	21.4	2.4
Magnolia kobus	Magnoliales	Magnoliaceae	Radial	12:06	06/05/2016	Wales	23.4	21.2	2.2
Euphorbia characias	Malpighiales	Euphorbiaceae	Radial	15:48	26/03/2015	Bristol	6.6	5.6	1
Cistus 'Snow Fire'	Malvales	Cistaceae	Radial	09:32	03/07/2016	Wales	28.7	18	10.7*
Cistus 'Snow White'	Malvales	Cistaceae	Radial	15:37	05/07/2016	Wales	25.6	18.3	7.3*
Cistus × *argenteus*	Malvales	Cistaceae	Radial	10:46	23/06/2016	Wales	26.5	20	6.5*
Cistus × *pulverulentus*	Malvales	Cistaceae	Radial	12:28	06/07/2016	Wales	24.1	21.3	2.8
Cistus × *purpureus*	Malvales	Cistaceae	Radial	16:24	08/06/2016	Bristol	30.8	24.4	6.4*
Cistus × *verguinii*	Malvales	Cistaceae	Radial	12:31	23/04/2016	Wales	29.3	20.7	8.6*
Helianthemum apenninum	Malvales	Cistaceae	Radial	11:52	26/06/2014	Bristol	23.5	19.2	4.3
Helianthemum nummularium	Malvales	Cistaceae	Radial	10:55	25/06/2014	Bristol	19.1	17.4	1.7
Alyogyne huegelii	Malvales	Malvaceae	Radial	12:16	23/04/2016	Wales	31	24.6	6.4
Malva sylvestris	Malvales	Malvaceae	Radial	10:47	26/06/2014	Bristol	19.2	18.6	0.6
Daphne odora	Malvales	Thymelaeaceae	Radial	14:26	18/02/2015	Bristol	11.1	10.7	0.4
Chamelaucium uncinatum	Myrtales	Myrtaceae	Radial	12:16	23/04/2016	Wales	24.7	23.1	1.6
Chamerion angustifolium 'Albino'	Myrtales	Onagraceae	Radial	11:32	26/06/2014	Bristol	19.3	19	0.3

Plant species	Order	Family	Floral Symmetry (Radial/Bilateral)	Time	Date	Location	Hot region temp. °c	Cold region temp. °c	Δ temp. °c
Fuchsia magellanica	Myrtales	Onagraceae	Radial	14:49	25/06/2014	Bristol	27.9	22.8	5.1
Nymphaea odorata	Nymphaeales	Nymphaeaceae	Radial	11:57	26/06/2014	Bristol	27.5	20.9	6.6
Eschscholzia californica	Ranunculales	Papaveraceae	Radial	13:08	04/06/2013	Bristol	33.5	22.5	11
Papaver cambricum	Ranunculales	Papaveraceae	Radial	11:55	25/06/2014	Bristol	26	21.4	4.6
Papaver rhoeas	Ranunculales	Papaveraceae	Radial	14:56	26/06/2014	Bristol	20.4	19.3	1.1
Anemone blanda	Ranunculales	Ranunculaceae	Radial	15:44	26/03/2015	Bristol	20.5	12.3	8.2
Anemone nemorosa	Ranunculales	Ranunculaceae	Radial	12:05	06/05/2016	Wales	22.6	21.7	0.9
Aquilegia vulgaris	Ranunculales	Ranunculaceae	Bilateral	13:27	04/06/2013	Bristol	29.3	22.4	6.9
Ficaria verna	Ranunculales	Ranunculaceae	Radial	13:45	04/05/2016	Wales	23.6	17.2	6.4
Helleborus orientalis	Ranunculales	Ranunculaceae	Radial	15:36	09/02/2015	Bristol	7.2	6	1.2
Helleborus × 'Snow White'	Ranunculales	Ranunculaceae	Radial	15:02	24/03/2015	Bristol	11.1	9	2.1*
Nigella damascena	Ranunculales	Ranunculaceae	Radial	15:06	25/06/2014	Bristol	27	24.5	2.5
Ranunculus aquatilis	Ranunculales	Ranunculaceae	Radial	14:17	18/02/2015	Bristol	11.9	10.8	1.1
Ranunculus auricomus	Ranunculales	Ranunculaceae	Radial	14:25	25/06/2014	Bristol	20.7	19.9	0.8
Ranunculus bulbosus	Ranunculales	Ranunculaceae	Radial	09:08	06/05/2014	Bristol	19.5	12.3	7.2
Ranunculus sp.	Ranunculales	Ranunculaceae	Radial	12:41	26/04/2016	Wales	17.6	11.8	5.8
Trautvetteria caroliniensis	Ranunculales	Ranunculaceae	Radial	11:04	26/06/2014	Bristol	19.3	18.6	0.7
Chaenomeles speciosa	Rosales	Rosaceae	Radial	11:47	24/03/2015	Bristol	17.2	12.2	5
Exochorda × *macrantha*	Rosales	Rosaceae	Radial	10:20	08/05/2016	Wales	24.3	23.3	1
Fragaria vesca	Rosales	Rosaceae	Radial	12:00	06/05/2016	Wales	21.6	20.9	0.7
Geum chiloense 'Lady Straheden'	Rosales	Rosaceae	Radial	13:52	25/06/2014	Bristol	34	27.1	6.9*
Geum chiloense 'Mrs Bradshaw'	Rosales	Rosaceae	Radial	13:45	25/06/2014	Bristol	38.3	30.9	7.4*
Geum 'Kariskaer'	Rosales	Rosaceae	Radial	11:50	06/05/2016	Wales	25.3	23.3	2

Plant species	Order	Family	Floral Symmetry (Radial/Bilateral)	Time	Date	Location	Hot region temp. °C	Cold region temp. °C	Δ temp. °C
Kerria japonica 'Flore Pleno'	Rosales	Rosaceae	Radial	11:56	06/05/2016	Wales	24.2	20.4	3.8
Potentilla fruticosa 'Gibson's Scarlet'	Rosales	Rosaceae	Radial	14:02	25/06/2014	Bristol	24.4	22.4	2
Prunus sp.	Rosales	Rosaceae	Radial	10:30	08/05/2016	Wales	24.3	23.4	0.9
Spiraea prunifolia	Rosales	Rosaceae	Radial	13:56	25/06/2014	Bristol	21.9	21.1	0.8
Paeonia peregrina	Saxifragales	Paeoniaceae	Radial	13:22	04/06/2013	Bristol	31.1	22.5	8.6
Bergenia cordifolia	Saxifragales	Saxifragaceae	Radial	14:22	18/02/2015	Bristol	16.6	11.6	5
Roscoea sp.	Zingiberales	Zingiberaceae	Bilateral	15:01	26/06/2014	Bristol	20.2	19.5	0.7

A4: The parameter values of the best fitting humidity structure models

Appendix table 10.4: *The parameter values of the best fitting models of both x and z axis models from our analysis of humidity structure of the 42 flower species and 6 controls sampled in chapter 4 and four artificial flowers variants sampled in chapter 5. All values are given in scientific format ($g \cdot Ex = g \cdot 10^x$).*

	Abutilon x milleri hort.	*Achillea millefolium*	*Allium ursinum*	*Bellis perennis*	*Calystegia silvatica*	*Campanula* sp.	*Centaurea montanus*	*Centaurea segetum*	*Cistus* 'greyswood pink'	*Clematis chinensis*	*Convolvulus sabatius*	*Coreopsis* sp.	*Cosmos parviflorus*	*Epilobium hirsutum*
I_x	3.17 E-01	1.72 E+00	2.42 E-01	5.82 E-01	9.74 E-01	3.57 E-01	2.02 E-01	1.10 E+00	6.61 E-01	6.54 E-01	1.91 E-01	7.05 E-01	6.31 E-01	5.87 E-01
A_x		7.34 E-03			1.25 E-02		-1.37 E-03			-4.01 E-03		1.44 E-03	3.05 E-03	
B_x	-2.32 E-04	-2.17 E-03	-3.56 E-04	-7.41 E-04		-5.01 E-04	-3.65 E-04	-1.12 E-03	-8.10 E-04	-6.88 E-04	-2.49 E-04	-7.68 E-04	-5.10 E-04	-8.09 E-04
g_{2x}	-1.15 E-01				2.63 E-01		1.12 E-01	-3.56 E-01		-9.48 E-02	6.83 E-01		3.19 E-02	
g_{3x}	-3.93 E-02				8.60 E-01		1.11 E-01	-2.48 E-01		-3.12 E-02	5.02 E-01		-6.32 E-02	
g_{4x}	-1.52 E-01				4.95 E-01		7.68 E-02	-1.69 E-01		-2.92 E-01	5.07 E-01		-1.35 E-01	
c_{2x}					2.34 E-02									
c_{3x}					5.01 E-02									
c_{4x}					3.71 E-02									
r_{2x}											-6.99 E-04			
r_{3x}											-5.38 E-04			
r_{4x}											-5.89 E-04			
I_z	1.41 E-01	6.53 E-01	-1.48 E-01	1.09 E-01	4.44 E+00	9.76 E-02	-1.77 E-02	1.89 E-01	3.60 E-01	4.99 E-02	4.43 E-01	2.20 E-01	4.14 E-01	1.88 E-02
B_z		-1.50 E-01		-3.65 E-02	-1.32 E+00				-7.92 E-02		-1.65 E-01	-7.56 E-02	-9.03 E-02	
g_{2z}	-5.91 E-02		2.46 E-01	1.01 E-01		-7.46 E-02	5.46 E-02		4.82 E-02	4.47 E-02	3.11 E-01	1.90 E-01		-4.67 E-02
g_{3z}	4.26 E-02		3.40 E-01	1.01 E-01		-1.85 E-02	1.05 E-01		-6.41 E-02	1.07 E-01	2.15 E-01	1.72 E-01		5.44 E-02
g_{4z}	-1.32 E-01		2.24 E-01	7.99 E-02		-4.55 E-02	1.03 E-01		-2.61 E-02	-4.02 E-01	1.37 E-01	1.54 E-01		1.43 E-01
c_{2z}														
c_{3z}														
c_{4z}														

