

Pollination Behavior of Bombus diversus in Impatiens textori : Acceptance and Rejection of Flowers

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Pollination Behavior of *Bombus diversus* in *Impatiens textori*: Acceptance and Rejection of Flowers

(ツリフネソウにおけるトラマルハナバチの送粉行動: 花の受け入れと拒否)

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The United Graduate School of Agricultural Science,

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Abstract of the dissertation

Foraging behavior of bumblebee has been paid attention as a key in pollination biology and is one of the most important subjects to understand the evolution of various flowers. However, flower-visiting behaviors of bumblebee have not been investigated enough yet. Although behaviors of pollinators must have various effects on a flower during the entire anthesis, the time from flower opening to falling, the previous studies have been done only by the simple and discrete observations after the bee landings on a flower. Therefore, the sequential events of pollinators on a flower throughout anthesis have not been revealed so far. To reveal the flower-visiting behaviors of pollinator including approach flights to a flower during entire anthesis, I made observations of bumblebee behaviors before their landing on flowers as well as after the landings based on long-term video recording.

On the long-term video data, I observed 13 *Impatiens textori* flowers during their entire antheses, from opening to falling, in total and detected 1527 flower visits of *Bombus diversus* during 370 hours. I made timelines of their sequential behaviors on each flower and examined their all behaviors around a flower including their approach flights. I classified *B. diversus* visits on *Impatiens* flowers as four-types of behaviors: hovering, touching, landing and probing.

The most frequent behavior was probing, which was the perfect flower acceptance behavior. In the probing, the bumblebees showed a set of sequential behaviors; they flew close to a flower, landed on the petals, walked into the corolla tube and probed for the nectar inside a spur by their mouthparts. Beside the probing behavior, *B. diversus* showed three-types of the flower rejection behaviors: hovering, touching and landing behaviors. The hovering was the most apparent flower rejection behavior; bumblebees flew close to a flower but changed their direction just in front of the flower. The touching was another apparent flower rejection behavior; bumblebees set their forelegs upon the petal of a flower but did not weight it; their behavior was like the "touch and go" of an aircraft. The landing was a flower rejection behavior that might have been misread as a probing by naked eyes; bumblebees landed and weighted the petals with their all legs completely, but left from the flower quickly without probing. In addition, these three behaviors were not available for the pollination of *Impatiens* plants.

The flower rejection behavior of bumblebees has been recognized by many previous workers as an insect behavior to avoid the empty flowers (no-nectar flower). Moreover, as the cues for their rejection behaviors, several recent studies suggested the scent mark of the previous flower visitors and also noticed the scent of the nectar itself. However, the effect of the scent as the cue for the rejection has never been examined in detail.

To check the function of nectar scent in *Impatiens textori* flowers, I examined the behavior of *Bombus diversus* on nectar-less flowers in which the spurs had been artificially removed. Bumblebee visits to both natural flowers and spur-cut flowers were also captured using a long-term video recording system and analyzed in detail. Visiting behavior and frequency were compared between the controls and treatments.

As the results, many bumblebees visited both types of flower, and their visit frequencies were not significantly different between the natural flowers and spur-cut flowers. However, the bumblebees stayed shorter on the spur-cut flowers than on the natural flowers significantly. The results suggest that bumblebees cannot detect the absence of nectar in *I. textori* flowers before probing them. Therefore, the nectar scent of *I. textori* itself does not attract bumblebees although the presence of nectar detains bumblebees on flowers for longer periods. In addition, the difference in the length of stay may show that the probing behaviors by bumblebees occur as the connected two steps: searching and drinking nectar.

To examine the scent mark left by previous flower visitor as a cue for bumblebees' flower rejection behaviors, I analyzed the chemical traits of the scent and then clarified the behavioral effect of the scent on bumblebees' flower visits in the field. For the chemical analysis, I used Solid-phase micro extraction (SPME) fiber to extract the scent from the flowers before and after bumblebee probing. After the extraction in the field, SPME fibers had been transferred to a Gas Chromatography-Mass Spectrometry and GC-MS machine for analysis. Tetradecanoic acid and n-hexadecanoic acid were detected as the presumed main components of the scent mark as chemical cues. Since these compounds are known to be a repellant matter for bumblebees, the scent of the previous visitor may act as a cue to reject the recently visited flowers.

The behavioral effect of the previous visitor's scent in bumblebees' flower visits was examined in detail based on the timelines of their sequential behaviors on each flower. The occurrence of acceptance and rejection behaviors in each timeline was paid attention. I checked the time interval between the initial probing and the next visits (either acceptance or rejection). As the results, bumblebees rejected almost all of the flowers just after a probing but accepted almost all of the flowers at about 15 minutes after the initial probing. During the 15 minutes, the frequency of the acceptance increased gradually and that of rejection decreased. Therefore, these 15minutes may be the duration of scent mark effect by the previous flower visitor in this case. The flowers may have no nectar just after the probing and certain nectar can be refilled during the 15 minutes.

Finally, I attempted to examine the relationship between the rejection behaviors and floral nectar refill. However, it is not easy to clarify the temporal changes in nectar volume especially in *Impatiens* flowers without any floral damages, because *Impatiens* flowers secrete and preserve nectar in their long, curved thin spur. For such the nondestructive and continuous observation of the nectar volume, I modified and used an interval-shooting camera with a special flash system. I attached a polymer optical fiber to the built-in flash of a compact digital camera and bent the fiber towards the camera's lens to provide backlighting. To record the temporal changes in nectar volume during the entire process of anthesis, I took interval images of the nectar silhouettes created using the backlight and estimated the nectar volume from the size of the nectar silhouette in the spur. By using of this method for the estimation of the temporal changes in nectar volume, I believe that I can clarify the ecological relationship between the rejection behaviors of bumblebees and floral nectar secretion of *Impatiens* plants in the near future.

My studies clarified some new aspects of flower visit behaviors of *B. diversus* on *I. textori* plants, but the future analyses based on the sequential observation of pollinators and changes of nectar on a flower throughout anthesis are needed to reveal the real situation of acceptance and rejection behaviors of bumblebees.

Chapter 1

General Introduction

General Introduction

The flowering plants comprise about one-sixth of all described species and the insects almost two-thirds (Wilson, 1992). The interactions between these large species groups are essential functions in terrestrial ecosystems. The most prominent interaction is the plant-pollinator partnership (pollination syndrome): the flowering plants attract insects for pollination and the insects visit flowers for rewards. Insects, who transfer pollen from one flower to another and contribute in pollination, an important ecosystem "service" are pollinators (Costanza et al. 1997). Pollinators are mostly from the insect orders Hymenoptera (bumblebees and honey bees), Diptera (some flies), Lepidoptera (butterflies and moths), and Coleoptera (beetles), and some are from the vertebrates, in particular some birds and bats (Proctor *et al* 1996). The economic value of pollinators is high as they are responsible for pollinating plants that gives us maximum of the food. In North America, 30% of food for human consumption originates from bee pollinated plants (Heinrich, 1979).

Among the pollinators, bumblebees are the most familiar with their large size and aesthetic pleasing colorful furry coat and widely recognized as being valuable through their role as a pollinator. They are the main pollinator for several agricultural crop plants like apple, almond, tomato, canola, red clover and blueberry (McGregor 1976; Parker *et al.* 1987; Thomson 1993). They are used in glasshouse cultivation like cabbage pollination in Holland (Free & Butler, 1959), kiwi fruits and tomatoes ever more. They are good pollinators of many crops as they have the ability to fly at low temperatures, long tongues and dense hairs with branch that is perfect for picking up and transferring pollen. The

ecological importance of bumblebees are considered greater than the economic importance as they pollinate about 30% of flowering wild plants, including many endangered species. Many wild flowers in the temperate, arctic, and alpine zones of the northern hemisphere are pollinated mainly or entirely by bumblebees, and sometimes by particular species of bumblebee. For example, high-altitude populations of *Polemonium viscosum* possess a suit of adaptive features that have coevolved with their bumblebee pollinators (Galen 1989). The population of *Impatiens textori* also shows has also co-evolutionary relation *with Bombus diversus* bumblebees (Kato 1998). Therefore, it is important to study the foraging behavior of bumblebee for having their great economic and ecological role.

Foraging behavior of bumblebee as a pollinator has various effect on the reproductive success of plants throughout anthesis such as the number of pollen grain deposited, removed or the number of seeds produced per visit (Dieringer 1992; Vaughton 1992; Keys *et al.* 1995; Stone 1996; Olsen 1997; Muchhala 2003). Therefore, behaviors of bumblebee to a flower must be figured out as sequential events throughout anthesis. These behaviors must give us essential information not only about interactions between the flowers and the pollinators but also among the pollinators on a flower. However, the sequential events of pollinators on a flower throughout anthesis have not been investigated so far.

Bumblebee behavior is widely studied and considered as one of the most important keys to understand the evolution of flowers (Darwin 1862; Grant & Grant 1965; Proctor *et al.* 1996). In previous studies, bumblebee behaviors have been examined by the simple and discrete observations mainly by naked eyes, which made clear whether they came to a

flower. In fact, bumblebee behaviors are usually very quick, and almost impossible to investigate the all behaviors and their behavior patterns to a flower as sequential events during entire anthesis by the naked eyes.

Bumblebees often display rapid movements in front of flowers, sometimes merely touching or landing on the corolla without probing inside the flower. These rejected flowers generally contain less nectar, on average, than accepted flowers (Corbet et al. 1984; Kato 1988; Heinrich 2004). Therefore it appears that bumblebees have the capacity to recognize small volumes of nectar before probing and deciding to either accept or reject a flower. The scent mark left by previous visitors to flowers is considered to be a possible cue (Cameron 1981; Marden 1984; Kato 1988; Schmitt and Bertsch 1990; Goulson et al. 1998; Stout et al. 1998; Goulson 2010), although the substances contained within a scent mark are unknown. The nectar scent, which can be the scent of floral nectar itself or the fermentation products from yeasts in the nectar, has also been proposed as a possible cue in various flowers (Crane 1975; Williams et al. 1981; Goulson 2010). These two cues may operate for flowers that secrete and store their nectar invisibly, deep inside the corolla tube. *Impatiens* flowers are an example of this, as they secrete and store nectar invisibly inside their long, curved, thin spurs. However, whatever the clue of the rejection or acceptance behaviors for a flower, I need to analyze the sequential detail features of quick insect behaviors on a flower to reveal the mechanisms of interaction among the visits. After a probing on a flower, what kind of behaviors will occur there next? What about the relationship between the times elapsed since last probing and the next behavior? The answers of such questions may give

us the important information to illustrate the mechanisms of acceptance and rejection behaviors.

In this thesis, I outlined the flower-visiting behaviors of bumblebees as a pollinator of Impatiens revealed by long-term video recording during the entire anthesis and described ecological interaction between bumblebees and flowers. This thesis has been organized into seven chapters. Chapter 2 describes the flower-visiting behaviors of bumblebees including approach flights to a flower during entire anthesis revealed by long term video recording, which provide us the sequential events. In this chapter, I also discussed the relationship between the flower visits of bumblebee and aerial temperature-humidity and the relationship between the flower visits and probing. Chapter 3 presents whether bumblebees can recognize nectar through its scent in Impatiens textori flowers. The results of examining the presence of scent mark left by previous flower visitor in the field are described in the chapter 4. Chapter 5 describes the mechanism in occurrence of acceptance and rejection behaviors by bumblebees analyzing the relationship between the initial probing and the next visits especially in attention to the interval (the duration of no-visit) between these two visits. Efficient duration of the scent mark by bumblebees on Impatiens *textori* is also discussed in this chapter. Chapter 6 describes the modified interval-shooting camera for non-destructive and continuous observation of nectar volume of Impatiens flowers.

