Contrasting Strategies between Honest and Dishonest Flowers:

Floral Adaptation to Learning Behavior of Pollinators

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Abstract

Flower diversity has long been considered the outcome of interaction with different pollinators. However, flowers with different traits often receive visits by similar pollinators comprising several taxonomic groups, which calls into question why they exhibit marked divergence despite being likely to share common selection pressure from pollinators. This paradox may be explained by taking into account the effects of behavioral differences among flower-visiting animals on pollination success.

Floral color change—the retention of old, nonproductive, rewardless, but fully turgid flowers in an altered color—serves as a good example in exploring associations between flower diversity and behavioral characteristics of animals. This trait syndrome has been considered a plant strategy for enhancing pollinator attraction while minimizing visits to non-reproductive flowers. Considering the perceptive and cognitive abilities of flower-visiting animals, these advantages should apply in most systems. It seems paradoxical, therefore, that floral color change occurs in only a portion of angiosperms.

This divergence may be explained if floral color change adds as yet unknown costs or benefits under specific conditions. In Chapter 1, to explore this possibility, I compare details of trait combinations and pollination consequences between a floral color-changing (*Weigela coraeensis*) and a non-color-changing (*W. hortensis*) species in a botanical garden where both species are planted in a shared environment. Results revealed that flowers of *W. coraeensis* secreted nectar for an initial 2-3 days and remained white, after which they persisted for a few more days with discolored, red-purple petals and reduced nectar levels. Flowers of *W. hortensis* also secreted nectar for an initial 2-3 days. They persisted for a few more days with the same color and reduced amounts of nectar, but did so only when pollinators were scarce. In the garden, because only *W. hortensis* suffered from pollination difficulty, both species retained their flowers for 4-5 days. All the visitors to *W. coraeensis* and *W. hortensis* flowers were insects, and

bee pollinators comprised 92% of total pollinators. Bees strongly preferred *W. coraeensis*, leading to a greater proportion of seed set in *W. coraeensis* than in *W. hortensis*. In addition to their higher nectar productivity, only *W. coraeensis* plants offered bees a color difference as a visual cue for choosing rewarding flowers and, thus, improving both long-term and short-term rates of nectar intake. By learning where to return, bees may have responded to the difference in resource exploitability. These results suggest that floral color change yields the additional benefit of attracting bee-like pollinators that return preferentially to more easily exploitable plants. However, under conditions where the benefits of enhancing both conspicuousness and exploitability cannot outweigh the costs of doing so, retention of rewardless flowers in the same color and flexibility in floral longevity—as was adopted by *W. hortensis*—may become more advantageous in terms of saving metabolic and ecological costs.

The next step is to reveal how color changers and non-color changers differ in interactions with pollinators in natural habitats. In Chapter 2, to answer this question, I examine floral traits and visitor faunas in a floral color-changing (*Weigela decora*) and a non-color-changing (*W. hortensis*) species in their natural habitats, and also compare foraging behavior among the visitor groups with different lifestyles or life cycles. To this end, I selected two populations of *W. decora* in Tsukuba and Nikko, and two populations of *W. hortensis* in Sendai and Tone. The two plant species achieved similar levels of seed set, and they differed only in the presence or absence of a color cue for effective flower choice—that is, *W. decora* retained old, less rewarding flowers in an altered color, while *W. hortensis* did so in the same color. The majority of visits to both plant species were by large eusocial bees (*Apis* and *Bombus*), small/non-eusocial bees, Acroceridae flies, and Syrphidae flies. Large eusocial bees visiting *W. decora* in Tsukuba often exhibited both reward-based flower choice and repetitive use of the same plants. On the other hand, the bees made less frequent revisits in Nikko where *W. decora* had lower levels of nectar productivity and grew in larger stands. Visits to *W. hortensis* by large eusocial bees were largely occasional at the two sites, and they exhibited weak and inconsistent patterns of flower

choice. As for small/non-eusocial bees and flies, either or both of these trends were weak or insubstantial, suggesting that these insects are less efficiency-oriented foragers. These results suggest that *Weigela* plants are exposed to different flower-visiting insects because of the flexible behavior of large eusocial bees. That is, the two plant species were both successful in pollination, by utilizing taxonomically similar but behaviorally diverse pollinators.

The results presented in Chapter 2 suggest that flowering plants receive frequent revisits by efficiency-oriented pollinators such as large eusocial bees only when the plants exhibit high nectar productivity and grow in small or sparse stands. Maintaining such regular visits would be particularly advantageous for plants when interspecific competition for pollinators is intense. These conditions appear to be consistent with those of the botanical garden (Chapter 1) where the color changers are more successful in pollination than the non-color changers. In contrast, when plants grow in large or dense stands with few blooming species, opportunistic visitors could be effective pollinators because they inevitably move among conspecific flowers irrespective of foraging tactics. Under such conditions, plants would only need to have as many open flowers as possible in order for the strategy of non-color changers to be successful. This study thus highlights the importance of studying behavioral diversity among flower-visiting animals to understand trait diversity among flowers with similar visitor faunas.

Key-words

combination of floral traits, floral color change, *Weigela*, pollinator, foraging behavior, color-reward association, preference for rewarding flowers, revisitation to the same plants, plant reproduction, plant-insect interaction

General Introduction

Ecological interactions between plants and pollinators have been considered an important evolutionary force underlying the diversification of flowering plants (Willmer 2011; Patiny 2012). Integrated sets of floral traits (e.g., morphology, color, odor, size, reward, phenology) associated with particular pollinator groups are known as floral or pollination syndromes (Stebbins 1970; Fenster et al. 2004). Based on the concept of pollination syndromes, variations in floral traits among closely related species have been considered the results of adaptation to different pollinator groups. One obvious example is the interaction between spur lengths of flowers and tongue lengths of pollinators. Whittall and Hodges (2007) shows a significant evolutionary trend for increasing spur length during directional shifts to pollinators with longer tongues, using a species-level phylogeny of the columbine genus, *Aquilegia*: bumble bees with short tongues pollinate flowers with short spurs, whereas hawkmoths with long tongues pollinate flowers with long spurs. Like this phenomenon typically exhibiting a fine morphological match between pollinator traits and floral traits, floral divergence can be explained by associations with diverse taxonomic groups of pollinators.

However, flowers often diverge without excluding one group of pollinator in favor of another (Waser et al. 1996). In particular, closely related species with different floral traits often receive visitations by similar pollinators. For example, although blue or purple flowers are thought to attract bee pollinators (Rausher 2008), closely related species with different floral colors often receive frequent visits by bees (Willmer 2011). As for other orders of flower-visiting animals, previous studies have also failed to find strong relationships between animal orders and flower colors (Muchhala 2003; Woodcock et al. 2014). In this manner, plant species within the same genus often possess different sets of floral traits, although they are pollinated by similar taxonomic groups of animals (Alcantara and Lohmann 2010). In such cases it is unclear why even closely related plant species, which are likely to share common

phylogenetic constraint and selection pressure by pollinators, show marked divergence in floral traits.

To understand how flowers with different sets of traits could evolve in response to similar taxonomic groups of pollinators, it may be helpful to clarify variations in the foraging behaviors of flower-visiting animals. This is because animals often vary their foraging behavior depending on cognitive abilities, energy requirements, ecological contexts, etc., even within the same taxonomic groups. For example, bees are known to vary greatly in their lifestyle or life cycle, ranging from eusocial to solitary (Michener 2007). The former group often establishes massive colonies, as in bumble bees, while the latter builds no colonies, as in Andrenid bees. Therefore, eusocial and solitary bees may vary in their behavior according to differences in their food demand or levels of motivation to improve foraging performance, even though eye spectral sensitivity in these insects is phylogenetically conserved in Hymenoptera (Peitsch et al. 1992, Briscoe and Chittka 2001). Moreover, even the same pollinator species may exhibit different flower or plant choice behavior, depending on whether they learn to shift their preferences. Most flower visitors, including diverse groups of insects and vertebrates, are known to learn floral cues associated with reward quantity and quality, such as floral colors, odors, form, or overall display of flowering plants (Chittka and Thomson 2000). Such a capacity for flexible associative learning presumably allows pollinators to adjust their foraging efforts in response to floral rewards and quantity of blooming species that vary over space or time. For example, pollinators may develop a preference for plants with high-reward flowers as they gain experience in communities that include many blooming species, whereas they may haphazardly choose plants irrespective of reward level in communities with few blooming species. Pollinators in different foraging contexts, therefore, may exert different selection pressures on floral traits through their foraging behavior.

Floral color change, that is the retention of old, nonreproductive, rewardless, but fully turgid flowers in an altered color (Weiss 1995, Weiss and Lamont 1997), may provide a good

system to study floral diversity in terms of outcome of interactions with varied animal behavior. Previous studies have reported that some species of angiosperm exhibit ontogenic color changes in fully turgid flowers (at least 588 species from 330 genera from 90 families: Gori 1983; Weiss 1995; Lunau 1996; Weiss and Lamont 1997; Oberrath and Böhning-Gaese1999; Atluri et al. 2000; Willmer 2011; Ohashi et al. 2015). The color-changed flowers usually have only a small amount of pollen and nectar left in flowers and show reduced levels of stigma receptivity compared with young, pre-change flowers (Weiss 1995; Weiss and Lamont 1997). This trait syndrome has been interpreted as a strategy to attract pollinators from long distances by increasing floral display size (i.e., the number of open flowers per plant or inflorescence) and simultaneously to minimize the probability of illegitimate visits by pollinators to plants with old, non-reproductive flowers by changing their color and reward value (Weiss 1991; Oberrath and Böhning-Gaese 1999). However, the above scenarios do not provide a clear answer to the question of why it typically occurs in only a portion of species within a genus (Weiss 1995; Ohashi et al. 2015). This question seems legitimate because the proposed advantages of floral color change should be more broadly applicable. Plants with more open flowers, discolored or not, often receive more frequent visits by pollinators (reviewed by Ohashi and Yahara 2001). It is also known that flowers in many plant species last longer when pollination is insufficient, allowing plants to increase display size and enhance pollinator attraction (Harder and Johnson 2005). Moreover, most flower visitors, including diverse groups of insects and vertebrates, can learn color-reward associations (Giurfa et al. 1995; Weiss 1997; Kandori and Yamaki 2012; Healy and Hurly 2013; Woodcock et al. 2014), based on which it could be expected that any type of pollinator will learn to avoid old and rewardless flowers. Therefore, the prevalence of both color-changing and non-color-changing species within the same genus cannot be explained solely by considering the innate abilities of pollinators.