	Eschscholzia californica	*Euphorbia milii*	*Fuchsia* sp.	*Geranium* 'Roxanne'	*Geranium robertianum*	*Geranium sanguinemum*	*Lantana* sp.	*Lavandula angustifolia*	*Leucanthemum vulgare*	*Lilium* sp.	*Linum grandiflorum*	*Linum usitatissimum*	*Nepenthes* sp.
I_x	1.50 E+00	1.00 E-01	4.50 E-02	5.06 E-01	4.13 E-01	4.59 E-01	1.47 E+00	4.66 E-01	1.78 E+00	1.61 E-01	2.49 E-01	7.66 E-01	2.61 E-01
A_x	5.80 E-02			-2.02 E-03					7.86 E-03	4.78 E-03	-2.13 E-03		
B_x	-1.35 E-03	-2.33 E-04		-6.07 E-04	-5.97 E-04	-7.67 E-04	-1.81 E-03	-7.07 E-04	-1.81 E-03		-3.24 E-04	-1.24 E-03	-3.26 E-04
g_{2x}	1.05 E+00	4.17 E-02		1.21 E-01		3.33 E-01		9.55 E-02	-3.96 E-01	-1.49 E-02	9.47 E-02	3.16 E-02	
g_{3x}	8.39 E-01	1.90 E-01		1.64 E-01		1.42 E-01		1.11 E-01	-2.77 E-01	6.87 E-02	3.47 E-02	-2.79 E-01	
g_{4x}	1.11 E+00	1.46 E-01		2.83 E-02		2.45 E-02		2.58 E-01	-1.67 E-01	1.65 E-01	-1.26 E-01	-3.75 E-01	
c_{2x}										-3.31 E-03	6.80 E-03		
c_{3x}										-2.96 E-03	3.67 E-03		
c_{4x}										-1.54 E-02	2.24 E-03		
r_{2x}						-2.71 E-04					7.87 E-07	2.50 E-04	
r_{3x}						1.39 E-04					4.30 E-05	6.20 E-04	
r_{4x}						2.52 E-04					7.31 E-05	7.27 E-04	
I_z	1.54 E+00	-8.29 E-02	3.39 E-02	2.86 E-01	7.61 E-03	1.33 E-01	6.20 E-01	4.79 E-02	8.95 E-01	1.85 E-01	1.12 E-01	-3.48 E-02	6.59 E-02
B_z	-4.64 E-01			-9.52 E-02		-6.01 E-02	-1.46 E-01		-2.34 E-01		-4.63 E-02		
g_{2z}	4.23 E+00	1.27 E-01	-4.60 E-02	8.16 E-02	1.02 E-01	5.94 E-02		3.50 E-02	-3.54 E-01	-9.52 E-02	1.75 E-01	8.78 E-02	3.31 E-02
g_{3z}	4.65 E+00	2.33 E-01	6.45 E-02	1.25 E-01	6.26 E-02	2.05 E-01		1.63 E-01	-4.71 E-01	1.99 E-02	9.12 E-02	1.44 E-01	-1.08 E-02
g_{4z}	7.85 E+00	8.87 E-02	1.85 E-01	3.85 E-02	9.53 E-02	2.04 E-01		2.33 E-01	-3.01 E-01	-8.76 E-02	3.90 E-03	1.51 E-01	-1.65 E-02
c_{2z}	-1.19 E+00								2.54 E-02				
c_{3z}	-1.32 E+00								1.97 E-01				
c_{4z}	-2.29 E+00								1.53 E-01				

	Nicotiana tabacum	*Oenothera caespitosa*	*Osteospermum* sp.	*Papaver cambricum*	*Papaver rhoeas*	*Potentilla* sp.	*Ranunculus acris*	*Ranunculus lingua*	*Rudbeckia hirta*	*Scabiosa* sp.	*Taraxacum* agg.	*Trifolium pratense*	*Tulbaghia violacea*
I_x	1.02 E-01	1.78 E+00	6.24 E-01	5.79 E-01	2.80 E-01	6.94 E-01	1.53 E+00	3.09 E+00	1.08 E+00	1.36 E+00	1.49 E+00	6.11 E-01	5.24 E-01
A_x		9.17 E-03	1.11 E-03		-2.62 E-03	1.98 E-03		1.76 E-02	6.50 E-03	4.90 E-03			
B_x	-9.82 E-05	-1.81 E-03	-6.50 E-04	-6.15 E-04	-3.01 E-04	-8.05 E-04	-1.88 E-03	-3.37 E-03	-1.09 E-03	-1.52 E-03	-1.92 E-03	-6.32 E-04	-6.04 E-04
g_{2x}		-3.36 E-01	5.49 E-01			-2.60 E-02	1.88 E+00	4.86 E-02	1.61 E-01		3.90 E-01	1.48 E-02	-3.39 E-01
g_{3x}		-5.21 E-01	5.41 E-01			-9.94 E-02	1.62 E+00	-3.26 E-01	-1.40 E-01		1.27 E+00	-2.33 E-01	-1.06 E-01
g_{4x}		-8.45 E-01	1.31 E-01			-6.37 E-02	3.11 E-01	-3.31 E-01	-2.27 E-01		1.86 E+00	-2.59 E-01	-1.66 E-01
c_{2x}		-7.91 E-04	1.01 E-02										
c_{3x}		4.87 E-04	1.20 E-02										
c_{4x}		6.68 E-04	3.31 E-03										
r_{2x}		4.47 E-04	-5.33 E-04				-2.71 E-03				-4.51 E-04	-1.38 E-04	3.40 E-04
r_{3x}		6.31 E-04	-5.45 E-04				-2.33 E-03				-1.66 E-03	1.07 E-04	1.93 E-04
r_{4x}		1.09 E-03	-2.16 E-04				-5.41 E-04				-2.24 E-03	1.42 E-04	3.57 E-04
I_z	5.25 E-02	1.14 E+00	4.40 E-01	3.33 E-01	2.31 E-01	2.30 E-01	1.41 E+00	1.58 E+00	5.69 E-01	7.65 E-01	5.97 E-01	1.91 E-01	2.33 E-01
B_z		-2.53 E-01	-1.21 E-01	-6.91 E-02		-4.34 E-02	-4.44 E-01	-4.08 E-01	-1.34 E-01	-2.01 E-01	-1.64 E-01		
g_{2z}	-1.55 E-02		1.51 E+00		-1.45 E-01		1.97 E-01	-4.29 E-02			2.16 E-01	-7.95 E-02	-1.62 E-01
g_{3z}	1.29 E-02		1.43 E+00		-1.29 E-01		2.37 E-01	-5.73 E-02			7.37 E-01	-1.27 E-01	-8.42 E-02
g_{4z}	-1.38 E-02		3.07 E-01		-1.22 E-01		7.83 E-02	-2.49 E-02			2.62 E+00	-1.69 E-01	-2.78 E-02
c_{2z}			-4.44 E-01								-5.39 E-02		
c_{3z}			-4.20 E-01								-2.11 E-01		
c_{4z}			-9.16 E-02								-7.91 E-01		