Chapter 2

Flower-visiting Behaviors of Bumblebee to Impatiens textori

2.1 Abstract

For comprehensive understanding the flower-visiting behavior of bumblebees including approach flights to a flower during entire anthesis, I examined the behaviors of Bombus diversus on flower visit to an Impatiens textori based on long term video recording. I examined 1527 bumblebees' visits to 13 flowers and detected the obvious four behavior steps in the process of acceptance or rejection of the flowers. As the acceptance behavior, bumblebees landed on petals without any hesitancy and probed deeply into the spur to get nectar (Probing). The probing behaviors were observed at the frequency of 53%. On the other hand, in the rejection process (47%), three obvious behaviors were observed: the approach flights to flowers only (Hovering, 26%), the touches on petals (Touching, 7%) and the landing on petals without probings (Landing, 14%). It is well known that bumblebee often rejects flowers by using the scent marker. In this study, the sequential rejection behaviors in the field also indicate that bumblebees can stop their flower visits depending on the strength of the scent markers. In addition, there were no apparent correlation between the number of visits and probing per hour as well as the number of probing and rejection per hour. The stay length did not represent the amount of nectar directly since many bumblebees spent two to five seconds for a probing on a flower regardless of the probing frequency per hour.

2.2 Introduction

Foraging behavior of pollinator has various effect on the reproductive success of plants throughout anthesis such as the number of pollen grain deposited, removed or the number of seeds produced per visit (Dieringer 1992; Vaughton 1992; Keys *et al.* 1995; Stone 1996; Olsen 1997; Muchhala 2003). Therefore, behaviors of pollinators to a flower must be figured out as sequential events throughout anthesis. These behaviors must give us essential information not only about interactions between the flowers and the pollinators but also among the pollinators on a flower. However, the sequential events of pollinators on a flower throughout anthesis have not been investigated so far.

Among the pollinators, Bumblebee behavior is widely studied and considered as one of the most important keys to understand the evolution of flowers (Darwin 1862; Grant & Grant 1965; Proctor *et al.* 1996). Further, it is also important for the pollination system of many endangered plant species as well as common agricultural crops like apple, almond, tomato, canola, red clover and blueberry (McGregor 1976; Parker *et al.* 1987; Thomson 1993). In previous studies, bumblebee behaviors have been examined by the simple and discrete observations mainly by naked eyes, which made clear whether they came to a flower. In fact, bumblebee behaviors are usually very quick, and almost impossible to investigate the all behaviors and their behavior patterns to a flower as sequential events during entire anthesis by the naked eyes.

The aim of this study was to reveal the all foraging behaviors of bumblebees including approach flights to a flower during entire anthesis based on long term video recording, which will provide us the sequential events. I examined behavior patterns, frequency and variation in the stay length of bumblebee in detail. I also analyzed the relationship between the bumblebee visits and aerial temperature-humidity and the relationship between the flower visits and probing.

2.3 Methods and Materials

2.3.1 Study site

The flower visits of Bumblebees were examined on the *Impatiens* flowers. This study were carried out at the Research Forest of Gifu Field Science Center of Gifu University (near Mt. Kuraiyama, Gero-shi, Gifu, Japan; latitude 35.59 N, longitude 137.12 E, 757m alt.) in September 2009. The daylight hours of the site were approximately 12 hours. The temperature varied 10 to 25 °C and the humidity was around 50% during daytime except for the rainy days.

2.3.2 Species descriptions

In the study site, *Impatiens textori* grew in the riverside of the mountain stream, and *Bombus diversus* was the dominant bumblebee species foraging on a large patch (30 x 20m) containing approximately 50 plants (Fig. 2.1).

2.3.2.1 Impatiens textori

Impatiens textori is an annual herb that grows naturally in the side of a mountain stream of East Asia- China, Korea and Japan. It belongs to the Balsaminaceae (the Balsam family) and can grow to a height from 50 to 80cm. The leaves are broad lanceolate and

alternate with fine toothed edges. The long peduncles are borne on the axiles and the purple flowers blooms from August to October. The shape of flower is tubular corolla that is hooked at one end like Noah's ark (Fig. 2.2 & Fig. 2.3). The flower is hermaphrodite (has both male and female organs), chasmogamous (open and exposes the male and female part of the flower). This potentially allows the flower to be cross-pollinated. This flower has long nectar secreting spurs from which secreting nectar continuously and pollinated by long-tongued bumblebee species, *Bombus diversus* (Kato 1988). When the seed pods mature, they explode when touched, sending seeds away.



Figure 2.1 A patch of Impatiens textori



Figure 2.2 Side view of Impatiens textori



Figure 2.3 Front view of Impatiens textori

2.3.2.2 Bombus diversus

Among the insects, bumblebees are the most familiar pollinators and widely recognized as being beneficial through their role as a pollinator. Bumblebees are social insects. They are large, fuzzy and attractive bees. *Bombus diversus* is one of the commonest species at Honshu, Shikoku and Kyushu in Japan. The workers of *B. diversus* are 10-18mm in length. The chest is covered with yellow-brown fur (Fig. 2.4). Pollen sticks to the fur of the body while they probe to the flowers. Thus, they are picking up and transferring pollen. They have long flying ability and excellent foraging skills. *B. diversus* has co-evolutionary relationship with the *Impatiens* flowers (Kato 1988). At study site, *B. diversus* usually opened *I. textori* flower (Fig. 2.5).



Figure 2.4 A bumblebee worker of *Bombus diversus*. It's fairly large (body length 10-18 mm) and are covered in dense fur.



Figure 2.5 *Impatiens textori* are usually opened by *Bombus diversus* worker at the study site.

2.3.3 Long-term video recording

I built the original long-term video recording system (Fig. 2.6) and recorded the side views of the flowers in different *I. textori* plants continuously during their entire antheses. I used the digital video cameras of Sony, DCR-TRV900, HDR XR-500V and HDR XR-520V, and kept power supplies and dry conditions of cameras throughout the recording period in the field. The recordings were started at least one hour before the flower opening and continued until after falling petals. I fixed the recorded flowers softly by tapes to avoid big vibrations by wind. I recorded these flower views with many floral visitors for more than 600 hours and analyzed the video data of about 500 hours for this study.



Figure 2.6 Long-term video recordings in the study site

2.3.4 Bumblebee Marking

Bumblebees of the surrounding area were collected by insect net and anesthetized by CO2 and marked by different colors to trace their re-entrance and analyze other behavioral phenotypes (Fig. 2.7). We totally marked 27 bumblebees from 5th September 2008 to 12th September 2008 and 197 bumblebees from 5th September 2009 to 30th September 2009 in this way.



Figure 2.7 Bumblebee Marking by color pen after capturing bumblebees around the experiment place.

2.3.5 Detection of flower visits by UFO Capture

The videos were analyzed digitally by computer in the laboratory. All scenes of bumblebee flower visits were individually extracted from the movies by a motion detection software program, UFO Capture (SonotaCo, Japan), which had been prepared in advance to detect bumblebee flower visits. To avoid overlooking any flower visits, I set the sensitivity of the detection slightly higher. All of the captured scenes were also checked visually and invalid detections were removed from the analyses.

2.3.6 Analysis of flower visits

In this study, I considered all behavior including approach flight as "flower visit". The video data were captured into a computer and all the flower visits of various insects were analyzed. The video scenes of the visits which collected from the long-term video data were arranged as sequential data along the occurrence time on each flower. The insect and its behavior on video scenes were played in tenfold slow speed on a computer display and were observed by the naked eyes. If the bumblebee landed upon the flower and probed for nectar, the visit was recorded as an 'acceptance'. If the flowers were only approached or were just touched or were landed briefly, the visit was considered as a 'rejection'. In this study, this rejection behavior was examined by using the slow speed play of the video data. I analyzed these visits by dividing the entire anthesis of each flower hourly. Figure 2 shows the behaviors of *B. diversus* observed on the two flowers, as typical examples of 13 flowers, having antheses for three day period (see Table 2.1).

By the (long-term) video recording system earlier, all flower visitors during the entire antheses on 13 flowers of *I. textori* were completely recorded. The weather was almost fine during the video recording period. However, each of the five flowers (F7-F11) had a rainy day in its anthesis (Table 2.1). The rainy days were only two days, September

12, 15. These five flowers experienced the rain on either the second day or the third day of their antheses (Table 2.1). In this chapter, I analyzed the behaviors of the *B. diversus* only on the fine days as the rainfall affects the behaviors of flower visitors naturally.

2.4 Results

2.4.1 Flower visitors during the entire anthesis of each flower

The long-term video recordings revealed the floral ecological features and all flower visitors during the entire antheses of 13 *I. textori* flowers (Table 2.1). The flowers of *I. textori* opened at various time only during the daytime. In addition, *B. diversus* often let the mature flower buds open and start their anthesis (Table 2.1). Once *Impatiens* flowers are opened, they remained open for two or three days and were visited by many insects only at the daytime. The end of the anthesis occurred by falling of calyxes, petals and stamens, leaving a style. During the anthesis, 134 insect visitors were recorded on an average for a flower (1,741 visits in total on 13 flowers). On an average 117 visits of *B. diversus* on a flower (1,527 visits on 13 flowers in total) were recorded, and mostly the other visitors were small flies (200 visits in total). In addition, 13 visits of hawkmoth and a single visit of honey bee were also observed during the field Study (Table 2.1).

Table 2.1

Ecological features and flower visitors of the flowers of Impatiens textori. All data were obtained from the long-term video

records. The categories of bumblebee behaviors were shown in Figure 1 and text.

		Florence			Ц	and which the		-			Bumblebe	e behavio	S		Weather
		STAMOLI			LION		SIC		Acceptance		Rejection		Rejections		
Flower code	Flo (wer open 2009)	fall	# of vists	Fly	Hawk- moth	Honey bee	Bumble bee	Probing	Landing	Touching	Hovering	/ visits (%)	VISIUS / IUS	rain fall (mm)
F1	Sept. 5	12:47 ‡	2nd night	148	19	0	0	129	63	18	5	43	51.2	7.1	no
F2	Sept. 5	13:34	2nd night	111	21	0	0	90	56	14	4	16	37.8	5.2	no
$\mathbf{F3}$	Sept. 8	11:44 [‡]	7:23 on 3rd day	138	7	0	0	131	65	14	10	42	50.4	6.1	no
F4	Sept.8	12:10 ‡	10:13 on 3rd day	214	47	0	0	167	73	25	15	54	56.3	6.9	no
$\mathbf{F5}$	Sept.8	12:40 ‡	2nd night	138	28	0	1	109	49	29	5	26	55.0	5.9	no
F6	Sept. 8	13:20	3rd night	147	23	0	0	124	06	17	5	12	27.4	4.0	no
F7 +	Sept. 10	14:32 ‡	12:30 on 3rd day	125	4	2	0	119	62	21	4	32	47.9	5.2	3rd day (33)
F8+	Sept. 11	9:55 ‡	7:06 on 3rd day	92	1	2	0	68	42	12	8	27	52.8	3.8	2nd day (33)
F9 +	Sept. 13	15:35 ‡	10:08 on 3rd day	122	14	2	0	106	50	14	13	29	52.8	5.2	3rd day (8)
$F10_{\pm}$	Sept. 14	7:20 ‡	2nd night	127	3	4	0	120	59	7	10	44	50.8	5.1	2nd day (8)
F11+	Sept. 14	9:36 ‡	15:53 on 2nd day	116	11	1	0	104	49	17	12	26	52.9	5.4	2nd day (8)
F12	Sept. 16	9:32 ‡	2nd night	135	16	1	0	118	76	17	Э	22	35.6	5.5	no
F13	Sept. 16	9:50 ‡	2nd night	128	9	1	0	121	75	4	16	26	38.0	5.7	no

t: flowers had rainy days (Sept. 12 or 15) ‡: flowers were opened by bumblebees

§ : day time only; 5:00-18:00

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2.4.2 Flower-visiting behaviors of B. diversus

B. diversus showed four-types of flower visit behaviors on *Impatiens* flowers (Fig. 2.8). The most frequent behaviors were probing, flower acceptance behaviors. In the probing behavior, the bumblebees flew close to a flower, landed on the petals, walked into the corolla tube and probed for the nectar inside a spur by their mouthparts. Beside the probing behavior, *B. diversus* showed three-types of the flower rejection behaviors such as hovering, touching and landing behaviors (Fig. 2.8). The hovering was the most apparent flower rejection behavior; bumblebees flied close to a flower but changed their direction just in front of the flower. The touching was another apparent flower rejection behavior; bumblebees set their forelegs upon the petal of a flower but did not weight it; their behavior was like the "touch and go" of an aircraft. The landing was a flower rejection behavior which was sometimes misread as a probing by naked eyes; bumblebees landed and weighted on the petals by their all legs completely, but left from the flower quickly without probing. In addition, these three behaviors were not available for the pollination of *Impatiens* flowers.

