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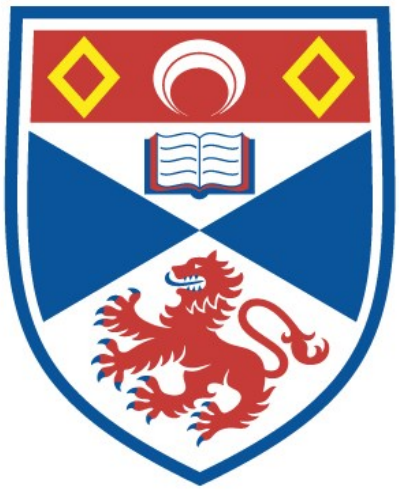

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The effects of insect visitation on floral colour change.

Clive Nuttman

Thesis submitted for the degree of Doctor of Philosophy, University of St. Andrews.

June 2003

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Declaration.

I, Clive Nuttman, hereby certify that this thesis, which is approximately 60000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Abstract.

This study investigated the effects of flower visitation on floral colour change and the subsequent influence of such change on insect foraging behaviour. Colour change was examined in six plant species; *Myosotis sylvatica*, *Echium vulgare* and *Lonicera periclymenum* were studied locally to St. Andrews, Fife, Scotland and *Echium judaeum*, *Lupinus pilosus* and *Alkanna orientalis* were studied in various locations in the eastern Mediterranean region.

Patterns of colour change were recorded both with natural insect visitation allowed and excluded, to establish whether the rate of colour change could be altered through visitation *per se* or rate of visitation. Detailed observation of the flower handling characteristics of all visiting insects allowed artificial floral manipulations to be devised that simulated the different aspects of visitor behaviour. This enabled the effects of simple mechanical handling on colour change to be separated from those of pollen deposition and post-pollination events. Floral reward was measured in relation to flower colour phases to assess whether the change in colour was acting as a functional signal to flower visitors; insect choice of flower colour was noted, to determine whether reward status affected foraging behaviour.

One or more factors significantly altered the characteristics of colour change in all species except *Lonicera periclymenum*. The triggering factor could be the exclusion of visitors, rate of natural visitation, floral manipulation, or aspects of the pollination process. In *Lupinus pilosus* pollen deposition and/or pollen tube growth was the trigger for colour change. Pollen deposition was also the most likely trigger in both *Alkanna orientalis* and *Myosotis sylvatica*, although the varied patterns of colour change in these species could be related to wound responses and/or senescence. Pollination processes were not involved in colour change in either species of *Echium*. The first recorded example of a 'reverse' colour change is reported for *Echium judaeum*.

Floral reward varied between colour phases in all plants except *Echium vulgare*, and visiting insects did not show any bias towards particular flower colour phases in this plant. In all other species a variety of flies and bees visited the most rewarding colour phase preferentially.

A model is presented that incorporates all influences on floral colour change in a single framework, potentially unifying the concepts of 'age-related' and 'inducible' change which have previously been thought to be distinct.

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Last, but by no means least, I dedicate this thesis to my parents, Don and Yvonne (don't worry, I don't expect you to plough through it!). Thank you for everything...you're probably still wondering what I am going to do when I grow up; 40 years on and still playing with bees!

Contents.

Chapter 1 - Introduction.

A flower is the reproductive element of an angiosperm and comprises modified leaves that can take elaborate and often highly coloured form. Colour is crucial because it, together with odour, is usually described as a secondary attractant for visiting organisms; primary attractants being nectar, pollen and other floral resources sought by such visitors (e.g. Faegri & van der Pijl, 1979; Vogel, 1983). The flowers of some plant species undergo dramatic changes in colour during their lives.

The subject of floral colour change has a long history within the scientific literature and has been thoroughly reviewed (Weiss, 1992, 1995a; Weiss & Lamont, 1997). The phenomenon was first formally described over 250 years ago (Weiss, 1995a). In the nineteenth century, in a letter to Charles Darwin, Műller (1877) noted the vivid colour change undergone by *Lantana camara* in Brazil and how the visitation patterns of butterflies were, apparently, influenced by such a change.

From the largely observational work of early scientists and naturalists, colour change studies in the twentieth century expanded to consider further aspects of the process, with particular reference to the ecological and evolutionary relevance of flower colour change. In addition, this work has been complemented by studies of the sensory abilities of a range of anthophilous visitors together with greater understanding of the physiological processes that underpin colour change within the plant.

The burgeoning literature has revealed that floral colour change is widespread in angiosperms; Weiss (1992, 1995a) reports the incidence in 20%

of flowering plant families and, more recently Weiss & Lamont (1997) revised this figure to 78 families encompassing over 450 species. A database included in Weiss (1995a) reveals that colour change in many of these species has been recorded through personal observation by, and personal communication to, the author; published work on the subject area in general, subsequent to this date, is scarce. Detailed studies of colour change at the species level remain relatively few and, while much attention has been given to this change as a visual signal for the visitor, there is also potential to investigate how rates of visitation and/or visitation behaviour might influence the change itself.

This thesis investigates floral colour change in relation to insect visitation; in particular the questions of if, and how, visitor activity can trigger such change are addressed. Here, I introduce the subject in terms of general pollination ecology, followed by a review of the understanding of floral colour change to date and the influence of colour on the behaviour of flower visitors.

1.1 Pollination Ecology: why do visitors visit flowers?

In describing biotic pollination as, simply, a mutualistic association, the reproductive benefit of pollen dispersal gained by the plant and the reward sought by the visitor mask a complex interaction of processes. The ecology of pollination involves many factors that may depend, to differing extents, on the phylogeny, morphology, physiology and behaviour of the organisms concerned; in addition, environmental conditions influence both plant and visitor. A wide range of invertebrates and vertebrates has been recorded as flower visitors and the association of plant and animal represents a mutual exploitation of resources.

The classic work of Faegri & van der Pijl (1979) summarised the categorisation of both flower type and visitor into pollination syndromes – matching morphological, physiological and behavioural traits of either, or both, organisms to produce 'typical' floral types visited by certain groups of pollinators. The variety of organisms that have been placed in these syndromes include bats, birds (with a distinction between passerines and hummingbirds) and certain non-flying mammals among vertebrates, and flies, beetles, wasps, bees, ants, butterflies and moths from invertebrate orders. Floral colour represents one aspect of these syndromes, along with scent, reward, morphology, size, and time of anthesis.

While various floral features provide an important cue for the attraction of anthophilous visitors, the purpose of visitation is to gain reward. This is usually food for energetic requirements in the form of nectar or pollen (Faegri & van der Pijl, 1979), either used directly by the visitor or for nest/larval provisioning. Flowers may offer solely pollen as a reward or both pollen and nectar; the latter is particularly susceptible to modification by environmental factors. Other rewards are less commonly recorded and include oils and waxes (Buchmann, 1987), floral scent used in mate attraction (Sazima et al.1993) and stigmatic exudate (e.g. Mathur & Mohan Ram, 1986). Flowers may also provide sites for shelter (and used as 'warm-up' sites in temperate regions), prey capture and mate-finding (Faegri & van der Pijl, 1979).

1.1.1. Pollen.

Pollen contains the male gametes and is not, unlike nectar, offered purely as an attractant or reward to foraging organisms; plant reproduction (unless a species is self-compatible) is dependent on successful dispersal of pollen (either biotically or abiotically) to conspecifics. As a highly proteinaceous material, pollen is of great nutritional value to visiting organisms; beetles, flies, some butterflies and some mammals feed on it. In particular, bees – especially solitary species – harvest pollen as a larval food and are thus highly committed to regular flower visitation. The bees are regarded as pollinators *par excellence* due to a combination of specialised pollen-collecting and nectar-feeding adaptations, social organisation in many groups, and their reliance on flowers for a source of food for their young (O'Toole & Raw, 1991).

1.1.2. Nectar.

Nectar comprises a sugary solution containing combinations that include sucrose, fructose and glucose (Proctor et al. 1996) as well as smaller, but occasionally significant, quantities of phenolics and amino acids (Baker & Baker, 1973; but see Willmer, 1980). The substance is secreted, with temporal variation, from the nectaries, glandular tissue within or, more rarely, external to the flower. Plants produce nectar for the simple function of attracting animals; it is sought, as a valuable high-energy food, by the full range of visiting organisms (Faegri & van der Pijl, 1979). The sugar composition of nectar sought by visitors broadly correlated with the type of visitor according to Baker & Baker (1983); for

example, 'long-tongued' bees were usually rewarded with sucrose-rich nectar and New World bats almost exclusively fed on hexose-rich nectar. Although not the sole determinant of foraging behaviour, the energetic value of nectar rewards could be used to predict the flower-visiting behaviour of insect pollinators (Heinrich, 1983). Hence the characteristics of nectar can influence visitation; sugar concentration has been shown to affect the pattern of visitation in bumblebees (e.g. Corbet et al.1979), and food choice in hummingbirds (Hainsworth & Wolf, 1976). These characteristics are also subject to alteration by environmental conditions.

1.1.3. How the environment affects nectar.

Although a plant may control temporal patterns of nectar secretion, a number of factors may affect the post-secretory characteristics of nectar. Corbet et al. (1979) highlighted the importance of temperature and humidity in determining nectar volumes and concentrations over time in *Echium vulgare.* Rising temperature and falling humidity correlated with increased concentration and decreased volume as evaporation of water took place. Conversely, a fall in temperature and rising humidity led to higher volumes and lower concentrations. In extremely humid tropical conditions, such a dilution might be expected to occur if nectar is freely exposed, or in the absence of features that help to maintain post-secretory nectar concentrations. Corbet & Willmer (1981) reported the presence of a lipid monolayer on the surface of standing nectar in the Costa Rican plant, *Justica aurea.* Willmer (1980), with regard to three temperate species, illustrated that nectar constituents could be altered by nectar removal by

insect visitors, and also highlighted the role of corolla morphology in the maintenance of nectar concentration. Plowright (1987) confirmed this latter effect in a range of tropical flowers.

Whilst any micro-environmental control of nectar characteristics by the plant that influences visitation may be beneficial, the visitors themselves are, in turn, affected by general environmental conditions.

1.1.4. How the environment affects visitors.

Environmental variables may control the activity patterns and behaviour of insects irrespective of nectar characteristics; temperature, humidity, radiation and wind represent the main factors on both a macro and micro-scale (Willmer, 1982). The elaborate relationships between insects and the weather hinge on the hygrothermal requirements of the former. Constant alteration of the temperature and relative humidity by variation in solar radiation and wind affects the temperature and water balance in insects, exacerbating the intrinsic vulnerability imposed by small size in relation to surface area (Willmer, 1982). Modifying behaviour in response to the above climatic variables may attain Hygrothermal balance in ectotherms. Willmer (1983) stressed the importance of the role of the weather in temperate insect activity patterns, solar radiation correlating with temporal patterns of activity of nectar-feeding insects and thermal costs being the crucial element influencing their foraging times.

There is considerable literature on environmental effects on the flower-visitor association that, while an important peripheral factor, for my studies, does

not require further detailed review in this work. However, of critical relevance is the visitor response to colour in general and colour change in particular.

1.2. Flower colour.

Colour is of particular relevance as an attractant to pollinators (Kevan, 1972; Scogin, 1983, 1988). Given that visual, olfactory (Faegri & van der Pijl, 1979) and, to a lesser degree, tactile senses (e.g. Kevan & Lane, 1985) must be employed by visitors to locate the presence of flowers from varying distances, the interaction between floral cues and sensory modalities underpins flower-visitor relations. Faegri & van der Pijl (1979) acknowledged that the importance, and use, of particular sensory modalities differed between pollinators, especially in relation to the time of day when foraging occurs; thus colour was a vital attractant to diurnal visitors.

The basis for floral colour is the presence of light-absorbing pigments within plastids and cell sap; the pigments comprise the flavonoids, carotenoids and (more rarely found) the betalains (Harborne, 1993). Flavonoids, which are responsible for blue, purple and pink coloration, are water soluble and comprise anthocyanins (that underpin colours at the blue end of the spectrum including ultraviolet), and anthoxanthins that give white to yellow coloration (Harborne, 1976); while fat soluble carotenoids underpin yellow, orange and red coloration (Goodwin, 1980). Betalains are restricted to a single plant order (Caryophyllales) and are also associated with red coloration (Mabry, 1980).

The distribution of pigment within floral parts may influence flower colour; more than one type of pigment may be present and occur in different cell

layers to give different colours to upper and lower surfaces of individual petals (Proctor et al. 1996). These authors also note the role of light refraction and reflection at the petal surface in producing 'white' coloration. The resultant colour, modified by absorbed and reflected light, is one of the means by which a flower attracts a visiting organism, and the differing perceptions of colour between organisms must therefore be borne in mind during any study that involves colour. The insect visual spectrum is of particular relevance to pollination ecology since two-thirds of angiosperms are pollinated by insects (Schoonhoven et al. 1997).

1.2.1. Perception of colour.

Bennett et al. (1994), in a review of colour and sexual selection that equally applied to flower colour, noted the basis of the problem; colour, *per se*, is not the property of an object, but is attributable to the nervous system of the organism perceiving the light. Endler (1990) studied the measurement and classification of colour in relation to animal vision and highlighted the physical properties of light and the visual and sensory processing capacities of an organism as the key factors in colour perception.

The human visual spectrum (400-700nm), based on blue, red, and green photoreceptors, is overlapped at the ultra-violet end of the spectrum (300-390nm) by many other animals (Mulligan & Kevan, 1973; Dafni, 1992; and see review of ultra-violet vision in animals by Silberglied, 1979). Many studies have contributed towards determining the general nature of colour vision in insects, especially hymenopterans. Daumer (1956, 1958) established the nature of colour

vision in honeybees (*Apis mellifera*), possessing a trichromatic visual system analogous to that of humans; these bees were sensitive to green, blue and ultra-violet. Also, honeybees could discriminate 'bee purple', a colour created from mixing light from the opposite ends of the bee spectrum, yellow and ultra-violet (Daumer, 1956). Further results collected for the honeybee, *Apis mellifera*, (the insect which has been examined in greatest depth for visual ability) showed that spectral sensitivity is greatest for the three types of photoreceptor at 344nm (ultra-violet), 436nm (blue), and 544nm (green) (Giurfa & Vorobyev, 1997).

The mechanisms of visual processing in insects have been evaluated (e.g. Backhaus, 1991, Chittka, 1997) and Menzel & Backhaus (1991) pointed out that behavioural testing of insects (using colour stimuli in training experiments) should be used to establish whether effective colour vision is present. These authors noted that the presence of different photoreceptors was not, in itself, indicative of colour vision.

Spectral sensitivity of different receptors showed similar patterns of colour vision in a limited sample of honeybees, bumblebees, solitary bees and wasps (Menzel, 1990). Further experimentation suggested that colour discrimination is a key factor in flower choice in hymenopterans in general (Chittka & Menzel, 1992). Complementary to this work, Vorobyev & Brandt (1997) reconstructed flower images 'as insects see them' through spectral reflectance analysis of a flower, using the honeybee visual system as a model.

Lepidopterans have also been found to be able to discriminate colour; physiological investigations regarding the nature of their receptors have been backed up by behavioural studies in both laboratory and field conditions. A study of 17 butterfly species reported the presence of red-absorbing pigments in the

receptors of 9 species, and that those also possessing green-absorbing pigments were suggested to have great visual acuity in the yellow/orange/red part of the spectrum (Bernard, 1979). The work on *Lantana camara* (see below) illustrates that butterflies are able to discriminate colour and this is confirmed in many other studies (e.g. Goulson & Cory, 1993).

Recent work has suggested that even nocturnal moths are able to discriminate colour; Kelber et al. (2002) showed that *Deilephila elpenor* could distinguish blue and yellow colours (they had previously been trained to feed at artificial flowers) from various grey colorations at even very low light intensities. This supplemented earlier research that showed diurnal moths were using colour cues when visiting flowers (Kelber & Pfaff, 1997) (although Brantjes (1978) showed that they use colour and odour cues in combination).

In dipterans the majority of work on vision has concentrated on hoverflies (Syrphidae). Ilse (1949) discovered that droneflies (*Eristalis tenax*) learned to discriminate particular shades of yellow following training on artificial flowers that provided sugar-water reward. Lunau & Wacht (1994) used naïve *Eristalis tenax* to confirm a behavioural bias towards coloured stimuli; the proboscis extension reflex (PER) could only be elicited in response to reflected green and yellow light of very narrow wavelength (520-600nm), and they attributed the reaction to the sensitivity of a particular photoreceptor type. Further work involving floral guides also reported the PER in response to yellow colour stimuli; Dinkel & Lunau (2001) found that guide lines of any colour were effective in directing *E. tenax* towards the central yellow spot of an artificial flower where the PER was elicited. However, the response was also noted

towards yellow guidelines and suggested that the flies were using the feeding response in error, having perceived the yellow signal as pollen.

Less is known about colour vision in other insect orders, though Menzel & Backhaus (1991) reviewed further studies of Hymenoptera, Lepidoptera and Diptera; together with work on Orthoptera, Hemiptera, Plannipennia, Coleoptera, Odonata and Blattoptera.

To date, there is a relative paucity of information concerning the visual capabilities whether of colour or other parameters, from distance, of a range of visiting organisms. In learning experiments with the honeybee, Giurfa & Vorobyev (1997) showed that angular size of an object was critical in detection tasks (whereby distance and diameter of an object are integrated). Their findings illustrated that distant objects of small angular size were detected by perception of 'green contrast', while closer objects of greater angular size were perceived on the basis of colour *per se*. However, this type of information is not available for the full range of flower visitors, and most studies assume that 'colour' as defined by human vision is an adequate descriptor of the cue being used. Thus, visual capabilities clearly vary between and even (as is known for humans) within species.

It is beyond the scope and technology of this study to quantify colour in relation to insect visitors; as in most other works to date, the human visual spectrum will be used as a starting point to assess colour change. Where such changes coincide with observed behavioural changes of visiting organisms, the precise cues for altered foraging patterns inevitably remain equivocal but perception of a visual signal can be safely assumed to have some role in subsequent behaviour.

1.3. Colour change.

The 'fine line' between colour change and the colour fading that may accompany floral senescence remains to be objectively defined. Senescence is usually described as a gradual deterioration that occurs between maturity and death of a plant, or plant part, resulting in abscission in the case of leaves, fruit and flowers. Colour change (although one of a number of visible cues that can signal the end of floral attraction (van Doorn, 1997)), as opposed to the sort of fading seen during wilting and senescence, appears to have a particular function in flowers. Given that attraction by colour is a powerful potential influence on visitor behaviour (Scogin, 1983), a selective advantage of continued visitation and, therefore, pollination opportunity (and, concomitantly, improved plant fitness) could result through the direction of visitors to pre-change flowers that remained receptive and/or rewarding (Gori, 1983). Several strands of evidence suggesting that colour change may have a salient biological function not attributable to age-related features of senescence were offered by Gori: -

i) Change can be induced by pollination.

ii) Pollination-induced change is rapid and is followed by retention of the flower on the plant for a considerable time prior to senescence and abscission.

iii) Change may involve only part of the flower or display.

iv) Change is frequently linked to alteration of reward status.

v) Colour change is one of several post-pollination alterations that, apparently, provide a signal resulting in modified visitor behaviour.

vi) Floral colour change is prevalent in diverse angiosperm taxa, which implies either independent convergent evolution, or repeated selective loss of ancestral colour change.

With reference to this final issue, Weiss (1995a) noted that colour change has not yet been documented in primitive angiosperm taxa, so the former explanation seems more likely. She found that a wide range of ancestral angiosperm taxa, including magnolid and hamamelid families, exhibited no evidence of floral colour change; the phenomenon was suggested to have evolved in response to selection pressure from visually oriented visitors that became increasingly specialised for flower visitation.

In my study at least one of the features outlined in i) to v) above must be satisfied for the purpose of defining colour change. The types, mechanisms and patterns of colour change and the ecological significance of these changes are reviewed below.

1.3.1. Prevalence of colour change.

Until relatively recently the plant kingdom had not been adequately surveyed for the presence of colour-changing species (Gori, 1983). However, this author noted a photographic survey that documented colour change in selective floral parts in 6% of 621 species, as well as evidence of the process in 26 out of 48 lupin species. Later more comprehensive reviews confirmed the extent of the phenomenon in angiosperms; approximately 500 species are now known to exhibit floral colour change (see appendix in Weiss (1995a) and also Weiss & Lamont (1997)). The widespread incidence of colour change across taxa from

order to species level, together with multiple types and mechanisms of change (see below), denoted probable convergent evolution (Weiss, 1995a).

1.3.2. Types of colour change.

Weiss (1995a) categorised 9 types of part-flower colour change as well as instances (recorded in 48 families) where the whole flower undergoes change. She documented localised change in 61 families of 9 separate floral parts or regions. These elements included the centre of the flower, the corolla tube, nectar guides/banner petal spots, nectaries and raised coronas/central eyes. Discrete petals could also change, as well as sundry petal appendages and reproductive parts, either filaments or parts of the pistil/ovary.

The pattern of types of localised colour change was inconstant within and between taxa (Weiss, 1995a). Change of the centre of the flower was documented in 27 families while change of petal appendages (e.g. fringed keels in Polygalaceae) was found in just 4 families. Within the family Verbenaceae, flowers from different species of the same genus (*Lantana*) show discrete types of change whereas in 41 other families colour change is restricted to change of a single part. Taxonomic representation of colour change is therefore disparate; a feature also found in the mechanisms of colour change.

1.3.3. Mechanisms of colour change.

Direct investigation of the mechanisms involved in colour change has been carried out in a limited number of species; pigments of plants from 26

families were analysed through microscopic examination or spectrophotometry by Weiss (1995a). Where colour change was observed from white/yellow/green/pink to orange/red/purple or vice versa, changes in anthocyanins were implicated, and the appearance of carotenoids was involved in white to yellow colour change (Weiss, 1995a). She identified seven probable sources of physiological process that contributed to floral colour change; the majority involved either synthesis or degradation of the main pigments.

i) Raised levels of anthocyanins were found in 25 plant families.

ii) Reduced levels of anthocyanins were found in 2 families.

iii) Two families showed increased levels of carotenoids.

iv) Two families showed reduced levels of carotenoids.

v) Synthesis of betalains occurred in 2 families (Aizoaceae and Cactaceae) of a single order (Caryophyllales).

vi) Colour change in 3 orders was suggested to occur due to altered pH. This type of change is, presumably, also linked to alteration in pigments; Pecket (1966) reported that anthocyanins were sensitive to change in pH during senescence in *Lathyrus hirsutus* and Fukada-Tanaka et al. (2000) mentioned the same association in a study of *Ipomoea nil.*

vii) Movement of floral parts accounted for 'apparent' colour change in one order. In two genera of the family Caesalpiniaceae (order Fabales) colour change was accompanied by a movement of floral parts that caused some part of the flower to be hidden from the visitor's view (Weiss, 1995a).

The pigment change mechanism for the majority of taxa included in the study by Weiss (1995a) was predicted by the researcher on the basis of these findings and the direction of colour change observed.

Weiss (1995a) concluded that evolutionary history alone cannot explain the occurrence of colour change across taxa. Colour change within closely related taxa may result from different mechanisms and affect different floral parts; conversely, unrelated species may exhibit similar mechanisms. Physiologically distinct processes suggest separate evolutionary origins and provide evidence for strength of selection for colour change.

1.3.4. Causes of colour change.

Whilst the trends and patterns of the types and mechanisms of colour change are varied, just two categories have been used to describe the causes of colour change. Gori (1983) separated floral change from simple senescence and described such change as either pollination-induced or non-induced. Hence colour changes were temporally variable during a flower's life following pollination, or occurred at a fixed time after opening and were, therefore, age-related. In both instances, the post-change flowers were retained on the plant prior to senescence. As a minor modification to this division, Weiss (1992) noted that "...colour changes are induced or hastened by pollinator visitation..." My study concentrates on which aspects of visitation trigger such changes if they are not simply age-related processes.

Although the mechanisms described in 1.3.3. are regarded as proximate causes (the physiological and biochemical bases) of colour change, the ultimate

triggers of such change remain unresolved for most species. Investigation of specific triggers of colour change is largely absent in the literature and the status of most species as undergoing age-related or inducible colour change is therefore rarely supported by detailed data. Both non-inducible age-related colour change and inducible change, resulting from some aspect of visitation, have been reported.

In an attempt to clarify and review some of these issues, the findings of recent studies of colour-changing species are summarised in Table 1.1 at the end of this chapter. In many cases these studies made no attempt to link precise aspects of visitation or pollination processes to the observed colour changes. Some research, in addressing disparate issues such as plant reproductive success and visitor behaviour, merely mentions the occurrence of a colour change as opposed to investigating causal explanations for the phenomenon.

Both the taxonomic distribution of inducible and non-inducible changes and the evolution of the types of change are indistinct (Weiss, 1992; Weiss & Lamont, 1997). These authors noted that although within plant families colour-changing taxa generally fell into a single category, in the genus *Lupinus* (Papilionaceae) both inducible and non-inducible colour change had been documented.

With regard to the conditions that could support the evolution of the different types of change, Weiss (1992) outlined two scenarios. Where little variance occurred in relative amounts of pollen removal or timing of pollination, or all pollen is removed over multiple visits, non-inducible change would be favoured. In contrast, inducible change would be selectively advantageous in less predictable circumstances; for example, where there was competition between

plants for pollinators, irregular visitation or a general dearth of pollinators, or where a single/few visits were required for depletion of all pollen. In the former case, a fixed temporal change would, presumably, ensure the end of the period of floral attraction at a point when sufficient visitation had occurred to effect successful pollination and further visitation would be guided towards still attractive pre-change flowers. In unpredictable conditions, a visitation- or pollination-induced change would prevent wasteful additional visitation to already fertilised flowers and, again, visitors would be directed away from these flowers.

1.3.5. Non-inducible colour change.

By comparing the time taken to commence and complete colour change between flowers receiving natural levels of visitation and those where visitation is prevented, it can be determined whether the process is simply related to the age of the flower. Where there is no difference in rate (or nature) of colour change between the two groups, the effects of visitation or other factors may be ruled out.

For example, colour change was found to be time-dependent in several lupin species, irrespective of visitation or other manipulation (e.g. Schaal & Leverich, 1980; Juncosa & Webster, 1989). Non-inducible change has also been reported in *Cryptantha humilis* (Caspar & La Pine, 1984), *Fuchsia excorticata* (Delph & Lively, 1985), *Banksia ilicifolia* (Lamont & Collins, 1988), *Mertensia paniculata* (Morris, 1996), *Myosotis colensoi* (Robertson & Lloyd, 1993), *Pulmonaria collina* (Oberrath & Bohning-Gaese, 1999), *Diervilla lonicera*

(Schoen, 1977), *Hibiscus mutabilis* (Amrhein & Frank, 1989), *Rhexia virginica* (Larson & Barrett, 1999a), *Cayratia japonica* (Kakutani et al.1989), *Calytrix glutinosa*, *Hypocalymma angustifolium*, *Verticordia huegelii*, *Grevillea pilulifera*, *Isopogon dubius* and *Petrophile biloba* (Lamont, 1985), *Chamelaucium uncinatum* (O'Brien, 1996), and *Aesculus carnea*, *Androsace lanuginosa*, and *Raphiolepis indica* (Weiss, 1992).

1.3.6. Inducible colour change.

Several of the 19 studies that have found colour change to be inducible (see Table 1.1) also extended their findings to indicate a possible trigger for such change. The remainder merely inferred that colour change was caused by visitation and/or pollination but did not define whether an aspect of a visitor's flower-handling activity or subsequent post-pollination events triggered that process.

From the evidence presented in some of these studies, a link can be made between pollen deposition as a potential trigger for colour change, the concomitant damage to floral tissues, and the subsequent role of plant growth regulators in the colour change process. In the orchid *Calypso bulbosa* Proctor & Harder (1995) found that colour change could be triggered by pollen deposition. Wounding of stylar tissue during the pollination process in *Carthamus tinctorius*, that was caused by scratches and tears of the stigmatic surface through pollen deposition and subsequent plant growth regulator (gibberellic acid) activity, was found to be the immediate precursor to colour change in this species (Fukushima et al. 1999).

The plant growth regulators auxin and ethylene were implicated in the fading of coloration in *Vanda* orchids (Burg & Dijkman, 1967); natural fading and pollinia removal (also, presumably, causing damage to floral parts) led to ethylene synthesis, as did exposure to auxin. Akamine (1963) had reported similar results for the same species. Although fading of colour does not strictly represent colour change as defined in this study, this example is regarded as colour change by Weiss (1995a).

Ethylene release has been linked to distinct colour change in other species. Stead & Reid (1990) found that colour change in the banner petal spot of *Lupinus albifrons* could be accelerated by exposure to exogenous ethylene, and also that endogenous ethylene was produced in greater concentrations in the style and ovary. Raised levels of ethylene could be induced through pollination, removal of the stigma, or squeezing the stigma. This coincided with colour change and the accumulation of anthocyanin pigment in the banner petal.

In *Cymbidium* orchids, Arditti et al. (1973) noted that pollination, emasculation and treatment with auxin could all lead to the production of ethylene within flowers, and that one effect of exposure to ethylene was colour change around wounded tissues. In the same species the effects of damage through emasculation, or ethylene treatment, brought about a change to red coloration in the labellae of flowers (Woltering & Somhorst, 1990); raised levels of anthocyanins (including cyanidin and malvidin) caused this change and followed increased activity of the enzyme phenylalanine ammonia-lyase (PAL). This enzyme was known to play a key role in the synthesis of anthocyanins and its activity could be elevated by the presence of ethylene (Woltering & Somhorst, 1990).

Rapid accumulation of anthocyanin pigments following increased activity of PAL had earlier been reported to be responsible for the white to red colour change in *Hibiscus mutabilis* (Amrhein & Frank, 1989). The synthesis of anthocyanins from a metabolic pathway that begins with PAL is now well understood (see review by Winkel-Shirley, 2001). Farzad et al. (2002) linked pollen deposition, raised levels of the anthocyanidin malvidin, and availability of light (a further requirement in anthocyanin production) to the colour change of *Viola cornuta*. These authors showed that when pollen was deposited on the stigma in flowers that were exposed to light, colour change occurred; this was not the case for plants kept in the dark. Hence both pollination and light were required as precursors to raised anthocyanin production. The presence of anthocyanins, in conjunction with co-pigments, is known to give red, purple and blue coloration in flowers (Farzad et al. 2002).

Thus ethylene production within plants has been clearly linked to anthocyanin synthesis (see above) and both are also involved in colour change. Whether as a result of pollination, damage caused to floral tissue by this process, or wounding of tissues during experimental treatments, colour change is hastened by the presence of ethylene.

Although these studies report some of the ultimate biochemical processes that may cause inducible colour change in some species, little attention has been paid to proximate triggers that do not involve pollination; only 2 examples have been published. In *Lupinus arizonicus* and *L. sparsiflorus* colour change of the banner petal spot was induced through either manipulation of the stigma or the removal of pollen from the stigmatic surface during the period of stigmatic receptivity (Wainwright, 1978). Although this example used manipulations that

simulated potential visitor activity, it is conceivable that either treatment caused tissue damage and thus colour change was instigated by the pathway illustrated above.

Pollinator visits to *Oenothora drummondii* were mimicked by Eisikowitch & Lazar (1987) to measure rates of colour change in relation to these mechanical handling effects. No alteration to naturally observed patterns of change was evident when touching various floral parts to simulate visiting insect activity, but removal of nectar did accelerate the rate of colour change.

The remaining studies detailed in Table 1.1 do not record what type of colour change might have occurred and a number report alternative findings in relation to the question of inducibility.

1.3.7. Conflicting evidence of causes of colour change.

In a number of studies colour change is suggested to be age-related but the reporting of this particular aspect of the work is equivocal (see column headed Age/Inducibility in Table 1.1). Here I pick out some examples to highlight the potential confusions.

a) Eight species were noted by Lamont (1985) to display non-inducible colour change following natural visitation, yet two of these species showed variable rates of colour change following floral manipulation by the experimenter: *Darwinia citriodora* exhibited more rapid colour change and *Verticordia chrysantha* much slower colour change when their stigmas had been removed.

b) Oberrath et al. (1995) suggested that colour change in several species of *Pulmonaria* (*P. officinalis*, *P. obscura* and *P.officinalis* x *obscura* hybrids) was age-related but conceded the possibility that handling effects on colour change could not be discounted. However, rates of colour change in *P. officinalis* and *P. rubra* were found to accelerate following artificial pollination in another study (Buchanan, 1995).

c) Colour change in *Lupinus nanus* was reported to be inducible by Weiss (1992), occurring in 1-2 days where visitation was allowed but being retarded to 5+ days when flowers were bagged. In contrast, Karoly (1992) related the change in the colour of the banner petal spot in this species to stigma receptivity that was purely age-related. In the congeneric, *L. texensis*, Weiss (1992) found an almost identical pattern to her findings for *L. nanus*, whereas Schaal & Leverich (1980) described a non-inducible colour change that happened after 5-6 days in the former species.

d) Colour change in *Lantana camara* occurs on the first day and has been assumed to be age-related by most researchers (e.g. Barrows, 1976; Weiss, 1992). However, Mohan Ram & Mathur (1984) suggested colour change was inducible in this species; they found that deposition of a single grain of pollen could lead to increased anthocyanin production and subsequent colour change.

1.3.8. Other correlates of colour change.

Simple temporal coincidence of alteration of floral reward with colour change has frequently been reported. In several instances nectar volume has been shown to be greater in pre-change flowers in comparison to post-change flowers

(for example, *Phyla incisa* (Cruzan et al.1988), *Melampyrum ciliare* (Kojima & Hori, 1994), and *Pulmonaria collina* (Oberrath & Bohning-Gaese, 1999)). More rarely, increased nectar reward in post-change flowers has been recorded (e.g. *Malvaviscus arboreus* (Gottsberger, 1971), and *Anchusa strigosa* (Kadmon et al. 1991)). Both nectar and pollen were found to be available in smaller quantities in post-change flowers of a number of plants (e.g. *Calytrix glutinosa* (Lamont, 1985), *Hamelia patens* (Lackie et al. 1986), and *Banksia ilicifolia* (Lamont & Collins, 1988)). Pollen availability (only) was greater in pre-change flowers in some species, including *Myosotis colensoi* (Robertson & Lloyd, 1993), *Aster vimineus* (Niesenbaum et al. 1998), and *Rhexia virginica* (Larson & Barrett, 1999a).

Post-change flowers in the majority of 97 plant species were found to have low nectar reward, less available pollen and non-receptive stigmas in comparison to pre-change flowers by Weiss (1995a).

Caspar & La Pine (1984) described change not only in reward status and ultraviolet reflectance characteristics, but also a qualitative change in odour between pre- and post-change flowers of *Cryptantha humilis*. Alteration of the profile of floral volatiles to coincide with colour change has also been shown to occur in two further plants. Relative quantities of scent compounds varied between first- and third-day flowers of *Cyphomandra sciadostylis*, accompanying a colour change of the corolla from deep violet to ochre (Sazima et al. 1993). Schiestl et al. (1997) noted both quantitative and qualitative changes in scent production coinciding with a colour change from brown to yellowish-white in *Ophrys sphegodes*.

Colour change in *Bauhinia monandra* (Caesalpiniaceae) is combined with mechanical movement of floral parts to dramatically alter the visual appearance of the flower (Weiss, 1995a).

The behaviour of visiting organisms, their response to colour change and possible ecological and evolutionary significance are covered in subsequent sections.

1.3.9. Colour change and pollination ecology; evolutionary considerations.

The superficial transaction of pollen dispersal in exchange for food hides many intricacies. The pollination of plants by animals is a striking example of a mutualism founded on exploitation; both participants receive a benefit from a 'transaction' born of selfish interests.

For selective benefits to accrue for both plant and visitor several conditions should ideally be met. A plant that offers an attractant cue that is recognised by potential visitors should present a physiologically inexpensive reward to regular visitors that is not subject to detrimental alteration by environmental conditions. The visitor, while possibly restricted by environmental conditions, should be active at times when a suitable reward is available; should be of a particular size and shape that enables access to reward; and must be behaviourally sensitive to the presence and to the condition of the reward-offering plant.

Gori (1983) offered a range of hypotheses that developed the ecological and evolutionary significance of colour change and that, while not mutually exclusive, might usefully be examined experimentally.

Reduced seed set may result from continued visitation to already fertilised flowers and this may be particularly relevant if a flower has reproductive parts that are fragile and susceptible to possible damage by visiting organisms or suffer from dislodgement of pollen. The plant would thus benefit from a colour change that directs visiting organisms towards pre-change, unfertilised flowers.

Non-rewarding flowers that are retained on the plant still act as a distance signal for visitor attraction, but then at close range the non-rewarding flowers are avoided. This will increase visitation frequency, and residence times, and benefit the plant by potential increased pollination and seed set as, at close range, visitors forage on the most rewarding (young) flowers. Gori (1989) suggested that retention may have evolved in circumstances where pollinators were scarce; increased display size through additional, albeit already pollinated flowers, encouraged further visitation whereas early abscission might reduce subsequent visits. The importance of retention of unrewarding, inviable flowers to provide long distance advertisement has been the subject of conflicting studies.

Jones & Cruzan (1999) recorded more visits of six bee species to *Lotus scoparius* for plants that had a combined display of pre-change yellow and post-change orange flowers, compared to displays where post-change flowers had been removed. Gori (1989) reported similar results for *Lupinus argenteus*; bees foraged more often at plants with greater display size than at those with fewer flowers, regardless of the proportion of pre- and post-change flowers available. Butterflies were shown to be attracted to larger displays of *Lantana camara*, and also approached such displays from greater distance, irrespective of available reward (Weiss, 1992). However, larger overall displays, incorporating significant

numbers of post-change flowers, did not promote increased visitation by flies, in *Cryptantha humilis* (Caspar and La Pine, 1984), or by birds in *Fuchsia excoricata* (Delph & Lively, 1989).

Gori (1989) mentioned the recovery of translocatable nutrients from senescing floral parts as a possible reason for the retention of post-change flowers. Delph & Lively (1989) put forward a further physiological constraint; the time required for the successful growth of pollen tubes beyond the point of floral abscission. Barrows (1976), in a study of *Lantana camara*, recorded nectar robbery by the meliponine bee, *Trigona fulviventris*, and suggested that the plant may benefit from the protection offered by peripheral 'rewardless' flowers to the inner flowers from this nectar thief. Schemske (1976) proposed that an enlargement in landing platform size of the whole inflorescence for butterfly visitors was a further utility of floral retention in the same species. Of benefit to plants, Gori (1983) also pointed out that if colour change signalled the cessation of viability of pollen or an unreceptive stigma, visitors are directed to viable, receptive flowers only.

Assuming that a visiting organism is gaining reward and, therefore, a selective advantage, all or any of these hypotheses (some specific to individual species) may contribute towards plant fitness through continued visitation, and thus both participants in the interaction benefit. Colour change, a signal that is clearly recognised, and responded to, by a wide range of visitors (see 1.4), is inextricably linked to floral retention and is thus a contributory factor in such associations.

1.4. Visitor behaviour in response to colour and colour change.

Reward type, temporal availability of reward, and the influence of the environment on both plant and visitor will affect visitor behaviour. However it is also linked to flower colour (see section 1.2.1) and therefore the colour change of flowers requires examination as a modifier of foraging behaviour. Colour preference that is apparently innate has been demonstrated to be modifiable by learned discrimination according to reward in bumblebees (Chittka & Waser, 1997). Since colour change may occur concomitantly with a change in reward and with further visual or odour signals (see section 1.3.8), there may be a combined influence of such cues, and discrimination of altered coloration could be a useful 'learned' cue to maximising reward. Here the issue of floral constancy is crucial.

Floral constancy, the apparent specialisation of certain visitors (both species and individual visitors) for particular flowers, has been acknowledged since the time of Aristotle (Proctor et al. 1996). In natural conditions, a high degree of flower constancy should benefit the plant in terms of increased likelihood of visitation to conspecifics by the visitor, while the visitor also benefits if it is not constrained by regular relearning of how to handle a range of reward-offering flowers that may differ in morphology (Waser, 1986). More recently, Chittka et al. (1999) highlighted the wider range of factors that may underlie floral constancy within insects. Both sensory (e.g. visual) and motor factors (e.g. flower handling) in relation to foraging were deemed important. Floral colour and population features (e.g. floral and interplant density) were also crucial influences on flower choice, although that review did not discuss colour

change as an additional possible influence on foraging behaviour and floral constancy.

Visitation patterns that alter with colour change have been described in diverse organisms (see review by Weiss, 1995a) and, in all studies included in Table 1.1 where a foraging preference was reported, the more rewarding flower colour phase was visited independently of their availability in the population. Colour-changing flowers effectively offer a test of floral constancy with a single trait having been altered naturally.

Such studies include both changes of nectar and pollen, and a range of different visitors. In research by Caspar & La Pine (1984) on *Cryptantha humilis*, over 90% of all visitors fed at pre-change, yellow-centred rewarding flowers even though these were displayed in almost equal proportions (52:48) to post-change white-centred flowers. Nearly half of all visits were made by empid flies and beeflies (Bombyliidae), though bees, especially anthophorids, also followed the above visitation trend.

A range of solitary and social bees had been noted to forage preferentially at particular flowers on colour-changing plants. *Anthophora acervorum*, *Apis mellifera*, and several species of bumblebee all made significantly more visits to rewarding red flowers of *Pulmonaria* sp. compared to blue flowers that contained less nectar (Oberrath et al. 1995). Honeybees and anthophorids showed similar visitation behaviour at *Lupinus pilosus*; flowers with pre-change, white banner petal spots contained greater quantities of pollen and visitation was disproportionately high given the presence of abundant post-change flowers (Ne'eman & Nesher, 1995).

In the field, a variety of insects, including honeybees and bumblebees, visited the pollen rich, yellow-centred flowers of *Aster vimeneus* and, in choice experiments using artificial flowers, continued to neglect the red-centred flowers that, under natural conditions, were usually depleted of pollen (Niesenbaum et al. 1998).

Butterflies from four families visited flowers of different colour morphs (pink and orange) of *Lantana camara*, with different species preferring one of the colour morphs to the other (Dronamraju, 1960). Subsequently, it was found that butterflies fed predominantly at the rewarding, pre-change yellow flowers of *L. camara* under natural (e.g. Barrows, 1976)) and experimental (Weiss, 1991) conditions. Further work with this plant by Weiss (1995b, 1997) showed that colour-related foraging behaviour of two butterflies, *Battus philenor* and *Agraulis vanillae*, was learned by association with available reward. *Battus* butterflies could be trained to visit the naturally 'unrewarding' colour if nectar was made available; and naïve individuals of *Agraulis* concentrated their foraging efforts on yellow, rewarding flowers according to previous experience.

Of vertebrate flower visitors, birds have been shown to respond to the availability of nectar reward in colour-changing flowers. In *Combretum farinosum* the older red flowers lack reward but are thought to act as attractants to visiting hummingbirds, which then feed almost exclusively at first-day green, nectar-secreting flowers (Schemske, 1980). Visits of several species of honeyeater were directed towards the yellow flowers of *Banksia ilicifolia*; older red flowers contained inviable pollen, unreceptive stigmata and less abundant nectar and were avoided (Lamont & Collins, 1988). A further example of birds avoiding red, post-change and less rewarding flowers was described by Delph &

Lively (1985); bellbirds and the nectar-robbing silvereyes both foraged preferentially at first- and second-day green flowers of *Fuchsia excorticata.* In an 'opposite' scenario, hummingbirds were the visitors to older, less intensely coloured and nectar-producing flowers of *Malvaviscus arboreus* in preference to the young brightly coloured but nectarless flowers (Gottsberger, 1971).

A colour-changing flower would appear to be adaptively 'fine-tuned' towards attracting a visitor that is able to modify its foraging and orientation behaviour according to the change in visual cue. The visual capability of visiting organisms is, therefore, of critical importance when considering this association.

1.5. Scope of this study.

Published work of recent years that relates to colour change is limited to 76 species from 27 families (Table 1.1). The scope of these studies encompasses visitor behaviour, floral reproductive biology and biochemical/physiological changes within the flower, as well as evolutionary and ecological considerations, but as yet little research has directly addressed the question of whether, and which, aspects of visitation might influence the colour change itself.

The effects of visitation on colour change can, initially, be explored at three levels. Firstly, there is exclusion of visitors, usually through bagging of plants, or of individual flowers; secondly, handling effects can be assessed through mechanical manipulation of flowers or floral parts; thirdly, visitation, *per se,* which may include variation in number and rate of visits, together with different flower-handling characteristics of visitors, can be manipulated and its effects examined.

1.5.1.Exclusion of visitors.

Of the studies included in Table 1.1, the exclusion of visitors through bagging was used in 34 species. In thirteen species the purpose of this methodology was simply to establish nectar profiles, without specific reference to colour change. Bagging was used for self-compatibility experiments in *Diervilla lonicera* (Schoen, 1977); for self-pollination studies in *Fuchsia excorticata* (Delph & Lively, 1985, 1989); and to determine seed set in *Banksia ilicifolia* (Lamont & Collins, 1988) and *Phyla incisa* (Cruzan et al. 1988). In all instances the effects of exclusion on plant success were being measured, as opposed to direct study of colour change. Oberrath & Bohning-Gaese (1999) used bagging to measure availability of nectar reward in relation to colour phase and Caspar & La Pine (1984) used it to determine inducibility of colour change. Weiss (1992) examined just 12 species, from 8 families through bagging experiments to determine inducibility of colour change and floral longevity and colour change was found to be inducible in 9 of these plants. She also pointed out possible pitfalls of using bags for exclusion; in particular, she highlighted the alteration of the floral microenvironment as a possible confounding variable, although no data were collected in this respect.

1.5.2. Floral manipulation.

Weiss (1992) described a bagged treatment group as 'unmanipulated' and an unbagged group as 'manipulated'; the last group, presumably, received natural manipulation via visitation but the former group were, presumably, manipulated

by the experimenter through handling and marking of individual flowers while being bagged.

The use of some more specific form of floral manipulation has been employed for differing reasons in research on colour-changing plants. Biochemical changes within floral tissue have been assessed, and linked to colour change, through damage to stamens and pistil in *Carthamus tinctorius* (Fukushima et al. 1997); damage to petals in relation to ethylene production in *Ipomoea tricolor* (Kende & Hanson, 1976); damage to pistil in relation to ethylene production in *Lupinus albifrons* (Stead & Reid, 1990); and excision of part-flowers in *Cymbidium* sp. (Woltering & Somhorst,1990).

Colour-changing flowers have been manipulated in several studies of reproductive success. Colour change was found to be accelerated following artificial pollination by hand in *Lotus scoparius* (Jones & Cruzan, 1982); *Calypso bulbosa* (Proctor & Harder, 1995); *Lupinus propinquus* (Weiss, 1992); and *L. pilosus* (Ne'eman & Nesher, 1995). In all but the final study cross-pollen was used to produce such an effect. In further studies (*Diervilla lonicera*, Schoen (1977), *Hamelia patens*, Lackie et al. (1986), and *Chamelaucium uncinatum*, O'Brien (1996)), both artificial pollination and emasculation of colour-changing flowers were used, but no effects on colour change were reported. Nectar robbery was investigated in relation to reproductive success (without recording possible colour change effects) by attaching plastic sleeves to *Mertensia paniculata* flowers that prevented bumblebees from piercing the corolla tubes (Morris, 1996).

In a study that assessed floral receptivity in a mixed-species community of colour-changing plants Lamont (1985) removed the stigmas from flowers of

eight species and found no effect on the rate of change in six, with conflicting directions in the rate of change in the remaining two (see section 1.3.7).

Visitor behaviour has sometimes been examined in response to floral manipulation of colour-changing flowers. Nectar was added to flowers of *Malvaviscus arboreus* during the 'unattractive' phase to see whether hummingbirds altered their visitation patterns; birds avoided the manipulated flowers (Gottsberger, 1971). Bumblebee behaviour was investigated by Larson & Barrett (1999a) with *Rhexia virginica* flowers, where visitation was simulated by removing pollen from flowers by tapping lightly with forceps; bee preference was then recorded for 'visited' or unvisited flowers. However, neither of these methodologies investigated alterations in the rates of colour change in the plants studied.

Clearly, very few studies have used floral manipulation to directly assess the link between effects of visitation and colour change. There are rare exceptions; Wainwright (1978) discovered that handling of the flower of two species of lupin during the period of stigmatic receptivity was implicated in colour change (see section 1.3.6), while Eisikowitch & Lazar (1987) tried a range of treatments in *Oenothora drummondii*, and found that colour change could be accelerated by nectar withdrawal but not through simple 'mechanical' touches.

1.5.3. Visitation.

Whilst many studies have inferred that visitation may influence colour change (through observation of the obvious visual change of the flower) and have shown that post-pollination events can be responsible for such change,

specific aspects of visitation that may underlie the process have been rarely researched. Although these observed effects often coincide with, and are assumed to be a result of, the biochemical and physiological mechanisms that promote the onset of colour change, there is little evidence to demonstrate the initial trigger of the mechanism for individual species. Major gaps exist in the literature to date concerning precisely how an insect contributes to the process of colour change.

The inducibility, or otherwise, of colour change has received relatively little attention (Weiss 1995a) and the possible effects of different visitors and their varied attempts at handling and manipulation of floral parts represent a further area of study. It would be useful to distinguish between features of visitation and post-pollination events as separate factors in inducible colour change; while the latter appears the most likely ultimate trigger for colour change other proximate factors may not be ruled out.

In a range of colour-changing plants local to St. Andrews, Fife, Scotland and further species found in the eastern Mediterranean (the reasons for choice of species are given at the beginning of each chapter) the following questions have been addressed:

1) Is there a definite trigger for change in flower colour in different species, rather than a simple temporal effect, and, if so, what is the trigger in each species? (i.e. inducible vs. non-inducible change will be investigated)
2) Is change (or rate of change) linked to any/all of the following factors?

- Reward status

- Visitation; physical contact/flower handling, pollen depletion, nectar withdrawal
- Pollination events e.g. pollen deposition, pollen tube growth, fertilization
- Other e.g. environmental cues

3) Do different visitors produce different effects e.g., through handling/manipulation, or visitation frequency?
4) How does colour change alter visitor behaviour, in terms of foraging and colour choices?

Although many of the links between flower and pollinator are understood (Figure 1.1), the influence of colour change requires further examination. Visiting organisms certainly respond to colour change but how prevalent is the role of the organism in producing that change? This study will use observation of natural visitation, allied to exclusion and manipulative experimentation, and attempt to link particular aspects of the visitation process with colour change in a range of plant species. Where practicable, plants have been studied that have not previously been the subject of detailed colour change research. It is anticipated that this type of study will reveal links between pollination and post-pollination events and visitor behaviour that are mediated by colour change. This will contribute to a fuller understanding of the ecological significance of colour change.

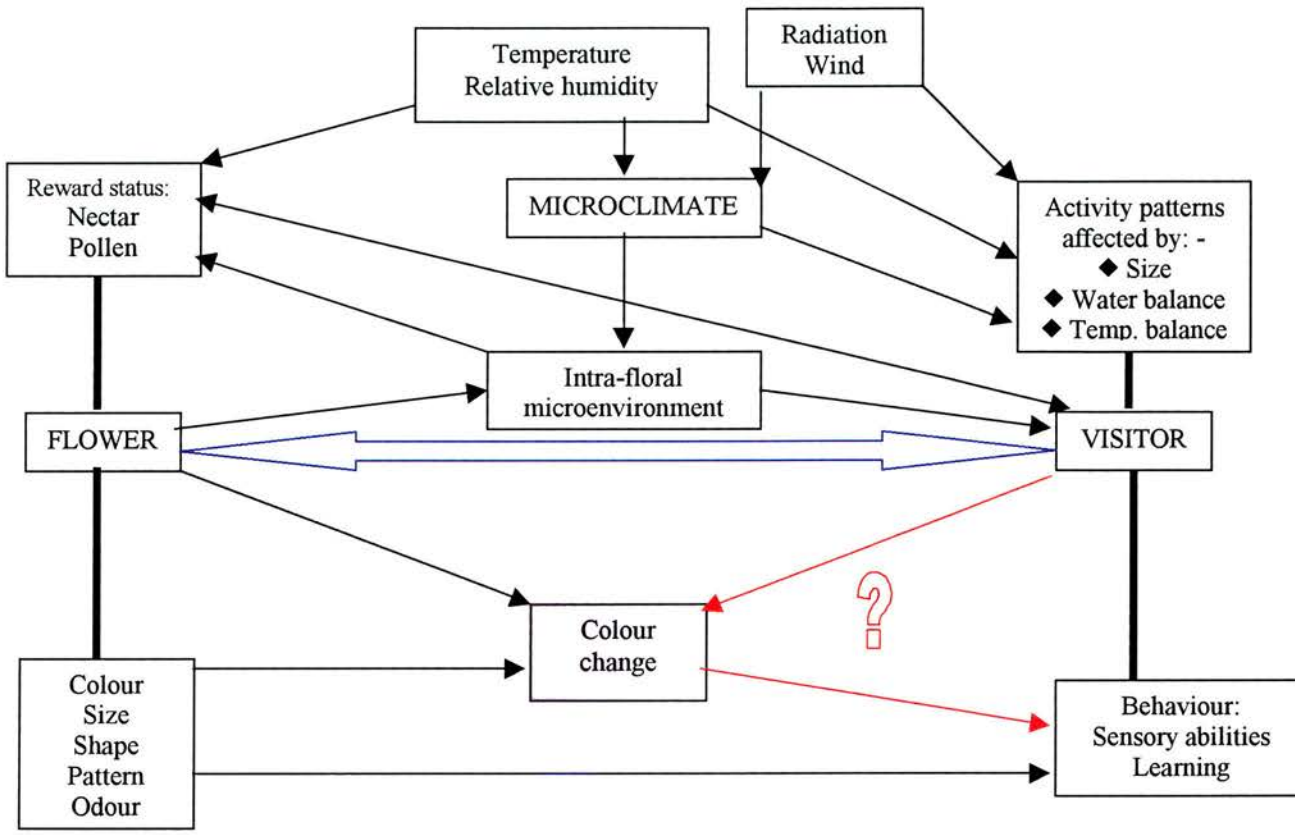


Figure 1.1. Complexity in flower-visitor associations. A wide range of factors impact upon the basic relationship (blue arrows); features intrinsic to either plant or visitor are linked by solid bars and these may be modulated by extrinsic influences, denoted by directional black arrows. The main issue addressed in this thesis is represented in red.

Taxon	Colour change	Floral longevity	Age (A) / Inducible (I)	Alteration in reward	Visitors	Visitation preference	Manipulation of floral parts	Bagging experiments	Reference
Bignoniaceae									
Catalpa sp.	Y→R	nr	nr	nr	Hb	✓pre-	N	N	1
Pachyptera hymenaea	Dl→W Wf 1d	3d	nr	N -	B, A, T	nr	N	N	2
Boraginaceae									
Anchusa capensis	W→R E	nr	nr	nr	Bb	✓pre-	N	N	1
A. strigosa	V→B Wf 4-8h	3-4d	nr	N +	B	✓post-	N	Y – nectar measurements	3
Cryptantha humilis	Y→W Cs 2d	4+d	A	N -	F, B	✓pre-	Y – artificial pollination	Y – to determine inducibility	4
Echium plantagineum	UV	nr	nr	N -	B, Bb	nr	N	N	5
E. vulgare	P→B Wf 1d	3d	nr	nr	B, Bb	nr	Y – nectar added in behavioural experiments	Y – nectar measurements	6, 7
Heliotropium anchusifolium	Y→Pu Cf 1-3d	3-6d	I	N -	B, Bb	✓pre-	Y – tagging and marking	Y – to determine inducibility and longevity	1
Mertensia paniculata	P→B Wf 12h	3-5d	A	?	Bb	nr	Y – effects of nectar theft	N	8
Myosotis colensoi	Y→W Cs 1d	nr	A	P -	F	✓pre-	N	N	9
Pulmonaria collina	R→B Wf	nr	A	N -	B, Bb	✓pre-	N	Y – nectar reward vs. colour phase	10
P. obscura	R→B Wf 3-5d	5-8d	A?	N -	B, Bb	✓pre-	N	Y – nectar measurements	11
P. officinalis	R→B Wf 3-5d	5-8d	A?	N -	B, Bb	✓pre-	N	Y – nectar measurements	11

Taxon	Colour change	Floral longevity	Age (A) / Inducible (I)	Alteration in reward	Visitors	Visitation preference	Manipulation of floral parts	Bagging experiments	Reference
Caesalpinaceae									
Caesalpinia pulcherrima	Y→R Pp 1d	2-3d	A?	nr	Bu	nr	N	Y – nectar measurements	12
Caprifoliaceae									
Diervilla lonicera	Y→R Wf 3d	nr	A	nr	B, Bb, Hm	nr	Y – self-compatibility experiments	Y – self-compatibility experiments	13
Lonicera periclymenum	W→Y Wf 1d	3+d	A?	nr	B, Bb, F, M, Be	nr	Y – artificial pollination experiments	Y – nectar measurements	14, 15
Combretaceae									
Combretum farinosum	G→R F & S 4d	5-7d	A?	N -	Hu	✓pre-	Y – artificial pollination experiments	Y – nectar measurements	16
Quisqualis indica	W→R 2d	3d	A?	N -	Hm, Sb, Hb, B	✓ass	N	Y – nectar measurements	17
Compositae									
Aster vimineus	Y→R Df →7d	nr	A?	P -	F, Hb, Bb	✓pre-	N	N	18
Carthamus tinctorius	Y→R Wf	nr	nr	nr	nr	nr	Y – damage and pollen deposition	N	19*
Convolvulaceae									
Ipomoea tricolour	B→Pu Wf 1d	1d	I	nr	nr	nr	Y – damage to petals	N	20*
Cruciferae									
Erysimum scoparium	nr	nr	nr	N -	nr	nr	N	N	1
Lobularia maritima	W→Pu Wf & A 1-2.5d	7-8+d	I	nr	F	✓pre-	Y – tagging and marking	Y – to determine inducibility and longevity	1

Taxon	Colour change	Floral longevity	Age (A) / Inducible (I)	Alteration in reward	Visitors	Visitation preference	Manipulation of floral parts	Bagging experiments	Reference
Geraniaceae									
Geranium dalmaticum	W→Pi Pf	nr	nr	N -	Hb	✓pre-	N	N	1
Hippocastanaceae									
Aesculus carnea	Y→R Ng 1-2d	3d	A	N -	Hy	✓pre-	Y – tagging and marking	Y – to determine inducibility and longevity	1
Loranthaceae									
Phragmanthera dshallensis	Y→R Wf 1d	3-4d	A?	N -	Sb	✖	N	N	21
Malvaceae									
Hibiscus mutabilis	W→R Wf	1d	A	nr	nr	nr	N	N	22*
Malvaviscus arboreus	S→fading	nr	A?	N +	Hu	✓post-	Y – nectar added for behavioural experiments	N	23
Melastomataceae									
Rhexia virginica	Y→R A 1d	2-3d	A	P -	B, Bb	✓pre-	Y – in relation to buzz pollination	N	24
Myrtaceae									
Calytrix glutinosa	W→R Cf	3-4d	A	P & N -	Bu, B	✓pre-	Y – stigma removal	N	25
Chamelaucium uncinatum	G→R H 10d	20+d	A	nr	B, Hb	nr	Y – in relation to pollen tube growth	Y – nectar measurements	26, 27
Darwinia citriodora	Y→R Wf	8-9d	A/I?	N -	Hb, M	nr	Y – stigma removal	N	25
D. fasiculata	W→R Wf	nr	nr	N -	nr	nr	N	N	1
Hypocalymma angustifolium	W→P Wf	3d	A	N -	Be, W, Hb	✓pre-	Y – stigma removal	N	25

Taxon	Colour change	Floral longevity	Age (A) / Inducible (I)	Alteration in reward	Visitors	Visitation preference	Manipulation of floral parts	Bagging experiments	Reference
Verticordia chrysantha	Y→R Wf	18-30d	A/I?	N -	B, W	✓pre-	Y – stigma removal	N	25
V. hueglii	W→R Wf	10-11d	A	N -	B, F	✓pre-	Y – stigma removal	N	25
Onagraceae									
Fuchsia excorticata	G→R Wf 4d	15d	A	N -	Bi, Bb	✓pre-	N	Y – self pollination experiments	28, 29
Oenothora drummondii	Y→R Wf	nr	I	nr	Hm, Hb	nr	Y – to determine inducibility of colour change	N	30
Orchidaceae									
Calypso bulbosa	Variable Wf	nr	I	nr	nr	nr	Y – pollen deposition and removal	Y – colour change effects	31
Cymbidium sp.	Y→O/R Pp	nr	I	nr	nr	nr	Y – flowers handled when exposed to ethylene	N	32*, 33*, 34*
Epidendrum radicans	W→R Pf 1-8d	8+d	I	P -	nr	nr	Y – tagging and marking	Y – to determine inducibility and longevity	1
Ophrys sphegodes	Db→Y/W	nr	I?	nr	B	nr	N	N	35
Vanda sp.	L→W Wf	nr	I	nr	nr	nr	N	N	36*, 37*
Papilionaceae									
Errazurizia megacarpa	Y→R Wf 3-7d	10+d	I?	nr	Hb, Hu, B	✓pre-	Y – self-pollination	Y – self-pollination	38
Lotus scoparius	Y→O Wf 1d	nr	I	P -	B, Hb	✓pre-	Y – self-pollination	Y – self-pollination	39, 40
Lupinus albifrons	W→M Bps 1-3d	5+d	I	nr	Bb	✓pre-	Y – measured ethylene production	N	41*
L. albifrons var. *collinus*	Colour not described, 1d	5+d	I	nr	Bb	nr	N	Y – to determine inducibility and longevity	1

Taxon	Colour change	Floral longevity	Age (A) / Inducible (I)	Alteration in reward	Visitors	Visitation preference	Manipulation of floral parts	Bagging experiments	Reference
L. argenteus	W→Y→Pu Bps 4d	9-11d	A	P -	Bb	✓pre-	Y – artificial pollination experiments	N	42, 43
L. arizonicus	Y→Pu/R Bps	nr	I	nr	B, Hb, Bb	✓pre-	Y – handling during stigmatic receptivity	Y – to determine inducibility and longevity	1, 44
L. bicolor ssp. *microphyllus*	W→L Bps 1d	5d	A	nr	Hb, Bb	✓pre-	Y – pollination experiments	N	45
L. nanus	W→Pu Bps 1-2/2-5d†	nr	A/I†	nr	Bb, Hb	✓pre-	Y – artificial visitation	Y – to determine inducibility, longevity and reproductive success	1
L. nanus ssp. *menkarae*	W→Pu Bps	nr	I	nr	B	nr	Y – handling to mimic visitation for self-pollination	N	45
L. nanus ssp. *latifolius*	W→M Bps 2d	nr	A	nr	B, Bb	nr	Y – pollination experiments	N	45, 46*
L. pilosus	W→Pu Bps 3d	7d	I	P -	Hb, B	✓pre-	Y – pollination experiments	N	47
L. propinquus	W→L→Pu Bps 3d	4.6d	I	P -	Bb	nr	N	Y – to determine inducibility and longevity	1
L. sparsiflorus	Y→Pu/R Bps 4h from visit	nr	I	nr	Bb	nr	Y – handled during stigmatic receptivity	N	44
L. texensis	W/Y→R/Pu Bps 5-6d†	5+d	A/I†	nr	Bb	nr	N	Y – to determine inducibility and longevity	1, 48
Primulaceae									
Androsace lanuginosa	Y→R Cf 5-7d	10-11d	A	N -	Bf, F	✓pre-	N	Y – to determine inducibility and longevity	1

Taxon	Colour change	Floral longevity	Age (A) / Inducible (I)	Alteration in reward	Visitors	Visitation preference	Manipulation of floral parts	Bagging experiments	Reference
Proteaceae									
Banksia ilicifolia	Y→R Wf 4-7d	To 27d	A	P & N -	Bi, A, Be, B	✓pre-	N	Y – to determine seed set	49
Grevillea pilulifera	Y→R Pf 2d	nr	A	P -	B	✓pre-	Y – stigma removal	N	25
Isopogon dubius	Y→O/R Pf 2d	nr	A	P -	B	✓pre-	Y – stigma removal	N	25
Petrophile biloba	Y→O/R Pf 3d	nr	A	P -	B	✓pre-	Y – stigma removal	N	25
Rosaceae									
Raphiolepis indica	W→R A 4-6d	8-10d	A	N -	nr	nr	Y – tagged and marked	Y – to determine inducibility and longevity	1
Rubiaceae									
Hamelia patens	O→R Wf 1d	2d	A?	P & N -	Hu, Bu	✓pre-	Y – flowers probed in relation to selfing experiments	Y – nectar measurements	50
Sapindaceae									
Koelreuterea paniculata	Y→R Pa	nr		N -	Hb	✓pre-	nr	nr	1
Scrophulariaceae									
Melempyrum ciliare	W→R/Pu Ps	nr	A?	N -	Bb	✓pre-	N	Y – nectar measurements	51
Solanaceae									
Cyphomandra diplonocus	V→Oc Wf 2d	3d	A?	nr	Eb	nr	N	N	52
C. endopogon	Dl→G/W Wf 1-3d	3d	?	S -	Eb	✓pre-	Y – to determine stigmatic receptivity	N	52

Taxon	Colour change	Floral longevity	Age (A) / Inducible (I)	Alteration in reward	Visitors	Visitation preference	Manipulation of floral parts	Bagging experiments	Reference
C. sciadostylis	V→Oc Wf 2d	3d	A?	nr	Eb	nr	N	N	52
Verbenaceae									
Lantana camara	Y→R Wf 1d	3-4d	A/I†	N -	T, Bu, Hb, B	✓pre-	N	Y – nectar measurements	1, 53, 54, 55, 56, 57, 58
L. hirta	Y→W E	nr	nr	N -	Bu	✓pre-	N	N	1
L. montevidense	W/Y→Pu E	nr	nr	N -	Bu	✓pre-	N	N	1
Phyla incisa	Y→Pu Ng 1d	3d	A?	N -	F, Bu, Hb, B	✓pre-	Y – butterfly visitation mimicked for pollination experiments	Y – pollination experiments	59, 60
P. nodiflora	nr	nr	nr	nr	Hb, F, Bu	✓pre-	N	N	1
Violaceae									
Viola cornuta	W→Pu Wf 5-8d	8d	I	nr	nr	nr	Y – emasculation for pollination experiments	Y – with foil for light effect experiments	61
Vitaceae									
Cayratia japonica	R→Pi/O Pf 12h	3-5d	A	? not in relation to colour change	A, W, B, Be, Bu	✖	N	Y – nectar measurement	62

Table 1.1. Summary of findings of studies on colour changing plants. Key: - Colour change; first line gives direction of change, second line the type of change, and the last line the time to change in days (d) or hours (h). i) Colours; Y = yellow; R = red; Dl = dark lavender; W = white; UV = ultra-violet; V = violet; P = pink; B = blue; Pu = purple; G = green; S = scarlet; O = orange; Oc = ochre; Db = dark brown; L = lavender; M = magenta. ii) Flower parts that change; Wf = whole flower; Cf = centre of flower; Cs = coronal scales; Pp = part petal; F = filaments; S = style; H = hypanthium; Pf = part flower; A = androecium; Df = disc florets; Bps = banner petal spot; Ng = nectar guides; Pa = petal appendages; Ps = petal spots. Visitors; Hb = honeybees; Bb = bumblebees, Eb = euglossine bees; B = other bees (solitary or unspecified); A = ants; T = thrips; F = flies; Bu = butterflies; Hm = hawkmoths; Be = beetles; M = moths; Hu = hummingbirds; Su = sunbirds; Hy = unspecified hymenopterans; W = wasps; Bi = other birds; Bf = beeflies. Pre- or post- refers to the colour stage of the flower that is preferentially visited. In the 'Alteration of reward' column, N = nectar; P = pollen; and S = scent (direction of arrow denotes whether available reward increases (+) or decreases (-) following colour change). nr = not recorded. * indicates purely laboratory-based research. † denotes conflicting findings between cited studies. All numbered references in the right-hand column are denoted by a superscript figure in the main reference list.

Chapter 2 – Materials and Methods.

This short chapter contains details of general materials and methods used throughout all experimental work, together with statistical software and tests used for data analysis. Thereafter, each chapter has full information on the particular techniques used for the individual species.

2.1 Chronology and location of fieldwork.

Fieldwork was carried out between February 2000 and July 2002 and the timetable of research is summarised in Table 2.1.

Year	Month(s)	Species	Location
2000	February – April	*Echium judaeum*	Mt. Carmel, Israel
	May – June	*Myosotis sylvatica*	St. Andrews
	July	*Echium vulgare*	Deal, Kent
	August - September	*Lonicera periclymenum*	St. Andrews
2001	March – April	*Lupinus pilosus*	Lesbos, Greece
	May – June	*Myosotis sylvatica*	St. Andrews
	July	*Echium vulgare*	Elie, Fife
	August	*Lonicera periclymenum*	St. Andrews
	September	*Aster tripolium*	St. Andrews
2002	March – April	*Alkanna orientalis*	St. Catherine, Egypt
	May – June	*Myosotis sylvatica*	St. Andrews
	July	*Echium vulgare*	Elie, Fife

Table 2.1 Chronology of field work 2000-2002. Work on *Aster tripolium* was discontinued due to time constraints.

2.2 Measurement of colour.

The equipment necessary for quantitative measurement of colour, such as the use of a spectrophotometer to record spectral reflectance/absorption characteristics of flowers (e.g. Oberrath & Bohning-Gaese, 1999) was not available for my study; nor does such technology solve all the 'problems' of assessing colour as seen by a flower visitor as discussed in Chapter 1. Therefore a qualitative assessment has been used. In every chapter flower colours have been given nominal descriptions that refer to perception by the human eye.