	Vinca herbacea	*Xerochrysum bracteatum*		*EmptyLidBlue*	*EmptyLid*	*Empty*	*WaterLidBlue*	*WaterLid*	*Water*		Active Humidity Humid	Active Humidity Dry	Passive Humidity Humid	Passive Humidity Dry
I_x	2.28 E-01	3.66 E+00		1.37 E-01	7.96 E-02	2.72 E-02	3.82 E-02	4.57 E-01	1.17 E+00		2.55 E+00	9.25 E-01	3.49 E+00	2.13 E+00
A_x	-1.07 E-03	1.27 E-02			-5.68 E-04	-4.42 E-04	-8.09 E-04				1.37 E-02	-2.96 E-04		-3.50 E-03
B_x	-2.15 E-04	-4.51 E-03				-4.41 E-05		-5.98 E-04	-1.53 E-03		-2.55 E-03	-1.26 E-03	-4.05 E-03	-2.85 E-03
g_{2x}	1.11 E-02			-1.81 E-01	-7.16 E-02	-4.55 E-04	-5.18 E-02				-3.50 E-01	-8.52 E-01	-1.01 E-01	-4.89 E-01
g_{3x}	-2.95 E-02			-8.72 E-02	-4.39 E-02	3.14 E-02	2.30 E-02				-3.10 E-01	-8.52 E-01	-6.91 E-01	-9.35 E-01
g_{4x}	-3.20 E-02			-1.56 E-01	-7.59 E-02	1.38 E-02	-8.80 E-02				-4.94 E-01	-9.34 E-01	-9.11 E-01	-1.28 E+00
c_{2x}												-2.64 E-03		-2.24 E-03
c_{3x}												-1.98 E-03		-2.26 E-03
c_{4x}												-2.23 E-03		-1.40 E-03
r_{2x}												1.06 E-03	3.17 E-04	7.83 E-04
r_{3x}												1.18 E-03	1.16 E-03	1.60 E-03
r_{4x}												1.20 E-03	1.51 E-03	1.92 E-03
I_z	6.28 E-02	1.46 E+00		9.38 E-02	7.14 E-02	-1.30 E-02	9.06 E-02	6.05 E-02	0.53 E+00		1.45 E+00	4.04 E-01	1.27 E+00	9.39 E-01
B_z		-3.74 E-01				1.73 E-02			-0.13 E+00		-3.94 E-01	-1.12 E-01	-3.86 E-01	-3.16 E-01
g_{2z}				-1.46 E-01	-4.51 E-02		-4.64 E-02				-2.23 E-01	-5.87 E-01	1.17 E-01	-2.32 E-01
g_{3z}				-3.59 E-02	-4.26 E-03		-3.98 E-02				-2.50 E-01	-5.89 E-01	1.93 E-01	-3.68 E-01
g_{4z}				-8.78 E-02	-4.10 E-02		-1.31 E-01				-2.97 E-01	-5.96 E-01	2.17 E-01	-7.36 E-01
c_{2z}											5.94 E-02	1.76 E-01		1.09 E-01
c_{3z}											7.37 E-02	2.01 E-01		1.97 E-01
c_{4z}											6.50 E-02	1.71 E-01		2.87 E-01

A5: AIC tables and sampling dates of control tube humidity analyses

The AIC tables, and sampling dates, relating to our analyses of humidity structure of controls as documented in chapter 4. For each individual control the date and time at which the first x axis transect replicate began is given (YYYY-MM-DD-hh-mm-ss). In each AIC table, each species having one for x and z axis models, AIC and degrees of freedom 'df' are given. Difference in ΔAIC, here calculated as AIC of model with the lowest AIC minus that of the current model, is also provided. Within each AIC table, shaded and in bold are the best fitting models as per the guidelines given in (Richards, 2008).

EmptyLidBlue

X axis model	df	AIC	ΔAIC
m7	8	-450.55	0.00
m5	7	-449.11	-1.44
m6	7	-448.99	-1.56
m4	**6**	**-447.59**	**-2.96**
m8	10	-447.32	-3.23
m9	10	-443.31	-7.24
m10	14	-443.14	-7.42
m3	5	-354.51	-96.04
m1	4	-354.04	-96.51
m2	4	-353.95	-96.60
m0	3	-353.51	-97.05

Z axis model	df	AIC	ΔAIC
z2	**6**	**-192.59**	**0.00**
z3	7	-191.89	-0.69
z4	10	-186.75	-5.84
z0	3	-169.15	-23.43
z1	4	-168.21	-24.38

Sampling dates
2017-08-16-15-38-03
2018-04-23-15-01-12
2018-04-23-11-08-51
2018-04-24-11-09-10
2018-04-24-13-44-02
2018-04-25-15-14-30

EmptyLid

X axis model	df	AIC	ΔAIC
m7	8	-927.86	0.00
m5	**7**	**-922.57**	**-5.30**
m6	7	-920.07	-7.79
m8	10	-917.40	-10.46
m10	14	-917.18	-10.69
m4	6	-914.97	-12.89
m9	10	-914.53	-13.34
m3	5	-861.53	-66.33
m1	4	-857.56	-70.30
m2	4	-855.51	-72.35
m0	3	-851.68	-76.18

Z axis model	df	AIC	ΔAIC
z3	7	-380.63	0.00
z4	10	-380.41	-0.22
z2	**6**	**-377.64**	**-2.99**
z1	4	-373.31	-7.33
z0	3	-370.71	-9.93

Sampling dates
2017-08-10-15-18-02
2017-08-16-14-20-40
2018-04-05-13-27-41
2018-04-09-14-30-30
2018-04-10-11-06-45
2018-04-11-13-11-22
2018-02-12-11-45-17

Empty

X axis model	df	AIC	ΔAIC
m7	**8**	**-966.68**	**0.00**
m10	14	-965.88	-0.80
m6	7	-959.90	-6.78
m9	10	-955.15	-11.53
m3	5	-951.44	-15.24
m8	10	-948.35	-18.33
m2	4	-945.24	-21.44
m5	7	-945.21	-21.47
m4	6	-939.07	-27.61
m1	4	-931.50	-35.18
m0	3	-925.85	-40.83

Z axis model	df	AIC	ΔAIC
z3	7	-480.40	0.00
z1	**4**	**-479.29**	**-1.12**
z4	10	-476.64	-3.76
z2	6	-474.86	-5.54
z0	3	-474.12	-6.29

Sampling dates
2017-08-10-14-00-37
2018-04-04-15-20-27
2018-04-05-12-10-17
2018-04-10-14-59-09
2018-04-11-10-36-30
2018-04-12-10-27-54

WaterBlueLid

X axis model	df	AIC	ΔAIC
m7	8	-541.52	0.00
m5	**7**	**-538.67**	**-2.84**
m8	10	-536.90	-4.62
m6	**7**	**-535.99**	**-5.52**
m10	14	-534.00	-7.52
m4	6	-533.27	-8.25
m9	10	-530.17	-11.34
m3	5	-490.54	-50.97
m1	4	-488.52	-53.00
m2	4	-486.28	-55.23
m0	3	-484.34	-57.18

Z axis model	df	AIC	ΔAIC
z3	7	-206.10	0.00
z2	**6**	**-204.41**	**-1.69**
z4	10	-200.47	-5.62
z1	4	-187.86	-18.24
z0	3	-186.75	-19.34

Sampling dates
2017-08-16-13-03-15
2018-04-23-13-43-47
2018-04-23-12-26-20
2018-04-24-12-26-35
2018-04-24-15-01-31
2018-04-25-11-22-09

WaterLid

X axis model	df	AIC	ΔAIC
m3	5	-97.88	0.00
m7	8	-95.56	-2.32
m2	**4**	**-92.55**	**-5.32**
m6	7	-90.15	-7.73
m10	14	-87.02	-10.86
m9	10	-85.75	-12.13
m1	4	96.22	-194.10
m0	3	98.10	-195.98
m5	7	100.29	-198.16
m4	6	102.19	-200.07
m8	10	105.34	-203.21

Z axis model	df	AIC	ΔAIC
z0	**3**	**-377.22**	**0.00**
z2	6	-376.97	-0.24
z1	4	-376.14	-1.08
z3	7	-375.93	-1.29
z4	10	-370.82	-6.40

Sampling dates
2017-08-10-17-52-48
2018-04-04-12-45-36
2018-04-05-10-52-48
2018-04-10-13-41-41
2018-04-11-14-28-51
2018-04-12-13-02-45

Water

X axis model	df	AIC	ΔAIC
m3	5	484.15	0.00
m2	**4**	**485.09**	**-0.94**
m7	8	488.66	-4.51
m6	7	489.61	-5.46
m9	10	494.89	-10.74
m10	14	499.74	-15.59
m0	3	680.47	-196.32
m1	4	680.93	-196.78
m4	6	685.70	-201.54
m5	7	686.16	-202.00
m8	10	692.06	-207.90

Z axis model	df	AIC	ΔAIC
z1	**4**	**-282.72**	**0.00**
z3	7	-280.48	-2.24
z4	10	-276.03	-6.69
z0	3	-204.04	-78.68
z2	6	-200.12	-82.60

Sampling dates
2017-08-10-16-35-25
2018-04-04-11-28-05
2018-04-05-14-45-08
2018-04-10-12-24-12
2018-04-11-11-53-57
2018-04-12-14-20-16

A6: AIC tables and sampling dates of flower species floral humidity analyses

The AIC tables, and sampling dates, relating to our analyses of floral humidity structure as documented in chapter 4. For each individual of each species the date and time at which the first x axis transect replicate began is given (YYYY-MM-DD-hh-mm-ss). In each AIC table, each species having one for x and z axis models, AIC and degrees of freedom 'df' are given. Difference in ΔAIC, here calculated as AIC of model with the lowest AIC minus that of the current model, is also provided. Within each AIC table, shaded and in bold are the best fitting models as per the guidelines given in (Richards, 2008).