I detected 1527 visits of *B. diversus* on 13 *I. textori* flowers. Bumblebees showed probing behaviors 809 times (53.0% of visits) in total and from 42 to 90 times on each flower (Table 2.1). On the other hand, they rejected flowers 718 times; hovering, touching and landing behaviors were detected 399, 110 and 209 times respectively. The rejection percentages varied from 27.4% to 56.3% for flowers whereas on an average 47.0% of *B. diversus* visits were rejection behaviors.



Figure 2.8

Four-types of flower visit behaviors of Bombus diversus on the Impatiens textori flowers. The bumblebees often changed their behaviors on the visits. I detected three-types of rejection behaviors of bumblebees visiting on the flowers due to probe into nectar secreted inside spurs. The definitions of these categories were explained in text. *B. diversus* visited the flower almost constantly in the entire anthesis (Fig. 2.9). In these three days, they showed both the probing and rejection behaviors. However their flower visits on the first day and the second day were both active and slightly more frequent than the visits on the third day. Bumblebees also visited in similar patterns on the five flowers that had three day antheses (Table 2.1). The decrement visits at the last day (the second day) were not observed on the other six flowers which had two day antheses (Table 2.1).



frequencies of bumblebee visits (see Table 2.1 and text). The height of each rectangle means the number of bumblebee flower visits per hour. The number of x-axis means the time interval: "6" means that one hour from 6:00 just before 7:00. The patterns in each rectangle show the frequencies of the bumblebee behaviors as shown in Figure 1.

2.4.3 Flower visits in relation to temperature and humidity

In the study site, the low temperature and the high humidity were recorded in the early morning. The humidity varied in the range from 50% to 100% (Fig.2.10A), and no relationship was observed between the frequencies of bumblebee flower visits and the humidity (Fig. 2.10B). The temperature varied from 10°C to 25°C and the flower visit activities of the bumblebees were also apparently different depending on the temperature (Fig. 2.10C, D). The numbers of flower visits in an hour were few around 10°C and widely varied in the range of higher temperature (Fig. 2.10C). The high frequencies of bumblebee visits, more than ten times in an hour, were often observed around 20°C, (Fig. 2.10C). The probing behaviors were also observed in the temperature range from 10°C to 25°C, and the outline of the distribution of data in the graphic area was almost the same with the case of flower visits although the number of probing in an hour were almost half of the visits (Fig. 2.10D); the high probing frequencies were also recorded around 20°C.





Relationships between the bumblebee visits and the aerial conditions. The temperaturehumidity dots were drawn from the data of average values at each hour.

2.4.4 Probing behaviors of B. diversus

Six visits in an hour were the most often observed case in the 215 hours video of 19 flowers (Fig. 2.11). The flower visits less than 3 times in an hour usually occurred during either early morning or evening and were not frequent cases. Moreover, the visits of seven times or less in an hour constituted 78.1% of the overall cases (168 hours). On the other hand, the bumblebees probed flower ten times in an hour in maximum, and the three probing in an hour were observed most frequently. The probing of five and less times in an hour constituted 90.7% of overall cases (195 hours).



Figure 2.11

Flower visits and probings of bumblebees per hour. The number of the flower visits and probings of bumblebees were counted per hour at each flower. The data were obtained from 13 flowers and their 215 hour antheses in total.

2.4.5 Relationship between probing and flower visits

The significant correlation was not detected between the number of flower visits and the number of probings (Fig. 2.12). The circles on the broken line indicate the cases in which bumblebees probed for nectar inside of the flower in their each visit. Such cases, bumblebee did not reject the flowers. However, when the flowers had more than eight visits in an hour, I did not observe the similar cases. On the other hand, bumblebees visited on a flower three or less in an hour for some cases and they rejected the flower all the times. It was the most frequent case that three bumblebees probed nectar inside spur of a flower in an hour when five bumblebees visited on the flowers at that time.



Figure 2.12

Relationship between the flower visits and the probings of bumblebees. The circle areas indicate the number of each combination. The broken line indicates the cases in which all bumblebees robed nectar inside spur of the flower.

2.4.6 The length of stay in flower probing

The bumblebees needed at least more than one second for a probing behavior but stayed on a flower rarely for more than ten seconds (Fig. 2.13). Many bumblebees spent two to five seconds for a probing on a flower, and the probing behaviors within these second range accounted for 66.6% (539 probings) of the whole.

The relationship did not appear as a simple trade-off line between the average stay length for a probing during a certain one hour and the number of probings at that time (Fig. 2.14). The bumblebees usually spent three to five seconds for a probing in average regardless of the probing frequency in an hour. Moreover, even when floral nectar was exposed by the high frequency of the probings in an hour, the bumblebees spent three to four seconds for their probings.



Figure 2.13

Stay length on a flower for a probing behavior of the bumblebee. The length (sec.) of each stay on a flower was calculated from the number of video frames in which one probing behavior was recorded.



No. of probings per hour

Figure 2.14

Relationship between the average stay length for a probing during a certain one hour and the number of probings at that time. The circle areas indicate the number of each combination.

2.5 Discussion

2.5.1 Flower rejection behaviors of B. diversus

It is now the well-known that bumblebees reject flowers by using the scent mark of previous visitor (Cameron 1981; Marden 1984; Kato 1988; Schmitt & Bertsch 1990; Goulson et al. 1998; Stout et al. 1998). The scent mark is consist of volatile chemical components (Schmitt et al. 1991) and recognized as the most important cue used to decide whether to probe or reject the flowers (Goulson 2010). In this study too, Bumblebees canceled their flower visits at a different timing on a series of behavior to probing. I classified these rejection behaviors into three patterns: hovering, touching and landing. Hovering was the most frequent rejection behavior that might be a timesaving way, by which they can increase their reward in a unit of time (Kato 1998; Schmitt & Bertsch 1990; Goulson et al. 1998). The hovering occurs mainly in recently emptied flowers that have stronger scent mark than flowers that have not been visited for a long while. The strength of the scent marks should show various levels on each flower in the field. Therefore, the bumblebees can stop their flower visits depending on the strength of the scent marks. When the scent strength becomes weak, bumblebees may show touching and then landing behaviors. Although these rejection behaviors, touching and landing, had not been detected precisely by the naked eyes, I revealed that these behaviors were not rare and occupied approximately 21% of all flower visits of bumblebees (319!1527 visits). If these two behaviors are recognized as the intermediate ones between the typical probing and the

typical hovering, it may provide a new viewpoint in relation to the threshold evaporating volatile chemicals which affect the bumblebee behaviors.

In this study, the air temperature varied from 10°C to 25°C (Fig. 2.10). If the repellent scent marks are volatile, the evaporation rate may depend on the temperature. Under such circumstances, when temperature is high, scent mark may evaporate rapidly, and the number of probing in an hour should increase. However, even around noon when air temperature becomes highest on a day generally, an apparent high probing rate (or a low rejection rate) did not occur (Fig. 2.9). Such small amount of air temperature range (10°C to 25°C) may not promote evaporating the scent marks. In other words, the strength of scent marks may not depend on air temperature but may depend on the stay length of previous visitor.

2.5.2 Floral longevity and flower visitors of *I. textori*

Depending on the ecological habits of pollinators, the time of flower open and longevity of a flower must reflect the reproductive success of its plants (Primack 1985; Ashman & Schoen 1996). Floral longevity influence the number of pollinator visits that can affect the amount and diversity of pollen deposition or removal, and, finally contributes to plant fitness (Primack 1985; Ashman & Schoen 1996). If floral longevity is less than one day, late opening may affect reproduction seriously. As shown in Table 2.1, the flowers of *I. textori* had the different flower opening times and two-three day floral longevities. In spite of such differences, all the flowers attracted many bumblebees (more than 89 visits), and kept the enough frequencies of visits and probings until flowers end (Fig. 2.9). Since the ecological relationship between *Impatiens* plants and the bumblebees is substantial, more than one hundred visits on a flower may not be so large (Table 2.1). The large number of bumblebee probings may affect the outcrossing rate of *I. textori* directly. In addition, the number of visits declined a little on the third day (Fig. 2.9) though the visits and probings were frequent until the flower end. It suggests that *I. textori* continues nectar secretion as the enough reward for pollen vectors until the end. At the same time, the frequent probings of bumblebee on a flower suggested the need of remarkable structural strength of flowers for the reproductive success *of I. textori* by the outcrossing.

2.5.3 The efficiency of the long-term video recordings

In pollination biology, the video recordings of flower visitors are now one of the usual methods. However, the long-term video recording covering the entire anthesis of a flower had probably never done, especially in the field with the weather changes. The long-term video recording on the *Impatiens* flowers recorded the floral ecological features and all flower visitors on each flower. By these video data, I could observe and analyze the flower visitor behaviors in the both fine and long scale in time. One second video movie data consist of 29.97 frames, and the long-term recordings supported by long-life batteries besides the wired power supply. Moreover, I could reconfirm the observation results repeatedly on the display monitor only by using the replay. The present study could not be

done only by naked eyes and I believe the long-term video recording method must be one of the powerful tools in the pollination biology.

2.5.4 Relationship between the number of Visits and Probing

The number of probing by bumblebees does not depend on the number of visits (Fig. 2.11). If bumblebee visits increase, probing do not necessarily increased. Again, if bumblebee visits very few, there are no guarantee to probe in all the case. In addition, no apparent correlation has been observed between the frequency of probing and rejection per hour. Therefore, obviously it depends on the other factors like the nectar scent - scent of floral nectar itself or fermentation products from yeasts in the nectar (Crane 1975; Williams *et al.* 1981; Goulson 2010) or scent of bumblebee itself (Cameron 1981; Marden 1984; Kato 1988; Schmitt & Bertsch 1990; Goulson *et al.* 1998; Stout *et al.* 1998). In *I. textori*, the number of probing does not depend on scent of nectar (Raihan & Kawakubo 2013). Till to date, I do not know the efficiency of the scent mark in detail.

2.5.5 Stay length of Flower Visits

The stay length does not represent the amount of nectar directly since many bumblebees spent two to five seconds for a probing on a flower regardless of the probing frequency in an hour (Fig.2.14). In other words, Nectar does not exist much than the amount that is drinkable within 2 to 5 seconds. Bumblebees need at least two seconds in probing behavior in an once even if quantity of nectar is little. I found some long stay length probably having differences in drinking speed of each bumblebee. The amount of nectar at the time of each probing is unidentified. Recently I also reported the method of measuring nectar in entire anthesis without destructing the flower (Raihan & Kawakubo 2013). Therefore, it will be interesting if any further study attempt to make relationship between the real amount of nectar and bumblebee behavior.

