

Ecophysiological study on the alternative life cycles of males in the  
Japanese common grass yellow *Eurema mandarina*

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## Contents

<b>General Introduction</b> .....	3
<b>Chapter 1. Reproductive phenology of overwintering females</b>	
Introduction.....	6
Materials and methods.....	8
Results.....	10
Discussion.....	13
<b>Chapter 2. Overwintering success in autumn-form adults</b>	
Introduction.....	16
Materials and methods.....	18
Results.....	21
Discussion.....	25
<b>Chapter 3. Late autumn occurrence of non-diapause male adults in various geographical populations</b>	
Introduction.....	29
Materials and methods.....	31
Results.....	35
Discussion.....	37
<b>Chapter 4. Effect of mating on survival at low temperature in autumn-form females</b>	
Introduction.....	41
Materials and methods.....	43
Results.....	45

Discussion.....	46
<b>General Discussion.....</b>	<b>50</b>
<b>Acknowledgements.....</b>	<b>55</b>
<b>References.....</b>	<b>56</b>
<b>Tables.....</b>	<b>64</b>
<b>Figures.....</b>	<b>78</b>

## GENERAL INTRODUCTION

Seasonal adaptation is a base of the prosperity of insects in the terrestrial ecosystem (Tauber *et al.* 1986; Danks 1987; Tanaka *et al.* 2004). Environmental elements such as temperature, humidity, abundance of foods and predators vary with time, especially for small poikilotherms like insects (Tauber & Tauber 1978; Danks 1994). In the Temperate Zone, most insects evolved diapause to survive winter, which is an unfavorable season for growth and reproduction because of low temperature, fallen snow, and food limitation (Tauber *et al.* 1986; Danks 1987). Diapause is a hormonally controlled arrest of development, and diapause and non-diapause insects often show drastically different morphological, physiological and behavioral features even within a species (Shapiro 1984; Tauber *et al.* 1986; Danks 1987).

Insects show a remarkable evolutionary plasticity and endless diversity in seasonal adaptation (Masaki & Wipking 1994). Although diapause is the core of seasonal adaptation, its incidence, maintenance, termination, intensity and developmental stage differ from species to species. Geographic variation in seasonal adaptation is also common in insects (Danilevsky 1966), and a polymorphic strategy has been often reported even within a population (Pener 1992; Masaki & Wipking 1994). However, adaptive significance of many intraspecific variations remains unclear (Ishihara & Seko 2007), although knowledge of the evolution of seasonal adaptation is a fundamental component for overall understanding of insects (Tauber & Tauber 1978).

The evolution of male reproductive diapause is an unsolved problem. Reproductive diapause is apparently important for both females and males when they overwinter as adults. However, male adults can produce offspring without diapause by pre-overwintering copulation, contrary to females (Kubrak *et al.* 2016). Males that have mated in autumn die before winter, but their sperm overwinter in the spermatheca of females and fertilize eggs in the following spring. Therefore, adult-overwintering insects can be classified into three types, i.e. species in which only females enter diapause, species in which all males and females enter

diapause, and species in which females and a part of males enter diapause (Pener 1992). The first type of insects is common in social wasps, some mosquitoes and predatory bugs (Tauber *et al.* 1986; Kobayashi & Osakabe 2009). The second type is the major strategy in adult-overwintering insects (Tauber *et al.* 1986). A few species in Orthoptera and Lepidoptera are classified into the third type (Fukuda *et al.* 1982; Pener 1992; Leong *et al.* 2012). Although Pener (1992) noted that non-diapause males emerging in late autumn may relate to female receptivity before winter, it is unclear which factors are responsible for the evolution of the mating system characterized by emergence of non-diapause males and female receptivity before winter.

The Japanese common grass yellow *Eurema mandarina* (de l'Orza) is a typical butterfly species classified into the third type, in which a part of males enter diapause. This species shows seasonal polyphenism consisting of the non-diapause summer-form and diapause autumn-form adults (Fukuda *et al.* 1982). *Eurema mandarina* has three direct developing generations and an overwintering generation in central Japan, and all males and females emerge as summer-form adults in the direct developing generations (Kato 1986). Autumn-form adults emerge only in the overwintering generation. Although short-day conditions and low temperature induce autumn-form adults (Yata 1974), approximately a half of males are insensitive to such conditions and emerge as summer-form adults, whereas all females emerge as autumn-form adults (Kato & Sano 1987). Therefore, summer-form males coexist with autumn-form males and females in late autumn in central Japan. Autumn-form females mate with summer-form males before winter, and re-mate with autumn-form males in the following spring (Kato 1989).

Here I aimed to reveal factors that have favored the evolution of non-diapause males in late autumn in *E. mandarina*. I particularly focused on an abiotic factor, winter severity, and two social factors, adaptive significance of pre-overwintering copulation in females and competition between summer- and autumn-form males. Winter severity is an important factor inducing the evolution of diapause in general. Adaptive significance of pre-overwintering

copulation of females is also important to understand the evolution of non-diapause males in late autumn, because summer-form males cannot obtain offspring when females have no receptivity for pre-overwintering copulation. Competition between summer- and autumn-form males must determine the fitness of each type. In Chapter 1, I described reproductive phenology of autumn-form females. Schedule of mating and oviposition is related to adaptive significance of pre-overwintering copulation of females and the pattern of male-male competition. In Chapter 2, overwintering success of autumn-form adults was estimated to understand winter severity for *E. mandarina*. In Chapter 3, geographic patterns in frequencies of summer-form males in late autumn and pre-overwintering copulation of females were examined by a laboratory experiment and a field survey. In Chapter 4, adaptive significance of pre-overwintering copulation of females was revealed by a laboratory experiment. From the results, I suggested that low overwintering success of autumn-form adults may have favored the evolution of pre-overwintering copulation of females. The frequency of both forms of males in late autumn depends on the paternity in sperm competition rather than overwintering success of autumn-form adults.

## Chapter 1.

### Reproductive phenology of overwintering females

#### INTRODUCTION

Winter is an unsuitable season for temperate insects to be active because of the low temperatures and intermittent snow, mortality increases greatly during overwintering, and many species including butterflies remain in inactive stages such as eggs or pupae in order to hibernate. Host plants for larvae are not widely available during the winter season in temperate zones, nor are nectar plants available for adults. Consequently, any reproductive behavior of adult butterflies during the winter is not adaptive. However, some species of butterflies, such as *Polygonia c-album* (L.), overwinter in the adult stage with reproductive diapause, involving the arrest of reproductive organs by hormonal control (Nylin 1992; Tatar & Yin 2001).

The Japanese common grass yellow *Eurema mandarina* (de l'Orza) is a butterfly that shows a seasonal polyphenism in wing color consisting of summer-forms and autumn-forms. Low temperature and short day conditions during larval stage induce autumn-form adults, and the sequential low temperature leads them to overwinter with reproductive diapause (Yata 1974). Only autumn-form adults are observed in the following spring (Fukuda *et al.* 1982). Because approximately a half of males are insensitive to short day conditions (Kato & Sano 1987), summer-form males but not females fly with both autumn-form males and females in October to November (Kato 1986). While autumn-form males show little mating activity in the autumn (Kato 1989), mating between summer-form males and autumn-form females has frequently observed (e.g., Tomioka 1970). Kato (1986) reported that before overwintering most autumn-form females already contained a single spermatophore in their bursa copulatrix, and the number of spermatophores was found to have increased after overwintering. Therefore, autumn-form females re-mate with autumn-form males during the

following spring.

Without examining substances transferred by males in detail, Kato (1986) proposed two hypotheses for the adaptive significance of the multiple mating of females with both summer- and autumn-form males: the nuptial gift hypothesis and the risk-hedge hypothesis. Because the spermatophore transferred from the male contains accessory gland secretions (Leopold 1976), the mated females absorb nutrients such as protein from the secretions and probably the entire spermatophore for egg maturation and/or somatic maintenance (Boggs & Gilbert 1979; Boggs & Watt 1981). Although autumn-form females of *E. mandarina* are sometimes active at sunflecks in deciduous forests during the winter (Fukuda *et al.* 1982), they must not obtain nutrients or energy because of the absence of flowering plants. Thus, the spermatophore derived from the summer-form males might be an important nutrient source for the females during overwintering. In addition, there is a risk for females that, after overwintering, they may encounter no males to mate with until the sprouting of the host plants because of the spring weather conditions or biological factors. Thus, it might be adaptive for such females to copulate with the summer-form males and lay eggs inseminated by their sperm.

The hypotheses proposed by Kato (1986) could be examined by dissecting wild autumn-form females collected before and after overwintering, because the number of spermatophores transferred, the amount of sperm stored and the number of eggs contained in each female provide information about the timing of remating and the onset of oviposition. However, there have been no data on the changes in the number of spermatozoa in the spermatheca or the number of eggs loaded in the autumn-form females after overwintering, possibly because of the low number of autumn-form females captured in the fields in early spring.

The timing of the remating with autumn-form males and the onset of oviposition in autumn-form females is also important for summer-form males that had mated before winter. When the autumn-form females re-mate with the autumn-form males before the



onset of oviposition, sperm derived from the summer-form males must be exposed to severe sperm competition, because last-male precedence is dominant in butterflies (Simmons 2001). Thus, when autumn-form females re-mate with autumn-form males before the onset of oviposition, summer-form males lose their advantage. If summer-form males overcome the last-male precedence, they fertilize eggs in the following spring without their own overwintering. Therefore, a counter-adaptation to increase paternity against the re-mating of the autumn-form females might evolve.

To clarify the reproductive strategy of the autumn-form females, the fate of the spermatophores from the summer-form males should be investigated. When the females remate after overwintering, the spermatophores in the bursa copulatrix could be identified with regard to the male by which they had been transferred. In this chapter, I captured autumn-form females before and after overwintering and dissected them to examine the number of eggs, number of spermatozoa in the spermatheca, and number and shape of the spermatophores in the bursa copulatrix. In addition, males of both forms were also collected, and their sperm bundles were examined. The adaptive significance of polyandry in autumn-form females and the possible counter-adaptation of summer-form males are also discussed.

## **MATERIALS AND METHODS**

In autumn and spring, *E. mandarina* is a ruderal butterfly that mainly inhabits open grasslands near deciduous forests, the banks of rivers and so on, where its host plants, *Lespedeza juncea* (L. f.), *L. bicolor* (Turcz.) and *Albizia julibrissin* (Durazz.), are found. In Tsukuba City, Ibaraki Prefecture, Japan, on sunny days in November and from March to May in 2013, I patrolled along the margins of deciduous forests around Mt. Tsukuba to search for female butterflies in flight. When encountered, I gently collected the butterflies using a net and put them into glassine envelopes individually. They were

dissected under a stereoscopic microscope on the day after their capture, after the forewing length had been measured by digital vernier calipers (accuracy 0.1 mm) as an indicator of body size.

The number of spermatophores in the bursa copulatrix was counted. Each spermatophore mass was measured using an electric balance (Mettler Toledo, AE240, accuracy 0.01 mg). The largest spermatophore in the bursa copulatrix was identified to be the spermatophore derived from the last male copulating. Because the females absorb the spermatophores gradually after copulation, the stage of spermatophore collapse in the bursa copulatrix can be used to determine the relative length of the time since the spermatophore was transferred from the male. The largest spermatophore among the spermatophores found in the bursa copulatrix of each female was classified into one of four stages on the basis of its shape to assess when the last mating occurred (Fig. 1). A spermatophore at stage I was defined as an intact one that might have been recently transferred by a male and had not yet been depleted by the female. A spermatophore at stage II was spherical without the neck. Because the spermatophore body was intact, it might also have been derived from a male with which the female had recently mated. A spermatophore at stage III had a concave shape, like a bowl, resulting from female absorption. A spermatophore at stage IV was largely destroyed, suggesting that a considerably long time had passed since the mating.

The spermatheca was dissected in a saline solution (Ringer's solution for insects) on a glass slide. All of the ejaculates in spermatheca were washed out into a small tube using a known volume of saline solution. The tube was gently stirred in order to homogenize the mixture. Six 10- $\mu$ l subsamples were removed from each sample using an autopipette and allowed to dry on glass slides under dust covers. The dried slides were dipped for 5 s in distilled water and allowed to dry again. Eupyrene and apyrene spermatozoa in each subsample were counted on black paper under a stereoscopic microscope ( $\times 100$ ). The numbers of eupyrene and apyrene sperm in the spermatheca were calculated by multiplying the average sperm count in 10  $\mu$ l of the suspension.

Eggs in the ovaries were counted and classified into three groups: mature eggs, submature eggs and immature eggs as reported by Watanabe and Ando (1993). A mature egg was white with a hard eggshell. A submature egg was filled with white yolk and without an eggshell. An immature egg included oocytes and had little yolk. The numbers of mature eggs and submature eggs were counted directly. The total number of immature eggs was estimated from the number of immature eggs per ovariole and the number of ovarioles.

Summer- and autumn-form males collected in November 2013 were dissected in saline solution to remove eupyrene sperm bundles from their duplex. Photographs of five eupyrene sperm bundles per male were taken under the microscope, and their lengths were measured using Image J 1.48 (Rasband 2014) (accuracy 10  $\mu\text{m}$ ).

All statistical analysis was carried out using R version 3.0.2. (R Developmental Team 2013). The Mann-Whitney *U* test with Bonferroni correction was used to compare the number of eggs and sperms between the pre-overwinter period and each period of post-overwinter. The Kruskal-Wallis test was used in the analysis on the number of eupyrene sperm among females classified by collection period and the stage of the largest spermatophore. For the comparison of sperm lengths between forms, the Mann-Whitney *U* test was used.