Abutilon x milleri hort.

X axis model	df	AIC	ΔAIC
m6	**7**	**-134.30**	**0.00**
m9	10	-132.48	-1.83
m7	8	-132.33	-1.97
m10	14	-127.38	-6.93
m2	4	-109.86	-24.45
m3	5	-107.89	-26.42
m4	6	-93.36	-40.94
m5	7	-91.39	-42.92
m8	10	-87.84	-46.46
m0	3	-72.73	-61.57
m1	4	-70.76	-63.55

Z axis model	df	AIC	ΔAIC
z2	**6**	**-39.16**	**0.00**
z3	7	-37.37	-1.79
z4	10	-31.86	-7.30
z0	3	-30.41	-8.75
z1	4	-28.60	-10.56

Sampling dates: 2017-10-11-12-14-25
2017-10-12-14-11-55
2017-10-17-12-26-17
2017-10-25-12-03-34
2018-06-21-14-48-05
2018-07-04-15-11-38

Achillea millefolium

X axis model	df	AIC	ΔAIC
m7	8	560.46	0.00
m3	**5**	**564.58**	**-4.12**
m10	14	565.05	-4.59
m6	7	576.52	-16.06
m9	10	576.99	-16.53
m2	4	580.07	-19.61
m1	4	831.78	-271.32
m5	7	833.62	-273.16
m0	3	837.15	-276.70
m8	10	839.00	-278.54
m4	6	839.09	-278.64

Z axis model	df	AIC	ΔAIC
z3	7	-109.95	0.00
z4	10	-106.58	-3.37
z1	**4**	**-104.12**	**-5.84**
z2	6	-73.85	-36.10
z0	3	-70.78	-39.17

Sampling dates 2018-07-19-10-28-34
2018-07-19-11-46-03
2018-07-20-10-03-55
2018-07-20-12-38-48
2018-07-23-14-00-20
2018-07-23-15-17-50

Allium ursinum

X axis model	df	AIC	ΔAIC
m3	5	-98.78	0.00
m4	6	-96.81	-1.97
m8	10	-96.34	-2.44
m2	**4**	**-93.67**	**-5.11**
m1	4	-38.05	-60.73
m0	3	-34.77	-64.01
m5	7	102.18	-200.96
m9	10	179.73	-278.51
m10	14	183.55	-282.33
m6	7	185.13	-283.91
m7	8	194.66	-293.44

Z axis model	df	AIC	ΔAIC
z3	7	-93.15	0.00
z2	**6**	**-93.04**	**-0.11**
z4	10	-88.08	-5.07
z0	3	-42.94	-50.21
z1	4	-42.11	-51.04

Sampling dates 2018-05-31-12-45-26
2018-06-01-14-41-55
2018-06-04-10-53-20
2018-06-05-13-02-36

Bellis perennis

X axis model	df	AIC	ΔAIC
m2	**4**	**-157.19**	**0.00**
m3	5	-155.38	-1.81
m9	10	-154.87	-2.33
m6	7	-154.59	-2.61
m7	8	-152.78	-4.42
m10	14	-147.95	-9.25
m0	3	136.20	-293.40
m1	4	138.13	-295.32
m4	6	140.91	-298.11
m5	7	142.84	-300.04
m8	10	148.51	-305.71

Z axis model	df	AIC	ΔAIC
z3	**7**	**-448.07**	**0.00**
z4	10	-445.11	-2.96
z2	6	-425.75	-22.32
z1	4	-372.01	-76.05
z0	3	-360.07	-88.00

Sampling dates 2018-05-03-10-02-36
2018-05-03-12-37-33
2018-05-03-13-54-58
2018-05-09-10-28-54
2018-05-09-11-46-21
2018-05-21-10-13-25

Calystegia silvatica

X axis model	df	AIC	ΔAIC
m10	14	1105.23	0.00
m8	**10**	**1105.77**	**-0.55**
m7	8	1116.62	-11.39
m5	7	1119.70	-14.47
m3	5	1126.50	-21.27
m1	4	1129.33	-24.10
m6	7	1195.07	-89.84
m4	6	1196.99	-91.76
m9	10	1198.83	-93.61
m2	4	1201.35	-96.13
m0	3	1203.12	-97.89

Z axis model	df	AIC	ΔAIC
z1	**4**	**495.56**	**0.00**
z3	7	500.63	-5.07
z4	10	503.99	-8.43
z0	3	535.30	-39.74
z2	6	540.61	-45.05

Sampling dates 2018-06-25-11-11-10
2018-06-28-11-29-34
2018-06-29-13-34-47
2018-07-02-13-42-12
2018-07-03-15-40-32
2018-07-06-10-28-25

Campanula sp.

X axis model	df	AIC	ΔAIC
m3	5	-267.34	0.00
m7	8	-267.30	-0.04
m2	**4**	**-262.39**	**-4.96**
m6	7	-262.21	-5.13
m9	10	-256.64	-10.70
m10	14	-255.87	-11.47
m1	4	-47.02	-220.32
m0	3	-45.64	-221.71
m5	7	-43.89	-223.45
m4	6	-42.47	-224.87
m8	10	-37.95	-229.39

Z axis model	df	AIC	ΔAIC
z2	**6**	**-371.83**	**0.00**
z3	7	-371.73	-0.10
z4	10	-365.82	-6.01
z0	3	-351.18	-20.65
z1	4	-350.74	-21.09

Sampling dates 2018-08-01-10-22-14
2018-08-01-12-57-07
2018-08-01-11-39-41
2018-08-02-11-05-06
2018-08-02-12-22-31
2018-08-03-15-08-04

Centaurea montanus

X axis model	df	AIC	ΔAIC
m7	**8**	**-342.32**	**0.00**
m10	14	-337.73	-4.59
m9	10	-333.98	-8.34
m6	7	-333.14	-9.19
m3	5	-314.36	-27.96
m2	4	-306.33	-35.99
m5	7	-177.40	-164.92
m4	6	-172.86	-169.46
m8	10	-171.57	-170.75
m1	4	-163.26	-179.06
m0	3	-159.14	-183.18

Z axis model	df	AIC	ΔAIC
z2	**6**	**-202.33**	**0.00**
z3	7	-201.05	-1.28
z4	10	-195.66	-6.67
z0	3	-189.42	-12.91
z1	4	-188.06	-14.27

Sampling dates 2018-06-25-15-03-29
2018-06-28-10-12-11
2018-06-29-10-59-52
2018-07-02-14-59-35
2018-07-03-14-23-09
2018-07-06-11-45-50

Centaurea segetum

X axis model	df	AIC	ΔAIC
m6	**7**	**587.29**	**0.00**
m9	10	587.69	-0.40
m7	8	589.16	-1.87
m10	14	594.92	-7.63
m2	4	595.90	-8.61
m3	5	597.77	-10.48
m4	6	676.59	-89.30
m5	7	678.50	-91.21
m0	3	681.50	-94.21
m1	4	683.40	-96.12
m8	10	684.03	-96.74

Z axis model	df	AIC	ΔAIC
z0	**3**	**5.29**	**0.00**
z1	4	5.78	-0.50
z2	6	8.77	-3.48
z3	7	9.24	-3.95
z4	10	14.26	-8.97

Sampling dates
2018-05-31-15-20-22
2018-06-01-10-49-30
2018-06-04-12-10-45
2018-06-05-09-10-11
2018-06-05-10-27-38
2018-06-18-14-28-24

Cistus 'greyswood pink'

X axis model	df	AIC	ΔAIC
m3	5	172.88	0.00
m7	8	173.30	-0.42
m2	**4**	**175.22**	**-2.34**
m6	7	175.71	-2.84
m9	10	179.56	-6.68
m10	14	182.49	-9.61
m1	4	331.19	-158.31
m0	3	331.77	-158.89
m5	7	333.90	-161.02
m4	6	334.50	-161.63
m8	10	339.54	-166.66

Z axis model	df	AIC	ΔAIC
z3	**7**	**-257.35**	**0.00**
z4	10	-256.01	-1.34
z1	4	-237.68	-19.67
z2	6	-230.01	-27.34
z0	3	-214.90	-42.45

Sampling dates
2018-05-11-11-50-45
2018-05-11-14-25-43
2018-05-14-14-17-28
2018-05-14-13-00-01
2018-05-15-10-26-31
2018-05-15-13-01-23