This divergence may be explained if the strategy of color-changing plants is favored under specific conditions that have not yet been discussed in the literature. A probable

explanation is that color-changing species may benefit from attracting particular taxonomic groups of pollinators, while non-color-changing species may focus on attracting other pollinator groups. Alternatively, even though color changers and non-color changers are visited by similar animal groups, pollinators of different plant populations may exhibit divergent behavioral characteristics. As I explained above, animals may differ in foraging behavior, depending on resource exploitability, the amount of blooming plants, or food demand, which all vary over space or time. Pollinators of different plant populations, therefore, may exert different selection pressures on floral traits through their foraging behavior.

To explore such possibilities, in Chapter 1, I compare behavioral responses of pollinators to color changers and non-color changers, using the two species of *Weigela* (Fig. 1.1) growing in a botanical garden. Because retention of flowers and resource exploitability affect the visitation rate of animals (Chittka and Thomson 2000), I compare the combinations of floral traits (floral longevity, reward productivity, perianth color and their ontogenetic changes) and behavioral responses of visitors to the different trait combinations. Moreover, I examine differences in pollination success of the two species in the shared environment. Considering the effects of flower retention and its color change suggested by previous studies (Oberrath and Böhning-Gaese1999), I could predict two possible scenarios. First, flowers of color changers possibly last for longer durations than those of non-color changers, which may allow the color-changing plants to increase display size and enhance pollinator attraction (Harder and Johnson 2005). In this case, the advantage of color changers is caused by the retention of old flowers, rather than the floral color change itself. On the other hand, the retention of old flowers may be less advantageous in terms of minimizing the costs of floral maintenance (Ashman and Schoen 1994). Second, if both species retain old flowers with few rewards and the floral color change is effective, the behavioral responses of flower visitors will vary between the two plant species. That is, flower-visiting animals are likely to choose more rewarding flowers on the plants with color-changing flowers by discriminating floral colors, whereas they will have

difficulty in choosing rewarding flowers on the plants with non-color-changing flowers. If such a visual guidance is beneficial for the color changers, they will be more successful in enhancing visitations by pollinators. In particular, the visual guidance may enhance visits by particular groups of pollinators such as bumble bees and honey bees, considering that they often develop a preference for more easily exploitable plants as they gain experience (Cartar 2004; Menzel et al. 2005; Makino and Sakai 2007). To examine such hypotheses, it is necessary to clarify the differences in the floral trait combinations, behavioral responses of visitors, and pollination consequences between the two plant species. Based on the differences described in Chapter 1, I discuss whether and how the two species adapt to different pollinators.

In Chapter 2, to examine whether the hypothesis proposed in Chapter 1 applies in natural habitats, I compare faunal compositions of pollinators between a color-changing and a non-color-changing species of *Weigela*. At the same time, I explore diversity in behavioral characteristics among taxonomic groups of flower-visiting animals, as well as compare foraging behaviors of each animal group among the plant populations. In Japan, natural habitats of color changers and non-color changers of *Weigela* usually differ from one another (Hara 1983). Visitor fauna, therefore, may also vary between *Weigela* species. However, previous studies have reported that the color-changing and the non-color-changing species were primarily visited by bees and flies belonging to several families that largely overlap between species of *Weigela* (Kato et al. 1990, Yamazaki and Kato 2003). This implies a difficulty in explaining the floral divergence solely by obtaining lists of taxonomic groups of pollinators. To solve this problem, I try to clarify variations in behavioral characteristics involved in floral display size and resource exploitability by observing the forging behaviors of all flower-visiting animals. I then discuss how the two *Weigela* species are exposed to different flower-visiting animals in their own habitats.

By integrating the findings discussed in Chapters 1 and 2, I provide a novel, comprehensive hypothesis to explain why color changers and non-color changers are both

prevailing. I suggest specific conditions in which floral color-changing plants will be successful. This study describes divergent reproductive strategies of flowering plants via pollinators: plants are exposed to different flower-visiting animals, laying the groundwork for contrasting but successful strategies of advertisement.

Chapter 1

How does a floral color-changing species differ from its non-color-changing congener? – a comparison of trait combinations and their effects on pollination

1.1 Introduction

Ecological interactions between plants and pollinators have driven the evolution of floral traits and their combinations (Willmer 2011; Patiny 2012). Especially, the varying balance between the costs and benefits associated with pollination process has often produced complex and sometimes counterintuitive assemblages of multiple trait dimensions (Ohashi 2002). Floral color change—the retention of old, non-reproductive, rewardless, but fully turgid flowers in an altered color—is a good example of such pollinator-mediated floral evolution. The color-changed flowers usually have only a small amount of pollen and nectar left in flowers, and show the reduced levels of stigma receptivity compared with young, pre-change flowers. This trait syndrome has been interpreted as a strategy to attract pollinators at long distances by increasing flower number or display size, and simultaneously minimize the probability of illegitimate visits by pollinators on plants to old, non-reproductive flowers by changing their color and reward value (Weiss 1991; Oberrath and Böhning-Gaese 1999). A few other authors have suggested that the rewardless, color-changed flowers encourage pollinators to leave plants and reduce geitonogamous self-pollination (Jones and Cruzan 1999; Ida and Kudo 2003).

While the distribution of relevant species across 330 genera and 90 families (at least)

suggests that floral color change is a widespread functional convergence within angiosperms (Gori 1983; Weiss 1995; Lunau 1996; Weiss and Lamont 1997; Oberrath and Böhning-Gaese 1999; Atluri et al. 2000; Willmer 2011; Ohashi et al. 2015), the above scenarios do not provide a clear answer to the question of why it typically occurs in only a portion of species within a lineage (Weiss 1995). This question seems legitimate because, according to previous studies, the proposed advantages of floral color change should be more broadly applicable. Plants with more open flowers, discolored or not, often receive more frequent visits by pollinators (reviewed by Ohashi and Yahara 2001). It has also been known in many plant species that flowers last for longer duration when pollination is insufficient, allowing plants to increase display size and enhance pollinator attraction (Harder and Johnson 2005). Moreover, most flower visitors including diverse groups of insects and vertebrates can learn color-reward associations (Weiss and Lamont 1997; Willmer 2011), from which I could expect that any type of pollinator will learn to avoid old, rewardless flowers, as well as to depart earlier from plants with more color-changed flowers. It is thus unclear why even closely related plant species, which are likely to share common phylogenetic constraints, show marked divergence in this respect.

A probable explanation for this interspecific variation is that the combinations of floral traits in color-changing plants can yield additional costs or benefits under specific conditions that have not been indicated in literature. In other words, I have difficulties in explaining the prevalence of color changers and non-color changers, possibly because the potential strength and weakness of these strategies in varying interactions with pollinators have not yet been exhausted. To explore such possibilities, it will be fruitful to compare between color changers and non-color changers within a lineage in terms of their ecological costs and benefits. The critical issues in conducting such comparisons are twofold. First, because floral traits are often plastic and function in concert with one another, an extensive examination is needed for floral longevity or the duration of flower retention, reward productivity, perianth color, and their ontogenetic changes at different pollination levels, together with their effects on reproductive success.

Second, because floral traits and their interactions with pollinators are likely to vary with the surrounding environment, data should be collected at the same time and location. Although the significance of such within-lineage comparisons has been emphasized by previous authors (Weiss 1995), these dual requirements appear to have precluded a full comparison of color-changing versus non-color-changing species. In particular, no real effort has been attempted so far to determine the trait combinations of non-color-changing species in a way comparable to those of color-changing species.

Here I try to remedy this lack of knowledge by comparing trait combinations and their pollination consequences between floral color changers and non-color changers, using a pair of native species in an Asian genus of Caprifoliaceae, *Weigela coraeensis* Thunb. and *W. hortensis* C.A. Mey (Fig. 1.1) growing in a botanical garden. By focusing on quantification of the combined patterns in floral longevity, nectar productivity, petal color, their ontogenetic changes at different pollination levels, as well as of their influences on pollination, I specifically address the following questions: (i) How do the two species differ in the combination of floral longevity, nectar productivity, petal color, and their ontogenetic changes at different pollination levels? (ii) How do flower visitors respond to the differences in trait combination, and how does that influence the pollination and reproductive success of these plants? (iii) How do the observed differences illustrate the potential costs and benefits received by these plants in their natural habitats, as well as the possible conditions that determine which strategy is favored by selection?

1.2   Materials and Methods

Weigela coraeensis and *W. hortensis* are both deciduous shrubs that occur in the coastal and mountain ranges of Japan. In late spring (May-June), both species produce 5-15 bell-shaped flowers that are perfect, homogamous, and self-incompatible (M.F. Suzuki, unpublished data) in small inflorescences or corymbs. Although each corymb is indeterminate and the order of

anthesis is centripetal by definition, the relation between age and spatial position of individual flowers is rather equivocal due to the multi-dimensional structure they form with one another and with nearby inflorescences. Flowers of both species appear turgid as long as they are retained on plants. Nectar accumulates at the bottom of the 2.0-2.5 cm long corolla tube. My preliminary survey shows that the number of ovules per flower is 89 ± 2.1 (n = 5, mean $\pm$ SE) in *W. coraeensis* and 68 ± 3.4 (n = 4) in *W. hortensis*, respectively. To human eyes, the corolla of a *W. coraeensis* flower turns from white to red-purple as days elapse from anthesis, whereas the corolla of a *W. hortensis* flower remains pale-pink until it falls off (Fig. 1.1).