Chapter 3

The Function of Nectar Scent in Acceptance and Rejection of

Flowers

3.1 Abstract

To clarify if bumblebees can recognize nectar through its scent in *Impatiens textori* flowers, I examined the behavior of *Bombus diversus* on nectar-less flowers in which the spurs had been artificially removed. Bumblebee visits to both natural flowers and spur-cut flowers were captured using a long-term video recording system. Visiting behavior and frequency were compared between the two flower types. Many bumblebees visited both types of flower, and their visit frequencies were not significantly different. However, the length of stay on each flower type did differ, with the bumblebees remaining on the spur-cut flowers for a significantly shorter time than on the natural flowers. The results suggest that bumblebees cannot detect the absence of nectar in *I. textori* flowers before probing them. Therefore, the nectar scent of *I. textori* does not serve to attract bumblebees although the presence of nectar will detain bumblebees on flowers for longer periods.

3.2 Introduction

Foraging bumblebees often display rapid movements in front of flowers, sometimes merely touching or landing on the corolla without probing inside the flower. These rejected flowers generally contain less nectar, on average, than accepted flowers (Corbet *et al.* 1984; Kato 1988; Heinrich 2004). Therefore it appears that bumblebees have the capacity to recognize small volumes of nectar before probing and deciding to either accept or reject a flower. However, the rejection mechanism used by bees in their recognition of nectar has not been determined (Goulson *et al.* 2001; Julie Thakar *et al.* 2003).

The scent mark left by previous visitors to flowers is considered to be a possible cue (Cameron 1981; Marden 1984; Kato 1988; Schmitt and Bertsch 1990; Goulson *et al.* 1998; Stout *et al.* 1998; Goulson 2010), although the substances contained within a scent mark are unknown. The nectar scent, which can be the scent of floral nectar itself or the fermentation products from yeasts in the nectar, has also been proposed as a possible cue in various flowers (Crane 1975; Williams *et al.* 1981; Goulson 2010). However, it was determined that nectar scent does not act as a cue for rejection in *Aconitum* flowers (Heinrich 1979). Floral nectar generally contains scented compounds (Raguso 2004), although no previous studies have concluded that bumblebees can recognize nectar through nectar scent.

Bumblebees are main the pollinators of Japanese *Impatiens*, which secrete floral nectar continuously inside their curved spurs. Bumblebees have been shown to use a scent mark left by previous visitors to a flower as a cue for the rejection of almost empty flowers

(Kato 1988). However, whether or not bumblebees perceive nectar through nectar scent in *Impatiens* flowers has not been investigated.

In this study, I examined the possibility that bumblebees can use nectar scent to decide either to accept or reject *Impatiens textori* flowers. I prepared artificial nectar-less flowers and examined the visiting behavior of bumblebees to both natural and artificial flowers. I also analyzed the length of stay of the bumblebees on both types of flowers to investigate the validity of artificial flowers and the effect of the nectar itself on stay length.

3.3 Materials and Methods

I examined the behavior of *Bombus diversus* on the pink flowers of *I. textori*, which secrete nectar deep inside their long, curved, thin spurs. The flowers produce nectar continuously as a reward for pollinators, which are mainly the nectar-gathering workers of a bumblebee species, *B. diversus* (Kato 1988).

3.3.1 NF and CF (natural and spur-cut flowers)

To clarify whether bumblebees recognize the nectar scent, I prepared artificial nectar-less flowers and placed them in the field. The artificial flowers (i.e., spur-cut flower, CF) were rendered nectar-less by cutting off the spur just before flower opening in the field (Fig. 3.1). As nectar refill was completely prevented in the CFs, I regarded them to be nectar-less for their entire antheses. I compared the flower-visiting behavior of bumblebees to the CFs to their flower-visiting behavior to natural flowers (NFs) in *I. textori*.



Figure 3.1

Spur-cut flower (CF) of *Impatiens textori* was prepared by cutting off the long, thin spurs just before flower-opening in the field. Black arrow shows the point of cutting.

3.3.2 Bumblebee marking

I confirmed that the artificial cutting of petals did not affect bumblebee behavior due to odors released from damaged areas of the flower. I analyzed the revisits of bumblebees that had been individually marked to examine whether a complete absence of spurs after cutting influenced the likelihood of revisits. Several bumblebees were captured around the study site and their backs were marked using paint of different colors. I marked 27 bumblebees in this way in September 2008, and 197 bumblebees in September 2009.

3.3.3 Long-term video recording

For the analysis of the flower-visiting behavior of bumblebees to both NF and CF, I recorded their approach flights to flowers using digital video cameras (DCR-TRV900, HDR XR-500V, and HDR XR-520V: Sony, Tokyo, Japan). I used a progressive video format that produced 30 picture frames per second. For comparison between NFs and CFs, I selected two flowers, ca. 350mm apart, on the same plant and captured the two flower types. For each pair of flowers, a continuous recording was made for the entire anthesis. Approximately 370 hours of video recordings were made for eight NF and CF pairs.

3.3.4 The place and period of video recording

The field observations and video recordings were undertaken at the Research

Forest of Gifu Field Science Center, Gifu University (near Mt. Kuraiyama, Geroshi, Gifu, Japan; 35.59°N, 137.12°E, altitude 757 m) in September 2008 and 2009. There were approximately 12 hours of daylight per day at the study site. The temperature varied from 10 to 25°C, and the humidity was around 50% in the daytime on dry days. At the study site, dense patches of *I. textori* grew along the side of a mountain stream and the main flower visitor was *B. diversus*.

3.3.5 Detection of flower visits by UFO Capture

The videos were analyzed digitally by computer in the laboratory. All scenes of bumblebee flower visits were individually extracted from the movies by a motion detection software program, UFO Capture (SonotaCo, Japan), which had been prepared in advance to detect bumblebee flower visits. To avoid overlooking any flower visits, I set the sensitivity of the detection slightly higher. All of the captured scenes were also checked visually and invalid detections were removed from the analyses.

3.3.6 Calculation of co-flowering time

From the recordings, I were able to determine the time of flower opening and fall, flower visit frequencies, and the length of stay of bumblebees on each NF and CF. The time of flower open was defined when flower buds were flourished naturally or were opened forcibly by bees . The time of flower fall was defined as the point when the dry corollas became disconnected with the main stalk of a flower. I estimated the time available for bumblebee visits to an individual flower by deducting the night time from the entire anthesis, and I then calculated the co-flowering time (i.e., when both NF and CF flowers were open).

3.3.7 Acceptance and rejection behaviors

I treated bumblebee probing behavior on flowers as an acceptance. Landings with no probing behavior, or rapid movements away from the front of the flowers in approach flights, were treated as a rejection. Therefore, all flower visits of bumblebees were defined as either an acceptance or a rejection. The stay length was measured by counting the picture frames in a video that included acceptance behavior from landing to leaving.

3.3.8 Statistical analysis

The number of flower visits and the acceptances and rejections of NFs and CFs were analyzed using a chi-squared goodness-of-fit test to determine the significance of any differences between the two flower types. I used a non-parametric method, the Mann-Whitney U test, to check the significance of any differences between stay lengths on the two flower types.

3.4 Results

3.4.1 Bumblebee visits to natural and spur-cut flowers

I detected 1274 bumblebee visits to 16 *I. textori* flowers, comprising eight NF and CF pairs (Table 3.1). The bumblebees often opened flower buds by force and they remained open for 2-3 days. All of the bumblebees were *B. diversus* and visited the flowers only in the daytime. Therefore, flowers had specific hours of availability for bumblebee visits, which varied from 18 to 32 hours (Table 3.1). The bumblebees visited eight NFs 602 times in total, with a range of 29 to 121 visits to each flower, and eight CFs 672 times with a range of 28 to 182 visits to each flower (Table 3.2). Because the number of available flowers varied at certain times (Table 3.1), I compared the number of visits (Table 3.2) and the number of acceptances in co-flowering time (Table 3.3). There were no significant differences (at the 5% level) in the number of bumblebee visits between NFs and CFs for each pair of flowers (Table 3.2). There were also no significant differences observed (at the 5% level) in the number of acceptances between NFs and CFs for each pair of flowers (Table 3.3).

Numbers of visits to each flower by bumblebees. Their visits were counted on digital video at eight pairs of natural flowers (NF) and spur-cut flowers (CF) in September 2008 and 2009.

Year	Flower Pair	NF/CF	Flower			No. of bumblebee visits
		111,01	Open	Fall	Hourst	
	1	NF	10:05:51	2nd Night	20	81
		CF	10:05:08	9:23:49 on 3rd day	24	100
2008	2	NF	11:26:16	2nd Night	22	68
2008		CF	10:10:33	6:30:00 on 3rd day	22	62
	3	NF	15:15:31	2nd Night	16	36
	3	CF	12:33:34	5:54:41 on 3rd day	17.5	37
2009	4	NF	14:32:44	12:30:24 on 3rd day	24.5	121
		CF	6:38:18	16:38:10 on 3rd day	23.5	108
	5	NF	9:32:33	2nd Night	20.5	118
		CF	12:42:21	3rd Night	32	182
	6	NF	8:01:42	3rd Night	22	64
		CF	1st Night	3rd Night	25.5	65
	7	NF	14:17:43	3rd Night	28	85
		CF	13:23:48	3rd Night	29	90
	8	NF	13:42:43	10:07:44 on 3rd day	22	29
		CF	14:17:27	2nd Night	18	28

+Available hours for visits

Numbers of bumblebee visits per hour to natural flowers (NF) and spur-cut flowers (CF). Chi-square goodness-of-fit test showed no significant difference (at the 5% level) between the two flower types (NF and CF).

Flower Pair	No. of Visits in total		No. of Visits per hour				
	NF	CF	NF	CF	X^2	P-value	
1	81	100	4.1	4.2	0.001657	0.968	
2	68	62	3.1	2.8	0.012587	0.911	
3	36	37	2.3	2.1	0.00422	0.948	
4	121	108	4.9	4.6	0.012342	0.912	
5	118	182	5.8	5.7	0.000411	0.984	
6	64	65	2.9	2.5	0.023754	0.878	
7	85	90	3.0	3.1	0.000747	0.978	
8	29	28	1.3	1.6	0.019607	0.889	

Numbers of acceptances (probings) by bumblebees per hour on natural flowers (NF) and spur-cut flowers (CF). Chi-squared goodness-of-fit test showed no significant difference (at the 5% level) between the flower types (NF and CF) separately in all of the samples, indicating no influence of nectar on flower acceptance.

	No. of Acceptances	No. of Acceptances per hour				
Flower Pair	NF	CF	NF	CF	X ²	P-value
1	64	68	3.2	2.8	0.022284	0.881
2	39	38	1.8	1.7	0.00059	0.981
3	23	17	1.4	1.0	0.090174	0.764
4	62	46	2.5	2.0	0.073198	0.787
5	76	92	3.7	2.9	0.105244	0.746
6	33	34	1.5	1.3	0.009804	0.921
7	19	27	0.7	0.9	0.039598	0.842
8	16	17	0.7	0.9	0.028213	0.867

3.4.2 Flower revisits by marked bumblebees

Of 224 marked bumblebees, many individuals were seen revisiting both NF and CF flowers. The marked bumblebees made a total of 272 visits (51 individuals) to eight NFs and 230 visits (50 individuals) to eight CFs (Table 3.4). Furthermore, 36 individual marked bees reaccepted 195 times on the eight NFs, and 27 individual marked bees reaccepted 150 times on seven CFs (Table 3.4). Flower pair No. 5 received the most visits by different marked bees, with at least 17 and 21 different bees visiting the NF and CF respectively, and 12 (41 times) and 10 (34 times) of these bees revisiting the flowers, respectively.

Moreover, several marked bees frequently revisited the CFs of different flower pairs. Table 3.5 shows the number of revisits (acceptances) of six marked bees (individual bee codes: B1, B5, and B10 in 2008 and B22, B40, and B157 in 2009), which were observed frequently on the flowers. From the observations, it was clear that bumblebees accepted and rejected CFs in the same manner that they did for NFs. There were no significant differences in the number of acceptances between NFs and CFs (Table 3.5).

Flower visits by marked bumblebees. I carried out a follow-up survey of flower visits by 27 marked bumblebees in 2008 and 197 marked bumblebees in 2009. Their revisits were observed commonly to both natural flowers (NF) and spur-cut flowers (CF).