Categories were most often devised in comparison to a colour matching chart used in the printing industry to produce specific ink coloration (Pantone Inc., New Jersey, USA). Each colour chip within this chart has a reference number and these are given within the text of the chapters. The relevant colours from this chart were matched by eye to successive phases of colour change, and small chips (approximately $1cm^2$) were attached to the edge of a ruler, against which the colour of individual flowers could be easily assessed and categorised. This type of colour categorisation is used in Chapters 3, 5, 6 and 7 and is similar to methodologies used for characterising colour in other studies of colour change (e.g. Farzad et al. 2002).

For the work in Chapters 4 and 8 the matching chart was not available and a simple assessment and categorisation 'by eye' was used; this method has also been used regularly in studies of colour change (see for example, Woltering, 1990; Obberrath et al. 1995).

2.3 Nectar reward.

Nectar volume was measured by withdrawing nectar from individual flowers with a glass microcapillary tube (Drummond Scientific Co., Pennsylvania, USA) of known volume (0.5, 1.0 or 5.0μl). Tubes were 32mm in length and therefore by measuring the length of the column of nectar within the tube, a volume could be calculated by multiplying the length by the known volume per mm (e.g. for a 1.0μl microcapillary, 1mm = 0.031μl).

Nectar concentration per flower was measured by transferring a drop of nectar from the microcapillary to a pocket refractometer (modified to take small volumes of *c.*0.1μl by the manufacturers, Bellingham & Stanley, Tunbridge Wells, UK). This instrument measures refractive index as the nectar forms a film between 2 glass prisms; since refractive index is a colligative property, it can be directly translated into an osmotic concentration, here as g sucrose per 100g solution.

Nectar sugar content per flower in mg (S) was calculated using the following equation (Prŷs-Jones & Corbet, 1991): -

$$S = dvC/100$$

where v is the volume, calculated as above in μl, and d refers to the sucrose solution density at the concentration (C%) reading from the refractometer. The density is calculated as:

$$d = 0.0037921 \times C + 0.0000178 \times C^2 + 0.9988603$$ (Prŷs-Jones & Corbet, 1991)

2.4 Environmental variables.

Both temperature (°C) and relative humidity (%) were measured at field sites using a hand held meter (HMI 31, Vaisala, Finland). These measurements were taken in the shade and close to ground level (usually < 30cm) within focal patches of the plant species being studied.

2.5 Statistical Analysis.

All data sets were tested for normality of distribution using the Anderson-Darling test (Dytham, 1999). If data were normally distributed (either as raw data, or following $\log_{10}$ transformation), standard parametric statistical tests were applied to compare the means of independent pairs of samples (t-test), or variance around the mean between more than two groups (ANOVAs). In the latter instance homogeneity of variance of data sets was tested using the Bartlett test (Zar, 1996).

Where the assumptions for parametric tests could not be met, non-parametric statistical tests were used in all cases. Mann-Whitney tests were used to test for differences between the medians of 2 samples, and Kruskal-Wallis tests for multiple comparisons of medians between 3 or more groups. For the latter tests, where a significant difference was found, a non-parametric Tukey-type multiple comparisons test was applied to determine where differences between particular groups occurred (Zar, 1996).

In Chapter 3 contingency table analysis was applied to the ranked proportions of colour-changed flowers in different treatment groups at particular

time intervals. Where these proportions were not equal a Tukey-type test for multiple comparisons (using an arcsin transformation to transform proportions) was used to test differences among proportions (Zar, 1996).

In Chapter 8 the data for presence of pollen tubes at the stigma were not distributed normally and the effects of time and treatment were analysed using a Scheirer-Ray-Hare test, a non-parametric equivalent of a 2-way ANOVA. The null hypothesis (H_0) was that there would be no effect on pollen tube growth of either time or treatment (visited vs. not visited). Data were ranked and a two-way ANOVA was carried out; the sums of squares of the ranks were divided by the total mean squares and the resulting ratio (H) determined as a chi-squared-distributed variable (Scheirer et al. 1976; Sokal & Rohlf, 1995).

All tests were conducted using Minitab Release12.21 (State College, Pennsylvania, USA).

Error bars on all figures are $\pm$ 1 standard error of the mean.

Chapter 3 – *Myosotis sylvatica.*

3.1 Introduction.

The wood forget-me-not (*Myosotis sylvatica*) is one of several plants in the family Boraginaceae that exhibit the phenomenon of floral colour change. Weiss (1995a) described nineteen species that undergo part- or whole-flower change, including 4 species and 1 subspecies from the genus *Myosotis* (*M. alpestris*; *M. alpestris* ssp., *asiatica*; *M. australis*; *M. sauveolens* and *M. sylvatica*). Weiss & Lamont (1997) added a further species from this genus, *M. colensoi*, to the database of colour-changing flowers.

Forget-me-nots comprise close to forty species found in temperate regions of both northern and southern hemispheres. Annual or perennial herbs, they grow to 2-60 centimetres and most display blue corollas after a pink bud stage (Clapham et al. 1962). In the Southern Hemisphere forget-me-nots have been the subjects of several studies. Robertson & Lloyd (1991) investigated the breeding system of six *Myosotis* species and all were found to be self-compatible and protogynous.

Subsequent research into one of these species, *M. colensoi*, reported a yellow to white colour change of the scales at the entrance to the corolla tube (see below and Figure 3.1); the main visitor, a tachinid fly, only visited pre-change flowers (Robertson & Lloyd, 1993). Colour change occurs in the same direction in this restricted part of the flower in *M. alpestris* and *M. sauveolens* (Weiss, 1995a).

Myosotis sylvatica Hoffm. grows to a height of up to 50 centimetres and the plant produces corollas that vary in width from 6 to 10 millimetres (Clapham et al. 1962). Flowers are bright blue and basically 'saucer-shaped' with a corolla tube that encloses the stamens (pollen dehisces introrsely) and style, nectar reward is present. Five coronal scales form the 'eye' of the flower (Figure 3.1) and restrict the entrance to the corolla tube (Clapham et al. 1962). Knuth (1906-9) described these scales as "yellow, pocket-like involutions at the entrance of the flower [that] serve both as nectar guides and nectar covers" when detailing *M. sylvatica*. Individual flowering cymes of *M. sylvatica* can display several open flowers, of which only those at the distal part have yellow-centred flowers; up to 7 closely grouped flowers may be present as a 'landing platform' for visitors.

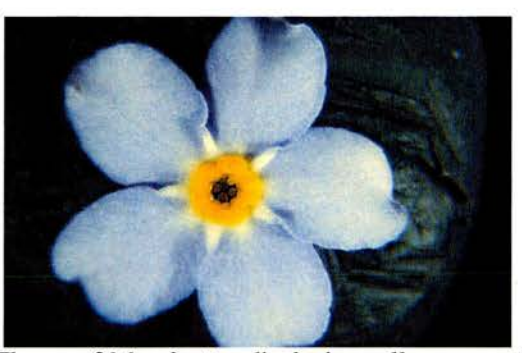


Figure 3.1 Flower of *M. sylvatica* displaying yellow coronal scales at centre.

The nature of colour change in *M. sylvatica* is poorly documented. Weiss (1995a) reported a change of the 'eye/corona' in this species from yellow to red and attributed the reference to Welsh & Ratcliffe (1986) (untraceable).

In Britain, *M. sylvatica* Hoffm. is a locally common inhabitant of damp and wooded areas, flowering from May to June throughout central and northern regions (Stace, 1991). The plant is a perennial and the early literature suggests it

is self-pollinating (producing up to 4 seeds), although attracting a wide range of insect visitors, including beetles, flies, bees and butterflies (Knuth, 1906-9).

Any of the visitors noted above could be an efficient pollinator of *M. sylvatica*, regardless of whether nectar or pollen was sought as a reward. The five anthers are clustered around the stigma at the narrow opening of the corolla tube and it appears unlikely that access to nectar (if secreted at the base of the tube) could be gained without contacting the anthers and stigma. Cross-pollen could be deposited in this way or self-pollen dislodged onto the stigma during flower handling.

The aim of this study was to establish the nature of colour change in *M. sylvatica*; the range of visitors to the plant at a site close to St. Andrews, Scotland; and possible links between colour change and visitation. The species was chosen due to its local abundance and as an example of a flower that exhibits distinct colour change in a localised part of the flower; in contrast, in the other members of the same family researched in this thesis the whole flowers change colour.

3.2 Materials and Methods.

3.2.1 Study plant and site.

A locally abundant population of these plants was studied at the Gilmerton Estate, St. Andrews, Scotland (NO 511119 (351152, 711942)) from May to June in 2000, 2001 and 2002. Dense patches (up to 30-40 plants, Figure 3.2) were present along roadside verges and hedgerows, a wooded burn adjacent to the main estate garden, and at the edge of a small spruce plantation among young sycamores and mature pines.

Figure 3.2 Patch of *M. sylvatica* at the Gilmerton Estate, Fife (May 2002).

3.2.2 Floral longevity, colour change and flower size.

At the start of each field season plants were selected at random and the uppermost, unopened flower on an individual cyme was marked with cotton thread. These flowers were checked each morning from the day of opening through to abscission to allow calculation of longevity, and petal colour and colour of eye/coronal scales was recorded.

The yellow coloration of the centre of the flower was limited to the coronal scales at the base of the petals. The visible upper surface of these scales was covered in dense, spiky hairs but immediately adjacent to the corolla tube entrance the surface appeared glabrous. These scales were bi-lobed and appeared 'raised' in pre-change flowers. This was apparently due to increased turgidity, and the loss of this turgidity caused a difference in appearance in post-change flowers irrespective of the colour change occurring.

Colour of the coronal scales was scored through the use of a five point categorical scale devised from a printer's ink colour chart (Figures 3.3 and 3.4): -

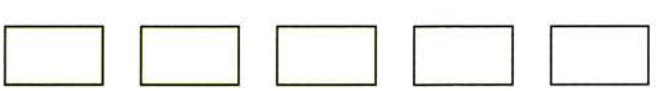
1 = Hexachrome yellow U

2 = Intermediate between 1 & 3

3 = 100U

4 = Intermediate between 3 & 5

5 = Cool grey 1U



1 2 3 4 5

Figure 3.3 Colour phases of the coronal scales of *M. sylvatica*; see text for full details.

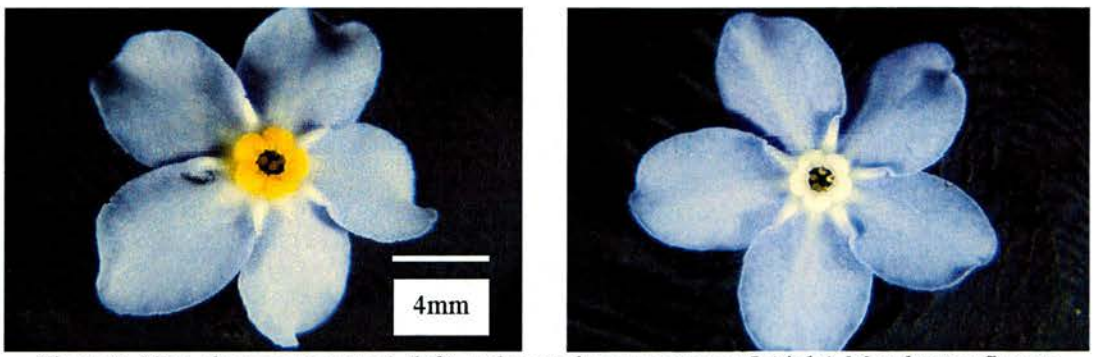


Figure 3.4 Pre-change category 1 (left) and post-change category 5 (right) *M. sylvatica* flowers.

The coronal scales changed from bright yellow to white over the course of the flower's life; a bright yellow appearance was maintained for 2 – 5 days, followed by a phase of faded-lemon yellow of 1 – 3 days and then a 2 – 5 day period of white, post-change coloration.

These categories were easily identifiable for laboratory work, in conjunction with use of a binocular microscope and constant light source (KW1500e, Visual Inspection Technology, UK). By affixing small samples of the colour chart to the edge of a ruler that could be held adjacent to the flower being examined, the coronal scale colour could be assigned to one of the above categories. Where close comparison could be made with coloured squares cut from the chart (e.g. during microscopic examination of pollen and nectar availability (see below)), all five categories were utilised. For field counts and observations of insect foraging choices the categories were 'collapsed' into yellow (stages 1 and 2), intermediate (stages 3 and 4) and white (stage 5) to allow reasonably objective evaluation of colour under varying light conditions.

The pink coloration noted in the bud stage (see section 3.1) was also evident in newly opened flowers and was also categorised for colour (Figure 3.5) and phase length measured. Flowers were initially pinkish-violet (256U)

followed by a blue phase (279U) that remained unchanged until floral abscission. Proportions of the petal and coronal scale colour phases within the population were calculated by randomly selecting plants and counting the number of flowers available within each category.

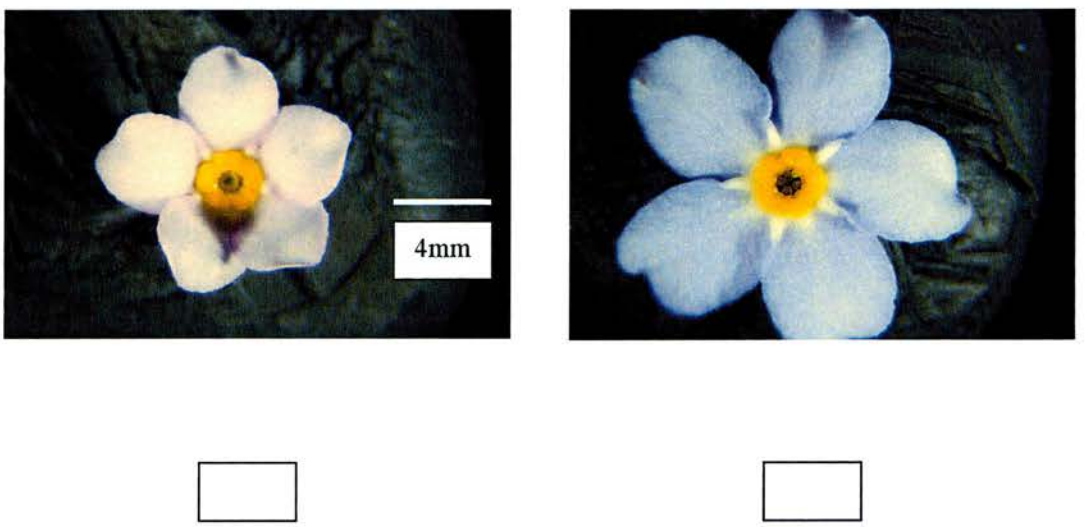


Figure 3.5 Petal colour phases in *M. sylvatica*. Left, in bud stage and, usually, for up to 1 day from opening the petals are pinkish-violet (256U). Right, fully open, turgid flowers remain blue (279U) until abscission.

In 2001 and 2002 longevity and colour change of coronal scales were recorded in bagged and open treatment groups, to assess possible effects of exclusion of visitors on rate of colour change and effects of 'bagging' *per se*. Petal colour change was assessed similarly in 2000 and 2002. Randomly selected flowers from individual plants were marked and left 'open' to natural rates of visitation. Concurrently, a one metre square patch of plants was covered with fine (<1mm) nylon mesh netting to exclude potential flower visitors. To avoid perturbation by movement of the net itself, the netting was held away from the enclosed plant by plastic supports and lengths of soldering wire to produce a 'cloche-effect'. Within the enclosure, twenty flowers were marked; taking care not to handle the flower itself by gently looping cotton thread over the corolla

and tightening at the pedicel. Laminated paper tags were attached and marked with a waterproof pen to allow identification of individual flowers. Data, as above, were collected on a daily basis.

A number of morphometric measurements were taken to establish the basic dimensions of the flower, in general, and the position of the anthers and style within the corolla tube, in particular (Figure 3.6).

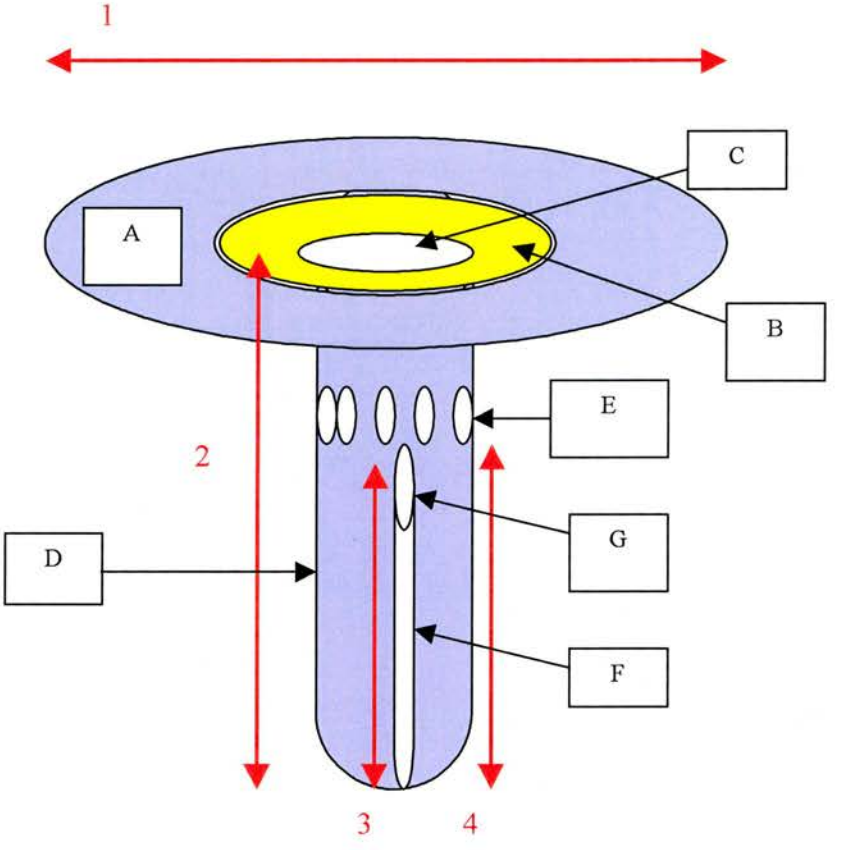


Figure 3.6 Schematic of *M. sylvatica* flower to illustrate morphometric measurements (red arrows); 1. Flower width 2. Corolla tube depth 3. Position of stigma 4. Position of anthers (see text for details). Key: - A = petals; B = ring of coronal scales; C = entrance to corolla tube; D = corolla tube; E = anthers; F = style; G = stigma.

Fifty flowers from each of the 'collapsed' coronal scale colour categories, were chosen at random and the following measurements taken. Flower width (1) was measured across the flattened surface of the petals and corolla tube depth (2) from the raised surface of the coronal scales to the point of insertion into the calyx.

As anther dehiscence occurred shortly after flowers opened (and therefore coincided with the yellow phase of the coronal scales) measurements were also taken to determine the position of the stigma in relation to the anthers at that time. A further 50 flowers with yellow (category 1 and 2) coronal scales were selected at random for measurement of two parameters within the corolla tube. By gently tearing apart the corolla tube longitudinally, the length of the style (3) and the distance between the base of the corolla tube and the lowermost tip of the anthers (4) were also recorded. All measurements were taken in the field using battery-operated digital callipers (Absolute Digimatic CD-6"C, Mitutoyo (UK) Ltd.).

3.2.3 Visitation.

To establish the identity of visiting organisms, all visitors to focal patches of *M. sylvatica* were recorded. Each patch comprised 8-10 plants within a 1 metre square area, displaying approximately 300 flowers. Data were recorded for 30 minutes in every hour between 08.00 and 17.00; trial observations both before and after these times had suggested that early morning and/or late evening foragers (e.g. bumblebees) were absent. Environmental variables (temperature and relative humidity) were recorded with a hand held recorder at the start of each observation period; measurements were made with the instrument held close (20-30cms) to ground level within the focal patch (and in shade for the temperature recording). Daily temperature and rainfall data for the April-June period (2000-2002) were supplied by the Scottish Meteorological Archive, Edinburgh, and referred to measurements taken at the Leuchars weather station,

approximately 8 miles from the study site. Observations were not made on days when climatic variables (particularly increased wind speed or rainfall) were likely to be detrimental to insect activity.

A visit was scored as a landing on a single flower within the focal patch that incorporated feeding or a feeding attempt; contact with either male or female reproductive parts could not be scored, due to the morphology and size of the flower. Insertion of mouthparts into the entrance of the corolla tube was considered to represent feeding or a feeding attempt, probing either for pollen or nectar.

Foraging preferences of the main insect visitors were recorded on six days between 18/5/01 and 13/6/01. Individual insects were followed on foraging bouts of up to 360 seconds in duration, exclusively within patches of *M. sylvatica*. Number of flowers visited and duration of probing time were noted, together with colour category of the coronal scales of all flowers visited. Individual flower-handling characteristics of all main insect visitors were also noted; this included movement across flowers where feeding did not take place as well as orientation of head and movement of mouthparts when feeding.

Sixty-one individuals of the hoverfly *Rhingia campestris* were followed on foraging bouts of between 6 seconds and 360 seconds and the following data recorded: length of feeding bout on individual flower (residence time); inspection behaviour (landing on or moving over the centre of flower without feeding response); and colour of centre of flower ('collapsed' categories). Data were collected in both May and June of 2001 and 2002; however a combination of scarcity of visitors and poor weather conditions led to a low sample size.

Individual insects were not marked, but very few foraging bouts were observed on any one day and independence of data was, therefore, assumed.

On each day that data were collected, patches of *M. sylvatica* were selected at random and the proportional availability of flower colour phases (in the same categories for visitor preference) within the population was calculated.

3.2.4 Floral resources in relation to colour change.

Availability of pollen and nectar were measured in relation to colour phase of the coronal scales of individual flowers.

i) Pollen.

Between 18/5/00 and 29/5/00 plants were picked and transported to the laboratory. Individual flowers were removed from their calyces and, using two pairs of watchmaker's forceps, the corolla tubes were split longitudinally to reveal the anthers. Flowers were viewed under a binocular microscope (Meizi EMZ, Japan) with a fibre optic light source (KL1500, Visual Inspection Technology, UK). Each of the five anthers was assessed for pollen depletion by means of a five-point categorical scale:

1) Surface of anther + pollen yellow; pollen covering both sides of anther with little or no surface visible.
2) Colour as 1 but pollen depleted and some areas of anther surface (still yellow) clearly visible.
3) Anther surface now brown; pollen pale yellow, particularly visible on sides of anther.

4) Anther brown; pollen as 3 with much of surface of anther visible.
5) Anther brown with very little cream-yellow pollen visible, mainly along edges of anther.

One hundred and twenty flowers were examined for pollen depletion using the above scale. Lloyd & Robertson (1991) noted that anther dehiscence is sequential in a number of *Myosotis* species; where the 5 anthers within a flower showed more than one stage of depletion, the category into which the majority of the anthers could be placed was used. Coronal scale colour was noted for each flower according to the 5-point scale prior to dissection.

In 2002, a more quantitative measure of pollen availability was used. Thirty-seven flowers from each of the 3 'collapsed' coronal scale colour categories were collected in the field and stored in individual vials containing 0.6ml of 70% ethanol for examination in the laboratory. Three counts of individual pollen grains were then made from each sample by the following method. Prior to each count, the vial was shaken vigorously to ensure pollen was dispersed throughout the liquid and the aliquots were taken from mid-way down the overall sample, adjacent to the preserved flower. A 0.15µl aliquot was removed from the vial and placed on a microscope slide; this amount was found to distribute evenly beneath a 22x22mm cover slip, and the total number of grains was easily counted using a light microscope with x10 objective lens. A mean of the three counts was calculated for each flower and multiplied by 40 to give a score per flower in the 0.6ml sample.

ii) Nectar.

In 2000 nectar reward was recorded primarily on a presence/absence basis. Plants were collected and prepared as in (i) above. When examined under a binocular microscope, droplets of nectar could be seen at the base of the corolla tube; taking care to score only droplets that were not directly associated with the longitudinal split of the corolla and which might, therefore, have been, or have included, cell sap/intercellular material. Coronal scale colour for each flower sampled was also recorded as above. Measurement of nectar volume per flower was attempted by using a 0.5µl microcapillary tube; individual droplets were collected from single or a number of flowers and transferred to a sugar refractometer to measure nectar concentration.

In 2001 a modified methodology was employed for measurement of nectar characteristics. Between 30/5 and 11/6, plants were collected as previously described and, within 1-4 hours of collection, nectar was extracted as below. The minimum time between sample collection and first measurement, allowing for travel from field site and laboratory set-up, was no less than 1 hour. Nectar characteristics of those flowers sampled first and those used later (up to 4 hours maximum from time of collection) did not appear to alter during this time; no variation was evident between volumes and concentrations of samples over this time period.

Equal numbers (usually fifteen) of flowers with similarly coloured coronal scales (yellow stages 1 and 2; white stage 5) were removed from their calyces and, taking care not to damage floral tissues, placed in separate 0.2ml Eppendorf tubes (Camlab, UK). Tubes were then simultaneously centrifuged (Sanyo MSE Micro Centaur) for 5 minutes at 7000rpm. Floral tissue was

removed and using a 0.5µl microcapillary, residual fluid that had collected at the bottom of the tube was collected and measured in millimetres, converted to a volume (1mm = 0.03µl), and transferred to a refractometer for sugar concentration measurement.

3.2.5 Floral manipulation; effect of differing levels of mimicked visitation rates.

A series of experiments was set up to determine whether rate of visitation, as mimicked by artificial flower handling, might influence onset and rate of colour change.

i) Laboratory.

A pilot study was used in 2000 to establish possible effects. At least 40 freshly cut cymes (with stalks up to 10cm in length) were placed in glass vials containing 15-20ml tap water; twenty cymes were randomly assigned to a control or treatment group. In both cases all flowers except first-day flowers were removed and the colour of the coronal scales of that flower confirmed as category 1 yellow. No further treatment was made to the control group flowers. However, the corolla tube of each of the treatment group flowers was probed ten times, each for a three-second period, using a dissected bee's tongue attached to a pair of watchmaker's forceps with Blu-tac to enable easy manipulation.

Long-tongued bees were selected from a reference collection that included *Bombus* spp., *Anthophora* spp., and *Eucera* spp.; the distal mouthparts, comprising labial palpi and glossa, were inserted to the base of the corolla tube

(c. 3-4mm). Bumblebees had been noted as occasional visitors to *M. sylvatica* and were recorded probing for nectar. The robustness of their mouthparts was the rationale behind this choice for mimicked manipulation; the more delicate nature of the mouthparts of syrphids (the most abundant visitors; see below) made them unsuitable for the proposed use.

The groups of flowers were arranged in matched pairs to enable close comparison of coronal scale colour at each data collection time; colour phase according to the five-point scale was noted at 24-hour intervals. Where wilting of a flower occurred within the first 24 hours (possibly as a result of damage to the cyme during cutting) no data were collected.

The subsequent finding (see Results) that colour change occurred more rapidly in manipulated flowers underpinned the fieldwork in 2001. Two possible confounding variables of laboratory work were thereby avoided; flower life was greatly reduced in laboratory conditions (perhaps due to raised temperature in the latter part of the above experiment), and was also affected by damage through removal of older flowers from the cyme.

ii) Fieldwork.

In 2001 exclusion 'cloches', (see above), were constructed over metre square patches of up to 20 plants; 30 buds (one day prior to opening) were marked within the cloche. On the morning of opening 10 of these marked (and now open) flowers were randomly assigned to one of three treatment groups; single manipulation per flower, 5 manipulations per flower or 20 manipulations per flower. Probing of the corolla tube was carried out as before with a bee tongue, and the exclusion netting replaced; onset of colour change of coronal

scales according to the 'collapsed' colour categories was recorded on subsequent days until wilting or abscission of marked flowers. Concurrently, as a control group, 20 buds from within the exclusion patches were marked and checked daily as above. An "open visitation" group of 20 buds outside the 'cloche' were also marked and data collected in the same way. For these experiments freshly dissected tongues of one species of bumblebee, *B. pascuorum*, were used. Three replicates of this full treatment regime were carried out.

The experiment was repeated in 2002, but with sample sizes increased for all treatment groups; "open visitation" n = 40 flowers; "bagged" n = 60 flowers; "bee tongue manipulation" (all levels) n = 20 flowers. Also, a further type of probe was used to manipulate a group of 60 flowers. A single nylon bristle from an artist's paintbrush was removed and mounted on a dissection probe as before. Each sample flower was probed 20 times and, between flowers, the nylon filament was washed in acetone and then distilled water and dried with soft tissue paper. This ensured that no pollen was transferred between flowers. This test was designed to confirm whether pattern of colour change might vary with type of manipulation; additionally, dislodgement of self- versus deposition of cross-pollen could have differing effects on colour change.

3.2.6 Seed set.

To confirm the self-compatible status of *M. sylvatica,* a simple visitor exclusion experiment was set up. On 24/5/01 20 buds one day prior to opening, on 20 individual plants, were tagged and left open to natural levels of visitation;

20 similar-aged buds beneath exclusion cloches were also marked. All marked flowers were collected on 4/6/01 and developing seeds counted.

In 2002, seed set was recorded for plants in several treatment groups used in some of the above experiments (3.2.6.ii) to compare reproductive success with type of manipulation and possible correlation with colour change. If colour change were linked with an aspect of successful pollination alone, colour change should not occur in those groups where seed set was absent, or low. On 18/6/02 the dried calyces from tagged flowers in the 'open, bagged, bee tongue' (x1 and x20 manipulations) and 'brush' groups (see above) were collected and counted. As an additional control to check for positional differences in seed production in the open group, further calyces from positions ten places below the marked flower were also collected and examined.

3.3 Results.

3.3.1 Flower longevity, floral colour change and morphometric measurements.

i) Longevity.

Under conditions of natural visitation flowers were open for between 4 and 12 days (mean longevity 7.7 ± 0.2 days, n = 77 flowers). However, there was a significant difference between the longevity of flowers between years (Figure 3.7); Kruskal-Wallis test, using medians, $H = 43.04$, df 2, $p < 0.001$ (in 2000 (n = 18) and 2002 (n = 41) median longevity = 8 days; 2001 (n = 18) median longevity = 5 days). This anomaly may have been linked to unusually dry and slightly warmer weather conditions during May (the peak flowering season) in 2001 compared to the same period in both of the other years. Significant rain (>0.1mm) fell on only 7 of 31 days in May 2001, with a total monthly rainfall of 14.9mm. Mean daily temperature for this period was 15.6°C. In contrast, similar amounts of rain fell on 18 days in May 2000 (total monthly rainfall 75.05mm) and on 21 days in May 2002 (total monthly rainfall 43.15mm). The mean daily temperature for these periods was 14.0°C and 14.7°C, respectively. The conditions in 2001 could have caused reduced floral longevity either a) due to scarcity of water during plant growth, or b) because of increased visitation, thus underpinning rapid and successful pollination, followed by earlier abscission upon fertilisation. The latter could have occurred if a key visitor flourished in the drier, warmer conditions of 2001 but was present in reduced numbers during the other years (no data collected).

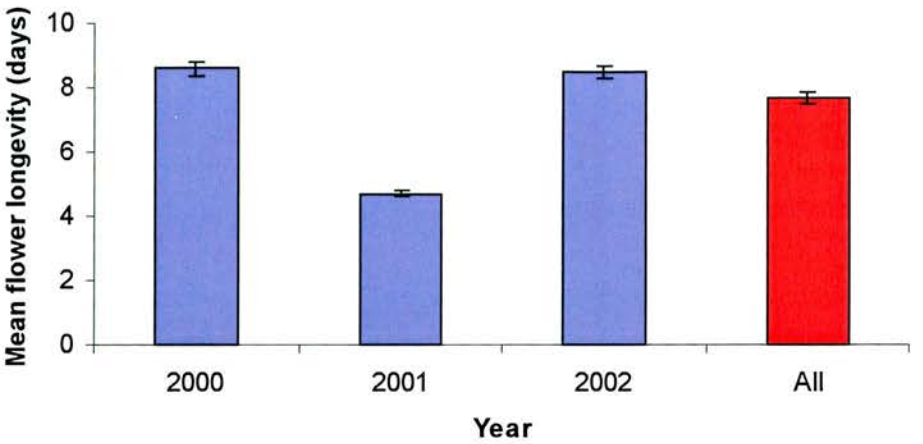


Figure 3.7 Mean longevity of flowers of *M. sylvatica* in 2000 (n = 18), 2001 (n = 18) and 2002 (n = 41).

Floral longevity was also influenced by exclusion of visitors within years (Figure 3.8). Mean longevity of 'bagged' flowers was 5.5 ± 0.2 days (n = 19) and that of 'open' flowers 4.7 ± 0.1 days (n = 18) in 2001; Mann-Whitney test, using medians, W = 241.0, p = 0.0008 (median longevity bagged 6 days; open 5 days). This pattern was repeated in 2002. Mean longevity of 'bagged' flowers was 11.0 ± 0.2 days (n = 39) and that of 'open' flowers 8.5 ± 0.2 days (n = 41); Mann-Whitney test, using medians, W = 993.0, p < 0.0001 (median longevity bagged 11 days; open 8 days).

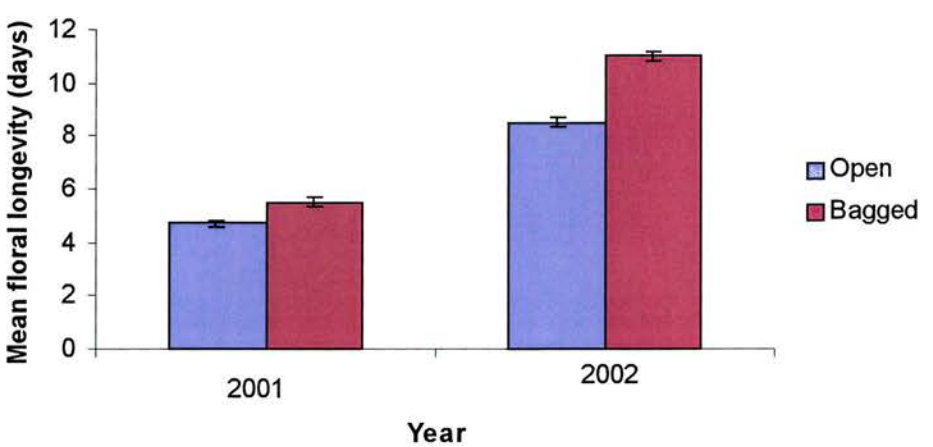


Figure 3.8 Floral longevity in *M. sylvatica* under natural visitation and with visitors excluded.

While there was a clear difference in longevity between open and bagged flowers the data illustrated in Figure 3.8 suggest that, of the earlier alternatives for reduced longevity in 2001, the constraint on flowering time was plant-related. Increased visitation could account for between-year patterns in open groups, but the exclusion of visitors should not have altered floral longevity between 2001 and 2002; longevity was significantly different between years in bagged flowers; Mann-Whitney $W = 190.0$, $p < 0.0001$ (median longevity 2001, 6 days; 2002, 11 days).

ii) Colour change.

a) Petals.

Length of petal colour phases was recorded in 2000 for 'open' flowers and 2002 for both 'open' and 'bagged' flowers. In 2000, all flowers ($n = 18$) were pink in bud and the petals remained pinkish-violet (colour 256U) upon opening. This phase lasted up to 3 days (mean 1.4 ± 0.2 days) followed by a blue phase (colour 279U) of 5 to 10 days (mean 7.4 ± 0.3 days).

In 2002, in open flowers ($n = 49$; a single flower did not change colour during its 9-day life span and was excluded from the data set as an outlier), the pinkish-violet phase lasted up to 2 days with a mean duration of 1.0 ± 0.1 days (Figure 3.9). This was followed by a blue phase of 6.4 ± 0.3 days. The initial phase in bagged flowers lasted 0.9 ± 0.1 days ($n = 50$) followed by a blue phase of 9.1 ± 0.2 days.

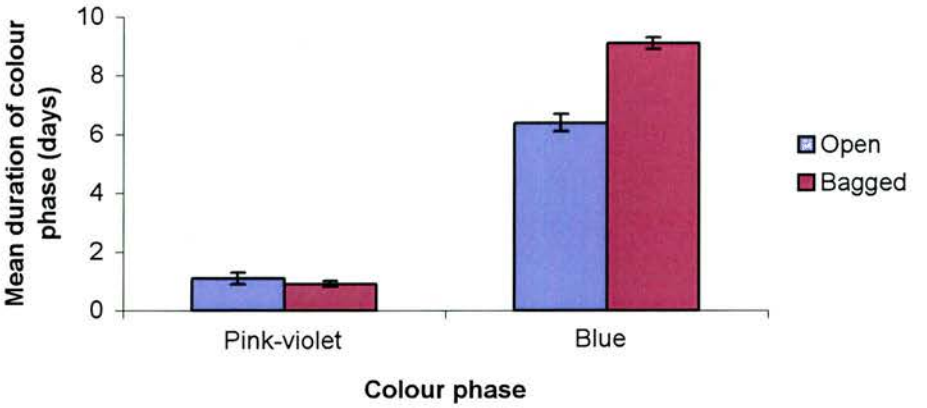


Figure 3.9 Duration of petal colour phases (see text for details) in *M. sylvatica* under visited and non-visited conditions.

The increased longevity of bagged flowers did not, therefore, reflect the duration of the pinkish-violet phase but was underpinned by an extended blue phase.

b) Coronal scales.

In 2001 there was a significant difference between the duration of the yellow coronal scale phase of open (mean 2.0 ± 0.1 days, n = 18 flowers) and bagged flowers (mean 2.7 ± 0.2 days, n = 19 flowers); Mann-Whitney test, W = 271.5, p = 0.0142 (median open flowers = 2 days; bagged flowers = 3 days). This pattern was also found in 2002 when a larger sample size was used and the durations of both the intermediate and white phases were also recorded (Figure 3.10). Mean length of the yellow phase was 2.1 ± 0.1 days in open flowers (n = 48) and 3.1 ± 0.2 days in bagged flowers (n = 44); Mann-Whitney test, using medians, W = 1608.5, p < 0.0001 (median phase; open 2 days, bagged 3 days). For the intermediate stage, the mean duration of the phases were 2.1 ± 0.2 days and 3.3 ± 0.2 days for open and bagged flowers, respectively; Mann-Whitney test, using medians, W = 1292.5, p < 0.0001 (median phase; open 2 days, bagged

3 days). In both open and bagged flowers the mean duration of the white phase was 3.4 ± 0.2 days. There was no significant difference between the median length of the white phase in these groups; Mann-Whitney test, W = 1578.5, p = 0.8311 (median phase 3 days in both groups).

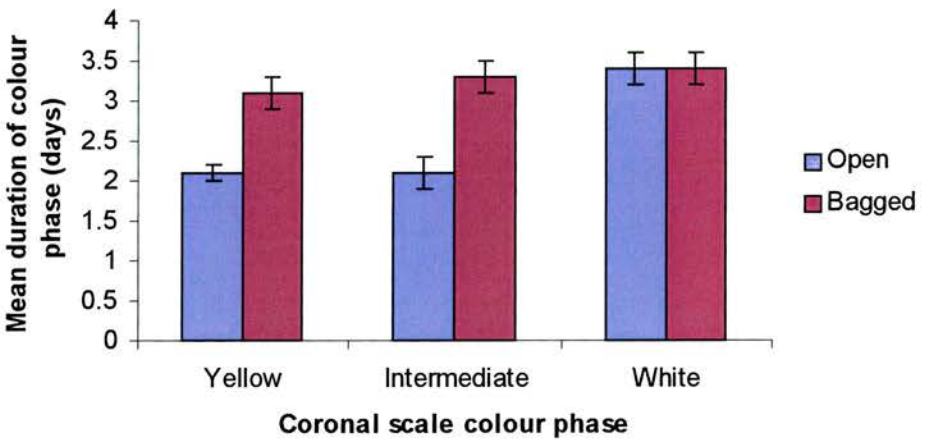


Figure 3.10 Duration of coronal scale colour phases of *M. sylvatica* under visited and non-visited conditions.

Longevity in *M. sylvatica* was, therefore, increased through the exclusion of visitors; duration of colour phase of the petals was not affected by this manipulation but duration of the coronal scale colour phase was, with both the yellow and intermediate phases increased by over a day in each case. This showed that some aspect of visitation accelerated colour change in normal conditions. The colour change of the petals occurred exclusively within the yellow phase of the coronal scales.

c) Colour phase availability within the population.

Table 3.1 illustrates the proportion of scale colour phases present in a random sample of the population. Ten patches of *M. sylvatica*, measuring between 0.5-1.0 square metre, were chosen and all flowers scored for petal and

coronal scale ('collapsed' category) colour. Of 2385 flowers counted, 189 (7.9%) were pink to pink-violet in colour and the remainder, 2196 (92.1%), were blue.

Patch no.	Number of cymes counted	Coronal scale colour		
		Yellow	Intermediate	White
1	51	99	50	102
2	40	91	42	98
3	49	102	62	71
4	46	86	49	71
5	52	111	67	61
6	51	74	78	79
7	56	104	79	77
8	55	83	74	99
9	52	134	79	25
10	52	97	72	69
Total		**981**	**652**	**752**
%		**41.1**	**27.3**	**31.6**

Table 3.1 Coronal scale colour of 2385 *M. sylvatica* flowers.

iii) Morphometric measurements.

Flower width ranged from 7.34 to 12.28mm with a mean of 10.39 ± 0.08mm (n = 150 flowers). There was no significant difference between colour phases; one-way ANOVA, $F = 0.31_{2, 147}$, $p = 0.735$. Corolla tube depth ranged from 2.48 to 4.75mm with a mean of 3.55 ± 0.03mm. Again, no difference was found between colour phases; one-way ANOVA, $F = 2.75_{2, 147}$, $p = 0.067$.

The distance between the base of the corolla tube and the lowermost tip of the anthers ranged from 1.22 to 2.09 mm with a mean of 1.63 ± 0.03mm. The length of the style varied between 1.07 and 2.08 mm with a mean of 1.40 ± 0.03mm. This illustrated the very close proximity of the stigmatic surface and the anthers (and see Figure 3.11) and, together with the restricted entrance to the

corolla tube, suggested that any probing by visiting insects or, possibly, even the act of landing might dislodge pollen and lead to successful pollination.

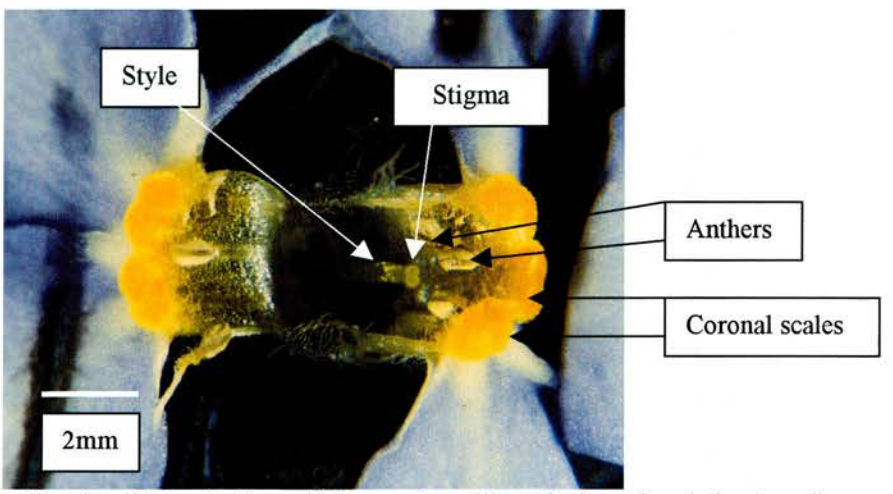


Figure 3.11 Corolla tube of *M. sylvatica* split to reveal position of stigma in relation to anthers. Dusting of pale yellow pollen can be seen just below stigma.

3.3.2 Flower visitors.

i) Overall visitation and visitation rates.

Visitation was recorded on ten days between 4/5/00 and 25/5/00; in 17 hours of observation, 252 visits were noted, all of which were by insects from 3 orders (Diptera, Hymenoptera and Lepidoptera; see Figure 3.12).

Hoverflies (Syrphidae) were the most abundant visitors, comprising 71% of all visitors. These were identified to species level on the wing where possible (e.g. *Episyrphus balteatus*, *Rhingia campestris*, *Leucozona lucorum*) and others were captured with a hand held net, killed using ethyl acetate and identified in the laboratory using the keys provided in Stubbs & Falk (2000). Less abundant visitors were only identified to generic level (e.g. *Melanostoma* spp. and *Platycheirus* spp.).

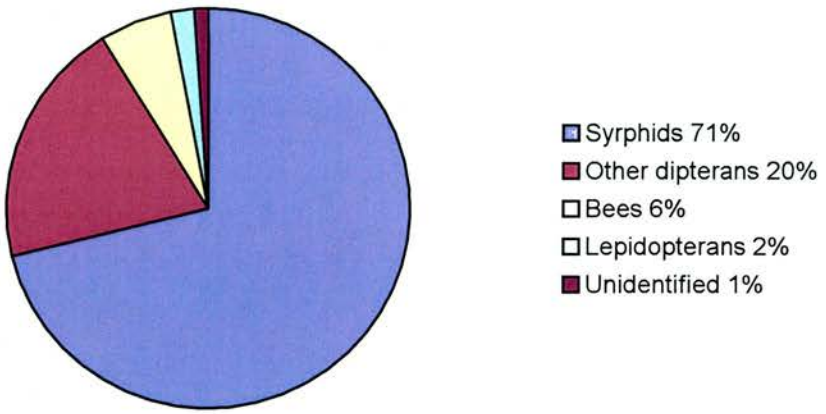


Figure 3.12 Proportions of 252 insect visitors to patches of *M. sylvatica*.

Mean visitation rate (for all visitors summed) per 100 flowers per 30-minute observation period peaked at 4.78 between 15.00 and 16.00 hours (Figure 3.13). At this focal patch shadows from nearby trees led to an abrupt cessation of visitor activity by mid-afternoon.

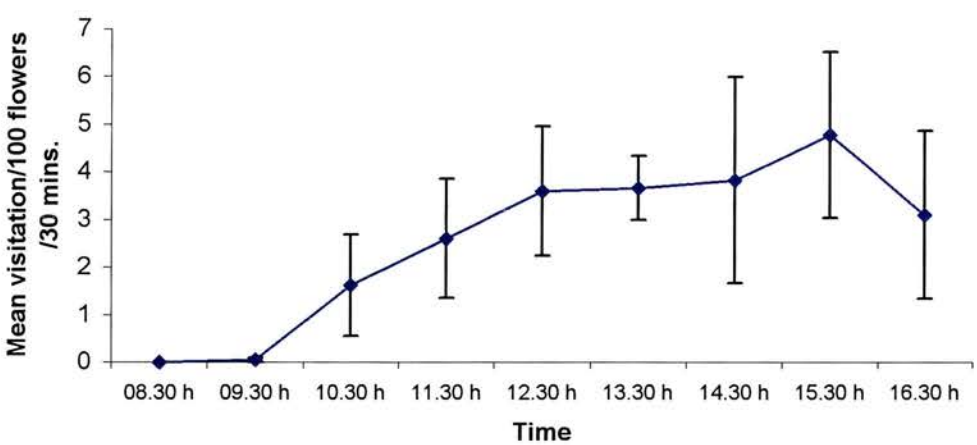


Figure 3.13 Mean visitation rate to focal patches of *M. sylvatica*. Between 1 and 7 focal watches were carried out for each time period; patches comprised c.300 flowers on 8-10 plants.

ii) Foraging by syrphids.

Data were collected on the foraging behaviour of *R. campestris* and *Melanostoma/Platycheirus* spp; as a 'grouped taxon' these latter flies were the next most abundant visitors and exhibited similar foraging behaviours but were

not identifiable to individual species on the wing. Most visiting syrphids were observed 'grooming' during foraging bouts; this activity almost certainly involved the collection of pollen that had adhered to various body parts during flower handling. Although the type of floral resource sought could not be confirmed for all visitors (because both nectar and pollen were hidden within a short corolla tube), where close-range observation did not result in disturbance of the visitor, inference as to potential reward could be made from specific flower-handling behaviours.

The main syrphid visitor, *R. campestris*, fed by probing gently with its long tongue without further movement of the head. This species has a proboscis measuring up to 12mm and a diet comprising 95% nectar and has previously been observed feeding mainly on the flowers of ground ivy (*Glechoma hederacea*) which have a corolla depth of approximately 12mm (Gilbert, 1981). Gilbert described a close match between corolla depth and proboscis length of 12 species of syrphid. Here, *R. campestris* exploited a nectar resource from a flower with a short corolla tube relative to its proboscis; the mean depth of the corolla in *M. sylvatica* was 3.55 ± 0.03mm. A 'pumping' action of the proboscis could be seen once a *Rhingia* was positioned over the centre of the flower. This movement underpins a particular aspect of syrphid feeding behaviour; these insects spit saliva onto their food as well as sucking the food with their mouthparts (F. Gilbert; personal communication).

All species of visiting syrphids exhibited a strong preference to feed at flowers with yellow centres. Hoverflies were frequently noted to land on, or crawl over, white-centred flowers without exhibiting a feeding response. Figure 3.14 illustrates that the flower choice of *R. campestris* is non-random in respect

to the availability of coronal scale colour phases within the population; χ^2 goodness of fit = 115.0, df 2, $p < 0.001$.

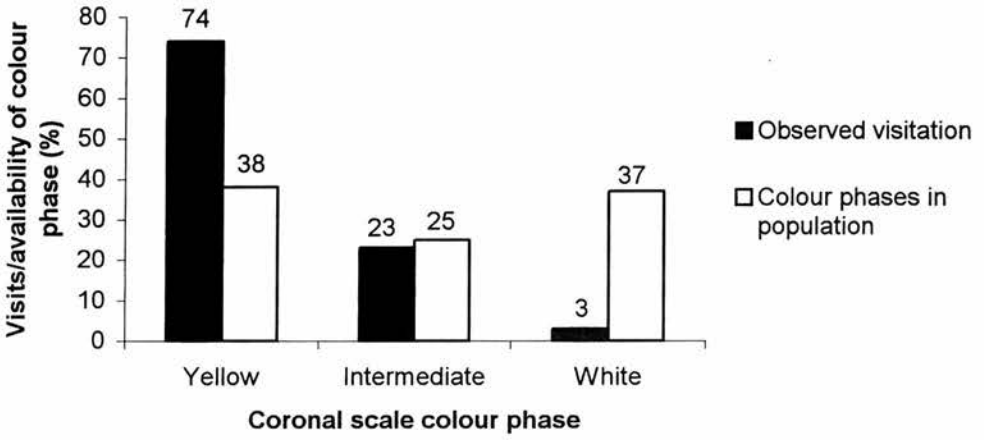


Figure 3.14 Floral colour phase preference of 61 individual *R. campestris* in feeding visits to 278 flowers. Colour categories as per text.

Hoverflies strongly preferred to feed at the younger, yellow-centred flowers; feeding visits were made to intermediate flowers but only 3% of all feeding attempts were made at white-centred flowers although this phase accounted for nearly 32% of flowers in the overall population (see Table 3.1).

Non-feeding inspections were not made on yellow-centred flowers by *R. campestris*; all landings on these flowers by 61 foraging individuals always resulted in the extension of the proboscis into the corolla tube. However, twenty-seven of these individuals inspected intermediate and/or white flowers without making an attempt to feed; flies crawled over these flowers (on occasions orienting themselves over the centre of the flower) but did not extend their proboscides. Thus 10% of visits to intermediate flowers and 90% of visits to white-centred flowers were inspections only (Figure 3.15).

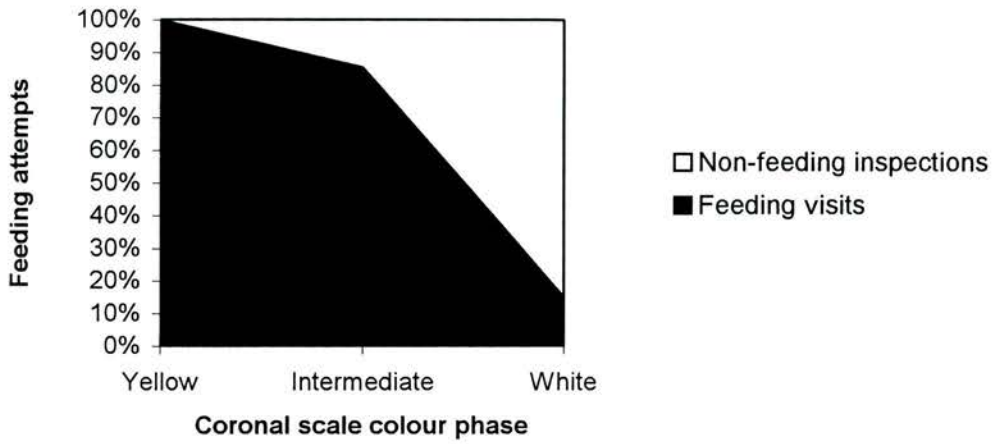


Figure 3.15 Percentage of non-feeding inspections to *M. sylvatica* by *R. campestris* compared to feeding visits as determined through extension of proboscis into corolla tube.

Residence times (including duration of probing) of *R. campestris* on individual yellow-centred flowers ranged between 1 and 53 seconds (mean 6.9 ± 0.5 seconds, n = 210). For intermediate flowers residence time varied between 1 and 24 seconds with a mean of 5.7 ± 0.6 seconds (n = 66). Probing was only recorded on 7 white-centred flowers and lasted from 1 to 5 seconds (mean 2.1 ± 0.5 seconds). Flies spent considerably more time feeding at yellow-centred and intermediate flowers than at white-centred flowers (Figure 3.16); Kruskal-Wallis test, using medians, H = 11.36, df = 2, p = 0.003.

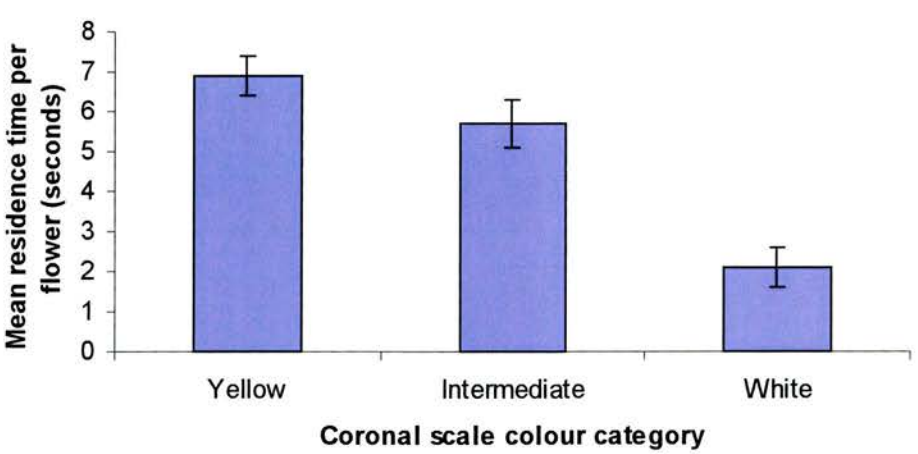


Figure 3.16. Residence times (duration of probing) of *R. campestris* at flowers of *M. sylvatica*.

Foraging behaviour of other syrphid visitors followed similar patterns to those recorded for *R. campestris*. The following data refer to individuals identified on the wing to either the genus *Melanostoma* or *Platycheirus*. These insects had noticeably shorter proboscides than *R. campestris* and, with the use of a hand held magnifying lens (x10), their labellae were visible as pad-like structures at the tip of the proboscis. Feeding behaviour of both taxa involved orienting over the centre of the flower and forcing the head towards (and therefore the mouthparts into) the entrance to the corolla tube. Pollen could certainly have been collected from this position; but whether nectar, at the base of the corolla tube, could be reached was uncertain just from observation.

Forty flies were followed on foraging bouts of between 6 and 138 seconds; data were collected as for *R. campestris*. Figure 3.17 illustrates that the flower choices, when feeding, of these other syrphids were non-random with respect to the availability of floral colour phases within the population (χ^2 goodness of fit = 7.45, df 2, p = 0.024) and as before they strongly preferred yellow-centred flowers. Only 2 visits were observed to a white-centred flower out of a total of 182 feeding visits. Non-feeding inspections were made to only 4 yellow-centred flowers out of 147 (3%) visited by 40 individuals, whereas visits to the remaining 143 flowers always resulted in the extension of the proboscis into the corolla tube. Twenty-two of the 40 individuals also inspected intermediate and/or white flowers without making an attempt to feed (Figure 3.18). Forty-seven per cent of visits to intermediate flowers and 93% to white-centred flowers were inspections only.

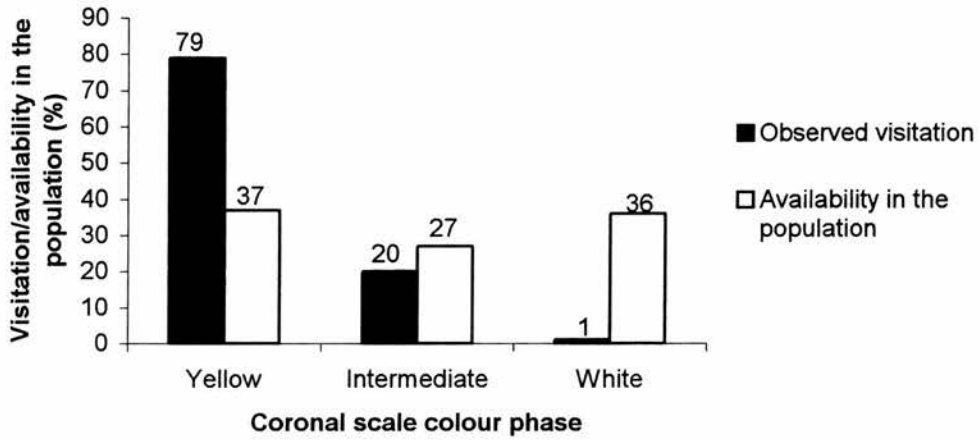


Figure 3.17 Floral colour phase preference of 40 individual *Melanostoma* and *Platycheirus* spp. in visits to 182 flowers. Colour categories as in text.

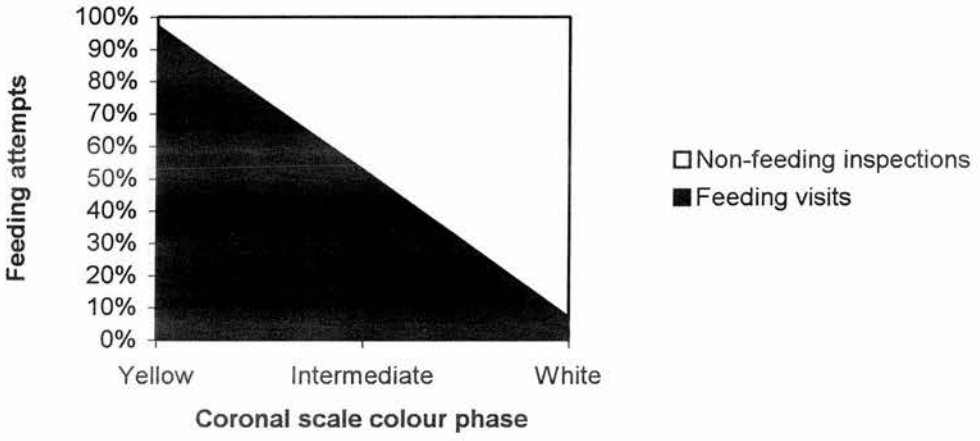


Figure 3.18 Percentage of non-feeding inspections to *M. sylvatica* by *Melanostoma*/*Platycheirus* spp. compared to feeding visits as determined through extension of proboscis into corolla tube.

Residence time (duration of probing) at 143 yellow-centred flowers ranged between 1 and 36 seconds (mean 5.7 ± 0.4 seconds, n = 38 insects). Twenty-one insects fed at 37 intermediate flowers for between 1 and 14 seconds (mean 4.1 ± 0.5 seconds); two of these flies did not feed at yellow-centred flowers. Just two flies fed at a single white-centred flower for 1 and 2 seconds respectively.

iii) Other visitors.

Quantitative foraging data were not collected on a range of other insects that were either scarce or irregular visitors, or that could not be followed during foraging bouts due to small size, or that were easily disturbed. These included a number of relatively short-tongued bees (including at least one species of andrenid and two apids from the genus *Nomada*), and the longer-tongued megachilid bee, *Osmia rufa*. The butterfly (*Artogeia napi*) was an occasional visitor as were empid flies, robber flies (Asilidae) and the yellow dung fly (*Scathophaga stercoraria*).

3.3.3 Floral resources in relation to colour change.

i) Pollen.

a) Depletion.

Table 3.2 shows the pollen status of 120 dissected flowers in 2000. Flowers where fading of the coronal scales had commenced (i.e. colours 2 to 5) contained less pollen than colour 1 flowers. Conversely, depletion of pollen to categories 4 and 5 was rarely found in colour 1 or 2 flowers. Both the mean and median score increased progressively as the flower-centre colour changed. This presumably reflected pollen removal through visitation over time.

Coronal colour scale	n	Pollen depletion scale					Mean score	Median score
		1	2	3	4	5		
1	37	24	2	10	1	0	1.7 ± 0.2	1
2	15	2	0	11	2	0	2.8 ± 0.2	3
3	12	1	0	7	2	2	3.3 ± 0.3	3
4	31	0	0	24	6	1	3.3 ± 0.1	3
5	25	0	0	10	11	4	3.8 ± 0.1	4

Table 3.2 Pollen depletion in relation to coronal scale colour category in 120 *M. sylvatica* flowers. Here, least depletion is found at '1' and most pollen is depleted at '5' on the scale.

b) Counts.

The direct pollen counts in 2002 produced results that conflicted with the findings in (a) above. Yellow-centred and intermediate flowers contained fewer pollen grains per flower than white-centred flowers (Figure 3.19), mean number of grains per flower being 564 ± 140, 616 ± 183, and 1524 ± 329 for yellow-, intermediate-, and white-centred flowers respectively. There was, however, no significant difference between the median number of grains per flower; Kruskal-Wallis test, $H = 4.50$, $df = 2$, $p = 0.105$. Possible reasons for these conflicting results are discussed later.

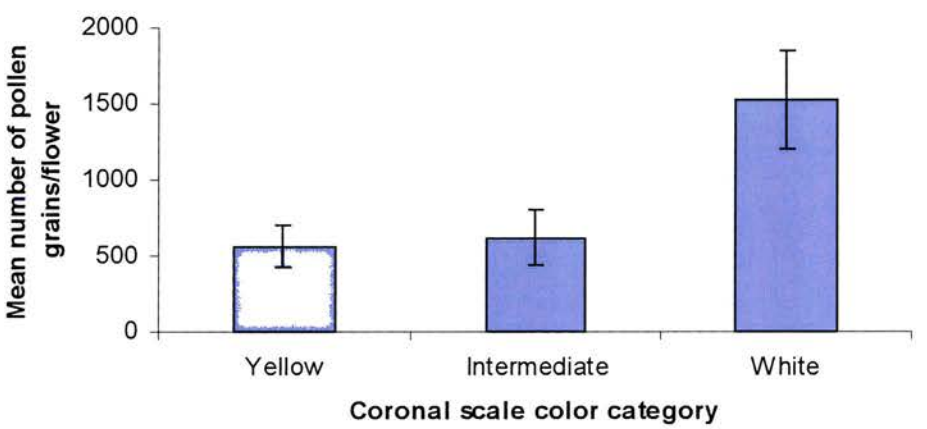


Figure 3.19 Pollen counts for different coronal scale colour phases of *M. sylvatica*.

ii) Nectar.

a) Presence/absence.

The 120 flowers dissected for scoring of pollen depletion in 2000 were also used to determine the presence/absence of nectar (Table 3.3).

Coronal scale colour	n	Nectar		% offering nectar reward
		Present	Absent	
1	37	35	2	95
2	15	14	1	93
3	12	4	8	33
4	31	12	19	38
5	25	3	22	12

Table 3.3 Nectar availability in 120 *M. sylvatica* flowers in relation to coronal scale colour.

High proportions of yellow-centred flowers (categories 1 and 2) contained nectar when compared to flowers where fading of the coronal scales had occurred. Only 12% of white-centred flowers contained nectar.

b) Volume measurements.

Nectar volumes and concentration were successfully measured using a modified methodology in 2001. Flowers were divided into two categories: yellow incorporated categories 1,2 and 3; white comprised categories 4 and 5. Yellow-centred flowers were sampled in 37 batches each numbering between 10 and 17 individuals (Table 3.4), giving 552 in total.

To establish a mean volume per flower, a mean of means was calculated using the values in Table 3.4 for the 37 sample batches. Mean nectar volume per flower was 4.4 ± 0.8nl. Concentration values were pooled from 24 sample batches (see note in table 4 legend) to produce a mean nectar concentration per flower of 45.6 ± 1.0%.

A measurable quantity of nectar was only available from a single sample batch of white-centred flowers (Table 3.5), out of 14 batches of between 10 and 15 flowers. In 13 of these batches barely visible quantities of nectar were

extracted which could not be successfully transferred to the refractometer. No concentration measurements were, therefore, possible.

No. of flowers	Total vol.(µl.)	Vol./flower (nl)	Conc. (% sucrose)
17	0.21	12	-
10	0.30	30	42
15	0.06	4	-
15	0.06	4	-
15	0.09	6	-
15	0.03	2	-
15	0.06	4	41
15	0.015	1	-
15	0.045	3	48
15	0.045	3	45
15	0.03	2	42
15	0.015	1	50+
15	0.075	5	48
15	0.03	2	47
15	0.12	8	50+
15	0.06	4	48
15	0.045	3	49
15	0.045	3	41
15	0.06	4	48
15	0.045	3	46
15	0.06	4	50+
15	0.045	3	47
15	0.03	2	46
15	0.045	3	50+
15	0.045	3	49
15	0.045	3	50+
15	0.045	3	55
15	0.045	3	53
15	0.06	4	50+
15	0.075	5	54
15	0.06	4	<40
15	0.03	2	41
15	0.06	4	37
15	0.045	3	41
15	0.12	8	36
15	0.045	3	41
15	0.045	3	49
Mean		4.4 ± 0.8	45.6 ± 1.0

Table 3.4. Nectar volume and concentration in yellow-centred flowers of *M. sylvatica*. (Where symbols + or < are shown, the concentration measurements are not definitive; a pair of refractometers were used (0-50% and 40-85%) and on these occasions measurement was attempted with the 'wrong' instrument).

No. of flowers	Total vol. (μl.)	Vol./flower (nl)	Conc. (% sucrose)
10	0.03	3	-
10	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-
15	-	-	-

Table 3.5. Nectar volume and concentration in white-centred flowers of *M. sylvatica* (- indicates absence of nectar or trace quantities that were to small to measure).

Greater quantities of nectar were therefore clearly present in yellow-centred flowers.

3.3.4 Floral manipulation; effect of differing levels of mimicked visitation rates.

i) Pilot study.

Preliminary data from the laboratory-based experiments in 2000 suggested that floral manipulation could hasten the rate of change of the coronal scale colour (Figure 3.20a-f). In three of six replicates (Figure 3.20b,c and d), colour change of the coronal scales was significantly more advanced in manipulated flowers when compared to matched control flowers two days after the initial manipulation (Table 3.6). There was no difference in the rate of colour change in the remaining replicates (Figure 3.20a, e and f), though only in the last of these replicates did colour change appear accelerated in control group flowers. Reduced sample size due to rapid floral wilting (thought to be related to greatly increased laboratory temperature) may have compromised the validity of the

results for replicates 'e' and 'f', where n was particularly reduced (see Table 3.6). The diminishing sample sizes (all groups started with at least 20 flowers per treatment at the start of each experiment) led to the choice of 'day 2' for statistical analysis; at this time at least 9 flowers of the original 20 were still available for comparison within each replicate (see Table 3.6).

Replicate	**Mean coronal colour scale score**		**Median coronal scale colour score**		**n**		**Mann-Whitney C vs. M**	
	C	**M**	**C**	**M**	**C**	**M**	**W**	**p**
a	2.8 ± 0.1	3.0 ± 0.1	3	3	22	20	430.5	0.2902
b	2.6 ± 0.2	3.1 ± 0.2	3	3	20	20	342.0	0.0499*
c	2.2 ± 0.2	3.3 ± 0.2	2	3	19	19	242.0	0.0001**
d	3.0 ± 0.2	4.2 ± 0.2	3	4	20	19	281.5	0.0006**
e	3.3 ± 0.3	3.7 ± 0.3	3	4	11	12	118.0	0.3880
f	3.4 ± 0.3	3.1 ± 0.2	3	3	10	9	108.5	0.4620

Table 3.6. Coronal scale colour score in *M. sylvatica* 2 days after start of experiment. **C** = control group, **M** = manipulated group, **W** = Mann-Whitney statistic, **p** = significance level (* $p<0.05$, ** $p<0.001$).