In the Tsukuba Botanical Garden, Ibaraki Prefecture, Japan (36°6'7"N, 140°6'38"E), I selected five of 19 *W. coraeensis* plants and four of 11 *W. hortensis* plants as focal individuals throughout this study, all of which had been planted in sunny locations and reached approximately 2-3 m in height. The focal plants of *W. hortensis* started blooming on average five days earlier than those of *W. coraeensis*, but data were collected only during the overlapping period.

Patterns of anthesis and flower retention

During 2009 and 2010, I haphazardly selected 1–3 inflorescences of average size from each of the focal plants and marked them with plastic tape, in order to assess floral longevity under open-pollinated conditions. I checked these inflorescences every day and marked all the newly opened flowers on their pedicels using oil-based, pigmented paint with little or no penetration into plant surface. Paints of different colors were used to distinguish anthesis dates. When a marked flower fell, I recorded the duration of flower retention by subtracting the flower's opening date from the closing date. For example, I considered that a flower persisted for two days when I found it opened on June 1st and disappeared (fell off) on June 3rd. During 2010, I also counted the newly opened flowers on each inflorescence to determine the average daily frequency of anthesis per inflorescence, i.e., anthesis rate. I then compared floral longevity and

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anthesis rate between the two species by using generalized linear mixed model (GLMM) analysis with an identity link function and a Gaussian error distribution, in which I considered species as the fixed factor and individual plant as the random factor. I fit all models by maximum likelihood using the lmer function in the lme4 package for R (Bates et al. 2011). The significance of the effect of plant species was tested by using the likelihood ratio chi-square statistic (Morrell 1998). In this study, all statistical analyses were conducted using R version 2.10.1 (R Development Core Team 2009).

Nectar productivity

During 2009 and 2010, I measured 24-h nectar productivity of *W. coraeensis* and *W. hortensis* as follows. I haphazardly selected 1-3 inflorescences from each focal plant and covered them individually with a polyester mesh bag to prevent pollinator visits. At each daily census, I unbagged them and painted all the newly opened flowers with a unique color, so that I could determine their flower age which was defined as zero-day-old on the day of anthesis. I then quantified the volume of nectar in zero-day-old flowers, as well as the nectar accumulated for 24 h in one-day or older flowers, by inserting a microcapillary tube (1-5 μl) into each flower. During 2010, I also measured sugar concentration (%, v/v) for each nectar sample with a refractometer, and calculated daily sugar production per flower. One microliter of pure water was added when the nectar amount was too few for the measurement.

For each species, I compared the three measures of nectar production—volume, concentration, and sugar mass—among flower cohorts (= groups of flowers in the same age) by repeating pairwise *t*-tests and adjusting *P*-values with false discovery rate (FDR) controlling procedures (Benjamini and Hochberg 1995). In addition, I compared these variables between the two species by fitting a GLMM with an identity link function and a Gaussian error distribution. In each model, I considered plant species and flower age as the fixed factors, together with their interaction term (species x age), and plant individual as the random factor. I tested the

significance of the effect of plant species by using the likelihood ratio chi-square statistic. Prior to these analyses, the variables were logarithmically transformed to correct for skewness, i.e., $\ln(x + 0.5)$ (Yamamura 1999).

Daily changes in petal color

During 2009, I sampled 22-40 flowers from each age cohort by arbitrarily selecting several inflorescences for every focal plant under open-pollinated conditions, and brought them to the laboratory. I then measured the spectral reflection functions of petals using BRC112E CCD array spectrometers and fiber optic reflection probe (B&W Tek inc., Newark, USA). The probe held at 45˚ to the petal surface and the bulb illuminated the measuring area of a petal (diameter = 5 mm). The bulb was connected with BDS-100 deuterium/tungsten light source (B&W Tek inc., Newark, USA) with 200-1100 nm spectral range. Each flower was mounted on a black sheet during the measurement to eliminate stray reflectance. Spectral reflectance functions were recorded 1-nm steps from 300 to 700 nm and were expressed relative to a white standard, i.e., a reference for 100% reflection.

The measured spectra were averaged at 5-nm intervals, and used for calculating the color loci in the hexagon color space considering the spectral sensitivity functions of honeybee (*Apis mellifera*) photoreceptors (Chittka 1992; Chittka and Kevan 2005). These sensitivity functions are phylogenetically conserved in Hymenoptera (Peitsch et al. 1992; Briscoe and Chittka 2001). For each plant species, I tested whether the color loci varied between successive flower age cohorts, by using multivariate analyses of variances (MANOVAs; Dytham 2011) with the adjustments of *P*-values with FDR controlling procedures. I also calculated the Euclidean distances between the average color locus of zero-day-old flowers and the other age cohorts as the measures of color change (Chittka and Kevan 2005). For reference, 0.09 color distance (in hexagon units) is required for approximately 60% correct discrimination in bumble bees (Dyer

2006), although this threshold may vary among individual foragers with their motivation and reward histories (e.g., Dyer and Chittka 2004).

To assess whether the changes in spectra of each species could be perceived by Diptera, I used the model proposed for fly color vision (Troje 1993; Arnold, Savolainen and Chittka 2009) and the spectral sensitivity functions of the hoverfly (*Eristalis tenax*) photoreceptors (Lunau and Wacht 1994). The color perceived by Diptera species is determined by the receptor stimulated most strongly in p-type receptors (R7p and R8p) and y-type receptors (R7y and R8y). So I took the difference in relative excitations between p-type receptors (R7p - R8p) and y-receptors (R7y - R8y), and considered them as x- and y-coordinates in an opponent color space. I then computed the average color loci for individual age cohorts and classified them into four categories according the signs of the coordinates: fly-UV (x+ y+), fly-blue (x- y+), fly-yellow (x- y-), and fly-purple (x+ y-). Changes in the color loci between successive flower age cohorts were also tested for significance using Wilk's MANOVAs with the FDR controlling procedures. I regarded that Diptera could perceive a color change only when the average loci of different age cohorts fell into separate color categories and significantly differed from one another.

Because the petals of *W. coraeensis* hardly have any reflectance in UV (300-400 nm) region (Fig. 1.2a), all the spectral information should be encompassed in RGB values that can be easily derived from digital images. To measure floral color in situ, therefore, I developed linear regression calibration models for estimating x- and y-coordinates in the hexagon space, by considering their corresponding RGB values from digital images as the explanatory variables. First, I photographed 30 flowers of different colors on the five focal plants with a digital camera (FinePix S8100fd, FUJIFILM, Tokyo, Japan). Individual flowers were shot with a piece of green paper used as a color reference, and then measured their spectral reflectance in laboratory. For each image, I obtained RGB values of the petal and the green reference by using Photoshop CS3 (Adobe Systems inc., San Jose, USA). To standardize the effect of varying light conditions, I divided the RGB values of petals (P_r, P_g, P_b) by those of the green reference (R_r, R_g, R_b) in the

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same image, and multiplied them by those of the green reference in an arbitrarily chosen "standard" image (S_r, S_g, and S_b). I thus obtained 30 sets of standardized RGB values of petals

$$\left(R_s = \frac{P_r \cdot S_r}{R_r}, \quad G_s = \frac{P_g \cdot S_g}{R_g}, \quad B_s = \frac{P_b \cdot S_b}{R_b} \right)$$ with their corresponding color loci (x, y) in the hexagon

space. By exhausting all possible models including these variables and their interaction terms, I selected the most parsimonious pair with the smallest AICs to estimate x and y: $x = 1.7e^{-3}G_s + 1.2e^{-3}B_s - 5.3e^{-6}G_sB_s - 2.5e^{-1}$ ($R^2 = 0.72$, $P < 0.001$) and $y = -4.8e^{-5}R_s - 4.8e^{-4}G_s + 3.2e^{-4}B_s + 2.2e^{-7}R_sB_s - 1.5e^{-6}G_sB_s + 2.5e^{-1}$ ($R^2 = 0.80$, $P < 0.001$).

During 2010, I used these calibration models to address how petal color of a *W. coraeensis* flower can translate into its nectar productivity. Before measuring nectar in individual flowers (see *Nectar productivity*), I photographed them to determine their own color locus. I then tested for the significance of correlation between the color distance from the day of anthesis and the daily sugar production with the following Monte Carlo randomization method. I shuffled the data on sugar production and calculated a Kendall's rank correlation coefficient (tau) between color distance and sugar production. This procedure was iterated 10000 times to get a null distribution of Kendall's tau expected by chance from the same dataset. The *P*-value was computed as $(N + 1)/(10000 + 1)$, where N represents the number of randomized sequences yielding tau values that are smaller or equal to the observed one.

Responses of floral traits to pollination level

I addressed whether and how the patterns in flower retention, nectar productivity and petal color in both species change in response to different levels of pollination in the following way. First, I examined the effect of bagging (i.e., a complete lack of pollinator visits), which was conducted primarily for nectar measurements (see *Nectar productivity*), on the duration of flower retention during 2009 and 2010. During 2009, I also examined the effect of bagging on petal color of *W. coraeensis* based on spectral reflectance curves (see *Daily changes in petal color*), using 25-29

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flowers of each age cohort collected from arbitrarily chosen inflorescences. Second, I examined

the effects of hand crossing and hand pollen removal (i.e., enhanced pollination) on the duration

of flower retention and nectar productivity in both species. I bagged 10 arbitrarily chosen

inflorescences from four of the focal plants in each species, and checked them at daily census.

For each newly opened flower with fully dehisced anthers, I simultaneously performed hand

cross and pollen removal. I deposited outcross pollen—collected from all the other focal plants

of the same species—onto its stigma with a paintbrush. I subsequently brushed pollen off from

its anthers as much as possible to simulate pollen removal by flower visitors. I then examined

these pollination-enhanced flowers for their floral longevity and nectar production rate. All these

results were compared among different levels of pollination (i.e., bagged, enhanced and open

pollination) using pairwise *t*-test with the FDR controlling procedures.