			No. of visits	No. of bees		
Flower pair	NF/CF	Total	Marked bees (%)	Marked	Revisited† (%)	
1	NF	81	57 (70.4)	9	5 (55.6)	
1	CF	100	69 (69)	10	7 (70.0)	
2	NF	68	42 (61.8)	8	5 (62.5)	
۷	CF	62	31 (50)	5	4 (80.0)	
2	NF	36	22 (61)	5	5 (100)	
	CF	37	11 (29.7)	3	2 (66.7)	
4	NF	121	60 (49.6))	12	9 (75.0)	
4	CF	108	63 (58.3)	14	10 (71.4)	
5	NF	118	50 (42.4)	17	12 (70.6)	
5	CF	182	47 (25.8)	21	10 (47.6)	
6	NF	64	28 (43.7)	6	4 (66.7)	
0	CF	65	0 (0)	0	0 (0)	
7	NF	85	1 (1.2)	1	0 (0)	
	CF	90	1 (1.1)	1	0 (0)	
8	NF	29	12 (41.4)	8	3 (37.5)	
	CF	28	8 (28.6)	5	2 (40.0)	
Total	NF	602	272	51	36 (70.6)	
TOLAL	CF	672	230	50	27 (54)	

† Number of marked bumblebees which visited twice or more.

Behavior of marked bumblebees on natural flowers (NF) and spur-cut flowers (CF). The top three marked bumblebees in terms of visiting frequency are shown. I carried out a follow-up survey of flower visits by 27 marked bumblebees in 2008 and 197 marked bumblebees in 2009. No significant difference in "Acceptance" (at the 5% level) was observed, even at the individual bee level, between NF and CF flowers by chi-squared goodness-of-tit test.

		No. of Ac	ceptances		
Year	Bee	NF	CF	X2	P-value
	B1	36	38	0.054054	0.816
2008	B5	8	15	2.13043	0.144
	B10	11	8	0.473684	0.491
	B22	15	8	2.13043	0.144
2009	B40	8	13	1.19048	0.275
	B147	9	14	1.08696	0.297

3.4.3 Stay length during flower visits

I accurately determined the length of stay of 1274 bumblebees that visited NFs and CFs in 2008 and 2009 by counting the number of video frames. Figure 3.2 shows the length of stay during acceptance behavior based on 422 visits in 2009. Similar results were obtained in 2008. Many bumblebees undertook probing behavior for 3-4 and 1-2 seconds on NFs and CFs, respectively. Although the maximum length of stay for NF and CF acceptors was almost the same, 69% of CF acceptors left the flowers within three seconds. The distribution of length of stay times differed significantly between the NF and the CF (P<0.0001 Mann-Whitney U test) (Fig. 3.2).



Figure 3.2

Stay length of bumblebees on NF (natural flowers) and CF (spur-cut flowers) in 2009. The distribution patterns of stay length differed significantly between NF and CF (P<0.0001) by non-parametric Mann-Whitney U test.
3.5 Discussion

3.5.1 Ineffectiveness of nectar scent as a clue used by bumblebees

There were no significant differences in bumblebee visits between the NF and the CF in each flower pair, and the bumblebee acceptance behavior also did not differ significantly for any flower pair (Tables 3.2 and 3.3). The study using marked bees revealed that revisits to both NF and CF were frequent (Table 3.4). These results suggest that *B. diversus*, in terms of both their approach flight to flowers and landing behavior on the petals, does not discriminate due to floral damage resulting from spur-cutting and also cannot recognize the presence of floral nectar of *I. textori*. The results also suggest that only after probing inside the flowers do bumblebees recognize the spur-cut or nectar-less conditions, and thus become aware that these flowers offer no reward. The bumblebees appeared not to remember spur-cut flowers even after they have previously visited them, as many of the marked bees revisited the same flowers (Table 3.4).

The overall observations indicated that bumblebees showed acceptance and rejection behavior for any type of *Impatiens* flower, irrespective of the presence of nectar. This behavior was also confirmed by observations of the same individual bumblebee visiting many flowers (Table 3.5). Therefore, nectar scent cannot act as an indicator of a reward in the relationship between *B. diversus* and *I. textori*.

However, *B. diversus* displayed apparent rejection behavior in visits to both NFs and CFs, although it did not exhibit remote perception of *I. textori* floral nectar. Therefore,

the scent mark left by previous visitors can be a more likely cue for rejection behavior on nectar-less flowers than is a previous recollection of the absence of nectar (Cameron 1981; Marden 1984; Kato 1988; Schmitt and Bertsch 1990; Goulson *et al.* 1998; Stout *et al.* 1998; Goulson 2010)

After alighting on a normal flower (i.e., NF in the present study), bumblebees must probe for nectar and take time to drink it before leaving the flower. The observations revealed that bumblebees remained on both NFs and CFs for a short period after acceptance of the flower (Fig. 3.2). If bumblebees were able to recognize the spur-cutting treatment during their probing behavior, they would be expected to display different behavior. Some bumblebees might become confused and spend a longer time on the CFs before departing. However, many bumblebees stayed on the NFs longer than on CFs and not such different behavior was recorded in bees on CFs. The distribution of the length of stay times differed significantly between NFs and CFs (Fig. 3.2). The general patterns of the histograms for the two flower types were markedly similar (Fig. 3.2). Therefore, any artifacts resulting from spur-cutting appear to be sufficiently negligible to allow a discussion of the significant differences in stay length.

3.5.2 Function of nectar detaining bumblebee on a flower

The length of stay on CFs may include an orientation period for the probing and detection of the nectar-less situation, before the bumblebee eventually abandons its attempt to obtain nectar. Therefore, the difference between the length of stay (1-2 seconds) may be

equivalent to the time required to drink nectar. Moreover, this difference may account for the function of nectar, which detains the bumblebee on a flower as a pollinator, as a longer stay on a flower might enhance pollen deposition and removal (Thomson & Plowright 1980; Feinsinger 1983; Galen & Plowright 1985; Thomson 1986; Lanza et al. 1995).

However, even if nectar has an effective function as a reward, the study has shown that the presence of nectar cannot directly influence the visit of a bumblebee. It is unlikely that the nectar scent provides bumblebees with information to make decisions whether to reject an *Impatiens* flower. Although I do not have enough information regarding the ecological conflict between the foraging strategy of bumblebees and the concealment of nectar in *Impatiens* flowers, bumblebees can clearly reject nectar-less flowers by using the scent marks left by previous visitors and then wait for a refill of nectar.

Chapter 4

The Effect of Scent Mark Left by Previous Visitors in

Acceptance and Rejection of Flowers

4.1 Abstract

To detect the presence of scent mark left by previous flower visitor in the field, I analyzed the scent of flower before and after bumblebee probing. I used Solid-phase microextraction or SPME fiber to extract the scent from the flowers before and after bumblebee probing. I extracted the scent form a flower by one SPME fiber that is not probed yet. I waited until a probing by a bumblebee in that flower, and as soon as after a probing I extracted scent again by another SPME fiber. After extraction, SPME fibers had been transferred to a Gas Chromatography-Mass Spectrometry or GC-MS machine for analysis. I compared the peak numbers of the compounds between the two fibers and I found major changes in the peak number of two compounds at the retention time of 38.7s and 45.8s. These two compounds are "Tetradecanoic Acid" and "n-Hexadecanoic Acid" and are used as repellant compositions. I assume that scent mark contain these two compounds that act as a repellant mark for bumblebees to reject the recently visited flowers.

4.2 Introduction

Foraging bumblebees can distinguish more or less rewarding flowers of the same plant species without sampling the reward available (Goulson et al. 2001). By this ability, when bumblebees approach flowers, they often show quick turns in front of flowers to reject them. These rejected flowers contain less nectar, on average, than accepted flowers (Heinrich 1979; Corbet et al. 1984; Wetherwax 1986; Kato 1988; Duffield et al. 1993). Previous studies have suggested some possible mechanisms to explain this. Some have suggested that bumblebees reject flowers assessing the extent of the reward directly by visual means (Thorp et al. 1975; Kevan 1976; Zimmerman 1982; Cresswell & Robertson 1994), whereas others have suggested that the scent of floral nectar itself, or that of fermentation products from yeasts in the flower, acts as a positive reward signal (Crane 1975; Williams et al. 1981; Heinrich 1979, Marden 1984; Goulson et al. 2001; Raguso 2004). Conversely, the most possible mechanism are scent mark left by previous insect visitors to flowers that works as a negative signal indicative of decreased nectar (Cameron 1981; Free & Williams 1983; Marden 1984; Kato 1988; Schmitt & Bertsch 1990; Giurfa 1993; Goulson et al. 1998; Stout et al. 1998; Giurfa & Núñez 1992, 1993; Goulson et al. 2000; Saleh et al. 2006).

Direct assessment by visual means may not operate for flowers that secrete and store their nectar invisibly, deep inside the corolla tube. *Impatiens* flowers are an example of this, as they secrete and store nectar invisibly inside their long, curved, thin spurs. Bumblebees' flower rejection behavior has been examined in *Impatiens textori*, and scent markers left by previous visitors have been strongly inferred to be the main cue for rejection (Kato 1988; Raihan & Kawakubo unpublished). The use of scent marks increases foraging efficiency by reducing the time spent handling unrewarding flowers (Kato, 1988; Schmitt and Bertsch, 1990; Goulson et al., 1998).

Although most of the recent studies have suggested the possibility of scent mark as a clue in this mechanism, there are so many unknown issues regarding the scent mark. Therefore, some workers have recently investigated on these unknown issues that are as follows: the scent mark is attractant or repellant, the origin or source of scent mark, the process of scent marking (active or marked automatically), the differentiation of scent mark with body scent etc. The scent mark has always been found as a repellant effect in the field experiments (Goulson et al., 1998, 2000; Stout et al., 1998; Gawleta et al., 2005), whereas an attractant effect in the laboratory experiments (Cameron, 1981; Schmitt and Bertsch, 1990; Schmitt *et al.*, 1991). These both types of marks are secreted from the tarsal glands in bumblebee (Schmitt et al.1991; Stout et al. 1998). At a high concentration, the scent mark may repel the foragers but at lower concentration attract them (Stout et al 1998).

However, the probable repellant effect of scent mark has been discussed in the field but the presence of scent mark has never been detected yet. In this study, I attempted to detect the presence of scent mark left by previous visitor in the flowers. Can bumblebees deposit scent mark while visit? What are the compounds of scent mark? I examined the scent of flower before and after the bumblebee visit to clarify these issues.

4.3 Materials and Methods

In this study, I compared the extracted scent of *I.textori* flower before and after the probing of a bumblebee by a chemical analysis (fig. 4.1).



Figure 4.1

Experimental design of the extraction of scent from the *Impatiens textori* flower before and after the bumblebee probing

4.3.1 Study Flowers

I carried out the study on the pinkish flowers of *Impatiens textori*, which secrete nectar deeply inside their long, curved, thin spurs. The flowers produce nectar continuously as a reward for pollinators, which are mainly the nectar-gathering workers of a bumblebee species, *Bombus diversus* (Kato 1988). At the study site, *I. textori* grew thickly along the side of a mountain stream.

4.3.2 Study Site

The study samples were collected at the Research Forest of Gifu Field Science Center, Gifu University (near Mt. Kuraiyama, Geroshi, Gifu, Japan; latitude 35.59 N, longitude 137.12 E, 757m alt.) in September 2009. There was approximately 12 hours of daylight at the site. The temperature varied from 10 to 25°C, and the humidity was around 50% in the daytime, except on rainy days.