## **RESULTS**

Twelve and 49 females were collected in the pre- and post-overwintering periods, respectively (Table 1). The average length of the forewing was around 22 mm in each collection period. Since the forewing length was regarded as an indicator of the body size of the females, no biased body size variation was found throughout the survey period. Therefore, the mortality of females during winter did not depend on the body size.

Out of 12 females collected in November 5 had very fresh wings with fine tears and lustrous scales, and may have just emerged. They had no spermatophores and were

identified as virgins. On the other hand, seven females contained a single spermatophore in the bursa copulatrix, although they still had fresh wings (Table 1). The number of spermatophores in the bursa copulatrix significantly increased after overwintering, and the total spermatophore mass was also significantly different from that in pre-overwintering females. The females collected in early May were aged compared with females collected in the pre-overwinter period, judging from bodies and wings with broken or extensive tears and highly frayed scales. They contained 2.23 spermatophores on average, with a maximum mating number of four in one female. Since I sometimes detected very tiny traces of spermatophore in the bursa copulatrix, females might often absorb spermatophores completely. Thus, the number of spermatophores in the females tended to be underestimated.

Each female collected in the pre-overwintering period contained no mature and no submature eggs (Table 1). They had 390 immature eggs. After overwintering, most females loaded mature eggs and submature eggs in late March, indicating that they started to produce mature eggs after overwintering. The number of mature and submature eggs then decreased somewhat in the females collected in late April and early May. A drastic change in the number of immature eggs was observed in the females collected in late April. Females collected in early May loaded 186 immature eggs. The number of immature eggs significantly decreased beginning in late April ( $P < 0.001$ ). Therefore, the onset of oviposition in the autumn-form females occurred in mid-April, and their lifetime number of eggs actually laid was about 200 eggs.

For mated females in the pre-overwintering period, spermatophores at stages II and III were identified (Fig. 2). Because most autumn-form males do not show sexual activity, spermatophores in the pre-overwintering females were derived from the summer-form males. On the other hand, in late March, the largest spermatophore contained in the bursa copulatrix in 58 % of females was in stage I or II. These females were identified as females that had copulated with the autumn-form males after overwintering. In 29 % of females, the

largest spermatophore contained in the bursa copulatrix was in stage III. Although a spermatophore would not have reached this stage of collapse until more than 1 week after copulation, there was no definitive information on whether these females re-mated or not after overwintering, because cool weather might inhibit adult activity (particularly mating behavior) and the adult physiology responsible for the metabolism. In the other 13 % of females, the largest spermatophore was at stage IV. These spermatophores were received before overwintering, i.e., the spermatophore was derived from the summer-form males.

In a half of the females collected in early April, the largest spermatophore was at stage I or II and was derived from autumn-form males with which they had mated recently. Spermatophores at stage III in this period were also derived from autumn-form males, probably including a few summer-form males. Out of 14 females, only 1 did not re-mate, judging from the fact that its largest spermatophore was at stage IV. Therefore, most females had already re-mated before the onset of oviposition. After the onset of oviposition (late April and early May), the largest spermatophores found were at stages I and II in 53 % (late April) and 62 % (early May) of females, respectively, showing that they had repeatedly mated during the oviposition period.

Females that contained spermatophores at stage I or II stored apyrene sperm as well as eupyrene sperm in the spermatheca (Fig. 3). Females in the pre-overwintering period that contained a spermatophore at stage II stored about 1,030 eupyrene and 220 apyrene spermatozoa, while those that contained a spermatophore at stage III had about 1,340 eupyrene spermatozoa without any apyrene spermatozoa. After overwintering, females in each period stored more than 1,000 eupyrene spermatozoa in the spermatheca. However, the females in which the largest spermatophore was at stage I stored 140 apyrene spermatozoa. After the onset of oviposition, the number of eupyrene spermatozoa stored in each female was also around 1,000. Change in the number of eupyrene spermatozoa was not significant throughout all collection periods and the stage of the largest spermatophore (Kruskal-Wallis test, n.s.).

The eupyrene sperm bundles of summer-form males were significantly longer than those of autumn-form males. The average lengths of the eupyrene sperm bundles were  $1,410 \pm 20 \mu\text{m}$  ( $n = 7, \pm \text{SE}$ ) and  $1,319 \pm 20 \mu\text{m}$  ( $n = 9, \pm \text{SE}$ ) for summer-form males and autumn-form males, respectively (Fig. 4).

## DISCUSSION

Pre-overwintering copulation by females was observed in several insect taxa, and its adaptive significance was discussed in the context of available nutrients or sperm for the females. For example, Roth and Reinhardt (2003) pointed out that females of the predatory bug, *Nabis rugosus* (L.), might evolve a sperm storage organ system to prevent the depletion of sperm received in the pre-overwintering period, because few males are encountered after overwintering. On the other hand, Koshiyama *et al.* (1994) suggested that females of the stink bug, *Menida scotti* (Puton), utilize the male-derived secretions as a nutritional source to enhance their survival and/or in egg oogenesis during hibernation. In most species hitherto reported, females copulate with males in both the pre- and post-overwintering periods, and post-overwintering copulation often occurs before the onset of oviposition.

In butterflies, females that received heavier or a larger number of spermatophores showed greater longevity and higher reproductive output in *Pieris napi* (L.) (Wiklund *et al.* 1993) and *Colias eurytheme* (Boisduval) (Rutowski *et al.* 1987). Prudic *et al.* (2011) suggested that females of *Bicyclus anynana* (Butler) in their dry seasonal form prolonged their longevity using nuptial gifts from males. Females of *Danaus plexippus* (L.) might use nutrients in the spermatophore during the overwintering period (Leong *et al.* 2012). Nuptial gifts to overwintering females are believed to be beneficial for males with which they had mated before overwintering because the “overwintering success of sperm” depends on the mortality of females (Stevens & McCauley 1989). Because overwintering insects suffer a

lack of nutrition (Danks 1987), females could use the spermatophores as nutrition for body maintenance during the winter. Therefore, spermatophores derived from summer-form males in *E. mandarina* may reduce the mortality of females during the winter.

Kato (1986) reported that the mean numbers of spermatophores in the bursa copulatrix of autumn-form females before and after overwintering were 1.0 and 1.5, respectively, suggesting that all autumn-form females mate with summer-form males before overwintering. In this chapter, some virgin females with very fresh wings had recently emerged and might still have been going to copulate before overwintering. They would then re-mate with autumn-form males after overwintering. Although they had stored a sufficient number of eupyrene spermatozoa to inseminate all of the eggs they had loaded, most autumn-form females re-mated before the onset of oviposition. The re-mating activity of autumn-form females might increase throughout the oviposition period. Thus, females that have mated in the pre-overwintering period may use spermatophores for egg maturation and as sources of nutrition during the overwintering period, supporting the nuptial-gift hypothesis.

In this chapter, most autumn-form females re-mated with autumn-form males before the onset of oviposition. After overwintering, the autumn-form males increased the activity on movement to search for autumn-form females (Kato 1989). Therefore, after overwintering, the females may have many opportunities to encounter autumn-form males, making the risk-hedge hypothesis unlikely.

Avoiding sperm competition is adaptive for males in polyandrous species (Simmons 2001). In butterflies, males attempt to reduce female receptivity to re-mating through the physical stimulus of ejaculating a large spermatophore (Sugawara 1979). However, the spermatophore derived from summer-form males was observed to have declined (stage IV) in the bursa copulatrix of females after overwintering, suggesting that it is difficult for summer-form males to prevent their mates from re-mating after overwintering using spermatophore size.

The cheap filler hypothesis, in which apyrene sperm in the spermatheca reduce female receptivity for re-mating, is a dominant explanation of the adaptive significance of apyrene sperm in the spermatheca. Cook and Wedell (1999) supported the hypothesis in *P. napi*: females storing a larger number of apyrene sperm in the spermatheca showed lower receptivity for re-mating. If apyrene sperm remain in the spermatheca after overwintering, summer-form males of *E. mandarina* might reduce the receptivity of females to re-mating with autumn-form males the following spring. However, apyrene sperm that arrived in the spermatheca gradually disappeared within 2 days after copulation in *Papilio xuthus* (L.) (Watanabe *et al.* 2000). In the females that might have mated recently in this chapter, apyrene spermatozoa might not have remained in the spermatheca for long. Consequently, there was little chance for summer-form males to avoid sperm competition against autumn-form males after overwintering. The larger sperm of summer-form males in comparison to that of autumn-form males might be a counter-adaptation to the mating strategy of autumn-form females. Detailed study of the sperm activity and insemination process in females after overwintering is needed.



## Chapter 2.

### Overwintering success in autumn-form adults

#### INTRODUCTION

Reproductive diapause is the hormonally induced arrested development of reproductive organs, and has evolved as a seasonal adaptation in overwintering adult insects (Nylin 2013). Overwintering insects are subjected to unfavorable conditions such as low temperatures, snow, and limited food and water availability (Calvert *et al.* 1983; Danks 1987; Brower *et al.* 2004), and, therefore, successful overwintering requires nutrients such as lipid reserves (Tuskes & Brower 1978; Pullin 1987; Alonso-Mejia *et al.* 1997). Reproductive diapause is important for females, because they must endure such conditions and invest in their own survival during winter to lay eggs in the following spring. However, it is not always important for male insects, because two alternative strategies are possible (Pener 1992; Kubrak *et al.* 2016). One is a combination of reproductive diapause and mating after overwintering, similar to the strategy in females. The other is production of offspring without a reproductive diapause by pre-overwintering copulation. In Lepidoptera, males of the pierid butterfly *Gonepteryx rhamni* (L.) show the former strategy (Wiklund *et al.* 1996), whereas most males of the lycaenid butterfly *Curetis acuta* Moore show the latter strategy (Shirouzu 2006). In the monarch butterfly *Danaus plexippus* (L.) (Leong *et al.* 2012) and the Japanese common grass yellow *Eurema mandarina* (de l'Orza) (Kato 1986), mating occurs in both autumn and spring. Factors for the evolution of male reproductive diapause are controversial.

*Eurema mandarina* is a suitable species for investigating the evolution of male reproductive diapause, because direct comparisons are possible between males emerging in the same season with and without reproductive diapause. This species shows multivoltinism and seasonal polyphenism, consisting of summer and autumn forms (Fukuda *et al.* 1982).

*Eurema mandarina* adults emerge as the summer form from June to September. A short

photoperiod and low temperature during the larval stage induce the development of autumn-form adults with reproductive diapause (Yata 1974). Some males, however, are insensitive to short photoperiods and low temperatures, and emerge as the summer-form even under these conditions (Kato & Sano 1987). Thus, the considerable number of summer-form males emerge with autumn-form adults from October to November in central Japan (Kato 1986), even though they are from the same cohort. In contrast to autumn-form adults, summer-form males cannot overwinter (Kato 2005).

Summer-form males and autumn-form females show high mating activity in late autumn (Kato 1989), although ovary development are clearly arrested until the following spring in autumn-form females (Chapter 1). Thus, most autumn-form females accept courtships of summer-form males before winter (Kato 1986). On the other hand, most autumn-form males show low mating activity in late autumn (Kato 2005), and their mating activity increases in the following spring (Kato 1989). In Chapter 1, I reported that most autumn-form females re-mate with autumn-form males before the onset of oviposition in spring. Therefore, sperm competition must occur between the summer- and autumn-form males. Because higher second-male sperm precedence is generally shown in Lepidoptera (Simmons 2001), autumn-form males may have an advantage over summer-form males. Deterioration of sperm derived from summer-form males is also possible during winter, but a half of males emerge as the summer-form under autumn-like conditions in the laboratory (Kato & Sano 1987), suggesting that summer-form males benefit from avoiding overwintering.

Emerging as a summer-form male in autumn seems advantageous because it improves the “overwintering success of sperm” (Watanabe 2016). If males suffer higher mortality than females during winter, overwintering success of sperm is higher in females than in males. Under these conditions, natural selection may favor males that copulate before overwintering, resulting in an increase of summer-form males. Although information on overwintering success in *E. mandarina* is deficient, Kato (1986) reported that the sex ratio of

autumn-form adults did not differ before and after winter. However, the sex ratio in individuals collected from the field is too sensitive to catchability of both sexes, depending on the activity of each sex and field conditions (e.g., weather and time of the day). Therefore, experiments under semi-natural conditions, such as using cages outdoors, are required to examine sexual differences in overwintering success in this species. In addition, the mark-release-recapture technique is valuable to estimate the degree of overwintering success in wild *E. mandarina* populations.

## MATERIALS AND METHODS

### *Overwintering success under semi-natural conditions*

In central Japan, autumn-form adults usually enter diapause in late November (Kato 1986). To obtain autumn-form adults immediately before overwintering, I patrolled the margins of rice fields that were surrounded by deciduous and coniferous forests in the afternoon of November 16, 2015 in Iwakura, Kyoto, Japan (35.09°N, 135.79°E). Seventeen males and 15 females of *E. mandarina* that were flying alone or foraging on nectar plants were caught with a net, individually placed into glassine envelopes, and brought to the laboratory at room temperature.