Flower visitors and their flower choice behavior

I performed three daily observations of flower visitors for each species during 2010 (*W.

coraeensis*: 25, 28 May and 2 June; *W. hortensis*: 14, 17, 21 May). In each observation, I

randomly picked a pair of focal plants and set up a HDD video camera (Everio GZ-MG575,

Victor Company of Japan, Ltd., Kanagawa, Japan) for each of them, by which all the flower

visitors entering a 30 x 30 cm frame were recorded during periods of peak forager activity

(0830-1130 and 1400-1700). Flower number per frame did not significantly vary between the

species (10-51 flowers per frame, $U = 10.0$, $P = 0.24$; Mann-Whitney U-test). I took care to

select each of the nine focal plants at least once for the observation. For all the flowers

monitored, I also recorded their ages and digital images for color evaluation. From the

videotaped images, I later collected data on visitor frequencies and taxa for individual flowers.

To address whether the flower visitor fauna differed between *W. coraeensis* and *W.

hortensis*, I compared the frequency of visits per flower by each visitor taxon between the two

species, by fitting a GLMM with a logarithmic link function and a Poisson error distribution. I

included the fixed and random factors in a model as described in *Nectar productivity*, and tested

the effect of plant species. I also examined whether the number of visits to individual flowers

was affected by their flower age, sugar production, and petal color. First, I compared the number

of visits between flower cohorts in each species by using pairwise *t*-tests with the FDR

controlling procedures. Prior to the analyses, the visit frequencies were log-transformed to

correct for skewness. Second, I calculated the mean visitation rate per flower and the mean daily

sugar production per flower for each age cohort in each species, and performed a randomization

test (10000 permutations) to test for the significance of Kendall's tau between these variables

(see *Daily changes in petal color* for more details on randomization procedures). Finally, I tested

for the correlation between the number of visits to individual *W. coraeensis* flowers and their

color distances from the zero-day-old flowers, by using the same randomization procedure for

Kendall's tau.

Daily changes in pollen receipt and removal

During 2009 and 2010, I haphazardly selected several inflorescences from each focal plant of *W.*

coraeensis and *W. hortensis*, and examined a total of 7-10 flowers per age cohort in each species

for pollen receipt and removal. I collected stigmas with forceps and mounted them on glass

slides with basic fuchsin gel (Kearns and Inouye 1993). Later, the number of pollen grains

deposited on the stigma of each flower was counted under an optical microscope at 40X. During

2009, I also collected five anthers from each flower and placed them in a 1.5-ml microcentrifuge

tube to examine the number of pollen grains left in flowers at each age. Back in the laboratory, I

added to each tube 1 ml of medium containing 50% (v/v) ethanol and 1.0% (v/v) tensioactive,

and shook it on a vortex mixer for 3 min (Dafni, Pacini, and Nepi 2005). I then sampled 10 μl of

the pollen suspension with a pipette and transferred it to a glass slide, and counted pollen grains

within arbitrarily selected 25 grids under an optical microscope. The average number of pollen

grains per grid was used to estimate total number of grains on a slide and, in turn, the original

amount left in the five anthers. During 2010, I attempted an improved approach, in which pollen grains in five anthers were suspended in 0.9% (w/v) saline water by immersing the tube in an ultrasonic bath for two hours. I then diluted the pollen suspension with 0.9% (w/v) saline water to 10 ml, and counted the number of grains in the sample with an automated particle counter (Z1 type Coulter counter, Beckman Coulter inc., Brea, USA). I then compared the number of pollen grains deposited on a stigma per ovule and left in anthers, between the two species or among flower cohorts for each species, using the same procedures described in *Flower visitors and their flower choice behavior*.

Seed production

To address whether seed production was limited by pollination in open-pollinated flowers, I compared the proportion of seed set between open-pollinated and hand-crossed flowers. During 2010, I arbitrarily selected three of the focal plants for *W. coraeensis* and *W. hortensis*, respectively, and bagged an inflorescence bearing 5-15 buds on each plant. I then performed hand crossing on a total of 27 newly opened flowers for each species, in the same manner as described above (see *Responses of floral traits to pollination level*). After the season, I collected the fruits produced by these flowers, and counted the number of mature seeds per fruit. For comparisons, I also collected a total of 27 fruits produced by open-pollinated flowers on the same focal plants, and counted the number of mature seeds. Results for both species were compared between open-pollinated and hand-crossed flowers by fitting GLMMs with a logarithmic link function and a Poisson error distribution. I considered treatment (hand-crossed or open-pollinated) as the fixed factors, individual plant as the random factors, and the number of ovules per flower as an offset variable. The significance of the effect of treatment was tested with the likelihood ratio chi-square statistic.

1.3 Results

Patterns in floral traits and their combinations

Flowers of *W. coraeensis* and *W. hortensis* did not significantly differ in the duration of retention under open-pollinated conditions both during 2009 (*W. c.*: n = 25, mean ± SE = 4.8 ± 0.22 days; *W. h.*: n = 34, mean ± SE = 5.1 ± 0.15 days; χ^2 = 1.3, P = 0.25; GLMM) and 2010 (*W. c*: n = 12, mean ± SE = 4.2 ± 0.47 days; *W. h.*: n = 14, mean ± SE = 4.3 ± 0.29 days; χ^2 = 0.068, P = 0.80; GLMM). Also, no significant difference in anthesis rate was detected between the two species during 2010 (*W. c.*: n = 31, mean ± SE = 1.3 ± 0.24 flowers/inflorescence/day; *W. h.*: n = 19, mean ± SE = 1.9 ± 0.34 flowers/inflorescence/day; χ^2 = 2.4, P = 0.12; GLMM).

Flowers of *W. coraeensis* and *W. hortensis* offered different levels of reward value in terms of nectar volume and its energy content. The volume of nectar produced per day was significantly greater in *W. coraeensis* than in *W. hortensis* flowers (2009: df = 1, 215, χ^2 = 4.0, P = 0.044; 2010: df = 1, 149, χ^2 = 5.5, P = 0.019; GLMM; see also Fig. 1.3a). On the other hand, nectar sugar concentration was slightly lower in *W. coraeensis* than in *W. hortensis* (df = 1, 125, χ^2 = 2.0, P = 0.16; GLMM; see also Fig. 1.3b). Taken together, daily sugar production per flower was greater in *W. coraeensis* than in *W. hortensis*, although the trend was not significant (Fig. 1.4; df = 1, 143, χ^2 = 3.5, P = 0.060; GLMM). In contrast to these differences in nectar productivity, daily changes in nectar (sugar) production rate in the two species were characterized by nearly identical ascent and descent—both peaked at one-day old and significantly declined thereafter (Fig. 1.4).

Daily change in petal color of *W. hortensis* was neither significant nor perceivable for insects in terms of relocations of color loci (Fig. 1.5). In contrast, daily change in petal color of *W. coraeensis* showed a corresponding pattern with that in nectar production. The average color distance from zero-day-old flowers reached 0.15 (in hexagon units) in three-day-old flowers (Fig. 1.5a), which would in most cases allow discrimination by bees (Dyer 2006). A similar

pattern held in the eyes of Diptera, as shown in the categorical color change (fly-yellow to fly-blue) at two-day-old flowers (Fig. 1.5b). These relocations of color loci were all statistically significant at 0.1% level (Wilk's MANOVA). Moreover, changes in petal color from zero-day-old flowers showed a significant negative correlation with daily sugar production in these flowers (n = 115, Kendall's tau = -0.36, $P < 0.001$; randomization test; see also Fig. 1.6a).

Responses of floral traits to pollination level

Whereas the durations of flower retention were nearly equal between *W. coraeensis* and *W. hortensis* under open-pollinated conditions (see the last section), these species responded differently in this respect when faced with altered pollination levels. In *W. coraeensis*, the enhanced pollination treatment, i.e., hand crossing combined with hand pollen removal, did not significantly affect the duration of flower retention (Fig. 1.7). In contrast, the bagging treatment increased flower retention for up to two days compared with the open pollination, although this trend was significant only during 2010 (Fig. 1.7). In *W. hortensis*, on the other hand, the enhanced pollination treatment significantly decreased flower retention by one to two days compared with the open pollination, whereas the bagging treatment did not significantly affect the flower retention (Fig. 1.7). Figure 1.7 also shows that flowers of *W. coraeensis* retained one to two days longer than those of *W. hortensis* both in terms of the maximum (under enhanced pollination treatment) and the minimum (under bagged treatment) longevities.

In contrast to the flexibility in flower retention, daily changes in neither nectar (volume) production rate of *W. coraeensis* and *W. hortensis* nor petal color of *W. coraeensis* significantly varied among different levels of pollination (data shown in Table 1.1).

Flower visitors and their flower choice behavior

All the visitors to *W. coraeensis* and *W. hortensis* flowers were insects, including bees (Hymenoptera), hoverflies (Diptera), beetles (Coleoptera), and butterflies (Lepidoptera) (Table

1.2). Bumble bees, honeybees, small bees, and butterflies mainly foraged for nectar, and only 3.9% of the observed bumble bees were accumulating corbicular pollen loads. I could not determine what beetles were feeding on, but they actively crawled back and forth in flowers and moved between plants. These visitors were thus considered as pollinators, i.e., their visits were generally legitimate. As for hoverflies, I often observed that they were licking pollen directly from the stamens, but I tentatively categorized them as pollinators because it seemed plausible that they occasionally touch stigmas and transfer pollen. In contrast, *Xylocopa* bees were excluded from pollinators, because they primarily robbed nectar from outside the tube by piercing it near the base.

Overall bee pollinators (i.e., all the bee visitors except *Xylocopa*) showed a strong preference for visiting *W. coraeensis* over *W. hortensis* flowers (Table 1.2; df = 1, 293, $\chi^2 = 4.2$, $P = 0.041$; GLMM). This trend was especially pronounced for the most frequent pollinators on both plant species, bumble bees (*Bombus ardens* and *B. diversus*) and honeybees (*Apis mellifera* and *A. cerana*). On the other hand, hoverflies showed a weak preference for *W. hortensis* flowers, although the trend was not significant (Table 1.2; df = 1, 293, $\chi^2 = 3.5$, $P = 0.061$; GLMM).