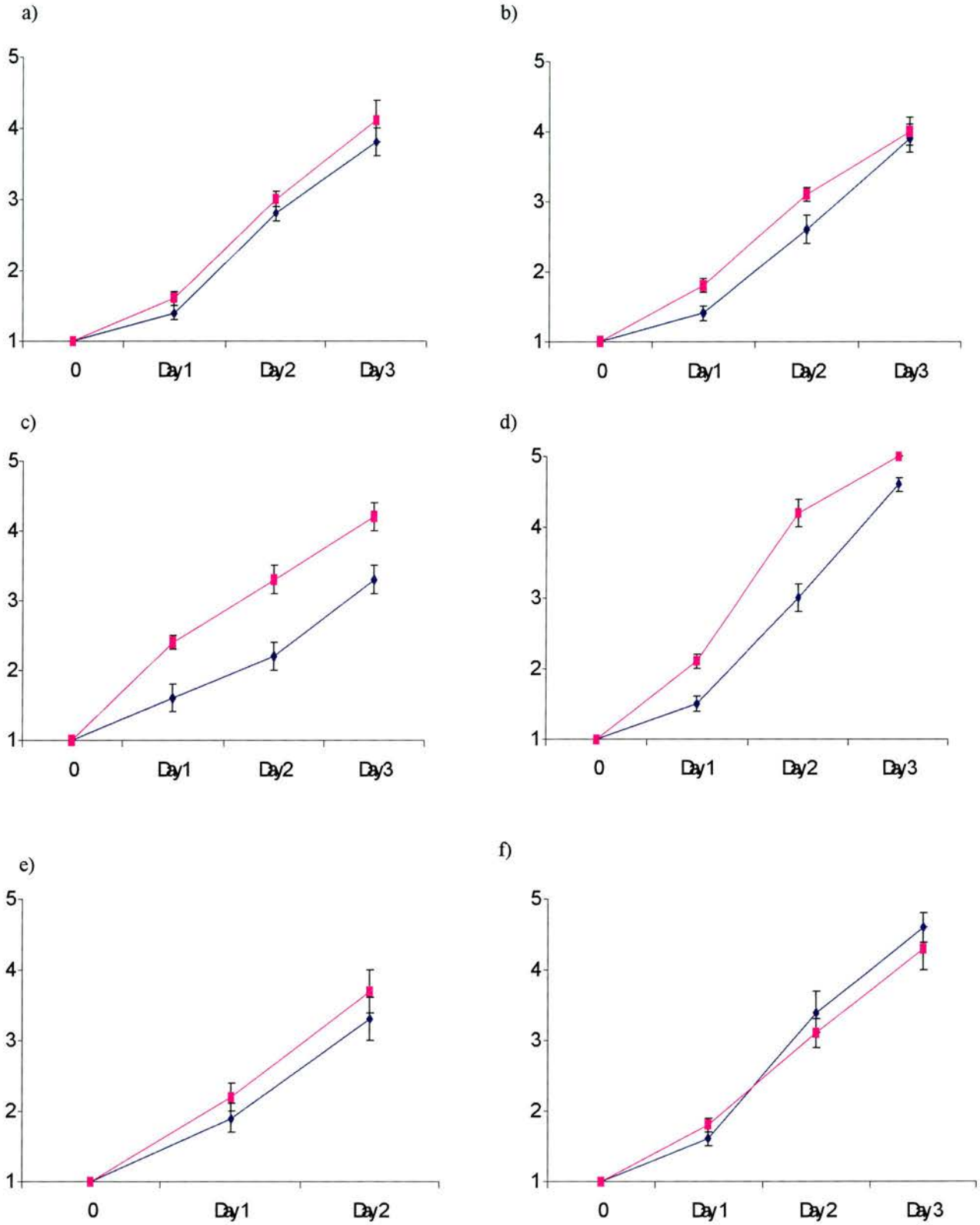


Figure 3.20a-f. Mean coronal scale colour score in *M. sylvatica* for six replicates (a-f) of paired treatment groups (see text). Y-axes; mean coronal scale colour score. X-axes; time in days from manipulation. ♦ = control; ■ = manipulated.

ii) Field manipulations 2001.

The results of floral manipulations under field conditions showed that coronal scale colour change was retarded through exclusion of insect visitors and could be induced by bee-tongue manipulations that mimicked natural visitation (Figure 3.21).

Two days after treatment, the majority of coronal scales of flowers (94%; n = 18) receiving natural visitation had begun to change. This was also the case for all the experimental groups (n = 30 in all cases); single manipulation (80%), five manipulations (80%) and twenty manipulations (83%). Scale colour change had commenced in all flowers in each of these groups after 3 days. In contrast, where visitors were excluded, only 47% of flowers (n = 19) had changed colour after 2 days, 74% by day 3, and the remainder after 4 days.

At day 2, differences among the proportions were tested by contingency table analysis (Zar, 1996). The proportions of flowers where coronal scale colour change had commenced were not equal, $\chi^2_{0.05,4} = 13.863$. A Tukey-type test for multiple comparison testing among the five proportions was used to determine which populations were different from the others. The only difference between ranked sample proportions was between bagged flowers and those that received natural visitation ($p<0.05$).

Therefore, natural visitation led to faster onset of colour change than either mimicked visitation or where visitors were excluded; there was no difference in the rate of change between mimicked visitation groups (regardless of level of manipulation) and bagged flowers.

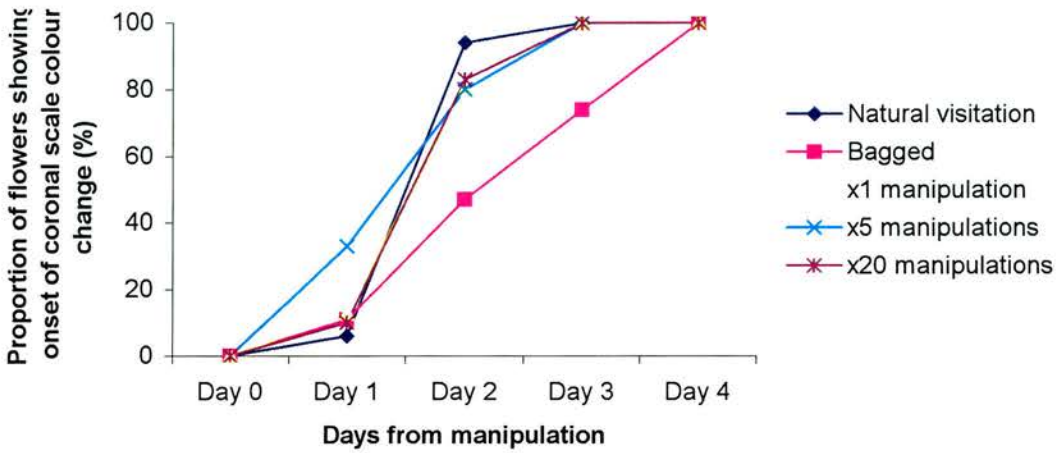


Figure 3.21. Onset of colour change in *M. sylvatica* following bee-tongue manipulation (May 2001). All treatments were carried out on the first morning of flower opening.

iii) Field manipulations 2002.

The repetition of the bee-tongue manipulations produced a similar pattern of results to those of 2001 (Figure 3.22), although the time taken for colour change to occur (in all groups) was slightly extended. This was possibly linked to the warmer, drier conditions of the earlier year (see section 3.3.1.i); as floral longevity was greater in 2002, the durations of the colour phases of the coronal scales might also be expected to be greater.

Three days after treatment groups were set up, flowers that were open to natural visitation (n = 40) and those that had received x5 manipulations (n = 20) exhibited the greatest proportions of flowers with colour-changed coronal scales (95%). Eighty-five per cent of flowers that had received x20 manipulations (n = 20) had commenced colour change, while the figures for single manipulations (n = 20) and bagged flowers (n = 58) were 68 and 66%, respectively. All flowers had commenced colour change in the x20 manipulation, x5 manipulation and natural visitation groups by day 4 and by day 5 this stage had also been reached in flowers in the bagged and x1 manipulation group.

Contingency table analysis was applied to the proportions of changed flowers in all treatment groups on day 3. The proportions of flowers where coronal scale colour change had commenced were not equal, $\chi^2_{0.05,4} = 10.774$. Post-hoc tests showed there was a significant difference between ranked sample proportions between bagged flowers and those that received natural visitation, and also between bagged flowers and the group receiving 5 manipulations ($p<0.05$).

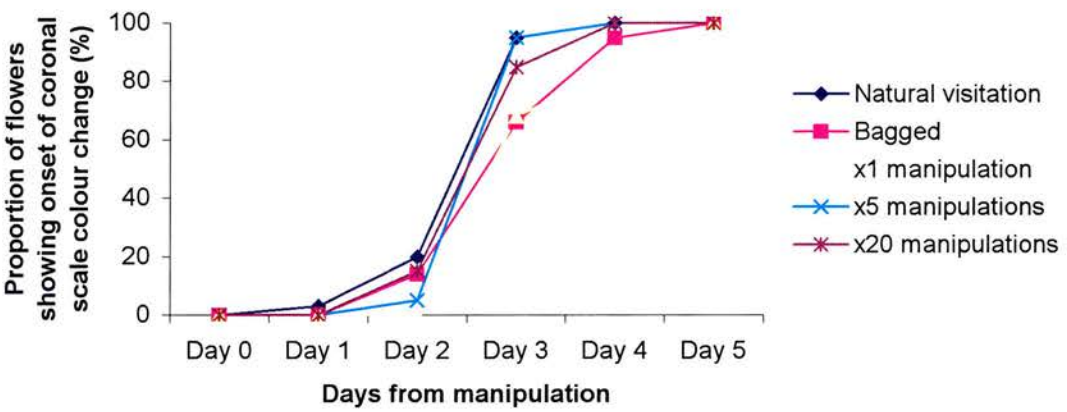


Figure 3.22 Onset of colour change in *M. sylvatica* following bee-tongue manipulation (May 2002).

As in 2001, natural visitation led to faster rates of change than that observed in bagged flowers, or in most of the flowers that had received mimicked visitation. However, in this year's trials faster rate of change was also evident in flowers that had received 5 manipulations compared to bagged flowers.

Particularly rapid rate of colour change was observed in flowers where manipulations were carried out with a nylon brush filament (Figure 3.23). After 2 days, 81% of flowers (n = 58) had commenced colour change compared to 14% and 20% of flowers in bagged and open groups, respectively. All manipulated flowers showed the onset of colour change after 3 days.

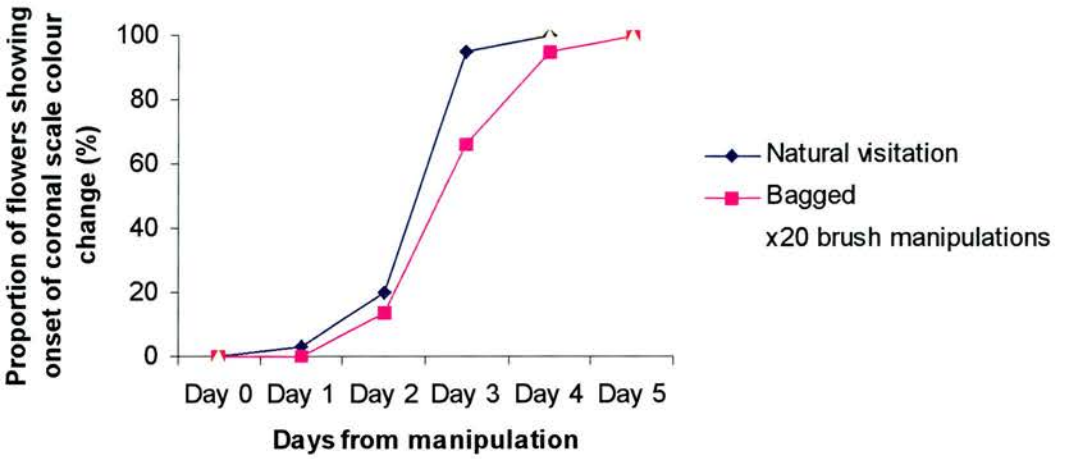


Figure 3.23 Onset of colour change in *M. sylvatica* following manipulation with a nylon filament (May 2002).

Contingency table analysis was applied to the ranked proportions of changed flowers in all treatment groups on day 2 and day 3 (Table 3.7).

Treatment group	No. of days after manipulation					
	2			3		
	Natural visitation	Bagged	Brush	Natural visitation	Bagged	Brush
Natural visitation	-	ns	*	-	*	ns
Bagged	ns	-	*	*	-	*
Brush	*	*	-	ns	*	-

Table 3.7 Differences in the ranked proportions of number of flowers where onset of colour change of coronal scales had commenced according to treatment group. * = significant difference between pairs ($p<0.05$).

Therefore, different types of artificial manipulation produced altered rates of coronal scale colour change in *M. sylvatica*. 'Brush' manipulations produced a hastened onset of change compared to bagged flowers on both days 2 and 3 and this was also the case in comparison with naturally visited flowers on day 2 but not day 3; increased natural visitation on day 3 might explain this pattern. Mimicked visitation with a nylon filament led to even more rapid change than in

those flowers receiving natural levels of insect visitation or where visitation had been simulated with bees' tongues (see Figures 3.21 and 3.22).

3.3.5 Seed set.

Seed set in 2001 occurred in both open and bagged groups. Individual flowers produced up to 4 seeds (typical of Boraginaceae); all flowers in the open group (n = 17) produced seeds (mean 2.9 ± 0.2 seeds per flower; 69.4% seed set) and all but one flower in the bagged group (n = 19) set seed (mean 2.0 ± 0.2 seeds per flower; 51.3% seed set). The median number of seeds per flower was significantly greater in open flowers (3) than in bagged flowers (2) (Mann-Whitney W = 396.5, p = 0.007).

Seed set in 2002 again took place in all treatment groups, although sample size was reduced in some groups due to seed predation. Seed set in open flowers did not vary according to position on cyme. Marked flowers (n = 41) produced a mean of 2.5 ± 0.2 seeds (62.2% seed set) and those ten positions lower (n = 46) on the same cyme produced a mean of 2.3 ± 0.2 seeds (59.9% seed set) (Mann-Whitney test, using medians, W = 1853.0, p = 0.6702). Overall mean number of seeds per flower in the open group (n = 87 flowers) was 2.4 ± 0.1 (60.6% seed set). Of these 87 flowers 7 failed to set seed at all. In bagged flowers (n = 55) seed set was 38.6% with a mean seed production per flower of 1.5 ± 0.2 seeds; 14 flowers produced no seeds.

Seeds were produced at similar levels in all manipulated groups. Where a single bee-tongue manipulation was carried out, the overall seed set was 31.2% (n = 12 flowers) with a mean number of 1.3 ± 0.3 seeds per flower. For flowers

receiving 20 manipulations (n = 18) the respective figures were 31.9% and 1.3 ± 0.2. Thus there was no difference in median seed production between different levels of bee-tongue manipulation (Mann-Whitney W = 186.0, p = 1.0; 6 of 30 flowers did not set seed). Flowers that received manipulations with nylon brush filaments set a mean of 1.2 ± 0.1 seeds per flower (n = 55 flowers) and overall seed set was 30.4%; 14 flowers did not set seed.

Colour change of the coronal scales ultimately occurred in all flowers of all treatment groups in these seed set trials; however, 41 of 227 flowers (18.1%) from all groups failed to set seed despite undergoing such change. Coronal scale colour change was not, therefore, indicative of the latter stages of successful pollination (i.e. fertilisation and/or seed production).

3.4 Discussion.

Colour change from yellow to white of the coronal scales in *M. sylvatica* is accurately determined for the first time in this study. The change occurs in the absence of visitation but is not simply related to the age of the flower; natural visitation and a range of artificial manipulations can hasten the onset of colour change. My study suggests that some event that is post-pollination but prior to fertilisation triggers commencement of floral colour change; deposition of pollen (either through visitor activity at the flower or via other mechanical perturbation) and pollen-pistil interactions that follow this episode are therefore the most likely precursors of this change. The change of colour of the whole flower is age-related and does not influence visitor behaviour.

A range of hoverflies, including the main visitor *Rhingia campestris*, preferentially visited the younger, yellow-centred flowers. This insect also spent longer feeding at these young flowers, and this ties in with the finding that greater nectar reward is available in yellow-centred flowers. The extension of the attractive 'yellow' phase in longer-lived unvisited flowers therefore illustrates an ecological utility to colour change in this species; the foraging bias of the key visitors ensures ongoing visitation to yellow-centred flowers that, concomitantly, have increased probability of successful pollination.

3.4.1 Longevity and colour change.

Data from two field seasons showed that where visitors were excluded floral life was extended for an additional 1 to 3 days (Figure 3.8); early wilting

and/or abscission (that might otherwise follow pollination) did not occur, and the overall visual display was retained for a longer period. The initial pink to blue colour change of petals was not influenced by bagging; the pink phase was purely age-related and the extended period of longevity incorporated a longer blue phase (Figure 3.9). Colour change in this direction is found in several forget-me-not species (Clapham et al. 1962) and in other members of the family Boraginaceae. Data were not collected on visitation to pink flowers; all pink flowers had yellow coronal scales and visitors were noted foraging at both pink and blue flowers indiscriminately

Colour change of the coronal scales from yellow to white, as found in my study, has been recorded in two further species of Boraginaceae, *Cryptantha humilis* (Caspar & La Pine, 1984), and *Myosotis colensoi* (Robertson & Lloyd, 1993). The coronal scales of *M. sylvatica* (which appear to act as the salient close-range foraging cue (see below)) exhibited an important difference according to treatment; the yellow- and intermediate-centre colour phases were of greater duration in bagged flowers when compared to their 'open' counterparts (Figure 3.10).

The duration of the white-centred phase remains unchanged (Figure 3.10) between treatments, and whether the retention of the older white-centred flowers has a role in maintaining visitor attraction remains equivocal. Whilst these flowers can be part of a 'landing platform' of up to 7 closely grouped flowers at the tip of the cyme, they frequently become isolated further down the cyme as new growth occurs. Insect visitors rarely land at these individual flowers, preferring to land at the upper groups of flowers and forage at the yellow- and intermediate-centred flowers within these upper clusters. Further work, involving

the manipulation of patches (and the distribution and availability of white-centred flowers within patches) may resolve whether floral retention has a utility to either plant or visitor. In other species where colour change of part of the flower takes place, findings suggest that long distance floral attraction is enhanced by such retention. Gori (1989) recorded that visitation was reduced to *Lupinus argenteus* if the flowers where change of the banner petal spot colour (see Chapter 8) had taken place were removed.

3.4.2 Visitation and reward.

Yellow- and intermediate-centred flowers receive disproportionately high levels of visitation by hoverflies (Figures 3.14 to 3.18), and this presumably relates to the visual acuity of these insects (see 3.4.3) in combination with a learned association of increased nectar reward within the pre-change colour phase (Tables 3.3 to 3.5). Although very few feeding visits were observed at white-centred flowers, in *Rhingia campestris* these bouts were 2-3 times shorter than those recorded at yellow- and intermediate-centred flowers (Figure 3.16). This most likely reflects the absence of nectar in these flowers (Table 3.5), although the possibility that nectar is still present but has become extremely concentrated (or, perhaps, crystalline) and, therefore, difficult to extract is a further consideration. Correspondence of increased reward with pre-change flowers is widespread in colour-changing taxa (see, for example, Weiss, 1991 and Table 1.1 and Chapters 4 and 7, this thesis).

Patterns of reward availability have been shown to be similar in other Boraginaceae that exhibit coronal scale colour change. In *Cryptantha humilis*,

nectar was rarely available in white-centred flowers (Caspar & La Pine, 1984) and pollen was found in reduced quantity in post-change flowers of *Myosotis colensoi* (Robertson & Lloyd, 1993). In my study, different methods of assessing pollen quantity produced different results (Table 3.2 and Figure 3.19); yellow-centred flowers contained more pollen than white-centred ones through visual inspection in 2000, whereas actual counts produced the opposite result in 2002. The 2000 results are probably more accurate. Re-inspection of photographs from 2000 confirmed that the visual inspection of anthers had correctly assessed availability of pollen but the counts made in 2002 were almost certainly affected by changes in the pollen with time. Pollen in yellow- and intermediate centred flowers was sticky and clumped, and the methodology used probably did not disperse the pollen within the ethanol, leading to the low figures recorded in comparison to white-centred flowers that contained dry and discrete pollen grains.

3.4.3 Hoverfly foraging.

The proboscis extension reflex (PER) of the drone fly (*Eristalis tenax*) has been shown to be elicited in response to reflected light of a very narrow wavelength in the yellow part of the spectrum (Lunau & Wacht, 1994) and is related to perceived food sources (Dinkel & Lunau, 2001). The latter study reported that these flies (when presented with artificial flower stimuli) followed nectar guides of various colours towards a hidden, central yellow disc; the PER was observed both at this location and towards the nectar guides if coloured yellow, but not in response to other colours. Although detailed information on

the visual capabilities of *Rhingia campestris* is not available, in my study these flies showed the PER almost exclusively in response to yellow- and intermediate-centred flowers (97% of all flowers visited; Figure 3.14). Feeding was not attempted at most white-centred flowers encountered (90% of white-centred flowers inspected did not elicit the PER, Figure 3.15) and, where this was observed, it is possible that residual yellow pollen was visible within the corolla tube. Similar patterns recorded for other hoverflies (Figures 3.17 and 3.18) indicate that the same visual cues are interspecific, and suggest that the hoverfly PER is commonly related to yellow wavelength perception.

Other dipterans respond to identical, extremely localised colour change cues in the same way. Ninety per cent of all visits, chiefly by empid and bombyliid flies, were recorded to yellow-centred flowers of *Cryptantha humilis* (Caspar & La Pine, 1984). Tachinid flies were the main visitors to *Myosotis colensoi* and were again rarely recorded at post-change, white-centred flowers; just 16% of all visits were made to this colour phase despite white-centred flowers accounting for over 50% of flowers within the population (Robertson & Lloyd, 1993).

Clearly yellow is not the only visual cue used by hoverflies, although tests with naïve insects have demonstrated an innate preference for this colour (Ilse, 1949; Lunau & Maier, 1995). At my field site *R. campestris* was observed feeding at pink flowers of *Silene dioica* and the reddish flowers of *Geum rivale*. The same species has been noted to feed at the purplish-blue flowers of *Glechoma hederacea* (Gilbert, 1981). Two other hoverflies, *Episyrphus balteatus* and *Syrphus ribesi*, exploited flowers of various colours according to Goulson & Wright (1998). Nor is colour the only important cue of course; the role of scent

attractants from flowers in general, and from pollen in particular, also requires determination in relation to foraging choice and patterns in hoverflies.

3.4.4 Manipulations, pollen deposition and effects on rate of colour change.

The general pattern found with all types and levels of manipulation was a more rapid onset of colour change in *M. sylvatica* and this confirms, together with retardation of colour change in bagged flowers, that the process is not simply related to the age of the flower. A handling effect, as opposed to a post-pollination effect, may be ruled out as a trigger for colour change; bagged flowers were neither visited by legitimate potential pollinators nor touched by me (or contacted by the exclusion netting). These flowers were, however, subject to mechanical perturbation by wind action (as in 'open' conditions where actual visitation may or may not occur) which could influence the movement of pollen within the flower.

The position of the anthers in relation to the stigma in *Myosotis* make some 'internal dispersion' of pollen inevitable (see Figure 3.11) and deposition on the stigma likely; as *M. sylvatica* is self-compatible this type of process must underlie the successful fertilisation and subsequent seed set found in bagged flowers. This type of action has been noted in certain flowers as a result of insect movement that does not necessarily deposit cross-pollen and yet induces self-pollination; for example, Pazy (1984) described this action in relation to the more complex flowers of two lupin species. In my study, *M. sylvatica*, displaying a more simple morphology than the latter flowers (but less likely to be pollinated

by the more simple 'mess and spoil' activities seen in composite- and umbelliferous-type flowers), benefits from such accidental pollen dispersal.

However, deposition is not guaranteed and the temporal variation in onset of colour change in bagged flowers (up to 5 days; Figures 3.21 and 3.22) may be explained by the random nature of pollen movement within the flower. Flowers would be subject to differential perturbation over time and, therefore, a uniform number of pollen grains would not land on every receptive stigma. If pollen deposition and/or subsequent pollen-pistil interactions triggered colour change, then either a threshold of number of grains could be required to initiate the process, or an additive effect could be important. However, the small size of the flower, and the restricted access to the corolla tube, prevents experimentation that incorporates varying the level of pollen deposition to determine the effects on colour change.

If it is assumed that by mimicking visitation through manipulation pollen is more likely to be deposited then a link between deposition and colour change can be made. Increased levels of visitation or manipulation should lead to earlier onset of colour change. This appeared to be the case when such trials were compared to bagged flowers. In both years, flowers open to natural visitation exhibited significantly faster onset of colour change in comparison to bagged flowers, but not to manipulated flowers; rate of change showed little variance between bagged and manipulated flowers. Although all levels of bee-tongue manipulation clearly seemed to hasten colour change (figures 3.21 and 3.22), statistical significance between manipulated and bagged flowers was only found in 2002 for the x5 manipulation group. This indicates that the 'quality' of visit or

manipulation may be the critical factor; only in this latter group were the simulated visits of similar quality to natural visitation (see below).

Open flowers might receive a single natural visit of sufficient quality (in terms of pollen deposition or removal) to effect colour change. In the field study, at peak visitation, the focal patch received fewer than 5 visits per 100 flowers during a 30-minute period (Figure 3.13), and it is therefore highly unlikely that any individual flower would be visited more than once a day, particularly given the absence of any visitors in early morning and late afternoon. Although flowers receiving a single mimicked visit, or increasing numbers of mimicked visits, did change colour, the rate of change was not significantly different to either bagged or open flowers (other than the single example above). Flowers of all groups were presumably equally likely to experience accidental deposition of self-pollen subsequent to the start of the trials, so a qualitative facet of natural visitation must have been responsible for the more rapid colour change that was recorded. Possibilities include pollen removal, pollen deposition and/or subsequent pollen-pistil interactions, and differences between the mechanical influence of a live insect visit and that of my attempts at manipulation.

Pollen removal, although evident both during natural and simulated visitation, is a doubtful trigger for colour change since maternal fitness could be severely compromised if all, or most, pollen could be removed without effecting pollination. As a colour change usually directs visitors towards the viable and most rewarding flowers (Weiss, 1991), in *M. sylvatica* such a trigger would be detrimental to overall plant fitness. Flowers that had changed colour following pollen removal (but without being pollinated) would not receive further visits and thus be less likely to receive any more pollen. Additionally, bagged flowers

changed colour without pollen removal, other than possible dislodgement within the corolla tube.

Pollen deposition and possible cumulative effects of increasing quantity of pollen deposited represent the most probable trigger for colour change, and this would be affected by both quantity and quality of visitation. Inevitably, self-pollen is dislodged through any type of visit, manipulation or wind action and could lead to self-pollination. Increasing number of visits or manipulations could add further self-pollen and/or introduce cross-pollen and accelerate pollination processes.

If a pollen-pistil interaction were a precursor to colour change, such a cumulative effect of pollen deposition would explain the patterns reported here. Pollen-pistil interactions can lead to internal changes in plants within 4 hours of the initial episode (e.g. processes in the ovaries (Deurenberg, 1976)). Further investigation is needed to establish whether variation in the amount of pollen deposited can influence the rate at which processes such as colour changes take place. In my study the 'brush' manipulations caused a more rapid colour change than any level of bee-tongue manipulation and actual deposition following these treatments could be compared to deposition following natural visitation. Differing amounts of pollen could be deposited according to the different type of 'probe' attempted and variation in the pollen load on the stigma could therefore result in the rates of colour change recorded.

Because the brush filament was cleaned after every insertion, transfer of cross-pollen could not have taken place. Deposition of cross-pollen has often been implicated in colour change (e.g. *Lupinus propinquus* (Weiss, 1992); *Alkanna orientalis*, see Chapter 4 of this thesis). However, the use of such a

relatively 'clumsy' probing instrument as the nylon filament could have led to a gross dislodgement of self-pollen onto the stigma (compared to the less robust bees' tongues) and this could, therefore, have accounted for accelerated colour change in this group. Hence, if the differences in the observed rates of colour change are related to variations in levels of pollen deposition, the cause of such differences may lie purely in my attempts at simulating visitation/probing of flowers with implements very different to the flies' proboscides that usually effect pollination. Insects could be guiding their mouthparts in a particularly subtle manner (dictated by the nature of the entrance to the corolla tube) that was not replicated by my actions.

Hence the type of implement used for probing could affect the rate of colour change; the most rapid onset of colour change being recorded when manipulations were effected with a nylon brush filament (Figure 3.23). Clearly, this manipulation produced a quantitatively different response; but it could also be qualitatively different, since the filament was neither as flexible nor as delicate as a bee's tongue. One possibility is that the 'harsher' surfaces and more rigid texture of the filament could have damaged floral parts within the corolla tube and instigated ethylene production as a plant wound response; this type of potential effect is more fully discussed in Chapter 9.

3.4.5 Further considerations.

Insect visits to *Myosotis sylvatica* could be regulated by a cue not yet discussed that encourages better dispersal of pollen both within and between flowers. Flower opening or anther dehiscence, both of which could be

accompanied by odour cues as well as offering a visual signal, could prompt actual visitation to individual flowers at slightly different times to those when my manipulations were carried out on the afternoon of opening. During dissection of flowers for visual inspection of pollen availability, one or more anthers on some flowers had not dehisced and pollen on newly dehisced anthers was sticky and clumped in comparison to older flowers. My manipulations could have included flowers in this state and, therefore, compromised dislodgement of self-pollen. Additionally, if an odour advertisement cue does operate in this species, visitation could occur more frequently after dehiscence of the final anther when the attractant cue is most intense, pollen has begun to lose its adhesive qualities, and greater dislodgement of pollen is likely to occur. In *Myosotis colensoi*, Robertson & Lloyd (1993) reported sequential dehiscence of the five anthers over approximately 10 hours following an initial 1 to 2 hour protogynous stage. Although in that species the stigma remains receptive and pollen viable for several days, most flowers were visited (and over 50% pollinated) prior to anther dehiscence. This suggests that a pollen odour cue is absent for *M.colensoi*, but a change in whole flower odour that coincides with scale colour change cannot be ruled out. Qualitative change in the floral volatile profile of *Cryptantha humilis*, a species of Boraginaceae that undergoes an identical colour change, has been reported between colour phases (Caspar & La Pine, 1984) and could also apply to both *M. colensoi* and *M. sylvatica*.

A number of confounding variables, mainly associated with the small size and 'difficult' spatial morphology of the flower, make unequivocal resolution of the trigger for colour change in *Myosotis sylvatica* problematical. Further observation of natural visitation, together with analysis of pollen deposition and

subsequent reproductive processes in individual flowers (having received varying levels of visitation by specific insects or different types and levels of manipulation), is required.

My study does, however, show that both plant and visitor benefit from colour change. Foraging insects are guided to rewarding flowers, and the plant gains additional reproductive success from natural visitation to yellow-centred flowers. Self-pollination through any perturbation of small and delicate flowers represents a 'fail-safe' mechanism for these plants; pollinators are limited and seemingly subject to fluctuation, but when they do visit they offer an 'improved service' in terms of greater seed set and, where cross-pollen is introduced, perceived fitness benefits.

Chapter 4 – *Alkanna orientalis.*

4.1 Introduction.

Alkanna orientalis (L.) Boiss. (Boraginaceae) is a perennial plant that is widely distributed in the eastern Mediterranean and Middle East and is locally abundant at higher elevations. In southern Sinai *A. orientalis* is found at medium elevations (1500-2400m) and is relatively common throughout the region, growing on rocky ground at the bottom, and along the steep sides, of wadis (dry river valleys) (see Figure 4.2 for typical habitat).

At the sites used, the plant was virtually the only species in flower for the duration of the study and was present in patches of up to $2m^2$ throughout the locations described below. Flowering occurs from late February to May; flowers are initially bright yellow (changing to pale yellow/whitish prior to abscission), borne on scorpioid cymes, and they remain on the plant for several days. The retention of pale flowers, and hence the total number of flowers on an inflorescence, have been found to vary between populations in this area (Gilbert et al. 1996). The flowers are protandrous, hermaphroditic and contain up to 6μl of nectar per flower that collects at the base of the corolla tube as a floral reward (Gilbert et al. 1996). Pollen is borne on five anthers, three of which are clustered just beneath the corolla tube entrance, with the remaining pair lower down within the corolla tube (Gilbert et al. 1996). *A. orientalis* is an obligate outcrosser (Semida, 1994; Gilbert et al. 1996); fruit set occurs within three weeks of floral abscission and up to four nutlets are produced.

The main visitor in the early part of the flowering season is the solitary bee *Anthophora pauperata* Walker 1871 (Hymenoptera: Anthophoridae) and the

close relationship between this bee and *A. orientalis* has been the subject of several studies. This association was described by Semida (1994), and the territoriality of male bees, and foraging behaviour of females, were established by Willmer et al. (1994).

Activity patterns varied diurnally between the sexes. Male bees patrolled territories that included patches of *Alkanna* throughout the morning and early afternoon, feeding occasionally on nectar but spending most time defending clumps of *Alkanna* from visitation by all other insects (Willmer et al. 1994). In contrast, although females foraged at the same plants, they visited large numbers of flowers on each plant for nectar (across the same time period as male visitation but with fewer visits during late morning/midday) and/or pollen. Pollen, which was gathered by buzz pollination (a low amplitude vibration of the flight muscles that dislodges pollen), was collected almost exclusively before 10.00 and after 13.30 (Willmer et al. 1994). The male behaviour ensured protection of resources for resident females and potential fitness advantages for territorial males (Willmer et al. 1994). Activity patterns of female bees were found to be closely linked to diurnal trends of pollen dehiscence (Stone et al. 1999).

Further work determined variation of plant characters between populations. Gilbert et al. (1996) reported differences between sub-populations from neighbouring wadis; larger flowers, with greater nectar reward and wider corollas, were retained for longer on plants in one population. This altered the overall floral display and was reported to be of selective advantage to plants where pollinator visits were infrequent. More recently, Wolff et al. (1997)

confirmed significant genetic differentiation between these same sub-populations through the use of RAPD analysis.

This plant was chosen to enable an extended field season in 2002 in a region of considerably different climate and topography to other study sites. The early flowering period (see above) ensured that field work could be carried out on an additional species of Boraginaceae that is known to change colour, and at a time when no flowering plants were naturally available for study in the UK. The small flowers of *Alkanna* are morphologically similar to those of *Myosotis sylvatica* (Chapter 3) and the direction of colour change from yellow to white is shared, although the type of colour change is different; in *Alkanna* the whole flower changes as opposed to a localised region. This direction of colour change is also different to the pink to purple-blue changes of whole flowers documented for Boraginaceae species elsewhere in this thesis (Chapters 5 and 6).

4.2 Materials and Methods.

4.2.1 Study site.

Fieldwork was carried out in the vicinity of the University of Suez Canal Field Station, St. Katherine, Sinai, Egypt between 20th March and 24th April 2002. The village of St. Katherine (approximately 28° 34′ N) lies to the south west of the Plain of El-Raha at an elevation of approximately 1600m; numerous steep-sided wadis encircle the plain, separated by mountain ridges of up to 2400m. Data were collected at four sites (Figure 4.1, see legend).

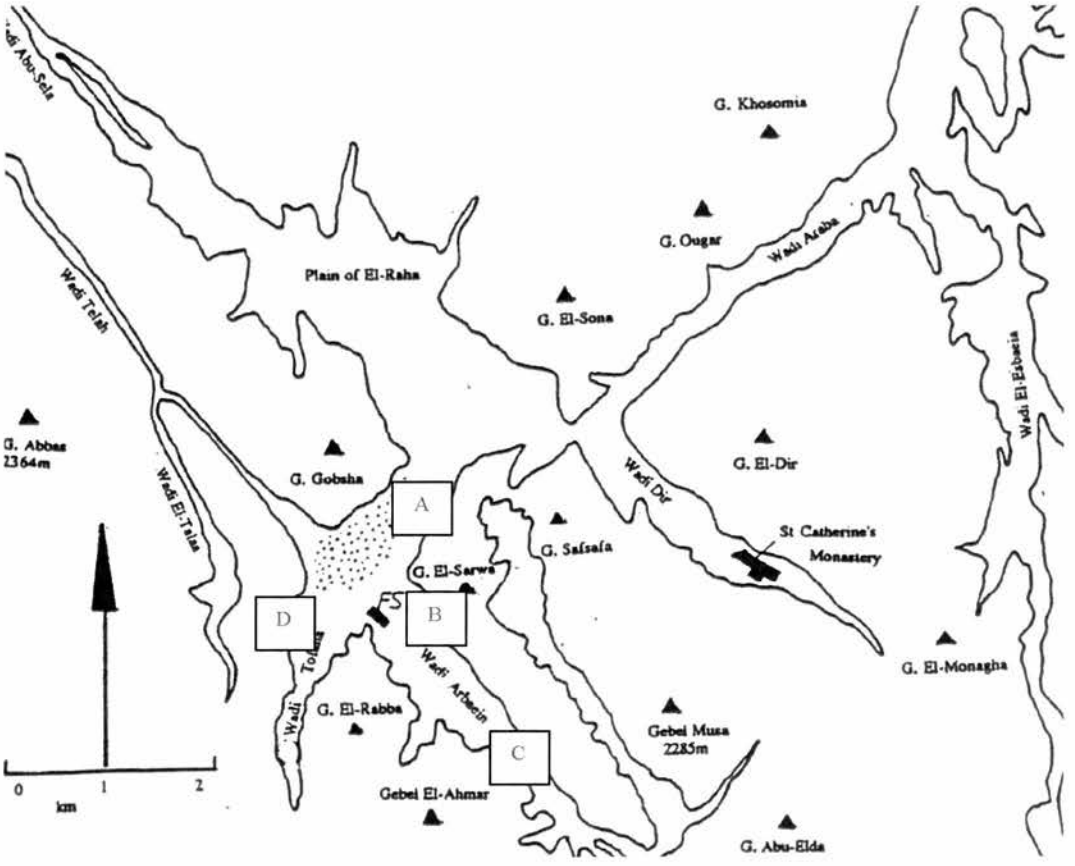


Figure 4.1 Map of study site. A = plain (extent of village of St. Katherine stippled); B = field station; C = Wadi Arbaein; D = Wadi Tofaha (modified from Gilbert et al. 1996 with permission of P. Willmer).

The chosen sites were the plain at the edge of the village (A); in and around the field station at the mouth of Wadi Arbaein (B) (Figure 4.2); approximately halfway (2km) along Wadi Arbaein (C); and at the mouth of Wadi Tofaha (D). Choice of sites was made on the basis of absence of potential disturbance of experimental trials; on most days herds of goats were brought through the area of study and these animals grazed on *Alkanna* as well as removing pollination bags.

Figure 4.2 Field site at mouth of Wadi Arbaein.

4.2.2 Floral longevity and colour change.

Five flowers on each of ten plants (and on separate cymes) were marked as buds one day prior to opening and checked daily through to abscission; thus floral longevity and rate of colour change under conditions of normal visitation were assessed. Colour was measured by assigning flowers to one of three

categories (Figure 4.3); bright yellow (as seen in newly opened flowers); intermediate (where the onset of colour change was determined against the next day's newly-opened flower); or pale yellow (clearly faded flowers that often appeared cream-white and could lack any yellow coloration).

Concurrently, five flowers on each of ten plants (and on separate cymes) were marked as buds one day prior to opening and bagged with fine nylon netting to prevent insect visitation for the duration of the flowers' life. These flowers were checked as above to enable comparison with the first group.

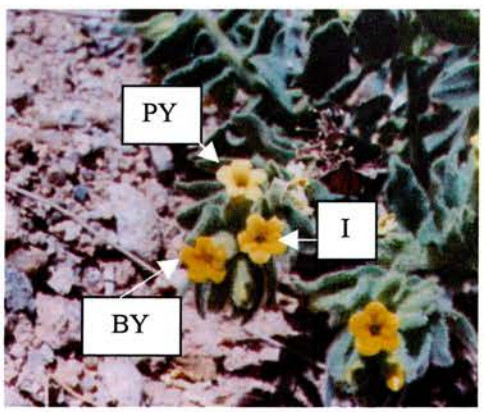


Figure 4.3 Colour phases in *A. orientalis*. BY = bright yellow, I = intermediate, PY = pale yellow.

4.2.3 Effects of manipulation on floral longevity and colour change.

Possible effects of different aspects of flower handling, together with artificial cross-pollination, were examined to see how visiting insects might trigger floral colour change. The bee *Anthophora pauperata* was virtually the only visitor to the plants throughout the study (a single visit by a nymphalid butterfly was observed, and bee flies (*Exoprosopa* sp.) were seen on a few occasions), and the nectar visits by this visitor were mimicked by hand, both with and without actual nectar withdrawal.

Pollen removal was considered as a further potential trigger but could not be satisfactorily mimicked because this flower is buzz pollinated; female bees would often force open flowers just prior to natural opening to remove pollen by buzzing (Stone et al. 1996). No type of artificial pollen removal could be carried out without producing possible confounding effects of damage to floral tissue in these unopened or partially opened flowers.

Several manipulations were possible however:

a) Hand cross-pollination. Sixty flowers distributed between 16 randomly chosen plants at site C were marked with numbered plastic tags and bagged one day prior to opening. On the day of opening the bags were taken off and cross-pollination attempted by taking open flowers from a nearby plant, inserting a fine artist's paint brush into the corolla tubes to remove pollen, and then inserting the paint brush into the corolla tube of the marked flower.

b) Nectar withdrawal. Forty-two flowers on 14 plants at site B were marked and bagged one day prior to opening. On the morning of opening the bags were taken off and each flower was probed 4 times with a 1µl microcapillary tube to remove any accumulated nectar. Earlier trials had shown that all nectar could be extracted with a maximum of 4 probes.

c) Probing without nectar withdrawal. Forty-two flowers distributed on the same plants as (b) above were probed 4 times with a sealed microcapillary (one end was held in a flame and then allowed to cool before use). Probing was thus effected without nectar withdrawal.

d) Repeated nectar withdrawal. Twenty-eight flowers on 11 plants at site B were marked and bagged one day prior to opening. The following morning at 07.00 the bags were taken off and each flower was probed with a 1µl

microcapillary tube until all nectar had been removed. The process was repeated at 11.00 and 15.00.

e) Repeated probing without nectar withdrawal. As (d) above but with a sealed microcapillary tube. On each plant the mean number of probes taken to remove all nectar from an individual flower at each treatment time (as in group (d)) was calculated and this figure used for the number of probes with the sealed microcapillary tube.

All groups were checked daily as in 4.2.2 above. Individual flowers were on separate, randomly chosen cymes in all treatment groups and exclusion bags were replaced after each treatment/data collection to prevent subsequent visitation.

4.2.4 Nectar.

Nectar volume and concentration were measured (using standard techniques detailed in Chapter 2) by removing each flower from its calyx and withdrawing nectar from the base of the corolla tube. Data were collected from five flowers of each colour category, from separate cymes on each of four plants, on three days at 06.00-08.00, 11.00-13.00 and 16.00-18.00.

4.2.5 Pollen.

Availability of pollen according to colour category was scored by visual inspection of the same 20 flowers used in collection of nectar data in 4.2.4. The methodology used by Stone et al. (1999) was modified to assess the depletion of

pollen in an individual flower, each of which was pulled gently apart and examined under a hand-held magnifying glass (x10). For each anther, pollen depletion was scored on a four point scale: 0 = no depletion; 1 = up to 50% depletion; 2 = 50-100% depletion; 3 = 100% depletion. *A. orientalis* has three anthers visible near the entrance of the corolla tube and two within the corolla; these 'sets' of anthers were estimated separately and the respective means added to give a total depletion score. Each flower could therefore have a potential maximum score of 6. The resultant scores were calculated as a proportion of maximum depletion and, as they are unsuitable for statistical testing, are displayed graphically.

4.2.6 Visitation.

Visitation by *A. pauperata* was observed over six days to assess whether foraging behaviour was influenced by change of floral colour. A visit was scored as a landing that incorporated a feeding attempt; either the insertion of the proboscis into the corolla tube to remove nectar or the distinctive noise produced by the female bees when carrying out buzz pollination. Patches were single *Alkanna* plants and were matched within trials for approximate area.

a) Colour choice on 'normal' patches. A different patch at site C was chosen at random on each of three days and the number of flowers of each colour category within the patch was noted. Visits by all foraging *A. pauperata* to a single patch of *A. orientalis*, together with the flower colours visited, were recorded for fifteen minutes within each hour between 08.00 and 16.00. The

site used became shaded shortly after 15.00 and, following a rapid fall in temperature, bee activity would cease at around 16.00.

b) Foraging at manipulated patches. On each of three days at different sites (A, B and D), two patches of *A. orientalis* were chosen that were of similar size and, additionally, was the same distance from their nearest neighbouring conspecific. On one patch all intermediate and pale yellow category flowers were removed, and on the other all bright yellow and intermediate category flowers removed, thus creating a patch where all flowers were bright yellow and a patch where all flowers were pale yellow/white. The patches were then balanced for flower number. Total residence time (in seconds) of foraging *A. pauperata* was recorded, together with number of flowers visited, for 8 fifteen-minute focal observation sessions of each 'colour' patch.

4.2.7 Seed set.

To confirm the status of *A. orientalis* as an obligate outcrosser seed set was assessed under conditions of natural visitation, where no visitation was allowed and following hand cross-pollination.

In these respective groups individual flowers were marked and either left open to visitors, bagged to prevent visitation or cross-pollinated by hand (as 4.2.3(a) above). After three weeks the marked calyces were removed and the number of nutlets counted. Where potential self-pollination (in bagged flowers) or hand cross-pollination was being assessed, the seed set of the flower four positions proximal to the base of the cyme from the marked flower was counted as an additional control.

To test whether floral colour change might simply be caused by successful cross-pollination, 100 pale flowers distributed between 16 plants (and on separate cymes) were marked at random; all should contain seeds if it is seed set that triggers colour change.

4.2.8 Stigmatic receptivity.

The receptivity of stigmas of varying ages was tested to examine possible correlation between floral colour and sexual phases. Thirty flowers in each of five age cohorts (modified from those identified by Stone et al. 1999) were used:

1 = unopened buds (bud)

2 = partly-opened flowers (male)

3 = fully opened, first-day, bright yellow flowers (female 1)

4 = fully opened, second-day flowers where colour change had begun to occur (female 2)

5 = three-day-old pale yellow flower (female 3)

Stone et al. (1999) had deemed category 2 flowers to be male phase (as indicated by pollen dehiscence), category 3 female and category 4 (here separated to distinguish between intermediate and pale yellow flowers) as post-reproductive.

Dafni (1992) noted that the presence of esterase (or enzymatic activity) which is associated with stigma receptivity could be detected experimentally by a range of chemical staining tests. Two such tests were employed here.

a) Perextesmo Ko. The methodology used by Dafni & Maues (1998) was followed to test for the presence of esterase activity. A single Perextesmo Ko test paper (Camlab, UK) was soaked in 1ml distilled water and a droplet placed on the distal tip of an excised style. The stigmas were bifid and each branch appeared bi-lobed. The preparation was evaluated under a binocular microscope and scored for intensity of stain on each of the four lobes using the following scale:

1. Light; commencement of light reddish-brown coloration on any part of the lobe surface.
2. Medium; deeper and more extensive staining of similar coloration to 1.
3. Heavy; complete and darker brick-red staining of all surfaces.

Each flower could, therefore, have a maximum score of 12.

b) MTT. The methodology used by Rodriguez-Riano & Dafni (2000) to test for the presence of dehydrogenase in pollen grains was followed. A few droplets of a 1% concentration of MTT (3[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma, UK) in 5% sucrose were placed on the distal tip of the excised style. The preparations were evaluated and scored on a 3-point scale as in (a) above, although staining with this chemical produced a blue-black coloration. (No other chemical stain was available for use at the St. Catherine Field Station as a comparison to Perextesmo Ko. MTT has been used in trials elsewhere to test stigmatic receptivity and produced results that were not entirely satisfactory (T. Rodriguez-Riano, personal communication). Results gained here, although matching those found with Perextesmo Ko (see below), should, therefore, be treated with caution.)

Prepared stigmas of both treatment groups were examined after 1, 5 and 15 minutes from the application of the staining chemical. The resultant scores were calculated as a proportion of maximum staining and a mean proportion calculated for the 30 flowers of each age cohort. As these resultant scores are unsuitable for arithmetical statistical testing they are presented graphically.

4.2.9 Pollen tube growth.

Sample flowers of comparable age but from different treatment groups were collected for analysis of pollen tube growth by fluorescence microscopy to attempt to establish whether colour change in *A. orientalis* might correspond with various stages of reproductive development. Fifty-one buds, on separate cymes and distributed between 10 plants, were marked 1 day prior to opening and enclosed in fine nylon netting to prevent insect visitation. Two days after opening all marked flowers were removed, fixed in 70% ethanol and stored at 4°C. A further 51 flowers that had been open to visitation were also collected; in these flowers the commencement of colour change was evident and they shared the same position (and, therefore, were of similar age) as a bagged counterpart (Figure 4.4).

Figure 4.4 Cyme position of 'open' flowers used for pollen tube growth analysis (see text). Those collected were 2nd/3rd day flowers (centre of photo) and had clearly commenced colour change in comparison to 1st day flowers. The example shown illustrates the full range of colour phases through to almost white.

All samples were stored until prepared for fluorescence microscopy and examined at the laboratories of the Sir Harold Mitchell Building, University of St. Andrews. The protocol used was modified from Gibbs & Bianchi (1999). The carpels were dissected from the remaining floral tissue and placed in 8M NaOH and incubated at 65°C for 12 minutes to soften the stylar tissue. Residue NaOH was washed off with distilled water and the carpel placed in 1% aniline blue for 5 minutes prior to squash preparation on a microscope slide. Slides were examined under a Leitz Laborlux 12 binocular microscope with fluorescent light source and were assessed (x25) for the following features: -

- Pollen grains attached to stigma
- Evidence of pollen tube growth in stigma and style

4.3 Results.

4.3.1 Floral longevity and colour change.

Under conditions of open visitation mean time from opening to floral abscission was 4.3 ± 0.1 days (n = 48 flowers) (Figure 4.5). Abscission occurred between 2 and 7 days after opening. In contrast, mean longevity of bagged flowers was 6.3 ± 0.2 days (n = 43 flowers). In this group abscission was noted from 2 to 8 days from opening. The original sample size of 50 flowers in each treatment group was reduced due to grazing by goats that appeared to select the young tips of vegetation and flowers as forage and also removed a significant number of nets during this and other experiments.

There was a significant difference between the median longevity of open and bagged flowers; Mann-Whitney W = 1368.0, $p<0.0001$ (median days to abscission; open flowers = 4 days, bagged flowers = 7 days).

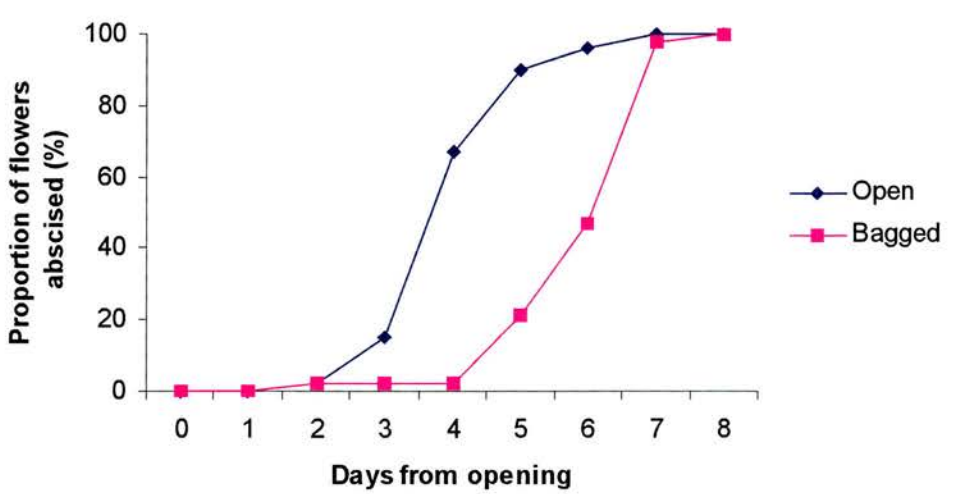


Figure 4.5 Floral longevity in *Alkanna orientalis*.

Onset of colour change and length of floral colour phases were significantly affected by preventing insect visitation. The length of all three floral colour phases was extended in bagged flowers (Figure 4.6 and table 4.1).

Group	n	Length of colour phase (days)					
		Bright yellow		Intermediate		Pale yellow	
		Mean	Median	Mean	Median	Mean	Median
Open	48	2.9 ± 0.1	3	0.9 ± 0.1	1	0.5 ± 0.1	0
Bagged	43	3.8 ± 0.1	4	1.2 ± 0.1	1	1.2 ± 0.1	1
Mann-Whitney	W		1556.0		1958.0		1697.0
	p		<0.0001		0.0284		<0.0001

Table 4.1 Duration of colour phases under different treatments in *A. orientalis*.

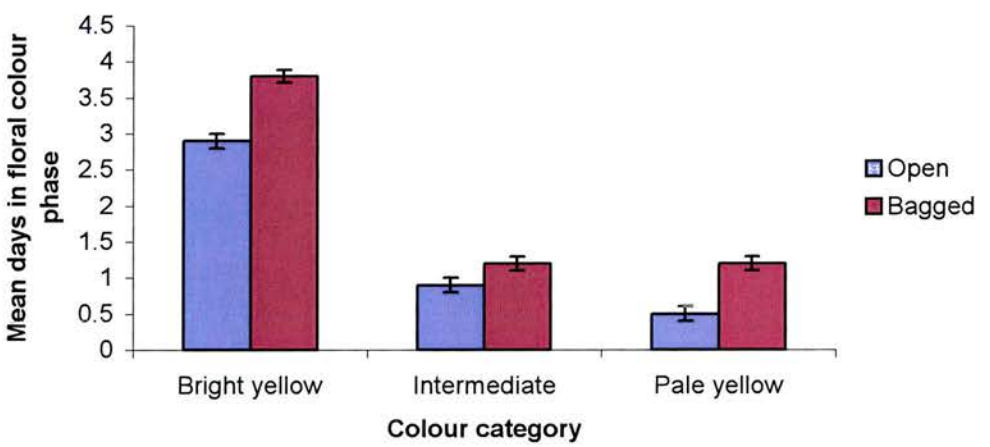


Figure 4.6 Length of floral colour phases in open and bagged flowers in *A. orientalis*.

These results indicated that where visitation was absent there was an extension of each colour phase, and this accounted for greater longevity in bagged flowers. In particular, the bright yellow phase was extended by roughly 1 day and the pale yellow/white flowers were retained for more than double the period seen in open flowers.

4.3.2 Effects of manipulation on floral longevity and colour change.

Floral longevity was clearly affected by certain types of manipulation (Figure 4.7).

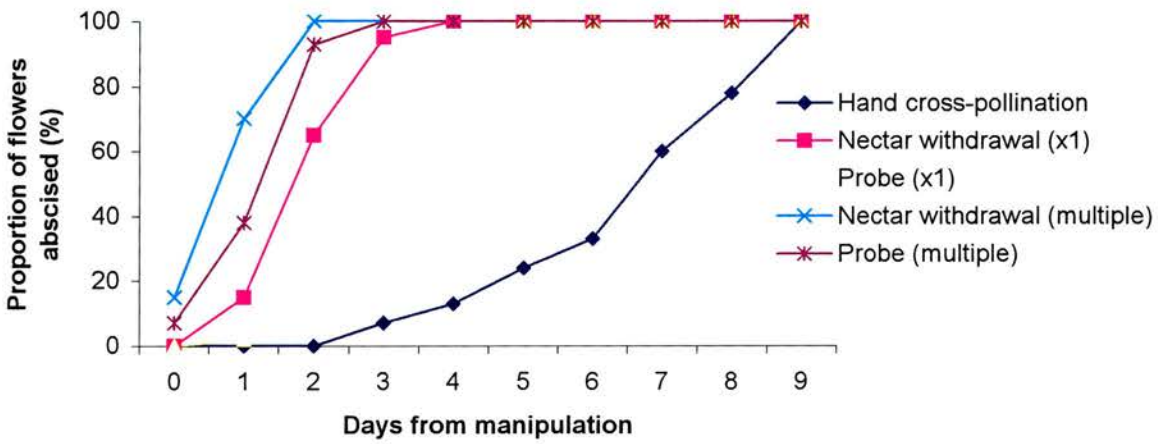


Figure 4.7 Floral longevity of *A. orientalis* flowers after various types of manipulation (see text for details). In multiple withdrawal/probe groups colour change commenced on the day of manipulation i.e. 'Day 0'.

The group of flowers that had been cross-pollinated by hand abscised between 3 and 10 days from opening and displayed a similar pattern to the bagged flowers (see Figure 4.5). Mean longevity was 6.8 ± 0.3 days; given the results below for other manipulated groups and above for open (and, therefore, presumably naturally cross-pollinated) flowers, this result was unexpected. Possible factors contributing to this result are detailed later.

Abscission had occurred in all flowers in all other manipulated groups by the fifth day following manipulation. Flowers receiving a single manipulation had a greater life span than those receiving multiple manipulations. A single withdrawal of nectar resulted in a mean longevity of 2.2 ± 0.1 days per flower and a single probe led to a mean longevity of 2.9 ± 0.1 days. In contrast, mean

longevity following multiple manipulations was 1.2 ± 0.1 days and 1.7 ± 0.2 days for nectar withdrawal and probe treatments respectively. Figure 4.7 illustrates that abscission can take place as early as the day of manipulation in these latter two groups.

Figure 4.8 highlights a considerable shortening of the floral colour phases following a single manipulation when compared to bagged flowers (see Figure 4.6). Similar durations of phases were recorded in bagged and hand cross-pollinated flowers. Cross-pollinated flowers exhibited floral colour phases of mean duration 3.0 ± 0.1 days, 2.3 ± 0.2 days and 1.0 ± 0.2 days for bright, intermediate and pale categories, respectively.

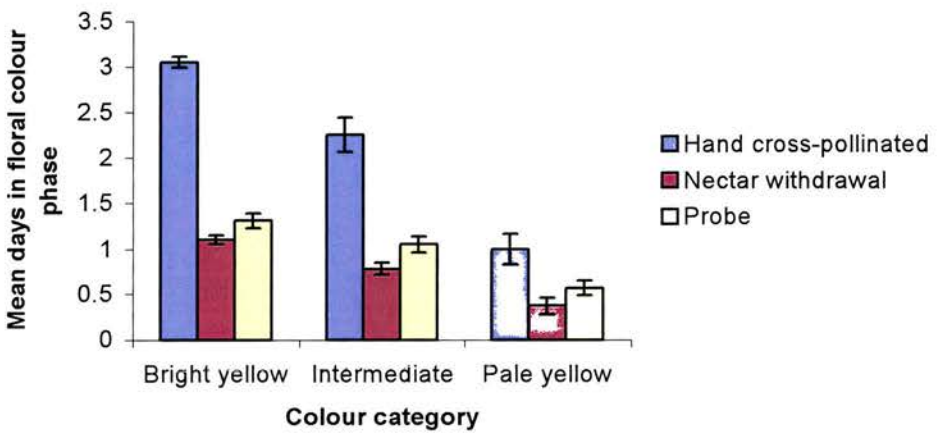


Figure 4.8 Length of floral colour phases in *A. orientalis* following single manipulation (see text for details).

Following nectar withdrawal, mean lengths of colour phases were 1.1 ± 0.1 days (bright yellow), 0.8 ± 0.1 days (intermediate) and 0.4 ± 0.1 days (pale yellow). In flowers receiving a single probe the mean lengths of these phases were 1.3 ± 0.1, 1.0 ± 0.1 and 0.6 ± 0.1 days.

Results for flowers in the multiple manipulation treatments are not displayed graphically because most flowers had changed colour by the final

treatment at 15.00 and all had changed colour by the first morning following manipulation. Fifteen of 29 flowers in the nectar withdrawal group and 17 of 29 probed flowers had changed colour by the final treatment time; two flowers from each group showed onset of colour change by 11.00. A feature noted rarely in earlier treatments – withering of flowers *in situ* prior to abscission – was frequently evident in flowers from both these experimental groups. Hence repeated manipulations led to rapid colour change and early withering and/or abscission of flowers when compared to all other treatments.

4.3.3 Nectar.

Nectar characteristics varied both with time of day (Figure 4.9) and flower colour category (Figure 4.10). Mean nectar volumes increased from 0.07 ± 0.01μl at 06.00-08.00 to 0.17 ± 0.03μl by 11.00-13.00, followed by a fall to 0.09 ± 0.02μl at 16.00-18.00 (n = 180 flowers at all collection times). There was a significant difference in nectar volume over time; Kruskal-Wallis test using medians, $H = 34.94$, $df = 2$, $p<0.001$. Nectar concentration decreased over time; an early morning mean figure of 49.7 ± 2.1% (n = 28) fell to 39.7 ± 1.0% (n = 78) by the middle of the day and further decreased to 36.5 ± 1.0% (n = 40) by the evening; (Kruskal-Wallis; $H = 26.44$, $df = 2$, $p<0.001$).

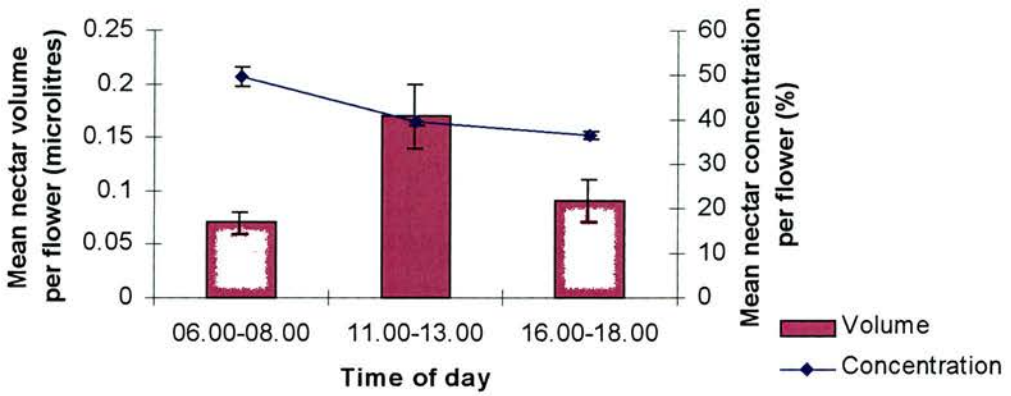


Figure 4.9 Nectar characteristics of *A. orientalis* over time of day; all colour categories summed.

Volume and concentration varied with floral colour category (Figure 4.10) and this, presumably, reflected the change in nectar secretion characteristics of flowers of different age. Bright yellow flowers contained a mean volume of 0.21 ± 0.02µl; intermediate flowers 0.10 ± 0.03µl; and pale yellow flowers 0.03 ± 0.01µl (all data for colour categories were summed over time of day; n = 180 flowers for each group). The respective concentrations for these categories were 39.0 ± 0.9% (n = 93), 40.0 ± 1.4% (n = 43) and 55.1 ± 4.4% (n = 10).

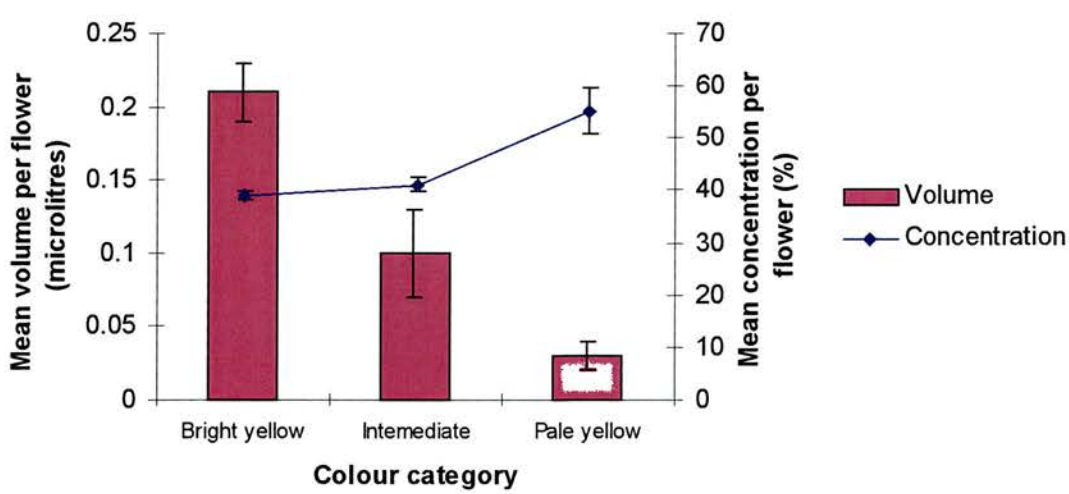


Figure 4.10 Nectar characteristics of different floral colour phases in *A. orientalis*. All data collection times summed.

This pattern was repeated at all data collection times (Figure 4.11), and presumably indicated reduced secretion with increasing age of flower.

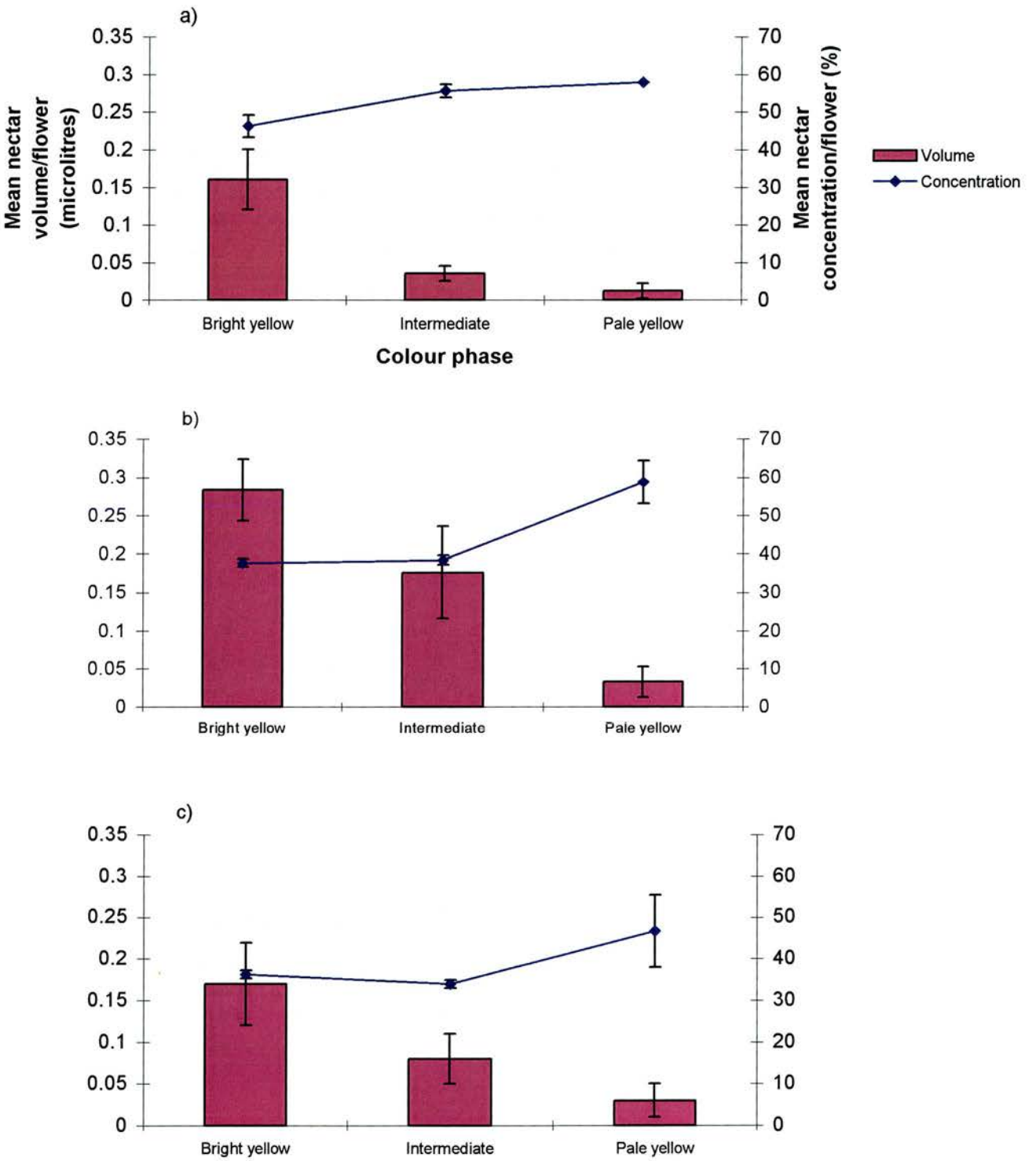


Figure 4.11a-c. Nectar characteristics of colour phases of *A. orientalis* over time. a) Morning; b) middle of day; and c) late afternoon. Volumes are a mean of 60 flowers of each colour phase at each data collection time. See text for sample sizes of concentration data points.

Due to large numbers of 'empty flowers' being sampled, particularly older pale yellow flowers, the sample sizes for concentration measurements were greatly reduced. In the morning respective sample sizes for bright yellow, intermediate and pale yellow flowers were 18, 9 and 1. Corresponding sample sizes for midday collections were 49, 23 and 6, and in late afternoon 26, 11 and 3 flowers.

Thus pale older flowers were much less rewarding than either intermediate or bright yellow flowers.

4.3.4 Pollen.

Figure 4.12 shows the pattern of pollen depletion in the different floral colour phases of *A. orientalis*. Bright yellow flowers contained more pollen than either intermediate or pale yellow flowers when scored as a proportion of maximum depletion; 0.80 ± 0.01 compared to 0.90 ± 0.01 and 0.91 ± 0.01 respectively. The high levels of depletion in all groups suggested very efficient pollen gathering on the part of visiting female *A. pauperata*.

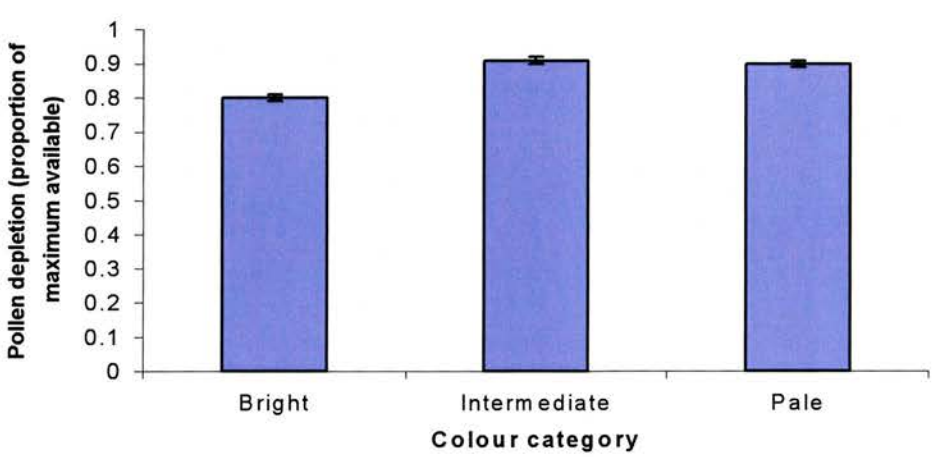


Figure 4.12 Pollen depletion in *A. orientalis*; depletion is scored as a proportion of the maximum possible available (see text for details).