On November 17, their forewing length was measured as an index of body size using digital vernier calipers (0.1 mm accuracy), and their body mass was measured using an electric balance (0.01 mg accuracy; Mettler Toledo AG285). The overwintering experiment was started on November 17 based on Wiklund *et al.* (2003). Butterflies were individually placed in plastic cups (101 mm diameter and 44 mm depth) with net caps. The cups were placed in plastic insect cages (210×320×180 mm<sup>3</sup>, with a netlike lid) with shallow water for keeping high humidity. To avoid wind and direct sunlight, the cages were covered with newspaper and placed under the eaves of a building on Yoshida Campus of Kyoto University

(35.03°N, 135.78°E). The butterflies were not allowed to feed during the experiment.

The butterflies were observed once a week, and dead individuals were removed. The water was replenished as necessary to maintain high humidity. The experiment finished on the 16th week (March 7, 2016), when the maximum temperature exceeded 20°C, because autumn-form adults increase their flight activity at this temperature. Fisher's exact test was used to compare overwintering success between the sexes.

For butterflies that died before March 7, the factors that affected their survival were examined using generalized linear mixed models, with sex, forewing length, and body mass included as factors. Body mass was expected to be correlated with forewing length, and the models included only either body mass or forewing length to avoid multicollinearity. The most appropriate model was selected based on the small-sample-size-corrected version of the Akaike Information Criterion (AICc), and forewing length and body mass were compared by Student *t*-test. The analyses were performed using R version 3.0.2 (R Development Core Team 2013).

#### *Mark-release-recapture experiment*

A field survey for the mark-release-recapture experiment was conducted at the foot of Mt. Hokyo (461 m above mean sea level), Tsukuba, Japan (36.15°N, 140.11°E; Fig. 5). The survey area mainly consisted of deciduous forest, rice fields, and abandoned fields that were dominated by the Japanese silver grass *Miscanthus sinensis* Andersson and the goldenrod *Solidago canadensis* L. Mountains and a few residential areas with a two-lane road surrounded the survey area. The survey area contained many sunny slopes, where autumn-form adults of *E. mandarina* overwinter (Itoh 2014). Several small ponds for irrigation were scattered over the survey area, and the host plant *Lespedeza juncea* (L.f.) grew along the margins of the forests and rice fields. Nectar plants for adults, *Bidens frondosa* L. and *Viola* sp., appeared in autumn and spring, respectively. I defined seven survey sites within the

survey area to apply the mark-release-recapture method within 30 min, due to the area's topographical and vegetational features. Survey sites A–E were near rice paddy fields or grasslands, and sites F and G were in a deciduous forest. Adult butterflies cannot enter most deciduous forests, because of dense bamboo grasses on the forest floor. However, mountain trails supplied a suitable space for flying at sites F and G.

The field survey was conducted from October to November 2013 and from March to May 2014. Each sampling effort was performed from 10:00 to 15:00 JST, on sunny days with gentle or moderate winds. One to four persons surveyed each site for at least 15 min in the order A, B, G, C, D, E, and F. When I encountered butterflies, I captured them gently using a net and put them in a cylindrical net cage. After sampling at each site, I recorded their forewing length, sex, and seasonal form. The seasonal form was determined by the width of black band on the tip of forewings according to Kato and Handa (1992). The wing wear of each individual was used to classify the butterflies into five age classes (see Watanabe & Ando 1993): FF, intact wings; F, fresh wings with fine tears but less lustrous scales; B, some wing tears and scale loss; BB, notched tears and many scales lost; BBB, broken or extensive tears and many scales lost. After being marked with a black felt-tip pen, the butterflies were anesthetized with carbon dioxide to avoid “capture-release trauma”, such as escape reactions or other abnormal dispersal behaviors, and released (Watt *et al.* 1977).

Tukey's test was used to compare forewing length among summer-form males, autumn-form males, and autumn-form females before and after overwintering. The statistical analyses were performed using R version 3.0.2 (R Development Core Team 2013).

The POPAN procedure in the program MARK (ver. 8.x) was used to generate linear models with constraints under the Jolly-Seber method, which assumes an open population (Cooch & White 2017). POPAN estimated primarily the daily apparent survival rate ( $\phi_i$ ), catchability ( $p_i$ ), and probability of entrance ( $Pent_i$ ). The probability of entrance is an index of recruitment. Then, daily population size ( $N_i$ ) was also estimated. Because summer-form and autumn-form adults have obviously different demographics, the analysis was conducted

separately for each form.

The apparent survival rate, catchability, and probability of entrance may vary with time. For apparent survival rates and probabilities of entrance, a constant model ( $\cdot$ ), a full-time dependent model ( $t$ ), a linear trend model ( $T$ ), and a quadratic trend model ( $TT$ ) can be used. These parameters may also differ among autumn, winter, and spring (*season*), or between winter and the other seasons (*winter*). Effects of seasons or winter on these parameters could coincide with the linear or quadratic trend. In contrast, I assumed that catchabilities are always full-time dependent, because the detailed environment of the study area and the sampling effort may vary with time.

These parameters may also differ between the sexes (*sex*) and, therefore, models including sex and interactions between sex and time components were also run for the apparent survival rate and probability of entrance of autumn-form adults. Catchability and population size were assumed to be sex-dependent, based on the general pattern observed in butterflies (e.g., Watt *et al.* 1977). The most appropriate model was selected based on AICc values.

## **RESULTS**

### *Overwintering success under semi-natural conditions*

During the experiment, the maximum temperature reached 20°C on November 20, February 14, and March 5 and 6 (Japan Meteorological Agency 2017). The survival rate in the 12th week of the experiment (just before the week including February 14) was 11.8% (95% CI: 1.46–36.44%) and 20.0% (95% CI: 4.33–48.09%) for males and females, respectively (Fig. 6). At the end of the experiment, the survival rate was 5.88% (95% CI: 0.15–28.69%) and 6.67% (95% CI: 0.12–31.95%) for males and females, respectively. The survival rates were not significantly different between the sexes, neither in the second week of February ( $P =$

0.645), nor at the end of the experiment ( $P > 0.999$ ).

The optimal model revealed that winter survival depended only on body mass (Table 2). In this model, the mean body mass regression coefficient was  $0.017 \pm 0.007$  ( $\pm$  SE), indicating that heavy butterflies had longer survival times than light butterflies. The mean male body mass was  $51.16 \pm 13.76$  mg ( $\pm$  SD), and the mean female body mass was  $57.42 \pm 15.01$  mg ( $\pm$  SD); the difference was not statistically significant ( $t = 1.231$ ,  $d.f. = 30$ ,  $P = 0.228$ ).

Forewing length may also affect survival, but models including forewing length as an independent variable had higher AICc values than those including body mass, and the null model (Table 2). Therefore, survival during winter depended on body mass rather than forewing length. The mean male forewing length was  $22.50 \pm 1.65$  mm ( $\pm$  SD), and the mean female forewing length was  $23.21 \pm 1.48$  mm ( $\pm$  SD); the difference was not statistically significant ( $t = 1.255$ ,  $d.f. = 30$ ,  $P = 0.219$ ).

#### *Recapture rate, forewing length, and wing wear*

I captured 53 summer-form males, 111 autumn-form males, and 96 autumn-form females in the autumn. After the winter, 80 autumn-form males and 83 autumn-form females were captured. Four autumn-form males captured in the autumn were recaptured in the following spring. Summer-form males, autumn-form males, and autumn-form females were handled 88, 266, and 219 times, respectively. Of the individuals released, 23 summer-form males (43.4%), 48 autumn-form males (26.8%), and 34 autumn-form females (19.5%) were recaptured at least once.

The forewings of summer-form males were significantly shorter than those of autumn-form males and females in the autumn (Table 3), indicating that autumn-form adults were larger than summer-form males. For autumn-form adults, no significant difference was found between sexes in any season. Forewing length did not significantly differ before and after overwintering in both sexes of autumn-form adults, suggesting that overwintering

success was not dependent on forewing length.

A few summer-form males were age FF, but over half of them were age B, BB, or BBB (Fig. 7a). In contrast, almost all of the autumn-form adults collected in the autumn were age FF or F (Fig. 7b, c), and over half of adults had fresh wings even in mid-April. After late April, many adults were classified as age BB or BBB without any obvious differences between the sexes, suggesting that autumn-form adults fly with damaged wings exclusively in spring.

#### *Abundance, apparent survival rate, probability of entrance, and catchability*

The optimal model revealed that there were no sexual differences in survival rate or the probability of entrance for autumn-form adults (Table 4), although these parameters differed between winter and the other seasons. Catchability was full-time dependent, indicating that the parameter changed from day to day. The parameter was also sex dependent, but without an interaction with time. Differences in AICc values between the most appropriate model and the other models were greater than 2, suggesting considerable support for real differences (Cooch & White 2017).

For summer-form males, the optimal model had a linear trend survival rate, a full-time-dependent catchability, and a constant probability of entrance (Table 5). The top three models had the same structure for catchability and probability of entrance. Although different structures for survival rate among the top 3 models were reported, differences in AICc values between the most appropriate model and the other models were less than 2.

In the autumn, the number of summer-form males and autumn-form adults of both sexes gradually decreased with time (Fig. 8). The maximum population size was 259.003 individuals (95% CI: 180.730-371.175) and 391.396 individuals (95% CI: 254.482-601.970) for autumn-form males and females, respectively, and that of summer-form males was 52.722 individuals (95% CI: 30.693-90.561) on the same day; autumn-form comprised 83.1% of



males. The sex ratio of summer- and autumn-form males to autumn-form females was estimated as 0.796.

The population in the spring was clearly smaller than that in the autumn for both sexes of the autumn-form (Fig. 8). The population size during the first survey in the spring was 5.2% of that during the last survey in the autumn, for both sexes. Although the number of autumn-form males and females increased with time, the maximum population size in the spring was only 37.7% (males) and 38.2% (females) of each population size immediately before overwintering. No sexual difference was detected in the decrease pattern of the population size.

The daily apparent survival rate during the winter was estimated as 0.979 (95% CI: 0.970-0.986) for both sexes of the autumn-form in the most appropriate model (Table 6), which was higher than the rate of 0.923 (95% CI: 0.896-0.944) that was estimated for the autumn and spring. Because the interval between the last survey in the autumn and the first survey in the spring was 141 days, the apparent survival rate during the winter was calculated as 5.0% (95% CI: 1.3-13.7%) for both sexes of the autumn-form.

According to the optimal model, the probability of entrance in the autumn and spring was estimated as 0.017 (95% CI 0.013-0.022) for both sexes of the autumn-form (Table 7). Summer-form males had a relatively high probability of entrance (0.054, 95% CI: 0.019-0.144) in the autumn. On the other hand, the probability of entrance during winter approached zero (95% CI: 0.000-0.000) for both sexes of autumn-form adults, suggesting higher residency during the winter.

Summer-form males had higher catchabilities than autumn-form adults in the autumn (Table 8), and autumn-form males always had higher catchabilities than autumn-form females. The catchability of both sexes of the autumn-form tended to be higher in spring than in autumn, indicating that changes in behavior had occurred between before and after overwintering.

## DISCUSSION

The objective of this chapter was to ascertain the adaptive significance of the production of summer-form males in late autumn in *Eurema mandarina*. I hypothesized that summer-form males increase the overwintering success of their sperm by engaging in pre-overwintering copulations with autumn-form females. If autumn-form females show higher overwintering success than autumn-form males, the hypothesis can explain why summer-form males emerge in late autumn. However, both the results of the experiment under semi-natural conditions and those in the mark-release-recapture experiment showed that overwintering success was similar between autumn-form males and females. Therefore, another hypothesis is required to explain the emergence of summer-form males in autumn. A possible hypothesis is the lack or relaxation of last-male precedence in the sperm competition in the following spring. Although paternity of eggs laid by autumn-form females remain unclear, eupyrene sperm derived from summer-form males showed its motility in the spermatheca of autumn-form females after overwintering (T. Konagaya, unpubl. data, 2013). No sexual differences in overwintering success have been reported in laboratory experiments conducted in other adult overwintering butterflies, such as *Polygonia c-album* (L.) and *Inachis io* (L.), and adults of these species generally mate after overwintering (Wiklund *et al.* 2003).

In this chapter, I examined the overwintering success of autumn-form adults in *E. mandarina* not only in an experiment under semi-natural conditions in 2015-2016, but also in a mark-release-recapture experiment in 2013-2014. The apparent survival rate as estimated by the Jolly-Seber method is a function of the true survival rate and the residence rate. Although autumn-form adults sometimes fly on warm days during winter (Itoh 2014), emigration during winter is expected to be low because of the low temperature. My analysis also revealed a low probability of entrance during winter. Therefore, the apparent survival rate during winter is an appropriate indicator of the true survival rate. I found that the overwintering

success estimated for the wild population (1.3-13.7%) was similar to that found under semi-natural conditions (males, 0.15-28.69%; females, 0.12-31.95%).

The field census by Kato (1986) also suggested the low overwintering success in autumn-form adults of *E. mandarina*. *Eurema mandarina* produces three direct developing generations and an overwintering generation throughout a year in central Japan. All males and females emerge as summer form in the former three generations. Without any mark-release-recapture survey, Kato (1986) reported a strong increase in the abundance with generations from spring to autumn, suggesting a low mortality rate during these seasons. Failure of overwintering may be a main mortality factor in *E. mandarina* throughout a year.