Clematis chinensis

X axis model	df	AIC	ΔAIC
m10	14	100.74	0.00
m7	**8**	**100.98**	**-0.24**
m6	7	112.24	-11.50
m9	10	112.34	-11.61
m3	5	124.24	-23.51
m2	4	133.78	-33.05
m5	7	187.58	-86.84
m8	10	189.87	-89.13
m4	6	194.27	-93.53
m1	4	201.01	-100.27
m0	3	206.92	-106.18

Z axis model	df	AIC	ΔAIC
z2	**6**	**113.79**	**0.00**
z3	7	114.92	-1.13
z4	10	120.68	-6.90
z0	3	129.12	-15.33
z1	4	130.43	-16.64

Sampling dates
2017-10-11-14-49-10
2017-10-12-15-29-18
2017-10-17-15-01-02
2017-10-25-14-38-21

Convolvulus sabatius

X axis model	df	AIC	ΔAIC
m9	**10**	**83.92**	**0.00**
m10	14	86.46	-2.53
m6	7	108.10	-24.17
m7	8	110.10	-26.17
m2	4	193.23	-109.30
m3	5	195.23	-111.30
m4	6	257.84	-173.91
m5	7	259.84	-175.91
m8	10	262.83	-178.91
m0	3	310.49	-226.57
m1	4	312.49	-228.57

Z axis model	df	AIC	ΔAIC
z4	10	-32.22	0.00
z3	**7**	**-29.94**	**-2.27**
z2	6	-5.68	-26.53
z1	4	2.97	-35.18
z0	3	21.22	-53.44

Sampling dates
2017-10-11-13-31-47
2017-10-12-12-54-32
2017-10-17-13-43-38
2017-10-25-15-55-46
2018-06-21-10-55-44
2018-07-04-11-19-20

Coreopsis sp.

X axis model	df	AIC	ΔAIC
m7	8	-140.37	0.00
m10	14	-137.87	-2.51
m9	10	-137.00	-3.37
m6	7	-135.84	-4.53
m3	**5**	**-135.80**	**-4.58**
m2	4	-131.49	-8.89
m1	4	158.99	-299.37
m0	3	159.40	-299.77
m5	7	161.02	-301.40
m4	6	161.46	-301.84
m8	10	166.24	-306.61

Z axis model	df	AIC	ΔAIC
z3	**7**	**-78.13**	**0.00**
z4	10	-72.56	-5.57
z2	6	-71.68	-6.44
z1	4	-58.49	-19.64
z0	3	-53.44	-24.69

Sampling dates
2017-09-18-11-41-06
2017-09-19-12-03-28
2017-09-20-11-53-45
2017-09-26-13-45-40
2017-09-27-10-37-20
2017-11-22-12-32-03

Cosmos parviflorus

X axis model	df	AIC	ΔAIC
m7	**8**	**189.04**	**0.00**
m3	5	196.26	-7.21
m6	7	197.61	-8.57
m10	14	199.86	-10.82
m9	10	202.74	-13.70
m2	4	204.39	-15.34
m5	7	260.35	-71.30
m1	4	264.79	-75.75
m8	10	266.12	-77.08
m4	6	266.69	-77.65
m0	3	270.86	-81.82

Z axis model	df	AIC	ΔAIC
z3	7	-131.70	0.00
z1	**4**	**-129.25**	**-2.46**
z4	10	-126.66	-5.05
z2	6	-116.37	-15.34
z0	3	-114.88	-16.82

Sampling dates
2017-07-18-12-32-34
2017-07-19-10-13-07
2017-07-20-16-35-40
2017-07-24-13-25-34
2017-07-25-15-34-23
2017-08-09-13-28-00

Epilobium hirsutum

X axis model	df	AIC	ΔAIC
m3	5	228.15	0.00
m2	**4**	**228.45**	**-0.30**
m7	8	232.70	-4.55
m6	7	233.00	-4.85
m9	10	234.97	-6.82
m10	14	239.85	-11.70
m0	3	365.60	-137.45
m1	4	366.14	-137.99
m4	6	370.68	-142.53
m5	7	371.22	-143.07
m8	10	376.74	-148.59

Z axis model	df	AIC	ΔAIC
z2	**6**	**-55.78**	**0.00**
z3	7	-55.50	-0.28
z4	10	-49.67	-6.12
z0	3	-42.31	-13.47
z1	4	-41.80	-13.98

Sampling dates
2018-07-09-15-44-44
2018-07-10-09-58-00
2018-07-10-12-32-52
2018-07-13-10-08-58
2018-07-13-11-26-23
2018-07-16-12-50-14

Eschscholzia californica

X axis model	df	AIC	ΔAIC
m7	**8**	**1394.98**	**0.00**
m10	14	1395.98	-1.00
m8	10	1400.17	-5.19
m3	5	1402.16	-7.18
m5	7	1404.65	-9.67
m1	4	1411.35	-16.37
m6	7	1463.97	-68.99
m2	4	1468.47	-73.49
m9	10	1469.89	-74.91
m4	6	1471.26	-76.29
m0	3	1475.45	-80.47

Z axis model	df	AIC	ΔAIC
z4	**10**	**513.73**	**0.00**
z3	7	524.82	-11.09
z1	4	539.47	-25.73
z2	6	577.74	-64.01
z0	3	585.94	-72.21

Sampling dates
2018-05-31-11-27-59
2018-06-01-12-06-57
2018-06-04-13-28-12
2018-06-15-09-58-53
2018-06-15-12-33-51
2018-06-18-10-36-01

Euphorbia millii

X axis model	df	AIC	ΔAIC
m6	**7**	**60.38**	**0.00**
m7	8	62.29	-1.90
m9	10	66.24	-5.86
m10	14	72.82	-12.44
m2	4	80.56	-20.18
m4	6	81.63	-21.25
m3	5	82.47	-22.09
m5	7	83.54	-23.16
m8	10	88.31	-27.93
m0	3	99.97	-39.58
m1	4	101.88	-41.50

Z axis model	df	AIC	ΔAIC
z2	**6**	**33.13**	**0.00**
z3a	7	35.09	-1.96
z4a	10	40.74	-7.62
z0	3	41.70	-8.57
z1a	4	43.66	-10.54

Sampling dates
2017-11-22-13-49-26
2017-11-22-16-24-17
2017-11-23-10-59-28
2017-11-23-13-34-18
2017-11-27-12-31-06
2017-11-27-13-48-27

Fuchsia sp.

X axis model	df	AIC	ΔAIC
m2	4	-52.52	0.00
m0	**3**	**-50.88**	**-1.64**
m3	5	-50.60	-1.93
m1	4	-48.96	-3.57
m10	14	197.82	-250.35
m8	10	198.27	-250.79
m5	7	202.92	-255.44
m9	10	204.31	-256.84
m4	6	204.79	-257.32
m7	8	207.08	-259.61
m6	7	208.96	-261.48

Z axis model	df	AIC	ΔAIC
z3	7	-156.31	0.00
z2	**6**	**-153.66**	**-2.65**
z4	10	-152.58	-3.73
z1	4	-111.30	-45.00
z0	3	-110.07	-46.24

Sampling dates
2018-07-10-13-50-21
2018-07-16-11-32-50
2018-07-16-14-07-41
2018-07-17-10-28-02
2018-07-17-11-45-27
2018-07-17-13-02-53

Geranium 'Roxanne'

X axis model	df	AIC	ΔAIC
m7	**8**	**-108.97**	**0.00**
m10	14	-100.40	-8.57
m6	7	-99.10	-9.87
m9	10	-94.55	-14.41
m3	5	-79.43	-29.54
m2	4	-70.84	-38.13
m5	7	98.86	-207.83
m4	6	102.90	-211.86
m8	10	103.90	-212.87
m1	4	111.28	-220.24
m0	3	114.96	-223.93

Z axis model	df	AIC	ΔAIC
z4	10	-235.40	0.00
z3	**7**	**-232.76**	**-2.64**
z1	4	-210.06	-25.34
z2	6	-199.12	-36.28
z0	3	-182.45	-52.96

Sampling dates
2017-09-18-14-15-54
2017-09-19-10-46-02
2017-09-26-15-03-03
2017-09-27-13-12-08
2017-09-28-12-13-11
2018-05-16-10-52-19

Geranium robertianum

X axis model	df	AIC	ΔAIC
m2	**4**	**286.70**	**0.00**
m3	5	288.69	-1.99
m9	10	290.32	-3.62
m6	7	292.53	-5.83
m7	8	294.52	-7.82
m10	14	296.59	-9.89
m0	3	354.70	-68.00
m1	4	356.69	-69.99
m4	6	360.57	-73.87
m5	7	362.56	-75.86
m8	10	367.22	-80.52

Z axis model	df	AIC	ΔAIC
z2	**6**	**-207.72**	**0.00**
z3	7	-206.08	-1.64
z4	10	-202.65	-5.07
z0	3	-195.47	-12.25
z1	4	-193.79	-13.93

Sampling dates
2018-06-25-13-46-00
2018-06-28-14-04-32
2018-06-29-14-52-12
2018-07-02-11-07-14
2018-07-03-11-48-13
2018-07-06-13-03-17

Geranium sanguinemum

X axis model	df	AIC	ΔAIC
m9	**10**	**69.36**	**0.00**
m10	14	74.68	-5.31
m6	7	80.61	-11.24
m7	8	82.45	-13.08
m2	4	109.45	-40.08
m3	5	111.31	-41.94
m4	6	254.58	-185.22
m5	7	256.49	-187.13
m8	10	261.16	-191.79
m0	3	268.67	-199.31
m1	4	270.59	-201.22

Z axis model	df	AIC	ΔAIC
z3	**7**	**-187.40**	**0.00**
z4	10	-184.64	-2.77
z2	6	-178.00	-9.41
z1	4	-126.40	-61.00
z0	3	-121.27	-66.13

Sampling dates
2018-06-28-12-47-03
2018-06-29-12-17-19
2018-07-02-12-24-43
2018-07-03-13-05-40
2018-07-06-14-20-47
2018-07-09-11-52-24

Lantana sp.