4.3.3 Scent Extraction by SPME Fiber

SPME or solid-phase microextraction is a sample preparation technique, involves the use of a fiber coated with an extracting phase which extract different kinds of analytes (including both volatile and non-volatile) from liquid or gas phase. I used SPME Fiber (fig. 4.2) Assembly 50/30um DVB/CarbonexTM/PDMS StableFlexTM (SUPELCO; 57328-U) to extract scent from the flowers. First, I chose fresh flowers that are not visited yet by foragers. I fixed the chosen flower to minimize the vibration effect occurred by strong wind. Then, I took the scent of fresh flower that is not probed yet by using one SPME fiber (fig. 4.3). The scent extraction by this fiber was continued for two minutes to confirm the extraction of scent in sufficient amount. To avoid the leak of scent, I capped the fiber as soon as possible after the end of extraction. After then, I waited until a probing by a bumblebee in that flower, and as soon as after a probing I extracted scent again by using another SPME fiber. At September 24th, I extracted scent from a fresh flower before 12:30pm, when a probing by bumblebee occurred. I extracted scent twice using by another two fibers at 12:31pm and 12:37pm after the probing.

4.3.4 Chemical Analysis by GC-MS machine

After extraction, SPME fibers had been injected to a Gas Chromatography-Mass Spectrometry or GC-MS machine (HP 5890) (fig. 4.2) for analysis. This analysis had been conducted at the Laboratory of Biochemistry of Wood Components and its applications, Faculty of Applied Biological Sciences, Gifu University with the help of an expert on chemical analysis. I measured retention time from the injected SPME fiber sample after the compound elutes from the column. I compared the retention times and compounds among the several samples. I identified a compound by comparing the compound's mass spectrum with known compounds.



GC MS Machine

Figure 4.2 GC-MS Machine & SPME Fiber



Figure 4.3 A scent taking scene by the SPME fiber in the field



Figure 4.4

The two major changes in the peak number of two compounds at the retention time of 38.7s and 45.8s. I extracted scent from a fresh flower before 12:30pm, when a probing by bumblebee occurred. I extracted scent twice using by another fibers at 12:31pm and 12:37pm after the probing.

Retention Time:38.7s Molecular formula:C14H28O2 Molecular Mass: 228 Name of Chemical: Tetradecanoic acid



Figure 4.5 Tetradecanoic acid at retention time 38.7 s

Retention Time:45.7s Molecular formula:C16H32O2 Molecular Mass: 256 Name of Chemical: n-Hexadecanoic acid



Figure 4.6 n-Hexadecanoic acid at retention time 45.7s

4.4 Result and Discussion

4.4.1 Existence of scent mark in the field

I compared the scent of flower before and after the probing of a bumblebee by a chemical analysis. Scent extraction from the flower in the field was a complex one having some factors that includes extracting other scent by strong wind, touching flower parts by hand vibration etc. Therefore, the output of chemical analysis often exhibited unsatisfactory result showing broad, overlapping, or unevenly formed peaks. However, some of the output showed satisfactory results with symmetrical, narrow, separate (not overlapping), and made with smooth lines. Based on these data, I compared the retention time of two samples and found major changes in the peak number of two compounds at the retention time of 38.7s and 45.8s (Fig. 4.4). The mass spectrums of these two compounds indicate that the compounds are "Tetradecanoic Acid" and "n-Hexadecanoic Acid" (fig. 4.5 and fig.4.6 respectively). These compounds are used as repellant compositions for repelling animals such as deer according to United States Patent (Patent Number: 4818535, Date of Patent: April 4, 1989).

The Dufour's gland secretion of *Xylocopa virginica texana* possesses short-term repellency for conspecifics when applied to passion flowers. This secretion contains a number of straight-chain hydro- carbons. The two major components are the methyl esters of palmitic (n-Hexadecanoic acid) and myristic acid (Tetradecanoic acid). Therefore,

I assume that scent mark of bumblebee also contain these two compounds that act as a repellant mark for bumblebees to reject the recently visited flowers.

4.4.2 Intensity of the scent mark

The numbers of peaks of "Tetradecanoic Acid" and "n-Hexadecanoic Acid at the retention time of 38.7s and 45.8s were highest at after the 4 minutes than the after 1 minute and after 7 minutes of probing (Fig. 4.4). I assume that just after deposition of scent mark, the concentration of scent are comparative low as it may take little time to emit the scent around. In other words, the intensity of scent mark becomes highest at one moment after the emancipation of scent mark at around. In this result, I got highest intensity of scent mark at around 4 minutes after the bumblebee probing than after the 1 minute and 7 minutes. The degree of scent intensity may become weaker and weaker with the time. The efficiency of repelling may also be weaker and weaker. These issues require further details investigations to reveal the real facts as I had lack of consistent data.

Chapter 5

Efficiency of Scent Mark Left by Previous Visitors in Acceptance

and Rejection of Flowers

5.1 Abstract

To understand the mechanism in occurrence of acceptance and rejection behaviors by bumblebees, I analyzed the relationship between the initial probing and the next visits especially in attention to the interval (the duration of no-visit) between these two visits. For analysis, I recorded the entire antheses of 19 *Impatiens textori* flowers and detected 1927 bumblebee behaviors in sequence that classified into acceptance (probing) and rejection (landing, touching and hovering) behaviors. The analysis shows that the number of rejection was occurred just after an acceptance, and the frequency of rejection was gradually decreased and became very small at about 15 minutes after the initial probing. These 15 minutes must be the effect duration of scent mark by the previous flower visitor in this case.

5.2 Introduction

As a pollinator behavior, bumblebee behaviors have been figured out as the continuous and sequential events on the *Impatiens textori* flowers by our previous study. As a result, bumblebee shows sequential behaviors on a flower, Hovering, Touching, Landing and Probing, broadly categorized into Acceptance and Rejection Behavior. This study may provide us essential information about the interactions not only between the flowers and pollinators but also among the pollinators on a flower.

When foraging bumblebees approach a flower, they often show quick turns to reject it (Cameron 1981; Free & Williams 1983; Marden 1984; Kato 1988; Schmitt & Bertsch 1990; Giurfa 1993; Stout *et al.* 1998; Goulson *et al.* 1998; Raihan & Kawakubo 2013). Several studies have been tried to reveal the causes of this rejection behavior by bumblebees though not yet clearly understood. Previous studies have mentioned mainly three types of possible cues related with this rejection behavior showing by bumblebees: (1) the direct assessment of rewards by visual mean(Thorp *et al.* 1975; Kevan 1976; Zimmerman 1982; Cresswell & Robertson 1994), (2) the scent of floral nectar itself, or scent of fermentation products from yeasts in the flower as a positive reward signal(Crane 1975; Williams *et al.* 1981; Heinrich 1979, Marden 1984; Goulson *et al.* 2001; Raguso 2004)and, (3) the scent mark left by previous insect visitors to flowers as a negative signal indicative of decreased nectar (Cameron 1981; Free & Williams 1983; Marden 1984; Kato 1988; Schmitt & Bertsch 1990; Giurfa 1993; Goulson *et al.* 2006).

The latter two mechanisms may operate for flowers that secrete and store their nectar invisibly, deep inside the corolla tube. *Impatiens* flowers are an example of this, as they secrete and store nectar invisibly inside their long, curved, thin spurs. However, I recently reported that bumblebees may not use nectar scent to decide either to accept or reject *Impatiens textori* flowers (Raihan & kawakubo 2013). Then, Bumblebees may possibly use scent mark left by previous visitors to an *Impatiens textori* flower as a cue for the rejection also supported by Kato 1988.

However, whatever the clue of the rejection or acceptance behaviors for a flower, I need to analyze the sequential detail features of quick insect behaviors on a flower to reveal the mechanisms of interaction among the visits. To The knowledge there was no report on mechanism in details in occurrence of accepting or rejecting after a probing based on the observation of entire flower anthesis. After a probing on a flower, what kind of behaviors will occur there next? What about the relationship between the times elapsed since last probing and the next behavior? The answers of such questions may give us the important information to illustrate the mechanisms of the rejection behaviors.

In this paper, I outline the mechanism in details occurrence of accepting or rejecting by analyzing the relationship between the initial visits and the next visits especially in attention to the interval (the duration of no-visit) between these two visits. I also measure the duration of the scent efficiency by bumblebees on *Impatiens textori*.

5.3 Methods and Materials

I analyzed the relationship between the initial probing and the next visits especially in attention to the interval (the duration of no-visit) between these two visits. For analysis, the flower visits of Bumblebees (*Bombus diversus*) were examined on the flowers of *Impatiens textori*. The field observations and video recordings were undertaken at the Research Forest of Gifu Field Science Center, Gifu University (near Mt. Kuraiyama, Geroshi, Gifu, Japan; 35.59°N, 137.12°E, altitude 757 m) in September 2009. There were approximately 12 hours of daylight per day at the study site. The temperature varied from 10 to 25°C, and the humidity was around 50% in the daytime on dry days. At the study site, dense patches of *I. textori* grew along the side of a mountain stream and the main flower visitor was *B. diversus*.

I built the original long-term video recording system and recorded the side views of the flowers continuously during their entire antheses. I used the digital video cameras (DCR-TRV900, HDR XR-500V, and HDR XR-520V: Sony, Tokyo, Japan). I used a progressive video format that produced 30 picture frames per second. Therefore, the recordings started at least one hour before the flower opening and continued until after the falling of petals. I fixed softly the recorded flowers to avoid big vibrations by wind.

The videos were analyzed digitally by computer in the laboratory. All scenes of bumblebee flower visits were individually extracted from the movies by a motion detection software program, UFO Capture (SonotaCo, Japan), which had been prepared in advance to detect bumblebee flower visits. To avoid overlooking any flower visits, I set the sensitivity of the detection slightly higher. All of the captured scenes were also checked visually and invalid detections were removed from the analyses. The video scenes of the visits were arranged as sequential data along the occurrence time on each flower. The behaviors on video scene were played in about tenfold slow speed on a computer display and were observed by the naked eyes. Then all flower visits of bumblebees were defined as either an acceptance or a rejection. If the bumblebee landed on the flower and then probed for nectar, the visit was recorded as an 'acceptance'. If the flowers were approached and then not landed upon, or were landed upon only briefly, the visit was recorded as a 'rejection'.

By the long-term video recording system mentioned above, all flower visitors during the entire antheses on 19 flowers of *Impatiens textori* were completely recorded. I examined the behaviors of the *Bombus diversus* only on the fine days in this paper.

In this observation from the recorded video, I counted the number of events either acceptance or rejection occurred after a bumblebee probed on a flower during the entire anthesis according to the experimental design in figure 5.1. I also counted the times elapsed since last probing and the next behavior, and then I converted the numerical value of frames into second. After that, I checked temporal change in the proportion of acceptances and rejections after the initial probing.



Figure 5.1

Experimental design for analyzing relationship between the initial probing and the next visits either acceptance (probing) or rejection (landing, touching and hovering) during the entire anthesis. I analyzed time since last probing and next visits.

5.4 Results

I detected 909 next visits just after the initial probing during the entire antheses of 19 *Impatiens* flowers. Bumblebee showed acceptances and rejections behaviors, 473 and 436 times respectively and the landing, touching and hovering behavior has been found 133, 83 and 220 times respectively in rejection behaviors after initial probing (Fig. 5.2).

Frequency of visits regardless of whether acceptances or rejections occurred declined after one last Probing event with time (Fig. 5.2). The number of the rejection behaviors of bumblebees remarkably decreased during the first 5 minutes and then became much lower at 15 minutes after the initial probing (Fig.5.2). But, the more time passed after initial Probing, the proportions of Acceptances and Rejections increased and decreased respectively up to a certain point of time as evident in the figure 5.3. Within 30 seconds of one acceptance event (Probing), bumblebees rejected the flower in more than 80% of the total approaches and accepted in less than 20% occasions. But with gradual increase of non-visit time period, acceptances increased and rejections decreased. At around 15 minutes of time interval, proportion of acceptances reached almost 100%.



Fig.5.2

Frequency of next visits (probing, landing, touching & hovering) within each 30s after an initial probing of bumblebee on *Impatiens* flowers.