Autumn-form adults must endure unfavorable conditions during winter, and have evolved more suitable traits for survival in such conditions than have summer-form adults (Brakefield & Zwaan 2011). It is important for butterfly activities to maintain the wings in good conditions without any visible damage. Kato (1986) reported that wing wear in *E. mandarina* summer-form adults increases linearly with age. I found that most autumn-form adults had undamaged wings until early April, despite their emergence before winter, in contrast to summer-form males. Because autumn-form adults show higher foraging activity than summer-form males in autumn (Kato 1989), the undamaged wings of overwintered adults may be due to the greater durability of their wings. Alternatively, reproductive activities such as searching females or oviposition might induce wing wearing. There was no clear sexual difference in the wing-wearing process in autumn-form adults throughout their lifespan.

Body size affects overwintering success in insects, and I used forewing length and body mass as indices of body size in this chapter. Although autumn-form adults had longer forewings than summer-form males, forewing length did not affect their overwintering success under semi-natural conditions. In addition, the mark-release-recapture experiment showed a slight or no effect of forewing length on the overwintering success of autumn-form adults. However, survival during the winter did depend on body mass under semi-natural

conditions. This agrees with the results in Pullin (1987) that heavy *I. io* and *Aglais urticae* (L., 1758) butterflies have high lipid contents and a high overwintering success. Considering the fact that a half of the lipid reserve of adult *D. plexippus* is consumed during winter (Tuskes & Brower 1978; Alonso-Mejia *et al.* 1997), lipid content may also be important for overwintering for autumn-form *E. mandarina* adults.

For overwintering butterflies, investing in survival by increasing their lipid contents may negatively affect their investment in reproduction. Karlsson *et al.* (2008) reported that autumn-form females have a lower fecundity than summer-form females in *P. c-album*. Although the cost of overwintering has not been examined in *E. mandarina*, summer-form males should invest most of their resources in reproduction, because they avoid overwintering. This is supported by the fact that summer-form males produce slightly longer eupyrene sperm than autumn-form males (Chapter 1). The avoidance of overwintering may improve ejaculate quality, and result in the relaxation of second-male precedence in the sperm competition in the following spring.

The overwintering success of *E. mandarina* recorded in this chapter (approximately 5%) was considerably lower than the 90–95% overwintering success reported in *P. c-album* and *I. io* in Sweden (Wiklund *et al.* 2003), even though the same methods were used to estimate overwintering success under semi-natural conditions. In *A. urticae*, Pullin (1987) reported an overwintering success in the laboratory of higher than 80% when the butterflies were fed on a sufficient amount of honey solution before overwintering. On the other hand, *D. plexippus* is another butterfly species that has been reported to have a low overwintering success (Calvert *et al.* 1983; Culotta 1992). Brower *et al.* (2004) reported that 75% of an overwintering *D. plexippus* colony was killed by a storm. *Danaus plexippus* and *E. mandarina* have similar overwintering success rates, and their mating systems are also similar: In *D. plexippus*, some males copulate with diapause females before winter (Leong *et al.* 2012). *Curetis acuta* also shows pre-overwintering copulation and relatively low winter survival (< 20%) (Umedzu 2016). Therefore, the considerably low overwintering success may

relate to the presence of non-diapause males (summer-form males) in late autumn.

In general, female receptivity for mating before winter may relate to the evolution of summer-form males in autumn (Pener 1992), especially when overwintering success is low. Severe winter requires many resources reserved for overwintering adults and reduces chances to encounter mates in the following spring due to the reduction of population density. Therefore, females in such species might evolve to increase receptivity for mating in autumn to obtain sperm or nutrients (Kato 1986), because a butterfly male transfers a spermatophore containing spermatozoa and nutrition as a nuptial gift during copulation (Boggs & Gilbert 1979). Conversely, if winter mortality is low, females are expected to have low receptivity to males before winter, because sperm storage during winter often brings costs for insect females (Roth & Reinhardt 2003). The degree of overwintering success may be more important than the sexual differences of overwintering success in the evolution of male reproductive diapause.

## Chapter 3.

### Late autumn occurrence of non-diapause male adults in various geographical populations

#### INTRODUCTION

Diapause is a hormonally controlled developmental arrest and is important for overwintering in insects in the Temperate Zone, because winter is an unfavorable season for their growth and reproduction (Tauber *et al.* 1986; Danks 1987). Low temperature, fallen snow and food limitation can inhibit growth and reproduction of insects during winter. Diapause of adult insects is characterized by a suppression of reproductive function, and is called as reproductive diapause (Perner 1992).

The evolution of male reproductive diapause is controversial. Unlike females that must overwinter to lay eggs, males can fertilize eggs without overwintering by pre-overwintering copulation, indicating the two fundamental strategies for males (Pener 1992; Kubrak *et al.* 2016). One strategy is pre-overwintering copulation without reproductive diapause, and is common in some predatory bugs, mosquitoes and social wasps in temperate zones (Tauber *et al.* 1986; Kobayashi & Osakabe 2009). The other strategy is the combination of diapause and mating in the following spring, and is predominant in adult overwintering insects (Tauber *et al.* 1986). The presence or absence of male reproductive diapause is ecologically important, because the demography in spring depends on the number of males that have overwintered.

In the Japanese common grass yellow *Eurema mandarina* (de l'Orza) both diapause and non-diapause male adults emerge in late autumn. This species shows a seasonal polyphenism consisting of non-diapause summer-form and diapause autumn-form adults (Fukuda *et al.* 1982). Short-day conditions and low temperature during the larval stage induce autumn forms in adults (Yata 1974). Kato (1986) showed that *E. mandarina* has three direct

developing generations and an overwintering generation in central Japan. In the direct developing generations, all males and females emerge as summer forms, and autumn-form adults emerge only in the overwintering generation. However, approximately a half of males are insensitive to short photoperiods and low temperature, and emerge as summer-form adults even in the conditions in which most females emerge as autumn-form adults (Kato & Sano 1987). Summer- and autumn-form males and autumn-form females coexist in late autumn in wild populations of central Japan (Kato 1989; Chapter 1).

Pre-overwintering copulation of autumn-form females is a key to understand the presence of non-diapause summer-form males in *E. mandarina* in late autumn. Most autumn-form females have high sexual receptivity even before winter and mate with summer-form males in autumn (Kato 1986). Most autumn-form males increase mating activity in the following spring (Kato 2005). After overwintering, most autumn-form females re-mate with autumn-form males before the onset of oviposition (Chapter 1). Therefore, fitness of summer- and autumn-form males depends on the paternity in sperm completion.

Effects of abiotic factors such as winter severity on the evolution of male reproductive diapause remain unclear. In Chapter 2, I pointed out that non-diapause males in autumn have been shown in butterfly species of which diapause adults suffer low overwintering success. The result of Chapter 2 also showed that autumn-form adults of *E. mandarina* suffer a considerably low overwintering success (approximately 5%) in central Japan (Chapter 2). Thus, if the fitness ratio of both forms of males in late autumn is sensitive to overwintering success of autumn-form adults, overwintering success might determine the frequency of summer-form males in late autumn in *E. mandarina*. This hypothesis predicts that populations in longer winter show higher frequencies of summer-form males. In this chapter, therefore, I examined frequencies of summer-form males by a rearing experiment in the laboratory and a field survey in several geographic populations. Furthermore, pre-overwintering copulation frequencies, potential fecundities, and the number of eupyrene sperm stored in the spermatheca were also examined for several geographic populations by

dissecting field-caught females.

## MATERIALS AND METHODS

### *Rearing experiment*

Summer-form females were caught by a net in six localities in Japan (Fig. 9): Iwanuma City/Watari Town, Miyagi Prefecture (June 15, 2016 and July 8, 2017), Kashiwa City, Chiba Prefecture (July 7, 2016), Kameoka City, Kyoto Prefecture (June 14, 2016), Kyoto City, Kyoto Prefecture (July 29, September 11, September 16, 2016), Ise City, Mie Prefecture (June 26, 2016) and Shibushi City, Kagoshima Prefecture (August 1, 9, September 24, 2016)).

These butterflies were put into glassine envelopes individually and brought to the laboratory. They were kept in paper-framed cages (22×22×30 cm<sup>3</sup>) individually under a long photoperiod of 16-h light and 8-h darkness (LD 16:8) at 25.0 ± 1.0°C, and fed 10% sucrose solution *ad libitum*. A foliated branch of a food plant *Lespedeza juncea* (L.f.) was placed as oviposition substrate in each cage. The foliated branch was exchanged 2-day intervals.

When eggs were laid on the foliated branch collected, it was transferred to a clear plastic cage (13 cm diameter, 19 cm height) and kept at 25.0 or 20.0 ± 1.0°C under a short photoperiod (LD 10:14). Hatched larvae were transferred to small plastic cups (10 cm diameter, 4.5 cm depth), and reared under the same conditions. Each plastic cup contained 1-5 larvae. Larvae were fed an artificial diet according to Kato and Sakakura (1994). The plastic cup and artificial diet were exchanged at 5- or 6-day intervals. When adult butterflies emerged, their sex, forewing length and seasonal form were recorded. Forewing length was measured by a digital caliper (accuracy 0.1 mm). Seasonal form was determined by width of black band on the tip of forewings according to Kato and Handa (1992).

All statistical analyses were performed using R, version 3.4.0 (R Development Core Team 2017). The proportion of summer-form adults was analyzed by generalized linear mixed



models (GLMM) using cbind function in the package glmmML with lineage ID as a random factor. Binomial error distribution and logit link function were assumed. The winter length where the maternal females were collected was defined as the number of days that the average maximum temperature is below 15°C in 1981-2010 (Japan Meteorological Agency 2017, see Fig. 9). The winter length, sex, rearing temperature, and interactions among these parameters were examined for explanatory variables. The most appropriate model was selected based on the Akaike Information Criterion (AIC).

### *Field survey*

Frequency of summer- and autumn-form adults in wild populations was examined in eight localities in Japan: Morioka City, Iwate Prefecture (October 1, 2016), Iwanuma City/Watari Town (October 7, 2016), Kameoka City (October 24, 2016), Joyo City, Kyoto Prefecture (October 27, 2016), Shimanto City, Kochi Prefecture (November 6, 2016), Nichinan City, Kagoshima Prefecture (November 13, 2016), Shibushi City (November 12, 2016) and Okinoerabu Island (December 9 and 10, 2016), Kagoshima Prefecture (see Fig. 9). Morioka City is near the northern limit of the range of *E. mandarina* (Shirouzu 2006). *Eurema mandarina* has a sibling subtropical species *E. hecabe*, and it is difficult to distinguish between these two species especially in old specimens. The latter species is distributed widely in the Ryukyu Islands, but not in Okinoerabu Island (Kato & Yata 2005). Therefore, I selected Okinoerabu Island as the southern limit of my collection sites.

The proportion of summer-form adults in the field depends not only on the numbers of summer- and autumn-form adults emerging in the generation, but also the time after the onset of adult emergence of the generation, because only the number of summer-form adults decrease gradually in autumn (Chapter 2). However, the onset of adult emergence of the overwintering generation in 2016 is unclear for all local populations. Thus, I examined the proportion of summer-form adults on a sunny day when the average maximum temperature in

1981-2010 was 20.5-21.5°C for each site (Fig. 9). I patrolled each survey area 10:00-15:00 JST and tried to collect all individuals of *E. mandarina* encountered. The forewing length, sex and seasonal form of the butterflies collected were examined. Their behavior immediately before collection was also recorded to avoid sampling bias caused by different behavioral patterns between summer- and autumn-form males (Kato 1989). The behavior was classified into flying, foraging, resting, puddling, and mating.

The effect of winter length on the proportion of summer-form adults was examined by GLMM. The winter length of each collection site was defined as the number of days when the average maximum temperature in 1981-2010 was below 15°C. In this analysis, binomial error distribution and logit link function were assumed. The behavior of butterflies immediately before collection was taken as a random factor. The *P*-value for the GLMM was obtained from the Wald test. Because most females collected in the field were the autumn form, sex did not apply to the analysis as an explanatory variable. The multiple linear regression analysis was applied to examine effects of the winter length and seasonal form in the forewing length of males. The linear regression analysis was used to examine the effect of the winter length in the forewing length of females.

#### *Dissection of field-collected females*

Female adults collected in the field were brought to the laboratory of Kyoto University in glassine envelopes. They were frozen for 1 day or more in a freezer (-20°C), and dissected in an insect saline (128.4mM NaCl, 4.7mM KCl and 1.9mM CaCl<sub>2</sub>) under a stereoscopic microscope with a ring light (LEICA S8AP0). All spermatophores were removed from the bursa copulatrix, and the number of spermatophore was counted to estimate the frequency of pre-overwintering copulation of the females. Each spermatophore was weighted using an electric balance (Shimadzu AUW220D, accuracy 0.01 mg).

Eggs in the ovaries were classified into 3 stages: a mature egg with a hard egg shell,

a submature egg filled with white yolk but without an egg shell, and an immature egg with no egg shell and a little or no yolk. The potential fecundity was estimated by the number of immature eggs in the ovaries for each autumn-form female. The number of immature eggs in an ovariole was counted under the stereoscopic microscope after eosin staining. Because a female has eight ovarioles, the number of immature eggs in an ovary was estimated as eight times the number of immature eggs in an ovariole. Although autumn-form females usually do not have mature or submature eggs in autumn because they are in reproductive diapause (Kato 1986), a few autumn-form females with damaged wings showed oviposition behavior in autumn and had mature or submature eggs (T. Konagaya, unpubl. Data, 2017). In these females, the number of immature eggs does not reflect the potential fecundity, because the number of immature eggs decreases with oviposition in *E. mandarina* (Chapter 1). Therefore, autumn-form females with mature or submature eggs were excluded from the analysis.