Because bee pollinators comprised 92% of total pollinators and mainly foraged for nectar, I performed my analyses of flower choice behavior using only data from these animals. On *W. coraeensis* plants, bees strongly preferred to visit young flowers with higher reward (Figs 1.4 and 1.8). This was more directly demonstrated by a positive correlation between the mean visitation rate per flower and the mean sugar production rate on *W. coraeensis* (n = 5, Kendall's tau = 0.80, $P = 0.039$; randomization test). On *W. hortensis* plants, in contrast, bees did not show such a strong preference for high-rewarding flowers—no significant correlation was detected between the mean visitation rate and the mean sugar production rate (n = 5, Kendall's tau = 0.20, $P = 0.41$; randomization test). Four-day-old flowers with the lowest nectar secretion were less favored by the bees (Figs 1.4 and 1.8), but this trend was not significant either.

The bees' preference for young flowers on *W. coraeensis* was based on the petal color, rather than the nectar productivity itself. The color distance from zero-day-old flowers was significantly correlated with both the nectar sugar production (see above) and the bee visitation rate per flower (n = 107, Kendall's tau = -0.51, $P < 0.001$; randomization test; see also Fig. 1.6c). However, if I use data only from two- or three-day-old (transitional) flowers whose petal color was not significantly correlated with their nectar productivity (two-day-old: n = 21, Kendall's tau = -0.11, $P = 0.25$; three-day-old: n = 20, Kendall's tau = 0.011, $P = 0.55$: randomization test), only the bee visitation rate significantly decreased with the petal color (two-day-old: n = 18, Kendall's tau = -0.55, $P < 0.001$; three-day-old: n = 20, Kendall's tau = -0.41, $P = 0.0084$; randomization test; see also Fig. 1.6b and d).

Pollination success

Under open-pollinated conditions during both seasons, the overall level of pollen deposition was significantly higher in *W. coraeensis* than in *W. hortensis* (Fig. 1.9a; 2009: df = 1, 98, $\chi^2 = 22$, $P < 0.001$; 2010 df = 1, 68, $\chi^2 = 6.1$, $P = 0.014$; GLMM). Furthermore, stigmas of *W. coraeensis* accumulated pollen far more quickly than *W. hortensis* during the initial three days (Fig. 1.9a). On the other hand, the overall level of pollen removal, i.e., the amount of pollen grains left in five anthers, did not significantly vary between the species (Fig. 1.9b; 2009: df = 1, 98, $\chi^2 = 0.45$, $P = 0.50$; 2010: df = 1, 118, $\chi^2 = 1.7$, $P = 0.19$; GLMM). The rate of pollen removal was slightly higher in *W. coraeensis* than in *W. hortensis* during the initial three days, but not significantly so (Fig. 1.9b).

The proportion of seed set per fruit in open-pollinated flowers of *W. coraeensis* reached almost 95%, and was not increased by hand crossing (open-pollinated: n = 27, mean ± SE = 95 ± 1.7%; hand-crossed: n = 27, mean ± SE = 93 ± 5.1%; $\chi^2 = 0.012$, $P = 0.91$; GLMM). In contrast, open-pollinated flowers of *W. hortensis* had a lower proportion of seed set than hand-crossed

flowers (open-pollinated: n = 27, mean ± SE = 27 ± 5.3%; hand-crossed: n = 27, mean ± SE = 46 ± 5.0%; χ^2 = 98, P < 0.001; GLMM).

1.4   Discussion

Differences in combinations of floral traits

My comparison has revealed that how the color-changing species, *W. coraeensis*, differs from the non-color-changing species, *W. hortensis*, in the combination of the flower retention with ontogenetic changes in petal color and nectar productivity. Flowers of *W. coraeensis* actively secreted nectar for initial 2-3 days and remained white regardless of pollination level, after which they persisted with red-purple petals little nectar production (Figs 1.4, 1.5 and 1.7). Considering that artificially enhanced pollination altered none of these trends (Fig. 1.7, see also Table 1.1), open-pollinated flowers of *W. coraeensis* would have received sufficient pollination service at my site. During pollinator scarcity, only the duration of red-purple flowers was extended for yet another day or two (Fig. 1.7). This implies that flower retention in *W. coraeensis* primarily functions to enlarge the overall display and attract passing pollinators at a distance, rather than to compensate insufficient pollen receipt or removal at an individual-flower level. Such rather inflexible trait combinations have also been reported in some color-changing flowers, although pollination accelerated discolouration in other species (Weiss and Lamont 1997; Willmer 2011).

On the other hand, flowers of *W. hortensis* actively secreted nectar for initial 2-3 days, after which they persisted for another day or two with the same color and significantly reduced nectar production (Figs 1.4, 1.5 and 1.7). When pollination was enhanced, no notable change was observed for the initial few days, and then flowers rapidly fell off before starting nectar reduction (Fig. 1.7, see also Table 1.1). In contrast, artificially reduced pollination did not extend the retention of rewardless flowers (Fig. 1.7, see also Table 1.1), suggesting that open-pollinated

flowers of *W. hortensis* were experiencing pollination difficulty at my site. Considering that the insects hardly discriminated against aged flowers (Fig. 1.8), the extended flower retention in *W. hortensis* would function both to compensate for insufficient pollination at an individual-flower level, and to increase pollinator attraction at the whole-display level.

It seems rather unexpected that *W. hortensis* flowers hardly produced nectar during the extended phase (Fig. 1.4). Previous studies on floral color change have often assumed, implicitly or explicitly, that rewardless flowers change their colors to repel pollinators, thereby minimizing visitation loss, body-surface pollen loss, or geitonogamous self-pollination for reproductive flowers (Oberrath and Böhning-Gaese 1999; Jones and Cruzan 1999). A naive expectation from this is that poorly pollinated flowers of *W. hortensis* will continue nectar production as long as they remain reproductive. Apparently, this expectation failed here. Retention of rewardless flowers without color change has little been described in literature, but plants may benefit by increasing the advertisement without excessive costs of nectar production (Pyke 1991). Frequent encounters with empty flowers may also encourage pollinators to leave plants and reduce geitonogamy (Harder and Barrett 1995; Biernaskie et al. 2002). And more importantly, the observed trait combination of *W. hortensis* provides a unique opportunity to evaluate the significance of color change in rewardless flowers of *W. coraeensis* in their interaction with pollinators (see below).

Differences in flower choice by pollinators

Bees harvesting nectar from *W. coraeensis* preferred to visit young, rewarding flowers (Fig. 1.8). This preference appears to have based on the difference in petal color, rather than the nectar productivity itself (see **Results**). Whereas the overall correlations of the petal color with the visitation rate and the nectar productivity were both highly significant, the correlation between the petal color and the nectar productivity was not significant in two- or three-day-old (i.e., transition phase) flowers. Bees frequented white flowers in such situations, suggesting that they

learned to use the color as a most reliable, if not perfect, cue for finding the reward. Because white flowers have a stronger color purity or contrast against the background than red-purple ones (Fig. 1.5), the observed preference may also reflect the insects' innate preference for color purity (Lunau 1996). Considering that bees usually learn color-reward associations after a few visits (Menzel and Erber 1978), however, it is unlikely that such innate preference had a greater impact on flower choice than learned preference. Moreover, *W. hortensis* with lower color purity received more frequent visits by flies than *W. coraeensis* (Table 1.2, Fig. 1.5), suggesting that the observed flower choice of pollinators cannot be explained solely by innate color preference.

In contrast, bees showed only a weak, mostly nonsignificant flower selectivity on *W. hortensis* (Fig. 1.8). This suggests that bees failed to find a reliable cue for the detection of reward on non-color changing *W. hortensis* (Figs 1.4 and 1.5). The slight decrease in visits to three- and four-day-old flowers (Fig. 1.8; see also Fig. 1.10) may indicate that pollinators used less distinctive changes in floral scent (Schiestl et al. 1997; Morinaga et al. 2009) or anther appearance in the absence of color cues.

Effects of different trait combinations on pollination
W. coraeensis yielded a greater proportion of seed set than *W. hortensis* (see **Results**). This difference can largely be attributed to the contrast between the two species in pollinator attraction. Flowers of *W. coraeensis* received more pollinator visits than those of *W. hortensis* (Table 1.2). Consequently, stigmas of *W. coraeensis* rapidly accumulated pollen and reached the maximum within the initial three days, while those of *W. hortensis* slowly accumulated pollen and reached only a half of *W. coraeensis* at most (Fig. 1.9a). Similar trends were observed for pollen removal, but not significantly so (Fig. 1.9b).

These results are consistent with the trend that nectar-collecting bees—the primary pollinators at my site (Table 1.2)—strongly preferred flowers of *W. coraeensis* (Fig. 1.8). The difference in bee visits cannot be attributed to interspecific variation in display size. The focal

plants I chose for each species bore approximately equal numbers of inflorescences (M.F. Suzuki, personal observation). Moreover, the average floral longevity and the anthesis rate did not significantly vary between the species (see **Results** and Fig. 1.7). Even if the differences were meaningful, the estimated inflorescence size was larger in *W. hortensis* (8.2 = 1.9 flowers/day x 4.3 days; Harder and Johnson 2005) than in *W. coraeensis* (5.5 = 1.3 x 4.2). In other words, *W. hortensis* attracted fewer bees than *W. coraeensis* despite its barely larger inflorescences.