Fig. 5.3

Temporal changes in the proportion of next visits (acceptances and rejections) within 30s after the initial probing. Black and white area shows the proportion of rejections and acceptance respectively. The rejection behaviors of bumblebees obviously interchanged with that of acceptance during these 15 minutes.

5.5 Discussion

The result shows that bumblebee rejected almost all of the flowers just after initial probing and gradual increase of non-visit time period, the acceptances increased and the rejections decreased gradually in *I. textori* and at around 15 minutes after the initial probing, the rejection became very small. To the knowledge, this is the first attempt to clarify the mechanism of accepting and rejecting of a flower analyzing bumblebee behaviors after initial probing at each 30s.

It is well known that bumblebee shows rejection behavior in recently probed flowers (Cameron 1981; Marden 1984; Kato 1988; Schmitt and Bertsch 1990; Goulson *et al.* 1998; Stout *et al.* 1998; Goulson 2010). In the study, almost half of the next visits were rejections (436 times). Among the rejections, hovering was the most frequent rejection behavior that might be a timesaving way, by which they can increase their reward in a unit of time (Kato 1998; Schmitt & Bertsch 1990; Goulson *et al.* 1998). Bumblebees also showed a notable number of other rejection behaviors, touching (83) and landing (133). The scent mark left by previous visitors to flowers is considered to be a possible cue for the rejection behavior (Cameron 1981; Marden 1984; Kato 1988; Schmitt and Bertsch 1990; Goulson *et al.* 1998; Stout *et al.* 1998; Goulson 2010). If bumblebees use scent mark as a clue, the result interprets that the strength of scent marks wanes over time as rejection gradually disappeared. I assume that the hovering occurs mainly in flowers that have stronger scent mark than flowers that have not been visited for a long while. Therefore, the bumblebees may stop their flower visits depending on the strength of the scent marks. When the scent

strength becomes weak, bumblebees may show touching and then landing behaviors. It may provide a new viewpoint in relation to the threshold evaporating volatile chemicals which affect the bumblebee behaviors.

In this study, the rejection became very small at around 15 minutes in *I. Textori* that prompt us to determine this duration as the repellency duration of scent mark for the bumblebees. However, the repellency duration of scent mark varies from 2 minutes on Borago officinalis (Williams 1998) to 24 hours on Lotus corniculatus (Stout & Goulson 2002). Borago officinalis has an unusually high rate of nectar secretion while Lotus corniculatus has a low nectar secretion rate. In another study on Symphytum officinale, Bombus terrestris, the rejection response had disappeared by 40 min nearly equals the time of refilling nectar (40-60 min) in S. officinale (Stout et al. 1998). Thus, the duration of repellency duration may have relationship with nectar secretion rate (Williams 1998; Stout & Goulson 2002; Goulson 2010). The data strongly suggested that repellency duration for the bumblebees at I. textori was around 15 minutes. Therefore, the amount of nectar refilled within 15 minutes in *I. textori* may be the ESS (Evolutionary Stable Strategy) point for the foraging bumblebees in a competition for gathering reward. Again, the intensity of insect scent may vary according to nectar secretion rate as bumblebee may learn appropriate concentration of scent mark (Stout et al. 1998). Therefore, the relationship between nectar secretion rate and repellency duration may be indirect relationship. The further study will focus on nectar secretion rate of *Impatiens textori* as I recently developed the method of non-destructive and continuous observation of nectar volume (Raihan & Kawakubo 2013).

Although the proportion of acceptance was few, I found some number of

acceptances just after initial probing. It may happen for the unmatured bumblebee workers who did not learn yet to associate the intensity of scent mark with the reward available. Again, If visitation rates are high or flowers are scarce, bees would be less choosy (i.e. have a lower threshold for acceptance of a flower) and hence be more likely to accept flowers which were visited quite recently.

Chapter 6

Nondestructive and Continuous Observation of Nectar Volume

6.1 Abstract

To observe temporal changes in the nectar volume of *Impatiens* flowers, I modified and used an interval-shooting camera with a special flash system. *Impatiens* flowers secrete and preserve nectar in their long, curved spur. Therefore, the former method of measuring the nectar volume inevitably necessitated destruction or damage of the floral parts. As a consequence, accurate continuous measurements of nectar volume under natural conditions have been difficult. While considering how to overcome this problem, I noticed that when flowers were observed against transmitted light from the sun, a silhouette of nectar was visible inside the spur. To exploit this phenomenon, I attached a polymer optical fiber to the built-in flash of a compact digital camera and bent the fiber towards the camera's lens to provide backlighting. To record the temporal changes in nectar volume during the entire process of anthesis, I took interval images of the nectar silhouettes created using the backlight and estimated the nectar volume from the size of the nectar silhouette in the spur. To The knowledge, this is the first reported method involving the use of an interval-shooting camera for accurate measurement of nectar volume *in situ*.

6.2 Introduction

The volume of floral nectar, as well as the sugar content and energy value of the nectar have often been measured in ecological studies of flowers (Zimmerman 1988; Kearns & Inouye 1993; Dungan *et al.* 2004). Moreover, attention has been drawn to the temporal changes in nectar volume, especially when attempting to understand plant-animal interactions in pollination biology (Bolten & Feinsinger 1978). For measurement of nectar volume, various techniques for sampling of nectar directly from flowers have been developed.

In many studies, nectar was withdrawn from flowers using a micropipette or micro-capillary tube (Collins & Newland 1986; McKenna & Thomson 1988; Kearns & Inouye 1993; Lanza *et al.* 1995; Corbet 2003; Tschapka 2004), or a power-driven aspirator (Armstrong & Paton 1990). In some other studies, the flower was centrifuged (Swanson & Shuel 1949; Armstrong & Paton 1990) or washed in a fixed amount of distilled water (Käpylä 1978; Grünfeld *et al.* 1989; Mallick 2000). Absorption of nectar using filter-paper wicks has also been reported (Kearns & Inouye 1993; Dósa 2003; Dósa 2008; Dósa & Matheisz 2001).

The effectiveness of these techniques naturally differs according to floral morphology, nectar characteristics and field conditions (Bolten & Feinsinger 1978; Kearns & Inouye 1993; Lloyd *et al.* 2002). Furthermore, the accuracy of nectar volume measurement employing some of these techniques is questionable, as destruction or damage of floral parts is unavoidable. Therefore, it is important to choose a technique that is appropriate for the aim of each individual study, and to apply it carefully for accurate nectar sampling (Lloyd *et al.* 2002).

However, these techniques may not preclude minor damage to *Impatiens* flowers because their nectar is concealed within a curved and thin spur. For example, probing with a glass capillary tube or absorption using paper wicks might damage the nectary tissue, even if these materials make only slight contact with the tissue. Even minor damage to the cell walls of nectary tissue might cause the cytoplasmic content to flow out into the nectar, thus altering the physiological function of the nectary (Wilmer 1980). Moreover, these techniques require the removal of nectar, which limits the possibility of observing temporal changes in nectar volume.

The temporal changes in nectar volume have been recorded to clarify the nectar secretion pattern of flowers. Ideally, the same flower is used continuously or repeatedly for such measurements without removing the nectar. In fact, up to now, very little information has been obtained about temporal changes in the nectar volume of a single flower throughout its anthesis in the absence of floral damage. Especially in the case of *Impatiens* flowers with their curved thin spurs, it has not been possible to clarify temporal changes in nectar volume without some degree of floral damage.

Against this background, I attempted to measure temporal changes in the nectar volume of *Impatiens* flowers without actually collecting the nectar. In order to do so, I specially modified the flash system of an interval-shooting camera to obtain silhouette images of the nectar using transmitted light from the flash. Here I describe the design of the special interval-shooting camera I employed, and its use for continuous observation of

nectar volume in Impatiens textori.

6.3 Materials and Methods

6.3.1 Impatiens flowers with curved, thin spurs

I used the flowers of *Impatiens textori* to measure temporal changes in the volume of nectar in their spurs. The nectar of the *Impatiens* flower is invisible from outside because it is stored nectar within the curved, thin spur. However, during observations in the field, I had often noticed that the nectar was visible as a silhouette when illuminated by transmitted light from the sun. The technique described here was developed from this concept.

6.3.2 Modification of a compact digital camera

I attempted to modify a compact digital camera (PENTAX Optio W10) to facilitate backlight-illuminated photography at any time or location. Although the camera employed is widely available commercially, it is waterproof and can take photographs at various time intervals. I attached a polymer optical fiber (Eska 0.75 mm: Mitsubishi Rayon) to the built-in flash of the camera and bent the fibers toward the camera lens to facilitate backlight illumination (Fig. 6.1). Approximately one hundred optical fibers 40 cm in length were bundled up by aluminum wires (Fig. 6.1A). Both ends of the bundle were sharply cut off, and one end was placed in contact with, and fixed to, the surface of the built-in camera flash using epoxy-forming paste (CEMEDINE epoxy paste for plastics) (Fig. 6.1C). The

wire bundling was flexible, allowing the fiber optics to direct the flash in various directions (Fig. 6.1B). Backlighting from this extended flash device allowed us to obtain a silhouette of the nectar stored inside the curved, thin spur of the *Impatiens* flower. In the field, the camera was fixed to a tripod, and an *I. textori* flower was positioned so that it faced the lens (Fig. 6.2A). A small plastic light diffuser was used to obtain a soft backlight suitable for a good-quality silhouette (Fig. 6.2B). To observe temporal changes in the nectar volume of *Impatiens* flowers, nectar silhouette photographs of seven flowers were taken using the modified camera at ten-minute intervals during the entire process of anthesis. As direct incident light from this extended flash light device is capable of damaging the image sensor of the camera, care was taken to use the camera only after the flower had been placed between the lens and the apex of the light device.

6.3.3 Estimation of nectar volume

I measured the length of the outside curve between the upper level of the nectar and the end of the spur (this was designated the nectar silhouette size (NSS): Fig. 6.3) in each interval photograph. The nectar volume was then estimated from this NSS value based on a regression curve (y=0.5773e0.172x, R2=0.9744; Fig.6.4) that was obtained by injecting various volumes of water into the empty spurs of flowers and measuring the NSS repeatedly (Fig.6.4).


Figure 6.1

The modified compact digital camera with the polymer optical fibers extending from the built-in flash. Front and top views of the modified camera are shown in A and B, respectively. For the modification, about one hundred optical fibers 40 cm in length were bundled up by aluminum wires. The wires allowed the bundle to be bent in various directions easily. Epoxy-forming paste (CEMEDINE epoxy paste for plastics) was used for fixing the fiber bundle.



Figure 6.2

The camera with the modified flash device set on a tripod in the field. A flower of *Impatiens textori* was set facing the camera lens. A: an *Impatiens textori* flower, B: a small plastic light diffuser. The small plastic light diffuser created good lighting conditions for silhouetting the nectar.





The silhouette of the nectar stored inside the curved spur of an *Impatiens textori* flower. The square in A shows the spur of a flower. B: The nectar silhouette inside the spur. The arrow in B indicates the upper surface of the nectar. The gray area in C shows the nectar inside the spur. The bold black line shows the length of NSS (Nectar Silhouette Size; see text).



Figure 6.4

A regression curve for estimation of the nectar volume based on the NSS (Nectar Silhouette Size). The NSS was measured repeatedly by adding various known volumes of water to the empty spurs.



Figure 6.5

Temporal changes in nectar volume in a typical *Impatiens textori* flower. A sawtoothshaped line was evident in the daytime (from 5:30 to 17:30), indicating increases and decreases in the nectar volume of the flower. The increasing line indicates continuous secretion of nectar and a sudden drop of the line indicates probing by bumblebees. At night, the line was flat and there was no nectar secretion after refilling. The maximum volume of nectar refilling was about .3 μ l in this flower.