The spermatheca was dissected in the insect saline on a glass slide. All the ejaculates in the spermatheca were washed out into a small tube with approximately 2 mL saline. The exact volume of saline including ejaculate was calculated as the difference in weight between an empty tube and the tube with saline. After homogenizing, six 10- $\mu$ L subsamples were removed from each sample using an autopipette, and allowed to dry on a glass slide under dust covers. Dried slides were dipped for 5 s in distilled water and allowed to dry again. Spermatozoa in each subsample were counted on black paper under the stereoscopic microscope. The number of sperm in the spermatheca were calculated by multiplying the average sperm count in 10  $\mu$ L of the suspension. Although butterfly males transfer fertile eupyrene sperm and non-fertile apyrene sperm to females (Swallow & Wilkinson 2002), apyrene sperm was not counted, because apyrene sperm disappears from the spermatheca in several days of copulation in *E. mandarina* (Chapter 1).

The proportion of mated females was examined by a generalized linear model (GLM) with winter length and forewing length as explanatory variables. Binomial error distribution and logit link function was assumed. The *P*-value for the GLM was obtained from

the Wald test. The number of eupyrene spermatozoa stored and the number of immature eggs were examined by multiple regression analyses to avoid over dispersion when the GLM with the Poisson error distribution was used. The winter length and forewing length were used as explanatory variables. Although they were counted data, multiple regression analyses can be applied, because the mean values of explained variables were sufficiently large.

## RESULTS

### *Rearing experiment*

I collected 10, 5, 5, 13, 3, 15 summer-form females in Iwanuma/Watari, Kashiwa, Kameoka, Kyoto, Ise and Shibushi, respectively. The proportion of summer-form in offspring varied from 0 to 100% among the lineages, and seemed to depend on the sex and rearing temperature (Fig. 10). Males showed a clearly higher proportion of summer-form adults than females, and the higher temperature induced a higher proportion of summer-form adults.

The optimal model selected by AIC showed that the proportion of summer-form adults depended on winter length, sex, rearing temperature and the interaction between sex and rearing temperature (Table 9). The difference in AIC values between the optimal and second optimal models was less than 2, suggesting inconsiderable support for real differences (Cooch & White 2017). The second optimal model included only sex and rearing temperature and the interaction between them. Therefore, sex, rearing temperature and the interaction between them were suggested important factors inducing summer-form adults in *E.*

*mandarina*. In the optimal model, the coefficient of effect of winter length in the proportion of summer-form adults was  $0.007 \pm 0.004$  (mean  $\pm$  SE). Its *P*-value obtained by the Wald-test was 0.090 ( $z = 1.638$ , *d.f.* = 95), although the other explanatory variables had small *P*-values ( $P < 0.05$ ). The analysis suggests a slight effect of the winter length on the proportion of summer-form adults.

### *Field survey*

Flying and foraging were major behaviors in males at collection (Table 10). The proportion of summer-form adults was approximately 10% in all populations except the most northern population (Morioka) in foraging males. In flying males, the proportion of summer-form adults varied from approximately 35% to 70% among the populations. However, the effect of winter length on the proportion of summer-form males was not statistically significant in the GLMM analysis considered the effect of behavior ( $z = 0.359$ ,  $d.f. = 20$ ,  $P = 0.720$ ).

In contrast to males, only 2 of 78 females collected were summer forms, and one of them rested on a plant in Iwanuma/Watari, and the other was puddling on a puddle near a small river in Okinoerabu. The proportion of summer-form adults was clearly different between the sexes.

The forewing length was different between summer- and autumn-form males (Table 11,  $t = -4.333$ ,  $d.f. = 299$ ,  $P < 0.001$ ). Males collected in localities with longer winter had longer forewing length ( $t = 4.344$ ,  $d.f. = 299$ ,  $P < 0.001$ ). However, when the males collected in Okinoerabu that is a small island and locates in a subtropical zone were excluded from the analysis, the effect of winter length was not statistically significant ( $t = 0.955$ ,  $d.f. = 279$ ,  $P = 0.340$ ), although the effect of seasonal form was still significant even in this analysis ( $t = -4.059$ ,  $d.f. = 279$ ,  $P < 0.001$ ). The result suggests no geographic variation of the forewing length of males throughout the temperate zone. Autumn-form females had longer forewing length than both of summer- and autumn-form males in all local populations, and showed no geographic variation in the forewing length in the temperate zone ( $t = -0.796$ ,  $d.f. = 73$ ,  $P = 0.249$ ).

### *Dissection of field-collected females*

Most autumn-form females (90.5%) contained 1 or 2 spermatophores in the bursa copulatrix (Table 12). Out of them, only 1 autumn-form females had 2 spermatophore. The total spermatophore mass was smaller in populations with longer winter length ( $t = -2.896$ ,  $d.f. = 57$ ,  $P < 0.01$ ), but did not depend on the forewing length ( $t = -0.032$ ,  $d.f. = 57$ ,  $P = 0.975$ ). The proportion of mated females did not depend on the winter length ( $z = -0.416$ ,  $d.f. = 71$ ,  $P = 0.677$ ) and the forewing length ( $z = 0.416$ ,  $d.f. = 71$ ,  $P = 0.259$ ). Therefore, the frequency of pre-overwintering copulation was similar among all local populations examined.

The number of immature eggs varied among individuals from 320 to 832. This number positively related to the forewing length ( $t = 2.934$ ,  $d.f. = 58$ ,  $p < 0.05$ ). Furthermore, autumn-form females collected in populations with longer winter length contained smaller number of immature eggs ( $t = -2.062$ ,  $d.f. = 58$ ,  $p < 0.01$ , Fig. 11a), showing geographic variation in the potential fecundity of autumn-form females.

Dissecting of spermatheca was succeeded in 58 autumn-form females. The forewing length did not affect the number of eupyrene sperm stored ( $t = 1.080$ ,  $d.f. = 56$ ,  $P = 0.285$ ). On the other hand, females would experience longer winter stored lower number of eupyrene sperm ( $t = -3.726$ ,  $P < 0.001$ ), showing geographic variation in the number of eupyrene sperm stored (Fig. 11b). In a separate analysis, the number of eupyrene sperm did not depend on the number of immature eggs in the ovary (Linear regression analysis,  $t = 1.507$ ,  $d.f. = 47$ ,  $P = 0.139$ ).

## **DISCUSSION**

The major objective of this chapter was to examine the effect of winter severity on the frequency of non-diapause summer-form males of *E. mandarina* emerging in late autumn. Non-diapause males in late autumn have been observed in butterfly species of which diapause adults suffer low overwintering success (Chapter 2). Therefore, I predicted that summer-form males emerge in late autumn more frequently in northern populations than in southern

population even in a species, *E. mandarina*. However, the field survey revealed no geographic variation in the frequency of summer-form males in late autumn. The rearing experiment under a short photoperiod showed that the frequency of summer-form males only slightly decreased with the winter length of their original habitat. Photoperiodism in insects is one of the traits sensitive to natural selection (Tauber *et al.* 1986), as shown in the example of geographic variation in the critical day length (e.g. Danilevskii 1966; Gomi 1997). Therefore, adaptive explanation should be explored for the lack of remarkable geographic variation in frequency of summer-form males in late autumn.

No or a slight geographic variation in the frequency of summer-form males in late autumn can be explained by the fact that most autumn-form females mated before winter in all local populations examined. The present results indicate that autumn-form females have high sexual receptivity before winter in all geographic populations explained. Therefore, there are frequent opportunities for summer-form males to mate in late autumn, irrespective of the winter severity in the local habitat. Because the fitness of summer- and autumn-form males depends on the number of mating and paternity in sperm competition between the males of the two forms (Chapter 1), the similar frequencies of summer-form males are not surprising, if sperm mortality in the spermatheca of overwintering females does not depend on winter severity.

Kato (1986) proposed two hypotheses to show adaptive significance of pre-overwintering copulation of females in *E. mandarina*. One is the risk hedge hypothesis, and the other is the nuptial gift hypothesis. The former hypothesis explains that pre-overwintering copulation of females as a preparation for less chances of mating before the onset of oviposition in the following spring. The latter hypothesis assumes that autumn-form females use male ejaculates as nutrition to improve overwintering success. In Chapter 1, I pointed out that the nuptial gift hypothesis is more probable in *E. mandarina*, because most autumn-form females that have overwintered re-mate with autumn-form males before the onset of oviposition. Butterfly females generally digest the spermatophore in the bursa copulatrix and

use it as nutrition for somatic maintenance and maturation of eggs (Boggs & Gilbert 1979). Leong *et al.* (2012) pointed out the possibility that females use spermatophores as nutrition during overwintering in the Monarch butterfly, *Danaus plexippus* (L.). Autumn-form females of *E. mandarina* also may increase their overwintering success by pre-overwintering copulation in all local populations examined.

In this chapter, the number of immature eggs contained in the ovary depended on the forewing length and winter length. The positive correlation between the body size and fecundity has been shown in various insects (Danks 1994). The present result, however, first demonstrated geographic variation in the potential fecundity of adult overwintering insects. The number of immature eggs in autumn-form females was larger in the populations with shorter winter. The trade-off between survival during winter and reproduction in the following spring can explain this difference. Autumn-form females in northern populations must invest much more resources in survival to succeed in overwintering than those in southern populations, resulting in decrease of the number of immature eggs in northern populations. In a nympharid butterfly *Polygonia c-album* (L.), autumn-form females lay less number of eggs after overwintering than summer-form females (Karlsson *et al.* 2008).

Autumn-form females in southern populations stored larger numbers of eupyrene sperm in the spermatheca than those in northern populations. This pattern may relate to the geographic variation in potential fecundity. Because the females in the southern populations contained larger numbers of immature eggs, they may have motivation to store larger numbers of eupyrene sperm. However, the number of eupyrene sperm stored did not depend on the potential fecundity. The other possibility relates to the winter length that they would experience. Roth and Reinhardt (2003) pointed out that sperm storage often brings a cost to overwintering female insects. Because autumn-form females in southern populations experience shorter winter, they could store larger numbers of eupyrene sperm than those in northern populations.

In summary, I compared the mating system of the overwintering generation in *E.*



*mandarina* among local populations including cool-temperate and subtropical zones in this chapter. In every local population, most autumn-form females mated before winter and a considerable number of males emerge as summer-form in late autumn. Although winter severity is drastically different between the cool-temperate and subtropical zones, the mating system of *E. mandarina* is robust geographically. This fact suggests less contribution of winter severity on fitness of diapause and non-diapause males emerging in late autumn when diapause females have mating activity before winter.

## Chapter 4.

### Effect of mating on survival at low temperature in autumn-form females

#### INTRODUCTION

Insects evolved diverse strategies to survive winter in temperate zones (Tauber *et al.* 1986; Danks 1987). Because winter is unfavorable for their growth and reproduction, diapause, a hormonally controlled developmental arrest, is important in seasonal adaptation of insects. Differences among species and populations are usual in incidence, maintenance, termination, and intensity of diapause (Danielevskii 1966). Inter- and intra-sexual differences within a single population are also reported in some insects (Pener 1992). These variations bring good opportunities to understand the evolution of seasonal adaptation (Masaki & Wipking 1994).

Adult overwintering insects show diversity in occurrence of male reproductive diapause. Whereas females cannot lay eggs in spring without overwintering, males can reproduce without overwintering by pre-overwintering copulation. Their sperm overwinter in the spermatheca of females, and fertilize eggs in the following spring. On the other hand, males also can obtain offspring by the combination of diapause and mating in the following spring. Therefore, adult-overwintering species can be classified into three types, i.e. species in which only females enter diapause, species in which all males and females enter diapause, and species in which a part of males and all females enter diapause (Pener 1992). The first type is common in social wasps in the Temperate Zone and often observed in mosquitoes and predatory bugs (Tauber *et al.* 1986; Kobayashi & Osakabe 2009). The second type is predominant in adult-overwintering insects (Tauber *et al.* 1986). The third type was reported in a few species in Orthoptera and Lepidoptera (Fukuda *et al.* 1982; Greenfield & Pener 1992).

Adaptive significance of pre-overwintering copulation of females is important to understand the evolution of male reproductive diapause, because non-diapause males cannot

obtain offspring without female receptivity for pre-overwintering copulation. Two hypotheses have been proposed as adaptive significance of pre-overwintering copulation of females. One is the risk-hedge hypothesis that explains pre-overwintering copulation of females as a preparation for less chances of mating before the onset of oviposition in the following spring (Kimura 1980; Taylor 1984; Kato 1986; Stevens & McCauley 1989; Naess & Nilssen 1991; Roth & Reinhardt 2003; Socha 2010; Leong *et al.* 2012; Golec & Hu 2015). The other is the nuptial gift hypothesis assuming that females increase overwintering success by pre-overwintering copulation using the nutrition in male ejaculate (Kato 1986; Koshiyama *et al.* 1994, 1996; Kobayashi & Osakabe 2009; Leong *et al.* 2012; Golec & Hu 2015). A prolonged longevity of mated females during overwintering was reported in predatory bugs *Orius sauterii* (Poppius), *Orius nagaii* Yasunaga, and *Orius strigicollis* (Poppius), in which no males enter diapause (Kobayashi & Osakabe 2009), supporting the nuptial gift hypothesis. However, the pre-overwintering copulation also functions as the preparation for no mating chance in spring in these species. On the other hand, higher longevity of mated females has not been observed in any insect species, in which a part of males enter diapause.