This unexplained gap in bee visitation cannot be filled solely by considering the difference in mean nectar productivity (Fig. 1.4). The proportion of each flower cohort within an inflorescence can be estimated from the same equation used above (e.g., number of age-zero flowers of *W. coraeensis* = 1.3 flowers/day x 1 day = 1.3). Assuming that floral display size was invariable and each flower in one cohort produces the average amount of nectar shown in Fig. 1.4, therefore, I can estimate the ratio of the whole-plant nectar production rate (= $\sum_i$ [(display size) x (prop. of the i-th cohort) x (average nectar productivity of the i-th cohort)]) as *W. c./W. h.* = 1.85. If pollinators distributed themselves on plants according to the ideal free distribution (Fretwell and Lucas 1970), therefore, bees should visit *W. coraeensis* 1.85 times as frequently as *W. hortensis* to gain equal reward from flowers on both species (e.g., Dreisig 1995). This expectation deviates from my finding, however, that bees visited young flowers of *W. coraeensis* 2.17 times as frequently as those of *W. hortensis*, as was calculated from the observed bee visits (Fig. 1.8) and the estimated proportions of flower cohorts.

The complementary explanation for the bees' preference for *W. coraeensis*, therefore, would have to be looked for in the reward profitability at the whole-plant level. Bees foraging on *W. coraeensis* could have been benefited by the color-based flower choice in at least two ways. First, they could increase the average nectar harvest rate by effectively avoiding rewardless flowers. More precisely, I could calculate the ratio of the whole-plant nectar productivity

experienced by the selective bees ($= \sum_i$ [(display size) x (prop. of the *i*-th cohort weighted by the observed visitation rate) x (average nectar productivity of the *i*-th cohort)]) as *W. c./W. h.* = 1.96. Second, the selective visits on *W. coraeensis* plants should have greatly reduced the variation in nectar crop among flowers, in addition to the inherent effect of the increased nectar level on interflower variability (i.e., CV = SD/mean). Considering that many nectarivores prefer less variable food sources (Harder and Real 1987; Perez and Waddington 1996), this would have further enhanced the attractiveness of *W. coraeensis*. *Weigela coraeensis* thus offered bees with a visual cue to improve both long- and short-term rates of energy intake. Bees often start their foraging career by visiting more conspicuous displays, but develop a preference for more profitable ones as they gain experience (Makino and Sakai 2007). I therefore suggest that *W. coraeensis* were successful at my site, because their large and easily exploitable displays attracted both inexperienced and experienced bee foragers, while *W. hortensis* mostly attracted inexperienced ones that served only temporarily.

Because my species bloomed in the same location, one might suspect an artificial interaction between them—once bees learned to avoid reddish flowers on *W. coraeensis*, they may have also avoided similarly coloured flowers of *W. hortensis* (Fig. 1.5) without inspection. This is not the case here, however, because *W. hortensis* started blooming several days earlier than those of *W. coraeensis*, and bees apparently had more chances to associate reddish color with reward on *W. hortensis* prior to the blooming of *W. coraeensis*.

Implications for the evolution of floral color change

Although I compared the two species in the same environment, their natural habitats usually differ from one another: *W. coraeensis* commonly occurs in temperate regions below an altitude of 700 m, whereas *W. hortensis* grows on snowy hills and mountains up to an altitude of 1800 m (Hara 1983). Results obtained here, together with this elevation difference, provide an

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opportunity to address the potential strength of non-color-changing *W. hortensis*, thereby leading us to a deeper understanding of the evolution of floral color change.

The plastic longevity of *W. hortensis* flowers should allow plants to adjust their display size to pollination environment, by enhancing the conspicuousness when pollinators are rare, while limiting self-pollination when pollinators are common (Harder and Johnson 2005). Furthermore, the retention of rewardless flowers with invariable color may help *W. hortensis* to save metabolic and ecological costs. The metabolic cost includes the resource allocation to nectar production (Pyke 1991). Also, I found that old flowers of *W. hortensis* accumulate less anthocyanins than those of *W. coraeensis* (S. Shimokawa et al., unpublished data), suggesting that the cost of anthocyanin production—a considerable investment for plant cells (Gould 2004)—is alleviated in *W. hortensis*. The ecological cost refers to an increased geitonogamy caused by extended stays on larger displays (Harder and Barrett 1995). Considering that an increased variability of nectar per flower encourages pollinators to leave plants (Biernaskie et al. 2002), *W. hortensis* may benefit from reduced geitonogamy by presenting variably rewarding flowers in the same color. When conditions severely limit photosynthesis or pollinators are risk averse, therefore, *W. hortensis* may become more adaptive.

The advantages of cost saving should be particularly important if floral color change does not greatly increase return visits by experienced pollinators. This would occur, for example, when pollinators have poor cognitive abilities or lack fidelity to learned plants. Alternatively, pollinators may exhibit little selectivity if few other plant species bloom in the vicinity. In such conditions, *W. hortensis* may surpass *W. coraeensis* in exploiting pollinators at lower costs.

These considerations suggest that necessary conditions for the evolution of floral color change would be 1) favourable environment for photosynthesis, 2) intense competition for pollinators among neighbouring plants, and 3) strong dependency on visits by both experienced and inexperienced foragers. The elevation difference between the natural habitats of my species appear to meet these conditions—*W. coraeensis* grows in more favourable environments for

photosynthesis, competes more intensely for pollinators, and depends on more cognitively sophisticated pollinators such as bees than *W. hortensis*. In future studies, I plan to examine how these conditions vary among natural habitats of *W. coraeensis*, *W. hortensis*, and the other color-changing and non-color-changing species.

Conclusions

This work provides a novel, comprehensive hypothesis to explain why species with color-changing and non-color-changing flowers are both prevailing: color changers will be successful only when the benefits of enhancing both conspicuousness and exploitability by pollinators outweigh the costs of doing so. When plants under environmental stress can increase pollinator visits solely by enhancing conspicuousness, in contrast, my hypothesis predicts that cost saving can become advantageous—as in the non-color changer, *W. hortensis*. These results strongly support my idea that I need to compare trait combinations and their cooperative functions between closely related species to understand the evolution of floral color change.

Chapter 2

Keep regulars, attract vagabonds – floral color change by plants for a full exploitation of behaviorally diverse pollinators

2.1   Note

Because this chapter is in preparation for the submission to a scientific journal, I show the summary in page 2, instead of the full manuscript.

General Discussion

In this section, based on the results presented in Chapters 1 and 2, I discuss why color changers and non-color changers are both prevailing. In Chapter 1, I clarified the differences in combinations of floral traits between a color-changing species of *Weigela* and its non-color-changing congener, as well as effects of the trait combinations on the behavioral responses of pollinators and pollination success. In Chapter 2, I revealed that the two plant species are both successful in pollination in their natural habitats, by utilizing taxonomically similar but behaviorally diverse pollinators. Here, I highlight different aspects of pollination via flower-visiting animals between the two plant species, after which I discuss why color changers and non-color changers are both successful in pollination in their own habitats.

In Chapter 1 (see also Suzuki and Ohashi 2014), I showed that the color changers retained old and rewardless flowers in an altered color, while the non-color changers did so without changing the petal color. In other words, the color changer provided visitors with a visual cue for choosing high-reward flowers on plants, whereas the non-color changer offered no such cue. As a consequence, the color changers received more frequent visits by bees than the non-color changers. Based on these results, I hypothesized that color-changing species have adapted toward bee-like pollinators that preferentially revisit more easily exploitable plants as they gain experience, while non-color-changing species focus on attracting less efficiency-oriented insects such as flies.

The results presented in Chapter 2, however, show that the two *Weigela* species in their natural habitats had similar visitor faunas in that both were dominated by bees and flies: more precisely, large eusocial bees, small/non-eusocial bees, Acroceridae flies, and Syrphidae flies. As a result of interactions with these visitors, flowers of both species produced sufficient seeds. This similarity in visitor fauna suggests difficulty in explaining the floral divergence in *Weigela* solely by obtaining lists of taxonomic groups of pollinators. To my knowledge, no study

has reported distinctive differences in taxonomic groups of flower-visiting animals between color-changing and non-color-changing species in other genera. A meta-analysis including various lineages suggests that faunal composition of flower visitors often overlaps between color-changing and non-color-changing species within the same family or genus, although color changers tend to bloom during seasons when bees are more abundant than non-color changers (Ohashi et al. 2015).

Taken together with the behavioral diversity of flower visitors, however, the two *Weigela* species are exposed to different flower-visiting insects in their own habitats. Large eusocial bees (bumble bees and honeybees) on color changers often exhibited repetitive use of the same plants, while small/non-eusocial bees and flies rarely revisited the same plants. Moreover, large eusocial bees tended to restrict their foraging to small areas of a plant population only when flowers exhibited high levels of nectar productivity and the plant population size was small. On the other hand, both types of bees and flies on non-color changers seemed to visit wider areas in a non-repeating fashion. The flexible behavior of large eusocial bees suggests that flower visitors within the same order or even the same species exhibit greatly varied responses to reward levels and spatial use of plant resources in different foraging contexts.

By considering such variations in the foraging behaviors of flower visitors and their contribution to pollination, I will discuss why color-changing and non-color-changing plants are both successful in their own habitats. In natural habitats, the non-color-changing species succeeded in achieving outcross pollination, although visits by bees and flies to these plants were largely occasional. This is likely because the non-color changers grow in large or dense stands with few neighboring species in bloom, where interspecific competition for pollinators is weak. In such large or dense plant populations, animals will inevitably move between conspecific flowers irrespective of their foraging tactics, so that even opportunistic visitors could be effective pollinators. Consequently, the absence of intense competition with other flowering species will

make it easier for these plants to attract pollinators without providing much reward or visual guidance to more rewarding flowers. Under such conditions, plants only need to have as many open flowers as possible for the strategy of non-color changers to be favored. On the other hand, the high seed set of color-changing flowers in the botanical garden (Chapter 1) and in their natural habitat in Tsukuba (Chapter 2)—where the plants exhibited high nectar productivity and grew in small or sparse stands with many other plant species blooming in the surrounding area—suggests that these conditions would have put color changers at a great advantage. Under such conditions, large eusocial bees would have sufficient opportunities to become familiar with individual plants of one plant species that provides adequate rewards, while other opportunistic foragers such as small/non-eusocial bees and flies would make frequent heterospecific moves because they probably pay less attention to differences in reward levels. Therefore, when large eusocial bees restrict their foraging to limited areas with a high rate of repeat visitation, the strategy of color changers will be particularly favored because they can retain bees as regular visitors by providing visual guides to high-reward flowers, as well as attracting passing foragers by retaining old flowers. In other words, although I proposed that floral color change yields the benefit of maintaining regular visitation by bees in Chapter 1, this benefit would be realized only when necessary conditions are met: (i) favorable environment to produce sufficient rewards, (ii) small plant population, and (iii) intense inter-specific competition for pollinators among neighboring plants.