The pattern of pollen depletion in different floral colour phases over time of day (Figure 4.13) confirmed published research on the timing of dehiscence in this species. The lower levels of depletion found in bright yellow flowers in the afternoon was a consequence of greater availability of freshly dehisced pollen in first-day flowers; Stone et al. (1999) reported peak availability of pollen during the early afternoon that coincided with female *A. pauperata* foraging activity.

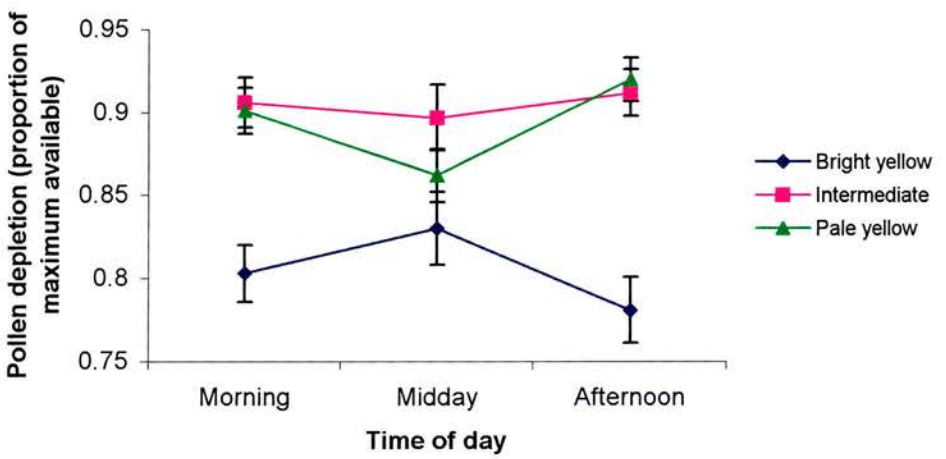


Figure 4.13 Pollen depletion over time in different floral colour phases of *A. orientalis*.

4.3.5 Visitation.

a) Colour choice on 'normal' patches.

In 6 hours of observation over 3 days a total of 99 individual foraging bouts by *Anthophora pauperata* were recorded. Due to the known foraging habits of this bee (see 4.1) these bouts were almost certainly not made by the same individual bees. Residence times on focal patches lasted from 4 to 130 seconds and visits were made to between 1 and 34 flowers. A total of 647 flower visits was noted. Figure 4.14(a-c) illustrates the number of observed visits by *A. pauperata* to flowers of different colour phase as compared to the expected

visitation given the proportion of flowers in each category available on patches on the three days of observation. Bees clearly preferred to visit the young bright yellow flowers. Day 1; $\chi^2 = 26.78$, df = 2, $p<0.001$; day 2; $\chi^2 = 14.89$, df = 2, $p=0.001$; day 3; $\chi^2 = 49.92$, df = 2, $p<0.001$.

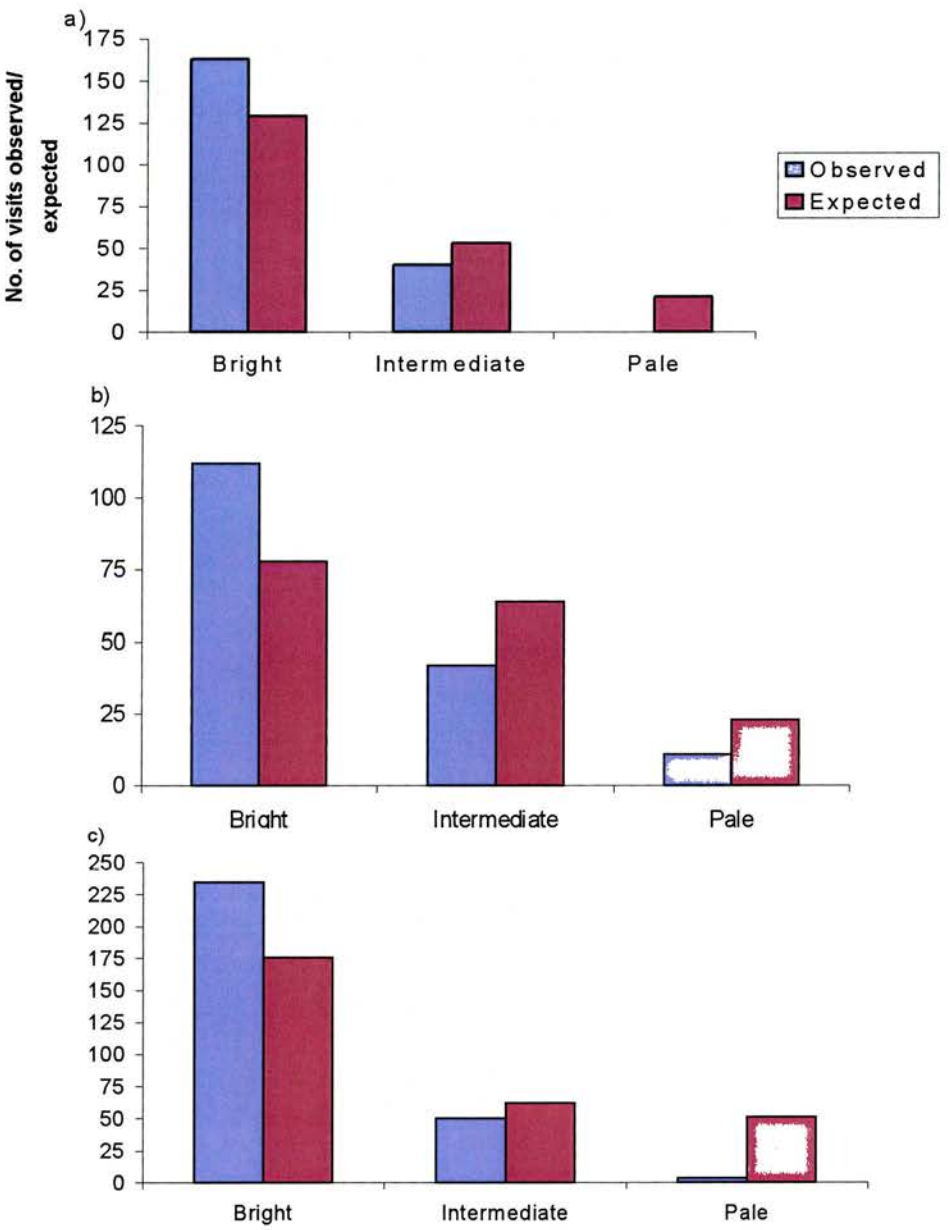


Figure 4.14 Observed and expected visitation by *A. pauperata* to different colour category flowers in patches of *A. orientalis* at Wadi Arbaein. a) 1/4/02, 203 flower visits in 28 trips; b) 3/4/02, 165 flower visits in 32 trips; c) 9/4/02, 289 flower visits in 39 trips.

b) Visitation to manipulated patches.

In six hours of observation of 'bright patches', 85 separate foraging bouts by *A. pauperata* were recorded of between 1 and 125 seconds with visits to between 1 and 60 flowers. For pale patches 49 foraging bouts lasting between 1 and 225 seconds with visits of 1 to 120 flowers were observed in a similar period. Mean residence times were 32.6 ± 3.0 seconds on bright patches and 19.9 ± 5.0 seconds on pale patches (Figure 4.15); Mann-Whitney test, W = 6572.5, p=0.0001 (median residence times; bright patches = 26 seconds, pale patches = 8 seconds).

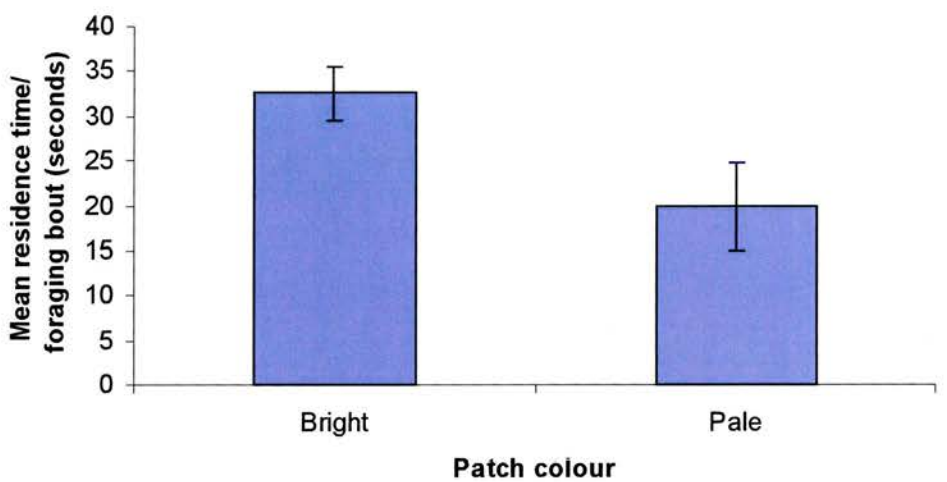


Figure 4.15 Residence time of visiting *Anthophora pauperata* on manipulated patches of *Alkanna orientalis*.

Mean numbers of flowers visited per bout were 14.6 ± 1.3 and 10.1 ± 2.8 flowers on bright and pale yellow patches respectively (Figure 4.16); Mann-Whitney test, W = 6607.0, p=0.0001 (median flowers visited, bright patches = 13, pale patches = 3).

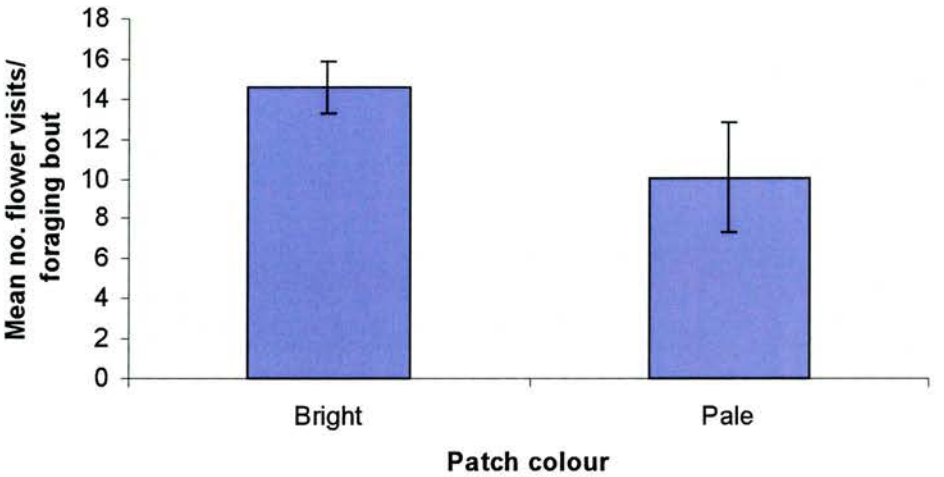


Figure 4.16 Flower visits by *Anthophora pauperata* to manipulated patches of *Alkanna orientalis*.

Therefore, foraging *A. pauperata* spent significantly greater time, and visited more flowers, on bright yellow patches than on pale yellow patches of *A. orientalis*. The considerable differences between the mean and median values for pale patches, for both flowers visited and residence times, are explained by outlying data for three bouts. Forty-six bouts to between 1 and 27 flowers lasted for periods of less than 60 seconds but 3 bouts lasted for up to 225 seconds and encompassed visits to a maximum of 120 flowers. There was less variance in foraging bouts at bright patches; foraging times ranged between 1 and 125 seconds with from 1 to 60 flowers visited. Thirteen of 85 bouts lasted for in excess of 60 seconds and 42 bouts included visits to more than 27 flowers.

4.3.6 Seed set.

The status of *A. orientalis* as an obligate outcrosser was confirmed; seed set, in all treatments, was calculated as the percentage of the total possible seeds per sample given a maximum of four seeds per flower. Seed set in bagged

flowers (n = 47) was confined to a single flower with 1 seed (0.1%); control flowers (n = 43), in positions where earlier visitation had been allowed, produced 39 seeds (23%) (four inflorescences lacked flowers at the lower position).

Successive attempts at hand cross-pollination were severely compromised by foraging goats. Just 12 flowers survived in the initial experiment of which 1 set a single seed an another 2 seeds (6%); of 12 control flowers 2 contained 3 seeds, 3 had 2 seeds, 6 a single seed whilst a single flower failed to set seed (38%). Of 29 flowers that remained at the end of a second attempt at cross-pollination, 10 set a single seed with the remainder containing none (9%). Control counterparts produced 21 seeds from 28 flowers (19%).

Successful cross-pollination (seen as seed set), following natural visitation, was ruled out as the causative agent of colour change; 25 of 48 marked pale flowers had failed to produce seeds. The remaining 23 flowers of this marked group contained 28 seeds in total, giving a net seed set of 15%.

4.3.7 Stigmatic receptivity.

Figure 4.17 illustrates a similar pattern of staining with both Perextesmo Ko and MTT tests; the figure represents the stain as a proportionate 'score' of the maximum possible after 15 minutes. MTT often produced staining within 1 minute. Maximum receptivity, as denoted by the heaviest staining with both tests, occurred in 2-day old flowers onwards, with intermediate (female 2) and pale yellow flowers (female 3) remaining receptive (>0.8) after 3-4 days and displaying no apparent withering of the style.

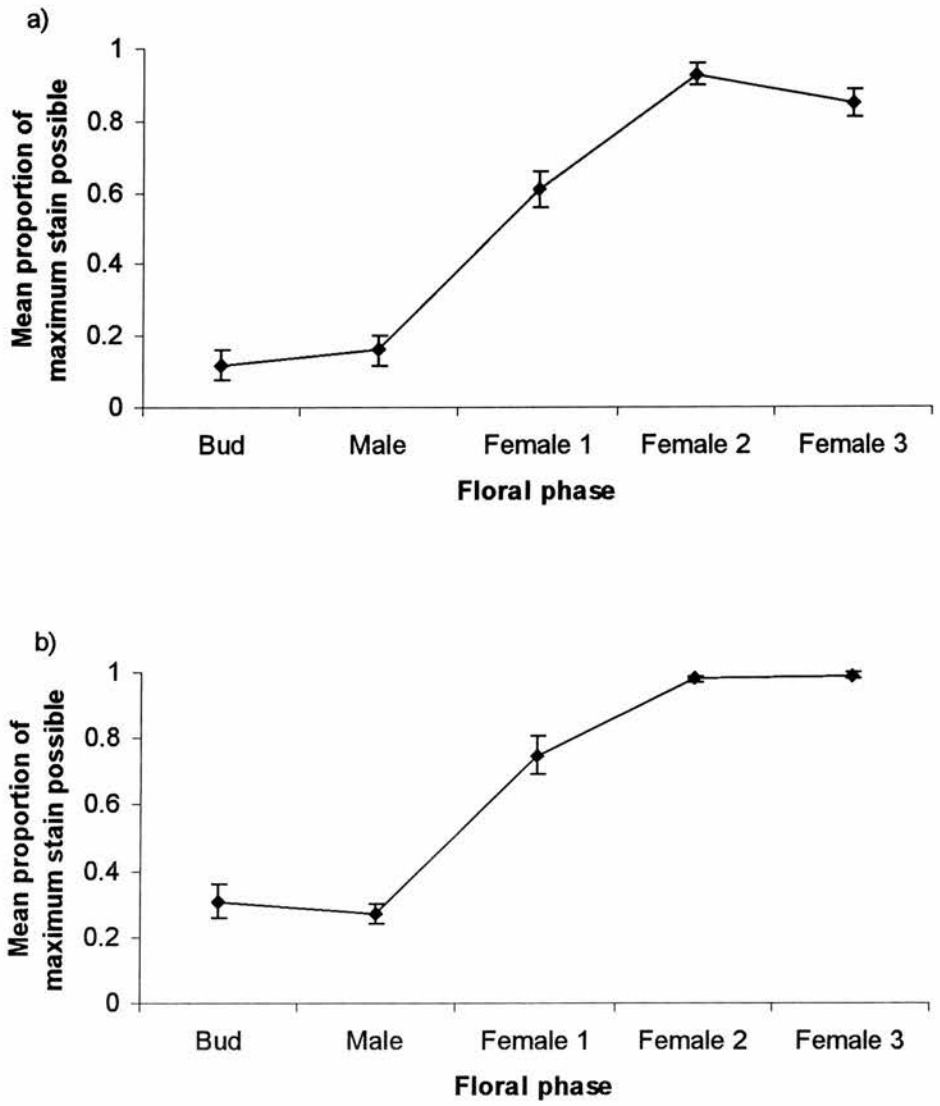


Figure 4.17 Stigmatic receptivity in *A. orientalis* as determined by chemical staining tests; a) Perextesmo Ko, b) MTT. See text for details of floral phase distinctions. n = 30 flowers for each data point.

Colour change, to the human eye, did not correspond with the change from male phase to female phase; flowers were bright yellow in both the male and female 1 phases, with the onset of change occurring later. However, maximum receptivity did coincide with onset of change (female 2).

4.3.8 Pollen tube growth.

Thirty-two of the 51 bagged and bright yellow flower preparations had pollen grains at the stigmatic surface. However, there was no definitive evidence of pollen tube growth in any of 51 samples. In one sample some fluorescence was noted within the mid-part of the style but the stigmatic surface had become detached and the source of the fluorescence could not be traced to a pollen grain at the stigma. Vascular tissue is a further source of fluorescence within stylar tissue (P. Gibbs, personal communication).

Forty-six of 51 open flowers, all of which had begun to change colour, had between 1 and 53 pollen tubes visible just below the stigmatic papillae or within the first few millimetres of the style; mean number of pollen tubes visible was 18.4 ± 2.0 per flower. Due to varying quality of preparation and, therefore, difficulty in resolving and counting precise number of tubes through the thick stylar tissue, these figures could be lower than the actual number of growing tubes. The 5 samples without visible pollen tubes all had pollen grains adhered to the stigmatic surface.

Therefore, pollen tube growth coincided with the onset of colour change in 2 day-old flowers that were open to visitation, although the precise trigger – pollen deposition and/or germination or commencement of pollen tube growth – was equivocal.

4.4 Discussion.

Visitation by bees and artificial manipulation of flowers accelerates colour change in *Alkanna orientalis*. Regular flower visits by *Anthophora pauperata* and a range of mimicked handling effects led to reduced periods in each of three identified colour phases, as well as shorter floral longevity. This study also suggests that the trigger for colour change is related to deposition of cross-pollen, either deposition *per se*, or successful germination, or pollen tube growth. Where these processes have not occurred colour change will still take place but over an extended time period; as discussed in other chapters, this could be explained by senescence processes, or damage to floral parts, both of which require further experimentation.

The foraging behaviour of the major pollinator, *A. pauperata*, is influenced by floral colour change. Under normal circumstances flower colour choice is biased towards younger, bright yellow flowers and, following manipulation of patches, residence time and number of flowers visited are increased further in bright yellow patches when compared to pale yellow patches of *A. orientalis*.

4.4.1 Floral longevity and colour change: attractiveness and visitation by bees.

Both floral longevity (Figure 4.5) and length of floral colour phases (Figure 4.6 and Table 4.1) are increased in the absence of visitation. A mean increase of two days in the life span of a non-visited flower represents approximately 50% greater longevity; this factor, combined with a further day in

the more rewarding bright yellow phase favoured by visiting bees, could prolong visual attraction and encourage further potential visits. Increased attractiveness of plants through retention of post-change flowers has previously been shown in several studies (e.g. Gori, 1989; Weiss, 1991; Jones & Cruzan, 1982; but see Caspar & La Pine, 1984 for alternative results).

Plant fitness, particularly in those species that are obligate outcrossers, is likely to be enhanced by further visitation. Gilbert et al. (1996) have previously suggested that, in sub-populations of *A. orientalis*, selection occurs for phenotypic reproductive characters (larger and more rewarding flowers) and shown that additional visits were gained by plants displaying greater numbers of flowers. Although no attempt was made to compare visitation levels in my study, increased longevity and a flexible colour change process are described, do contribute to extended attractiveness of the floral display, and can explain some of the patterns noted by Gilbert et al.

Where visitation was highest, as recorded by Gilbert et al. (1996) (in Wadis Arbaein and Tofaha) successful pollination is the most likely explanation for rapid colour change and early abscission of flowers, and would explain the observations of fewer flowers per plant, in general, and fewer pale flowers, in particular, as noted in that study. Greater number of flowers and more pale flowers per plant were recorded in populations on the Plain of El Raha (Gilbert et al. 1996) where fewer bees are present (Willmer et al. 1994) and smaller numbers of flowers are dislodged during foraging (Gilbert et al. 1996).

As a potential rule, then, where visitation is low more flowers are retained and the display size is increased, thus giving higher and more prolonged attractiveness that may lead to increased cross-pollination in the medium to long

term. Visitation may vary on both micro- and macro-scales. Where *A. pauperata* is abundant there could be a higher density of male territories, and plants on the edge of, or between, territories could receive fewer visits from both sexes of bee than those in the centre. Where the bees are scarce (e.g. Wadi Dir and the Plain (Gilbert et al. (1996)) all plants receive fewer visits. Both scenarios could ensure differential visitation both between and within plants, and might explain the differential colour phase proportions described by Gilbert et al. (1996).

4.4.2 Nectar.

Nectar characteristics of *A. orientalis* in this study are at variance with earlier research. Gilbert et al. (1996) cited a figure of up to 6µl per flower at a concentration of 35% but in reporting their results recorded mean volumes of 0.33 and 0.15µl per flower for unvisited and nectar-withdrawal groups respectively after 24 hours. Stone et al. (1999) found differences between male and female flowers and relatively high mean volumes (>4µl) in flowers from more than one population. Semida (1994) reported overall volumes in bagged flowers of approximately 2.5µl per flower over the course of a day. In this study overall mean volumes were low (Figure 4.9), 0.33 ± 0.06µl per flower standing crop, summed for all ages of flower across time of day. There are a number of possible reasons and a combination of these factors could account for the figures reported in my study:

a) High numbers of *A. pauperata*. A lack of rainfall in 2002 (see below) may have facilitated increased survival of nests in the wadi bottoms (through the absence of possible flash floods) and thus enhanced

numbers of emergent bees. As this bee appears to be an extremely efficient forager, with males defending floral resources and females foraging within these territories (Willmer et al. 1994), high numbers might lead to reduced nectar standing crop.

b) Water stress. No rain had fallen in the region since April 2001 (M. James; personal communication) and only a limited snowmelt occurred in the spring of 2002. Lack of available water might lead to low levels of nectar production.

c) Herbivory. Although earlier studies suggest otherwise (Gilbert et al. 1996; Stone et al. 1999), herbivory by goats on new inflorescences occurred on most days in Wadi Arbaein during my study. In addition camels have been observed to eat whole plants at some local sites (M. James, P. Willmer; personal communication). Damage by herbivores to plants has been shown to affect nectar availability; production was reported by Strauss et al. (1996) to be reduced in *Raphanus raphanistrum* following herbivory by butterfly larvae.

Nectar characteristics varied with flower colour and, therefore, age (Figure 4.10). As bright yellow flowers contained, on average, twice as much nectar as intermediate flowers and four times as much nectar as pale yellow flowers, bees might learn to associate colour with reward level and thus forage more efficiently. This is highlighted both in visits to normal patches (Figure 4.14), where nectar foraging bouts are concentrated at bright yellow flowers, and where patches were manipulated to produce longer residence times on bright yellow patches and visits to more flowers per patch (Figures 4.15 and 4.16). The

latter visitation patterns reflect, presumably, the higher nectar rewards available; bees spent longer times at rewarding flowers and re-visited the colour that was previously rewarding.

4.4.3 Pollen.

Pollen depletion was high in all colour categories and is therefore unlikely to be of importance either in triggering colour change or influencing visitor choice as a visual cue (Figure 4.12). Pollen dehiscence takes place early in the development of the flower, on the day of opening (Willmer et al. 1994; Semida, 1994; Gilbert et al. 1996; Stone et al. 1999) and female *A. pauperata* gather pollen by buzz pollination before the flower has fully opened. All buds and flowers visited for pollen are thus bright yellow, but they present a different visual cue from fully open flowers in terms of appearance. Stone et al. (1999) note that a pollen odour may be the most relevant visitation cue at this stage. With regard to colour change, as some or all pollen could potentially be removed without effecting pollination, a benefit to the plant of such a trigger for change (beyond paternal fitness) is absent. Furthermore, as peak stigmatic receptivity (Figure 4.17) occurs after pollen release and continues for several days, the plant will benefit from continued visitation, and potentially improved maternal fitness, well after most or all pollen is removed.

4.4.4 Post-pollination events.

If colour change signals reward level in general, but also corresponds with reproductive processes, both plant and visitor benefit; foraging activity is directed to the most rewarding flowers that are also those most likely to require further pollination. Deposition of cross-pollen, combined with successful germination, is shown to be the most probable trigger for the commencement of colour change in this plant. In bagged flowers only self-pollen could become adhered to the stigmatic surface; this had occurred in the majority of samples but onset of colour change had not commenced after 48 hours. Self-pollen could be deposited through perturbation by wind or disturbance by the experimenter when bagging the flower. Together with the absence of seed set in bagged flowers, this suggests that self-incompatibility is sporophytic in *A. orientalis*. In all but 5 open flowers pollen tube growth was visible within the style and coincided with the intermediate colour phase. The presence of pollen grains on the stigmata in the remaining flowers, all intermediate in colour, should not be regarded as conclusive evidence that deposition alone is the trigger of colour change.

Pollen-pistil interactions can result in rapid effects on plants, such as protein synthesis within the ovary (e.g. Deurenberg, 1976). Neither this study, nor other work to date, confirms the first stage of the interaction as causative of colour change. The time scale between deposition of pollen and onset of colour change requires confirmation; in my study only second-day flowers that had been open to visits (and where onset of change was evident) were analysed. The analysis of first-day, pre-change flowers needs to be undertaken to determine how close the link is between deposition and commencement of colour change.

Also, the possible effects of additive pollen deposition on colour change, and whether deposition or commencement of pollen tube growth in stylar tissue is the critical factor in colour change, all remain to be resolved in this species.

Previous attempts at hand cross-pollination in this species have resulted in low seed set (Gilbert et al. 1996, Semida, 1994), and here this was also the case. Both the longevity of the flower (Figure 4.7) and length of colour phases (Figure 4.8), following attempted hand cross-pollination, suggest that successful deposition of pollen by hand is difficult. In particular, the presence of hairs in the mouth of the corolla tube was felt to be a potential barrier to pollen transfer by brush.

4.4.5 Manipulations.

The attempts at mimicking visitation described here must be treated with caution; the extremely rapid colour change (Figure 4.8) and, in some cases, withering and abscission on the first day (Figure 4.7) may be due to damage to floral tissue rather than any natural process linked to flower handling. Nectar withdrawal has been found to be involved in colour change in at least one species, *Oenothera drummondi* (Eisikowitch & Lazar, 1987), but while the resultant colour change could influence foraging behaviour (and therefore be ecologically relevant) the value to the plant is difficult to ascertain. Nectar can be removed without pollen deposition or removal and thus neither maternal nor paternal fitness is enhanced. Similarly, the mechanical act of probing presents the same problem; if colour change is to be a reliable signal for both plant (directing visitors towards flowers still requiring pollination) and visitor (usually

corresponding to decreased, or even a lack of, floral reward e.g. Weiss, 1991), probing as a trigger may compromise plant reproductive success where colour change occurs in the absence of pollination. Either action could have led to deposition of self-pollen, a further possible trigger of colour change that is discussed in more detail above.

The most likely reason for the patterns described may be damage of floral tissue when attempting the manipulations; again the possibility of ethylene (via the known wound response) being implicated should be considered (see Chapter 9). Even allowing for gentle manipulations by the experimenter there is a great difference between the texture and action of a glass microcapillary and a bee's tongue; in addition the latter could effect successful delivery of cross pollen and the former was cleaned between flowers. Notably, in both the nectar withdrawal and probing groups, longevity was shorter and colour change more rapid where multiple manipulations were used; damage is more likely to be incurred with repeated manipulations.

The suggested patterns of colour change in *Alkanna* are presented in Figure 4.18. Our knowledge of the association between *A. orientalis* and *A. pauperata* is strengthened by an understanding of the role of floral colour change in this interaction. Future study might address the biochemical basis of colour change in this plant and whether other flower visitors respond to the same visual cue that, apparently, contributes to the relationship between bee and plant.

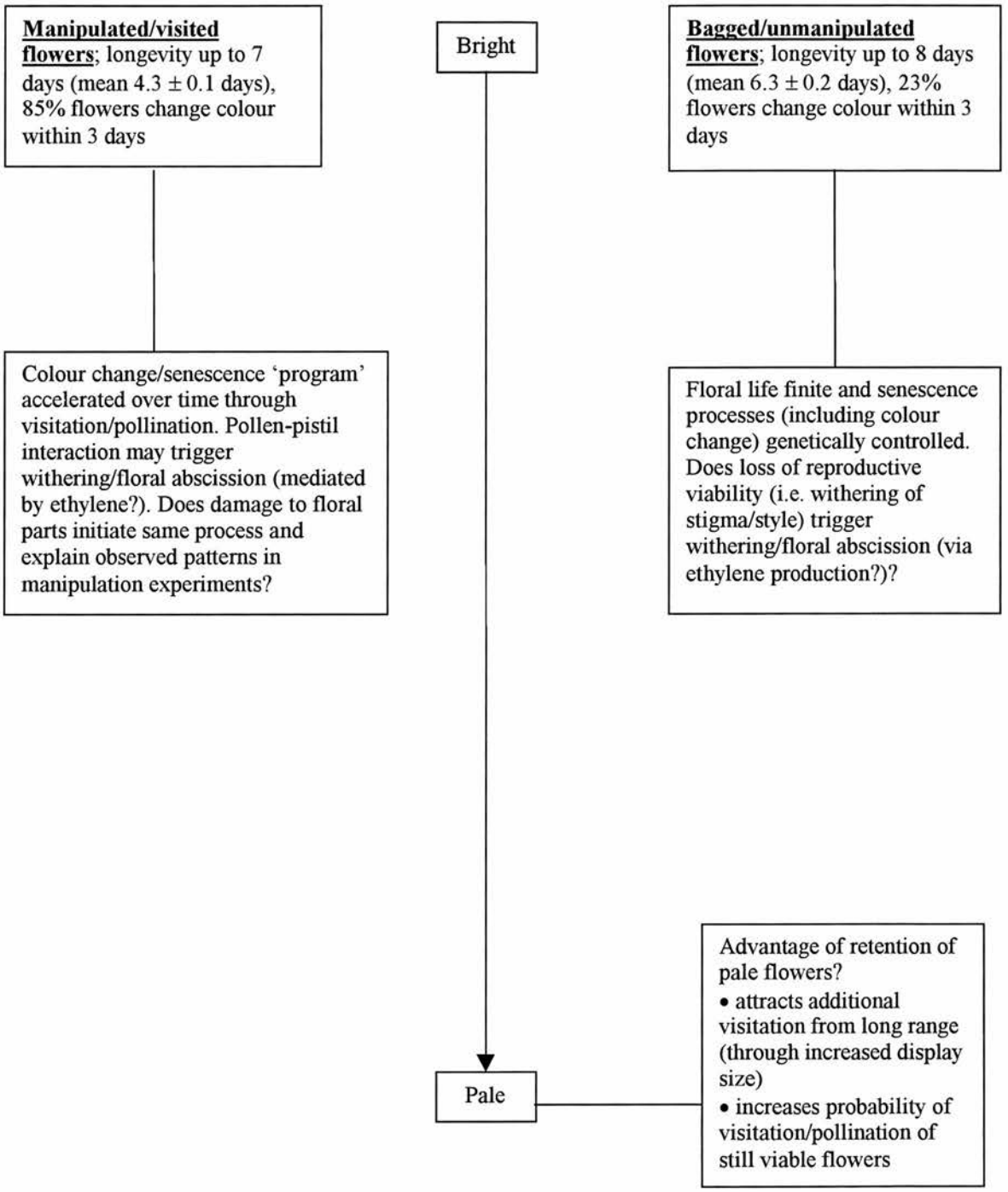


Figure 4.18 Schematic to illustrate effects of visitation on, and possible mechanisms of, colour change in *Alkanna orientalis*.

Chapter 5 – *Echium vulgare.*

5.1 Introduction.

The genus *Echium*, comprising annual, biennial and perennial herbs and shrubs, contains 18 species that are widespread and locally abundant throughout Europe (Tutin et al. 1976). Viper's bugloss (*Echium vulgare* L., Boraginaceae) is a self-compatible, perennial plant found commonly in a variety of habitats throughout Britain and with an extensive range in Europe (Polunin, 1988). The flowers are typical of bee-pollinated blossoms as described by Faegri & van der Pijl (1979); nectar is hidden at the base of a conspicuously colourful, zygomorphic corolla tube that offers a good landing platform.

Individuals of *E. vulgare* may grow to 90cm and have several flowering stems with inflorescences that are spike-like to paniculate (Tutin et al. 1976). Up to 50 cymes bearing *c.* 20 flowers have been recorded, hence a single plant can produce several hundred flowers although only a few are open on a cyme at any one time (Rademaker et al. 1999). Stems are covered in stiff hairs.

Corbet (1978) determined several stages of growth of the hermaphroditic flowers of E. vulgare. Initially the 4-5 stamens are exserted beyond the corolla tube, exceeding the style. During this phase, dark blue pollen dehisces from the anthers. The style then extends and, when level with the longest stamen, the stigma bifurcates and the surfaces, which are bi-lobed, turn rough and sticky; pollen presentation is almost finished at this time. Later, the style grows beyond the now shrivelling anthers prior to withering of stamens and/or corolla. The alteration of the character of the stigma has been reported as occurring at the end

of the first day and equated to the onset of stigmatic receptivity (Klinkhamer & van der Veen-van Wijk, 1999). These processes effectively make the flowers protandrous and, despite the slight temporal overlap of pollen availability and stigmatic receptivity mentioned by Corbet (1978), autogamous self-pollination is prevented by spatial separation of anthers and style (Rademaker et al. 1999). Flowers last for 3-4 days (Corbet, 1978).

Colour change in flowers of the Boraginaceae occurs in at least 19 species from 11 genera (Weiss, 1995a); the only member of the genus *Echium* listed by Weiss in this database is *E. decaisnei* which displays a red to white change of the androecium. However, colour change from purple to blue in *E. plantagineum* has also been described (Bos et al. 1983) and a change in the UV-patterns of this species has been reported (Kruijer, 1987; Moeliono, 1987). Furthermore, colour change has frequently been noted in *E. vulgare*; buds are pink and the tubular flowers are described as turning pink-blue during the first day and blue thereafter (Klemow et al. 2002; Klinkhamer & van der Veen-van Wijk, 1999; Klinkhamer & de Jong, 1990; Rademaker et al. 1999; Rademaker et al. 1997).

A number of studies have concentrated on visitation by insects and the relationship with the nectar characteristics of *E. vulgare*. A study by Corbet (1978) found altered sugar characteristics across time of day and attributed these to nectar secretion rates and microenvironmental changes, which were also linked with bee visits. The particular importance of humidity as an influence on nectar, and consequent effects on bumblebee visitation, were reported by Corbet et al. (1979). The earlier work by Corbet made no reference to variation in nectar production with respect to age of flower, and a subsequent study found

decreasing production over time, and higher nectar reward in younger 'male' flowers that underpinned visitation by bumblebees (Klinkhamer & de Jong, 1990). The latter research also demonstrated that larger plants with more flowers received an increased number of approaches by bumblebees than small plants with fewer flowers, and that isolated plants of any size received fewer approaches. By varying the volume of available nectar, Pappers et al. (1999) found visitation sequences by bumblebees could be manipulated (bagged plants, where nectar was allowed to accumulate, received longer visitation sequences) but the number of approaches did not change; the bees were basing their foraging decisions on nectar reward at individual flowers.

Bumblebees are the main visitors to natural populations of *E. vulgare*. Three studies of the plant at a coastal dune site in Holland reported *B. terrestris* and *B. pascuorum* as the main visitors (Klinkhamer & de Jong, 1990; Pappers et al. 1999; Rademaker et al. 1999). These, and several further species of bumblebee, were recorded by Corbet (1978), visiting large patches of *E. vulgare* in Norfolk, England, together with honeybees, cuckoo bees and diurnal hawkmoths.

This species was chosen because of its easy accessibility at convenient field sites and the flowering time in the wild allowed fieldwork to be done after *Myosotis sylvatica* finished flowering, and prior to the onset of flowering of *Lonicera periclymenum*. Additionally, the larger flower size enabled different types of artificial manipulation (e.g. pollen removal, see below) to be carried out that were not possible in the smaller flowered species of Boraginaceae (see Chapters 3 and 4). In contrast to *Myosotis sylvatica* and *Alkanna orientalis*, the colour change occurs from pink to blue and takes place comparatively early in a

flower's life (although, in common with the latter species, colour change affects the whole flower). Colour change in *E. vulgare* has not been fully documented, either in terms of the nature and length of the various colour phases, or with regard to whether alteration of floral reward coincides with the colour change and how visitation may be concomitantly influenced. This study examines these features together with possible triggers for colour change in relation to visitation.

5.2 Materials and Methods.

5.2.1 Study sites.

Two plant populations were used for this work. In the summer of 2000 a small population (35-40 plants) was studied at a patch of rough ground (a thin strip, approximately 20 x 60m), just above the high-water mark of a shingle beach at Kingsdown, near Deal, Kent (TR 379484 (637992, 148450)). Data were collected between 22nd July and 8th August. A site on the cliffs to the west of Elie, Fife (NO 473000 (347325, 700012)) (Figure 5.1), was used in both 2001 and 2002; data were collected between 4th and 17th July and 17th June and 4th July, respectively. Here the plant was growing on an area of ground (approximately 30 x 30m) with minimal grass cover where subsidence had previously occurred.

Figure 5.1 Field site at Elie, Fife, June 2002. Several plants of *E. vulgare* can be seen in the centre of the photograph, protruding above the skyline.

5.2.2 Floral development, longevity and colour change.

In the 2000 field season, one hundred buds were tagged at dawn on the morning of opening and monitored twice daily over 5 days. Data were collected for floral longevity (to time of abscission), colour, and floral morphology. Corolla tube length (from point of insertion into calyx to distal tip of upper limb; see Figure 5.2) and width (symmetrical plane of corolla limbs; see Figure 5.2) were measured, together with a note of development of reproductive parts in relation to flower growth.

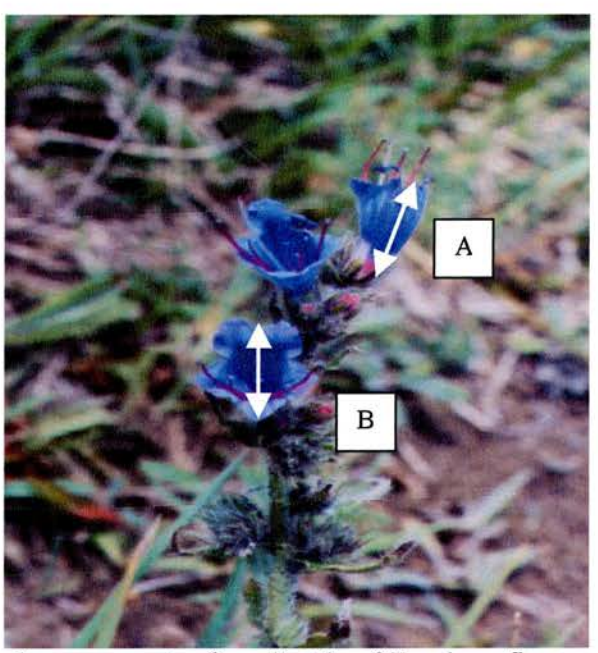


Figure 5.2 Morphological measurements of corolla tube of *E. vulgare* flowers. A = Length, B = width.

Flower colour was compared against a chart (Figure 5.3) devised from a printer's ink colour-matching book (see Chapter 2).

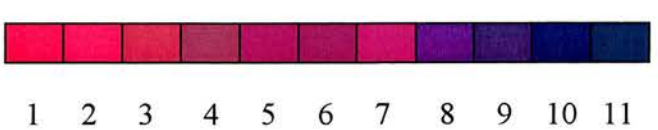


Figure 5.3 Chart for matching colour phases in *E. vulgare* (see text for details).

Eleven phases of colour were identified, the majority of which occurred from bud to initial opening. Stages 1-3 were pink to reddish-purple (colours 206U, 207U and 215U) and were found only in small buds. Reddish-purple to purple larger buds were noted in stages 4-6 (260U, 259U and 2602U) just prior to opening when flowers were purple (stage 7, 2592U). Fully turgid flowers were bluish-purple to blue (stages 8 and 9, 268U and 2748U, respectively), giving a general impression of a blue colour with a wash of purple. Older flowers lost their purple tinge (stage 10, 280U) prior to commencement of withering when they appeared darker blue with a brownish tinge (stage 11, 288U).

Initial observations suggested that, due to the time taken to collect data from the size of sample used, the speed of colour change (and any subtleties of colour change) might have been overlooked. Therefore, a further cohort of 30 buds were tagged at 07.30 and observed hourly to detect such features of change; the stages defined above were determined during this work. Owing to the rapid colour change of discrete flowers individual cymes could exhibit examples of several stages at any given time (Figure 5.4).

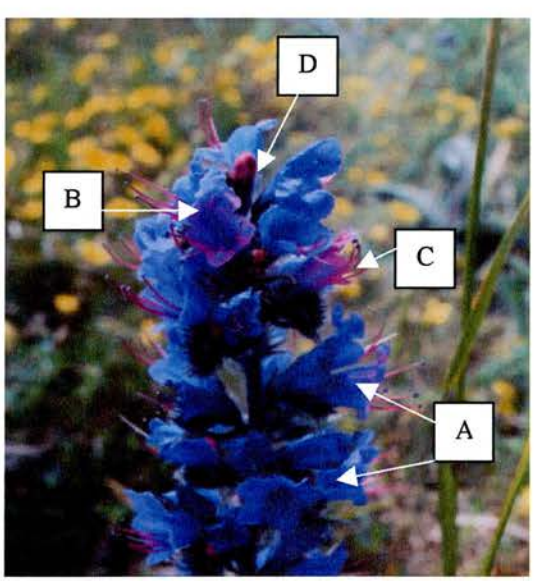


Figure 5.4 Individual cyme of *E. vulgare*. Majority of flowers are fully mature and blue (stage 9, A, see text) but some younger bluish-purple (stage 8, B), newly opened purple (stage 7, C) and pink buds (stage 1, D) are also visible.

Colour change occurred in individual flowers in a proximal-distal direction (Figure 5.5).

Figure 5.5 Partially open purple (stage 7) flower of *E. vulgare*. Base of corolla tube is already blue-purple with colour change occurring in proximal-distal direction.

For collection of visitation data (see below), colour phases were 'collapsed' into purple, intermediate and blue categories (stages 7, 8, and 9/10, respectively) for ease of scoring visits of short duration to multiple flowers.

5.2.3 Nectar profile.

Nectar volumes and concentrations were measured (see Chapter 2) in relation to age of flower and colour of flower. To extract all nectar from a flower it was necessary to gently pull the corolla tube from the calyx and probe from the base of the tube with a 1µl microcapillary.

A cohort of 100 buds was tagged in four groups of twenty-five to establish the length of the period of nectar production; on four successive days at 11.00 hours nectar from all flowers in one of these groups was sampled. Between 29th July and 8th August nectar was sampled from a further 10 'purple' (stage 7) and 10 'blue' (stage 9/10) flowers, chosen at random, at two-hour intervals between 07.30 and 17.30 to establish whether nectar characteristics varied with time of day and flower colour.

The period of data collection was extended due to rain on several days during this period; two sets of data were collected for each time session.

In both colour categories many 'empty' flowers are included in the data. A number of flowers contained trace quantities of nectar (below 1mm in the microcapillary and therefore < 0.03µl, see Chapter 2) and these were allocated a nominal volume of 0.015µl.

5.2.4 Visitation.

Insect visitors were recorded on 8 days between 28th July and 8th August 2000; observations were made hourly, between 07.00 and 19.00, over twenty-minute periods, to a focal plant displaying approximately 500 flowers. Data were only recorded during dry and sunny or bright conditions. A visit was defined as an approach that led to a feeding bout on either nectar, pollen, or both, or a landing that involved contact with reproductive floral parts; multiple flowers could be visited within each bout.

Solitary bees were grouped on the basis of size and behaviour. Small solitary bees (< 8mm) visited solely for pollen, restricting their movements to the anthers and not entering the corolla tube; contact with the style was not observed. Medium solitary bees (8-15mm) were almost exclusively a single species of halictid that foraged for both nectar and pollen; pollen-collecting female bees were frequently laden with the distinctive blue pollen of *E. vulgare*. Large solitary bees (*Anthophora* sp., > 15mm) were rare visitors and are included in the 'other' category; visitation was for nectar only and behaviour involved hovering to feed with no discernible contact with the floral reproductive parts.

Foraging data for visiting *Bombus* sp. were collected in 2001 and 2002. Individual insects were followed during feeding bouts to assess whether flower colour phase visitation was independent of colour phase availability in the general population. As well as the flower colour visited, the total number of flowers of each colour category present on the focal patch was also noted.

5.2.5 Manipulations.

i) Mimicked visitation effects.

The effects of two types of visit, pollen removal (as observed in syrphids and small solitary bees) and nectar withdrawal (for bumblebee visits) were investigated by simulating these processes. Buds were chosen at random and, as soon as corollas were partially open (and, therefore, both pollen and nectar were theoretically accessible to visitors) and while still purple, assigned to one of three experimental groups.

Control: - no treatment.

Pollen removal: - as soon as the anthers were exposed, all visible pollen was removed from anthers with a fine artist's paintbrush.

Nectar withdrawal: - flowers were probed *in situ* at each data collection time with a 1μl microcapillary until all available nectar had, apparently, been withdrawn (the earlier methodology of removal of the flower allowed removal of all nectar but was obviously unsuitable for this experiment).

All flowers were marked, and natural visitation to these flowers was prevented by driving away potential flower visitors as they approached focal plants. Flower colour was measured every 30 minutes against a colour chart as above.

ii) Visitor exclusion and bagging effects.

This was designed to see if colour change was affected by preventing visitation, and, additionally, the possible influence of the use of pollination bags. As opening had been noted to occur more rapidly by day than by night (Corbet, 1978), foil bags were also used to alter the level of light that the flowers received. Buds were chosen at random and assigned to one of three experimental groups.

Control: - no treatment. Flowers tagged with cotton thread and natural visitation allowed.

Bagged: - flower enclosed in fine (approximately 1mm aperture) nylon netting.

Foil: - flower enclosed in 5cm x 5cm squares of aluminium foil, fastened at the pedicel.

The flowers were re-examined after 4 hours and the stage of floral development and colour change recorded.

5.3 Results.

5.3.1 Floral development, longevity and colour change.

Flowers lasted between one and five days (mean longevity 3.9 ± 0.1days, n = 94) prior to withering and/or abscission; completely shrivelled brown flowers were sometimes 'held' on the cyme by still turgid styles. The flowers were clearly protandrous, with blue pollen available immediately upon opening but with the style not yet extended or the stigmatic surfaces exposed. Extension of the style usually took place on day 1 or 2. In 11 flowers, the style had grown beyond all five anthers by the end of the first day; a further 69 flowers had reached this stage during day 2, 3 more by day 3, and in the remainder of flowers (11) the style did not protrude beyond the corolla for the duration of floral life. Bifurcation of the stigma occurred after opening and before full extension of the style beyond the corolla limbs.

Maximal access for nectar-seeking visitors, as determined by size of corolla dimensions, occurred on day 1 or 2. Corolla size (mean length 13.05 ± 0.14mm; mean width 10.49 ± 0.17mm, n = 96) was greatest on the first day in 39 flowers, on day 2 in 56 flowers, and on day 3 in a single flower (the sample size differs from the longevity data set because 2 flowers were 'lost' after 2 days, as opposed to abscising/withering naturally). Although flowers remained on the plant for up to 3 further days only 5 remained 'accessibly' open by day five, withering and closure of the corolla having occurred in most instances.

The colour change was rapid; within one hour of the start of opening 2 of 30 flowers had reached the fully turgid blue state (stage 9), while 18 of 30 flowers were blue after two hours, and an hour later only two flowers had not reached this 'mature'

phase. All flowers had changed to blue within 5 hours of opening. As purple flowers (stage 7) were available throughout the day during nectar profile measurements it was confirmed that flower opening was not limited to certain periods of the day as reported earlier (Corbet, 1978).

Although protandrous, the sexual phase change of the flowers did not appear to correspond with colour change in *E. vulgare* (Figure 5.6).

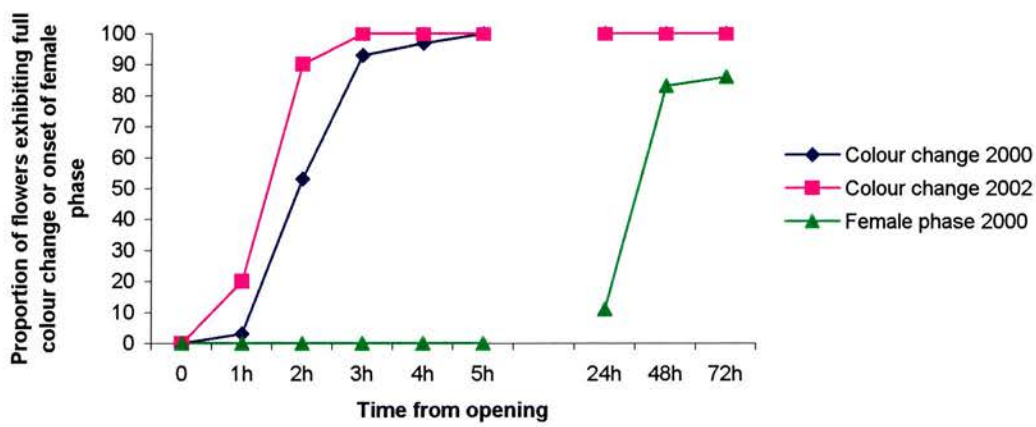


Figure 5.6 Flower colour and sexual phase in *E. vulgare*. Sexual phase data from 2000 were used; onset of female phase usually occurred on the second day (see above in this section) and always after completion of colour change. Colour change to blue-purple (stage 9) was complete within samples of the Kent population (2000, n = 30) and the Fife population (2002, n = 20, data from 5.3.5.ii used (see below and Figure 5.16)) within 5 hours of opening.

Hence, growth of the style occurred after the rapid colour change. Therefore, first-day blue-purple flowers (stage 9) could be either male or female, but thereafter all flowers were functionally female (apart from 11 out of 94 'abnormal' flowers where full development of the style did not take place).

5.3.2 Nectar profile.

i) Age of flower.

Nectar was available on the first three days of floral life (Figure 5.7). On day 1 mean volume was 0.86 ± 0.30µl (n = 22). Mean volume on day 2 was 0.24 ± 0.10µl (n = 16), and on day 3 0.06 ± 0.04µl (n = 4). Nectar could not be extracted from any of 22 day 4 flowers.

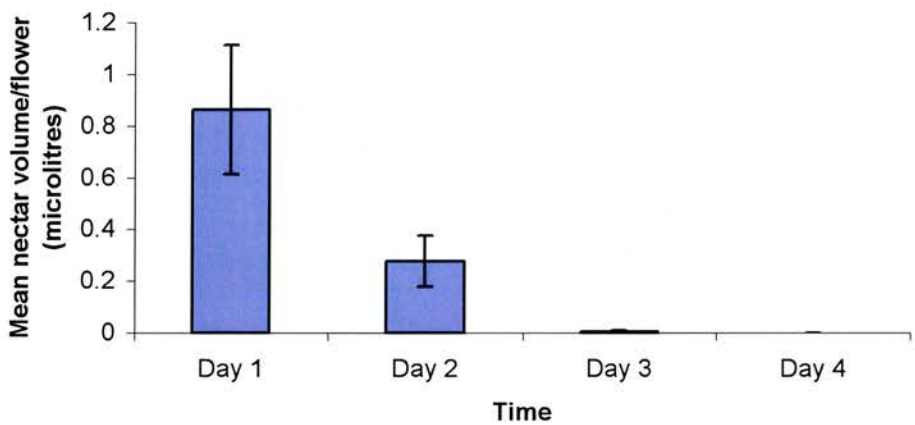


Figure 5.7 Mean nectar volume per flower with flower age in *E. vulgare* (measurements taken at 11.00h each day, these represented 24-hour multiples from time of tagging, see 5.2.3).

Mean concentration in day 1 flowers was 22.2 ± 4.3% (n = 17), and on day 2 24.5 ± 4.4% (n = 11). The median concentration in first-day flowers was not significantly different to that of second-day flowers (Mann-Whitney, W = 250.0, p = 0.2252, median concentration of first-day flowers 21.25%, and of second-day flowers 24.0%). The small quantities extracted from third-day flowers could not be measured for concentration.

ii) Time of day

Small quantities of nectar were found throughout the day (Figure 5.8) with a gradual rise from early morning (0.07 ± 0.03µl per flower, n = 40) to a peak of 0.10 ± 0.02µl per flower at 13.30 before a reduction during the afternoon. Mean nectar concentrations varied little during the day, recordings of 21.3 ± 1.7% (n = 14) at 13.30 and 28.7 ± 2.8% (n = 6) at 15.30 represented the low and high figures, respectively; this presumably reflected protected micro-environmental conditions within the corolla tube (Corbet et al. 1979).

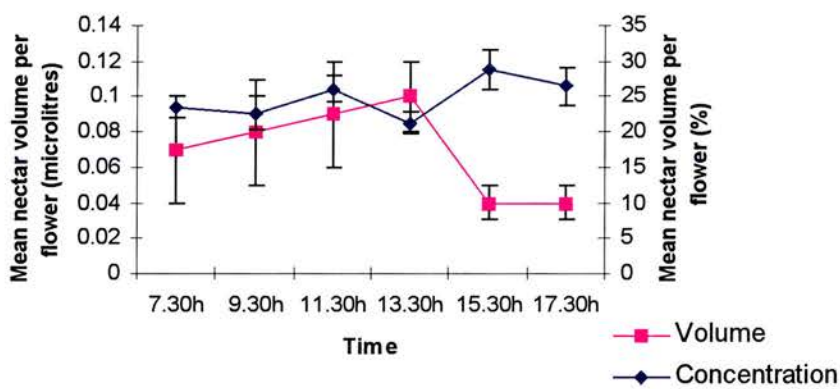


Figure 5.8 Nectar characteristics over time in *E. vulgare* (n = 40 at each data point).

Possible reasons for the low volumes in comparison with (i) above are not clear.

iii) Flower colour

The data used in (ii) above were separated into flower colour categories to establish whether there were differences in nectar characteristics between younger purple and older blue flowers. Mean nectar volume per flower for purple flowers (stage 7) was 0.07 ± 0.02µl (n = 120), with extracted volumes ranging from 0 to

0.96µl. In blue flowers (stage 10) mean nectar volume was 0.06 ± 0.01µl per flower (n = 120), with extracted volumes ranging from 0 to 1.11µl (Figure 5.9).

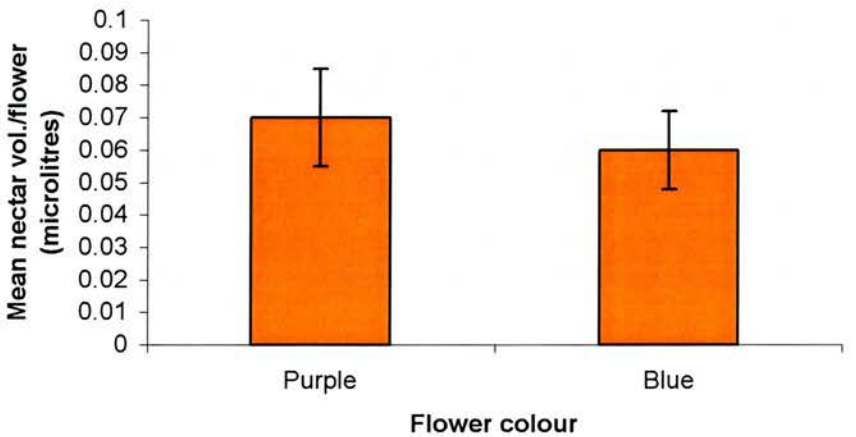


Figure 5.9 Nectar volume per flower in *E. vulgare* by flower colour.

There was no significant difference between the median values for purple and blue flowers; Mann-Whitney W = 14450.5, p = 0.99, median value for both blue and purple flowers = 0.015µl.

Mean nectar concentration of blue flowers was 26.8 ± 1.5% (n = 26) and that of purple flowers 24.3 ± 0.9% (n = 26) (Figure 5.10); these data were transformed to provide normal distribution but there was no significant difference between the mean values (Two-sample t-test; T = 1.16, p = 0.25, df = 41).

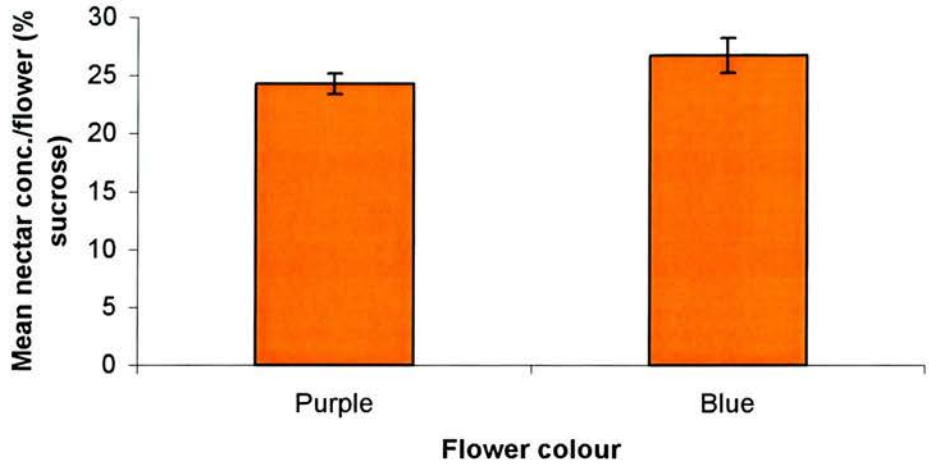


Figure 5.10 Nectar concentration per flower in *E. vulgare* flower colour categories.

Nectar characteristics of the different colour category flowers showed little difference over time (Figure 5.11) apart from a very low mean volume for purple flowers at 11.30 ($0.02 \pm 0.01\mu l$); generally the data for both groups followed the pattern for the combined data in Figure 5.8 above.

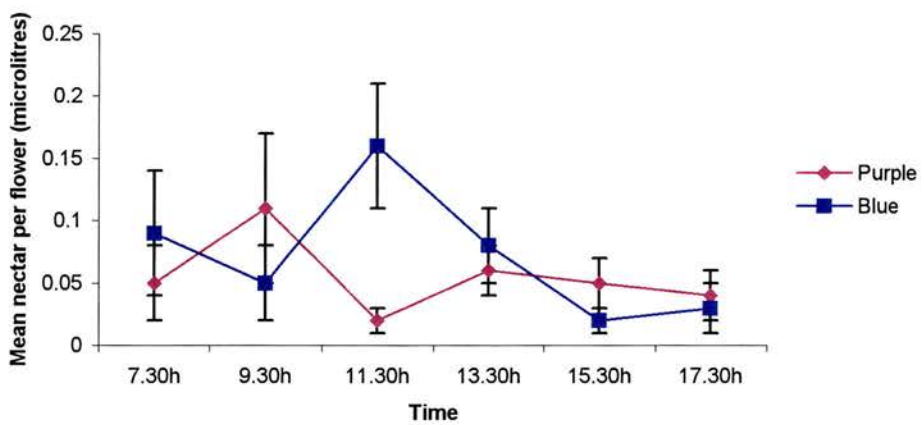


Figure 5.11 Nectar volume in different colour category flowers of *E. vulgare*.

A significant difference was found at 11.30, when mean volume per flower in purple flowers was $0.02 \pm 0.01\mu l$ (n = 20 flowers) and $0.16 \pm 0.05\mu l$ per flower (n =

20) in blue flowers (Mann-Whitney test, using medians, W = 323.0, p = 0.015 (median volume purple flowers 0.015µl, blue flowers 0.060µl)).

5.3.3 Visitation.

In total, 561 insect visits were recorded at the Kent site with the predominant visitors being bumblebees, solitary bees and hoverflies (Figure 5.12). The majority of insects were identified to species level on the wing and the dominant visitors were bumblebees and syrphids.

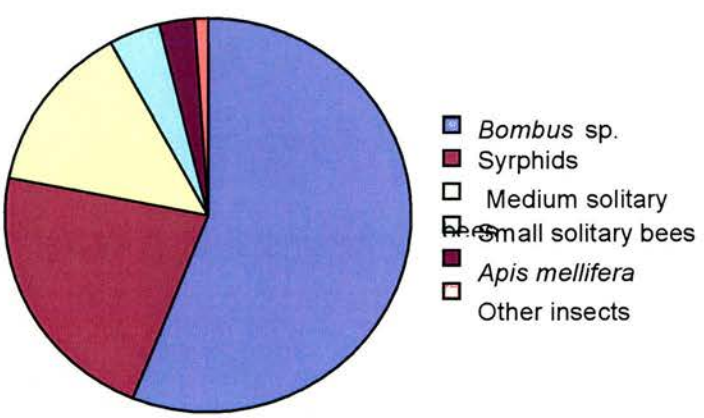


Figure 5.12 Proportion of 561 visitors to *E. vulgare*; *Bombus* sp., 56%, syrphids 22%, medium solitary bees 14%, small solitary bees 4%, *Apis mellifera* 3%, others 1%. (See 5.2.4 for size definitions for solitary bees.)

Of the visits by medium-sized solitary bees, 74 of 77 were made by a single species of halictid; small solitary bees were not captured for further identification.

Visitors categorised as 'other' comprised just 7 of 561 visits and included calliphorid flies, vespid wasps and a single lepidopteran.

Bumblebees fed by landing on the lower lip of the corolla tube and climbing over the stamens and style to probe the base of the corolla tube. Identified species included *Bombus lapidarius*, *B. pratorum*, *B. terrestris* and *B. pascorum*. Visits solely for pollen collection were not noted, although it was assumed that pollen would adhere to the ventral surface of the abdomen and thorax due to the feeding method.

Syrphids, mainly represented by *Episyrphus balteatus* and *Syrphus ribesii*, fed exclusively on pollen, gathering it by clinging to individual anthers and 'dabbing' the anther with the mouthparts.

Bombus sp. were most active between mid-morning and early afternoon (Figure 5.13). The site was east facing and at the base of a cliff and became heavily shaded after 17.00 each day. Observations after this time on a single day suggested an abrupt reduction in the number of foraging bees.

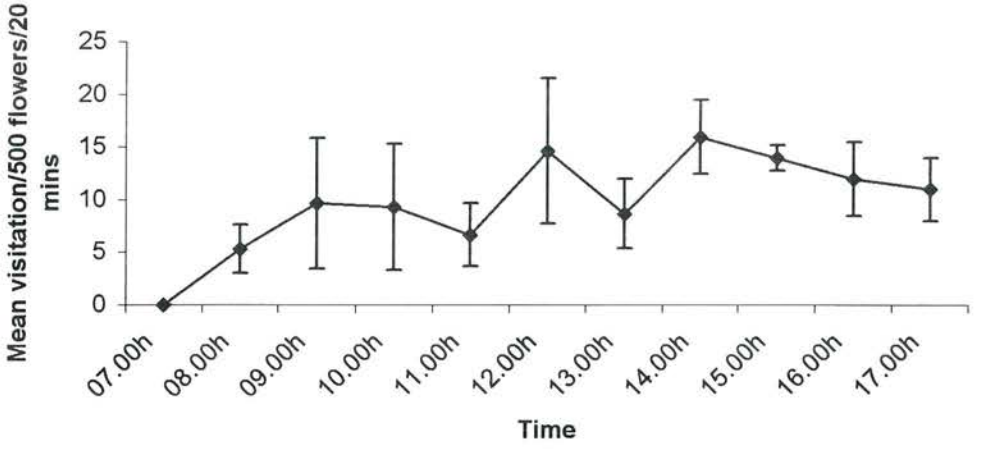


Figure 5.13 Mean visitation rate of *Bombus* spp., over time to focal patch of 500 *E. vulgare* flowers. Each observation period was for 20 minutes within the stated hour.

Visitation patterns across time exhibited few trends for the other dominant visitor groups. Syrphid visitation peaked during the early morning from 08.00 to

09.00 and generally declined thereafter. Medium-sized solitary bees showed increased visitation during late morning and reached peak visitation at 15.00. Thus visitation was relatively low in terms of approaches onto the focal patch per observation period, but all visitors were noted to visit multiple flowers in most trips (no data collected); in particular syrphids could spend the entire 20-minute period within the patch, visiting individual flowers continuously.

5.3.4 Foraging of *Bombus* sp.

In contrast to the variety of visiting insects to *E. vulgare* in the Kent population, nearly all visitors in Fife were bumblebees. Visits of 56 bumblebees to 1318 flowers were scored for colour category, and Figure 5.14 shows that visitation was in accordance with availability of colour phases within the population (χ^2, goodness of fit = 4.61, df = 2, p = 0.1, ns).

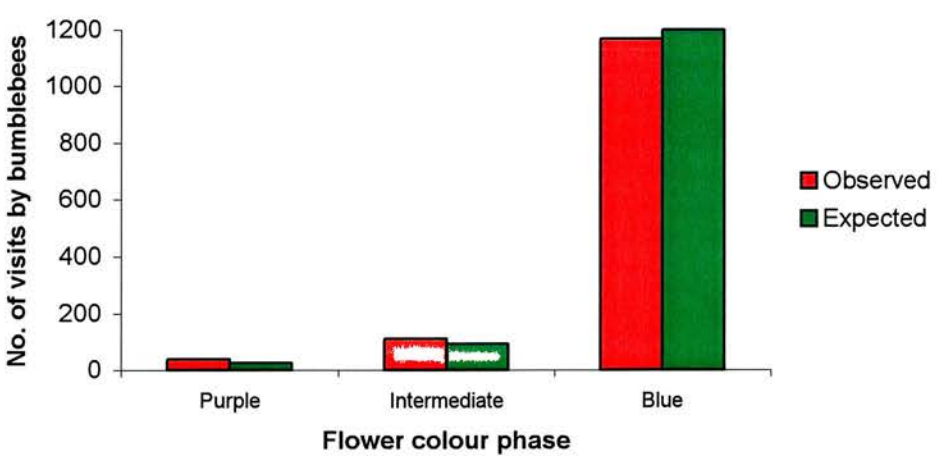


Figure 5.14 Observed and expected visitation of *Bombus* sp. (n = 56 bees) to 1318 flowers of different colour categories on *E. vulgare*.

Hence these bees, predominantly including individuals of *B. pascuorum* but also examples of *B. pratorum*, *B. terrestris* and *B. lapidarius*, appeared to show no response to the colour change in *E. vulgare*.

5.3.5 Manipulations.

i) Mimicked visitation effects.

Full colour change in all flowers occurred within 180 minutes of opening, and therefore of treatment, and in some flowers had taken place within 60 minutes (Figure 5.15), i.e. flowers had moved through both the purple and intermediate stages to blue-purple. Where visitation was prevented and flowers not manipulated in any way, the mean time to change colour was 105 ± 4 minutes (n = 61 flowers). The respective mean times for flowers undergoing pollen removal and nectar withdrawal were 98 ± 7 minutes (n = 27) and 138 ± 7 minutes (n = 15). There was a significant difference in the median time to colour change between treatment groups (Kruskal-Wallis test, $H = 11.72$, $df = 2$, $p = 0.002$, median time to change in nectar withdrawal flowers 120 minutes, and in control and pollen removal flowers, 90 minutes). A post-hoc test showed that there was a significant difference ($p < 0.05$) in rate of change between the nectar withdrawal and both control and pollen removal groups but not between the latter pair of treatments. Thus, removal of nectar retarded the colour change in *E. vulgare*.

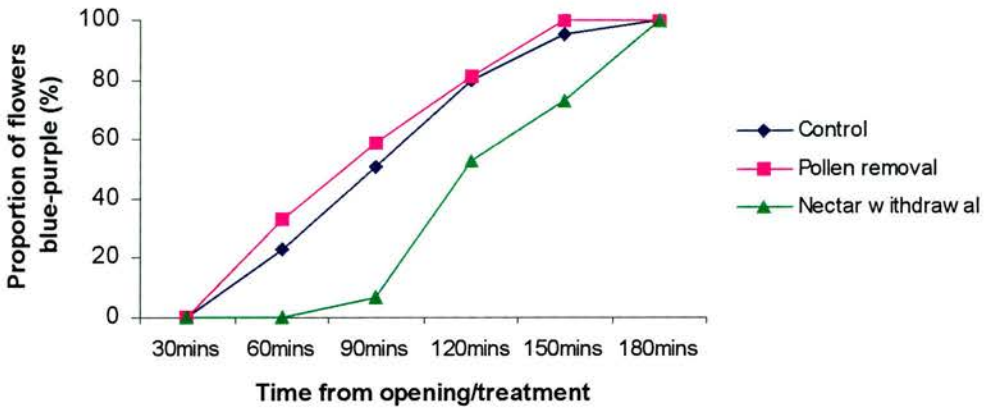


Figure 5.15 Rate of colour change in *E. vulgare* immediately following opening or experimental treatment.

ii) Bagging effects.