6.4 Results and Discussion

The modified camera recorded good-quality photographs of the nectar silhouette in the spur (Fig. 6.3) every ten minutes during the entire process of anthesis in seven flowers. Since these flowers remained open for two or three days, I obtained more than 350 sequential photographs for each flower. The cameras recorded a total interval of about 409 hours for the seven flowers successfully.

Figure 5 shows one example of the temporal changes in nectar volume during the entire anthesis period in *Impatiens* (Raihan and Kawakubo, unpublished). The sawtooth-shaped line in the daytime (from 5:30 to 17:30) apparently indicated increases and decreases in the nectar volume of the flower with time. The increase in the line represented continuous secretion of the nectar, and any sudden drop clearly represented probing by bumblebees. Moreover, surprisingly, the flat line evident at night showed that the volume of nectar did not continue to increase at that time, reaching a maximum of about 4.3 μ l.

The new recording system using this modified camera was able to reveal the dynamic status of nectar volume in *Impatiens textori*. A similar result would probably have been obtained using another backlighting system, but this may have required a more extensive and more complex setup. For example, major camera manufacturers already provide remote flash light devices. Recently, a wireless flash light commander capable of controlling remote flash light units at the same time has become available (e.g., Nikon SU-800, Canon ST-E3-RT), and this would make backlighting easier. However, continuous shooting in the field using such systems would not be suitable because the extra devices

needed for the power supply and waterproofing features would not be simple. The system using a modified compact digital camera did not need any extra batteries or rain-covers during field recordings. Since the system is compact and light, even a small tripod can maintain the position of the camera.

Although the model of camera on which I based this system has now been discontinued (PENTAX Optio W10, 2006 model), newer models with almost the same functions are still available for the same purpose. If a camera has both time intervalshooting and waterproofing features, any researcher would be able to modify it using the same method as that described here. In addition, such non-destructive and continuous observation with a simple backlighting flash may provide various opportunities for field research observers such as naturalists. The system may be applicable for recording changes within certain structures and/or motion within biological organs or tissues. I believe that this camera system can be applied for various study purposes over a wide range of biological fields.

Details of the nectar secretion patterns of *I. textori* will be presented and discussed in The next work, since I have already obtained good-quality data on temporal changes in nectar volume for six other flowers.

Chapter 7

General Discussion

General Discussion

In pollination biology, the video recordings of flower visitors are now one of the usual methods. However, the long-term video recording covering the entire anthesis of a flower had probably never done, especially in the field with the weather changes. The long-term video recording on the *Impatiens* flowers recorded the floral ecological features and all flower visitors on each flower. By these video data, I could observe and analyze the flower visitor behaviors in the both fine and long scale in time. Moreover, I could reconfirm the observation results repeatedly on the display monitor only by using the replay. The present study could not be done only by naked eyes and I believe the long-term video recording method must be one of the powerful tools in the pollination biology.

By observing flower-visiting behaviors of bumblebees based on long-term video recording, I recognized four-types of behaviors (fig.2.8) broadly sorted into two behaviors, acceptances or rejections considering their availability for the pollen transfer. The most frequent behaviors were probing, flower acceptance behaviors. Beside the probing behavior, *B. diversus* showed three-types of the flower rejection behaviors such as hovering, touching and landing behaviors. To my knowledge, it is a first take on to sort the bumblebee behaviors based on observations in the field by long-term video recording for the entire period of flower anthesis.

The flowers attracted many bumblebees (more than 89 visits), and kept the enough frequencies of visits and probings until flowers end despite having differences in floral longevities. Since the ecological relationship between *Impatiens* flowers and the

bumblebees is substantial, one hundred visits may not be so large (Table 2.1). The large number of bumblebee probings may affect the outcrossing rate of *I. textori* directly. In addition, the number of visits declined a little on the third day (Fig.2.9) though the visits and probings were frequent until the flower end. It suggests that *I. textori* continues nectar secretion as the enough reward for pollen vectors until the end. At the same time, the frequent probings of bumblebee on a flower suggested the need of remarkable structural strength of flowers for the reproductive success *of I. textori* by the outcrossing.

In this thesis, I discussed the relationship between the flower visits and probing. The number of probing by bumblebees does not depend on the number of visits (Fig. 2.12). If bumblebee visits increase, probing do not necessarily increased. Again, if bumblebee visits very few, there are no guarantee to probe in all the case. In addition, I did not observe any obvious correlation between the frequency of probing and rejection in an hour. Therefore, it may depend on the other factors like nectar scent - scent of floral nectar itself or fermentation products from yeasts in the nectar (Crane 1975; Williams *et al.* 1981; Goulson 2010) or scent of bumblebee itself (Cameron 1981; Marden 1984; Kato 1988; Schmitt & Bertsch 1990; Goulson *et al.* 1998; Stout *et al.* 1998).

However, bumblebees showed acceptance and rejection behavior for any type (NFs or CFs) of *Impatiens* flower, irrespective of the presence of nectar. I confirmed it by the observations of the same individual bumblebee visiting many flowers (Table 3.5). As a result, I understood that nectar scent cannot act as a cue in the relationship between *B*. *diversus* and *I. textori*. And so, I propose the scent mark left by previous visitors can be a more likely cue for rejection behavior on nectar-less flowers than is a previous

remembrance of the absence of nectar. It is now the well-known that bumblebees reject flowers by using the scent mark of previous visitor (Cameron 1981; Marden 1984; Kato 1988; Schmitt & Bertsch 1990; Goulson *et al.* 1998; Stout *et al.* 1998). The scent mark is recognized as the most important cue used to decide whether to probe or reject the flowers (Goulson 2010). In these circumstances, I tried to detect the presence of scent mark left by previous flower visitor in the field. The results was about to specify the presence of scent mark in the field and "Tetradecanoic Acid" and "n-Hexadecanoic Acid" may be the main components of scent mark. These compounds are used as repellent compositions for repelling animals such as deer according to United States Patent (Patent Number: 4818535, Date of Patent: April 4, 1989). I assume that scent mark of bumblebee may consist these two compounds in greater portion that act as a repellent mark for bumblebees to reject the recently visited flowers. These two components may be volatile as the scent mark is consisting volatile chemical components (Schmitt *et al.* 1991). Therefore, their strength may decrease gradually by evaporation.

The bumblebees stopped their visits probably depend on the strength of the scent marks. Hovering may occur in recently emptied flowers that have stronger scent mark than flowers that have not been visited for a long while. Hovering was the most frequent rejection behavior that might be a timesaving way, by which they can increase their reward in a unit of time (Kato 1998; Schmitt & Bertsch 1990; Goulson *et al.* 1998). When the scent strength becomes weak, bumblebees may show touching and then landing behaviors. Although these rejection behaviors, touching and landing , had not been detected precisely by the naked eyes, I revealed that these behaviors were not rare and occupied

approximately 21% of all flower visits of bumblebees (319!1527 visits). If these two behaviors are recognized as the intermediate ones between the typical probing and the typical hovering, it may provide a new viewpoint in relation to the threshold evaporating volatile chemicals which affect the bumblebee behaviors.

In this study, the air temperature varied from 10°C to 25°C (Fig. 2.10). If the repellent scent marks are volatile, the evaporation rate may depend on the temperature. Under such circumstances, when temperature is high, scent mark may evaporate rapidly, and the number of probing in an hour should increase. However, even around noon when air temperature becomes highest on a day generally, an apparent high probing rate (or a low rejection rate) did not occur. Such small amount of air temperature range (10°C to 25°C) may not promote evaporating the scent marks. In other words, the strength of scent marks may not depend on air temperature but may depend on the stay length of previous visitor.

The length of stay on CFs (empty flowers) may include a positioning period for the probing and detection of the no nectar situation, before the bumblebee eventually abandons its attempt to obtain nectar. Therefore, the difference between the lengths of stay (1-2 seconds) may be equivalent to the time required to drink nectar. Moreover, this difference may account for the function of nectar, which detains the bumblebee longer on a flower. A longer stay on a flower might enhance pollen deposition and removal (Thomson & Plowright 1980; Feinsinger 1983; Galen & Plowright 1985; Thomson 1986; Lanza et al. 1995). The length of stay does not represent the amount of nectar directly since many bumblebees spent two to five seconds for a probing on a flower regardless of the probing frequency in an hour. Bumblebees need at least two seconds in probing behavior in a once

even if quantity of nectar is little. I found some long stay length probably having differences in drinking speed of each bumblebee. The amount of nectar at the time of each probing is unknown. Therefore, it will be interesting if any further study attempt to make relationship between the real amount of nectar and bumblebee behavior.

In this thesis, I showed that bumblebee rejected almost all the flowers just after initial probing. Gradual increase of non-visit time period, the acceptances increased and the rejections decreased gradually in *I. textori* and around 15 minutes after the initial probing, the rejection became very small. To my knowledge, this is the first attempt to clarify the mechanism of accepting and rejecting of a flower analyzing bumblebee behaviors after initial probing at each 30s. The result interprets the strength of scent marks wanes overtime as rejection gradually disappeared. In this thesis, I also determined the duration of repellency of scent mark for the bumblebees. However, this duration varies from 2 minutes on Borago officinalis (Williams 1998) to 24 hours on Lotus corniculatus (Stout & Goulson 2002). Borago officinalis has an unusually high rate of nectar secretion while Lotus corniculatus has a low nectar secretion rate. In another study on Symphytum officinale, Bombus terrestris, the rejection response had disappeared by 40 min nearly equals the time of refilling nectar (40-60 min) in S. officinale (Stout et al. 1998). Thus, the duration of repellency of scent mark may have relationship with nectar secretion rate (Williams 1998; Stout & Goulson 2002; Goulson 2010). The data strongly suggested the duration of repellency of scent mark for the bumblebees at *I. textori* was around 15 minutes. Therefore, the amount of nectar refilled within 15 minutes in *I. textori* may be the ESS (Evolutionary Stable Strategy) point for the foraging bumblebees in a competition for gathering nectar.

The intensity of insect scent may vary according to nectar secretion rate as bumblebee may learn appropriate concentration of scent mark (Stout *et al.* 1998). Therefore, the relationship between nectar secretion rate and the duration of repellency of scent mark may be indirect relationship. The further study needs to focus on continuous nectar secretion rate of *Impatiens textori*.

Finally, I wanted to examine the relationship between the rejection behaviors and floral nectar refill. However, it is not easy to clarify the temporal changes in nectar volume especially in *Impatiens* flowers without any floral damages, because *Impatiens* flowers secrete and preserve nectar in their long, curved thin spur. For such the nondestructive and continuous observation of the nectar volume, I modified and used an interval-shooting camera with a special flash system. I attached a polymer optical fiber to the built-in flash of a compact digital camera and bent the fiber towards the camera's lens to provide backlighting. To record the temporal changes in nectar volume during the entire process of anthesis, I took interval images of the nectar silhouettes created using the backlight and estimated the nectar volume from the size of the nectar silhouette in the spur. By using of this method for the estimation of the temporal changes in nectar volume, I believe that I can clarify the ecological relationship between the rejection behaviors of bumblebees and floral nectar secretion of *Impatiens* plants in the near future.

My studies clarified some new aspects of flower visit behaviors of *B. diversus* on *I. textori* plants, but the future analyses based on the sequential observation of pollinators and temporal changes of nectar on a flower throughout anthesis are needed to reveal the real situation of acceptance and rejection behaviors of bumblebees.

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Abbreviations

- CF: Nectar-spur Cut Flower
- CO₂: Carbon dioxide
- GC-MS: Gas chromatography-mass spectroscopy
- H: Hovering
- L: Landing
- NF: Normal Flower
- P: Probing
- SPME: Solid Phase Microextraction
- T: Touching

Academic Papers Relating the Dissertation

1. Raihan J. & Kawakubo N. (2013) Nondestructive and continuous observation of nectar volume using time-interval photography *Plant Species Biology*

2. Raihan J. & Kawakubo N. (2013) Ineffectiveness of nectar scent in generating bumblebee visits to flowers of *Impatiens textori*. *Plant Species Biology*