In the Japanese common grass yellow *Eurema mandarina*, both diapause autumn- and non-diapause summer-form males emerge in a same generation in autumn (Fukuda *et al.* 1982). In central Japan, *E. mandarina* has three direct developing generations and an overwintering generation (Kato 1986). All individuals emerge as summer-form in the direct developing generations. Autumn-form adults emerge only in the overwintering generation, because short-day conditions and low temperature in the larval stage induce autumn-form adults (Yata 1974). However, approximately a half of males are insensitive to short-day conditions (Kato & Sano 1987). Therefore, summer- and autumn-form males and autumn-form females coexist in late autumn in Japan (Kato 1989; Chapter 3). Most autumn-form females mate with summer-form males before winter (Kato 1986), whereas most autumn-form males increase mating activity after overwintering (Kato 2005). In spring, autumn-form females re-mate with autumn-form males before the onset of oviposition (Chapter 1).

The nuptial gift hypothesis is more probable than the risk-hedge hypothesis as adaptive significance of pre-overwintering copulation of autumn-form females in *E. mandarina*, because most autumn-form females have sufficient chances for mating in the following spring (Chapter 1). In butterflies, spermatophores are transferred from males to females during mating and function as nuptial gifts (Boggs & Gilbert 1979). Butterfly females that have received a larger amount of spermatophore often show longer longevity and higher fecundity (e.g. Rutowski *et al.* 1987). In this chapter, therefore, the effect of pre-overwintering copulation on survival of females at low temperature was examined in the laboratory. The body size and termination of diapause were also examined as factors affecting overwintering success of females.

## **MATERIALS AND METHODS**

Summer-form female adults were caught by a net on August 8, 2017 in Kyoto Prefecture (Kyoto City 35.01°N, 135.77°E, Kameoka City 35.02°N, 135.59°E and Nantan City 35.07°N, 135.27°E), Japan. They were kept individually in paper-framed cages (22×23×30 cm<sup>3</sup>), and fed 10% sucrose solution *ad libitum* under 12-h light and 12- darkness (LD 12:12) at 25.0 ± 1.0°C and more than 65% R.H. A foliated branch of a food plant *Lespedeza juncea* (L.f) was placed in each cage as an oviposition substratum. The branch with eggs was collected at 1- or 2-day intervals, and transferred to a plastic cup (13cm diameter, 19 cm depth). Hatched larvae were individually reared in small plastic cases (36×36×14 mm<sup>3</sup>) under the same conditions. In these conditions, approximately a half of males emerge as summer-form adults, whereas most females emerge as autumn-form adults (Kato & Sano 1987). The larvae were fed an artificial diet by Kato and Sakakura (1994). Pupae were put on lids of plastic cups (13cm diameter, 8cm depth) by a double-sided tape for eclosion.

Seasonal forms, sex and forewing length were examined in newly emerging adults every day. Forewing length was measured by a digital caliper (accuracy 0.1 mm). Seasonal

forms were determined by the width of the black band on the tip of forewings (Kato & Handa 1992). Summer-form males and autumn-form females were reared separately in wood-framed cages (40×50×50 cm<sup>3</sup>) under the same conditions, and fed 10% sucrose solution *ad libitum*. A wood-framed cage contained less than 30 individuals. These adults were weighed once a week.

Newly-emerging females were divided into the mating and control groups. On the day of adult emergence, females in the mating group (1-5 individuals) were transferred to a wood-framed mating cage (40×50×50 cm<sup>3</sup>) containing 10-20 summer-form males, which were 7-day old or older and sexually matured. Mated pairs were transferred to a small plastic cup (10 cm diameter, 4.5 cm depth). After the end of copulation, mated females were put back to the rearing cage. The control group females were transferred to the rearing cage without mating.

On the third week, females of the both groups were transferred to an airtight plastic cage (40×20×20 cm<sup>3</sup>) under LD 10:14 at 10.0 ± 1.0°C after the final measurement of body mass. The butterflies were individually kept in envelopes within plastic cups (13cm diameter, 8cm depth) placed in the cage. The relative humidity in the cage was maintained at 90 ± 3% by water in two plastic cups placed in it. The butterflies were not allowed to feed in the cage.

Survival of the butterflies was recorded once a week. Dead butterflies were dissected in an insect saline (128.4mM NaCl, 4.7mM KCl and 1.9mM CaCl<sub>2</sub>) under a stereoscopic microscope. The presence of mature eggs with hard egg shell was examined for all females as an index of termination of reproductive diapause, because some autumn-form adults terminate diapause before overwintering at 25°C even under short-day conditions (Kato & Handa 1992). For mated females, the spermatophore derived from summer-form males was removed from the bursa copulatrix and weighted (accuracy 0.01 mg). Its morphological shape was classified into 4 stages according to Chapter 1: an intact one (Stage I), a spherical one without the neck (Stage II), a concave one like a bowl (Stage III), a largely destroyed one (Stage IV).

R, version 3.4.0 (R Development Core Team 2017) was used for all statistical

analyses. The survival time of females was examined by a generalized linear model (GLM) assuming Poisson error distribution and log link function. The diapause status (presence or absence of mature eggs in the ovaries at the time of death), mating status and body mass were used as explanatory variables. The *P*-value for this analysis was obtained from the Wald test.

## RESULTS

In the mating and control groups, 3 of 16 females and 4 of 19 females had mature eggs, respectively. I assume that in these females reproductive diapause terminated before or during the period at low temperature. The proportion of females with mature eggs did not differ between the mating and control groups (Fisher's exact test,  $P > 0.999$ ). The forewing length and the mating status also had no significant effect on the diapause status (Table 13).

The body mass was positively correlated to the forewing length at the first, second and third measurement (Table 13). The body mass depended on the mating experience at the first and second measurement, but not at the third measurement. There is no effect of the diapause status on the body mass. The body mass increased during the pre-overwintering period irrespective of the mating and diapause statuses. On the other hand, the increase in the body mass during the pre-overwintering period was significantly larger in females with longer forewing length, but did not depend on the diapause and mating statuses. Therefore, the performance to increase body mass did not correlate to the diapause or mating status.

The mean survival period was 13.4 weeks ( $n = 13$ ) and 5.0 weeks ( $n = 3$ ) in mated females that had maintained and terminated diapause, respectively (Fig. 12). In the control group, the mean survival period was 7.9 weeks ( $n = 15$ ) and 4.3 weeks ( $n = 4$ ) in females that had maintained and terminated diapause, respectively. The survival of females at the low temperature depended on the body mass ( $z = 4.261$ ,  $d.f. = 31$ ,  $P < 0.001$ ), the diapause status ( $z = 3.686$ ,  $d.f. = 31$ ,  $P < 0.001$ ), and the mating status ( $z = 3.198$ ,  $d.f. = 31$ ,  $P < 0.01$ ). Heavier females had longer survival times, and females that had maintained diapause had significantly

longer survival times than females that had terminated diapause. Furthermore, mating experience clearly resulted in prolonged longevity at the low temperature. Only mated females that had maintained diapause succeeded to survive the experimental period of 17 weeks.

The dissection of females succeeded in 1 of 3 females that had terminated diapause, and 12 of 13 females that had maintained diapause. The autumn-form female that had terminated diapause had a spermatophore of stage III, and its mass was 0.25 mg. In the females that had maintained diapause, 2 had spermatophores of stage II (1.95 and 2.51 mg), and 10 had spermatophores of stage III ( $0.82 \pm 0.49$  mg,  $\pm$  SD). This morphological change of spermatophores from stage I indicated the digestion of spermatophores by females.

## DISCUSSION

The main objective of this chapter was to examine the effect of pre-overwintering copulation on overwintering success of autumn-form females in *E. mandarina*. In Chapter 1, I pointed out that the nuptial gift hypothesis is probable as adaptive significance of pre-overwintering copulation of females. This hypothesis assumes that ejaculates derived from summer-form males improve overwintering success of autumn-form females (Kato 1986). The result of this chapter also supported this hypothesis, because the mated females showed prolonged survival at a low temperature in the laboratory. Morphological changes in spermatophores suggested their digestion by females.

Pre-overwintering copulation may be indispensable for autumn-form females of *E. mandarina* to overwinter in central Japan. Only mated females that had maintained reproductive diapause survived 4 months at a low temperature in the present study. Although the laboratory conditions of constant LD 10:14 and 10°C differ from the natural conditions in winter, the overwintering period is approximately 4 months in central Japan (Chapter 3). On the other hand, in southern Japan, the overwintering periods might be less than 2 months.

However, the result of Chapter 3 showed that most autumn-form females had a single spermatophore before winter even in southern Japan. Therefore, pre-overwintering copulation can improve overwintering success even in this area.

Physiological mechanisms of prolonged longevity in mated females remain unclear. Butterfly spermatophores contain nutrients such as sugars and proteins (Watanabe & Sato 1993; Bissoondath & Wiklund 1995; Blanco *et al.* 2009), and these nutrients may affect overwintering survival of females. In the present experiment, however, the females including those in the control group foraged the sucrose solution *ad libitum* before the transfer to low temperature. Therefore, proteins or other nutrients might be more important than sugars as the nuptial gift for females. Alternatively, the spermatophore derived from summer-form males may function as an additional nutrition storage organ in autumn-form females, if the females can digest the spermatophore at low temperature.

Quality of ejaculates as a nuptial gift may differ between summer-form males of the direct developing generations and the overwintering generation in *E. mandarina*. For direct developing generations of *E. mandarina*, Hiroki and Obara (1997) showed that the spermatophore mass in the bursa copulatrix did not affect the fecundity and longevity of summer-form females, although the maximum spermatophore mass corresponded to 4.8% of the male body mass on the day of eclosion (Konagaya & Watanabe 2013). This result contrasts with the general pattern in non-diapause adults of pierid butterflies (Rutowski *et al.* 1987; Watanabe & Ando 1993, Wiklund *et al.* 1993). In a nympharid butterfly *Bicyclus anynana*, females that have mated with dry-season-form males show longer longevity than females that have mated with wet-season-form males (Prudic *et al.* 2011). Furthermore, nuptial gift for overwintering females may be important for non-diapause males in late autumn, because overwintering success of sperm depend on that of his mates.

Maintenance of reproductive diapause must be important for autumn-form females to overwinter in *E. mandarina*. Females that had terminated diapause showed apparently short longevity at low temperature. Although I did not detect the time when females produced



mature eggs in the present study, the termination of diapause might be induced by high rearing temperature (25°C) before the transfer to low temperature. Because egg maturation clearly requires nutrients, this reproductive investment might sacrifice the resource for survival under the trade-off between reproduction and survival (Kirkwood & Rose 1991). As another possibility, females that have terminated diapause cannot reduce the metabolic rate at low temperature.

The body size is important for overwintering of autumn-form females in *E. mandarina*, because larger females showed longer longevity at low temperature. Overwintering adults are larger than summer-form adults (Chapter 3) as shown in many other insects (Danks 1994). The large size of overwintering individuals may be an adaptation to increase overwintering success. However, overwintering success in *E. mandarina* did not depend on the forewing length in a field survey (Chapter 1). In Chapter 2, I pointed out that the body mass is more important than the forewing length as a factor affecting overwintering success, because the body mass reflects lipid contents stored (Pullin 1987).

In a general perspective, the most important point of the present result is that the nuptial gift hypothesis can explain the pre-overwintering copulation of females. Although the nuptial gift hypothesis and the risk hedge hypothesis are not mutually exclusive, the latter does not seem to be important in *E. mandarina* (Chapter 1). Therefore, emergence of summer-form male adults in late autumn may have evolved together with pre-overwintering copulation of females to increase overwintering success. This scenario could apply to other insects such as the monarch butterfly *D. plexippus*. Females of this species probably have sufficient chances to mate immediately after overwintering because of their large-scale winter aggregation. Furthermore, 40% of diapause females mated with non-diapause males before winter (Leong *et al.* 1995, 2008), and mated females collected from winter aggregation sites showed longer longevity than unmated females of which diapause had been terminated in the laboratory (Leong *et al.* 2012). Although insufficient data are available in other insects, here I suggest that the nuptial gift is an important factor in the evolution of pre-overwintering

copulation and emergence of non-diapause male adults in late autumn.

## GENERAL DISCUSSION

Most insects have evolved diverse strategies to survive winter, which is an unfavorable season for their growth and reproduction (Tauber *et al.* 1986; Danks 1987). Reproductive diapause is usually important for adult overwintering in males and females. However, males can reproduce without overwintering and reproductive diapause by pre-overwintering copulation, indicating two fundamental ways to fertilize eggs for males when females overwinter as adults (Pener 1992; Kubrak *et al.* 2016). The occurrence of male reproductive diapause differs among species, in which all females enter reproductive diapause (Danilevskii 1966; Tauber *et al.* 1986; Pener 1992). In some insects including the Japanese common grass yellow *Eurema mandarina* (de l'Orza), only a part of males enter reproductive diapause with females (Table 14). In the present study, I approached to identification of the factors that have favored the evolution of non-diapause summer-form males in late autumn in *E. mandarina* by both laboratory experiments and field surveys.