X axis model	df	AIC	ΔAIC
m2	**4**	**475.45**	**0.00**
m3	5	477.15	-1.69
m6	7	479.41	-3.95
m7	8	481.10	-5.64
m9	10	483.35	-7.90
m10	14	490.95	-15.49
m0	3	730.76	-255.31
m1	4	732.63	-257.17
m4	6	735.88	-260.42
m5	7	737.75	-262.29
m8	10	743.71	-268.25

Z axis model	df	AIC	ΔAIC
z3	7	-115.16	0.00
z1	**4**	**-113.42**	**-1.74**
z4	10	-110.60	-4.56
z0	3	-80.24	-34.92
z2	6	-80.20	-34.96

Sampling dates
2017-07-18-16-24-44
2017-07-19-14-05-17
2017-07-20-14-00-54
2017-07-24-14-42-55
2017-07-25-11-40-20
2017-08-09-12-10-35

Lavandula angustifolia

X axis model	df	AIC	ΔAIC
m7	8	34.35	0.00
m6	**7**	**38.65**	**-4.30**
m10	14	40.51	-6.16
m9	10	42.99	-8.64
m3	5	70.06	-35.71
m2	4	73.56	-39.21
m5	7	216.61	-182.26
m4	6	218.08	-183.73
m8	10	220.36	-186.01
m1	4	234.15	-199.80
m0	3	235.36	-201.01

Z axis model	df	AIC	ΔAIC
z3	7	-73.62	0.00
z2	**6**	**-70.93**	**-2.69**
z4	10	-68.52	-5.10
z1	4	-42.00	-31.62
z0	3	-40.42	-33.20

Sampling dates
2018-07-27-11-31-18
2018-07-27-15-23-41
2018-07-30-10-40-46
2018-07-30-13-15-40
2018-07-31-10-21-43
2018-07-31-11-39-07

Leucanthemum vulgare

X axis model	df	AIC	ΔAIC
m10	14	402.76	0.00
m7	**8**	**408.59**	**-5.84**
m9	10	420.41	-17.65
m3	5	423.98	-21.23
m6	7	428.29	-25.54
m2	4	442.01	-39.25
m5	7	618.49	-215.73
m1	4	622.03	-219.27
m8	10	623.39	-220.63
m4	6	626.17	-223.41
m0	3	629.36	-226.60

Z axis model	df	AIC	ΔAIC
z4	**10**	**-120.65**	**0.00**
z3	7	-112.30	-8.36
z2	6	-80.97	-39.68
z1	4	-18.36	-102.29
z0	3	-5.20	-115.46

Sampling dates
2018-07-27-12-48-43
2018-07-27-14-06-12
2018-07-30-11-58-13
2018-07-30-14-33-05
2018-07-31-12-56-32

Lilium sp.

X axis model	df	AIC	ΔAIC
m10	14	429.76	0.00
m8	**10**	**431.30**	**-1.53**
m9	10	440.01	-10.25
m4	6	441.00	-11.24
m0	3	441.92	-12.16
m5	7	442.80	-13.03
m6	7	443.00	-13.23
m1	4	443.72	-13.96
m2	4	443.92	-14.15
m7	8	444.79	-15.03
m3	5	445.72	-15.95

Z axis model	df	AIC	ΔAIC
z3	7	-211.08	0.00
z2	**6**	**-210.26**	**-0.81**
z4	10	-207.27	-3.81
z1	4	-187.57	-23.51
z0	3	-187.30	-23.78

Sampling dates
2017-11-22-15-06-50
2017-11-23-12-16-53
2017-11-23-14-51-41
2017-11-27-11-13-41
2018-04-09-13-13-01
2018-04-13-11-42-49

Linum grandiflorum

X axis model	df	AIC	ΔAIC
m10	**14**	**-222.80**	**0.00**
m7	8	-208.48	-14.32
m6	7	-206.13	-16.68
m9	10	-201.15	-21.66
m3	5	-159.53	-63.27
m2	4	-157.89	-64.92
m8	10	-140.56	-82.25
m5	7	-127.53	-95.27
m4	6	-126.20	-96.60
m1	4	-90.76	-132.04
m0	3	-89.87	-132.94

Z axis model	df	AIC	ΔAIC
z4	10	-239.47	0.00
z3	**7**	**-238.41**	**-1.06**
z2	6	-230.82	-8.65
z1	4	-182.46	-57.01
z0	3	-178.26	-61.21

Sampling dates
2017-09-18-10-23-43
2017-09-19-13-20-49
2017-09-20-10-36-22
2017-09-26-12-28-17
2017-09-27-14-29-31
2017-09-28-13-30-35

Linum usitatissimum

X axis model	df	AIC	ΔAIC
m10	14	146.18	0.00
m9	**10**	**147.48**	**-1.31**
m6	7	170.30	-24.12
m7	8	171.55	-25.38
m2	4	189.73	-43.55
m3	5	191.04	-44.87
m4	6	340.82	-194.64
m5	7	342.40	-196.22
m8	10	344.03	-197.86
m0	3	349.55	-203.37
m1	4	351.14	-204.97

Z axis model	df	AIC	ΔAIC
z3	7	-161.84	0.00
z4	10	-161.44	-0.40
z2	**6**	**-160.41**	**-1.43**
z1	4	-137.78	-24.06
z0	3	-137.01	-24.82

Sampling dates
2018-06-21-13-30-34
2018-06-26-10-35-17
2018-06-26-11-52-44
2018-06-26-13-10-11
2018-06-26-14-27-36
2018-07-04-12-36-47

Nepenthes sp.

X axis model	df	AIC	ΔAIC
m6	7	-640.32	0.00
m7	8	-638.61	-1.71
m9	10	-637.05	-3.28
m2	**4**	**-636.65**	**-3.67**
m10	14	-636.25	-4.08
m3	5	-634.94	-5.39
m0	3	-360.19	-280.13
m1	4	-358.31	-282.02
m4	6	-358.05	-282.28
m5	7	-356.16	-284.16
m8	10	-352.85	-287.48

Z axis model	df	AIC	ΔAIC
z2	**6**	**-398.69**	**0.00**
z3	7	-397.04	-1.65
z4	10	-395.02	-3.67
z0	3	-389.03	-9.66
z1	4	-387.34	-11.35

Sampling dates
2018-05-04-10-39-51
2018-05-04-11-57-21
2018-05-04-13-14-50
2018-05-04-14-32-17
2018-05-10-11-38-47
2018-05-10-14-13-44

Nicotiana tabacum

X axis model	df	AIC	ΔAIC
m2	**4**	**-940.22**	**0.00**
m3	5	-938.23	-1.99
m6	7	-938.03	-2.20
m7	8	-936.03	-4.19
m9	10	-934.91	-5.31
m10	14	-928.70	-11.52
m0	3	-847.95	-92.27
m1	4	-845.96	-94.26
m4	6	-844.75	-95.48
m5	7	-842.75	-97.47
m8	10	-838.04	-102.18

Z axis model	df	AIC	ΔAIC
z3	7	-550.60	0.00
z4	10	-547.78	-2.82
z2	**6**	**-545.19**	**-5.42**
z1	4	-538.71	-11.89
z0	3	-534.18	-16.43

Sampling dates
2017-11-29-11-24-12
2017-11-29-12-41-33
2017-11-29-13-58-56
2017-11-29-15-16-23
2018-04-09-11-55-32
2018-04-09-15-47-53

Oenothera caespitosa

X axis model	df	AIC	ΔAIC
m10	**14**	**452.49**	**0.00**
m7	8	461.04	-8.55
m9	10	485.11	-32.62
m3	5	491.56	-39.07
m6	7	497.05	-44.56
m2	4	523.53	-71.04
m5	7	617.95	-165.46
m8	10	623.86	-171.37
m1	4	634.20	-181.71
m4	6	639.13	-186.64
m0	3	653.81	-201.32

Z axis model	df	AIC	ΔAIC
z1	**4**	**14.05**	**0.00**
z3	7	16.56	-2.52
z4	10	21.60	-7.56
z0	3	54.55	-40.50
z2	6	58.00	-43.95

Sampling dates
2018-08-02-13-39-59
2018-08-02-14-57-28
2018-08-03-11-15-43
2018-08-03-12-33-08
2018-08-03-13-50-37
2018-08-06-11-38-32

Osteospermum sp.