All flowers in the control group (n = 20) had changed colour within 240 minutes. In contrast, 7 of 39 (18%) flowers in the netted group were still intermediate in colour after the same time period, as were 9 of 36 (25%) flowers in the foil-covered group (Figure 5.16). Additionally, a single flower in this latter group remained purple after 4 hours. In both the netted and foil-covered groups several flowers had not fully opened after 4 hours; the process of opening appeared to be affected by bagging in general, and through enclosure with foil in particular (although the constriction of the flower during the latter process cannot be ruled out; it was possible to ensure, visually, that the netting was not touching the corolla, but this was not possible with the foil covering).

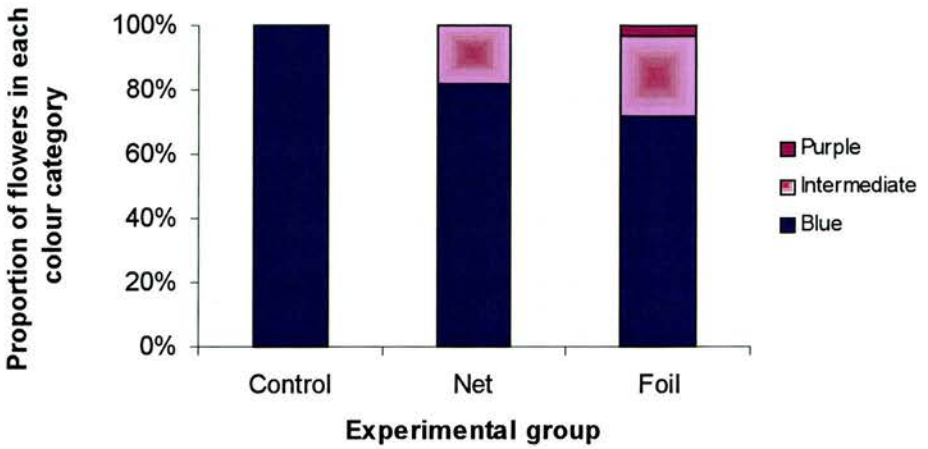


Figure 5.16 Colour change in *E. vulgare*; effect of bagging on colour category 4 hours after opening.

Hence, either an alteration in light availability, or possible other changes caused by bagging (e.g. raised temperature or humidity within exclusion bags, no data collected) led to a slower rate of colour change in *E. vulgare*.

5.4 Discussion.

5.4.1 Colour change.

Colour change in *E. vulgare* is rapid, and its rate and pattern are certainly unrelated to any post-pollination event, although slight retardation in the rate of change was found following withdrawal of nectar. Under natural conditions flowers can change from purple to blue in as little as 1 hour, but more usually take up to 4 hours. Minor differences within this time frame occurred between manipulation groups (Figure 5.15) although the proportion of the mean floral life span in the pre-change phase, following all types of manipulation, was very short.

Within the mean flower life of 3.9 days, the visitation 'window' (assuming maximum daylight of 16-18 hours but allowing a reduction for absence of foragers during early morning/late evening) is perhaps 12 hours per day or 2880 minutes in an 'attractive' state over this whole period (fully open corolla of any colour phase). The mean time in the purple stage (105 minutes), when visitors are excluded, represents only 3.6% of a flower's life; artificial removal of pollen (which could occur in the field through natural visitation or as a result of wind perturbation) resulted in a similar mean time in this phase (98 minutes). A slight extension of length of this colour phase following nectar withdrawal (138 minutes, the additional 33 minutes equating to 1.1% of a flower's life) appears insignificant in the light of this calculation, unless the colour acts as a signal to visitors that ensures increased or additional visitation.

5.4.2 Nectar reward and visitor behaviour.

General nectar characteristics (Figure 5.8) in this study agreed with earlier findings that greater quantities of nectar are available during the morning (Corbet, 1978), although the latter study did not measure nectar in relation to flower colour. Since no difference was found in reward status between pre- and post-change flowers, and the main visitors (*Bombus* sp.) foraged directly in relation to colour phase availability, this study suggests that there is no ecological function to colour change in this plant. Bumblebees from at least 4 species showed no bias towards visiting certain colour phases in *E. vulgare*, and this could be explained by the uniform characteristics of nectar reward across colour phase (Figures 5.9 and 5.10). Foraging bees gain equal reward from all flowers, and therefore do not discriminate between such phases (Figure 5.14).

My findings contradict those of Klinkhamer & de Jong (1990), who found greater visitation to first-day 'pink-blue' flowers than second-day blue flowers and lower nectar volumes in the older flowers. Whilst my data confirm that less nectar is available in second-day flowers (Figure 5.7), my data for nectar volumes in blue flowers (Figure 5.9) were, potentially, collected from both first- and second-day flowers. In both the Kent and Fife populations intermediate flowers (presumably the equivalent of pink-blue flowers) only lasted for a matter of a few hours after opening. Although I also found that second day flowers are predominantly female, Klinkhamer & de Jong (1990) reported that male and female flowers were different colours (although they present no clear data on this point), and found that 'male flowers' received more visits in comparison with 'female flowers' on the same plants. My study indicates that while purple and purple-blue flowers are always male phase, blue

flowers can be male or female on the first day. Colour was not an indicator of sexual phase in my study (Figure 5.6); individual plants had few purple and intermediate flowers, all of which were male and were visited in proportion to their availability in the population. The differences in flower colour described here could be accounted for by genetic differences between the Dutch and British populations or environmental factors e.g. soil properties.

Weiss (1991, 1995a) found that, in a range of plants, floral colour change coincided with a decrease in nectar reward that appeared to be a salient cue to visiting organisms; through learned behaviour foraging was directed away from flowers with reduced reward. Following colour change, visitors were, effectively, 'directed' towards flowers that were usually still sexually viable (Weiss 1991). Further pollination thus achieved could benefit the plant, and the visitors would forage more efficiently, thus ensuring mutual benefit from this 'control' of the plant-animal association. A range of insects have been shown to respond to colour change as a foraging cue, including lepidopterans (Weiss, 1995b); hymenopterans (e.g. Casper & La Pine, 1984; Ne'eman & Nesher, 1995, Obberrath et al. 1999); and dipterans (Caspar & La Pine, 1984). Further instances of this phenomenon in hymenopterans and dipterans are reported elsewhere in this thesis. In all cases the pre-change flowers contained greater levels of nectar (and in some cases, pollen reward), and were visited independently of their distribution in their populations. In two plants where increased nectar was available in post-change flowers (*Malvaviscus arboreus* (Gottsberger, 1971) and *Anchusa strigosa* (a member of the Boraginaceae that changes from violet to blue in under 8 hours (Kadmon et al. 1991)), the main visitors (hummingbirds and bumblebees, respectively) avoided the pre-change, less rewarding flowers. Hence

colour change has been found to be a reliable visual cue in a number of insect-plant interactions.

But in my study the process of colour change in *Echium* did not serve a similar function. Nevertheless, the presence of purple and intermediate flowers could be important; flowers of more than one colour could increase the overall visual attractiveness of individual plants, perhaps enhancing contrast perception for foraging insects. Future work might include comparing approaches and visits to plants both with and without pre-change flowers; if these flowers do influence visitation (either through visual or other cues e.g. olfactory), more visits could be expected to plants containing a mixed display of all colour phases.

5.4.3 Pollen removal and pollination.

Pollen is available in *E. vulgare* upon flower opening and therefore coincides with the purple and intermediate phases; but pollen remains present into the blue phase if visitation has not taken place. In the experiment to mimic visitor effects (Figure 5.15) all insects that approached focal plants were driven away prior to landing; pollen thus remained undisturbed, yet all flowers still changed colour fully (personal observation). In the same experiment, removal of all pollen did not influence the rate of colour change. It is noteworthy that Rademaker et al. (1997) reported that bumblebees could remove up to 44% of available pollen in this species in a single visit.

Activity of visitors that gathered solely pollen (e.g. syrphids and small solitary bees) was not recorded in relation to colour phase visited due to the scarcity of such foraging trips at the Fife site. This type of flower handling is unlikely to transfer

pollen to the stigma as both types of insect landed on individual anthers to feed and were not observed contacting the stigma. As the flowers are effectively protandrous through spatial, if not precise temporal, separation of anthers, pollen deposition and/or subsequent floral reproductive processes can be ruled out as a trigger for colour change. Full colour change took place where visitation was prevented and when all self-pollen was removed; thus pollen was definitely not deposited and the onset and rate of colour change remained unaffected by any pollen-related phenomena.

5.4.4 Sexual phase and colour.

Weiss (1992) reported the coincidence of sexual phase with colour change in a number of species. The timing of the female phase in *E. vulgare* may reveal a loose correlation with colour phase; full extension of the style occurred from the first day onwards and within the blue phase in my study (Figure 5.6). Klinkhamer et al. (1999) described *E. vulgare* as receptive when the style exceeded the longest stamen and the stigmatic lobes diverged, and Corbet (1978) documented a qualitative change in the stigmatic surface at this stage. However, neither of those studies carried out any testing of receptivity, so that unequivocal confirmation of the onset of the female phase in relation to colour change is still required. However, given the lack of any effects of floral manipulation on rates of colour change in *Echium vulgare*, such aspects were judged to be not worth pursuing for present purposes.

5.4.5 Bagging and light.

Floral development and rate of colour change of *E. vulgare* were influenced by bagging flowers (Figure 5.16), and this could reflect the alteration of light characteristics through such actions. Farzad et al. (2002) noted an absence of colour change in *Viola cornuta* where plants were kept in the dark, and linked this to reduced anthocyanin production; light is a known requirement for anthocyanin synthesis and these pigments are critical to effect colour change (e.g. Woltering & Somhorst, 1990).

In *E. vulgare*, rate of colour change was possibly influenced by subtle changes in light intensity as caused by enclosing flowers with pollination bags, and less subtly by using foil; additionally, floral development was retarded where foil was used. I could not tell whether this was due to the constriction imposed by the bagging manipulation or any additional features of the reduced light intensity concomitant with bagging. Alteration of micro-environmental conditions within the different types of exclusion bag, such as raised humidity and temperature, could influence physiological processes.

Further work on this species might include more in-depth investigation of the effects of light cues on colour change; the fact that most flowers under the 'foil' treatment did develop fully, and change colour, suggests that conditions within the pollination bags may not have been uniform. This should be repeated under laboratory conditions where whole plants could be used, light could be unequivocally prevented from entering, and environmental variables could be controlled; this was problematical in the field, particularly under strong wind conditions.

As discussed in Chapter 1, colour changes can be categorised as non-inducible, time-related processes, or as inducible changes resulting from visitor activity and/or post-pollination events within the plant. In *Echium*, the process is non-inducible, and the extreme rapidity of the event and short duration of the pre-change colour, together with an absence of bias in visitation towards these flowers, indicate that in *E. vulgare* colour change does not have an adaptive function. I report elsewhere in this thesis a similar process in the petals in a further member of the Boraginaceae, *Myosotis sylvatica* (Chapter 3). In both instances the pink to blue colour change seems to serve no ecological purpose as a signal to potential visitors and may reflect a purely ontogenetic phenomenon.

Chapter 6 – *Echium judaeum.*

6.1 Introduction.

The family Boraginaceae contains over 2000 species within about 100 genera; they have a worldwide distribution in temperate and subtropical regions with a major concentration in the Mediterranean area (Heywood, 1978). The genus *Echium*, comprising annual, biennial and perennial herbs and shrubs, contains about 40 species in total, and 5 that are found in Israel; *Echium judaeum* Lacaita is locally abundant throughout the country and is also found in southern Lebanon and southern Syria (Zohary, 1966); this author also notes the plant referred to as *E. judaicum*. The species is present on the coastal plain near Haifa, northern Israel and on the lower slopes of the Mount Carmel National Park and surrounding region; here it thrives on disturbed ground with sandy soils. The plant is an annual and, in common with other members of the genus, produces up to 4 nutlets per flower.

Individuals of *E. judaeum* may grow to 50cm and, during March and April, have several flowering stems with terminal inflorescences (Zohary, 1966); newly opened flowers are borne sub-terminally on scorpioid cymes. Corollas are between 25-35mm in length, over 10mm in diameter, and trumpet-shaped; coloration is described as purple in bud and then bluish-violet (Zohary, 1966). Three of the 5 stamens are usually included within the corolla and the remaining pair may be shortly exserted (Zohary, 1966).

Colour change in flowers of the Boraginaceae occurs in at least 19 species from 11 genera (Weiss, 1995a); the only member of the genus *Echium*

listed by Weiss in this database is *E. decaisnei* which displays a red to white change of the androecium. However, colour change from purple to blue in *E. plantagineum* has also been described (Bos et al. 1983) and a change in the UV-patterns of this species has been reported (Kruijer, 1987; Moeliono, 1987). *E. angustifolium*, a species sympatric with *E. judaeum* in the Middle East, has also been noted to change colour from red to purple/violet, although the timing of this change is unclear; Zohary (1966) deemed the change to occur only in withering flowers, whereas Fragman et al. (2001) suggests the flower changes as it matures. Furthermore, colour change has frequently been noted in *E vulgare*; buds are pink and the tubular flowers are described as turning pink-blue during the first day and blue thereafter (Klinkhamer & van der Veen-van Wijk 1999; Klinkhamer & de Jong, 1990; Rademaker et al. 1999; Rademaker et al. 1997; Klemow et al. 2002) (see also Chapter 5).

Echium judaeum has rarely been the subject of published work. In particular, floral colour change has been poorly documented in *E. judaeum*; Zohary (1966) noted the flowers to be purple in bud and blue-violet thereafter, whereas Fragman et al. (2001) reported a pink to blue change. An overnight change has been suggested (A. Dafni; personal communication), as has a change following handling that implies extreme sensitivity (G. Ne'eman; personal communication). This study describes the nature of colour change in this species, together with details of visiting insects, their foraging patterns and flower-handling behaviour.

The species was chosen because of its early flowering season. Spring flowers are present in this part of the Mediterranean region from mid-February onwards and therefore allow field study prior to the field season in the UK.

Additionally, as the suggested nature of colour change in *E. judaeum* has been the subject of conflicting descriptions (see above), full resolution of the process in this plant contributes to our knowledge of colour change in this genus, as well as offering a comparative example to *Echium vulgare* (Chapter 5). Further work on this species was planned for subsequent years but, owing to the worsening political situation in the Middle East, was cancelled following consultation with NERC (Natural Environment Research Council). As a consequence sample size is often rather low.

6.2 Materials and Methods.

6.2.1 Study Site.

A large population (several hundred plants) of *E. judaeum* was studied on a south facing slope of the Mount Carmel National Park, near En Hod, Israel (32° 42′ 40″ N, 34° 58′ 67″ E; GPS, Garmin Olathe, Kansas, US) between 24th February and 5th April 2000. The plants were found growing alongside a rough track on sandy soil (Figure 6.1).

Figure 6.1 Patch of *E. judaeum* near the village of En Hod, Mount Carmel, Israel, April 2000.

The plant community comprised typical Mediterranean phrygana; evergreen shrubs and sub-shrubs up to 1.5m and a range of perennials and annuals. Dominant species included *Cistus* spp., *Satureja thymbra*, *Sarcopoterium spinosum* and *Genista fasselata*, interspersed with *Pinus halepensis* seedlings. The surrounding habitat was part of a mosaic of vegetation

regenerating following the incidence of fire, and bees dominate the visiting fauna. The study site was last burnt in 1974 and is now subject to low levels of grazing by goats (Vulliamy, 2002).

6.2.2 Floral development, longevity and colour change.

Thirty plants were selected at random from the population and a single bud was marked; ten plants were placed into each of three experimental groups:

Control – no treatment and open visitation.

Bagged and manipulated – the whole plant was enclosed in fine nylon netting (aperture < 1mm) to prevent insect visitation. On the morning of opening the marked flowers were probed with a 1µl microcapillary and any accumulated nectar withdrawn. Flowers in this group were further handled when morphometric measurements were taken at each data collection session.

Bagged only – the whole plant was bagged as above and a wire framework was constructed around the marked flower to prevent any abrasion of the corolla by the netting. No further treatment was carried out and the flowers were not handled during measurement procedures (see below).

At two-hourly intervals from 06.30 to 16.30, from the day of opening to floral abscission, each marked flower was checked and the following details recorded.

a) time of opening
b) presence/absence of pollen
c) position of style relative to stamens
d) time of bifurcation of stigma

e) corolla length and width (see Chapter 5, measurement parameters as for *Echium vulgare*)

f) corolla colour compared to a printer's ink chart (Figure 6.2 and see below)

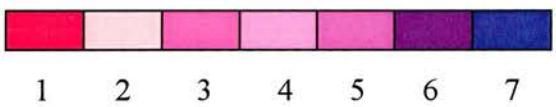


Figure 6.2 Colour matching chart for stages of colour change in flowers of *E. judaeum* (see text for details).

Seven colours were identified; 1, 214U; 2, 211U; 3, 244U; 4, 2577U; 5, 2587U; 6, 2736U; and 7, 2748U. Stages 1 and 7 were only found in buds and withered flowers, respectively.

As colour in fully open flowers of *E. judaeum* was extremely variable (some variation was even noted within individual flowers; see section 6.3.2 for further details) these stages were 'collapsed' into five categories (stages 2 to 6 in Figure 6.2) for the *predominant* appearance of any one flower. A 'colour score' could therefore be assigned to individual flowers on the following basis: -

1 – pink

2 – pink/intermediate

3 – intermediate

4 – intermediate/purple

5 – purple

Scores 2 and 4 thus took account of flowers that exhibited a particularly 'patchy' appearance that did not allow immediate categorisation into the upper or lower score.

A mean daily 'colour score' was produced for each treatment group by calculating the mean score for each flower across data collection times and combining the results to give a 'mean of means'.

These categories were collapsed further to enable rapid assessment of colour when recording multiple insect visits; pink (stage 2), intermediate (stages 3 and 4 combined), and purple (stages 5 and 6 combined). This allowed simple categorisation of all flowers even under varying light conditions and could, in particular, be utilised when following visitors to establish foraging patterns.

6.2.3 Nectar profiles.

Nectar volume and concentration were measured across time of day, and in relation to flower age and colour, for both open and bagged flowers. Individual flowers could not be re-sampled as repeated nectar withdrawal could not be effected without causing damage to floral tissue around the point of insertion into the calyx. Therefore, nectar was withdrawn by removing the corolla and opening out the corolla tube by tearing apart the petal tissue from lip to base; nectar was thus exposed as droplets (usually two) near the corolla base. A small number of flowers contained high volumes of nectar with concentrations <10%; it was thought that rainwater had entered the corolla to produce such dilute readings and these were excluded from any analysis. Greater numbers of flowers were 'empties' and some contained just a trace of nectar (<1mm in the microcapillary and, therefore below 0.03µl volume); in the latter instance a volume of 0.015 was assigned for subsequent analysis.

i) Nectar and flower age – cohorts of 100 buds on at least five 'open' or 'bagged' plants were marked prior to anthesis. Nectar volume and concentration were recorded for a sub-sample of open flowers from each treatment group between 09.00 and 11.00 on four successive days.

ii) Nectar characteristics over time – on each of three days at least 10 flowers were sampled for volume and concentration at two-hour intervals between 07.00 and 17.00. Open flowers were chosen at random and bagged flowers were taken from 10 plants enclosed at dawn on the day of data collection.

iii) Flower colour – where flower colour could be easily assigned to one of the three 'collapsed' categories (see above), data from (ii) were used.

6.2.4 Environmental data.

Microenvironmental variables were recorded for the duration of nectar profile measurements. Temperature and relative humidity were measured (see Chapter 2) at hourly intervals between 07.00 and 17.00.

6.2.5 Visitation.

Insect visitation was observed across four days to a focal patch of ten plants that displayed in excess of 300 flowers, to establish the identity of visitors and any temporal patterns of visitation. A visit was scored as a landing that incorporated a feeding bout either for nectar or pollen and/or involved contact with floral reproductive parts. Visits were recorded for 30 minutes in every hour

between 06.30 and 17.30; initial observations had confirmed an absence of insect activity before and after these times.

Where possible, visitors were identified to species level on the wing and consisted mainly of bees. Categories of bee, based on body length, were used where species could not be determined. Small solitary bees (< 8mm) visited solely for pollen; medium solitary bees (approximately 8-14mm) collected both pollen and nectar; and large solitary bees (>15mm) appeared to feed on nectar only.

6.2.6 Visitation to manipulated patches.

Small, adjacent patches of *E. judaeum* were manipulated to offer different colour cues to visitors. Focal patches of approximately $0.5m^2$ were selected; the distance to the nearest neighbouring patch of *E. judaeum* was standardised. Three separate pairs of 'colour choice patches' (with flower number per patch balanced) were created by removal of certain colour flowers. Data were collected on a single day for each of these pairs of manipulations.

i) Pink vs. Natural – all purple and intermediate flowers were removed from one patch to produce a 'pink only' display. The 'natural' patch retained flowers of all colour categories in normal frequencies.

ii) Pink vs. Purple – all intermediate and purple flowers were removed from one patch to ensure a 'pink only' display, and all pink and intermediate flowers removed from the other patch to produce a 'purple only' display.

iii) Purple vs. Natural – flowers were removed as above to produce these display categories.

All visitors were then recorded for 20-minute periods per patch in each hour between 08.00 and 16.00. A visit was scored as an arrival on the patch by any insect that led to a landing on a flower of any colour.

6.2.7 *Apis mellifera* – foraging.

Detailed data of the activity of the most numerous visitor, the honeybee (*Apis mellifera*, see below), were collected. Foraging patterns, including reward sought and colour of flowers visited, were recorded over three successive days. All honeybees visiting a focal patch of four plants, displaying approximately 100 flowers, were observed for 30 minutes in each hour between 07.00 and 17.00. Residence time on the patch was noted, together with foraging time at individual flowers; whether nectar, pollen or both were collected; and the colour of each flower visited or inspected (pink, intermediate or purple; see above). 'Inspections' were scored as non-landing 'visits' within 1-2cm of a flower.

The possible influence of colour change on foraging choice was also investigated. An individual plant was bagged overnight to ensure colour change in all newly opened flowers (for details, see below); the netting was removed the following morning and visitation by *A. mellifera* recorded between 08.00 and 16.00. All inflorescences displayed at least two fully open, purple flowers at the start of observations; flowers that opened during the data collection period (and were, therefore, pink) were removed carefully with forceps. Thus, visitation

(which was scored as above) was noted as being to either 'young', first-day flowers, or 'old', second/third-day flowers; this was easily assessed because the youngest flowers were always closest to the apex of the cyme.

6.2.8 Seed set.

Seed set was measured in bagged plants and in plants open to natural visitation. Thirty plants, with no open flowers, were selected at random and marked; additionally, a patch of ground approximately 4x2 metres and containing at least 20 plants of similar developmental stage was enclosed with nylon netting to prevent visitor access.

After 28 days the marked and bagged plants were re-visited and seed set counted by randomly selecting 15 dried calyces on each plant (these were taken from different cymes, and different positions on the stem where possible) and counting the number of nutlets in each calyx. As a further control, for each marked 'open' plant a nearby plant (<1m) was chosen at random and the number of nutlets in each of 15 calyces counted.

6.3 Results.

6.3.1 Floral development and longevity.

Flower opening was recorded in open and both bagged treatment groups throughout the day and was not, therefore, subject to any temporal pattern. A single cyme usually displayed (from the apex downwards in succession) one or more wilted flowers that were, apparently, held in place by still turgid styles, one wilting flower, one fully turgid flower, and one bud.

Under conditions of natural visitation, flowers lasted 2-4 days (mean longevity 3.1 ± 0.3 days, n = 8). Flowers that were manipulated had an identical range of longevity (mean 3.3 ± 0.3 days, n = 8). However, in contrast, bagged and unmanipulated flowers lasted from 3 to 5 days (mean 4.5 ± 0.2 days, n = 10) (Figure 6.3).

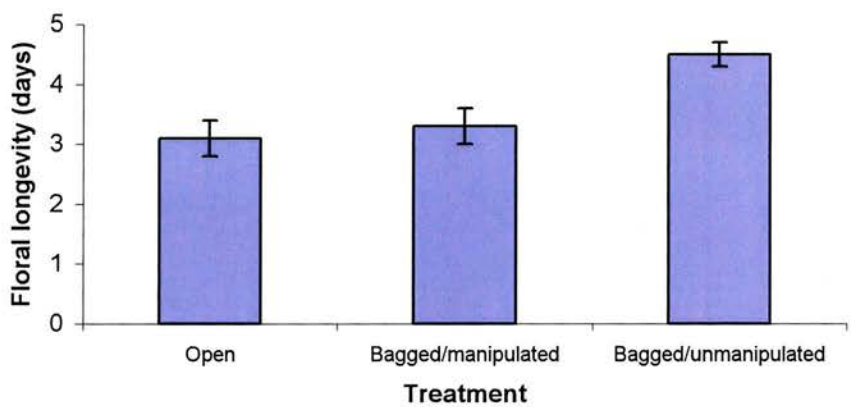


Figure 6.3 Floral longevity in *E. judaeum*; see text for details of treatment groups.

Flowers were hermaphroditic and protandrous. Dehiscence was synchronous with flower opening; pollen was blue-purple in colour and was frequently harvested rapidly by visiting insects. In the 'open' group pollen was

always fully depleted before the end of the second day; some depletion occurred in all flowers on the first day, and, in one flower, all pollen had been removed within 4 hours of opening. In the absence of visitation, pollen remained on the anthers beyond the second day, although was also evident as a 'dusting' on the inner surface of the corolla (presumably having been dislodged by wind/mechanical perturbation). The pollen faded to pale blue in the bagged groups after two days.

Bifurcation of the stigma had usually occurred within 24 hours of flower opening, by which time the style had reached a position level with, or slightly beyond, the 2 exserted stamens. Growth of the style was often rapid on the first day; increases of several millimetres in length took place between sampling times (personal observation, no data collected).

Flower size varied between 25 and 32mm in length (mean 28.8 ± 0.3mm) and 12-19mm in width (mean 15.7 ± 0.3mm) (n = 32 flowers), with maximum size being reached on the first day in 10 flowers, on day 2 in 21 flowers, and on day three in a single flower.

6.3.2 Colour change.

Flower colour, and colour change, in *E. judaeum* were extremely variable. All flowers were pink-red in bud (stage 1). In the field the overall impression of the population was that pink (stage 2) fully open, turgid flowers predominated but with considerable numbers of purple-blue (stages 5 and 6) flowers and wilted blue (stage 7) flowers (Figure 6.4). A range of intermediate (pink to purple, stages 3 and 4) flowers was also present, with varying colour

distribution within the flowers. Colour change appeared to occur in a proximal-distal direction from the base of the corolla but, occasionally, amorphous blue-purple 'blotching' was apparent around the lips of the corolla.

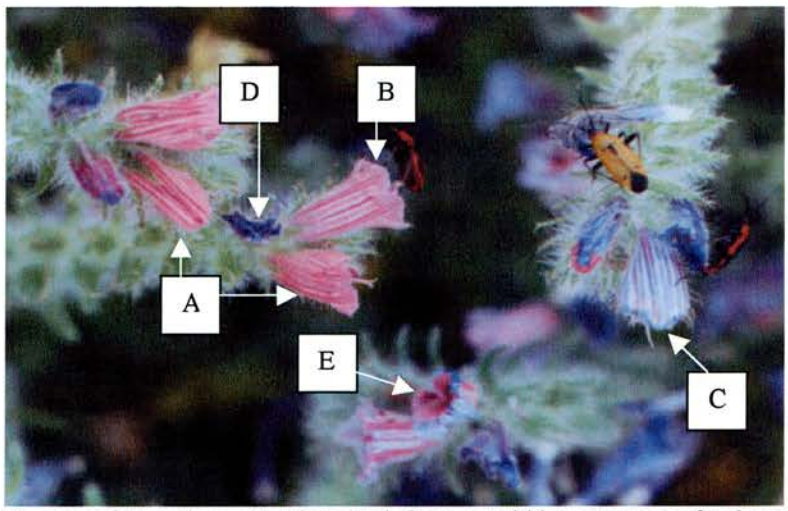


Figure 6.4 Flowers of *E. judaeum* (under attack from capsid bugs); range of colour categories are visible that illustrate variability in this species. Newly opened pale pink flowers (A) predominate, with intermediate (B) and purple (C) flowers also evident. Withered blue (D) flowers can also be seen and one flower (E) is pink with blue blotches around the mouth of the corolla.

Colour change in this species could be induced by bagging (Figure 6.5); in the bagged group where no manipulation or handling took place, all flowers (n = 10) had changed to purple within 4 hours of opening and remained purple thereafter. Of flowers that had been bagged and handled, seven of eight focal flowers changed to purple within the same time span. Although remaining predominantly purple through to wilting, these flowers displayed more variation in colour; all flowers being described as part or wholly intermediate at some stage of their lives. The remaining flower in this group was pink to intermediate throughout. In the control group (n = 8) a single flower underwent the rapid pink-purple change described above. Of the remaining flowers, four retained the pink coloration (stage 2) seen just after opening, through until wilting; three flowers were predominantly pink to intermediate throughout the observation period.

Although the sample size is low here, the finding is supported by the fact that the rapid pink to purple change was also observed in those plants that had been bagged for seed counts. The uniform purple coloration of all mature flowers within the bagged enclosure was not noted elsewhere at the field site for the entire duration of the study.

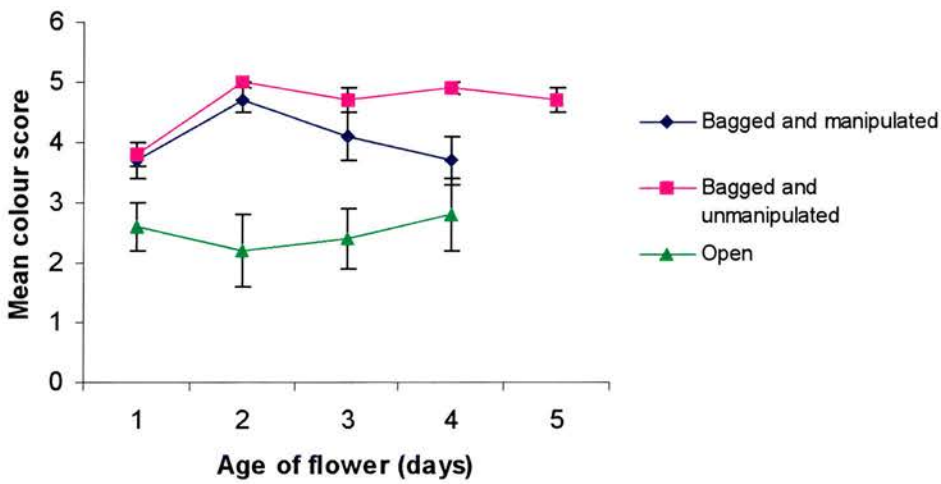


Figure 6.5 Mean daily 'colour scores' for flowers of *E. judaeum* in different treatment groups. Each data point represents a 'mean of means' for the total number of flowers (see text for sample sizes) across all data collection times for each day.

An unusual bi-directional colour change was also seen in *E. judaeum*; where exclusion bagging was *removed* again after 24 hours, first-day purple flowers could change from purple back to pink. All flowers (n = 33) on separate cymes of a single plant, bagged overnight for the *Apis* foraging experiments, exhibited this 'reverse' change the next day. This change was neither as rapid nor as 'complete' as the pink to purple, within 4-hour, change described earlier. The secondary/reverse change occurred over at least 8 hours and was first noticed three hours after removal of the exclusion netting as a subtle early 'pinkening' of the lips of the corolla tube and, in particular, of the nectar guides/veins. To the human eye, a qualitative difference between the youngest and the next youngest flower on a given cyme was evident in 20 pairs of flowers after 5 hours. The

flowers never regained the 'solid' pale pink coloration of newly opened flowers, or of those that were not bagged and remained pink for their whole life span. However, they were clearly showing some reversal of the original age-related bagging-induced colour change.

6.3.3 Nectar profiles.

i) Flower age.

Nectar was secreted for 2-3 days in open flowers and up to 4 days in bagged flowers and its characteristics are illustrated in Table 6.1 and Figures 6.6 and 6.7. Data were collected between 08.00 and 10.30 on consecutive mornings. The low sample size, in particular in the bagged group, was due to the destruction of bagged and marked plants by grazing goats; the entire cohort of marked flowers had been used by the fourth day and whether nectar was produced after this time is not known.

Day	Parameter measured	Open	Bagged
1	Volume (μl)	1.54 ± 0.20 (n = 23)	0.94 ± 0.22 (n = 12)
	Concentration (%)	24.8 ± 1.0 (n = 23)	30.5 ± 1.1 (n = 15)
2	Volume (μl)	2.75 ± 0.29 (n = 20)	1.88 ± 0.40 (n = 9)
	Concentration (%)	22.5 ± 1.2 (n = 20)	23.4 ± 1.8 (n = 9)
3	Volume (μl)	3.12 ± 0.34 (n = 34)	5.81 ± 1.42 (n = 16)
	Concentration (%)	17.5 ± 0.6 (n = 30)	28.1 ± 3.0 (n = 15)
4	Volume (μl)	- (n = 10)	8.22 ± 1.79 (n = 10)
	Concentration (%)	-	22.3 ± 5.5 (n = 10)

Table 6.1 Nectar characteristics of *E. judaeum* with age of flower.

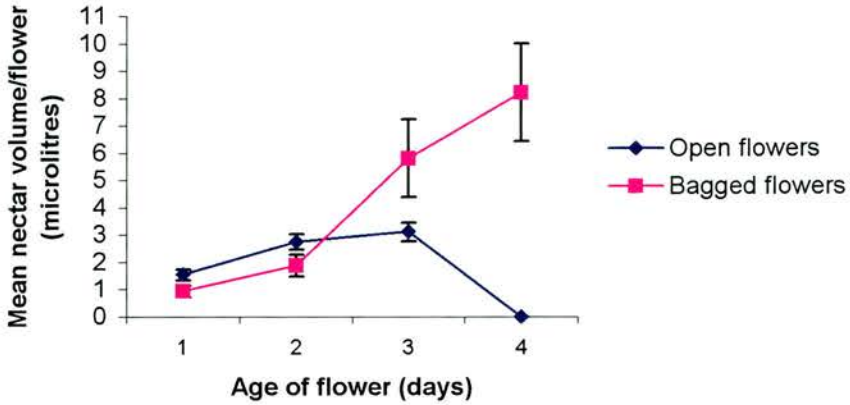


Figure 6.6 Nectar secretion in *E. judaeum*.

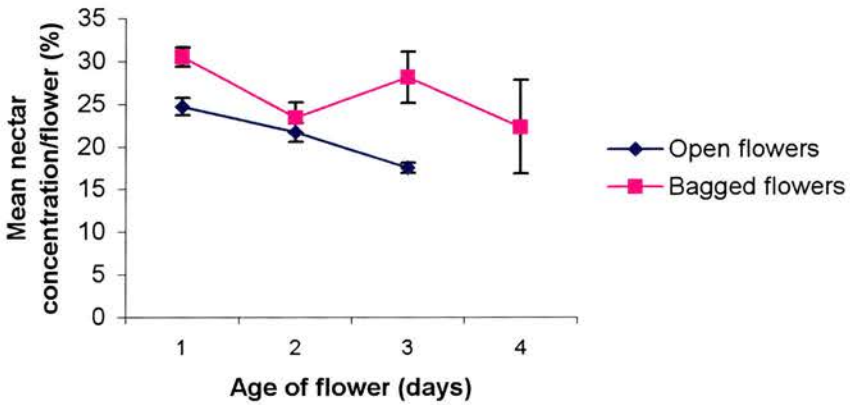


Figure 6.7 Nectar concentration in *E. judaeum*.

ii) Time of day.

Nectar availability in open flowers declined during the middle of the day and rose again during late afternoon (Figure 6.8), presumably mainly as a result of temporal trends in visitation patterns (see below). Total mean volumes were below 1µl at all times and varied from 0.18 ± 0.08µl (n = 30 flowers) at 11.00 to 0.37 ± 0.10µl (n = 40) at 17.00. In bagged flowers, nectar availability increased throughout the day to 15.00 (mean volume 4.4 ± 0.5µl; n = 40 flowers) and decreased marginally thereafter (Figure 6.8). There was a significant difference in nectar volume across time in bagged flowers (one-way ANOVA; $F = 13.11_{5,}$

$_{200}$, $p < 0.001$) but not in open flowers (one-way ANOVA; $F = 0.70$, $_{5,206}$, $p = 0.625$).

Nectar concentration in open flowers was greatest at 11.00 (mean 34.7 ± 2.7%; n = 9) in open flowers, which coincided with the hottest part of the day (24.5 ± 0.7°C). In bagged flowers, mean nectar concentration increased to 37.9 ± 2.0°C (n = 30) by 11.00 and remained close to this figure for the remaining data collection periods (Figure 6.8).

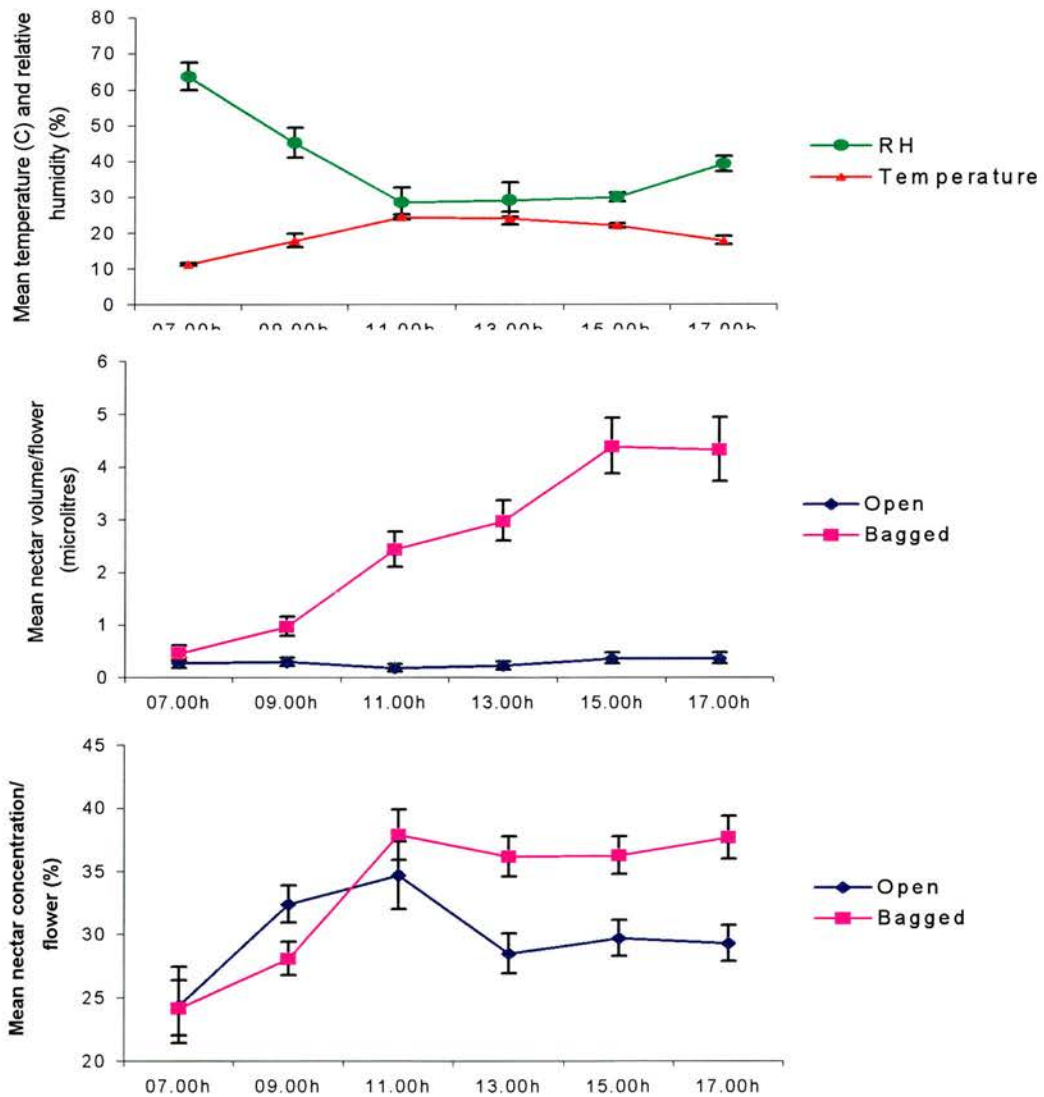


Figure 6.8 Top; mean environmental variables at En Hod, Mount Carmel, 24th February to 6th March 2000. Middle; mean nectar volume in *E. judaeum*. Bottom; mean nectar concentration in *E. judaeum*. All data summed for random mixed sample of flower ages.

iii) Flower colour.

The patterns for nectar characteristics varied between flower colours but showed the same overall trend between open and bagged flowers (Figure 6.9) and the results are summarised in Table 6.2.

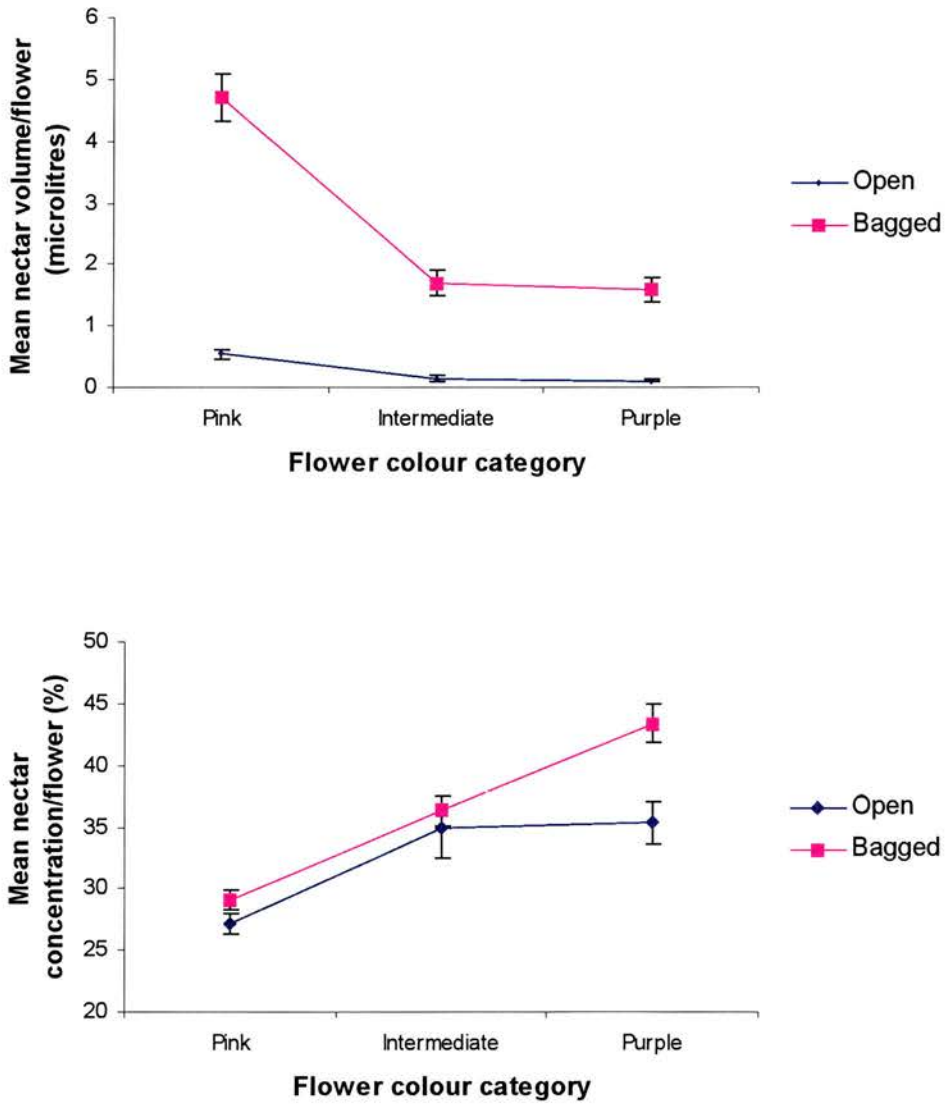


Figure 6.9 Nectar characteristics of different colour category flowers in *E. judaeum*. Top; mean nectar volume per flower. Bottom; mean nectar concentration per flower.

Group/colour		Volume (µl)	Concentration (%)	Kruskal-Wallis
Open	**Pink**	0.54 ± 0.08 n = 79	27.2 ± 0.8 n = 54	p<0.001
	Intermediate	0.14 ± 0.04 n = 78	35.0 ± 2.5 n = 22	
	Purple	0.11 ± 0.02 n = 53	35.4 ± 1.7 n = 18	
Bagged	**Pink**	4.7 ± 0.4 n = 81	29.1 ± 0.8 n = 78	p<0.001
	Intermediate	1.7 ± 0.2 n = 74	36.3 ± 1.2 n = 57	
	Purple	1.6 ± 0.2 n = 51	43.4 ± 1.5 n = 46	

Table 6.2 Nectar characteristics of different floral colour phases of *E. judaeum*.

Hence pink flowers of *E. judaeum* contained greater nectar reward than either intermediate or purple flowers in both open and bagged flowers.

6.3.4 Visitation.

Over 2000 visits were recorded over 6 days to a focal patch of the *Echium* plants; observation was abandoned on two days due to adverse weather conditions, but records were obtained on at least four days for each of the hourly data collection periods. Bees comprised over 94% of all visitors (Figure 6.10), with nearly three-quarters of all visits made by honeybees (*Apis mellifera*). Individuals of this species began to arrive at around 07.00; peak numbers were present between 09.00-10.00, with visitation trailing off by late afternoon (Figure 6.11).

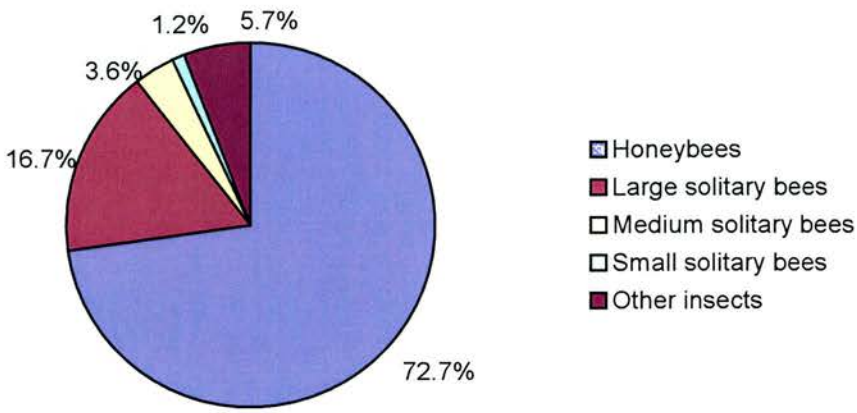


Figure 6.10 Proportion of visitors to *E. judaeum*; for details of bee size categories and identity of 'other insects', see text.

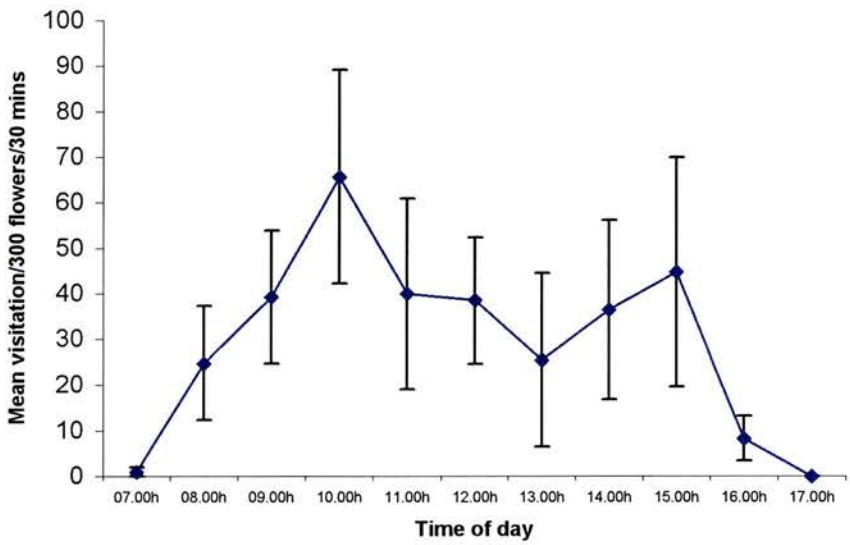


Figure 6.11 Daily visitation pattern of *A. mellifera* to *E. judaeum*. Mean visitation rate over a minimum of four 30-minute observation sessions.

Large solitary bees were most abundant in early morning and late evening (Figure 6.12) and identified taxa included the megachilid *Chalicodoma siculum*, a cuckoo bee *Melecta* sp., and *Anthophora* spp. The *Anthophora* appeared to be foraging for nectar only; individuals either hovered in front of the corolla and inserted their tongue into the base of the corolla, or occasionally climbed into the corolla. The former behaviour was not observed in *Melecta* or *Chalicodoma siculum* and, presumably, was dependent on tongue length in relation to corolla

depth; both these bees contacted floral reproductive parts and could, therefore, be responsible for pollen transfer.

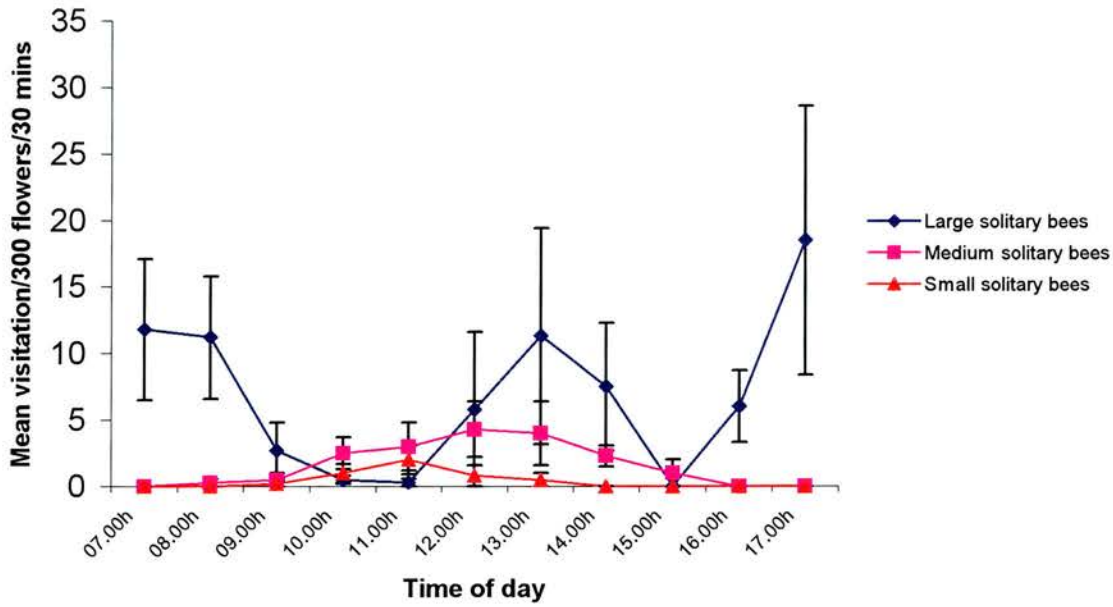


Figure 6.12 Daily visitation patterns of solitary bees to *E. judaeum*. Mean visitation rate over a minimum of four 30-minute observation sessions. See text for distinction between size of visitor.

Medium solitary bees showed increased visitation during the middle of the day and early afternoon; identified genera included *Eucera* and *Synhalonia*, with *Melliturga praestans* and *Mellitioides mellitioides* becoming especially common later in the study period. Flower-visiting behaviours included landing on the exserted stamens and dabbing at the anthers with their mouthparts, as well as landing and crawling into the corolla. Although not strictly a flower visitor, a further medium-sized solitary bee was noted collecting withered and abscised flowers from the focal patch. This bee is awaiting full identification but has been placed in the tribe Osmiini. It used the collected flowers to line nest holes at a nearby nest aggregation (S. Roberts; personal communication).

Small solitary bees, commonest between 10.00-12.00, were mainly halictids and andrenids, and accounted for just over 1% of all visits; these insects

fed solely on pollen by alighting on the anthers and were not noted to contact stigmas throughout the study.

Other insect visitors (5.7%) consisted of bee flies (*Usia bicolor* and other bombyliids), dipterans, and lepidopterans including the nymphalid *Cynthia cardui*, the pierid *Gonepteryx cleopatra*, the papilionid *Papilio machaon* and the sphingid moth *Macroglossum stellatarum*. The last of these, a diurnal forager, was responsible for over 90% of visits in this 'other' category, due to foraging by a few individuals during early and mid-morning on three days.

6.3.5 Visitation to manipulated patches.

i) Pink vs. Natural.

The artificial pink patch received fewer visits in the majority of observation sessions (Figure 6.13); mean number of visits to pink = 19.0 ± 1.7 (n = 8 periods), and to natural = 23.5 ± 2.0 (n = 8). However, the differences between the median number of visits to patches were not quite significant; Mann-Whitney W = 50.0, p = 0.0649 (median visits to pink = 18, natural = 23).

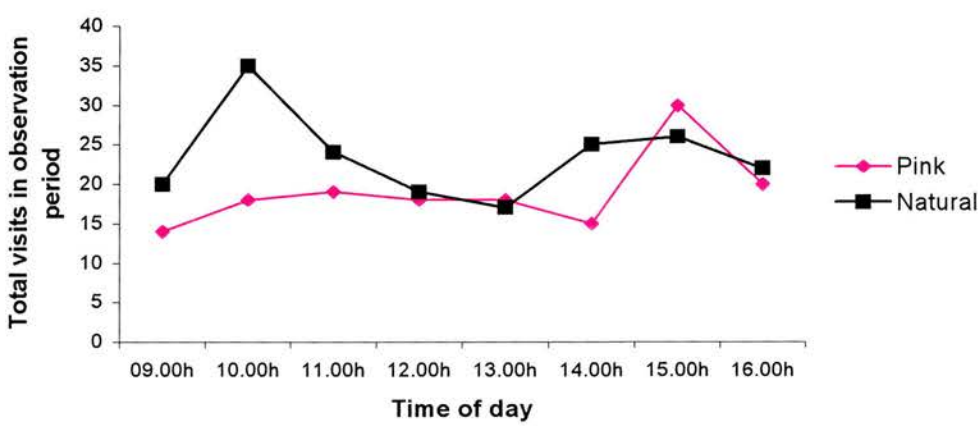


Figure 6.13 Total insect visitation to manipulated patches of *E. judaeum*, 2/4/2000.

ii) Pink vs. Purple.

The mean number of visits to the pink patch was 17.9 ± 3.3 (n = 8 observation periods) and the mean number of visits to purple patches was 16.8 ± 2.6 (n = 8) (Figure 6.14). There was no significant difference between the mean number of visits to patches, (2-sample t-test; T = 0.27, p = 0.79). However, a notable trend was a 'switch' from visits predominantly to purple flowers to predominantly to pink flowers; this coincided with the lifting/breaking of cloud cover after 10.00. The possible significance of the role of altered light conditions and this influence on colour choice is discussed later.

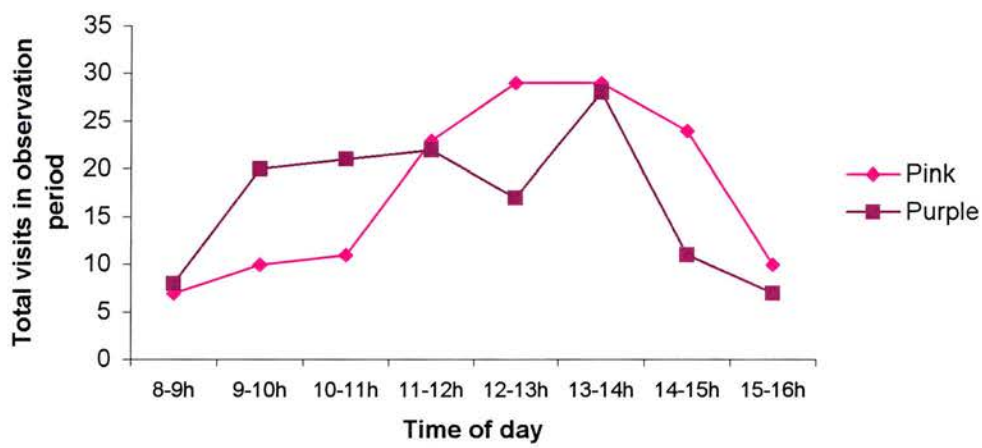


Figure 6.14 Total insect visitation to manipulated patches of pink and purple-flowered *E. judaeum*, 3/4/2000.

iii) Purple vs. Natural.

The mean number of visits to the purple patch was 18.7 ± 3.6 (n = 7 observation periods) and the mean number of visits to the natural patch was 19.7 ± 3.7 (n = 7) (Figure 6.15). There was no significant difference between the median number of visits to patches; Mann-Whitney W = 49.5, p = 0.7491 (median visits to purple = 17; natural = 21).

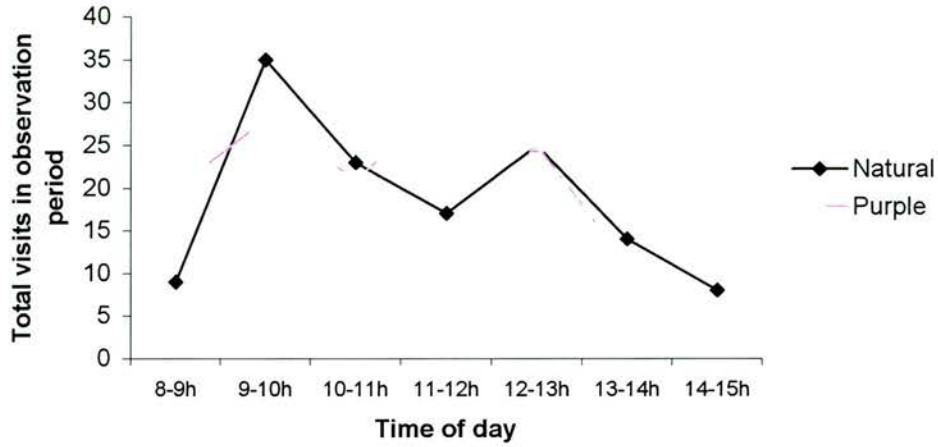


Figure 6.15 Total insect visitation to manipulated patches of *E. judaeum*, 5/4/2000.

Further replicates of these manipulations were prevented by poor weather conditions.

6.3.6 *Apis mellifera* – foraging.

One hundred and eighty-one foraging trips by honeybees were observed to a focal patch of *E. judaeum*. Of these, 81 were for nectar only, 38 for pollen only, 32 trips involved collection of both nectar and pollen, and 30 trips comprised flower inspection with no landing. The trips incorporated visits to 468 flowers and inspections of a further 612 flowers; in both cases, the majority of flowers visited were pink (Figure 6.16).

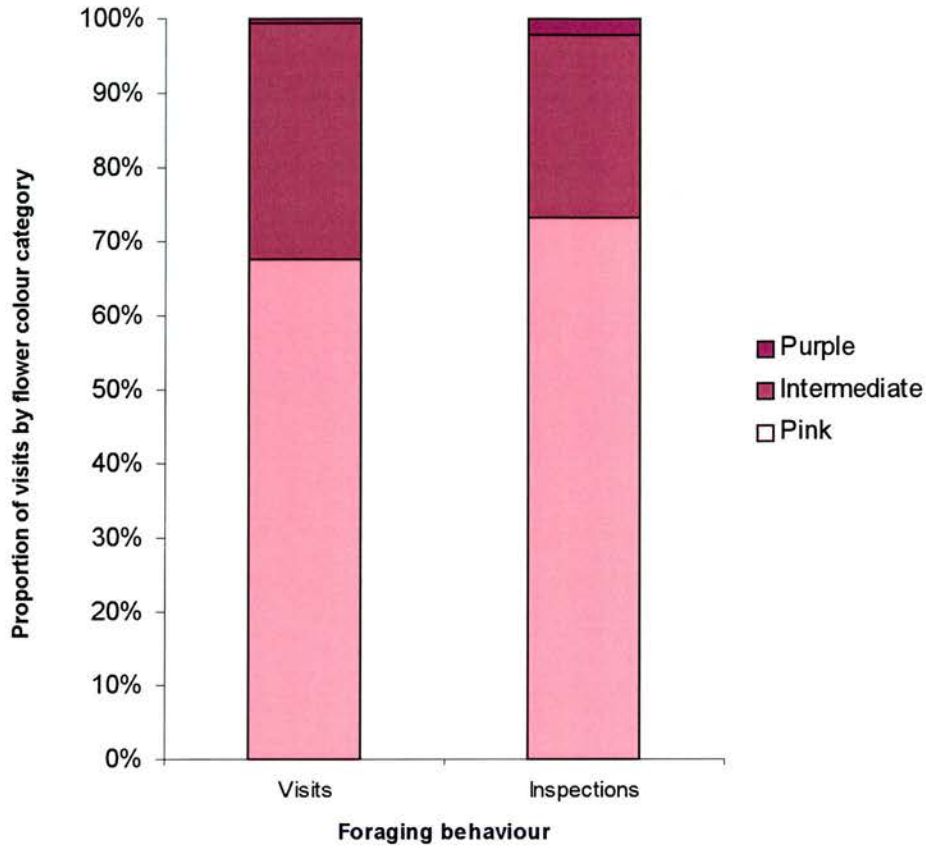


Figure 6.16 Foraging behaviour of *A. mellifera* (n = 181 foraging trips) in relation to flower colour at focal patch of *E. judaeum*. Visits; 316 pink flowers: 148 intermediate flowers: 3 purple flowers. Inspections; 449 pink: 149 intermediate: 14 purple.

Mean residence time for an individual bee on the focal patch was 42.1 ± 3.2 seconds; nectar visits lasted 7.1 ± 0.2 seconds per flower, pollen visits 9.3 ± 1.0 seconds per flower, and pollen + nectar visits 18.8 ± 2.8 seconds per flower. Up to 15 flowers were visited during each trip (mean 2.6 ± 0.2 flowers) and up to 17 flowers inspected (mean 3.4 ± 0.2 flowers). The majority of individual flower visits were for nectar (336 visits; 72%), followed by pollen only (118 visits; 25%), and then visits for both nectar and pollen at the same flower (14 visits; 3%).

Forty-six foraging trips by *A. mellifera* were observed between 08.00 and 16.00 on 4th April 2000 to a manipulated patch of *E. judaeum*. Thirty-three

flowering cymes that had been bagged overnight displayed at least two fully turgid purple flowers; on each the youngest, first-day flower exhibited commencement of 'reverse' colour change to a more pink appearance during the observation period. Four cymes had a third viable purple flower and thus the actual ratio of available flowers was 33 purple→pink: 37 purple.

Bees visited between 1 and 32 flowers on each trip (mean 8.7 ± 1.1 flowers); of 400 flower visits, 264 were made to purple→pink flowers and 136 to purple flowers. Eight bees visited more purple than purple→pink flowers, 6 bees visited equal numbers of the different category flowers and the remaining 32 bees visited more purple→pink than purple flowers. Fourteen bees made their first visit on the focal patch to purple flowers, with 11 then switching to a purple→pink flower for their second visit. Of 32 bees that visited a purple→pink flower first, 25 carried on to forage at purple→pink flowers for their second visit.

Figure 6.17 illustrates the observed and expected visitation to the manipulated patch; the observed visitation was significantly different from that expected given the availability of flower colour categories on the focal patch ($\chi^2 = 23.652$, df = 1, $p < 0.001$).

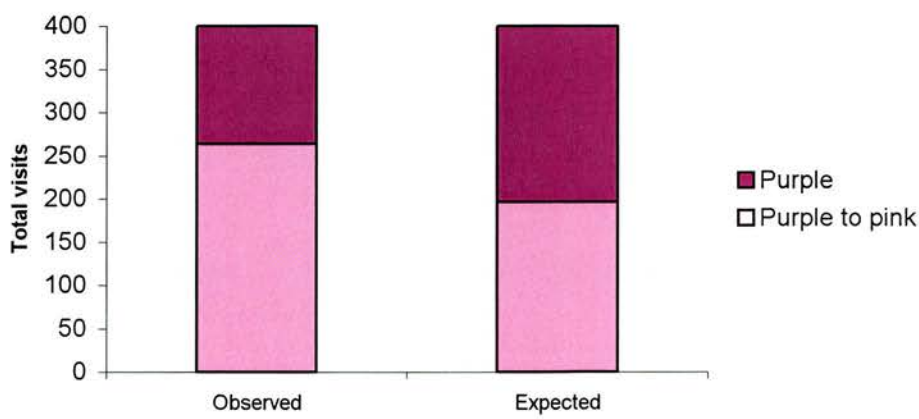


Figure 6.17 Visitation of *A. mellifera* to manipulated patches of *E. judaeum*. 400 flower visits were recorded from 46 foraging bouts in the ratio 264 purple→pink: 136 purple.

Therefore, where the 'reversed' change of colour had been induced, *A. mellifera* visited in a non-random pattern. The youngest flowers that displayed the gradual reversal to pink were chosen more frequently than expected from their availability on the focal patch.

6.3.7 Seed set.

Three tagged plants in the 'open' group failed to develop; therefore a total of 27 plants, and the same number of additional controls, were sampled, whilst in the bagged group, 18 plants were sampled. Mean seed set per flower was calculated for each plant and a mean of means used for further analysis; seed set is summarised in Table 6.3.

Treatment	No. of flowers	Total seed count/maximum seed set	Range of mean no. of seeds per plant	Overall mean	% seed set
Open	405	995/1620	1.6 – 3.1	2.5 ± 0.1	61
Open (additional control)	405	1072/1620	1.8 – 3.3	2.6 ± 0.1	66
Bagged	270	121/1080	0.1 – 0.9	0.5 ± 0.1	11

Table 6.3 Seed set in *E. judaeum*; count of seeds from 15 flowers per plant in each treatment group.

There was no significant difference between mean seed production in open and control groups; open 2.5 ± 0.1 seeds per flower (n = 405 flowers) and control 2.6 ± 0.1 seeds per flower (n = 405), (2-sample t-test; T = -1.70, p = 0.096). However, bagged flowers produced only 0.5 ± 0.1 seeds per flower (n = 270 flowers) and there was a highly significant difference in mean seed set between bagged and the combined open and control treatments, (one-way ANOVA; $F = 199.10_{2,69}$, $p < 0.001$).

The difference is largely because, of 270 flowers in the bagged group, 208 failed to set seed at all, although at least one flower on every plant did produce at least one seed. On three mornings between initial set-up and seed count (the enclosure was checked each morning), single bees (perhaps having emerged from nests in the soil) were found within the netting, although none was seen visiting the enclosed plants. Therefore, it is possible that *E. judeaum* is self-incompatible and that the 62 flowers that produced seeds were visited by trapped bees. The probability of this being the case, given multiple flowering on cymes of 18 plants over 28 days (and subsequent random choice of both cyme and flower position when counting seeds), is considered to be low; it is more likely that a degree of self-compatability exists.

6.4 Discussion.

Colour change in flowers of *E. judaeum* has been shown to be 'reversible', a character not previously reported in any other plant species. The initial change may be simply time-related, but it is clearly not the result of post-pollination events. However, the faster and potentially 'reversible' change can be induced by bagging the flowers, as discussed below, and reversed by unbagging them.

6.4.1 Normal 'forward' colour change.

Colour change in *Echium judaeum* from pink to purple in populations open to natural visitation is variable and does not take place in all flowers; however, where visitation is prevented this change takes place in all flowers in approximately 4 hours. Differential levels of visitation, and/or an aspect of visitor handling behaviour, may be linked to colour change in *E. judaeum*.

As discussed in Chapter 1, an ecological benefit may result from floral colour change. Post-change flowers (whether having changed 'naturally' over time, or been induced as a result of post-pollination events) may be visited less frequently than viable, pre-change flowers; thus favouring further pollination opportunity for the plant and efficient foraging by visitors (Gori, 1983; Weiss, 1991). In *Echium judaeum*, post-pollination events can be ruled out as a trigger for normal colour change, since bagged flowers rapidly changed colour (but see below regarding possible deposition of self-pollen). Neither is time

unequivocally linked to colour change; some flowers remained pink throughout their life.

Two aspects of insect visits can be ruled out as triggers for the initial colour change. Firstly, pollen depletion did not occur in bagged flowers where colour change did take place; therefore insect removal of pollen was not the crucial trigger. Secondly, the colour changes could not have been instigated by general 'mechanical' handling effects from visiting insects since these were excluded from bagged flowers. However, the effects of damage to floral parts, through repeated visitation, could mediate colour change, for example via the release of ethylene as part of the wound response, and this could also explain some of the unusual blotching described in some flowers (see Chapter 9).

Post-pollination processes can also be excluded as a potential trigger for colour change. Pollen deposition has been reported as a trigger for colour change (e.g. Mohan Ram & Mathur, 1984; and see elsewhere in this thesis); for *E. judaeum* deposition of self-pollen could have occurred in bagged flowers, but *all* bagged flowers that changed colour would have to have received such deposition. Given the 4-hour time course of change noted, this scenario is unlikely; although the period of stigmatic receptivity is not known for *E. judaeum*, self-pollen could only be deposited when the style is fully extended and the time course for this to occur usually exceeded the time taken for colour change. Furthermore, although there could be some temporal overlap of sexual phases (data not collected), transfer of self-pollen is effectively prevented by the spatial separation of anthers and style. It is conceivable that, in bagged flowers, wind action or passing animals (evidence of nocturnal activity by wild boars was noted) could aid short-distance pollen movement, deposition, and subsequent

colour change. However, this is equally unlikely as all flowers on all bagged plants changed colour even on windless days in daylight hours, and in the absence of any animals.