These results strongly suggest that clarification of the behavioral diversity of animals would provide insight into how flowers visited by similar pollinator groups could evolve different sets of traits. Flower visitors greatly varied in their behavior, depending on intrinsic factors such as the difference in energy demand, as well as on extrinsic factors such as plant population size and density, even if they belong to the same taxonomic groups. That is, flower visitors within the same orders or even the same species in different foraging contexts greatly varied in responses to reward levels and spatial use of plant resources. Such taxonomically

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similar but behaviorally diverse animals should be regarded as different pollinators, because they would exert different selection pressure on floral traits through their foraging behavior. Therefore, in order to understand the reproductive strategies of flowering plants, it is important to investigate the foraging behavior of visitors to each plant species in each community, as well as to examine how the plant maximizes its gain from pollination by adapting to flower-visiting animals.

The behavioral diversity of flower-visiting animals also helps explain diversity in floral traits of plants that receive visitations by various groups of pollinators. Besides the case of floral color change, closely related plant species with different floral traits often receive visitations by similar pollinators comprising several taxonomic groups (Ollerton 1996, Waser et al. 1996). The association of particular sets of floral traits with specific pollinators has been questioned given the apparent widespread generalization in pollination systems found in various studies (Waser et al. 1996, Petanidou et al. 2008). Moreover, evaluations of pollination syndromes, using several communities around the world, found support for pollination syndromes for only about 30% of the species in the communities (Ollerton et al. 2009). My study provides insight that will help resolve this problem: flower diversity not included in the concept of pollination syndromes may be explained by considering the effects of behavioral variations of flower-visiting animals, and not solely by obtaining lists of taxonomic groups. Comparisons of pollinator behaviors towards various plant species in natural foraging contexts will lead to breakthroughs in understanding the evolution of flowers in diffuse plant-pollinator relationships.

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Table 1.1 Daily changes in (a) 24-h nectar production (µl/flower) and (b) floral color in reduced (by bagging) pollination compared with those in enhanced (by hand crossing with hand pollen removal from anthers) and open pollination. Means and SEs are shown with sample sizes in parentheses, calculated for (a) *W. coraeensis* and *W. hortensis* and (b) *W. coraeensis*. (b) Color distance in the bee and fly vision was evaluated as the difference between the average color loci of zero-day-old flowers and that of each cohort. Significance of the calculated differences between pollination treatments in each cohort was tested with standard *t*-tests with the FDR controlling procedures.

a) Daily nectar production (*µ*l/flower)

Flower age	W. coraeensis			W. hortensis		
	Bagged	Enhanced	P	Bagged	Enhanced	P
0	1.50 ± 0.18 (23)	1.94 ± 0.31 (26)	0.84	0.73 ± 0.12 (13)	0.91 ± 0.10 (24)	0.59
1	2.97 ± 0.42 (23)	3.30 ± 0.32 (26)	0.84	1.38 ± 0.40 (7)	1.27 ± 0.22 (20)	0.89
2	2.75 ± 0.38 (22)	2.97 ± 0.44 (24)	0.84	1.39 ± 0.42 (11)	1.40 ± 0.34 (10)	0.89
3	2.00 ± 0.94 (21)	1.55 ± 0.60 (13)	0.84	0.34 ± 0.19 (10)	1.13 ± 0.47 (4)	0.45
4	0.80 ± 0.25 (16)	0.63 ± 0.14 (8)	0.84	0.27 ± 0.17 (5)	0 ± NA (1)	0.87

b) Colour distance from the average colour loci of zero-day-old flowers on *W. coraeensis*

Flower age	Bee colour distance (in hexagon units)			Fly colour distance (in units of colour space defined by x = R7p - R8p and y = R7y - R8y)		
	Bagged	Open	P	Bagged	Open	P
1	0.04 ± 0.00 (27)	0.05 ± 0.00 (33)	0.18	0.05 ± 0.01 (27)	0.07 ± 0.01 (33)	0.12
2	0.09 ± 0.01 (29)	0.08 ± 0.01 (40)	0.93	0.11 ± 0.01 (29)	0.11 ± 0.01 (40)	0.81
3	0.16 ± 0.01 (29)	0.16 ± 0.01 (30)	0.93	0.21 ± 0.01 (29)	0.24 ± 0.01 (30)	0.10
4	0.19 ± 0.00 (25)	0.21 ± 0.01 (29)	0.18	0.24 ± 0.01 (25)	0.27 ± 0.01 (29)	0.12

Table 1.2   Visitation rate per flower per five-hour-block for each taxonomic group of insects.

Means ± SEs for *W. coraeensis* flowers (n = 116) and *W. hortensis* flowers (n = 179) are shown.

Significance of differences was tested with GLMM analysis.

Visitor	Visitation rate per flower		Significance	
	W. coraeensis	*W. hortensis*	χ^2	*P*
Hymenoptera	2.58 ± 0.25	1.11 ± 0.11	10.42	0.001
Apidae	2.47 ± 0.24	1.07 ± 0.11	6.57	0.010
Bombus	1.62 ± 0.21	1.03 ± 0.11	3.15	0.076
Apis	0.65 ± 0.09	0.03 ± 0.01	17.98	< 0.001
Xylocopa	0.21 ± 0.05	0.01 ± 0.01	0.15	0.701
Small bees*	0.10 ± 0.03	0.03 ± 0.01	8.44	0.004
Subtotal (-*Xylocopa*)	2.37 ± 0.26	1.09 ± 0.11	4.17	0.041
Diptera	0.05 ± 0.02	0.23 ± 0.05	3.51	0.061
Coleoptera	0.01 ± 0.01	0.01 ± 0.01	1.45	0.229
Lepidoptera	0	0.01 ± 0.01	0	1.000
Total (-*Xylocopa*)	2.43 ± 0.26	1.34 ± 0.13	0.33	0.565

*Small bees possibly included Andrenidae, Colletidae, Halictidae, or Megachilidae, although they were merely identifiable on video recorded images.

Figure 1.1 Flowers of *Weigela coraeensis* (left) and *W. hortensis* (right). Photographs: Miki Suzuki.
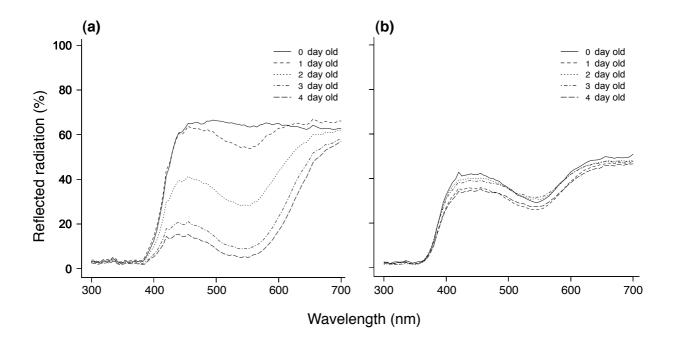
Figure 1.2 Spectral reflectance functions of (a) *W. coraeensis* and (b) *W. hortensis* flowers. Each line represents the mean for 29-40 *W. coraeensis* flowers per age cohort and for 22-27 *W. hortensis* flowers per age cohort.
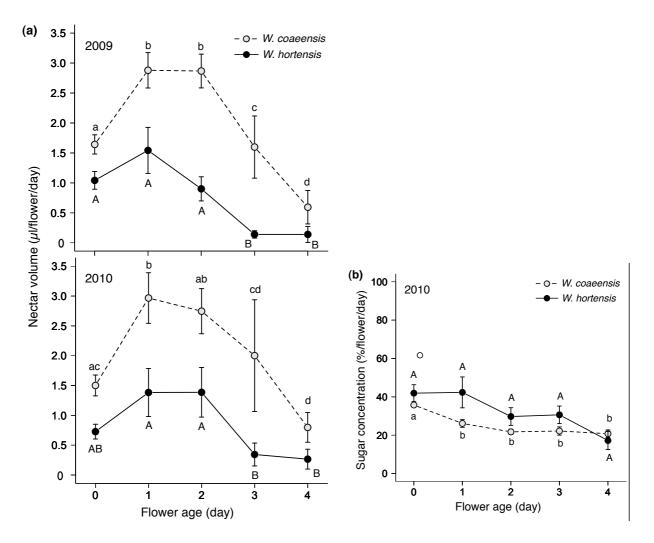
Figure 1.3 Daily changes in 24-h nectar productivity. (a) Nectar volume (μl) produced per day per flower. Means and SEs were calculated for 18-27 and 16-23 flowers during 2009 and 2010, respectively (gray circles, *W. coraeensis*), and for 14-27 and 5-13 flowers during 2009 and 2010, respectively (black circles, *W. hortensis*). (b) Sugar concentration (%, v/v) produced per day per flower during 2010. Means and SEs were calculated for 14-23 flowers (gray circles, *W. coraeensis*), and also for 4-13 flowers (black circles, *W. hortensis*). Different letters indicate significant differences at the 0.05 level (lower case for *W. coraeensis* flowers, and upper case for *W. hortensis* flowers), detected by pairwise *t*-tests with FDR adjustments.