X axis model	df	AIC	ΔAIC
m10	**14**	**329.77**	**0.00**
m7	8	345.02	-15.25
m9	10	375.93	-46.16
m6	7	378.77	-49.00
m3	5	388.25	-58.48
m2	4	416.95	-87.17
m8	10	478.53	-148.76
m5	7	483.01	-153.23
m4	6	504.11	-174.34
m1	4	509.07	-179.30
m0	3	527.96	-198.18

Z axis model	df	AIC	ΔAIC
z4	**10**	**-37.56**	**0.00**
z3	7	-4.89	-32.67
z1	4	26.37	-63.92
z2	6	76.34	-113.90
z0	3	92.20	-129.75

Sampling dates
2017-07-18-13-49-59
2017-07-19-11-30-31
2017-07-20-15-18-15
2017-07-24-12-08-11
2017-07-25-14-16-22
2017-08-09-14-45-23

Papaver cambricum

X axis model	df	AIC	ΔAIC
m7	8	768.48	0.00
m3	5	768.88	-0.39
m6	7	772.41	-3.93
m2	**4**	**772.68**	**-4.20**
m9	10	773.47	-4.99
m10	14	773.62	-5.14
m5	7	784.35	-15.87
m1	4	784.38	-15.90
m0	3	787.87	-19.38
m4	6	787.94	-19.46
m8	10	788.66	-20.17

Z axis model	df	AIC	ΔAIC
z1	**4**	**-144.20**	**0.00**
z3	7	-139.67	-4.53
z4	10	-137.02	-7.17
z0	3	-135.33	-8.86
z2	6	-130.69	-13.50

Sampling dates
2018-05-09-13-03-46
2018-05-09-14-21-15
2018-05-10-12-56-15
2018-05-10-15-31-09
2018-05-11-10-33-18
2018-05-11-13-08-14

Papaver rhoeas

X axis model	df	AIC	ΔAIC
m5	7	-99.49	0.00
m3	**5**	**-98.00**	**-1.49**
m8	10	-97.86	-1.64
m4	6	-82.76	-16.73
m2	4	-81.59	-17.90
m1	4	-37.87	-61.62
m0	3	-24.76	-74.73
m6	7	155.07	-254.56
m9	10	168.27	-267.76
m7	8	177.15	-276.65
m10	14	192.06	-291.55

Z axis model	df	AIC	ΔAIC
z3	7	-164.02	0.00
z4	10	-159.72	-4.31
z2	**6**	**-158.94**	**-5.09**
z1	4	-142.71	-21.31
z0	3	-138.87	-25.15

Sampling dates
2018-06-15-13-51-16
2018-06-18-13-10-59
2018-06-19-11-27-20
2018-06-19-12-44-49
2018-06-19-14-02-18
2018-06-19-15-19-43

Potentilla sp.

X axis model	df	AIC	ΔAIC
m7	**8**	**-207.04**	**0.00**
m10	14	-204.01	-3.03
m3	5	-197.37	-9.66
m9	10	-195.64	-11.40
m6	7	-193.91	-13.13
m2	4	-184.98	-22.06
m1	4	155.86	-362.90
m5	7	157.04	-364.08
m0	3	158.44	-365.48
m4	6	159.69	-366.73
m8	10	162.79	-369.83

Z axis model	df	AIC	ΔAIC
z1	**4**	**-329.74**	**0.00**
z3	7	-326.48	-3.26
z4	10	-321.83	-7.91
z0	3	-317.09	-12.65
z2	6	-313.55	-16.19

Sampling dates
2018-05-14-10-25-04
2018-05-14-11-42-32
2018-05-15-11-43-56
2018-05-15-14-18-52
2018-05-16-12-09-48
2018-05-16-13-27-15

Ranunculus acris

X axis model	df	AIC	ΔAIC
m9	**10**	**1128.43**	**0.00**
m10	14	1130.99	-2.56
m7	8	1139.72	-11.29
m6	7	1142.25	-13.82
m3	5	1158.13	-29.70
m2	4	1160.32	-31.88
m5	7	1265.50	-137.06
m4	6	1266.49	-138.05
m8	10	1271.12	-142.69
m1	4	1275.79	-147.36
m0	3	1276.63	-148.19

Z axis model	df	AIC	ΔAIC
z4	10	157.87	0.00
z3	**7**	**163.43**	**-5.55**
z1	4	165.57	-7.70
z0	3	209.32	-51.45
z2	6	209.52	-51.65

Sampling dates
2018-04-30-10-20-20
2018-04-30-11-37-45
2018-04-30-12-55-14
2018-04-30-14-12-42
2018-05-01-10-05-37
2018-05-03-11-20-04

Ranunculus lingua

X axis model	df	AIC	ΔAIC
m10	14	915.79	0.00
m7	**8**	**921.71**	**-5.91**
m3	5	925.22	-9.43
m9	10	946.93	-31.14
m6	7	951.34	-35.55
m2	4	953.93	-38.14
m1	4	1145.70	-229.91
m5	7	1147.14	-231.35
m8	10	1150.25	-234.46
m0	3	1158.93	-243.14
m4	6	1160.59	-244.80

Z axis model	df	AIC	ΔAIC
z1	4	13.05	0.00
z4	10	16.28	-3.23
z3	**7**	**17.87**	**-4.83**
z0	3	103.37	-90.32
z2	6	108.77	-95.72

Sampling dates
2018-07-09-13-09-53
2018-07-09-14-27-20
2018-07-10-11-15-27
2018-07-13-12-43-50
2018-07-13-14-01-21
2018-07-16-10-15-21

Rudbeckia hirta

X axis model	df	AIC	ΔAIC
m7	**8**	**655.28**	**0.00**
m10	14	663.03	-7.75
m6	7	664.14	-8.86
m3	5	665.04	-9.75
m9	10	668.20	-12.92
m2	4	673.36	-18.07
m5	7	729.08	-73.79
m8	10	733.34	-78.06
m1	4	735.44	-80.16
m4	6	735.59	-80.30
m0	3	741.62	-86.34

Z axis model	df	AIC	ΔAIC
z3	7	-51.52	0.00
z4	10	-49.12	-2.39
z1	**4**	**-46.23**	**-5.29**
z2	6	-30.94	-20.58
z0	3	-27.30	-24.22

Sampling dates
2017-09-18-12-58-29
2017-09-19-14-38-14
2017-09-20-13-11-08
2017-09-26-11-10-54
2017-09-27-11-54-43
2017-09-28-10-55-46

Scabiosa sp.

X axis model	df	AIC	ΔAIC
m3	**5**	**251.20**	**0.00**
m7	8	255.72	-4.52
m10	14	264.58	-13.39
m2	4	270.29	-19.09
m6	7	274.91	-23.71
m9	10	279.69	-28.49
m1	4	572.77	-321.57
m5	7	578.26	-327.06
m0	3	578.26	-327.07
m8	10	583.63	-332.43
m4	6	583.76	-332.57

Z axis model	df	AIC	ΔAIC
z3	7	-75.78	0.00
z1	**4**	**-70.03**	**-5.76**
z4	10	-69.80	-5.98
z2	6	-26.77	-49.01
z0	3	-24.54	-51.24

Sampling dates
2017-07-18-15-07-20
2017-07-19-12-47-54
2017-07-20-12-43-29
2017-07-24-16-00-20
2017-07-25-12-58-21
2017-08-09-10-53-12

Taraxacum agg.