Bagging may have effects other than excluding visitors. Three environmental variables, which could have been altered by the presence of bagging, might be implicated in the acceleration of colour change by enclosure and require further investigation. Light, humidity and temperature were, potentially, influenced by fully enclosing plants, despite the use of fine gauge (aperture < 1mm) netting. Microenvironmental measurements were not made within the enclosures in this study, but in humid tropical conditions (Corbet & Willmer, 1981) found higher temperatures within pollination bags in both sunny and shaded sites, and also reported variable humidity levels.

As a preliminary test of this environmental effect of bags, on one morning a raised net was fixed above a patch of c.10 plants. Normal visitation was thus allowed, and natural airflow would, presumably, have removed effects on either temperature or humidity, but light levels would be altered. The flowers in this patch did not take on the 'all purple' coloration of their fully enclosed counterparts and remained of 'normal' appearance, which may suggest that light is not the important trigger here.

6.4.2 Nectar accumulation as a possible trigger for colour change.

Although the actual trigger for this normal colour change remains unresolved, the pre-change pink coloration could actually be 'maintained' through the activities of regular visitors, with nectar withdrawal possibly

contributing to retention of 'pinkness'. The pink to purple colour change is hastened in the absence of regular visitation (Figure 6.5) and may be connected with nectar reward status.

Anecdotally, *E. judaeum* has been suggested to exhibit an overnight colour change (A. Dafni; personal communication, but no indication was offered as to the direction of change). This occurrence and some of the patterns reported here may be explained by visitation of the main nectar-feeder, *Apis mellifera*. Visits by honeybees to *E. judaeum* decline from mid-afternoon onwards (Figure 6.11), and visitation probably ceases completely for at least 12 hours from 18.00. If colour change is coupled physiologically to nectar withdrawal and/or further secretion, then both the apparent overnight change and the presence of 'naturally' purple flowers (which had changed overnight) are potentially explained. When nectar is not removed regularly (as would be the case during the night) the change to purple ensues; this particularly rapid change can also be induced when the flower is bagged.

The foraging behaviour of *A. mellifera* is influenced by flower colour and by colour change; preferring to visit pink flowers (Figure 6.16). The association of certain colours with particular reward levels may underlie this finding. Pre-change, pink flowers contain greater nectar reward than either intermediate or purple flowers (Figure 6.9), and there is a temporal change in nectar secretion with more produced during the morning and late afternoon (Figure 6.8).

Hence the major difference between visited and non-visited flowers is the lack of nectar depletion and subsequent replenishment of the latter. Nectar withdrawal has been demonstrated to be involved in colour change in *Oenothera drummondii* (Eisikowitch & Lazar, 1987); there, the yellow to red change was

accelerated by up to a day when nectar was removed by microcapillary. In my study flowers that were probed once for nectar on the morning of opening showed intermediate levels of colour change to those that were bagged and not handled at all, which turned purple, and those that were open to visitation, which remained predominantly pink (Figure 6.5). Thus withdrawal of nectar could be stopping the pink→purple change; in the open group differential levels of visitation could underlie the variable change recorded if those flowers that remained pink were visited, and nectar withdrawn, more regularly than the intermediate-colour flowers in the same group.

6.4.3 'Reverse' colour change.

The slower and most unusual 'reverse' colour change that occurred when bagged flowers were uncovered again requires investigation. It could again be related to environmental factors (temperature, humidity, and light), all of which would alter when bags were removed. In terms of the mechanism hypothesised above for the 'forward' change, this 'reverse' change could be underpinned by aspects of pigment synthesis needed to achieve the purple to pink alteration. Clearly, a flower has a maximum time period in which it is able to secrete nectar (in the case of *E. judaeum*, up to 3 days) and those flowers that were noted to change colour bi-directionally were the 'youngest' on the focal plants. Hence, perhaps, only the young flowers can 'afford' the cost of a more energetically expensive re-synthesis of pink pigments (or breakdown of purple pigments that mask pink coloration), in tandem with continued nectar secretion. Replenishment

of nectar removed by regular visitation could thus be coupled to pink coloration and higher reward found in these flowers.

6.4.4 Ecology of colour change in *E. judaeum.*

This raises the question of whether there is any benefit to the plant of any aspect of colour change in this species; is there an ecological significance to having pink and/or purple flowers? Purple flowers can form a substantial proportion of the overall display in the natural population (see Figure 6.1). This is most often in the form of the blue-purple wilted flowers which, in the accepted sense of colour change (restricting the term to fully turgid and, apparently, viable flowers), would not be considered part of the advertisement as turgidity has been lost. But the facts that some of the purple flowers are still 'functional', and that young flowers, when bagged and not handled, also change to a colour seen more usually towards the end of floral life, suggest that the purple coloration may have a functional purpose related to attraction.

Through personal observation (and, therefore, dependent on the human visual system), on dull mornings there appeared to be greater concentrations of purple flowers at the site. There is a slightly tenuous logic to this observation that would also help to explain the earlier note on colour change occurring overnight. It is implausible that a nocturnal change acts as a signal to a nocturnal forager; such an animal is unlikely to be discriminating visually. If it is assumed that nothing visits *E. judaeum* at night (no observations were made), and that the suggestion linking colour change to nectar depletion is also correct, it might follow that, in the early morning, a good proportion of flowers would be

intermediate/purple through lack of visitation. On dull and/or cool days, visitation by *A. mellifera* could be reduced due to thermal constraints; honeybees that are active in air temperature of 10°C or below suffer quick cooling of the thorax to a level below that required for vigorous flight (Heinrich, 1993). On 28/2/2000 (a day when observations were eventually abandoned due to rain and temperature ranged between 10.1 and 11.2°C) no honeybees had visited the focal patch by 10.00 whereas on most other days visitation began at around 07.00. Small and medium-sized solitary bees would be subject to similar constraints, and these were clearly less abundant in the early morning in general (Figure 6.12). The limited number of flowers visited by large solitary bees soon after dawn would also not lead to widespread nectar depletion. Hence the proportion of unvisited and therefore purple flowers would be greater on cool mornings, as observed. The effects of bagging in producing an exclusively purple display (Figure 6.5) would also fit with this argument. The result of an absence of visitation is a largely purple display, as demonstrated where plants were bagged.

Under more prevalent, warmer and/or brighter conditions the lower levels of purple flowers usually found may be testament to the foraging efficiency of *A. mellifera* i.e., honeybee visits keep the flowers pink for longer. The data collected suggest consistent visitation may be experienced by the majority of flowers; on 6/3, 721 trips were made by *A. mellifera* to the focal patch of *c.*300 flowers and each foraging bout could extend to multiple flower visits. The majority of flower visits were made to pink flowers and visitation to a bagged and uncovered patch resulted in a non-random distribution of flower visits in favour of youngest reverse-changing flowers (Figure 6.17). The observation of an initial change (to the human eye) of the nectar guides may also be important,

as could possible changes outside the human visual spectrum. Bos et al. (1983) reported a change in the UV characteristics of the congeneric *E. plantagineum* that influenced foraging patterns in bumblebees; in their study these bees consistently visited the youngest flower available. These flowers contained greater quantities of pollen and nectar, as well as displaying nectar guides under UV illumination. In my study, pink flowers of *E. judaeum* were consistently found to contain greater levels of nectar reward than either intermediate or purple flowers. Pink pigmentation, either in young flowers or following 'reverse' change (initially along the nectar guides), may therefore be a visual signal of reward availability at close quarters, whereas purple may contribute to the overall display from greater distance.

A study of a related species, *Cerinthe major* L. (Boraginaceae) found high levels of variation in available nectar in flowers both within and between plants (Gilbert et al. 1991). In excess of 75% of flowers in that study produced relatively little nectar, a pattern reported in other species (e.g. *Prosopis glandulosa* (Golubov et al. 1999)). If nectar secretion and floral colour change are physiologically linked, the reverse colour change in *E. judaeum* could, simply, be advertising re-commencement of nectar availability.

From an evolutionary perspective, the known spectral sensitivity of honeybee vision at the purple-UV end of the insect visual spectrum (e.g. Lunau & Maier, 1995; Menzel & Backhaus, 1991) might explain the utility of a floral display of mixed coloration. The presence of pink, intermediate and purple flowers could offer enhanced attractant qualities and enable better perception of patches of *E. judaeum* from long distances. There is evidence that foraging insects benefit from a mix of flower colours. Goulson (2000) discovered

increased flight times and, therefore, reduced foraging efficiency in bumblebees searching for *Lotus corniculatus*, when presented with a matrix of the same-coloured flowers (yellow to the human eye) of different species. That study found no difference in flight times of bumblebees searching for the purple flowers of *Vicia cracca* either with or without a background matrix of yellow flowers, but did not test the bees using other purple flowers as a background. Further tests of visitation to artificially manipulated patches of *E. judaeum* might reveal whether the ability of honeybees to detect flowers is affected by within-patch variation of available flower colours.

Given the known ability of honeybees to use achromatic cues for long-range detection (Vorobyev & Brandt, 1997), the differing perception of a range of flower colours (produced by colour change and, perhaps, modified by varying ambient light characteristics) could contribute to initial recognition of, and arrival at, *E. judaeum* plants. Once foraging at the patch of plants, the colour cue offered by rewarding pink flowers, in combination with a learned association of 'pinkness' with higher reward, ensures extended visitation and potential pollination of viable flowers.

The remarkable range of colour in mature flowers of *E. judaeum*, together with the speed of colour change and unique 'reverse' change, demands further study. In particular, the apparent link between nectar secretion, colour change and honeybee visitation reveals a hitherto unreported example of a very close-coupled association. Foraging bees rarely visit post-change purple flowers but, when offered a plant containing only this colour phase, will visit these flowers and could be inducing a 'reverse' colour change to the more regularly visited

pink flowers, regulated by nectar withdrawal. Thus the activity of bees appears to affect colour change in a manner not previously documented, and certainly not related to pollination or post-pollination events. Further research is required to establish precisely how this change visually influences the bee. These findings and possible mechanistic pathways are summarised in Figure 6.18.

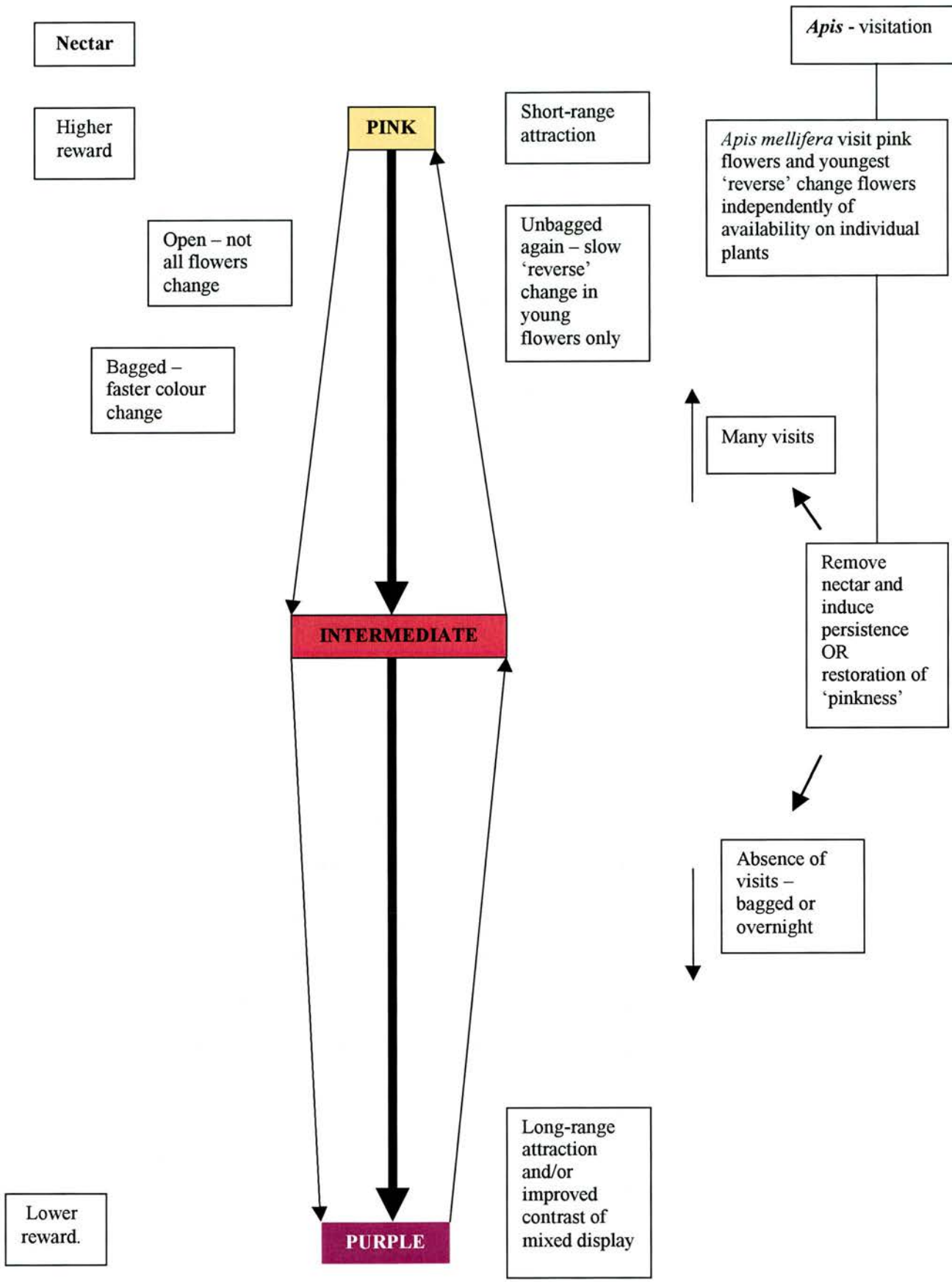


Figure 18. Colour change in *E. judaeum*; possible mechanistic pathways linking colour change, nectar withdrawal and visitation.

Chapter 7 – *Lonicera periclymenum.*

7.1 Introduction.

The plant family Caprifoliaceae has a global distribution throughout temperate and tropical regions, particularly the Americas and eastern Asia, and comprises over 450 species in 18 genera (Heywood, 1978). Nine members of the Caprifoliaceae, from 3 genera, have been noted to exhibit floral colour change; Weiss (1995a) listed these species, all of which displayed colour change from white or yellow to yellow or orange/red. The genus *Lonicera* consists of mainly deciduous shrubs and woody climbers, 17 of which are found in Europe. Only 5 species of *Lonicera* manifest floral colour change, always involving the whole corolla. Of these, 4 species alter from white to yellow including *L. periclymenum*, and the remaining species, *L. hildebrandiana*, also changes in this direction but, eventually, becomes orange (Weiss, 1995a).

Lonicera periclymenum L. is distributed throughout the region (Tutin et al. 1976). The plant is a climber and displays a terminal inflorescence of creamy-white to yellow flowers, each with 5 exserted stamens, that are up to 5.5cm in length, including deep corolla tube (Tutin et al. 1976). *L. periclymenum* is self-compatible and produces a few-seeded red berry.

Colour change in *L. periclymenum*, together with alteration in floral orientation, was detailed by Knuth (1906-09) and noted by Ottosen (1986). Flowers are zygomorphic, pinkish-red in bud and white upon opening (Figure 7.1), which usually occurs during the evening (Knuth, 1906-09). First-morning flowers remain white, but after 1-2 days the flowers change gradually through

cream-white to pale yellow and, from 2-3 days until withering and/or abscission, are brighter yellow (Knuth, 1906-09; Ottosen, 1986; and see Figure 7.1).

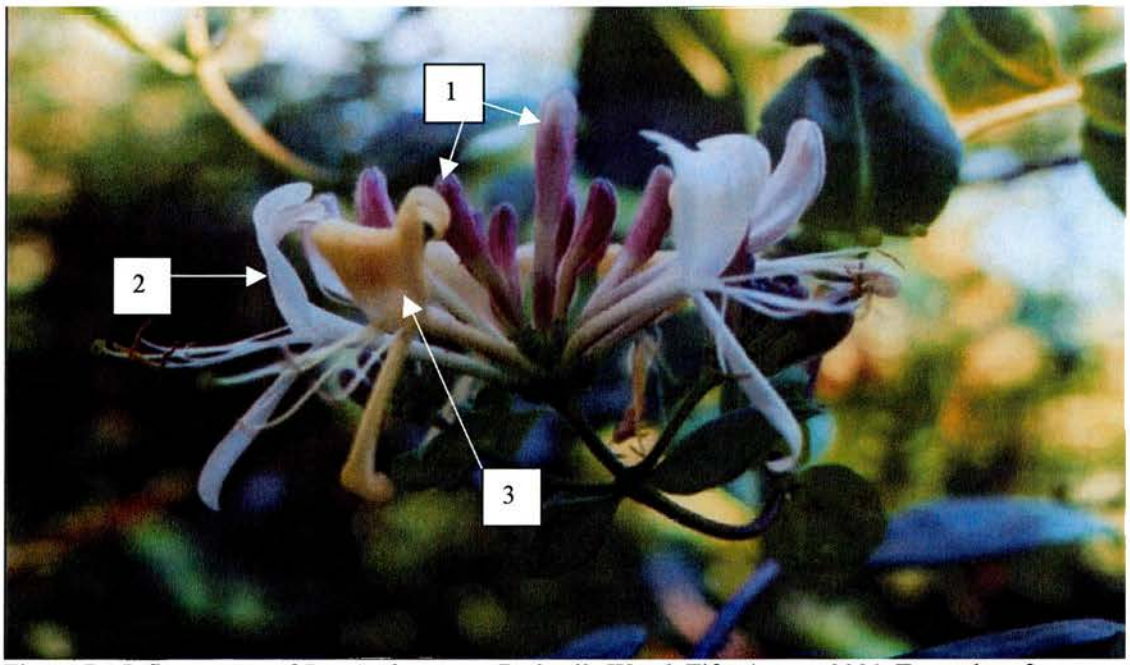


Figure 7.1 Inflorescence of *L. periclymenum*, Redwells Wood, Fife, August 2001. Examples of reddish-purple buds (1); white first-day flowers (2); and older yellow flowers (3) are clearly visible.

The change in orientation (see Knuth 1906-09 for full details and see Figure 7.6) coincides with an alteration in shape; the gradual rolling up of both upper and lower petals, together with a movement in the position of the anthers relative to the style (Figure 7.2).

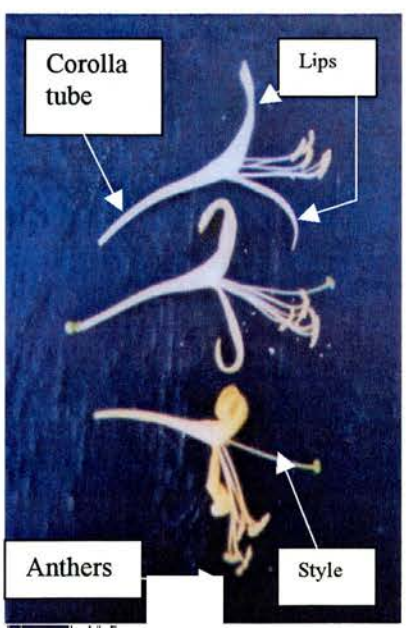


Figure 7.2 Change in appearance of flowers of *L. periclymenum*. Note progressive change in colour from white to yellow according to age of flower.
Top – newly opened flower; upper lip erect and lower limb gently curved. Style hidden amongst newly dehisced anthers.
Middle – 1-2 day-old flower, both lips curled inwards. Filaments and anthers beginning to droop and style clearly visible.
Bottom – 2+ day-old flower, both limbs completely curled. Filaments completely flaccid and style not yet started to wilt.

The flowers of *L. periclymenum* are described by Faegri & van der Pijl (1979) as typical of those usually pollinated by moths; the blossoms of the so-called 'phalaenophilous syndrome' often open at night, are strongly scented, contain copious nectar in long tubes or spurs, and, frequently, are white or faintly coloured. This suite of characteristics could attract nocturnal or crepuscular visitors in particular, and the floral morphology was felt to be unsuitable for diurnal foragers such as bumble bees (due to the absence of a landing platform) although dipterans, especially syrphids, could exploit pollen reward (Faegri & van der Pijl, 1979).

A wide range of visitors including sphingid and noctuid moths and several hoverfly species were reported to visit *L. periclymenum* by Knuth (1906-09), who also described nectar-feeding attempts by a bumble bee, *Bombus hortorum* L. This bee was found to be an important pollinator of *L. periclymenum* at the northern limits of the plant's range in Denmark; here, the activity of male *B. hortorum* effected pollination and compensated for the absence of nocturnal moths (Ottosen, 1987). The same researcher had previously reported *B. hortorum* as a regular visitor to *L. periclymenum*, together with syrphids and coleopterans (Ottosen, 1986).

L. periclymenum was included in a database of colour-changing flowers (Weiss, 1995a) but, although the change of colour in this species has previously been described, little attention has been directed towards the possible ecological significance of this colour change. As well as being present in accessible wild populations close to the University of St. Andrews, this species represented an ideal study plant for colour change in relation to floral manipulation. The large individual flowers allowed a variety of different handling effects that simulated

those observed in nature. In particular, corolla tubes could be easily probed (with or without withdrawal of nectar) and pollen deposition could be simply effected or prevented. The influence of confounding variables found in nature (e.g. wind or other mechanical perturbation of inflorescences) could also be avoided, in some parts of the work, through the use in a greenhouse of readily available cultivars from commercial growers.

7.2 Materials and Methods.

Study of this species was carried out initially in the field and subsequently, due to the practical problems described below, within a greenhouse using a commercial variety of the plant.

7.2.1 Field.

i) Study site.

Data were collected in the summers of 2000, 2001 and 2002 at Redwells Wood, nr. Anstruther, Fife (NO 555085 (355520, 708532)). The plants were found at a height of 1-2.5 metres, alongside a track through mixed woodland, climbing a variety of trees including birch (*Betula* sp.), spruce (*Picea* sp.), and willow (*Salix* sp.) (Figure 7.3).

Figure 7.3 Inflorescences of *L. periclymenum* amongst low birch (*Betula* sp.) saplings in Redwells Wood, Anstruther, Fife, August 2001.

ii) Longevity and floral colour change.

Twenty-five individual flowers, randomly chosen on five separate plants and from different inflorescences, were tagged with cotton thread one day prior to opening and left open to visitation. Floral development was monitored twice daily through to abscission; colour change was measured by comparison with a colour scale devised from a printers ink colour chart. Newly opened flowers were off-white (chart number 1U) and changed through pale yellow (127U) to deep yellow/ochre (115U) (Figure 7.4). These colour phases are referred to, hereafter, as white, intermediate and yellow, respectively.

Figure 7.4 Colour phases in *L. periclymenum* (see text for details).

iii) Nectar.

Measurement of nectar characteristics was made using the protocol described in Chapter 2. In this species the long corolla tubes (>20mm) varied in curvature and therefore, to ensure withdrawal of all nectar, individual flowers were removed from their calyces before sampling. Data were collected for flowers in the three colour phases to determine whether reward varied according to colour (and thus age) of flower.

iv) Visitation.

Floral visitors were recorded over twenty-minute observation periods each hour on 4th and 5th September 2000. A focal patch of 9 inflorescences comprising 43-47 flowers was used for these observations. A visit was scored as any landing by an insect that incorporated a feeding attempt for either nectar or

pollen and that included contact with floral reproductive parts. Floral reward sought, and variation in flower-handling behaviour between visitors, were noted.

Individuals of the only visiting nectar-feeder, *Bombus hortorum*, were followed during feeding bouts to assess whether flower colour phase visitation was independent of colour phase availability in the general population. Visits were scored as above; as well as the flower colour visited and sequence of visits, the total number of flowers of each colour category present on visited inflorescences was also noted.

v) Problems with field study of *L. periclymenum*.

A number of problems were evident in handling this species that made the flowers unsuitable for manipulative experiments in their natural habitat.

The use of pollination bags to exclude visitors was not possible due to the particular plant architecture of *L. periclymenum*. The species climbs on other vegetation and the distal parts of inflorescence-bearing stems are particularly delicate and hang free from such vegetation; bagging or netting tended to weigh down the stems and flatten individual flowers. Such consequences might damage flowers in general and protruding reproductive parts in particular, with possible confounding effects on floral development. Attempts were made to attach light wire frameworks around inflorescences, but the stems could not bear the additional weight that resulted.

A further source of error that could not be eradicated was the deposition of pollen through either wind action or perturbation when I handled the inflorescences. In the early part of a flower's life the flower (and, therefore, the reproductive parts) is almost horizontal but over time begins to droop and can

eventually hang almost vertically in comparison to the original position (Figure 7.5). As a result, self-pollen can be dislodged onto the stigma at this stage or, where inflorescences bear many flowers; pollen from surrounding flowers can be similarly transferred. Any slight perturbation, particularly from wind action, can aid this process. If pollen deposition, or a subsequent reproductive process, was implicated in colour change in *L. periclymenum* (a self-compatible species) any attempts at investigating possible cues for such change could be compromised under field conditions.

Figure 7.5 Older yellow flowers of *L. periclymenum* begin to droop; consequently, self- or geitonogamous-pollen is, potentially, easily dislodged from newly opened white flowers onto the stigmas below, and pollination effected.

Therefore, I decided to carry out all manipulative experiments within a greenhouse where some control of these problems could be attempted; exclusion of visitors and absence of wind action could be achieved and, in easier working conditions, the 'accidental' influences of handling by myself considerably reduced.

7.2.2 Greenhouse.

All experiments were carried out in the greenhouse of the Plant Sciences Department, Sir Harold Mitchell Building, University of St Andrews. Fourteen plants of the same cultivar (*L. periclymenum* cv. Graham Thomas) were used during these experiments; though it was conceivable that the plants were originally taken as cuttings from a single parent plant during their propagation for the commercial market. All plants, which were approximately 1m in height when purchased, were re-potted in compost (Levington M2 Potting Compost, Levington, Ipswich, UK) and kept in plastic pots (20cm high and 20cm in diameter) attached to central bamboo supports with plastic tags. Watering took place daily during the morning and 3 grains of nutrient supplement (Osmocote Plus, Scotts, Ohio, USA) were added to each of the pots at the beginning of the experiments. Both light (16 hours per day) and temperature (20 ± 3°C) were controlled and plants were placed free standing in a position sheltered from potential draughts.

i) Mimicked visitor effects.

Individual flowers were marked by fastening coloured cotton thread loosely around the base of the corolla tubes of white, first-morning flowers and randomly placed in one of six experimental groups

Control: - no treatment.

Pollen depletion: - to simulate the feeding activities of visiting syrphids (see below), a fine artist's paintbrush was used to remove pollen from all five anthers. Pollen was yellow in colour, and maximum depletion was indicated by full

exposure of the brownish inner surface of individual anthers. To ensure that pollen deposition did not occur in this and other experimental groups, flowers were manipulated while holding a small glass tube (5mm diameter) over the style and stigma of the flower being treated.

Emasculation: - all anthers were removed by cutting the filaments with scissors a few millimetres below the point of insertion into the anther.

Nectar withdrawal: - nectar feeding by *Bombus hortorum* was mimicked by inserting a 1µl microcapillary repeatedly until all available nectar had been withdrawn.

Corolla-pierced: - evidence of nectar robbing, in the form of irregular holes (approximately 5mm in length) torn in the basal few millimetres of corolla tubes, was noted in the natural population, although neither the visitor nor precise method of piercing the corolla tube was observed. Earlier research (Ottosen, 1986) identified *Bombus terrestris*, a wasp (*Vespa vulgaris*) and long horn beetles (*Strangalia* sp.) as the main robbers in Denmark, while *Bombus pratorum* fed at existing holes. To mimic this effect, using a dissecting needle, a slit 5-6mm in length was made within the basal half of the corolla tube. Nectar was not withdrawn from this opening, as more extensive damage to floral tissue could not be avoided by this method; a further group was used to simulate the combination of tissue damage and nectar withdrawal (see later).

Emasculation with nectar withdrawal: - anthers were removed and nectar withdrawn as above.

Flowers were checked daily until abscission and scored for colour change as above. Despite exercising great care in protecting the stigmas from pollen during these manipulations it became evident that deposition was still occurring;

inspection of flowers on the morning after manipulation with a handheld magnifying glass (x10) revealed the presence of pollen grains on stigmas. Presumably, this pollen had fallen from flowers above the marked individuals outside the time of manipulation (see (v) above and Figure 7.5). Pollen deposition, as a discrete factor, was therefore investigated.

ii) Pollen deposition effects.

Hand-pollination of marked flowers was carried out in three separate experimental groups. A single 'brush-load' of pollen was transferred in each case.

Autogamous: - self-pollen from the marked flower was brushed onto the stigma using a fine paintbrush.

Geitonogamous: - pollen from a flower of the same plant but on a different inflorescence was applied as above.

Cross-pollination: - pollen from a flower from a different plant was applied as above.

Two additional groups were also monitored to examine 'minor' handling effects.

Stigma contact: - the style was held with dissecting forceps and the stigma surface touched repeatedly with a clean dissection probe for a total of 5 seconds.

Protected stigma: - a small polythene sleeve, approximately 1cm x 2cm, was fastened around the stigma with cotton thread.

A control group of no treatment was also marked.

iii) Manipulations with protected stigmas.

To ensure that the effects of pollen deposition on longevity and colour change could be unequivocally separated from the influence of floral manipulation, a further experiment tested both factors with the stigmas protected from accidental pollen deposition. First-morning flowers were marked and polythene sleeves fastened around the stigmas (as 7.2.2ii above) prior to manipulation. Accidental pollen deposition was therefore prevented, both during experimental set-up and through coincidental transfer from surrounding flowers. In addition to a control group, where the only handling effect was the action of attaching the sleeve, five experimental groups were used, employing methodology as above; pollen depletion, nectar withdrawal, corolla piercing, emasculation, and pollen deposition (autogamous).

7.3 Results.

7.3.1 Field.

i) Longevity and flower colour change.

Twenty-five flowers were monitored under natural conditions between 29th August and 3rd September 2000. Three flowers had opened by 19.00 on the first day of observation but the majority of flowers (88%) opened overnight, as all were open at 09.00 the following morning. The initial opening of individual flowers coincided with a pulse of sweet-smelling floral scent; this became less obvious in older flowers and was absent (to the human nose) in inflorescences comprising solely older flowers (>2-3 days). Flowers lasted between 3 and 6 days (Figure 7.6) with a mean longevity of 4.4 ± 0.2 days.

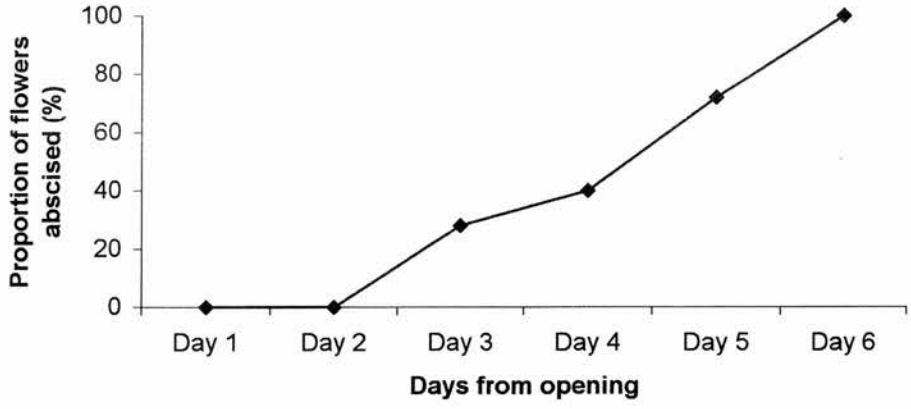


Figure 7.6 Floral longevity in *L. periclymenum* under natural conditions.

Colour change was very gradual in *L. periclymenum*. The reddish-purple coloration of the bud gave a slightly pinkish hue to some open flowers but the basically white colour of all first-day flowers contrasted noticeably with older

flowers on the same inflorescence. The white phase lasted up to 1.5 days with a mean phase length of 0.8 ± 0.1 days. The intermediate phase lasted between 1 and 2.5 days (mean 1.8 ± 0.1 days) and the yellow phase up to 4 days (mean 1.8 ± 0.3 days) (Figure 7.7).

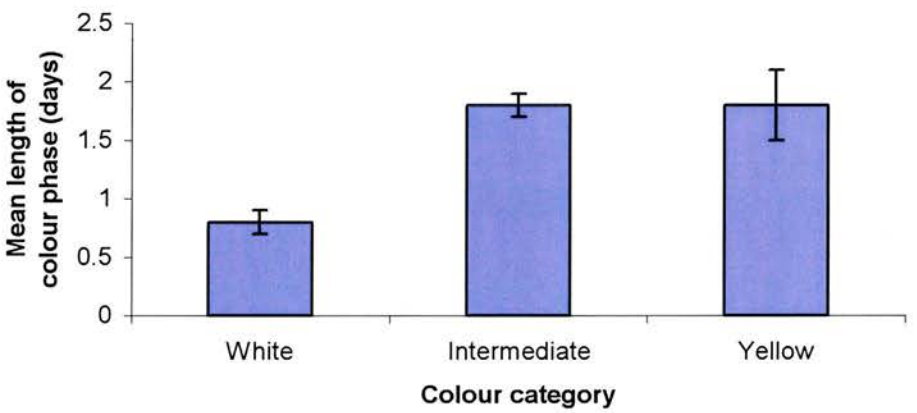


Figure 7.7 Mean length of floral colour phases in *L. periclymenum*; see text for details of colour categories (n = 25 flowers).

ii) Nectar.

Nectar characteristics were measured between 09.00 and 11.00 each morning between 2nd and 8th August 2001 and varied according to flower colour phase and, therefore, age of flower (Figure 7.8). Mean volume for white flowers was 0.81 ± 0.12µl per flower (n = 64, range 0 – 3.9µl), 0.91 ± 0.21µl per flower for intermediate flowers (n = 59, range 0 – 9.0µl), and 0.57 ± 0.11µl per flower for yellow flowers (n = 95, range 0 – 4.2µl). Thus, considerable variation was present within groups. Large numbers of empty flowers (19/64 white, 26/59 intermediate, and 49/95 yellow) probably account for the differing mean and median patterns, and may have been due to patchy visitation by nectar-feeding visitors.

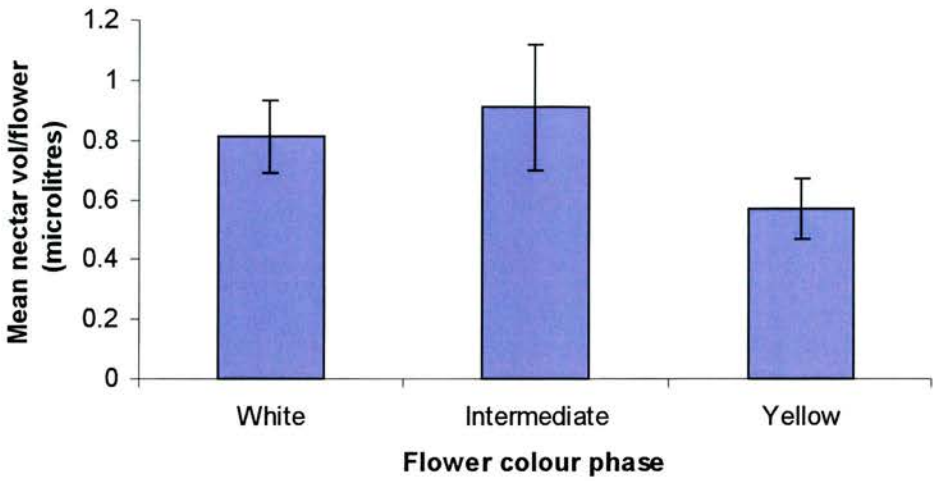


Figure 7.8 Nectar volume in *L. periclymenum* by flower colour category (see text for details).

There was a significant difference between the median nectar volumes for flowers of different colour categories; Kruskal-Wallis test, $H = 6.53$, $df = 2$, $p = 0.038$ (median values: white = 0.45µl, intermediate = 0.23µl, yellow = 0µl). In a Tukey-type post-hoc test for multiple comparisons of groups with unequal sample sizes (Zar, 1996), there was a significant difference between white and yellow flowers ($p<0.05$) but not between any other pair of groups.

Nectar concentration also varied with flower colour and age (Figure 7.9), though dilute nectar was found in all flowers. (NB.Where exceptionally low nectar concentrations were recorded (<10%) both volume and concentration measurements were not included for statistical analysis; these figures were frequently found following heavy overnight rain and it was very likely that rainwater had entered the corolla tube to produce the high volume, low concentration readings.) Mean concentration in white flowers was 19.38 ± 0.34% (n = 45 flowers, range 14 – 23%), 17.50 ± 0.45% in intermediate flowers (n = 33, range 11 – 21%), and 15.25 ± 0.48% in yellow flowers (n = 46, range 11 – 24%). There was a significant difference in the median nectar concentration of different

flower colours; Kruskal-Wallis H = 36.26, df = 2, $p<0.001$ (median values: white = 19.0%, intermediate = 18.5%, yellow = 15.0%). A Tukey-type post-hoc test found no difference between white and intermediate groups ($p > 0.05$), but a significant difference ($p<0.05$) between both of these groups and yellow flowers.

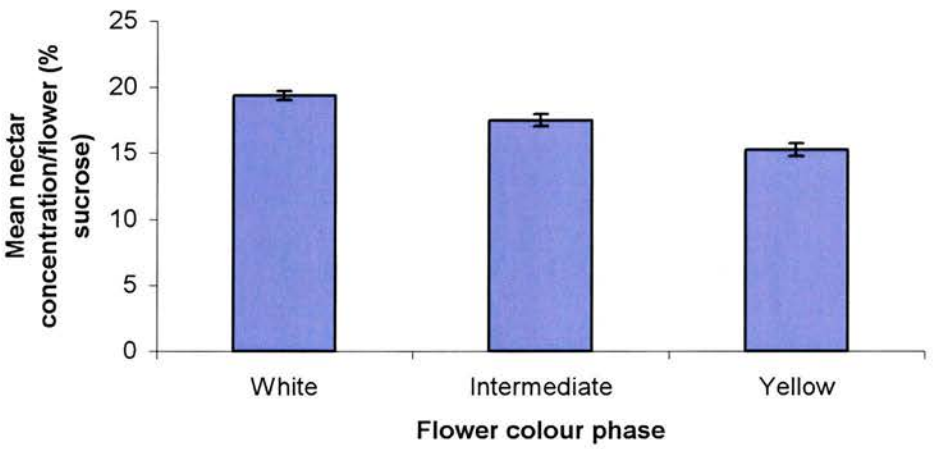


Figure 7.9 Nectar concentration in *L. periclymenum* by flower colour category.

Mean sugar content per flower in both white and intermediate flowers (0.17mg) was almost twice that of yellow flowers (0.09mg). Hence sugar reward was considerably higher in white and intermediate flowers.

iii) Visitation.

A total of 778 insects were recorded as floral visitors to *L. periclymenum* during the periods of observation; the proportions and patterns of these visitors are displayed below (Figures 7.10, 7.11 and 7.12). Figure 7.10 illustrates gross visitation to *L. periclymenum* over a two-day period. Of syrphid visitors (Figure 7.11), the great majority were *Episyrphus balteatus* and the remainder included *Syrphus ribesii* and *Eristalis* sp. All recorded bumblebees were *Bombus hortorum*, a particularly long-tongued species (Prŷs-Jones & Corbet, 1991) able

to reach the nectar. Other visitors included calliphorid flies, vespid wasps, *Apis mellifera* and a single unidentified moth.

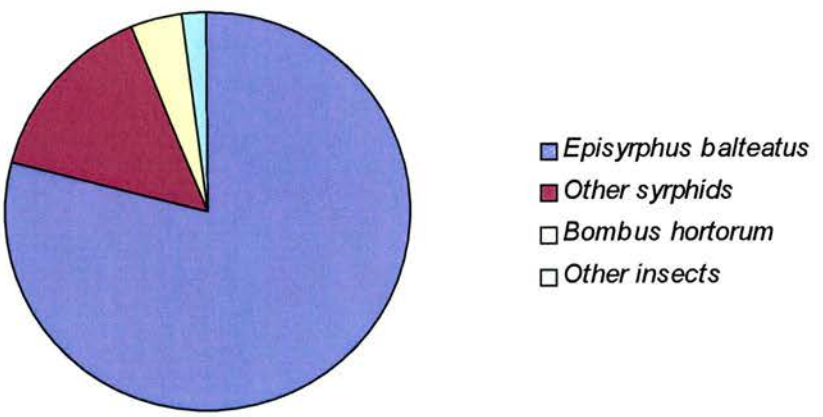


Figure 7.10 Proportions of 778 visitors to *L. periclymenum*. *Episyrphus balteatus* 613 (79%), other syrphids 117 (15%), *Bombus hortorum* 32 (4%), other insects 16 (2%).

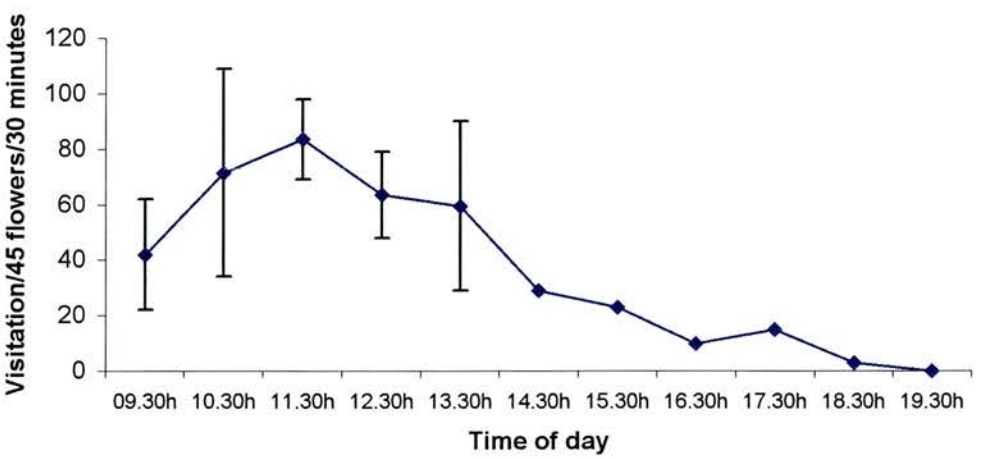


Figure 7.11 Visitation to *L. periclymenum* by all syrphids over a two-day period. Data points for time periods 09.30 to 13.30 are the mean values for two observation sessions; data for remaining periods are for a single observation owing to loss of raw data.

Visitation patterns show very low rates during daylight hours by *B. hortorum* and the group of insects defined as 'others' (Figure 7.12). The

maximum average visitation rate for either of these groups was 3 visits per 30-minute period at 10.30 by *B. hortorum*. Syrphid visitors, in contrast, were plentiful and reached a peak visitation of 83.5 visits/45 flowers in the 30 minutes at the 11.30 observation period (Figure 7.11).

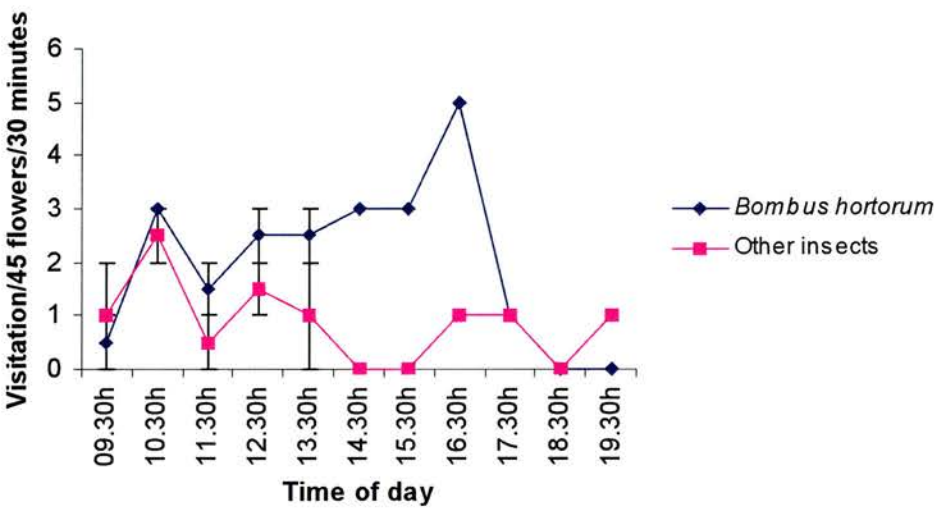


Figure 7.12 Visitation to *L. periclymenum* by *B. hortorum* and other insects (see text for details). (See legend for Figure 7.11 regarding data loss for afternoon observation sessions.)

Details of flower handling by visitors were noted to determine possible types of simulated visitation for later experimentation. All visiting syrphids displayed the same feeding method; landing on individual anthers, these insects would repeatedly dab at the anthers with extended mouthparts, presumably removing pollen which is the main food of the most abundant visitors, *E. balteatus* and *S. ribesii* (Gilbert, 1981). Individuals would often feed for several minutes on the anthers of a single flower and might spend an entire 30-minute observation period on the flowers of 2-3 inflorescences. Occasionally, individuals were noted clinging to and feeding, in the same manner, on a stigma. It was not clear whether these flies were feeding on pollen already deposited, or on stigmatic exudate.

Visiting calliphorid flies were observed crawling over both corolla lips as well as reproductive parts and appeared to be feeding on pollen. Vespid wasps also moved across all parts of the flower and, in addition, frequently inspected the base of an inflorescence; the identity of the visitor that chewed holes at the base of the corollas was not observed but these wasps, or *B. terrestris* (another occasional visitor), are possible nectar thieves (Ottosen, 1986).

Fast-flying moths were noted at dusk on three evenings feeding on the plants, but their feeding behaviour could not be described in detail due to their tendency to feed at inflorescences high in the canopy. As they were clearly hovering and not settling to feed they were presumably sphingid-type moths often associated with various honeysuckle species (e.g. Knuth, 1906-09; Ottosen, 1986). Detailed information is required on the feeding position of these visitors in relation to the reproductive parts of the flower in order to assess their possible role in pollen transfer.

iv) Foraging by *Bombus hortorum*.

Bombus hortorum was the sole confirmed and legitimate nectar-feeder observed at *L. periclymenum*. Access to the corolla tube was gained by landing below the upper limb and crawling the short distance along the anthers and style to the opening of the corolla tube before probing for nectar (Figure 7.13). Pollen could be either transferred to the stigma from abdominal hairs or dislodged from the flower being visited and from surrounding flowers on the same inflorescence. Visits lasted a few seconds and several inflorescences were usually visited on each foraging bout.

Figure 7.13 *B. hortorum* feeding at inflorescence of *L. periclymenum*, Redwells Wood, Fife, September 2001.

Thirty-three foraging bouts that incorporated visits to 241 flowers were recorded for visiting *B. hortorum*. Choice of flower colour was significantly different from availability of floral colour phases on those inflorescences visited (Figure 7.14), (χ^2 goodness of fit = 70.95, df = 2, $p<0.001$), with bees clearly preferring to visit young white flowers to old yellow flowers.

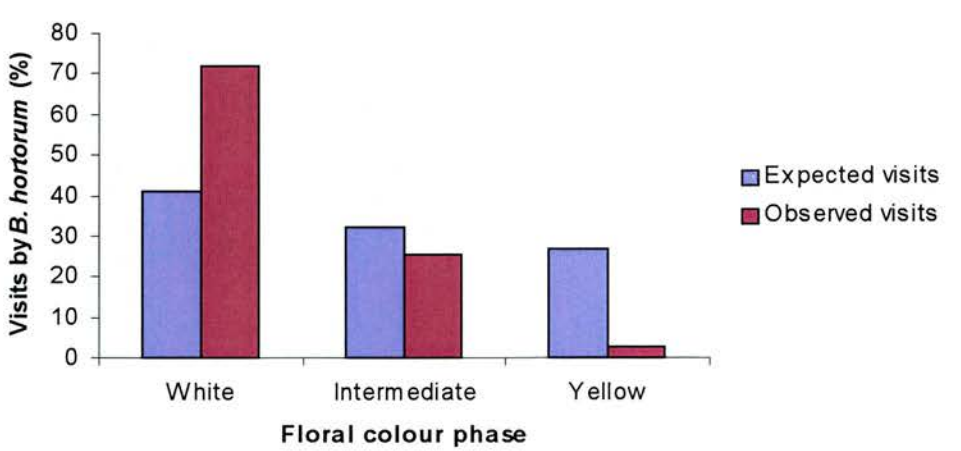


Figure 7.14 Visitation of *B. hortorum* to *L. periclymenum*; observed visits to flowers in three colour categories by 33 bees.

Visits by the 33 individual bees are illustrated in Figure 7.15. Twenty-eight bees fed at white flowers in greater ratios than their availability on visited inflorescences, of these bees 13 fed *solely* at white flowers. Most notably 2 bees (nos. 19 and 33) fed only at white flowers when this colour phase comprised under 25% of the total display. Of the remaining 5 bees, one individual (no. 3) did not feed at any white flowers and the other 4 (nos. 4, 23, 29 and 30) visited directly in proportion to availability of white phase flowers. In total, only 6 of 241 flowers visited were yellow phase and just 3 bees were responsible for these visits to yellow flowers.

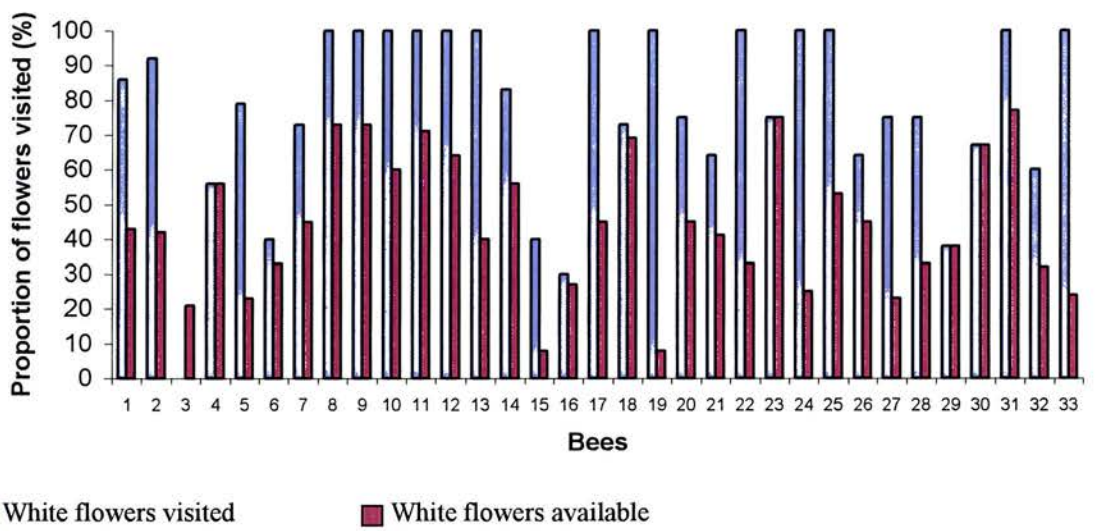


Figure 7.15 Foraging bouts of *B. hortorum* at *L. periclymenum*. Visits of 33 bees to between 1 and 6 inflorescences; feeding at white phase flowers shown as proportion of total feeding visits against proportion of white flowers available on visited inflorescences.

Thus visitation by *B. hortorum* was biased towards the more rewarding, first-day white flowers; even where these flowers were in the minority on visited inflorescences foraging was still concentrated on them.

7.3.2 Greenhouse.

i) Mimicked visitor effects.

Figure 7.16 illustrates longevity of flowers following experimental treatment of various types and this is summarised in Table 7.1. Floral life for the whole population varied between 2 to 13 days; Kruskal-Wallis test, using medians, showed a significant difference between treatments, $H = 21.28$, $df = 5$, $p = 0.001$.

Group	n	Floral longevity (days)	
		Mean	Median
Control	40	7.0 ± 0.3	7
Pollen depletion	23	8.7 ± 0.3	9
Emasculation	28	7.6 ± 0.3	8
Nectar withdrawal	15	7.5 ± 0.9	7
Nectar withdrawal + emasculation	21	8.4 ± 0.3	9
Corolla-pierced	31	7.4 ± 0.3	8

Table 7.1 Floral longevity in *L. periclymenum.*

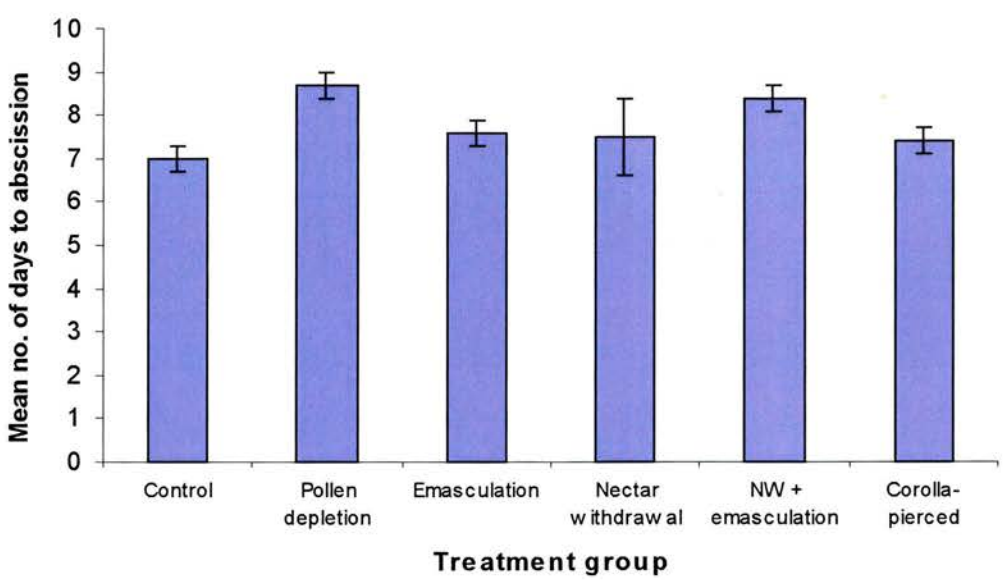


Figure 7.16 Longevity in *L. periclymenum* following floral manipulation (see text for details of individual treatments).

Tukey-type post-hoc tests revealed significant differences in longevity between pairs of treatment groups (Table 7.2).

Group	Control	Pollen depletion	Emasc.	Nectar withdrawal	Nectar withdrawal + emasculation	Corolla-pierced
Control		*	ns	ns	*	ns
Pollen depletion	*		ns	ns	ns	ns
Emasculation	ns	ns		ns	ns	ns
Nectar withdrawal	ns	ns	ns		ns	ns
Nectar withdrawal + emasculation	*	ns	ns	ns		ns
Corolla-pierced	ns	ns	ns	ns	ns	

Table 7.2 Post-hoc tests for pair-wise comparisons of floral longevity between treatment groups.
* denotes $p<0.05$, ns = not significant.

These results could be explained by the absence of pollen in both 'depletion' and 'withdrawal with emasculation' groups; accidental autogamous self-pollination could not take place (and thus reduce longevity through early abscission) in these treatments. However, there was no significant difference between the control and simple emasculation groups, so possible pollen deposition and/or subsequent events related to such deposition cannot fully explain this result.

Floral longevity in the greenhouse plants exceeded that of flowers in the wild. In natural conditions mean longevity was 4.4 ± 0.2 days ($n = 25$) and in the greenhouse 7.7 ± 0.2 days ($n = 158$), (median 4.5 and 8 days respectively, Mann-Whitney test, $W = 16020.0$, $p < 0.0001$). This could have been due to the commercial variety having been selected for greater floral life, or could be connected with the sheltered conditions of the greenhouse. For example, in the absence of natural visitors and perturbation by wind, pollen was less likely to be transferred and possibly trigger early abscission, following pollination.

Length of colour phase in the white category did not vary between treatment groups; 155 of 158 flowers in all groups had changed colour by the second morning (Kruskal-Wallis test; $H = 4.3$, $df = 5$, $p = 0.507$, median length of white phase in all groups = 1 day). The intermediate phase lasted between 1 and 3 days and there was a significant difference in the median length of this colour phase between treatment groups (Kruskal-Wallis test, $H = 19.23$, $df = 5$, $p = 0.002$). Post-hoc tests showed a difference between the nectar withdrawal group and the control, corolla-pierced and the nectar withdrawal with emasculation groups, respectively ($p < 0.05$).

Greater variation in floral longevity was apparent in the yellow phase of flowers (Figure 7.17) and is summarised in Table 7.3.

Group	n	Length of yellow phase (days)	
		Mean	Median
Control	40	4.2 ± 0.3	4
Pollen depletion	23	6.0 ± 0.4	6
Emasculation	28	5.0 ± 0.3	5
Nectar withdrawal	15	5.3 ± 0.9	5
Nectar withdrawal + emasculation	21	5.3 ± 0.3	5
Corolla-pierced	31	4.3 ± 0.3	4

Table 7.3 Length of yellow colour phase in *L. periclymenum* according to treatment group.

Both control and corolla pierced groups had shorter median yellow phases (4 days) than emasculation, nectar withdrawal and nectar withdrawal with emasculation flowers (all 5 days) and pollen depletion flowers (6 days) (Kruskal-Wallis test, $H = 20.92$, $df = 5$, $p = 0.001$). Post-hoc tests revealed significant differences between pairs of groups (Table 7.4).

Group	Control	Pollen depletion	Emasc.	Nectar withdrawal	Nectar withdrawal + emasculation	Corolla-pierced
Control		*	ns	ns	*	ns
Pollen depletion	*		ns	ns	ns	*
Emasculation	ns	ns		ns	ns	ns
Nectar withdrawal	ns	ns	ns		ns	ns
Nectar withdrawal + emasculation	*	ns	ns	ns		ns
Corolla-pierced	ns	*	ns	ns	ns	

Table 7.4 Post-hoc tests for pair-wise comparisons of length of yellow colour phase between treatment groups. * denotes $p<0.05$, ns = not significant.

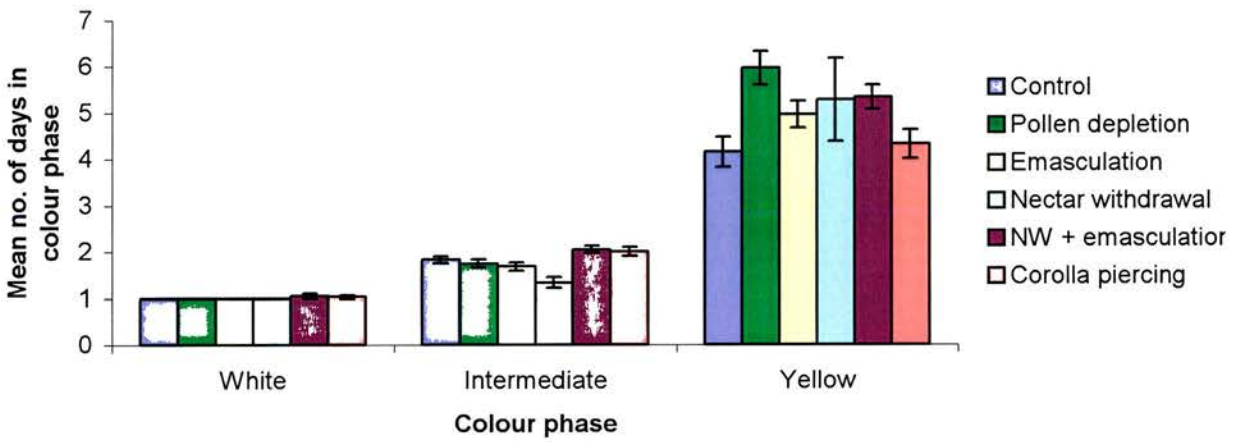


Figure 7.17 Length of colour phases in *L. periclymenum* following floral manipulation.

Hence where longevity varied with type of treatment, difference in length of colour phase occurred only in the yellow phase.

ii) Pollen deposition effects.

Longevity in *L. periclymenum* was significantly reduced by pollen deposition (Figure 7.18); where pollen (self or cross) was brushed on to the stigma, mean longevity was 3.9 ± 0.3 days (n = 32 flowers) compared to 7.0 ± 0.4 days in those groups where no pollen was deposited (n = 32 flowers), (Mann-

Whitney test, using medians: W = 1422.5, p<0.0001; median longevity in unpollinated groups (control, protected stigma and stigma contact), 7 days; in pollinated groups (autogamous, geitonogamous and cross pollen), 3 days).

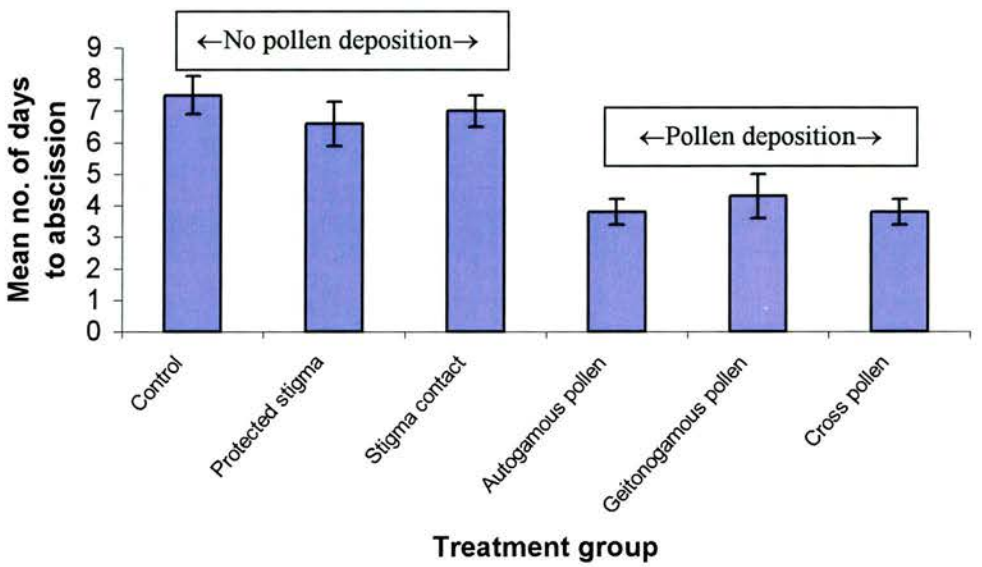


Figure 7.18 Effects of stigma manipulation and pollen deposition on longevity in *L. periclymenum*.

Neither white nor intermediate colour phases varied with these treatments (Figure 7.19); Kruskal-Wallis test, white phase, H = 0, df = 5, p =1.0; intermediate phase, H = 7.71, df = 5, p = 0.173. Median length of both colour phases = 1 day for all treatment groups.

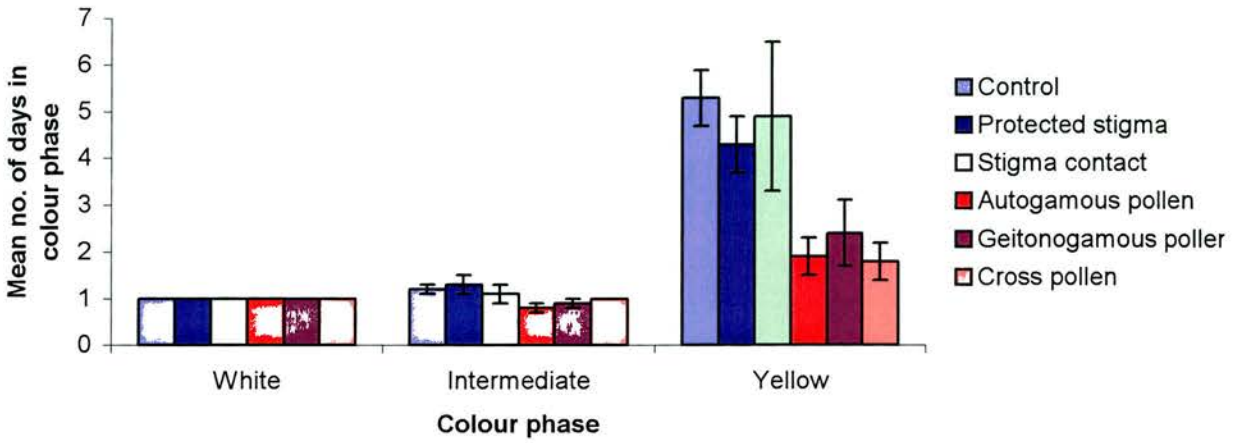


Figure 7.19 Length of floral colour phases in *L. periclymenum*, following stigma manipulation and pollen deposition.

However, the length of the yellow colour phase varied significantly according to whether pollen had been deposited onto the stigma (Mann-Whitney test: $W = 677.0$, $p < 0.0001$; median length of phase was 1 day for pollen deposition groups, and 5 days for groups where no pollen was deposited). Hence, the main effect here was that pollen presence on the stigma reduced floral longevity by reducing the *persistence* of the old yellow phase flowers, but did not hasten colour change at all.

iii) Manipulations with protected stigmas.

When stigmas were fully protected from pollen deposition, there was extended mean longevity of flowers in all treatment groups compared to flowers that had been hand-pollinated (with self-pollen) (Figure 7.20 and Table 7.5).

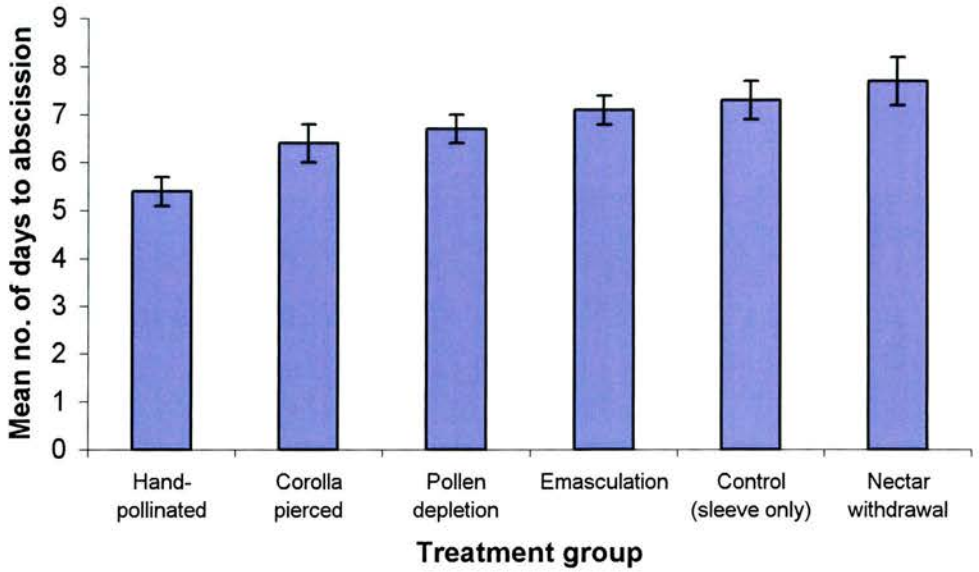


Figure 7.20 Longevity in *L. periclymenum*, following floral manipulation.

Group	n	Floral longevity (days)	
		Mean	Median
Hand-pollinated	51	5.4 ± 0.3	5
Corolla pierced	48	6.4 ± 0.4	6
Pollen depletion	47	6.7 ± 0.3	7
Emasculation	50	7.1 ± 0.3	7
Control (sleeve only)	46	7.3 ± 0.4	7
Nectar withdrawal	46	7.7 ± 0.5	8

Table 7.5 Floral longevity in *L. periclymenum* following different treatments.

There was a significant difference between the median longevity of flowers subject to different treatments; (Kruskal-Wallis test, $H = 29.09$, $df = 5$, $p<0.001$). Post-hoc tests confirmed that median longevity of flowers in the pollen deposition group was significantly different ($p < 0.05$) to that of all other treatment groups except the corolla-pierced group. There were no significant differences between any other pair of treatments.

In common with the earlier manipulations, the white and intermediate colour phases did not vary with treatment but the yellow phase was again extended to account for the greater longevity (Figure 7.21 and Table 7.6).

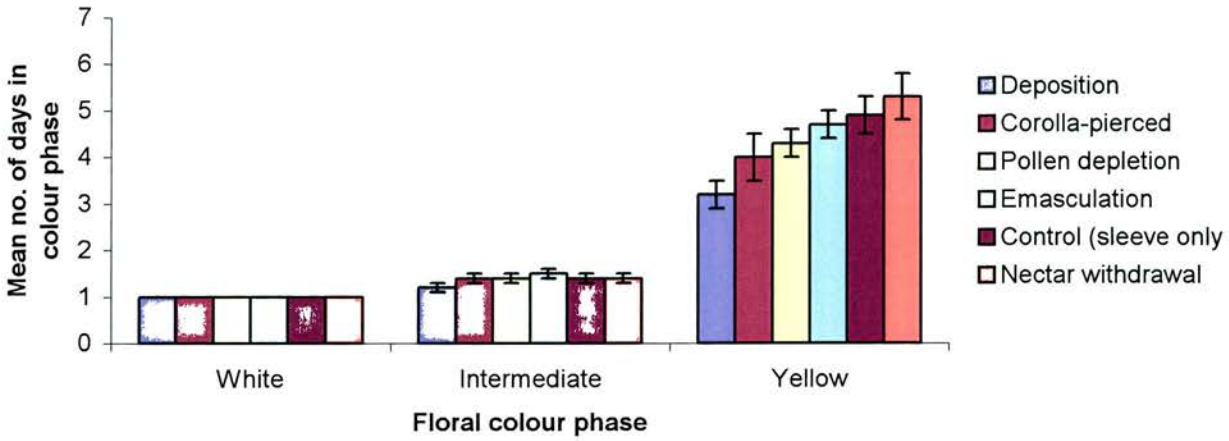


Figure 7.21 Length of floral colour phases in *L. periclymenum*, following floral manipulation.