Figure 1.4 Daily changes in 24-h nectar productivity. Sugar mass (mg) produced per flower per day during 2010. Means and SEs were calculated for 14-23 flowers (gray circles, *W. coraeensis*) or for 4-13 flowers (black circles, *W. hortensis*). Different letters indicate significant differences at the 0.05 level (lower case for *W. coraeensis* flowers, and upper case for *W. hortensis* flowers) in pairwise *t*-tests with the FDR controlling procedures.
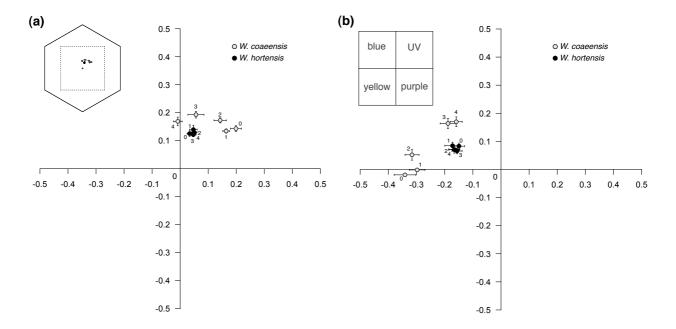
Figure 1.5 Daily color changes of *Weigela* flowers in the eyes of (a) bees and (b) flies, evaluated with the color vision model proposed for each insect type. (a) The x and y coordinates were calculated according to the relative stimulation of the UV, blue and green receptors elicited by the petals (Chittka 1992). The inset shows the overall view of the hexagon with the magnified area indicated by the dashed square. (b) The x and y coordinates were calculated as the differences in relative excitations between p-type receptors (R7p - R8p) and y-receptors (R7y - R8y). Colors in the same quadrant of the space (see the inset) are not discriminated by flies, meaning that all petals appear (clockwise, from top-left) fly-blue, fly-UV, fly-purple or fly-yellow (Troje 1993; Arnold, Savolainen and Chittka 2009). Means ± 2SEs of x and y coordinates were calculated for 29-40 flowers (gray circles, *W. coraeensis*) or for 22-27 flowers (black circles, *W. hortensis*). The numbers beside the symbols indicate flower age.
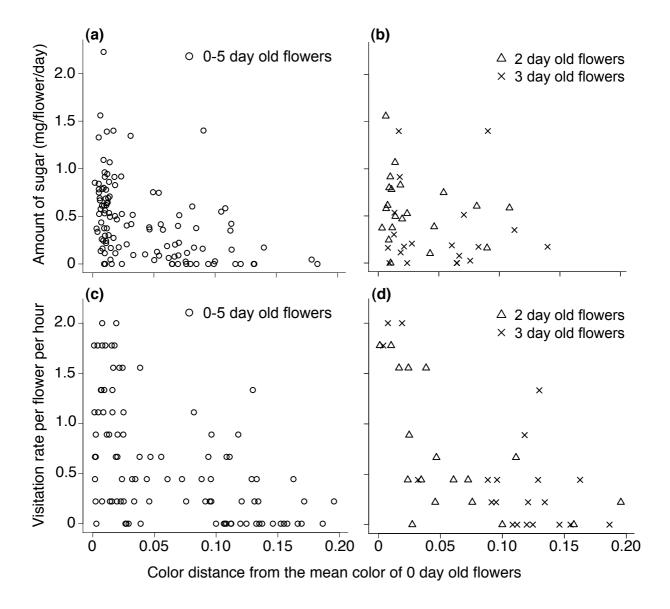
Figure 1.6 (a, b) Relationship between the nectar sugar production (mg/flower/day) and color distance from the average color of zero-day-old flowers in *W. coraeensis*. (a) Data of 0-5 day old flowers and (b) data of two- or three-day-old (transitional) flowers. (c, d) Relationships between hourly visitation rate by bees and floral color observed for individual flowers of *W. coraeensis*. (c) Data of 0-5 day old flowers and (d) data of transitional flowers.
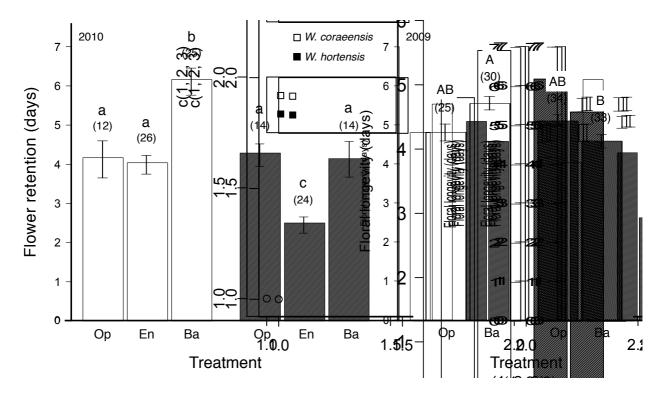
Figure 1.7 Changes in floral longevity in response to enhanced pollination (i.e., hand crossing combined with hand pollen removal) (En) and bagging (Ba) treatments, compared with those in open-pollinated (Op) condition. Enhanced pollination treatments were conducted only during 2010. White and black columns with error bars represent means and SEs for *W. coraeensis* and *W. hortensis*, respectively. Sample sizes are shown in parentheses. Different letters indicate significant differences at the 0.05 level (lower case for 2010, and upper case for 2009) in pairwise *t*-tests with the FDR controlling procedures.
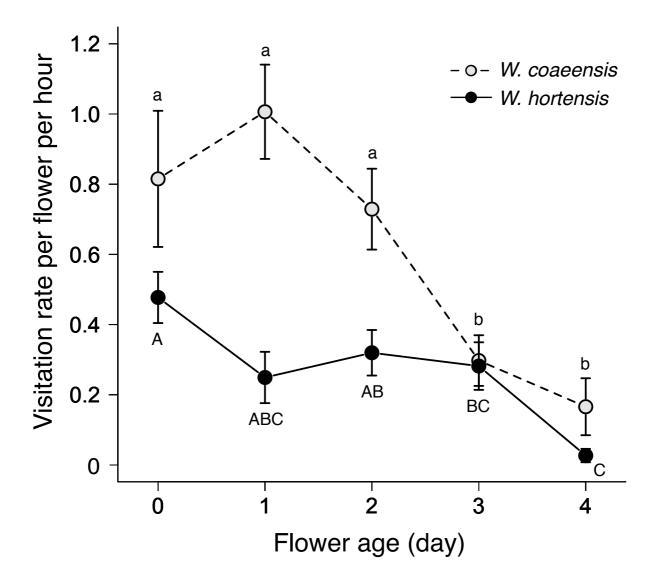
Figure 1.8 Relationships between hourly visitation rates by bee pollinators and flower age. Means and SEs were calculated for 10-31 flowers (gray circles, *W. coraeensis*) or for 14-51 flowers (black circles, *W. hortensis*). Different letters indicate significant differences at the 0.05 level (lower case for *W. coraeensis* flowers and upper case for *W. hortensis* flowers) in pairwise *t*-tests with the FDR controlling procedures.
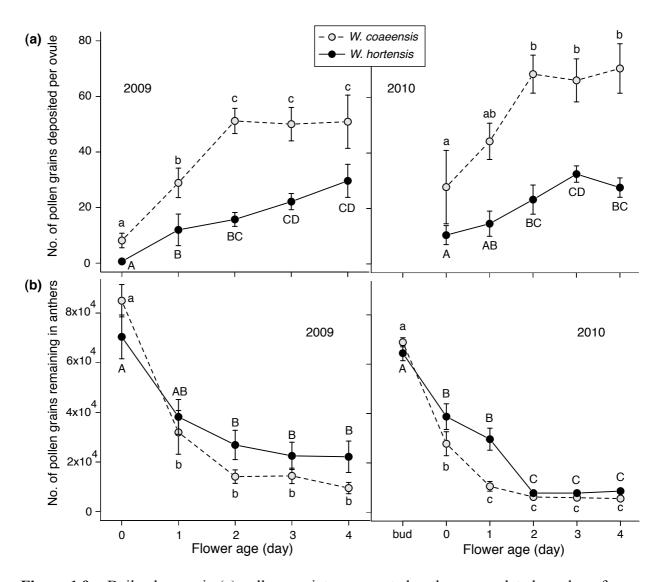
Figure 1.9 Daily changes in (a) pollen receipt, represented as the accumulated number of pollen grains deposited on stigmas divided by mean ovule number (2009: n = 10 flowers ; 2010: n = 7 flowers), and (b) pollen removal, represented as the number of pollen grains left on anthers (2009: n = 10 flowers; 2010: n = 7-10 flowers). Gray and black circles with error bars represent means and SEs for *W. coraeensis* and *W. hortensis*, respectively. Different letters indicate significant differences at the 0.05 level (lower case for *W. coraeensis*, and upper case for *W. hortensis*) in pairwise *t*-tests with the FDR controlling procedures.
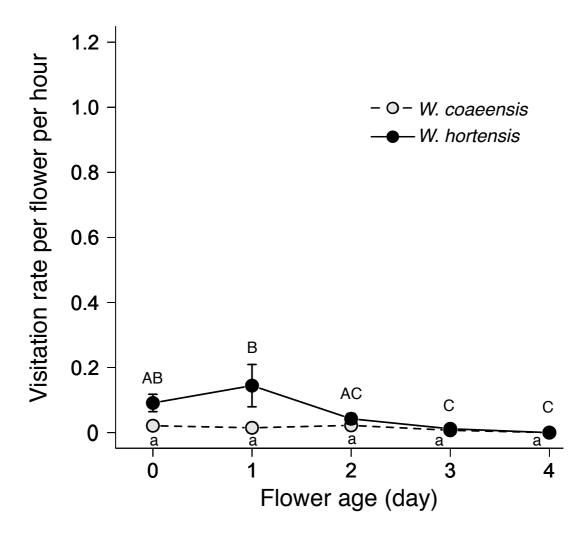
Fig. 1.10 Relationships between hourly visitation rates by fly pollinators and flower age. Means and SEs were calculated for 10-31 flowers (gray circles, *W. coraeensis*) or for 14-51 flowers (black circles, *W. hortensis*). Different letters indicate significant differences at the 0.05 level (lower case for *W. coraeensis* flowers and upper case for *W. hortensis* flowers) in pairwise *t*-tests with the FDR controlling procedures.