### *A new insight on the evolution of non-diapause males in late autumn*

Male-male competition and winter severity were potential factors determining the frequency of both forms of males. In Chapter 1, I showed that autumn-form females that had mated with summer-form males re-mated with autumn-form males before the onset of oviposition, suggesting that the fitness of both forms of males strongly depends on the paternity in sperm competition between summer- and autumn-form males. On the other hand, summer-form males in late autumn is a phenotype that avoids diapause (Kato 1989), and avoidance of overwintering might be adaptive for summer-form males. If autumn-form males show lower overwintering success than autumn-form females, summer-form males can increase overwintering success of sperm by pre-overwintering copulation. In Chapter 2, however, I reported no sexual differences in overwintering success of autumn-form adults. In addition,

the frequency of both forms of males did not change from the subtropical zone to cool-temperate zone (Chapter 3), indicating that the relative fitness of both forms of males did not depend on winter severity. Therefore, I concluded that sperm competition is more important than winter severity as a factor determining frequency of both forms of males.

Female receptivity for mating before winter is another potential factor affecting the evolution of non-diapause males in late autumn (Pener 1992). Only when females have receptivity for mating before winter, non-diapause males can evolve. In Chapter 4, I suggested that the adaptive significance of pre-overwintering copulation is improvement of overwintering success in *E. mandarina*. Nutrition derived from males must be important for overwintering females, especially when overwintering success is low. This idea corresponds to that pre-overwintering copulation has been observed in butterflies species with low overwintering success (Chapter 2). Therefore, I suggest that low overwintering success favors the evolution of pre-overwintering copulation of females, and indirectly enhances that the evolution of non-diapause males in late autumn.

#### *Unsolved problems in the evolution of non-diapause males in late autumn*

Detailed features of male-male competition that allowed the evolution of non-diapause males remain unclear. In *E. mandarina*, approximately a half of males emerge as summer-form adults in autumn (Kato & Sano 1987). Therefore, summer-form males should fertilize approximately a half of eggs laid by autumn-form females in spring, if seasonal forms of males under short-day conditions are genetically determined. Because autumn-form females that had mated with summer-form males in autumn re-mate with autumn-form males in the following spring before the onset of oviposition (Chapter 1), summer-form males must fertilize approximately a half of eggs even under sperm competition. This prediction did not correspond to the general pattern of last male precedence in sperm competition of butterflies (Simmons 2001). Although I have not estimated the paternity, summer-form males in late

autumn produced longer eupyrene sperm than autumn-form males (see Chapter 1). Long eupyrene sperm might buffer the last male precedence in the sperm competition (Gage 1994; Pattarini *et al.* 2006). This hypothesis should be examined in future.

The maintenance of polymorphic strategy in males of *E. mandarina* is another unsolved problem. The results in Chapter 3 that the frequency of summer-form males under short-day conditions varied among the lineages indicate a genetic variation for the sensitivity of males to a short photoperiod. Because natural selection has a general tendency to reduce genetic variation (Gray & McKinnon 2007), the maintenance of genetic variation should be explained specifically. Negative frequency dependent selection could maintain the polymorphic strategy of males in the final generation of the year in *E. mandarina*, when the rare form is more successful than the common form (Gross 1996). This assumption may be applicable to *E. mandarina*. The number of mating is a fundamental component of the fitness of summer- and autumn-form males, and is clearly frequency dependent. An autumn-form female usually mates with one summer-form male in autumn, and re-mate with one autumn-form male in the following spring before the onset of oviposition (see Chapter 1). Because a male can mate with several females, on the other hand, the average number of mating in summer-form males is estimated as the number of females divided by the number of summer-form males. This estimation can also be applied for autumn-form males. When the summer-form male is the rare form, the number of mating of an individual must be larger for summer-form males than for autumn-form males, and vice versa. This condition indicates a selective advantage of the rare form. To examine this hypothesis, theoretical studies and long term investigations on changes in the frequency of summer-form males in a population are required.

Another unsolved problem is the cost of mating for overwintering males in *E. mandarina*. If pre-overwintering copulation imposes only low cost to males, males may evolve to mate in both the pre- and post-overwintering periods. However, producing large spermatophores may be costly for butterfly males, especially under food limitation (Cordero

2000; Prudic *et al.* 2011; Takeuchi 2012; Carballero-Mendieta and Cordero, 2013), thereby sacrificing male longevity. In *E. mandarina* summer-form males transfer a large spermatophore in a copulation. The spermatophore mass is approximately 5% of the male body mass on the day of emergence (Konagaya & Watanabe 2013). Moreover, butterfly males transfer accessory substance as well as spermatophores to females during copulation. The total ejaculate mass is even larger than the spermatophore mass (Hiroki & Obara 1997). In Chapter 2, overwintering success of autumn-form males depended on the body mass, and thus it is highly likely that mating brings large costs for overwintering in butterfly males.

Uncertainty of winter conditions should be focused in future works. In Chapter 1, I pointed out that autumn-form females of *E. mandarina* have sufficient chances for mating in spring before the onset of oviposition. However, autumn-form females might suffer less chances for mating after exceptionally severe winter reducing population density. In this situation, pre-overwintering copulation of females functions as preparation of sperm, supporting the risk-hedge hypothesis in adaptive significance of pre-overwintering copulation (Kato 1986). A complete test of the risk-hedge hypothesis requires long-term studies.

Uncertainty of winter conditions might also affect the evolution of non-diapause males in late autumn. Non-diapause males can obtain offspring by pre-overwintering copulation when most autumn-form males failed in overwintering under exceptionally severe winter. Therefore, a genotype producing both forms of males in late autumn might be favored by natural selection, because it functions as bet-hedging that increases geometric mean of fitness (Hopper 1999). In Chapter 3, however, the frequency of summer-form males did not change from the subtropical zone to cool temperate zone, although the risk of exceptionally severe winter may be higher in populations with higher latitude.

#### *Future directions*

It is possible that pre-overwintering copulation of females with non-diapause males to

increase overwintering success under a low overwintering success are evolved in some other insects, in which a part of male adults enter diapause. For example, the monarch butterfly *Danaus plexippus* (L.) is a probable candidate. First, this species often shows low overwintering success (< 25%). Second, females have sufficient chances for mating after overwintering, because they make large scale aggregations during overwintering. In addition, mated females seem to use spermatophores for their somatic maintenance (Leong *et al.* 2012). On the other hand, higher longevity of mated females during overwintering was not shown in a predatory bug *Nabis rugosus* (L.), in which a part of males and all females enter reproductive diapause (Roth & Reinhardt 2003). In this species, the risk hedge hypothesis can explain pre-overwintering copulation of females, because males show lower overwintering success than females (Roth & Reinhardt 2009).

Females that have receptivity for mating both before and after winter have been reported in several insect species of Orthoptera, Heteroptera, Coleoptera, Diptera and Lepidoptera (Table 14). In only three species of them, diapause females mate with non-diapause males in autumn (Kato 1986; Greenfield & Pener 1992; Leong *et al.* 2012). The presence of non-diapause males in autumn is unclear in the other species. However, female bias in population sex ratio during overwintering has been reported in a predatory bug *N. rugosus* (Roth & Reinhardt 2009), a grasshopper *Stenocatantops splendens* (Thunberg) (Zhu *et al.* 2013), and the kudzu bug *Megacopta cribraria* (F.) (Goelc & Hu 2015). These female bias might result from the presence of non-diapause males that die before winter. If non-diapause males in late autumn may be more common in nature than we have believed, there are chances for comparative study to understand the evolution of male reproductive diapause. In the present study, I suggest importance of overwintering success of diapause adults and sperm competition between non-diapause and diapause males on the evolution of non-diapause males emerging in late autumn. Therefore, overwintering success and paternity in the sperm competition should be widely examined for the complete understanding of life-cycle evolution in adult overwintering insects.

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**Table 1** Forewing length, number of spermatophores in the bursa copulatrix, total weight of spermatophores in the bursa copulatrix and number of eggs in the ovaries for females of *Eurema mandarina* in the pre- and post-overwintering periods (mean  $\pm$  SD).

	Pre-overwinter			Post overwinter		
	12	7	14	15	13	13
No. females						
Forewing length (mm)	21.9 $\pm$ 1.6	22.8 $\pm$ 1.0	22.2 $\pm$ 1.7	22.5 $\pm$ 1.4	22.6 $\pm$ 1.3	
No. spermatophores	0.58 $\pm$ 0.51	1.57 $\pm$ 0.53**	1.21 $\pm$ 0.43*	1.26 $\pm$ 0.46*	2.23 $\pm$ 0.83****	
Total spermatophore mass ( $\mu$ g)	0.42 $\pm$ 0.19	0.58 $\pm$ 0.42	0.74 $\pm$ 0.63	0.65 $\pm$ 0.62	1.51 $\pm$ 1.39*	
No. mature eggs	0.0 $\pm$ 0.0	25.3 $\pm$ 17.0****	31.1 $\pm$ 16.8****	20.7 $\pm$ 4.6****	18.7 $\pm$ 4.8****	
No. submature eggs	0.0 $\pm$ 0.0	14.7 $\pm$ 8.3****	16.1 $\pm$ 6.2****	12.1 $\pm$ 4.6****	8.2 $\pm$ 4.4****	
No. immature eggs	390.7 $\pm$ 60.4	344.0 $\pm$ 50.0	348.0 $\pm$ 93.8	264.5 $\pm$ 55.7****	186.0 $\pm$ 87.1****	

Mann-Whitney *U* test with Bonferroni correction was used. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .

**Table 2** Results of model selection based on the small-sample-size-corrected version of the Akaike Information Criterion (AICc) for generalized linear mixed models of factors that affected the survival of wild-caught autumn-form *Eurema mandarina* adults under semi-natural conditions.

Model	AICc	$\Delta$ AICc	<i>d.f.</i>	Deviance
Body mass	49.58	0	27	42.66
Sex + Body mass	51.69	2.11	26	42.1
Null model	53.27	3.69	28	48.83
Sex * Body mass	54.25	4.67	25	41.75
Forewing length	55.14	5.56	27	48.21
Sex	55.62	6.04	27	47.71
Sex + Forewing length	57.59	8.01	26	47.99
Sex * Forewing length	60.46	10.88	25	47.96

**Table 3** Mean ( $\pm$  SE) forewing lengths (mm) of *Eurema mandarina* summer-form males, autumn-form males, and females in autumn and spring.

Survey period	Summer-form males	Autumn-form males	Autumn-form females
Autumn	21.3 $\pm$ 0.19 (53) <sup>a</sup>	22.1 $\pm$ 0.12 (109) <sup>b</sup>	22.1 $\pm$ 0.14 (95) <sup>b</sup>
Spring	-	22.2 $\pm$ 0.12 (75) <sup>b</sup>	22.2 $\pm$ 0.12 (81) <sup>b</sup>

Different superscript letters indicate significant differences ( $P < 0.01$ , Tukey test).

**Table 4** Demographic models of autumn-form *Eurema mandarina* adults selected by the small-sample-size-corrected version of the Akaike Information Criterion (AICc).

Model	AICc	$\Delta$ AICc	AICc Weights	Model Likelihood	No. Parameters	Deviance
$\varphi$ ( <i>winter</i> ) $p$ ( <i>sex</i> + <i>t</i> ) <i>Pent</i> ( <i>winter</i> ) $N$ ( <i>sex</i> )	1205.609	0	0.31363	1	35	-1308.38
$\varphi$ ( <i>winter</i> ) $p$ ( <i>sex</i> + <i>t</i> ) <i>Pent</i> ( $T$ + <i>winter</i> ) $N$ ( <i>sex</i> )	1207.961	2.3519	0.09676	0.3085	36	-1322.32
$\varphi$ ( <i>sex</i> * <i>winter</i> ) $p$ ( <i>sex</i> + <i>t</i> ) <i>Pent</i> ( $T$ ) $N$ ( <i>sex</i> )	1208.911	3.3015	0.06019	0.1919	37	-1323.73

$\varphi$ ,  $p$ , *Pent*, and  $N$  represent the apparent survival rate, catchability, probability of entrance, and population size, respectively, and terms in parentheses represent the model's structure. *winter* indicates that the parameter differed between winter and the other seasons, and *sex* indicates that the parameter differed between the sexes. *t* indicates that the parameter changed from day to day, and  $T$  indicates a linear trend.

**Table 5** Demographic models of summer-form *Eurema mandarina* males that emerge in autumn selected by the small-sample-size-corrected version of the Akaike Information Criterion (AICc).

Model	AICc	$\Delta$ AICc	AICc Weights	Model Likelihood	No. Parameters	Deviance
$\varphi(T)p(t)Pent(.)N(.)$	226.3241	0	0.30331	1	12	-112.27
$\varphi(.)p(t)Pent(.)N(.)$	226.447	0.1229	0.28523	0.9404	11	-109.461
$\varphi(TT)p(t)Pent(.)N(.)$	227.2252	0.9011	0.19329	0.6373	12	-111.369

$\varphi$ ,  $p$ ,  $Pent$ , and  $N$  represent the apparent survival rate, catchability, probability of entrance, and population size, respectively, and terms in parentheses represent the model's structure.  $T$  indicates a linear trend, and  $t$  indicates that the parameter changed from day to day. A dot (.) indicates that the parameter was constant throughout the experiment, and  $TT$  indicates a quadratic trend.

**Table 6** The daily apparent survival rates ( $\varphi$ ) estimated by the most appropriate model for summer-form males and autumn-form adults in *Eurema mandarina*.