X axis model	df	AIC	ΔAIC
m10	14	803.67	0.00
m9	**10**	**808.22**	**-4.55**
m7	8	829.61	-25.94
m6	7	835.27	-31.60
m3	5	887.53	-83.86
m2	4	891.76	-88.09
m5	7	1064.85	-261.18
m4	6	1066.40	-262.73
m8	10	1069.21	-265.54
m1	4	1089.96	-286.29
m0	3	1091.16	-287.49

Z axis model	df	AIC	ΔAIC
z4	**10**	**68.66**	**0.00**
z3	7	109.50	-40.83
z1	4	133.79	-65.13
z2	6	166.51	-97.85
z0	3	181.00	-112.34

Sampling dates
2018-04-26-10-58-07
2018-04-26-12-15-36
2018-04-26-14-50-30
2018-05-01-11-23-06
2018-05-01-12-40-30
2018-05-01-13-57-55

Trifolium pratense

X axis model	df	AIC	ΔAIC
m9	**10**	**-227.50**	**0.00**
m10	14	-221.32	-6.18
m6	7	-220.33	-7.17
m7	8	-219.52	-7.98
m2	4	-139.87	-87.63
m3	5	-138.77	-88.73
m4	6	33.85	-261.35
m5	7	35.33	-262.83
m8	10	41.09	-268.59
m0	3	68.86	-296.37
m1	4	70.40	-297.91

Z axis model	df	AIC	ΔAIC
z3	7	-212.80	0.00
z2	**6**	**-212.25**	**-0.55**
z4	10	-207.13	-5.67
z0	3	-177.36	-35.44
z1	4	-177.26	-35.55

Sampling dates
2018-07-19-13-03-30
2018-07-19-14-20-55
2018-07-20-11-21-23
2018-07-20-13-56-15
2018-07-23-11-25-28
2018-07-23-12-42-51

Tulbaghia violacea

X axis model	df	AIC	ΔAIC
m9	**10**	**-42.88**	**0.00**
m10	14	-36.69	-6.19
m6	7	-35.69	-7.19
m7	8	-34.31	-8.58
m2	4	2.47	-45.35
m3	5	3.94	-46.82
m4	6	42.15	-85.03
m5	7	43.67	-86.56
m8	10	48.81	-91.69
m0	3	70.72	-113.60
m1	4	72.30	-115.18

Z axis model	df	AIC	ΔAIC
z2	**6**	**-153.87**	**0.00**
z3	7	-153.03	-0.84
z4	10	-149.23	-4.64
z0	3	-130.12	-23.74
z1	4	-129.07	-24.81

Sampling dates
2017-10-11-16-06-35
2017-10-12-11-37-09
2017-10-17-11-08-54
2017-10-25-13-20-58
2018-06-21-12-13-09
2018-07-04-13-54-13

Vinca herbacea

X axis model	df	AIC	ΔAIC
m7	**8**	**-841.56**	**0.00**
m10	14	-838.34	-3.22
m3	5	-818.74	-22.82
m6	7	-810.45	-31.11
m9	10	-809.09	-32.47
m2	4	-790.46	-51.10
m5	7	-592.50	-249.06
m8	10	-588.05	-253.51
m1	4	-585.48	-256.09
m4	6	-579.48	-262.08
m0	3	-573.07	-268.50

Z axis model	df	AIC	ΔAIC
z4	10	-486.79	0.00
z2	6	-486.39	-0.40
z3	7	-484.58	-2.21
z0	**3**	**-482.43**	**-4.35**
z1	4	-480.62	-6.17

Sampling dates
2018-04-13-13-00-18
2018-04-13-14-17-41
2018-04-13-15-35-08
2018-04-25-12-39-36
2018-04-25-13-57-03
2018-04-26-13-33-05

Xerochrysum bracteatum

X axis model	df	AIC	ΔAIC
m3	**5**	**1117.21**	**0.00**
m7	8	1120.26	-3.05
m2	4	1124.41	-7.20
m6	7	1127.54	-10.33
m10	14	1128.29	-11.08
m9	10	1129.75	-12.54
m1	4	1335.68	-218.46
m0	3	1338.18	-220.97
m5	7	1340.24	-223.03
m4	6	1342.77	-225.56
m8	10	1346.21	-229.00

Z axis model	df	AIC	ΔAIC
z1	**4**	**4.94**	**0.00**
z3	7	6.10	-1.16
z4	10	10.65	-5.72
z0	3	90.70	-85.76
z2	6	94.16	-89.22

Sampling dates
2018-05-31-14-02-55
2018-06-01-13-24-26
2018-06-04-14-45-41
2018-06-05-11-45-07
2018-06-15-11-16-24
2018-06-18-11-53-32

A7: AIC tables and sampling dates of artificial flower floral humidity analyses

The AIC tables, and sampling dates, relating to our analyses of artificial flower humidity structure as documented in chapter 5. For each individual artificial flower of each variant the date and time at which the first x axis transect replicate began is given (YYYY-MM-DD-hh-mm-ss). In each AIC table, each species having one for x and z axis models, AIC and degrees of freedom 'df' are given. Difference in ΔAIC, here calculated as AIC of model with the lowest AIC minus that of the current model, is also provided. Within each AIC table, shaded and in bold are the best fitting models as per the guidelines given in (Richards, 2008).

Active Humidity Humid

X axis model	df	AIC	ΔAIC
m3	**5**	**1114.88**	**0.00**
m7	8	1119.73	-4.85
m10	14	1127.27	-12.39
m2	4	1151.32	-36.44
m6	7	1156.27	-41.39
m9	10	1158.71	-43.82
m1	4	1473.78	-358.90
m5	7	1479.30	-364.42
m8	10	1485.08	-370.20
m0	3	1488.09	-373.21
m4	6	1493.63	-378.75

Z axis model	df	AIC	ΔAIC
z1	**4**	**86.67**	**0.00**
z3	7	92.09	-5.42
z4	10	95.24	-8.57
z0	3	233.29	-146.62
z2	6	239.03	-152.36

Sampling dates
2017-11-16-11-08-52
2017-11-16-12-26-15
2017-11-16-13-43-38
2017-11-16-15-01-05
2018-02-01-11-54-27
2018-02-01-10-37-04
2018-02-01-13-11-52
2018-02-01-14-29-17

Active Humidity Dry

X axis model	df	AIC	ΔAIC
m10	**14**	**-29.10**	**0.00**
m9	10	-22.24	-6.85
m7	8	126.84	-155.94
m6	7	132.75	-161.84
m5	7	205.87	-234.96
m8	10	210.19	-239.28
m4	6	210.35	-239.45
m3	5	314.15	-343.25
m2	4	317.09	-346.18
m1	4	364.45	-393.55
m0	3	366.80	-395.90

Z axis model	df	AIC	ΔAIC
z4	**10**	**-347.76**	**0.00**
z3	7	-312.58	-35.18
z2	6	-310.97	-36.78
z1	4	-279.22	-68.53
z0	3	-278.31	-69.44

Sampling dates
2017-11-20-15-10-07
2017-11-20-16-27-30
2017-11-20-17-44-55
2017-11-20-19-02-19
2018-01-30-10-35-56
2018-01-30-11-53-23
2018-01-30-13-10-46
2018-01-30-14-28-06

Passive Humidity Humid

X axis model	df	AIC	ΔAIC
m9	**10**	**1208.27**	**0.00**
m10	14	1214.90	-6.62
m6	7	1260.35	-52.08
m7	8	1261.82	-53.54
m2	4	1298.20	-89.93
m3	5	1299.70	-91.43
m4	6	2047.90	-839.63
m5	7	2049.75	-841.48
m0	3	2054.29	-846.01
m8	10	2055.56	-847.28
m1	4	2056.14	-847.87

Z axis model	df	AIC	ΔAIC
z4	10	-125.11	0.00
z3	**7**	**-122.95**	**-2.15**
z1	4	-76.13	-48.98
z2	6	111.63	-236.74
z0	3	129.54	-254.64

Sampling dates
2017-10-03-10-34-17
2017-10-03-13-09-02
2017-10-04-10-58-13
2017-10-04-12-15-36
2017-10-05-11-36-06
2017-10-05-14-10-52
2017-10-10-13-23-13
2017-10-10-14-40-36
2017-10-23-12-10-47
2017-10-23-13-28-10
2017-10-24-10-17-19
2017-10-24-12-52-05

Passive Humidity Dry

X axis model	df	AIC	ΔAIC
m10	**14**	**872.95**	**0.00**
m9	10	890.60	-17.65
m7	8	1001.48	-128.54
m6	7	1019.29	-146.34
m3	5	1102.00	-229.05
m2	4	1116.68	-243.74
m5	7	1468.42	-595.48
m8	10	1474.10	-601.16
m4	6	1475.71	-602.76
m1	4	1514.24	-641.29
m0	3	1520.78	-647.83

Z axis model	df	AIC	ΔAIC
z4	**10**	**-156.34**	**0.00**
z3	7	-131.26	-25.08
z1	4	-103.48	-52.86
z2	6	-67.79	-88.56
z0	3	-46.80	-109.54

Sampling dates
2017-10-03-11-51-40
2017-10-03-14-26-27
2017-10-04-13-32-59
2017-10-04-14-50-22
2017-10-05-10-18-43
2017-10-05-12-53-29
2017-10-10-10-48-25
2017-10-10-12-05-48
2017-10-23-10-53-22
2017-10-23-14-45-33
2017-10-24-11-34-42
2017-10-24-14-09-28