Median length of both the white (Kruskal-Wallis, H = 7.84, df = 5, p = 0.165) and intermediate (Kruskal-Wallis, H = 7.09, df = 5, p = 0.214) phases was 1 day in all treatment groups. There was a significant difference in the median length of the yellow phase; (Kruskal-Wallis, H = 24.0, df = 5, p<0.001).

Group	n	Length of yellow colour phase (days)	
		Mean	Median
Hand-pollinated	51	3.2 ± 0.3	2
Corolla pierced	48	4.0 ± 0.5	3
Pollen depletion	47	4.3 ± 0.3	4
Emasculation	50	4.7 ± 0.3	5
Control (sleeve only)	46	4.9 ± 0.4	5
Nectar withdrawal	46	5.3 ± 0.5	6

Table 7.6 Length of yellow colour phase in *L. periclymenum* according to treatment group, with stigmas protected.

Post-hoc tests showed a significant differences in the median length of the yellow phase between the pollen deposition group and the emasculation, control (sleeve only) and nectar withdrawal groups ($p < 0.05$).

7.4 Discussion.

Colour change in *L. periclymenum* occurs independently of a range of manipulations, including pollen deposition, which simulated the behaviour of a range of insect visitors. Floral longevity, on the other hand, is influenced by manipulations that incorporate the deposition of pollen on the stigma; early abscission of flowers took place in response to such deposition. The length of the final yellow colour phase was the only variable, and this was reflected in the time to abscission; in the absence of pollen deposition yellow flowers were retained for an extended period. In the field, withered *L. periclymenum* flowers were not retained (presumably being dislodged by wind or perturbation by visitors), so that overall longevity was greatly reduced. Furthermore, the still turgid, post-colour-change flowers were rarely visited. Hence, the role of retention of these older flowers in attraction requires further research.

7.4.1 Flower visitors.

The plant attracted a wide suite of potential pollinators, and the limited data on one visitor, *B. hortorum*, suggest that floral colour may be relevant to foraging decisions (Figure 7.14 and 7.15); visitation by this bee was biased towards younger white flowers that also contained greater nectar reward (Figure 7.8 and 7.9). This insect was the only legitimate nectar-feeder to visit and, due to its flower-handling behaviour, the most likely diurnal pollinator.

Flowers of the genus *Lonicera* have been placed in the phalaenophilous pollination syndrome by Faegri & van der Pijl (1979); the combination of

evening opening with heavily scented flowers provides the necessary cues for visitation by moths. In the absence of abundant moths at the northerly edge of the plants' range in Denmark, Ottosen (1986, 1987) identified *B. hortorum* as the most important pollen vector in promoting outcrossing in *L. periclymenum*, although levels of self-pollination were high. The foraging behaviour of this bee was likened to that of tropical trap-lining species and, with the scarcity of the moths that are co-adapted to visit *L. periclymenum*, the importance of an opportunistic visitor as the chief pollinator was emphasised (Ottosen, 1987). Since Scotland is also close to the northern limit of distribution of *L. periclymenum* and very few moths were seen during my study, the same may apply.

Additionally, Ottosen (1987) reported that the only bees that could remove nectar legitimately from *L. periclymenum* were long-tongued bumblebees such as *B. hortorum* which has the longest tongue of any British bumblebee (Prŷs-Jones & Corbet, 1991). My study has found low levels of nectar standing crop in comparison with earlier research. Although white and intermediate colour phase flowers contained more nectar than yellow flowers (Figure 8), mean volumes were below 1µl in all groups and 'empties' were frequent in every category. The majority of flowers studied by Ottosen (1987) contained between 1 and 11µl; given the lack of abundance of *B. hortorum* in the present work, depletion by diurnal visitors cannot explain my low recordings (flowers that had pierced corolla tubes were excluded). A comparison of nectar secretion between populations receiving differential levels of visitation by nectar feeders might be instructive in resolving this problem; as nectar production imposes some cost on a plant (e.g. Estes et al. 1983; Pyke, 1991), reduced nectar

production may be selected for in this northerly population where exploitation is low.

Ottosen (1986) noted that nectar robbery combined with low numbers of *L. periclymenum* plants could compromise floral constancy in *B. hortorum*. My finding that this bee forages preferentially on white flowers (Figures 7.14 and 7.15) suggests that constancy may be occurring; bees are presumably learning by association of the presence of higher reward in these younger flowers, and visit independently of their availability in the population. The visual cue may be the colour change or the altered morphological appearance of the flower over time; alternatively, the cue may be olfactory with an altered floral volatile profile over time acting as the relevant factor in foraging choice.

Further experimentation should centre on these possibilities, together with an investigation of long-range visual attraction. Retention of post-change yellow flowers could increase the overall display size and attract additional visitors (e.g. Cruzan et al. 1988; Gori, 1989; Weiss, 1991). Although *B. hortorum* responds to an alteration in colour cue, the change in colour may not be adaptive for the plant over much of its range where moths are the important pollinators. The role of colour vision in nocturnal insects has recently been shown to be of greater relevance than previously assumed; Kelber et al. (2002) demonstrated that chromatic cues were used in foraging by the hawkmoth *Deilephila elpenor* in tests with artificial flowers where odour cues were absent. Weiss (1995a) and Weiss & Lamont (1997) emphasized the relevance of whole-flower colour change in moth-pollinated flowers (as opposed to localised part-flower changes that offer a relatively small, less perceptible visual signal). Lunau & Maier (1995) noted that consideration of the UV characteristics was also important;

human-white flowers that were visited by bees during the day absorbed UV-light, whereas reflectance of UV-light was found in several 'white' species visited by moths at night. If moths are the natural co-evolved visitors to *L. periclymenum*, the role of colour and colour change as purely visual cues requires further examination.

The importance of visual cues and the pollination effectiveness of other visiting insects remain unresolved. Syrphids could be valuable pollen vectors over short distances; their foraging activity was limited to small patches of inflorescences but did involve contact with stigmas. Whether feeding on sugary stigmatic exudate or removing pollen already deposited on a stigma, these insects could enhance pollen transfer. No data were collected on the colour phases visited by these insects.

7.4.2 Floral manipulations.

In the initial greenhouse experiments the finding that control flowers had the shortest mean longevity (Figure 7.16 and Table 7.1) appears paradoxical in the light of subsequent experiments linking the effects of pollen deposition with shorter life spans (Figure 7.18). However, all other group treatments incorporated some removal of pollen and there was, therefore, less chance of accidental self-pollination in these groups. All pollen was removed in three groups (pollen depletion, emasculation and nectar withdrawal with emasculation) and some pollen was dislodged during manipulation in the other two groups (nectar withdrawal and corolla-pierced). Control flowers were not touched after initial marking; with a full complement of pollen as the flower develops, contact

between anther and stigma could have occurred in these flowers and transfer of pollen was therefore possible. Early floral abscission was subsequently shown to occur following pollen deposition in this plant and could result from a pollen-pistil interaction and/or subsequent reproductive processes.

Deposition of pollen, either within- or between-plants, reduced the time to abscission by 45% in *L. periclymenum* (Figure 7.18) and longevity was increased where stigmas were protected during trials and pollen deposition was therefore prevented (Figure 7.20 and Table 7.5). A review by van Doorn (1997) highlights the effects of pollination *per se* on floral attraction in a range of species; changes that potentially alter visitor behaviour vary from a subtle colour change to perianth abscission. Further wide-ranging studies of floral longevity (van Doorn, 2001, 2002) have centred on the classification of senescence and petal abscission, and the role of ethylene in such processes. These are discussed in Chapter 9 in relation to the early abscission of *L. periclymenum* flowers recorded in my study

The rate of colour change is seemingly independent of visitation in *Lonicera*, being purely age-related and non-inducible. However, floral retention, and therefore potentially continued visitation, is certainly linked to pollen deposition and/or subsequent processes. Visitation by insects that are not necessarily well adapted to transfer pollen or accidental short-range pollen movement via any perturbation of the inflorescence could contribute to the alteration of the visual cue. In the absence of these influences individual flowers last longer and thus the display remains attractive to further visitors. Any floral character that increases the opportunity of further pollination could benefit the plant by the means suggested in Figure 7.22.

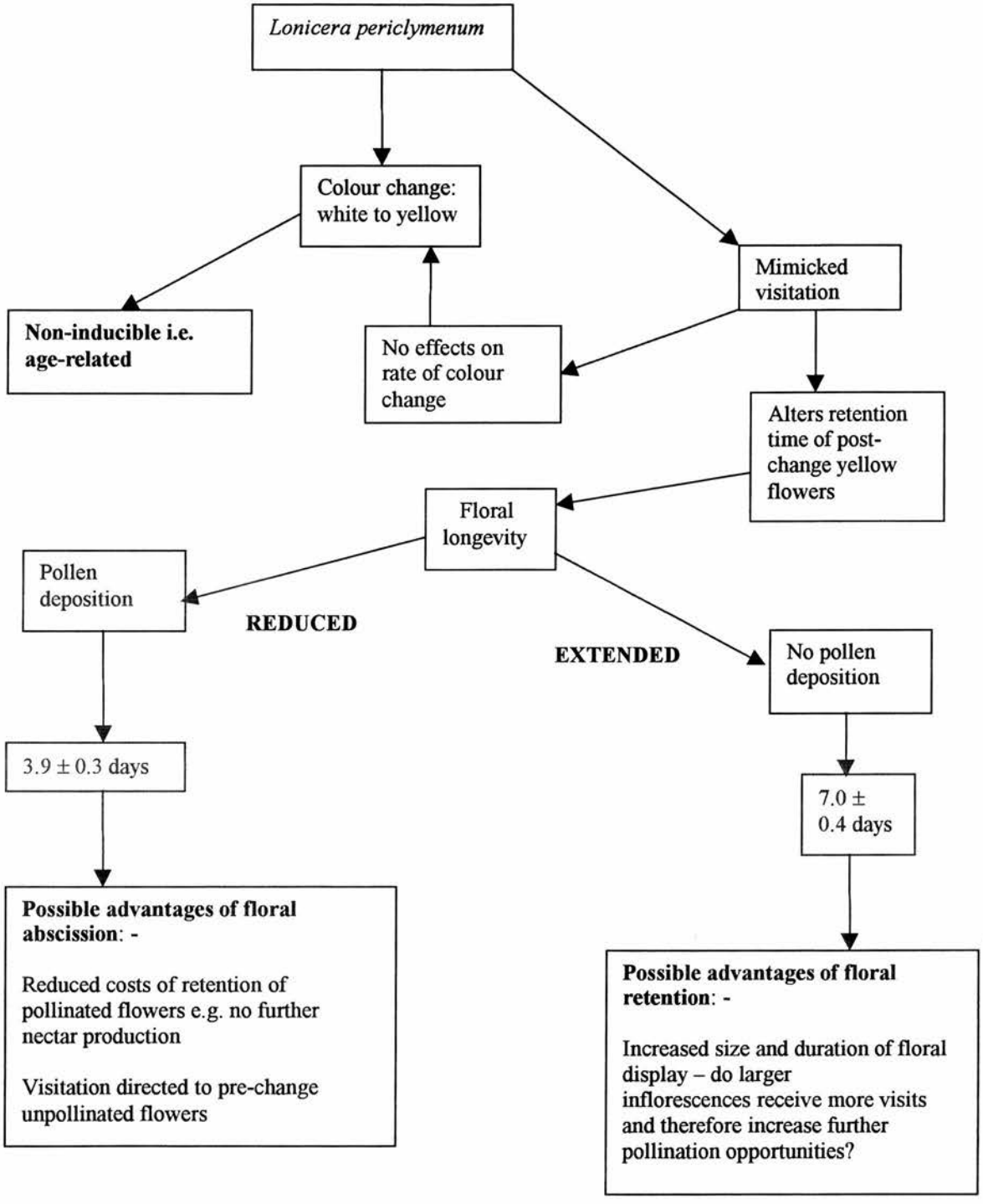


Figure 7.22 Schematic of possible benefits accruing to *L. periclymenum* from altered longevity of flowers following pollen deposition.

Chapter 8 – *Lupinus pilosus.*

8.1 Introduction.

Lupins display the keel blossoms that are widespread in many plant taxa (Westerkamp, 1997). An immovable vexillum (also referred to as flag, standard or banner petal and bearing a spot of varying colour) stands erect above a pair of wing petals and conceals partly fused keel petals within that are manipulated by visiting insects to expose pollen reward (e.g. Dunn, 1956; Faegri & van der Pijl, 1979; Westerkamp, 1997). Many species exhibit a localised colour change and the large conspicuous flowers can be easily manipulated to simulate insect visitation. The racemes of *L. pilosus* comprise acropetally developing whorls of five flowers that open on the same day (Ne'eman & Nesher, 1995). Hence, following visitation, differences in rate of colour change can be assessed straightforwardly in relation to adjacent flowers and whorls of different age and therefore lupins present an ideal plant to test the influence of visitation on floral colour change.

Several lupin species undergo a localised change in the colour of the banner petal spot (BPS). Weiss (1995a) includes 18 examples in her database while, earlier, Gori (1983) had reported that 26 of 48 species displayed colour change, and these have been the subjects of a number of studies. In the New World *Lupinus*, group *Micranthi* (Dunn, 1956), *L. texensis* (Schaal & Leverich, 1980), *L. arizonicus* and *L. sparsiflorus* (Wainwright, 1978), *L. argenteus* (Gori, 1989), *L. nanus* (Juncosa & Webster, 1989); *L. albifrons* (Stead & Reid, 1990); *L. nanus* (Karoly, 1992) and *L. arizonicus*, *L. nanus*, *L. albifrons* ssp. *collinus*, *L.*

texensis and *L. propinquus* (Weiss, 1992) have received such consideration. However, of Old World species, colour change research has been very limited, and has only concerned *L. pilosus* (Ne'eman & Nesher, 1995).

Between different members of the genus, and even within species, there have been conflicting results in respect of the pattern of colour change. Change was reported to be inducible, perhaps as a result of post-pollination changes (e.g. Stead & Reid, 1990; Weiss, 1992; Ne'eman & Nesher, 1995), or to be a non-inducible, age-related process (e.g. Dunn, 1956; Gori, 1989; Juncosa & Webster, 1989; Karoly, 1992). Colour change in *Lupinus nanus* was found to be inducible by Weiss (1992), occurring in 1-2 days where visitation was allowed but being retarded to 5+ days when flowers were bagged. In contrast, for the same species (Karoly, 1992) related a change in the colour of the banner petal spot to changes in stigma receptivity that were simply age-related. In *L. texensis*, Weiss (1992) found an almost identical pattern to her findings for *L. nanus*, whereas Schaal & Leverich (1980) described a non-inducible colour change that happened after 5-6 days.

Of the above studies, only one reported that manipulation of the flower led to colour change; in *L. arizonicus* and *L. sparsiflorus* removal of pollen from, or excision of, the stigma during the period of stigmatic receptivity triggered such change (Wainwright, 1978). Colour change of the BPS in *L. pilosus* in Israel was described by Ne'eman & Nesher (1995), whose other findings included greater pollen reward in pre-change flowers and a preference for visiting these flowers by foraging bees.

Nectar reward in lupins was said to be absent by Knuth (1906-09), Dunn (1956), Faegri & van der Pijl (1979), Gori (1989), and Yeo (1993) but

Westerkamp (1997) and Westerkamp & Weber (1999) suggested that nectar was available, although quantitative data were not presented. Pollen is always a reward in lupins, but is presented secondarily by a triggering mechanism; Westerkamp (1997) described a 'piston-like' mechanism whereby visiting insects cause a non-explosive release of pollen accumulated in the acumen of the keel petals (see also Yeo, 1993). Ne'eman & Nesher (1995) noted that in *L. pilosus* pollen could be squeezed beyond a collar of peristigmatic hairs to contact the stigma and effect self-pollination; however in some lupin species a further requirement was the rupturing of a cuticle around the stigma before pollen can contact it (Juncosa & Webster, 1989).

In *L. pilosus*, the wing petals and outer margins of the flag petal remain blue throughout opening and colour change is restricted to the BPS which is white at initial flowering before turning pink and then, eventually, purple prior to abscission (Ne'eman & Nesher, 1995). These authors showed artificial pollination accelerated colour change in this species and the change itself was suggested to be of benefit to both plant and visiting insect. The visitor forages on more rewarding (pre-change) flowers thus avoiding already pollinated (post-change) flowers, and thus enhances further pollination success (and outcrossing opportunity) for the plant (Ne'eman and Nesher, 1995).

A variety of bees, particularly anthophorids (Dunn, 1956; Wainwright, 1978), *Apis mellifera* (Knuth, 1906-09; Dunn, 1956; Schaal & Leverich, 1980; Wainwright, 1978), megachilids (Knuth, 1906-09; Dunn, 1956; Wainwright, 1978), and bumblebees (Knuth, 1906-09; Dunn, 1956; Schaal & Leverich, 1980; Wainwright, 1978) has been noted to feed on, or collect, pollen from various

lupin species. Anthophorids and *A. mellifera* were the most abundant visitors to *L. pilosus* in Israel (Ne'eman and Nesher, 1995).

Early spring flowering of *L. pilosus* on the island of Lesbos, Greece enables an extension of the field season relative to the UK. The current study addresses what the trigger may be for colour change in *L. pilosus* (separating the 'mechanical' effects of flower handling during visitation from subsequent pollination processes) through a combination of observational work and laboratory analysis of reproductive processes.

8.2 Materials and Methods.

8.2.1 Study plant and site.

L. pilosus L. (syn *L. pilosus* Murr.; *L. varius* L. ssp. *orientalis* Zohary) (Fabaceae) is a self-compatible annual legume that flowers from March to June, usually on acidic soils. It is found throughout the eastern Mediterranean (Blamey & Grey-Wilson, 1993; Plitmann et al. 1980) and on Lesbos is locally abundant (T. Petanidou, personal communication).

A population in excess of 10,000 plants was studied at a terraced roadside olive grove (Figure 8.1), between the villages of Remva and Chidira on the island of Lesbos (N 39° 12′ 32.5″, E 026° 01′ 54.7″). The site had a southwesterly aspect and light, sandy soil.

Figure 8.1. Part of study site showing patches of *L. pilosus*, near Chidira, Lesbos.

L. pilosus formed dense homogeneous carpets; specimens of *Asphodelus aestivus* occasionally grew within these patches but were more plentiful at the

edge of these lupin stands and amongst rockier soil where *L. pilosus* was largely absent. *Muscari* sp. was present in low numbers around the edge of the lupin patches.

The study was carried out between 13th March and 3rd April 2001.

8.2.2 Floral longevity, colour change and reward.

The nature and rate of colour change, together with floral longevity, were examined under natural conditions and, to confirm the effects of visitation, with visitors excluded.

Randomly chosen individual plants were tagged and individual flowers marked with cotton thread on the day prior to opening; subsequently, each morning for nine days the flowers were examined and notes were made on the colour and condition of each flower. Concurrently, individual plants were enclosed in fine gauze bags (< 1mm. apertures) to exclude insect visitors, and individual flowers were again tagged with cotton thread; data were recorded similarly.

Efforts to extract nectar from *L. pilosus* were unsuccessful (no data collected). In numerous attempts with different sizes of microcapillary (0.5 an 1.0μl) I failed to remove any nectar although co-workers reported small quantities (P. Willmer, personal communication); the behaviour of visiting bees did suggest the presence of nectar (see below).

8.2.3 Visitation.

Insect visitation to *L. pilosus* was recorded over a four-day period. Between the hours of 08.00 and 18.00 visitation to a focal patch of 16 plants (displaying between 79 and 142 flowers) was noted for 30 minutes within every hour. Preliminary observations confirmed an absence of visitors outside this time period; the aspect of the site led to shading and, concomitantly, reduced temperatures that were unsuitable for foraging insects. A visit was deemed to be a landing on a flower that involved possible nectar feeding (bees probed the base of the banner petal with their tongues), an attempt to feed, or collection of pollen that lead to contact with floral reproductive parts. Visiting solitary bees were grouped into two categories according to body length; 'small' bees were <8mm and 'medium' bees were approximately 10-15mm.

By far the most important visitor was *Bombus terrestris* L. (Apidae). Therefore, foraging individuals of this species were chosen at random and followed for periods of between 10 seconds and two minutes; data were collected on number of plants visited, colour of flower visited and residence time on individual flowers. On each day that data collection was undertaken, the proportion of flower colour phases available was noted in a random sample of up to fifty plants within the population.

8.2.4 Effects of visitation rate on colour change.

An observational experiment to assess whether visitation *per se*, or rate of visitation, might influence colour change was carried out by allowing limited visitation to individual flowers and then recording subsequent colour change in those flowers.

Whorls of flowers on up to ten closely grouped plants that were one day prior to opening were bagged overnight and opened to visitation the following morning. The focal plants were continuously observed until differing levels of visitation had been recorded; individual flowers were marked and plants 're-bagged' after single, 4-6 or 10+ visits by foraging bees. The flowers were examined daily for the five following days and any colour change noted. To act as control groups, two further cohorts of flowers were marked concurrently; one group was bagged to prevent visitation completely and the other left open to natural rates of visitation.

8.2.5 Mechanical manipulation experiments (i).

Following observations of visitation behaviour of *B. terrestris*, an attempt was made to simulate visitation and monitor the rate of subsequent colour change. Fifteen plants were tagged and five individual flowers on a single whorl marked a day prior to opening and randomly placed into one of five experimental groups: -

- Control – tagged only and natural visitation allowed throughout experimental period

• Bagged – covered with fine gauze bags to exclude visitors throughout the experimental period

• Manipulation x1 – single manipulation (see below) of marked flowers on day of opening, then bagged as above

• Manipulation x5 – five manipulations of marked flowers on day of opening, then bagged as above

• Manipulation x20 – twenty manipulations of marked flowers on day of opening, then bagged as above.

The 'manipulations' involved using a pair of dissection forceps to ease back the wing petals from the inner keel, then moving a dissection probe gently along the upper surface of the keel from the point of insertion at the base of the banner petal towards the acumen of the keel (Figure 8.2). Triggering of the keel mechanism and pollen release were not necessarily achieved during this manipulation.

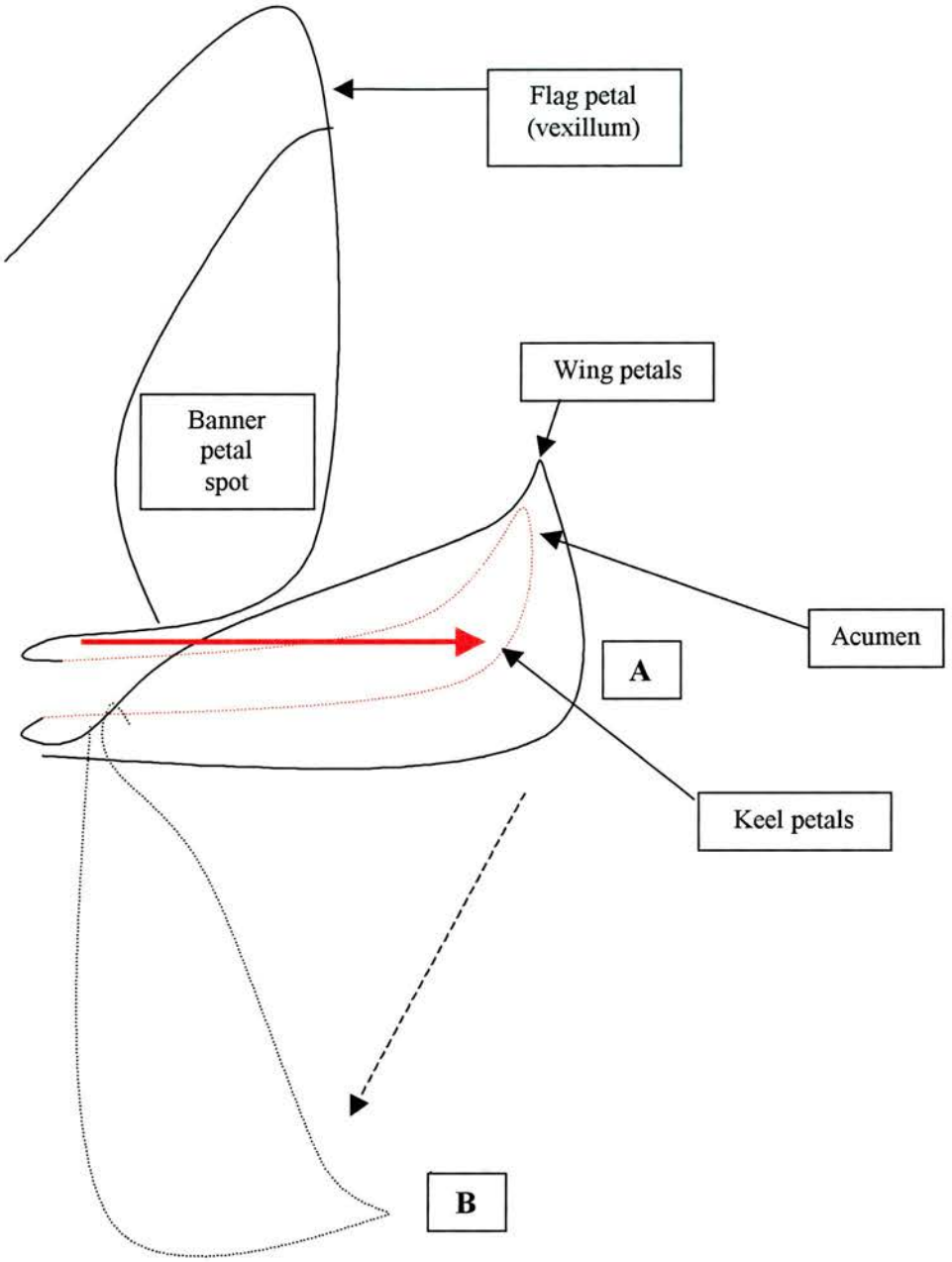


Figure 8.2 Schematic of lupin flower (side view) to show manipulation of wing and keel petals. Solid black line and point A denotes normal position of wing petals; wing petals eased back to position B with forceps to expose hidden keel petals (drawn in red and enclosing pistil and stamens within tip (acumen)). Solid red arrow describes direction of movement of dissection probe during manipulations.

Each flower was checked daily for nine days and any colour change noted. Three replicates of these manipulations were achieved. The first replicate was carried out on the first (i.e. basal) whorls of randomly chosen plants, whereas subsequent replicates used whorls of a 'higher' position on the raceme.

8.2.6 Mechanical manipulation experiments (ii).

Trends in section 8.2.5 did not distinguish sufficiently between 'handling effects' *per se*, and 'handling leading to triggering of pollen release'. Therefore, a set of manipulations was carried out to attempt to determine whether a simple handling effect could influence onset and rate of colour change compared to a full triggering of the keel mechanism and consequent depletion of pollen. The latter manipulation attempted to mimic the activity of bumblebees collecting pollen.

A control group of nine plants was chosen with their topmost whorl a day prior to opening. Individual flowers were tagged and monitored daily for the six-day duration of the experiment. Twenty-four further plants, with whorls at the same stage of development, were enclosed in fine gauze bags and manipulated, on the morning of opening, in the following manner: -

- Group A (12 plants) – all five flowers on a marked whorl received gentle manipulations as in section 8.2.5. Four plants received a single manipulation per marked flower, four received five manipulations per flower and four received twenty manipulations per flower. The piston mechanism was not triggered during these manipulations.
- Group B (12 plants) – all five flowers on a marked whorl received firmer manipulations whereby the triggering mechanism was clearly released; the anthers and style were exposed and pollen was removed by a firmer stroking action of the dissection probe in the manner previously described. Four plants received a single triggering per marked flower, four received five 'triggerings' per marked flower and four received twenty manipulations.

In the case of the latter group pollen was, on some occasions, fully depleted prior to the twentieth manipulation (visual inspection); all pollen was depleted on a single flower in as little as ten manipulations, while five flowers still yielded pollen after twenty manipulations.

All flowers from the control and experimental groups were examined each morning for five days; the onset and nature of colour change were recorded, and the proportion of flowers in each group that had undergone colour change was calculated.

8.2.7 Pollen tube growth.

Sample flowers of different ages were collected for analysis of pollen tube growth by fluorescence microscopy, to establish whether colour change in *L. pilosus* might correspond with various stages of reproductive development. Fifty-six plants were chosen at random and assigned to one of two experimental groups. Twenty-eight plants were left open to visitation and twenty-eight were bagged as before; in both instances the topmost whorl one day prior to opening was marked with cotton thread. At 24-hour intervals thereafter, four plants from each group were chosen at random, examined and scored for colour phase and then the five flowers from the marked whorl were removed and fixed in 70% ethanol. All samples were stored at 4°C until prepared for fluorescence microscopy and examined at the laboratories of the Sir Harold Mitchell Building, University of St. Andrews.

Floral tissue was removed to expose the carpel, and the distal 1cm of the style excised. Styles were softened by incubation for seventeen minutes at 65°C

in 8M NaOH, washed in distilled water, and stained with 1% aniline blue, prior to squash preparation on microscope slides (modified from Gibbs & Bianchi, 1999). Prepared material was examined using a Leitz Laborlux 12 binocular microscope fitted with a fluorescent light source. Fixed specimens were then scored for pollen grains attached to the stigma, and evidence of pollen tube growth in stigma and style. At least ten pistils from both visited and non-visited flowers from each 24-hour collection period were examined.

8.2.8 Effect of position of flower and colour phase on foraging preference.

The foraging preference for white BPS flowers could be based exclusively on colour, or might be related to the position of the flower on the plant. *L. pilosus* inflorescences open sequentially from the basal whorl and, consequently, white flowers become concentrated towards the top of the raceme with older, lower, flowers having predominantly pink and purple BPS (Figure 8.3).

To control for this possibility and ensure that only BPS colour was the cue, banner petals from individual flowers were carefully detached and 'inserted' into a second, intact, flower (Figure 8.4). By this manoeuvre it was possible to alter the visual signal to a visiting insect, and offer different colour phase flowers in novel positions on the plant, i.e. white BPS (rewarding) flowers on topmost whorls could be masked with purple banner petals from older flowers, and purple (unrewarding) flowers in positions further down the raceme could be masked with white banner petals. The hypothesis was that if colour were the most important cue bees would continue to forage predominantly on white flowers

irrespective of position on the plant. If, however, position were more important then foraging would continue on uppermost flowers that offered normal reward but with purple banner petals.

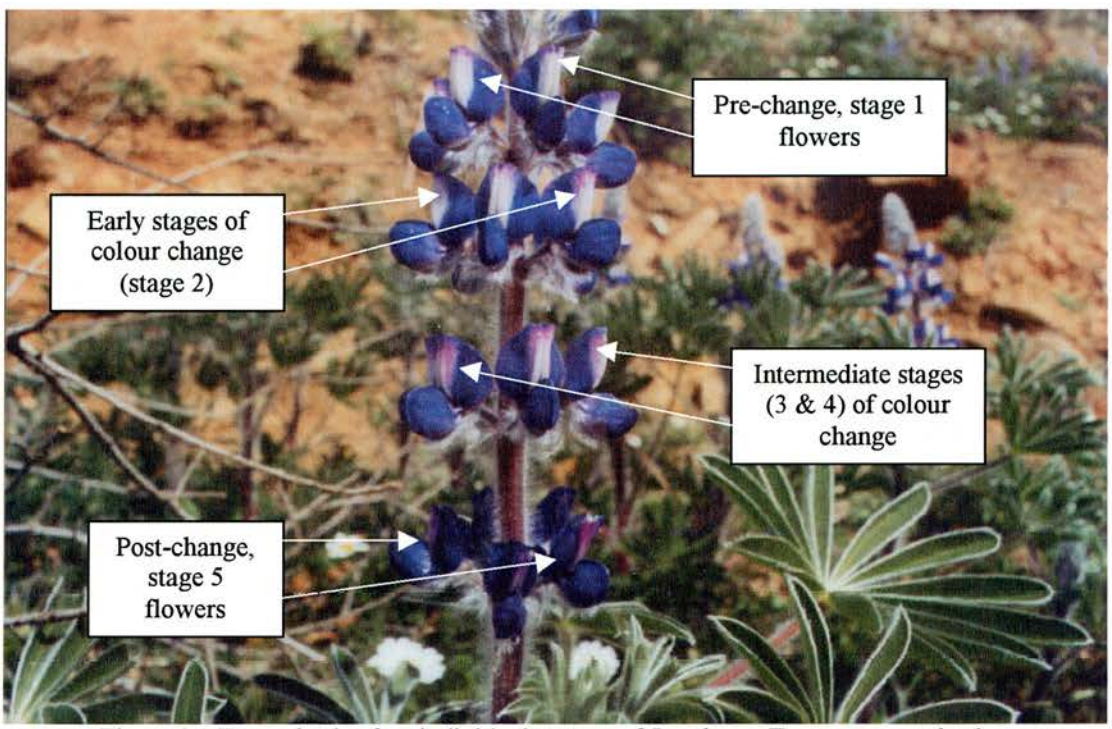


Figure 8.3 Four whorls of an individual raceme of *L. pilosus*. Topmost two whorls comprise flowers that exhibit no, or little, evidence of colour change; whorls 3 and 4 show flowers in advanced/completed phases of colour change (see 'Results' text for full description of stages of change).

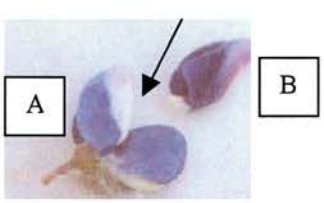


Figure 8.4 White BPS of an individual, intact flower (A) can be masked by insertion of complete, excised flag petal of a post-change flower (B).

Five plants were enclosed in fine gauze bags three days prior to observations; this ensured that the upper whorls on each plant received no

visitation between opening and observations. On the morning of observations the bags were removed and the following manipulation carried out:

- Three plants were randomly chosen as a control group and tagged only;
- Two plants, all white flowers on the uppermost whorls, had their banner petals 'masked' with purple petals removed from other plants as described above.

Sketches of flower positions on all five plants were made, and insect visits (as defined earlier) and inspections (non-landing approaches to within 2 cms.) were recorded over a five-hour period.

8.3 Results.

8.3.1 Floral longevity and colour change.

Mean longevity of *L. pilosus* flowers under natural conditions was 5.3 ± 0.1 days (n = 45), rising to 10.0 ± 0.1days (n = 43) with visitors excluded (Figure 8.5) (Mann-Whitney test, using medians, W = 1035.0, $p < 0.0001$).

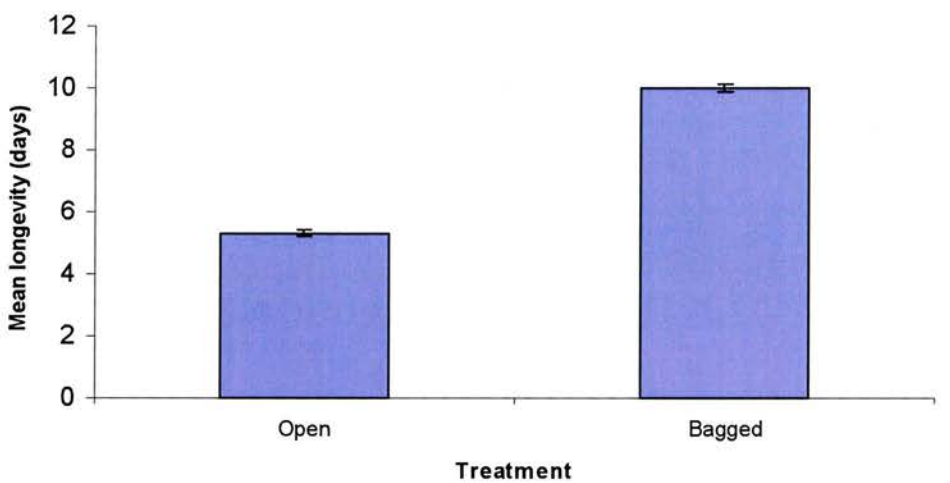
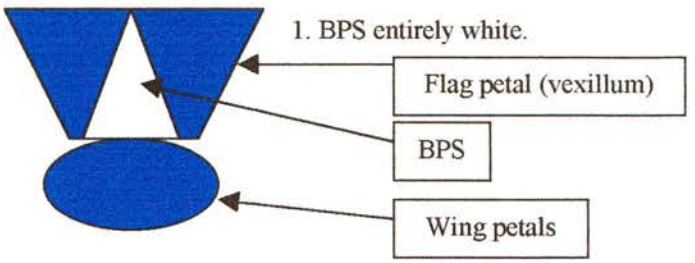
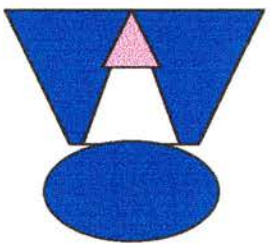


Figure 8.5 Floral longevity in *L. pilosus*.

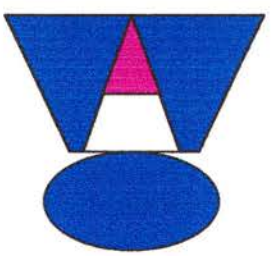
Clearly visitation and/or the presence of bags *per se* had an effect on longevity.

Five stages of floral colour change of the banner petal spot (BPS) were identified in *L. pilosus* (Figure 8.6). Stages 1 and 2 were recorded as white, 3 and 4 pink and stage 5 purple for comparison of phase lengths and subsequent scoring of bee visitation. Hereafter these are simply referred to as 'white flowers', 'pink flowers' and 'purple flowers' though only the BPS changed.

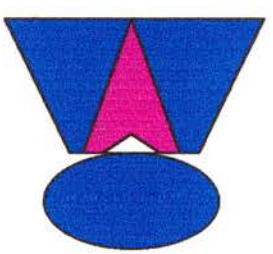




2. BPS predominantly white; first evidence of pinkish blush towards top of flag petal.



3. First intermediate stage; BPS predominantly pink and brighter than stage 2. Area of white coloration restricted to lower part of vexillum



4. BPS almost entirely bright pink. Small patch of white at lower centre of vexillum.

5. BPS entirely purple-blue.

Figure 8.6 Schematic of floral colour change in banner petal spot (BPS) of *L. pilosus*.

The duration of the colour phases of the banner petal spot (BPS) varied according to treatment (Figure 8.7); with normal visitation the BPS remained

white for 1.8 ± 0.1 days whereas with visitors excluded it remained white for 5.1 ± 0.2 days (Mann-Whitney test, using medians; W = 1089.0, $p < 0.0001$). The pink phase lasted 1.6 ± 0.1 days in open flowers and 2.3 ± 0.2 days in bagged flowers (Mann-Whitney test, using medians; W = 1622.5, $p = 0.0008$). The purple phase was recorded for 1.9 ± 0.1 days and 2.5 ± 0.1 days in open and bagged conditions, respectively (Mann-Whitney test, using medians; W = 1594.0, $p = 0.0002$). Clearly all phases were lengthened by bagging, but the main reason for increased longevity was an extension of the young/white phase.

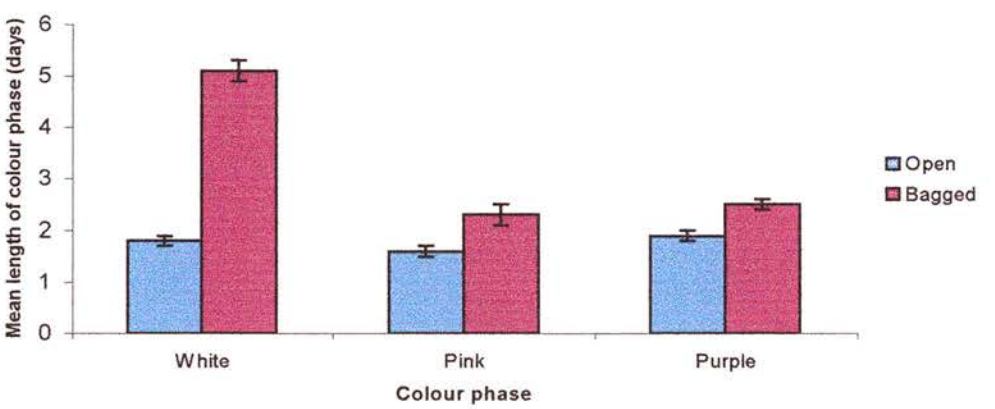


Figure 8.7 Duration of the colour phases of the BPS under different treatments in *L. pilosus*.

8.3.2 Visitation.

Visitation was solely by insects, and was dominated by hymenopterans. In twenty-two hours of observations 65 visitors were recorded of which 59 were bees (Figure 8.8). *B. terrestris* accounted for 60% of all visitors, and bumblebees were the only group at the focal patch noted to activate the trigger mechanism and gain access to pollen reward. A number of medium-sized (10-15mm) and small (<8mm) solitary bees were recorded, none of which were able to trigger the

pollen release mechanism. This was also the case for all other insect visitors including bombyliids, syrphids and other dipterans.

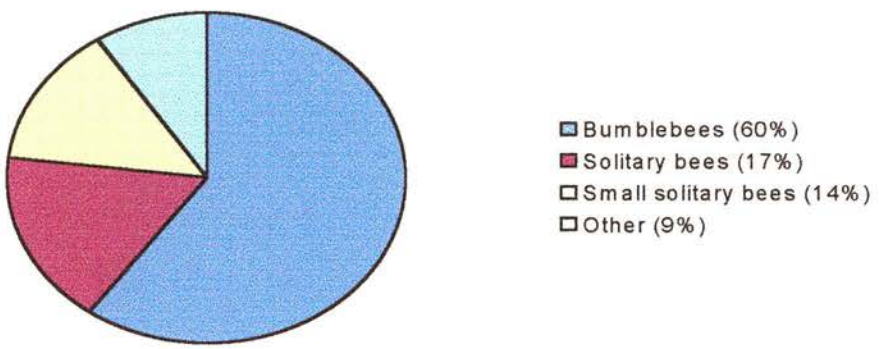


Figure 8.8 Gross visitation pattern to focal patch of *L. pilosus*. Total visitors comprised 39 *B. terrestris*, 11 medium/large solitary bees, 9 small solitary bees and 6 other insects.

Visitation behaviour within the focal patch suggested *B. terrestris* was the most likely pollen vector; in addition to being the only 'triggering' visitor, they made multiple flower visits per plant and to multiple plants within the patch. Solitary bees (31% of all visits) often only visited a single flower per inflorescence or per plant.

The BPS foraging preference during 150 foraging bouts by *B. terrestris* was recorded during visits to 2073 flowers. These observations were made across 6 days and, although individual bees were not marked following data collection, by working in different areas of the study site (and given that several hundred bees could be actively foraging at any one time) probably avoided pseudoreplication. Of these visits, 2041 (98%) were made to white flowers (mean residence time per flower was 1.87 ± 0.60 seconds; n = 78 bees) and 32 (2%) to pink flowers; no visits were noted to purple flowers. Sixteen individual bees made visits to pink flowers and the remainder (134) foraged exclusively on

white flowers. In the population, on 4 days on which observations were made, 3479 flowers were counted on 225 plants in the following proportions: 2028 white (58%), 770 pink (22%), 681 purple (20%) (Table 8.1).

Count/day	Number of plants	Total flowers	Colour phase		
			White	Pink	Purple
			Number of flowers (%)		
1	75	1233	705 (57)	287 (23)	241 (20)
2	50	787	461 (59)	176 (22)	150 (19)
3	50	711	409 (58)	162 (23)	140 (19)
4	50	748	453 (61)	145 (19)	150 (20)
Total	225	3479	2028 (58)	770 (22)	681 (20)

Table 8.1 Proportions of flower colour phases over 4 days in *L. pilosus*.

The observed visitation preferences thus differed significantly from those expected from colour phase availability within the population (Figure 8.9, $\chi^2 = 1032.0$, d.f. = 2, $p < 0.001$).

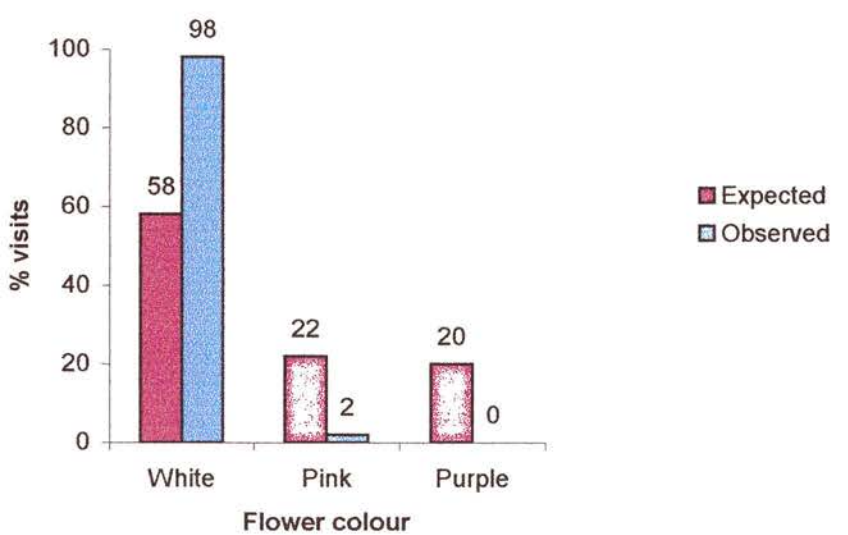


Figure 8.9 Observed visitation of 150 foraging *B. terrestris* to 2073 flowers of *L. pilosus*.

8.3.3 Effects of visitation on colour change.

Within the control group, under conditions of natural visitation, all flowers (n = 45) had changed colour by day 5, though no colour change was noted on the first day. In contrast, bagged flowers showed no colour change until at least day 4 and only 40% (n = 43) had changed colour by day 5 (Figure 8.10).

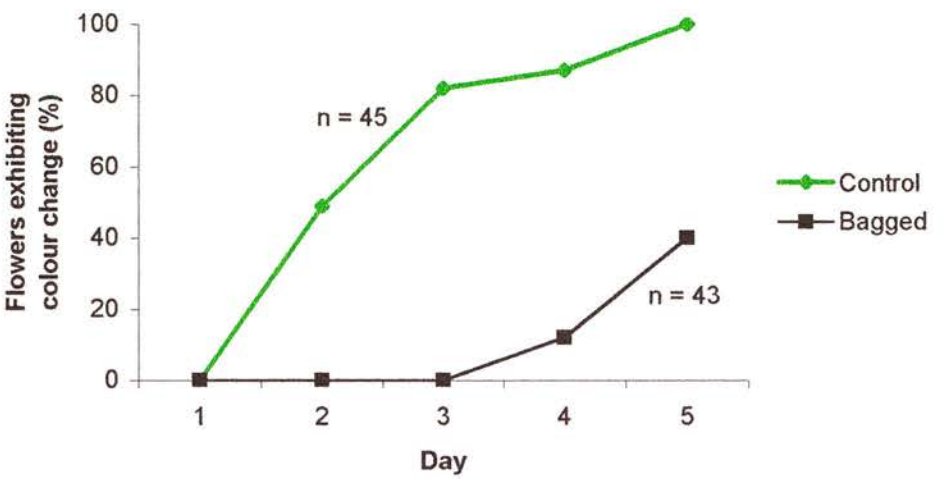


Figure 8.10 Rate of colour change in *L. pilosus*.

Figure 8.11 illustrates the effects of varying controlled levels of visitation by *B. terrestris* to *L. pilosus*. No colour change was noted in any group until day three. After five days, 48% of flowers receiving a single visit (n = 31) had changed colour; 82% of flowers (n = 34) receiving 4 to 6 visits had changed at this stage and 77% of flowers (n = 26) receiving over ten visits had reached the pink phase. In this latter group no flower received more than 18 visits. Due to the tendency of *B. terrestris* to visit more than a single flower on each inflorescence in rapid succession, plants were bagged after the 'last' focal flower received its tenth visit (adjacent flowers often having received more than this number of visits).

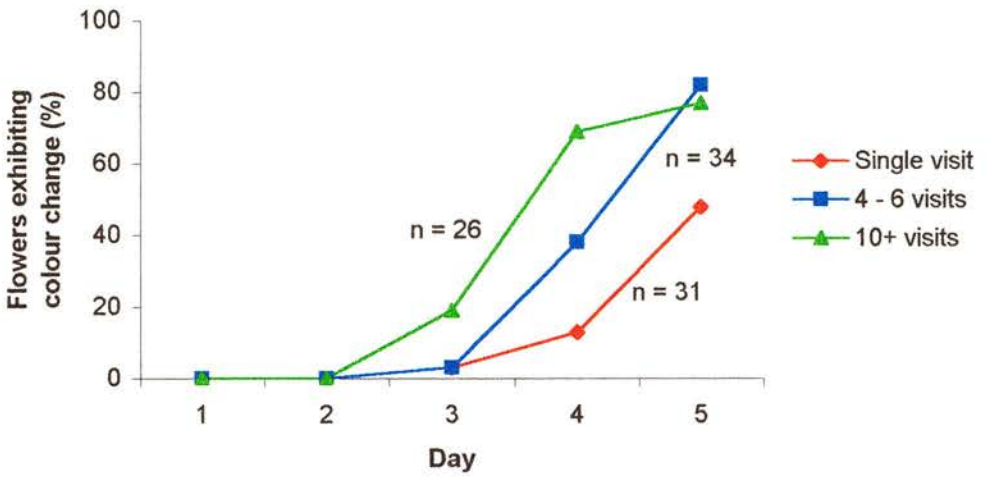


Figure 8.11 Effects of different rates of visitation by *B. terrestris* on colour change in *L. pilosus*.

Colour change was, therefore, affected by the rate of visitation *by B. terrestris*; although change could be triggered by a single visit, a greater number of visits was more likely to lead to colour change in most flowers. The rate of colour change following a single visit was almost identical to that found in bagged flowers (see Figure 8.10); this suggested that some autogamous self-pollination occurred in the latter group and is discussed later.

8.3.4 Mechanical manipulation experiments (i).

Commencement of colour change was first noted in the control group flowers (day 2 or 3) (Figure 8.12), and change had occurred in all these flowers by 3-5 days from initial opening. The onset of colour change was slowest in the bagged group, commencing from day 4 and evident in all flowers between day 6 and 9. The manipulated groups displayed intermediate onset and rates of change, but generally followed the pattern of earlier and faster change corresponding to greater number of manipulations.

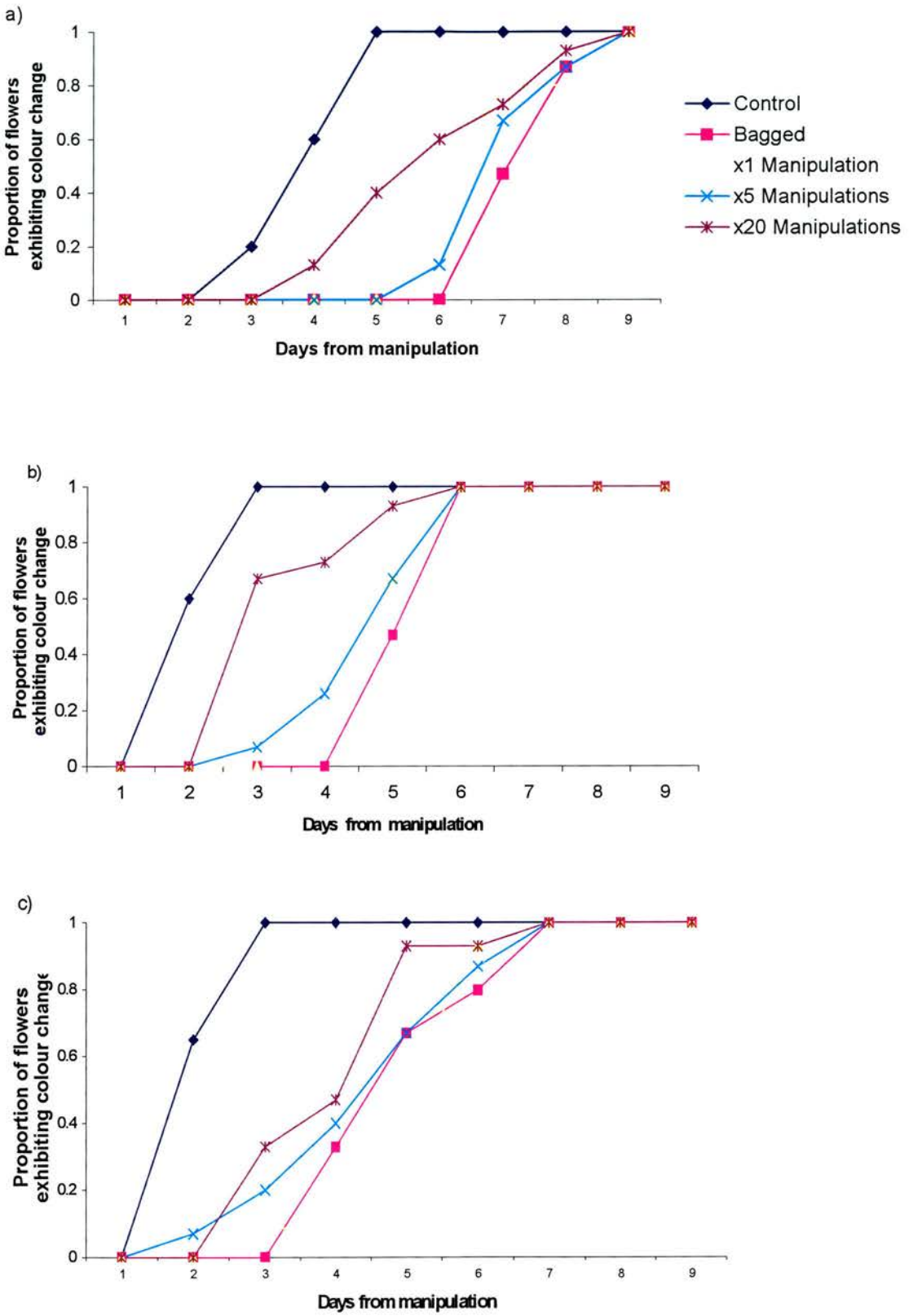


Figure 8.12 Colour change in *L. pilosus* (3 replicates) following mechanical manipulation (n = 15 in all treatment groups). Flowers in (a) were from basal whorls whereas those in (b) and (c) were from upper whorls.

Interestingly, basal whorl flowers (Figure 8.12a) changed colour more slowly than upper whorl flowers (Figures 8.12b and c) under all conditions. Even 20 manipulations of basal whorl flowers failed to induce as rapid a change as that seen following fewer manipulations in upper whorl flowers. This slower change and therefore prolonged attractive phase could have fitness benefits to the plant if further visitation results. Ne'eman & Nesher (1995) found that seed production varied with whorl position; basal whorl flowers produced significantly more seeds than those on higher whorls.

8.3.5 Mechanical manipulation experiments (ii).

In these experiments, manipulation *per se* was distinguished from manipulation with pollen release. The mimicking of a handling effect without causing triggering of the pollen release mechanism did not lead to the onset of colour change within a five-day period (Table 8.2); no change was noted in any of the experimental groups. The pattern of colour change in these experiments differed from that recorded in 8.3.4, with reduced numbers of flowers exhibiting change, even in the control group, when compared to earlier results (see Figure 8.12). This was probably related to a period of cold, wet weather (unfortunately, environmental data were not collected); control group flowers may have received fewer visits as a result of reduced foraging activity by bees (personal observation) and, given the conditions, physiological processes may have been retarded (and, therefore, rate of colour change slowed) in all plants.

Manipulation level	No. of flowers exhibiting colour change					
	Days after manipulation					
	1	2	3	4	5	6
Control (n = 45)	1	6	7	11	18	28
x1 manipulation (n = 20)	0	0	0	0	0	0
x5 manipulations (n = 20)	0	0	0	0	0	0
x20 manipulations (n = 20)	0	0	0	0	0	0

Table 8.2 Onset of colour change in *L. pilosus* following experimental handling effects with no triggering of pollen release mechanism (group A).

Where triggering was effected by heavier manipulations with pollen release, colour change was recorded in all experimental groups to varying levels (Figure 8.13). Where 20 manipulations per flower had been carried out, the proportion of flowers that changed colour within five days (60%) almost matched that of the control group undergoing natural levels of visitation (62%). Hence pollen release may be an important influence on rate of colour change in *L. pilosus*.

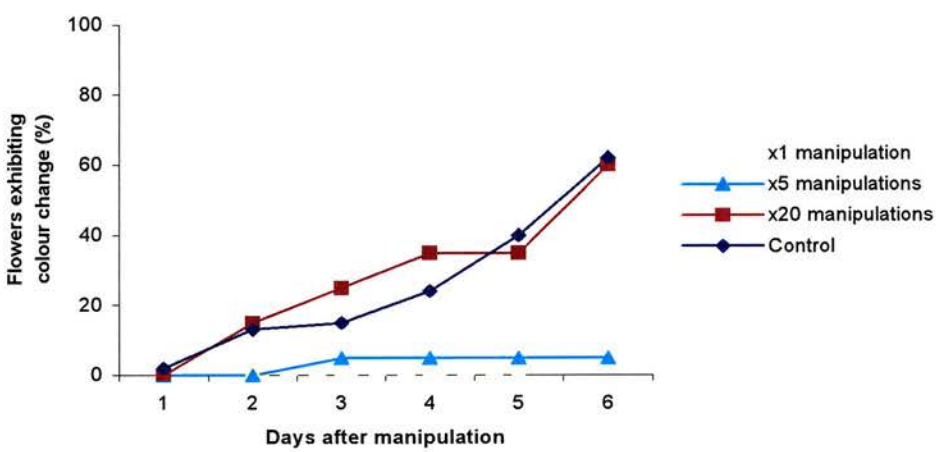


Figure 8.13 Onset of colour change in *L. pilosus* following handling effect with pollen release mechanism triggered (group B).

8.3.6 Pollen tube growth.

At least ten pistils from both visited and non-visited flowers from each 24-hour collection period were examined for evidence of pollen tube growth (Figure 8.14).

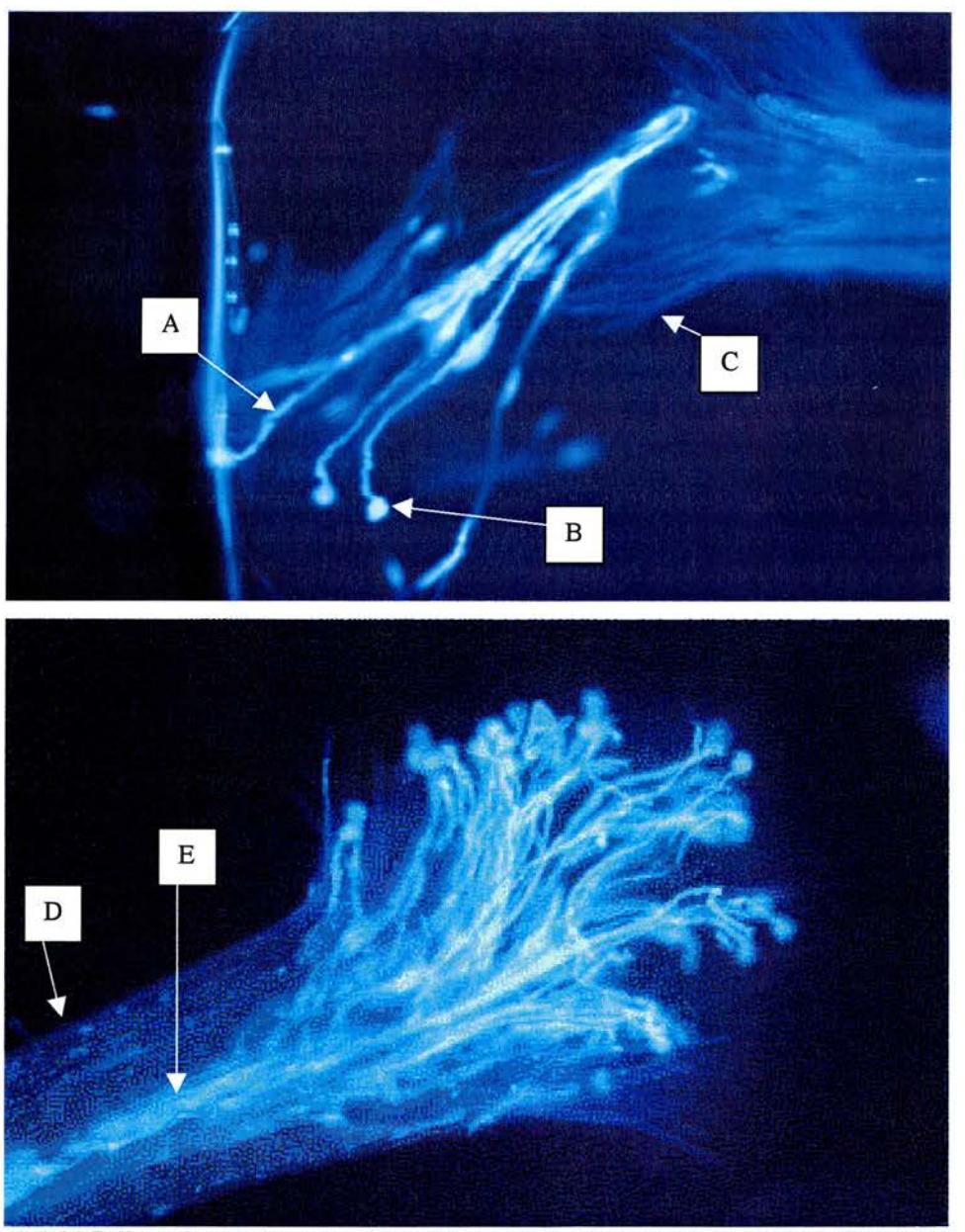


Figure 8.14 Pollen tube growth in *L. pilosus*. Top; sample from 'unvisited' group 168 hours after flower opening (5 pollen tubes visible). Bottom; sample from visited group after 144 hours (at least 35 pollen tubes visible) (photographed through Labor Lux binocular microscope with fluorescent light source). A = individual pollen tube; B = individual pollen grain; C = collar of peristigmatic hairs; D = style; E = bundle of pollen tubes growing towards ovules.

The presence of pollen tubes in preserved samples examined in the laboratory coincided fairly precisely with recorded colour change in *L. pilosus*. Pollen tube growth and colour change were also clearly related to visitation. Of 145 flowers inspected 60 exhibited colour change (Table 8.3), 53 of which were from the visited group and all of which had pollen tubes within the style. Four of the remaining 7 non-visited flowers had no pollen tubes visible either at the stigma or within the style whereas in the other 3 flowers pollen tubes were evident within the style. Eighty-five flowers showed no evidence of colour change; 64 of these (10 visited and 54 non-visited) had neither pollen grains adhered to the stigma nor any evidence of pollen tube growth. The remaining 21 (8 visited and 13 non-visited) had pollen grains adhered to the stigma and pollen tubes growing within the stigma but only one of these flowers (visited) had pollen tubes (two) visible within the first 1-2mm of the style.

Treatment	Number of flowers examined	Colour change commenced	Pollen grains evident at stigmatic surface	Pollen tube growth evident below stigmatic surface	Pollen tube growth evident within style
Visited	71	✓ (53)	✓ (61)	✓ (61)	✓ (54)
		✖ (18)	✖ (10)	✖ (10)	✖ (17)
Non-visited	74	✓ (7)	✓ (16)	✓ (20)	✓ (3)
		✖ (67)	✖ (58)	✖ (54)	✖ (71)

Table 8.3 Incidence of colour change in relation to pollen deposition and stages of pollen tube growth in flowers of *L. pilosus*. Figures in brackets refer to numbers of flowers in each category.

For the visited group, at each time interval, pollen tubes were present in the stigma and were apparent throughout the style in many cases. Mean numbers of pollen tubes at the stigma increased from 0.4 ± 0.4 after 24 hours to 32.9 ± 5.0 after 144 hours. Pollen tubes were rarely present in the non-visited group; none

were visible at the 24, 72 and 120 hour intervals with a maximum mean of 1.4 ± 0.6 per flower after 144 hours (Figure 8.15).

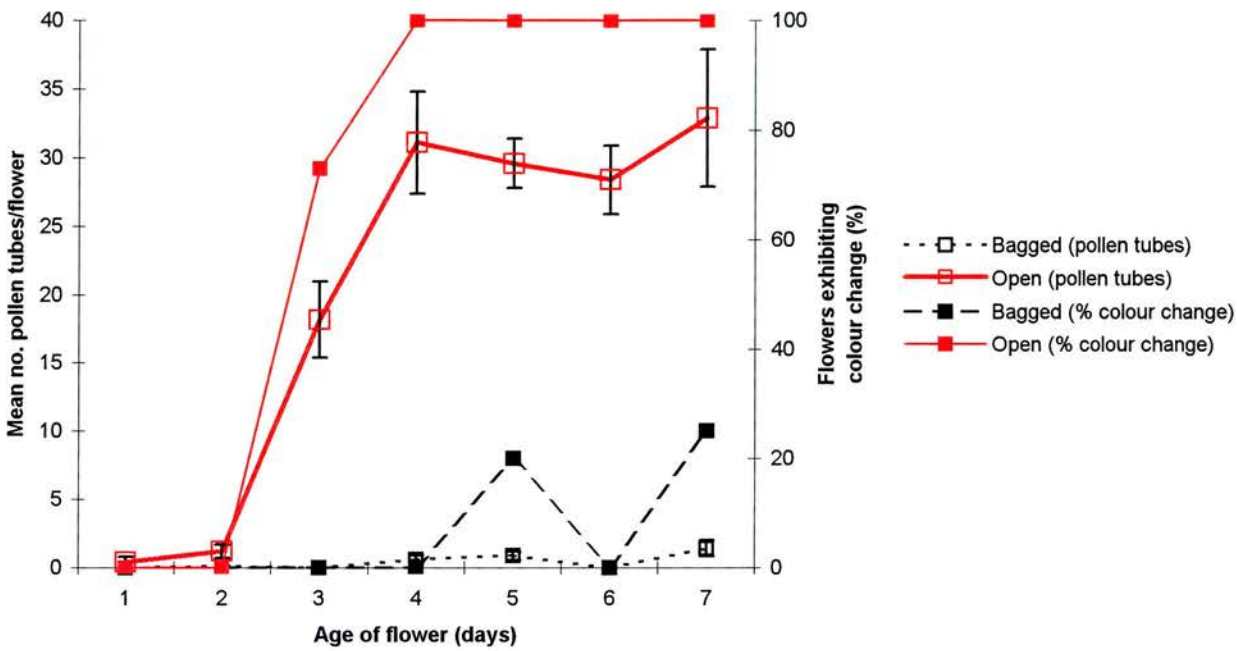


Figure 8.15 Mean number of pollen tubes visible at stigma and colour change, over time in *L. pilosus*.

In a two-factor Scheirer-Ray-Hare test of pollen tube growth, there was a significant interaction between time period and treatment (visitation/non-visitation); $H = 13.37$, df = 6, $p = 0.04$ (time; $H = 23.94$, df = 6, $p = 0.0005$, treatment; $H = 77.34$, df = 1, $p < 0.0001$).

These data highlight the close coincidence of colour change with post-pollination events in *L. pilosus*; pollen deposition and/or a very early stage of pollen tube growth were implicated in the onset of colour change.

8.3.7 Effect of position of flower colour and phase on foraging preference.

The three plants that were chosen as controls each displayed 15 flowers with a white BPS (5 on each of the uppermost three whorls). Eighteen flowers on the top two whorls of the remaining bagged plants were successfully manipulated to display an altered signal of purple flowers.

A total of 546 visits were recorded to flowers of both groups; 545 of these were to control group flowers (542 by *B. terrestris*, and three by assorted sized solitary bees). The single visit to a manipulated group flower was made by a syrphid. All 45 control group flowers were visited; flowers received between 2 and 24 visits (mean = 12.4 ± 0.8 visits). Inspections also occurred, with 197 in total (all by *B. terrestris*), of which 125 were made to control group flowers and 72 to manipulated flowers (Figure 8.16). Forty-one control flowers received between 1 and 8 inspections (mean 2.8 ± 0.4), and 16 of 18 manipulated flowers were inspected between 1 and 9 times (mean 4.0 ± 0.6 inspections).

There was no significant difference between the median number of inspections of individual control and manipulated flowers (Mann-Whitney test, $W = 1326.5$, $p = 0.08$, median number of inspections 2.0 to manipulated, 4.5 to control flowers). However, there was a significant difference between the median number of visits to individual control and manipulated flowers (Mann-Whitney $W = 1845.0$, $p<0.001$, median number of visits was 0 to manipulated, 13.0 to control flowers).

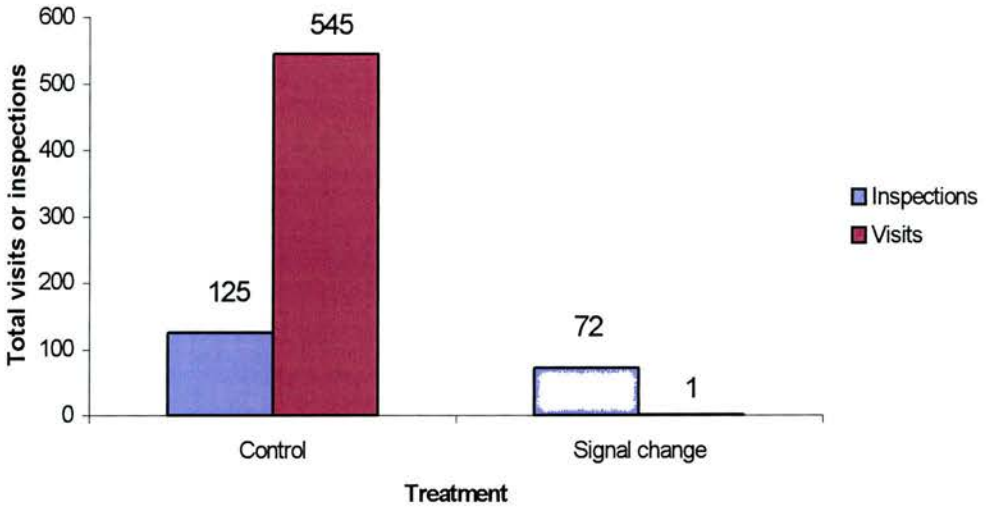


Figure 8.16 Total number of inspections and visits (see text for details) to control and 'signal-changed' flowers of *L. pilosus*.

Therefore, it is clear that position on the plant is less important than colour as a foraging cue. No visits to purple flowers on uppermost whorls were made by *B. terrestris* despite regular approaches to within 2cms of these flowers. Unfortunately, further replication and variation of this work was prevented by inclement weather and this compromised the validity of these results. It was intended to replicate these trials with the additional control of either handling all control flowers, or placing white petals in front of white petals to eliminate handling effects (e.g. odour) as a potential variable; the manipulation *per se* could have been causing the bees to avoid these flowers.

8.4 Discussion.

This study agrees with the findings of Ne'eman & Nesher (1995) that colour change in *L. pilosus* may be accelerated by pollination. However, it goes further and suggests that a specific aspect of pollination (either pollen deposition or, more likely, pollen tube penetration and subsequent growth within the style) corresponds temporally with colour change (Figure 8.15) and may act as the trigger for such change.

The colour change in this plant has ecological significance because it is shown to influence the foraging behaviour *B. terrestris* (Figure 8.9). The importance of increasing rates of visitation by these bees as an influence on colour change is also suggested (Figure 8.11). Through simulating aspects of the flower handling behaviour of *B. terrestris*, the tripping of the pollen release mechanism is shown to be a critical factor in triggering colour change (Figure 8.13) as opposed to handling without pollen release (Table 8.2). Where colour change occurred in bagged and, therefore, non-visited flowers (e.g. Figures 8.10, 8.12) autogamous self-pollination is assumed to have taken place. The change occurs more slowly than in visited flowers and may be effected by slight accidental or natural perturbation of flowers, or the mechanism may be tripped automatically towards the end of a flower's life and in the absence of visitation. A possible additive effect of multiple tripping of the pollen release mechanism (and/or continued pollen deposition) on colour change in visited flowers requires further investigation.

In some lupin species colour change has seemingly been shown to be age-related and independent of induction, visitation and/or pollination (Dunn, 1956;

Schaal & Leverich, 1980; Gori, 1989; Juncosa & Webster, 1989). Inducible colour change via artificial pollination has been demonstrated in *L. pilosus* (Ne'eman & Nesher, 1995), as confirmed in my study. Colour change has been suggested to be caused by the manipulation of the flower during stigmatic receptivity in both *L. arizonicus* and *L. sparsiflorus* (Wainwright, 1978) and, in *L. albifrons*, linked to ethylene production in the keel and pistil (Stead & Reid, 1990). The possibility of ethylene being implicated in colour change in the former study (released as a wound response during manipulations) and in my study is discussed in detail in Chapter 9. In my study specific aspects of flower handling by visiting *B. terrestris*, and a pollen-pistil interaction, are put forward as *precursors* to colour change in *L. pilosus*; though the precise *trigger* for such change at a physiological level requires further determination.

Westerkamp (1997) and Westerkamp & Weber (1999) have described in detail the foraging activity of bees in relation to the 'keel blossoms' of the Fabaceae, and lupins are a clear example of the "secondary pollen presentation" mechanism detailed. If colour change was simply related to the handling process of visitation and there was constancy in visitation behaviour, even a single visit might prompt colour change. However, from my results it would appear that both quantity and quality of visit play a role in the process.

The exclusion of insect visitors (Figure 8.10) delayed the onset of colour change and, in further experiments involving a light manipulation of individual flowers with no pollen release, such change had not commenced within six days of opening (Table 8.2). That colour change did occur in a few non-visited flowers may be explained by mechanical perturbation by wind/animal contact leading to triggering of the pollen release mechanism. If colour change is linked

to a post-pollination event, as discussed below, it can be assumed that these flowers had undergone autogamous pollination.

Trials suggested that increasingly rapid colour change is directly linked to visitation and in particular the triggering of the pollen release mechanism. Even a single visit by *B. terrestris* could lead to colour change (Figure 8.11) (although rate of change was similar to that recorded in bagged flowers). The main pattern of both natural visitation and manipulation that induced triggering showed greater proportions of post-change flowers with increased visitation/treatment levels (Figures 8.11 and 8.13). However, the finding that not all flowers that received visitation or were subject to manipulation changed colour points to a specific aspect of the visitation process being behind onset of colour change, i.e. the 'quality' of the visitor's behaviour at a flower influenced subsequent outcomes.

A number of factors within the visitor-plant interaction require consideration in relation to colour change.

a) "Simple" handling, *per se*, did not lead to colour change. Results in Table 8.2 showed that even up to 20 gentle manipulations of individual flowers could not trigger the onset of colour change within 5 days. This is of clear benefit to the plant if the pre-change flowers attract more visits through offering a specific colour cue, particularly where pollinators are limited. If visitors that merely landed on the flower (without receiving or donating pollen, i.e. not tripping the pollen release mechanism or exposing the stigma) were able to trigger colour change, further potential visits by "legitimate" manipulators of the release mechanism, foraging according to colour cue, would be precluded. Both paternal and maternal fitness of the plant would be

compromised by such a change: furthermore, the post-change colour phase would no longer advertise the reduced pollen availability that foragers, presumably, have learnt to associate with pink/purple coloration. The results shown in Figure 8.8 are equivocal; tripping of the pollen release mechanism was not fully controlled for in these trials and may have accounted for the recorded colour change.

b) Nectar withdrawal. In *Oenothera drummondii*, the removal of nectar has been shown to accelerate the rate of colour change (Eisikowitch & Lazar, 1978). The presence of nectar reward in *Lupinus* sp. is equivocal; keel flowers of the Fabaceae were described as nectar flowers (e.g. Westerkamp, 1997; Westerkamp & Weber, 1999), whereas lupins in general, and individual lupin species, have been noted as nectarless as outlined in section 8.1. In the present study, *B. terrestris* was observed probing the base of the vexillum, as were bombyliids and medium-sized solitary bees. Although the latter two groups were scarce visitors, the behaviours suggest an availability of nectar. While such a trigger for change cannot be excluded, the complex floral morphology of lupins may preclude investigation of the effect of nectar withdrawal on colour change even if nectar were found to be definitely present.

c) A correspondence of colour change to reduced stigmatic receptivity cannot be ruled out. Alon (1986) found the stigma in *L. pilosus* to be receptive from opening; data here appear to confirm this, as pollen was found attached to the stigmas of first-day flowers and commencement of pollen tube growth occurred within 24 hours of initial tagging in visited groups (Figure 8.15). Loss of receptivity could be signalled by colour change, but change appears

to be slightly delayed following pollen deposition and early stages of pollen tube growth (Table 8.3). In bagged groups, banner petal spots remained white for 3-4 days (e.g. Figures 8.10 and 8.12) and up to 6 days in some treatment groups (Table 8.2) before change commenced, highlighting that colour change is inducible and, therefore, plastic in *L. pilosus*. Loss of receptivity as a post-pollination event (even following deposition of a few pollen grains) could coincide with and, possibly, trigger colour change and requires further examination.

d) The utility to a plant of pollen depletion as a trigger to colour change is doubtful. Ne'eman & Nesher (1995) highlighted that the colour change in *L. pilosus* signalled a change in reward status to visiting insects, amounts of available pollen in pink and purple flowers being significantly less than in white flowers. In my experiments involving triggering of the pollen release mechanism, all pollen could be removed in a minimum of ten manipulations but pollen was still present in the acumen of the keels of some flowers after the maximum twenty manipulations. While the difference between artificial and natural tripping should be considered, a colour change dependent on pollen depletion might have detrimental consequences to female fitness. If a single or few visits removed all available pollen and triggered colour change without pollination occurring, further visitation would be directed away from the flower by that change without benefit to maternal success.

With the above factors either ruled out by experiment or of little utility to either plant or visitor, pollen-pistil interactions may represent the most likely source for the precise trigger for colour change in this species. In *L. pilosus* pollen deposition *per se*, or pollen tube growth within the style, appear to be the

triggers for colour change. Tripping of the pollen release mechanism in *L. pilosus* presumably ensures either that self-pollen is deposited (as pollen is squeezed into the acumen of the keel petal) or that cross-pollen from the visitor's ventral surface is dusted onto the stigma. A trigger for colour change at this point of the pollen germination/fertilisation pathway would be of great value to the plant, because colour change would then direct visitors away from flowers that had already received sufficient pollen grains to effect fertilisation. Thus only flowers with white banner petal spots would receive further visits; no dislodgement of pollen grains already on stigmas of previously visited, post-change flowers could occur, and no damage would be caused to growing pollen tubes through additional visitation. The probability of pollination of remaining white flowers would also be enhanced. Visiting bees would learn by association that little reward could be gained from post-change flowers and thus would both forage and pollinate more efficiently.

Whether pollen deposition *per se*, or pollen tube growth, or penetration of the ovule by the pollen tube and subsequent fertilisation, is the signal for colour change in *L. pilosus* remains uncertain. Some stigmas from white flowers (both visited and non-visited) showed the start of pollen tube growth but none had pollen tube bundles within the style; only in the visited groups were these bundles obvious throughout the style, and all these flowers had undergone colour change. Ovule penetration did not appear to be a cue for colour change because evidence of pollen tails at the ovule was found in only five flowers; for similar reasons fertilisation cannot be the trigger. On balance, the evidence suggests that significant pollen tube penetration into the style is the critical factor, presumably initiating a chemical signal that begins the biochemical changes needed for

pigment synthesis and consequent colour change. This idea will be explored further in Chapter 9.

Note.

Much of the content of this chapter has been accepted for publication under the following title: -

Nuttman C.V. & Willmer P.G. (2003) How does insect visitation trigger floral colour change? *Ecological Entomology* **28** (In press).

Chapter 9 - General Discussion.

9.1 Summary of experimental findings.

This thesis extends the knowledge of floral colour change in one species, *Lupinus pilosus*, and offers new information in relation to the same phenomenon in five further previously unexplored plants; *Alkanna orientalis*, *Myosotis sylvatica*, *Echium judaeum*, *Echium vulgare*, and *Lonicera periclymenum*. Most notably, one species (*E. judaeum*) displayed a 'reversal' of colour change, a feature not previously reported. Table 9.1 summarises the features of floral colour change in each of these plants, together with visitor behaviour in response to such change.

i) The nature of colour change; do the studied species qualify?

In all species studied the post-colour-change phase represented a considerable proportion of the floral life (see Table 9.1). Fully turgid flowers were retained on the plants without displaying further signs of senescence (e g. fading or withering) and, therefore, satisfied an important criterion that has been applied to colour-changing plants in earlier research (e.g. Gori, 1983; Weiss, 1995a). Furthermore, two species (*Myosotis sylvatica* and *Lupinus pilosus*) exhibited colour change that was restricted to a localised part of the flower, and all species except *Echium vulgare* showed an alteration in reward status that coincided with colour change (see below). Hence all the species studied presented one, two or three of the 'qualifying' characteristics described in relation to colour change in Chapter 1.

Species	Floral longevity (days)	Time to commencement of change (days)	Time post-change (%)	Type of change			Increased reward[1]		Primary visitor(s)	Foraging preference	
				'Age-related'	'Inducible'						
					Accelerated	Decelerated	Pre-change	Post-change		Pre-change	Post-change
Myosotis sylvatica	6.7 ± 0.3	2.1 ± 0.1	69	+	++	+	✓		Syrphids	✓	
Alkanna orientalis	4.3 ± 0.1	2.9 ± 0.1	33	+	+++	+	✓		Solitary bee	✓	
Echium vulgare	3.9 ± 0.1	1 – 4 hours	96	+++	-	+	nd	nd	*Bombus* spp.	np	np
Echium judaeum	3.1 ± 0.3	1 – 4 hours	95	?	+++	+++	✓		*Apis mellifera*	✓	
Lonicera periclymenum	4.4 ± 0.2	0.8 ± 0.1	82	+++	-	-	✓		*Bombus hortorum*†	✓	
Lupinus pilosus	5.3 ± 0.1	1.8 ± 0.1	66	+	+++	+	✓[2]		*Bombus terrestris*	✓	

Table 9.1 Features of floral colour change and visitation in 6 plant species. Floral longevity and times to commencement of colour change are mean values ± 1 standard error of the mean from earlier chapters.

Key: nd = no difference

np = no preference

- acceleration and deceleration of "inducible" colour change refers to effects on time of onset of change through some form of treatment
- [1]refers to findings in this thesis of the status of **nectar** reward, with exception of [2] where **pollen** availability has previously been shown to be greater in pre-change flowers (Ne'eman & Nesher, 1995)
- †not the primary visitor in terms of abundance, but the sole legitimate nectar feeder.

The rate of colour change was found to be unaffected by either visitation or artificial manipulation in one species, *Lonicera*, and therefore was related solely to the age of the flower. In contrast, flowers of all the other species exhibited rates of colour change that could be influenced through various artificial manipulations and/or aspects of visitation and post-pollination events; these species would traditionally be said to show 'inducible' colour change. The question of whether an age/inducibility dichotomy is strictly useful is discussed later.

ii) Reward status and direction of colour change.

Nectar reward was unaltered between pre- and post-change floral colour phases in a single species, *E. vulgare*. In that species both nectar volume and concentration in the ephemeral purple flowers were not significantly different to the values found in older blue flowers (Figures 5.9 & 5.10). Nectar volumes were greater in the pre-change phases of *Alkanna* (Figure 4.10) and *Myosotis* (Table 3.4); both of these flowers underwent a yellow to white change, the whole flower changing in *Alkanna* while only the coronal scales changed in *Myosotis*. The pink to purple change in *E. judaeum* coincided with reduced nectar volumes (Figure 6.9) and *Lonicera* contained more nectar and higher sugar reward in white and intermediate flowers than post-change yellow flowers (Figure 7.8 & 7.9). In *Lupinus*, the presence or absence of nectar remains equivocal and visitors appeared to forage for pollen; an earlier study by Ne'eman & Nesher (1995) found significantly more pollen in white pre-change flowers than in pink and purple post-change ones.

iii) Visitor preference and colour.

The visitation behaviour of a range of insect visitors was influenced by the availability of different floral colour phases in all but one species *(Echium vulgare*, see Figure 5.14) and was, presumably, linked to the correlation of reward and colour, modulated by learning.

Visitation to flowers was non-random in respect of availability of colour phase in the population in 5 species; the primary visitor foraged predominantly at pre-change flowers. In *Myosotis*, the syrphids *Rhingia campestris*, *Melanostoma* sp. and *Platycheirus* sp. made over 70% of feeding visits to yellow-centred flowers despite this colour phase representing under 40% of the total flowers that were available (Figures 3.14 to 3.18). The solitary bee *Anthophora pauperata* generally avoided older intermediate and pale yellow flowers of *Alkanna* and visited more bright yellow flowers than pale ones in manipulated trials (Figure 4.16). Where a 'reversed' colour change had been induced in flowers of *E. judaeum* following overnight bagging and subsequent bag removal, the honeybee (*Apis mellifera*) made 66% of visits to young flowers that were changing back to pink, although these flowers accounted for only 47% of those available on a focal patch (Figure 6.17). The only diurnal nectar-feeding species to visit *Lonicera*, the bumblebee *Bombus hortorum*, usually visited the more rewarding white phase flowers; over 70% of feeding attempts were made at these flowers, which accounted for under 40% of all flowers in the population (Figure 7.14). Another bumblebee, *Bombus terrestris*, made 98% of visits to white banner spot flowers of *Lupinus*; these bees were not observed feeding at older purple banner spot flowers, although this colour phase represented 20% of the overall floral display (Figure 8.9). For all these species, then, colour is a significant cue for the primary

visitor, and colour change is likely to have functional/ecological significance. In the sixth plant an exception was evident; at *E. vulgare*, several *Bombus* spp. were regular visitors and flower visitation was in accordance with colour phase availability within the population (Figure 5.14).

iv) Pollination, manipulation and colour change.

The types of artificial manipulation tested and the early stages of post-pollination event that were investigated are outlined in Table 9.2. Ideally, all types of manipulation would have been tried on all species but in some flowers this was not possible due to basic floral morphology, and for a variety of reasons (as detailed below) in others. Short flowering seasons in combination with adverse weather conditions limited fieldwork in the UK (e.g. *Echium vulgare*). Planned fieldwork to replicate experiments and carry out further work with *Echium judaeum* had to be cancelled due to unusual circumstances in Israel (see Chapter 6). Analysis of pollen tube growth was first used in 2001 and produced good results in *Lupinus* and, the following year, in *Alkanna*; time constraints prevented further use of this particular technique.

Manipulations using excised bees' tongues and nylon brushes accelerated colour change in *Myosotis* (Figure 3.23), probably in tandem with post-pollination events, whilst the process was slower following bagging (Figures 3.21 to 3.23). The small size of the flowers of this species made some of the manipulations tried with other flowers impossible. For example, any manipulation of the anthers was impractical because they are hidden within the corolla tube. The small entrance to the tube also prevented nectar withdrawal being tested as a potential trigger for colour change.

Species	Anther manipulations		Visitor mimicking					Bagging effects		Post-pollination events	
	Pollen removal	Emasculation	Corolla piercing	Nectar removal	Microcapillary insertion without nectar removal	Bee tongue insertion	Nylon brush insertion	Nylon bag – visitors excluded	Foil bag – visitors and light excluded	Pollen deposition	Pollen tube growth
Myosotis sylvatica						✓(+)	✓(+)	✓(+)			
Alkanna orientalis				✓(+)	✓(+)			✓(+)		✓(?)	✓(+)
Echium vulgare	✓(-)			✓(+)				✓(-)	✓(+)		
Echium judaeum				✓(+)				✓(+)			
Lonicera periclymenum	✓(-)	✓(-)	✓(-)	✓(-)	✓(-)					✓(-)*	
Lupinus pilosus	✓(+)							✓(+)		✓(+)	✓(+)

Table 9.2 Artificial manipulations and post-pollination events tested in relation to colour change in 6 plant species.
+ = effect on onset and/or rate of colour change
- = no effect (* no effect on colour change but clear effect on floral longevity)

In *Alkanna* colour change was retarded through bagging (Figure 4.6) and hastened through a range of other manipulations (Figure 4.7). The key factor in natural colour change in this species was probably deposition of cross-pollen, but particularly rapid change following manipulation could have been due to floral damage and a consequent wound response (see below). The small size of *Alkanna* flowers and similar morphology to those of *Myosotis* limited the potential range of manipulations that were attempted.

Minor effects of both nectar removal and light availability were found on colour change in *Echium vulgare* (Figures 5.15 & 5.16). The absence of light slightly retarded colour change in *Echium vulgare*. The effect was found towards the end of my final field season and is worthy of further attention in future work. The finding reported by Farzad et al. (2002) of a 'mesh-patterned' colour change on individual flowers of *Viola cornuta* (that resulted from covering plants with fine-mesh cloth) illustrates the potential effects of light on colour change.

In *Echium judaeum* colour change was accelerated through bagging (Figure 6.5) and apparently retarded by nectar removal, with the latter characteristic probably implicated in triggering the change. Anther manipulations of these flowers were not used; spatial separation of floral reproductive parts in both *Echium* species (preventing 'accidental' selfing) appears to rule out post-pollination effects as a trigger for colour change (since this also occurred in bagged flowers). *E. judaeum* in particular was extremely sensitive to handling effects with respect to colour change.

In *Lupinus*, pollen deposition, and/or subsequent pollen tube growth following visitation by a particular visitor (*Bombus terrestris*), and/or pollen release by artificial manipulation, accelerated colour change in (Figures 8.11,

8.13 & 8.15). Nectar withdrawal manipulations could not be tried on this species; unequivocal confirmation of the presence of nectar has yet to be established (see Chapter 8). Even if found, nectar could be particularly well-hidden and therefore difficult to extract without causing damage to floral tissue that could produce confounding effects of wound response in relation to colour change (see below).

In just one species, *Lonicera*, there was no influence on the rate of colour change following any type of manipulation, although early floral abscission was a feature of this species following pollen deposition (Figure 7.18). The large flowers enabled several types of manipulation to be carried out but bagging was not possible due to the position of inflorescences (see Chapter 7). Piercing of the corolla tube was used in *Lonicera* because this mimicked nectar robbery; this type of flower damage was only found in this plant and therefore was not a relevant 'type' of visit to simulate in other species.

Hence my study provides evidence for marked complexity in relation to the proximal triggers of colour change. In one species (*Lupinus*) a handling characteristic peculiar to one visitor is critical: in 2 others (both species of *Echium*) factors that are neither age- nor pollination-related can affect colour change; additionally, the effects of environmental conditions (as affected by bagging) could be important.

9.2 Rate of colour change: Is age vs. inducibility a useful concept?

Flowers that change colour have usually been categorised according to the temporal pattern of their change. Weiss & Lamont (1997) suggested that colour-changing flowers fall into one of two divisions; either in the absence of

flower visitors a fixed time-related alteration takes place, or an aspect of visitation leads to a colour change that can occur at any time in a flower's life. In the latter case only a hastening of change is suggested to occur. Thus colour change is either non-inducible or inducible. An earlier review (Gori, 1983) put forward a similar dichotomy. Gori highlighted non-induced and pollination-induced floral changes (including colour) that were distinct from simple floral senescence for the reasons listed in Chapter 1. (Pollination can induce rapid change that is succeeded by retention of flowers; the change can occur in localised parts of the flower and often coincides with change in reward; and colour change, sometimes in combination with other altered cues, can result in modification of the visitors' behaviour). He proposed no separation of particular aspects of visitation and/or pollination-related events.

A problem arises here in relation to those flowers where colour change is said to be inducible. 'Inducible' flowers do eventually change colour just before or during senescence (e.g. Weiss, 1992: Weiss & Lamont, 1997) and although some colour-changing flowers were reported to wither or abscise without changing colour (Weiss, 1992) no supporting data were presented. In my study, I found no indication of colour change failing to occur in those species where the process could clearly be hastened by extrinsic factors and which thus would normally be categorised as 'inducible' (*Myosotis*, *Alkanna* and *Lupinus*). Hence there is no clear evidence in my study or in the literature of colour change failing to occur in 'inducible' species in the absence of visitation.

Patterns of colour change in my study provide indications that the process of colour change could be far more flexible than previously suggested, and my data challenge the age-inducibility dichotomy of floral colour change. A

physiologically controlled temporal continuum is, perhaps, a more valid mechanism for *all* colour change able to account for both supposed 'age' and 'inducible' types of change, i.e. an explanation based on parsimony of physiological processes could unify these separate categories. This may be a simplified interpretation of observed patterns of colour change in this thesis and it requires confirmation through biochemical assay. However, the following section reviews the role of a particular compound in flowers that could link the acceleration of colour change reported following visitation/pollination in some flowers with the apparently fixed temporal colour change in others.

9.2.1 Age and visitation-related colour change: does ethylene provide one unifying mechanism?

Plant growth regulators are analagous to hormones found in animals, acting as chemical messengers, and contributing to the systemic regulation of the organism (Leopold, 1987). Ethylene (C_2H_4) is produced by plants in gaseous form and has been classified as a plant growth regulator; the gas is one of a number of 'growth substances' that are important modulators of development and growth in plants (e.g. Abeles, 1992). Among particular processes in which ethylene is known to be involved, the substance has a role in flowers; as well as the promotion or inhibition of flowering, ethylene can change the sex of developing flowers (see, for example Abeles, 1992).

The role of ethylene in the development of flowers following pollination, and the possible modes of signalling within plants, were reviewed by O'Neill & Nadeau (1997). Alteration in floral pigmentation was one of several

developments that occurred irrespective of pollination in some plants (i.e. it was related to the age of the flower), but that could be accelerated by pollination in others. They concluded that the role of ethylene was as a co-ordinating signal within senescing flowers, and that it was the direct causative agent of senescence of perianths in all climacteric flowers (such flowers exhibit increased respiration immediately prior to wilting).

I now cover the action of ethylene in more detail in relation to its possible links to floral colour change, before illustrating how the patterns described in my study could be explained by endogenously produced ethylene. The core of the argument is that ethylene has been implicated in both colour change and senescence processes and might therefore, via a wound response, explain some of the other patterns reported in my study.

i) Ethylene and wounding.

Ethylene can be produced by plants if damaged (e.g. in response to attack by insects or mechanical wounding), as a stress response (Abeles, 1992). The possible effect of 'wounding' of the stigma when penetrated by germinating pollen has been recorded to coincide with raised ethylene production by flowers. For example, Gilissen (1976), investigating stigma and style removal in *Petunia hybrida*, determined that the style was the critical 'sense organ' in wilting. Complete removal of the style did not result in wilting as rapidly as that seen following stigma or 'half-style' removal, which suggested that complete removal had interfered with the movement of information between floral parts. Gilissen & Hoekstra (1984) found both pollination and stigma wounding led to the generation of a 'wilting factor', and also noted that both events caused increased

production of ethylene (by whole flowers as measured through withdrawal of gas samples and analysis by gas chromatography). They suggested that the 'wilting factor' might be ethylene itself, or a precursor to ethylene synthesis; pollen tube growth caused the wounding of stigmatic cells, and the subsequent production of ethylene within the style was proposed as a possible early post-pollination event (Gilissen & Hoekstra, 1984).

Fukushima et al. (1999) noted, through scanning electron microscopy of *Carthamus tinctorius* flowers, how the process of pollen deposition led to damage to the surface of the stigma and style. Ketsa et al. (2001) reported the effects of pollen deposition on production of ethylene in *Dendrobium* sp., where even incompatible pollen placed on the stigma triggered C_2H_4 production, via the auxin precursor 1-aminocyclopropane-1-carboxylic acid (ACC). This latter compound had earlier been found to be present in *Petunia* pollen (Whitehead et al. 1983). However, later studies suggested that these pollen-borne quantities of ACC were too low to account for all ethylene production following pollen deposition and, additionally, the compound was absent in the pollen of some flowers such as orchids, and thus could not be a 'universal' cause of ethylene production (O'Neill & Nadeau, 1997). The precise identity of the pollination 'signals' that trigger subsequent floral changes, including ethylene production, have not been fully resolved but have been categorised as 'primary signals' (if connected with the first perception of pollen at the stigma), or as 'secondary signals' (if associated with reproductive processes in the ovary and floral senescence) (O'Neill & Nadeau, 1997). A correspondence of raised ethylene levels, synthesised *de novo* in the style, with various stages of pollen tube growth into the style, was found by De Martinis et al. (2002) in *Nicotiana tabacum*

flowers. These authors' results showed clear peaks of ethylene production within 3h of pollen deposition and after 48h when the pollen tubes reached the ovary.

Thus it is increasingly evident that ethylene production within the stigma/style can be a 'normal' consequence of some form of damage to those parts either by 'experimental wounding' or as a consequence of pollen germination and pollen tube growth.

ii) Ethylene production following pollination and during senescence.

Ethylene is also synthesised by flowers following pollination, and by flowers that are approaching senescence. O'Neill & Nadeau (1997) noted that patterns of senescence could be altered temporally by pollination; gradual senescence in some flowers (e.g. *Petunia*) could be accelerated by pollination, whereas in other species there was no evidence of senescence until after pollination (e.g. in many orchids). Raised levels of ethylene that could share the same basic mechanism accompanied both patterns (O'Neill & Nadeau, 1997).

In the absence of an experimental treatment, *Vanda* orchid blossoms showed no increase in ethylene production for at least 70 hours whereas rapid synthesis took place (<10 hours) following artificial pollination (Burg & Dijkman, 1967). In a further example, increases in endogenous ethylene accompanied features that included the irreversible wilting of petals and swelling of the ovaries of carnations (*Dianthus caryophyllus*) (Nichols & Frost, 1985). Nichols (1977) had earlier identified the style, ovary and petals as sites of ethylene production in this species and also noted a surge in levels either following pollination or during natural senescence. In *Ipomoea tricolor*, senescence of the flower (which included a colour change from blue to purple)

took place along with an increase in endogenously produced ethylene (Kende & Baumgartner, 1974). In subsequent experiments, excised segments of this species underwent normal senescent processes (the colour change and rolling up of the segment) only if cut 1 day prior to opening. If removed a day earlier these changes did not occur, which led to the suggestion that the system responsible for ethylene production developed as a feature of the ageing process itself (Kende & Hanson, 1976). Ethylene production in flowers of *Digitalis purpurea* was also linked to both pollination and normal patterns of senescence (Stead & Moore, 1983). In unpollinated flowers, ethylene levels reached 2nl h^{-1} after 6 days (the usual time to abscission) compared with a rise to over 5nl h^{-1} within 24 hours if artificially pollinated on the morning of opening.

Whether accelerated through pollination or occurring via a more gradual process, senescence reduces floral attraction.

ii) a) Ethylene and floral attraction.

The consequent cessation of floral attraction caused by senescence of the perianth minimises the cost of maintaining elaborate floral structures, as well as diverting visitors away from pollinated flowers (Stead, 1985). He pointed out in a later review (Stead, 1992) that further pollen deposition was wasted because competition between growing pollen tubes for food could occur within the style. This author also noted that pollination usually hastens patterns of floral senescence most often via corolla modification (especially through wilting), colour change, or floral abscission and that ethylene was implicated in all three processes.

Recent research by van Doorn (2001, 2002a, 2002b) has categorised floral senescence and abscission in terms of a plant's sensitivity or otherwise to ethylene. He showed that up to 300 species were extremely sensitive (as determined by petal wilting, withering or floral abscission) to exposure to exogenous ethylene (the gas, at a concentration of 3ppm, was injected into a chamber containing the plant and the response was measured after 24h) (van Doorn, 2001). In species where flower colour change could be induced by pollination, all were from families that commonly contained ethylene-sensitive species that exhibited the same effect from exposure to the gas. The absence of the 'opposite' association, between ethylene-induced colour change in flowers where similar pollination-induced effects have not yet been reported, could be due to a paucity of detailed research on the effects of pollination (van Doorn, 2002a). In one family, Crassulaceae, colour change, though not senescence, could be induced by exposure to ethylene in some genera but not in *Sedum* spp. (van Doorn, 2002a). The reported instances of colour change in this family (*Sedum villosum* and *Kalanchoe pumila*, Weiss (1995a)) have not yet been evaluated for the effects of pollination. Van Doorn (2001) also reported ethylene-insensitive senescence especially within the families Iridaceae and Lilaceae, however, possible differences in effect between exogenous application and endogenously produced ethylene should not be ruled out.

Hence, whether produced endogenously or applied exogenously, ethylene has clear links to both pollination and senescence.

iii) Ethylene and colour change.

In a review, Stead & van Doorn (1994) concluded that, despite the lack of a complete understanding of the physiology of floral colour changes, ethylene was implicated, somehow, in all of the small number of species where such change had been reported.

Akamine (1963) reported a fading of *Vanda* orchids that correlated with increased ethylene following removal of pollinia. Also in this genus, Burg & Dijkman (1967) discovered that the application of a further plant growth regulator (the auxin indole acetic acid) to the stigma caused ethylene production that was suggested to be linked to the fading process. They attributed the raised ethylene noted in the Akamine study to a wound response; pollinia removal in their research also caused a transient rise in ethylene. The banner petal spot of *Lupinus albifrons* underwent an accelerated colour change when exposed to ethylene for up to 24h in a sealed chamber, and the keel and pistil were found to be sites of endogenous ethylene production (Stead & Reid, 1990). Colour change was also influenced by ethylene in a range of other plants. The blue to purple change of the corolla that accompanies senescence was hastened in *Ipomoea tricolour* after an exposure to ethylene for only 20 to 40 minutes (Kende & Baumgartner, 1974). In *Cymbidium* sp. coloration of the lips of the flower changed from 'unpigmented' to dark red within 40h of initial exposure to ethylene (a pattern that was also found following emasculation of flowers) (Woltering & Somhorst, 1990). These authors also recorded that the change in coloration was caused by an increased accumulation of a range of anthocyanins in the lips following either of these treatments.

iv) Pigments, ethylene and colour change.

Anthocyanins are one of the major classes of pigments associated with floral colour change (Weiss, 1995a). Several studies have recorded an increase in the levels of anthocyanin pigments concomitant with ethylene synthesis. Craker et al. (1971) found that a combination of ethylene treatment and light conditions were responsible for controlling the anthocyanin content of seedling tissue in *Sorghum vulgare*; synthesis of these pigments was promoted or inhibited dependent upon the time of exposure to the gas. These results were repeated in other plant species (*Brassica rapa*, *Euphorbia pulcherrima* and *Vaccinium macrocarpon*) while monitoring the effects of the interaction of CO_2 and ethylene (because CO_2 can act as a competitive inhibitor of ethylene action) (Craker & Wetherbee, 1973). Chadwick et al. (1980) found that the application of auxins to the stigmas of *Cymbidium* orchids led to raised levels of ethylene and simultaneous anthocyanin production; the consequent effects were an induced colour change prior to senescence and wilting of the perianth. In *Lupinus albifrons*, the change in coloration of the banner petal spot from white to purple was due to increased concentrations of the pigment cyanadin that was associated with ethylene production in the style (Stead & Reid, 1990). These authors also found that, although colour change could still be induced in isolated banner petals through exposure to ethylene, the concentration of this pigment was less than half that measured in intact flowers (where the additional effect of endogenously produced ethylene from the style influenced pigment synthesis).

Thus the consequences of wounding, senescence and pollination are known to involve ethylene, and production of this gas can underpin the synthesis of pigments that leads to colour change. The schematic shown in Figure 9.1

offers a simplified mechanism for colour change incorporating age, visitation, pollination and wound response. Many of the links have been known for some considerable time but have not been drawn together as a potentially unifying explanation for all colour change. The key point is that colour change may not be either 'inducible' or 'age-related' but a plastic process, temporally mediated by ethylene production caused by a variety of triggers.

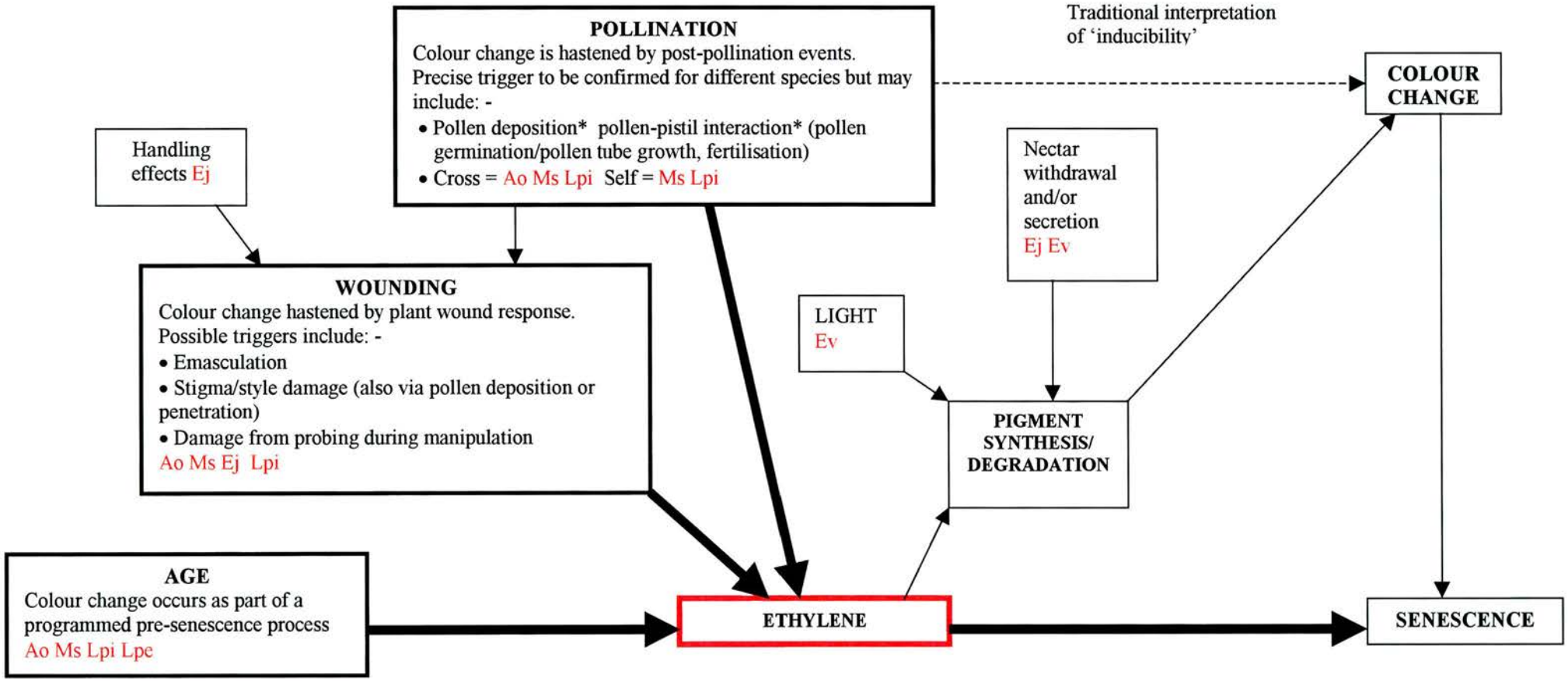


Figure 9.1 Schematic of suggested routes of colour change for six plant species.
Key: - Ao = *Alkanna orientalis*; Ms = *Myosotis sylvatica*; Ej = *Echium judaeum*; Ev = *Echium vulgare*; Lpi = *Lupinus pilosus*; Lpe = *Lonicera periclymenum*
* Pollination events indistinguishable from pollen-pistil 'wound' effect

A recent study by Farzad et al. (2002) challenges this model as it found no link to ethylene production in the colour change of *Viola cornuta*. They identified malvidin to be the key anthocyanidin (the precursors of anthocyanins that lack sugars) involved in the colour change from white to purple in an ornamental variety of *Viola cornuta*. They showed that, in the absence of light, this colour change did not occur; light acted as an initial trigger to synthesis of these pigments. Pollination was the key trigger for colour change, but the change here did not appear to be ethylene-mediated; plants treated exogenously with silver thiosulphate (STS, an ethylene synthesis inhibitor) changed colour at the same rate as control plants, but exhibited delayed senescence (Farzad et al. 2002).

The methodology in that study involved spraying all leaves and buds of treatment group plants with STS, which were then placed with control plants on the same bench; confounding effects of exposure to exogenous ethylene from these latter plants could have occurred. Some earlier studies suggested that sensitivity to exogenous ethylene is dependent on stage of flower development, as (for example) the buds of *Digitalis purpurea* were unaffected by such application (Stead & Moore, 1983). The same may be true of *Viola*, and the effects of STS could have diminished by the time colour change, possibly triggered by ethylene released from control plants, occurred in treatment plants. Also, the *Viola* study does not make clear whether plants in the experimental groups were emasculated and no detail is given as to whether styles were checked for pollen tube growth. If self-pollination had taken place and the effects of STS were diminished over time, endogenously produced ethylene could have accounted for the observed colour change, either as part of a wound response or

through pollen deposition and/or subsequent processes. Thus it remains unclear whether *Viola* can fit within the model in Figure 9.1.

9.2.2 How does this model relate to, or explain each of my test species?

In my study, in the absence of visitation (as effected by bagging flowers), different pathways for colour change may underlie the patterns reported following visitation and/or manipulation. Colour change and floral abscission could be triggered by post-pollination events including ethylene production where successful visitation occurs, but via programmed senescence processes where visitation is prevented.

i) *Myosotis sylvatica.*

Links between ethylene synthesis and colour change have already been reported occasionally in flowers where part-flower change occurs (e.g. the banner petal spot in *Lupinus albifrons* (see above) (Stead & Reid, 1990)). In *Myosotis*, significantly faster onset of colour change of the coronal scales (compared to bagged flowers) was effected by probing the corolla with a bee's tongue (Figure 3.22) or a nylon brush filament (Figure 3.23). Two possible explanations could account for this pattern, either of which could be mediated by increased ethylene production. Firstly there could be damage of floral tissue caused by the probing instrument, or secondly there could be gross deposition of pollen through the use of implements far less delicate than the usual visitors' mouthparts. In my study the former possibility can be ruled out because flowers in the 'brush' group set seed in similar quantities to both 'bagged' and 'bee-tongue manipulation groups', suggesting such damage had not occurred. A more

probable explanation is a difference between pollen deposition by the implements used for manipulation and deposition effected by natural visitation. Probing with a 'gross' implement almost certainly results in considerable deposition of pollen and successful pollination (as evidenced by seed set data). Both open and bagged groups eventually 'catch up', in terms of pollen deposition, through repeated 'quality' visitation in open flowers (resulting in further deposition of cross and self pollen, leading to even higher levels of seed set) or 'perturbation-induced' self-pollination in bagged flowers. Self-pollen could be moved over short distances within the small corolla tube by visitors and/or by mechanical perturbation (e.g. caused by wind action or handling attempt by any insect visitor). In all these scenarios colour change could be triggered by endogenous ethylene produced at some stage of the pollen-pistil/pollination pathway.

The floral morphology of *M. sylvatica*, together with its self-compatibility, makes the unequivocal establishment of a colour change trigger problematical. Hence artificial pollen deposition and/or emasculation could not be effected through the reduced corolla tube opening and, even if this were possible, the confounding influence of ethylene release (as a possible wound response from these actions) would prevent the isolation of the trigger for colour change. Colour change occurred more rapidly in visited flowers and following all types of manipulation in comparison with bagged flowers, but change still took place in the latter group and could have been due to the effects of ethylene following self-pollination or, in the unlikely event of no pollen deposition, as part of normal senescence (colour change eventually occurred in all flowers).

ii) *Alkanna orientalis.*

In *Alkanna* colour change took place in all flowers eventually, even where visitation had been prevented, but it could be hastened by natural visitation and various manipulations (Table 4.1 and see section 4.3.2). It may therefore be a case of programmed senescent colour change that can be accelerated by 'extra' ethylene resulting from visitation (and successful pollination) or manipulations. Several strands of evidence support the possibility of more than one ethylene-mediated pathway being responsible for the observed patterns in this species. An aspect of pollination, following deposition of cross-pollen, was the probable trigger for colour change in flowers open to visitation. As bagged flowers also ultimately changed, where pollen germination and pollen tube growth did not occur (due to self-incompatibility) ethylene could not have been synthesised solely as part of a post-pollination event. However, a senescence-linked release of ethylene could have accounted for the 'late' colour change found in bagged flowers (Table 4.1). On this model, the increasingly rapid colour change observed following probing and/or nectar withdrawal would then be due to damage of floral parts during manipulation. On occasions the rigid style was heard 'snapping' back into place as the microcapillary was inserted; such an action could have damaged the tissue of the pistil and thus led to ethylene release as a wound response leading to colour change. Repeated probing by visitors with soft mouthparts would be qualitatively different from my attempts to mimic the same action with a glass microcapillary and would elicit little or no 'wounding' effect.

Here, then, colour change occurred in three discrete temporal patterns: within hours where probable damage to floral parts had been inflicted (Figure

4.7); usually within 2 to 3 days when open to natural visitation (Figure 4.6); and retarded to 4+ days when flowers were bagged (Figure 4.6).

Further work is required to confirm that the only potential common proximate trigger for all pathways could be pollen deposition (including the 'wound effect' of germinating pollen penetrating the stylar tissue). This could certainly occur in all treatments. Self-pollen dislodged onto the stigma in a bagged flower could instigate pollen-pistil interactions that lead to ethylene production while, at the same time, effecting a self-incompatibility response.

If ethylene is not contributing to the colour change in the ways described above, the less parsimonious possibility of separate mechanisms for the 3 rates of the same process would need to be investigated.

iii) *Echium vulgare* and *E. judaeum*.

Both species of *Echium* exhibited patterns of change in colour that had little in common with other plants in my study. For both species a pollination-related pathway and a senescence-based pathway involving ethylene synthesis can probably be ruled out. The colour change always occurred within a few hours of flower opening in *E. vulgare* (Figure 5.15) and could be induced by bagging within a similar period in *E. judaeum*. Hence the change occurs too early in a flower's life to be part of a senescent process. As both flowers exhibit a spatial separation of anthers and stigma, it follows that where flowers are bagged, pollen deposition does not take place and subsequent pollen-pistil interactions and/or successful pollination are also absent, ruling out a pollination-related influence on the colour change in bagged flowers.

Individual plants of *E. vulgare* were observed for long enough periods (1 – 4 hours) in open conditions but in the absence of visitors, to exclude simple handling effects triggering colour change in some way. An aspect of nectar secretion or production cannot be ruled out as a factor in colour change; artificial nectar withdrawal from newly-opened flowers led to a slight retardation of colour change, as did the combined exclusion of light and visitors (Figure 5.16).

E. judaeum exhibited the most unusual pattern of colour change in this study. When visitors were excluded, flowers changed colour rapidly from pink to purple. This could have been due to an absence of normal nectar depletion. When bags were removed and visitation re-commenced the gradual change back to pink, rewarding coloration occurred, suggesting that nectar depletion could initially retard the colour change. This species was extremely sensitive to handling: floral herbivory by *Amphicoma* sp. (Coleoptera) was widespread and resulted in heavy purple-blue blotching and streaking around chewed areas which suggested that a mechanical effect of handling influenced flower colour, presumably through wounding of floral tissue.

Whether there is a causal link between nectar withdrawal and/or secretion and colour change requires further work. Several studies of colour change have noted a correspondence of reduced nectar reward with colour change. For example, Gori (1983) listed 21 plant species that had been noted to change colour; 16 of these offered negligible reward after colour change with no data available for the remainder. Two studies reported reduced nectar reward in 5 species (Lamont, 1985) and 26 species (Weiss, 1991), respectively, in post-change flowers.

A review by Mayak & Halevy (1980) noted that ethylene is involved in the translocation of sugars from the petals to the ovary following pollination. Whether this process could have any bearing on the alteration of nectar reward in conjunction with colour change that is seen in many species, or on the patterns I report for *Echium*, represents a future possible research subject.

iv) *Lonicera periclymenum*.

Lonicera was not susceptible to alteration of rate of colour change through visitation or manipulation of any sort, but floral longevity could perhaps be modified by ethylene. In my study the significantly early abscission of those flowers that had been artificially pollinated (whether autogamous, geitonogamous or cross) (Figure 7.18) suggests that an aspect of pollination could modulate ethylene production and, whilst not affecting colour change, could be responsible for abscission. An ornamental hybrid of *Lonicera*, *Lonicera* x heckrotti 'Goldflame', was found to be highly sensitive to ethylene prior to abscission (van Doorn, 2001).

In my study, in manipulations where pollination had been prevented, flowers with pierced corollas had shorter life spans than those flowers where tissue damage was either not incurred (sleeve only, nectar withdrawal), or was made on a different part of the perianth (e.g. the filaments of the emasculation group) (Figure 7.20). The piercing of petal tissue could clearly instigate ethylene production as a wound response and explain this result.

v) *Lupinus pilosus.*

Colour change in *Lupinus* in my study was related to post-pollination events (Figure 8.15); an earlier study had also reported accelerated colour change following artificial pollination (Ne'eman & Nesher, 1995). As this species is self-compatible, the deposition of either self or cross-pollen, and/or the commencement of pollen tube growth, could account for ethylene production and thence colour change. Where visitation was prevented but colour change still took place there could be a trigger from self-pollination through accidental tripping of the pollen release mechanism, or the change could merely be part of a natural aspect of senescence in unpollinated flowers. Stigma wounding has already been shown to lead to raised levels of ethylene in *L. albifrons* (Stead & Reid, 1990) (see above), and the colour change in my study, observed following the manipulations that incorporated pollen release (Figure 8.13), could also have been triggered by floral damage. This sequence of events most probably accounts for the 'inducible' colour change that Wainwright (1978) attributed to stigma manipulations in *L. arizonicus* and *L. sparsiflorus*.

9.3 Potential ecological benefits of colour change in my study species.

Whichever of the suggested pathways in Figure 9.1 leads to floral colour change, ecological advantages appear to be present; in general for the visitor (where colour change coincides with, and therefore signals alteration of, reward status), and in particular for the plant (with some variation in how these benefits might accrue).

The age-related pathway of colour change, which occurs in the absence of visitation (that accelerates the change), coincides with extended longevity in *Myosotis*, *Alkanna*, *Lupinus*, and *Lonicera*. All but the last of these have longer duration of the 'attractive' pre-change colour phase (compared to unvisited flowers) that could encourage further visitation to these flowers once a forager arrives at the plant. In contrast, the retention of older post-change flowers in *Lonicera* could act as a longer distance visual cue. Hastened colour change, following pollination in *Myosotis*, *Alkanna* and *Lupinus*, directs further visitation to pre-change flowers that require pollination.

If any flower is damaged, possibly to the point of no longer being reproductively viable, a colour change initiated by ethylene as a wound response could benefit the plant; visitors would be directed away from flowers that may not be capable of setting seed. For *E. judaeum*, where damage was caused by my manipulations or by insects, a wound response activating colour change in this way prevents further visitation.

The ecological benefits of colour change in the *Echium* species resulting from the effects of nectar withdrawal and the influence of light are unclear.

9.4 The role of floral constancy: why not visit a post-change flower?

Floral colour change, then, can have ecological benefits to the plant, which 'manipulates' its visitors away from pollinated (or damaged), unrewarding flowers towards younger, unpollinated and rewarding flowers, while maintaining a large attractive floral display. The visitors can also benefit by being able to select more rewarding flowers more easily at close range. But why do flowers

use colour change to manipulate their visitors, and why are the visitors so readily manipulated in this way? Perhaps some clues come from the issue of floral constancy in pollination.

Colour change in flowers is widespread, and visitor response, in terms of reduced visitation, to post-change flowers is well documented (e.g. Weiss, 1995a). Weiss & Lamont (1997) highlight that, in the vast majority of cases, pre-change flowers receive disproportionately high visitation in relation to their abundance in the studied populations and the same was true for five out of six of my study species. In most studies of colour change the visitation patterns have been linked to the presumed visual and cognitive abilities of the visiting organisms. A change of colour coincides with a change in floral reward and, through learning the association of colour and reward; visitors discriminate against post-change flowers.

The relationship of visitor and flower, in terms of causation of foraging patterns, has received considerable attention from the time of Darwin's remarks on the tendency of bees to repeatedly visit flowers of the same species (Darwin, 1876). Subsequent studies of insect visitors in general, and bees in particular, have revealed much about the physiological background to vision (e.g. Menzel & Backhaus, 1991) and the processing of visual information by flower visitors (e.g. Backhaus, 1991), all of which relate to this so-called 'flower constancy'. Additionally, data have been provided that confirm the reality of flower constancy; pollinators apparently do ignore potentially rewarding flowers while concentrating their foraging efforts at single species or morphs of species (e.g. Waser, 1986). That study separated 'fixed' and 'labile' preferences; in the former case flower visitors may be restricted in their foraging activities by particular

floral food requirements (oligolectic bees were used as an example), whilst in the latter case only the most energetically-rewarding flowers would be visited (as reported in both hummingbirds and bumblebees (Waser, 1983)). Waser concluded that behavioural constraints were critical in floral constancy; the phenomenon was more pronounced when foraging was amongst disparate flower types, because of the inability of foragers to learn to deal with several flower types. A more recent review by Chittka et al. (1999) emphasised that whilst memory constraints were important in maintaining flower constant behaviour, sampling different flower species could incur costs. These could include time delays due to inefficient handling of novel flowers in comparison with continued foraging at 'known' floral sources. Subsequently, Goulson (2000) found increased flight times by bumblebees when searching for their usual forage plant when these plants were placed within mixed arrays of similarly coloured flowers.

The learning constraints of switching to novel flowers (termed 'Darwin's interference hypothesis', Woodward & Laverty (1992)) represent a 'motor and memory' problem for bees, while sensory abilities are more likely to be the key factor in floral constancy if a 'visual search image' is being utilised (Gegear & Laverty, 2001).

To examine the competing hypotheses suggested to underlie constancy, colour was one of several floral traits manipulated in tests of floral constancy with bumblebees by Gegear & Laverty (1998, 2001). The results showed that constancy of visitation behaviour in bumblebees was increased when two or more traits (from colour, size and complexity) were varied. Trials with *Bombus impatiens*, trained to feed at artificial flowers on pure arrays of single flower types, showed that these bees became increasingly selective in choice of flower

type when confronted with arrays of flowers where the above traits had been manipulated (Gegear & Laverty, 2001).

Studies of floral constancy have usually paid scant attention to instances of floral colour change. However, the discrimination between pre- and post-change flowers would be particularly interesting in this respect, representing an intra-specific form of floral constancy rather different from studies of mixed flower communities (e.g. Goulson, 2000; Wilson & Stine, 1996)) or flower colour polymorphisms (e.g. Kay, 1976; Stanton, 1987)). Flower colour clearly represents an important aspect of constancy in many of these studies, and the relevance of colour to a flower preference is well-documented (see Chapter 1). Furthermore, a range of insect taxa showed foraging preferences for a particular colour phase in five of six plants in my study. Bees, and particularly large bees such as *Bombus*, are especially useful to studies of floral constancy and therefore 2 of my study plants may be particularly instructive here.

i) Bumblebees.

Bumblebees showed constancy to pre-change flowers in *Lonicera*, which exhibits a long corolla tube that prevents access to nectar for short-tongued bees, and in *Lupinus*, which presents pollen secondarily through the complex tripping mechanism described elsewhere in this work, and thus offers reward only to large (heavy) bees.

The possible use of a visual cue by *Bombus hortorum* for pre-change flowers of *Lonicera* is of particular note. Flowers of this type have traditionally been included in the sphingophilous pollination 'syndrome' (Faegri & van der Pijl, 1979) and the colour/scent/phenology all support the view that hawkmoths

would be the major pollinator; crepuscular, hovering feeders attracted by olfactory cues – honeysuckles emit sweet scents on the evening of opening (see Proctor et al. 1996)). However, Ottosen (1986) reported *B. hortorum* to be one of the dominant diurnal visitors to *L. periclymenum* at northern latitudes and suggested a shift away from the specialist 'co-evolved' pollinator to a more generalist scenario. At the latitudinal limits of the plant in Denmark, bumblebees appear to have become opportunistic visitors to, and concomitantly successful pollinators of, *L. periclymenum* (Ottosen 1986, 1987). Weiss (2001) notes that overlap of attraction cues between visiting taxa can influence such pollinator shifts. When visiting *L. periclymenum*, *B. hortorum* is responding to a visual cue (colour change) that presumably has little ecological relevance to scent-driven moths foraging at low light levels. In my study bumblebees rarely visited yellow flowers (Figure 7.14) and, although this phase represented over 40% of a flower's life (Figure 7.7), any possible ecological function of the retention of these flowers remains unresolved. Retention of yellow phase flowers that had not been pollinated could contribute to overall long distance attractiveness of the plant for a bee; however, this could not be tested in the field due to the general paucity of visitors to this population.

B. terrestris on *Lupinus* displayed the most significant level of colour-related floral constancy of all visitors recorded in this thesis; 98% of all visits were recorded to pre-change flowers of *L. pilosus* (Figure 8.9). Honeybees and an anthophorid were shown to visit pre-change flowers in similar fashion in another study of the same species and both could effect tripping of the pollen release mechanism (Ne'eman & Nesher, 1995). The complexity of this type of flower demands particular handling behaviours from visitors; only bees seem

able to handle keel blossoms in the required manner (Westerkamp, 1997). Bees do probe the base of the vexillum and cause outward and downward movement of the keel to expose the pollen; consistent positioning is critical to this process and application of weight alone will not cause operation of the piston mechanism (Westerkamp, 1997). In my study some *B. terrestris* used 'buzzing' (sonication of the anthers by vibrating the flight muscles) to release additional pollen from these flowers. Hence the particular handling characteristics required for this flower appear to demand considerable learning abilities.

Bumblebees have been trained in controlled conditions to be constant to rewarding artificial flowers of various colours (e.g. *Bombus impatiens*, Heinrich, 1983; Gegear & Laverty, 2001). Floral constancy was also investigated in *Bombus vagans* by Wilson & Stine (1996), where bees foraging freely in the wild were offered a choice of flowers that varied in colour and morphological complexity. When two complex red flowers (vetch and red clover) were available bees showed no significant constancy, switching regularly between the choices. Flowers of similar morphology but differing in colour (white and red clover) were also offered and bees rarely switched between the two. Constancy was thus stronger where colours were different but morphology was similar, and these findings suggested that the colour cue is most important (Wilson & Stine, 1996).

In the Gegear & Laverty study artificial flowers were used with 'unnatural' colour, and the Wilson & Stine research took no account of potential colour cues in flowers that appeared similar in colour to humans but could be different to bees. To avoid these problems the use of a colour-changing flower as

a test of constancy would control these potential variables, in a more natural experiment.

It might be the case that, where colour change occurs, visitors are 'confused' by the alteration of colour, and interpret that change as equivalent to an altered resource. The potential increase in time required to exploit a 'new' resource (e.g. Waser, 1986; Goulson, 2000) is avoided, and constancy to the pre-change flower phase results. To explore this idea, lupins represent the ideal test of constancy. Because colour of the banner petal spot and position on the plant can be varied as described in Chapter 8 by inserting a new petal (yet size, reward and handling complexity remain unaffected following petal replacement), the importance to floral constancy of colour relative to other floral traits might be established. Preliminary indications from my work on *Lupinus*, where differences in other floral traits are controlled, suggest that colour is indeed critical (Figure 8.16).

In contrast to the above examples, visitation by bumblebees to *E. vulgare* was dependent on the proportions of colour phases in the population (Figure 5.14), the only plant where this occurred. Perhaps coincidentally, this was the only species in which nectar reward did not vary between colour phase; visiting bumblebees do not base foraging choice on the association between colour and reward and hence do not exhibit colour-phase constancy. Wide variation in nectar content in flowers that had previously been visited was the main factor to influence bumblebee visit patterns to *E. vulgare* in previous research (Pappers et al. 1999). In that study plants were bagged to artificially vary the nectar standing crop between plants; the bees' decision to depart the plant was affected by varying reward, with longer visitation sequences at 'high reward' plants.

ii) *Apis mellifera.*

Colour certainly plays an important role in honeybee foraging; chromatic cues are discriminated at close range (Giurfa & Vorobyev, 1997) while Giurfa et al. (1995) demonstrated apparent innate colour preferences for 'bee uv-blue' and 'bee green' in naïve bees. In training experiments with artificial flowers Wells & Wells (1986) noted that individual constancy in *A. mellifera* was not influenced solely by increasing reward in a single colour morph, but that the bees could learn to discriminate; a switch of constancy did occur when reward quality or frequency was varied between flower types.

In my study, although the mechanism of colour change in *E. judaeum* remains difficult to resolve, the foraging behaviour of *A. mellifera* points to a clear ability to learn the association of higher reward in the pink, pre-change flowers and thus to forage mainly on these flowers. The finding that continued visitation seems to result in a retardation of colour change to purple (Figure 6.5) implies a link between flower handling in general (and nectar secretion and/or depletion in particular) and colour change. The reverse change exploits the bees' learned association of pink as the rewarding colour; the plant is effectively 're-advertising' the presence of nectar and, therefore, reinforces the constancy of the visitor.

iii) *Anthophora pauperata.*

Constancy in solitary bees has rarely been reported, perhaps because (as Weiss (2001) pointed out) solitary insects have to perform a range of tasks that in social insects may be subject to division of labour. Gross (1992) studied the foraging behaviour of *Trichocolletes* sp. (Colletidae) in a mixed community of

three plants offering flowers of similar colour and floral complexity. Although foraging was generally inconstant, some preference was recorded for movements between the two species that offered nectar reward (*Dillwynia hispida* and *D. uncinata*) as opposed to visits to the nectarless plant (*Pultenaea densifolia*), suggesting that a degree of constancy is possible (Gross 1992). (This example was deemed to represent constancy by Gegear & Laverty (2001) although the Gross study does not include details of temporal availability of the different floral rewards, nor how this could influence reported foraging patterns).

Anthophora pauperata was the only example of solitary bee in my study, and it accounted for 99% of bee visitation to *Alkanna orientalis* in a study by Stone et al. (1999); this solitary bee was not noted to forage at flowers of any other species in this habitat (P. Willmer, personal communication; personal observation). In my study, constancy in *A. pauperata* was exhibited as a preference for pre-change flowers (however the small number of individual bees observed should be borne in mind; male territoriality and limited foraging patterns of females in this species (Willmer et al. 1994; Stone et al. 1999) indicate that observation of focal patches in my study would have restricted the sample size of individual bees recorded). Further data need to be collected on individual bees to see if a form of intra-plant floral constancy, based on colour phase, is really occurring; given the absence of other flowering plants early in the spring, constancy of *A. pauperata* to *Alkanna* is ensured anyway.

iv) Syrphids.

A similar problem of low sample size of foraging insects also applied to my study of *Myosotis sylvatica*. The influence of poor weather conditions, and a

resultant limited number of foraging insects on most days of observation, compromised independence of data collection. Although late in the flowering season (towards the end of May and early June) there were occasional days when *Nomada* spp. and small dipterans (<5mm) were frequent visitors, the majority of visitation was by syrphids. *M. sylvatica* therefore presents a good opportunity to study floral constancy in hoverflies, a potentially important group of pollinators that has received relatively little study in this respect.

Goulson & Wright (1998) noted that constancy in syrphids was likely to be due to different constraints from those in some other flower visitors. As many syrphids forage for pollen, which is usually found more easily than nectar in most flowers, learning may play a less important role than visual acuity. In *M. sylvatica* a range of syrphids was observed, and did include one species that was foraging for nectar; *Rhingia campestris* has a diet of predominantly nectar and has mouthparts adapted for nectar feeding (Gilbert, 1981). Constancy to patches of *M. sylvatica* was virtually assured because there was a paucity of plant species flowering at this time at my study site. A few individuals of *Silene dioica* were present and *R. campestris* foraged occasionally on these plants. The only other flowering plant available was *Geum rivale*, which was assumed to have available nectar as *Bombus pascuorum* was noted as a regular visitor. *Rhingia campestris* was not observed feeding at these flowers and is possibly constrained from doing so by floral orientation and morphology; *Geum* flowers are downward-facing and exhibit a mass of anthers that possibly prevent small, lightweight visitors from gaining access to nectaries.

To examine whether constancy to particular species occurs in *R. campestris*, the absence of a mixed community of plants was compensated by the

opportunity to investigate within-species constancy to floral colour phases of *Myosotis*. Even the small data set in my study suggests that this hoverfly does feed preferentially at pre-change, yellow-centred flowers of *M. sylvatica* (Figures 3.14), and these flowers contained significantly more nectar than white-centred flowers (Table 3.4). Foraging *R. campestris* at *M. sylvatica* were seen to probe yellow-centred flowers preferentially; these insects would frequently land on an inflorescence and move across white-centred flowers without extending their proboscis before settling over yellow-centred flowers to feed.

In a further observation of this apparent preference for yellow coloration, I noted a range of hoverflies, including *Episyrphus balteatus*, *Eristalis* sp. and *Heliophilus* sp. discriminating between pre- and post-change flowers of the composite *Aster tripolium* (intended as a 7th study species, but not followed through due to time constraints). Here predominantly yellow-centred and pollen-rich disc flowers received significantly more visits than red-centred flowers that lacked pollen, in contrasting patches balanced for size and nearest neighbour distance (Table 9.3).

Patch	Approaches	Feeding visits	% of approaches converted to feeding visits
Yellow	82	69	84
Red	56	9	16

Table 9.3 Approaches and feeding visits to patches of manipulated patches of *Aster tripolium* at Balgove Bay, Eden Estuary, Fife (NO 491185 (349112, 718537)). Data were collected during sunny weather on 15/8 and 27 to 31/8/2001 inclusive. Results collated from 13 fifteen-minute observations to 0.5m^2 patches of *Aster* where either all yellow-centred or red-centred flowers had been removed. Approaches were non-landing flights to within 5cm of any flower within the patch and feeding visits incorporated landing on centre of flower and extension of mouthparts towards flower.

Lunau & Maier (1995) used naïve hoverflies (*Eristalis tenax*) to confirm a behavioural bias towards certain coloured stimuli. The proboscis extension reflex (PER) could only be elicited in response to reflected green and yellow light of very narrow wavelength (520-600nm) and Lunau and Maier attributed the reaction to the sensitivity of a particular photoreceptor type. Further work involving floral guides also reported the PER in response to yellow colour stimuli (Dinkel & Lunau, 2001).

Floral constancy within an array of flowers has been documented for two syrphid species foraging in natural conditions. *Episyrphus balteatus* and *Syrphus ribesii* visited *Pulicaria dysenteria*, *Eupatorium cannabinum* and *Centaurea nigra* most often in a community of 16 plant species, although neither exhibited constancy to white or purple colour morphs of *Lobularia maritima* in experimental arrays (Goulson & Wright, 1998). However, Stanton (1987) found constancy to pink flowers for syrphids foraging at white, yellow, pink and bronze morphs of *Raphanus sativus*; this suggested some degree of flexibility in learning to associate colour and reward, even given the known behavioural responses of hoverflies to yellow colour stimuli of very precise wavelengths (Lunau & Maier, 1995).

9.5 Concluding remarks.

My thesis presents new evidence that highlights further the complexities of floral colour change. Whilst I have proposed a simplified model that could account for the varied patterns of colour change I report, the main finding is that colour change is a flexible temporal process and has more than one proximate trigger. Additionally, foraging behaviour that is 'colour phase constant' is confirmed for a range of insect visitors and this illustrates the potential ecological benefits of colour change in five plants that have not previously been researched with regard to this question.

By applying a range of artificial manipulations that mimicked insect visitation I used new techniques to test whether various aspects of mechanical handling influenced colour change. Similar types of test had only been tried in relation to colour change twice before on just three plant species (see section 1.5.2) and therefore my study helps to address a gap in the literature.

One conclusion of my work relates to the issue of 'inducibility' or 'age-relatedness' of colour change; the background to this problem is highlighted in sections 1.3.4 to 1.3.7 (p16-23). The utility of the concept is challenged because the patterns found in my study species suggest that an 'either/or' dichotomy is inappropriate to explain the results observed.

Most notably, the colour change found in *Echium judaeum* shows that colour change can be 'reversed' and the initial change either hastened or retarded according to visitation, yet is unaffected by pollination (the traditional trigger for so-called 'inducible' change). In *Echium vulgare* pollination is similarly uninvolved in colour change; both species occupy somewhat isolated positions in

my model and require further work to clarify if and how ethylene is implicated in their colour change patterns. No research has been carried out on closely related species that helps with this problem; only the congeneric *E. decaisnei* has been noted to change colour, but displays only part-flower colour change and no further data are presented (Weiss, 1995a).

The other members of the family Boraginaceae in this thesis have more 'obvious' potential triggers for colour change but both still exhibit noteworthy patterns of colour change. *Alkanna orientalis* offers the best evidence for the temporal flexibility and likelihood of the same fundamental role of ethylene in colour change via different pathways in my model. As the species is self-incompatible and still changes colour when bagged (although retarded from the visited rate), the later aspects of pollination (i.e. after pollen deposition, because self-pollen is accidentally deposited in bagged flowers) are only triggering colour change in visited flowers. Bagged flowers change colour just prior to senescence, presumably as part of an ageing process. The hastened change of manipulated flowers, which also do not receive cross-pollen, points to a wound response triggering change in these flowers. Again, no members of this genus have been studied in relation to colour change to compare with my model.

The patterns of colour change in *Myosotis sylvatica* suggest colour can occur via pollination, wounding or age pathways, but due to its self-compatibility the change could simply be triggered by the deposition of pollen *per se* and the different rates of change merely reflect varying pollen load on the stigma. Colour change in one other closely related species, *M. colensoi*, is not fixed temporally (Robertson & Lloyd, 1993) and therefore could also be effected by the same

mechanisms as *M. sylvatica* (*M. colensoi* is also self-compatible, Robertson (1992)).

Colour change in *Lupinus pilosus* is triggered by pollen deposition and/or pollen tube growth and this accounts for the earlier finding of accelerated colour change following pollination by Ne'eman & Nesher (1995). Thus either the wound or pollination pathways (which may not be mutually exclusive) in my model explain the patterns of colour change I report. Earlier studies on lupins have placed the type of colour change into both 'age' and 'inducible' categories (see Weiss, 1995a, and Table 1.1). All those previously described as 'inducible' could also fit my model if pollination or wounding is the relevant trigger for colour change. As none of the 'age-related' colour-changing lupins have been reported not to change colour prior to senescence, these species could all follow the age-related pathway of ethylene production and colour change.

The four congenerics of *Lonicera periclyenum* that change colour (Weiss, 1995a) have not been the subject of detailed colour change study and therefore offer no further clues to the efficacy of my model. *Lonicera* is the only species I studied that has a fixed temporal pattern of colour change not influenced by any extrinsic triggers.

Future work could concentrate on a more precise evaluation of my model in terms of the timing of colour change in relation to aspects of wounding, pollination, senescence and measurement of concomitant endogenous ethylene production. This would enable determination of the definite triggers for colour change in each case; in particular, where colour change varies temporally, the influence of pollen deposition and amount deposited, pollen tube growth, and fertilisation could be assessed.

Hence a good proportion of the original aims of my thesis (see section 1.5.3, p35-36) have been achieved. The trigger for colour change is not universal for my study species although a basic mechanism may be shared. Alteration in reward certainly coincides with colour change in all but *Echium vulgare*, but is possibly involved in modulating change in *Echium judaeum*. The aspect of visitation that appears crucial to colour change in most of my study species is pollen deposition and/or subsequent reproductive processes; however, specific visitors can influence colour change (since in *Lupinus* a specific handling effect was critical to effect pollen deposition and colour change). Subsequent to floral colour change, visitor behaviour is certainly modified; insects from several taxa respond to the 'signal' given by such change, and forage more efficiently at pre-change flowers. This colour change is clearly functional in enhancing the opportunity for further successful pollination in most species.

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