Season	Period	Autumn-form males		Autumn-form females		Summer-form males	
		Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Autumn	Oct. 21 - Oct. 22	0.923	0.896 - 0.944	0.923	0.896 - 0.944	0.950	0.847 - 0.985
	Oct. 22 - Oct. 27	0.923	0.896 - 0.944	0.923	0.896 - 0.944	0.921	0.826 - 0.967
	Oct. 27 - Oct. 28	0.923	0.896 - 0.944	0.923	0.896 - 0.944	0.879	0.787 - 0.935
	Oct. 28 - Oct. 30	0.923	0.896 - 0.944	0.923	0.896 - 0.944	0.819	0.701 - 0.897
	Oct. 30 - Oct. 31	0.923	0.896 - 0.944	0.923	0.896 - 0.944	0.737	0.544 - 0.869
	Oct. 31 - Nov. 1	0.923	0.896 - 0.944	0.923	0.896 - 0.944	0.636	0.353 - 0.848
	Nov. 1 - Nov. 3	0.923	0.896 - 0.944	0.923	0.896 - 0.944	0.520	0.192 - 0.832
	Nov. 3 - Mar. 24	0.979	0.970 - 0.986	0.979	0.970 - 0.986	-	-
	Mar. 24 - Mar. 25	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Mar. 25 - Mar. 28	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
Spring	Mar. 28 - Mar. 29	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Mar. 29 - Mar. 31	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Mar. 31 - Apr. 1	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 1 - Apr. 2	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 2 - Apr. 7	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 7 - Apr. 8	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 8 - Apr. 10	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 10 - Apr. 14	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 14 - Apr. 15	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 15 - Apr. 23	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
Summer	Apr. 23 - Apr. 24	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 24 - Apr. 25	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 25 - Apr. 26	0.923	0.896 - 0.944	0.923	0.896 - 0.944	-	-
	Apr. 26 - Apr. 27	0.923	0.896 - 0.944	0.923	0.896 - 0.945	-	-
	Apr. 27 - May 2	0.923	0.896 - 0.944	0.923	0.896 - 0.946	-	-
	May 2 - May 8	0.923	0.896 - 0.944	0.923	0.896 - 0.947	-	-

The most appropriate model was different between autumn-form adults and summer-form males. The model is  $\{\varphi(\text{winter}) p(\text{sex}+\text{t}) Pent(\text{winter}) N(\text{sex})\}$  for autumn-form adults and  $\{\varphi(\text{T}) p(\text{t}) Pent(\cdot) N(\cdot)\}$  for summer-form males.

**Table 7** The probabilities of entrance ( $Pent$ ) estimated by the most appropriate model for summer-form males and autumn-form adults in *Eurema mandarina*.

Season	Period	Autumn-form males		Autumn-form females		Summer-form males	
		Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Autumn	Oct. 22 - Oct. 27	0.017	0.013 - 0.022	0.017	0.013 - 0.022	0.054	0.019 - 0.144
	Oct. 27 - Oct. 28	0.017	0.013 - 0.022	0.017	0.013 - 0.022	0.054	0.019 - 0.144
	Oct. 28 - Oct. 30	0.017	0.013 - 0.022	0.017	0.013 - 0.022	0.054	0.019 - 0.144
	Oct. 30 - Oct. 31	0.017	0.013 - 0.022	0.017	0.013 - 0.022	0.054	0.019 - 0.144
	Oct. 31 - Nov. 1	0.017	0.013 - 0.022	0.017	0.013 - 0.022	0.054	0.019 - 0.144
Winter	Nov. 1 - Nov. 3	0.017	0.013 - 0.022	0.017	0.013 - 0.022	0.054	0.019 - 0.144
	Nov. 3 - Mar. 24	0.000	0.000 - 0.000	0.000	0.000 - 0.000	-	-
Spring	Mar. 24 - Mar. 25	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Mar. 25 - Mar. 28	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Mar. 28 - Mar. 29	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Mar. 29 - Mar. 31	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Mar. 31 - Apr. 1	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Apr. 1 - Apr. 2	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Apr. 2 - Apr. 7	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Apr. 7 - Apr. 8	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Apr. 8 - Apr. 10	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Apr. 10 - Apr. 14	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
	Apr. 14 - Apr. 15	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-
Apr. 15 - Apr. 23	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-	
Apr. 23 - Apr. 24	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-	
Apr. 24 - Apr. 25	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-	
Apr. 25 - Apr. 26	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-	
Apr. 26 - Apr. 27	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-	
Apr. 27 - May 2	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-	
May 2 - May 8	0.017	0.013 - 0.022	0.017	0.013 - 0.022	-	-	

The most appropriate model was different between autumn-form adults and summer-form males. The model is  $\{\varphi(\text{winter})p(\text{sex}+t) Pent(\text{winter})N(\text{sex})\}$  for autumn-form adults and  $\{\varphi(T)p(t)Pent(.)N(.)\}$  for summer-form males.

**Table 8** The catchability ( $p$ ) estimated by the most appropriate model for summer-form males and autumn-form adults in *Eurema mandarina*.

Season	Period	Autumn-form males		Autumn-form females		Summer-form males	
		Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Autumn	Oct. 22	0.020	0.009 - 0.041	0.010	0.005 - 0.022	0.076	0.025 - 0.209
	Oct. 27	0.142	0.091 - 0.215	0.077	0.046 - 0.127	0.274	0.131 - 0.485
	Oct. 28	0.079	0.047 - 0.129	0.041	0.023 - 0.073	0.460	0.253 - 0.681
	Oct. 30	0.132	0.084 - 0.201	0.071	0.042 - 0.118	0.639	0.296 - 0.882
	Oct. 31	0.096	0.059 - 0.154	0.051	0.029 - 0.088	0.185	0.065 - 0.426
	Nov. 1	0.109	0.068 - 0.171	0.058	0.034 - 0.099	0.372	0.104 - 0.751
	Nov. 3	0.057	0.031 - 0.103	0.029	0.015 - 0.057	0.502	0.025 - 0.975
	Mar. 24	0.143	0.027 - 0.503	0.077	0.013 - 0.347	-	-
	Mar. 25	0.321	0.154 - 0.550	0.192	0.079 - 0.396	-	-
	Mar. 28	0.211	0.106 - 0.377	0.119	0.053 - 0.244	-	-
	Mar. 29	0.426	0.283 - 0.583	0.272	0.156 - 0.431	-	-
Spring	Mar. 31	0.101	0.044 - 0.215	0.054	0.022 - 0.126	-	-
	Apr. 1	0.196	0.113 - 0.318	0.109	0.057 - 0.200	-	-
	Apr. 2	0.208	0.125 - 0.325	0.117	0.064 - 0.204	-	-
	Apr. 7	0.102	0.047 - 0.209	0.054	0.023 - 0.121	-	-
	Apr. 8	0.211	0.125 - 0.333	0.119	0.065 - 0.207	-	-
	Apr. 10	0.179	0.103 - 0.294	0.099	0.053 - 0.179	-	-
	Apr. 14	0.145	0.076 - 0.258	0.079	0.038 - 0.154	-	-
	Apr. 15	0.231	0.140 - 0.357	0.131	0.073 - 0.224	-	-
	Apr. 23	0.321	0.179 - 0.507	0.192	0.098 - 0.343	-	-
	Apr. 24	0.174	0.088 - 0.315	0.096	0.046 - 0.190	-	-
	Apr. 25	0.207	0.113 - 0.348	0.116	0.060 - 0.214	-	-
Apr. 26	0.115	0.055 - 0.224	0.061	0.028 - 0.128	-	-	
Apr. 27	0.214	0.123 - 0.346	0.121	0.065 - 0.213	-	-	
May 2	0.112	0.051 - 0.231	0.060	0.026 - 0.133	-	-	
May 8	0.069	0.023 - 0.184	0.036	0.012 - 0.103	-	-	

The most appropriate model was different between autumn-form adults and summer-form males. The model is  $\{\varphi(\text{winter})p(\text{sex+t})\text{Pent}(\text{winter})N(\text{sex})\}$  for autumn-form adults and  $\{\varphi(\text{T})p(\text{t})\text{Pent}(\cdot)N(\cdot)\}$  for summer-form males.



**Table 9** Results of model selection based on the Akaike Information Criterion (AICc) for generalized linear mixed models of factors that affected the proportion of autumn-form adults in *Eurema mandarina* reared under short-day conditions at 20 or 25°C.

Model <sup>†</sup>	AIC	ΔAIC	<i>d.f.</i>	Deviance
<i>Win</i> + <i>Sex</i> * <i>Tem</i>	211	0	95	199
<i>Sex</i> * <i>Tem</i>	211.8	0.8	96	201.8
<i>Win</i> + <i>Sex</i> + <i>Tem</i>	213.7	2.7	96	203.7
<i>Win</i> * <i>Tem</i> + <i>Sex</i>	213.8	2.8	95	201.8
<i>Sex</i> + <i>Tem</i>	214.2	3.2	97	206.2
<i>Win</i> * <i>Sex</i> * <i>Tem</i>	215	4	92	197
<i>Win</i> * <i>Sex</i> + <i>Tem</i>	215.5	4.5	95	203.5
<i>Sex</i>	217.7	6.7	98	211.7
<i>Win</i> + <i>Sex</i>	218.1	7.1	97	210.1
<i>Win</i> * <i>Sex</i>	219.8	8.8	96	209.8
<i>Win</i> + <i>Tem</i>	604.1	393.1	97	596.1
<i>Win</i> * <i>Tem</i>	605.3	394.3	96	595.3
<i>Win</i>	606.2	395.2	98	600.2
<i>Tem</i>	607	396	98	601
Null model	608.1	397.1	99	604.1

<sup>†</sup>*Win* represents the winter length defined as the number of dates that the average maximum temperature in 1981-2010 is below 15°C (Japan Meteorological Agency 2017), and *Tem* indicates the rearing temperature.

**Table 10** Proportion of summer-form adults (%) for males and females with their behavior in *Eurema mandarina* collected in the field.

Collection site	Flying		Foraging		Resting		Puddling		Mating		Total	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Morioka	66.7 (9)	0.0 (2)	42.86 (7)	0.0 (2)	0.0 (1)	-	-	-	-	-	52.9 (17)	0.0 (4)
Iwanuma/Watari	60.0 (30)	0.0 (6)	10.0 (10)	0.0 (15)	10.0 (10)	25.0 (4)	85.7 (7)	-	100.0 (1)	0.0 (1)	46.6 (58)	3.8 (26)
Kameoka	51.9 (27)	0.0 (8)	10.0 (10)	0.0 (7)	25.0 (4)	0.0 (1)	-	-	-	-	39.0 (41)	0.0 (16)
Joyo	33.3 (27)	0.0 (5)	8.33 (12)	0.0 (8)	0.0 (4)	-	-	-	-	-	23.3 (43)	0.0 (13)
Shimanto	47.1 (34)	0.0 (3)	10.0 (10)	0.0 (5)	-	-	-	-	-	-	38.6 (34)	0.0 (8)
Nichinan	42.1 (19)	0.0 (2)	-	0.0 (1)	0.0 (1)	-	50.0 (2)	-	-	-	40.9 (22)	0.0 (3)
Shibushi	58.8 (51)	0.0 (2)	12.5 (8)	0.0 (3)	-	-	-	-	100.0 (2)	0.0 (2)	54.1 (61)	0.0 (7)
Okinoerabu	68.4 (19)	-	-	-	-	-	-	100.0 (1)	-	-	68.4 (19)	100.0 (1)

**Table 11** Forewing length of *Eurema mandarina* collected from eight localities in Japan in relation to seasonal forms.

Collection site	Male		Female	
	Summer form	Autumn form	Summer form	Autumn form
Morioka	22.7 ± 1.4 (9)	23.1 ± 1.1 (8)	-	23.3 ± 0.7 (4)
Iwanuma/Watari	21.6±1.1 (25)	22.8±1.0 (28)	22.2 (1)	23.2±1.1 (24)
Kameoka	21.8±1.2 (16)	22.9±0.9 (23)	-	22.9±1.1 (16)
Joyo	21.8±1.4 (10)	22.1±1.4 (33)	-	22.9±1.3 (13)
Shimanto	21.5±1.7 (19)	22.7±1.2 (29)	-	23.2±0.7 (8)
Nichinan	21.2±0.8 (9)	21.3±1.3 (12)	-	23.9±1.2 (3)
Shibushi	22.3±1.3 (33)	22.7±1.3 (28)	-	23.4±1.4 (7)
Okinoerabu	20.0±1.2 (13)	20.7±1.7 (7)	-	-
Total	21.7±1.4 (134)	22.5±1.3 (168)	22.2 (1)	23.1±1.1 (75)

Forewing lengths are shown as mean ± SD (mm) with sample sizes in parentheses.  
Five individuals were excluded in this table because of damage in the forewings.

**Table 12** Number of females classified by the number of spermatophore contained, the total spermatophore mass contained and the proportion of mated individuals in autumn-form females of *Eurema mandarina* collected from seven localities in Japan.

Collection site	No. spermatophores			Total spermatophore mass <sup>†</sup>	Proportion of mated females
	0	1	2		
Morioka	1	8	0	0.58 ± 0.43 (8)	88.9% (9)
Iwanuma/Watari	3	16	1	0.43 ± 0.46 (11)	85.0% (20)
Kameoka	1	14	0	0.33 ± 0.42 (14)	93.3 % (15)
Joyo	0	12	0	0.39 ± 0.26 (12)	100.0% (12)
Shimanto	1	8	0	0.70 ± 0.32 (8)	88.9% (9)
Nichinan	1	2	0	0.69 (1)	66.6% (3)
Shibushi	0	6	0	1.25 ± 0.85 (6)	100.0% (6)

<sup>†</sup>Values are shown as mean ± SD with sample sizes in parentheses.

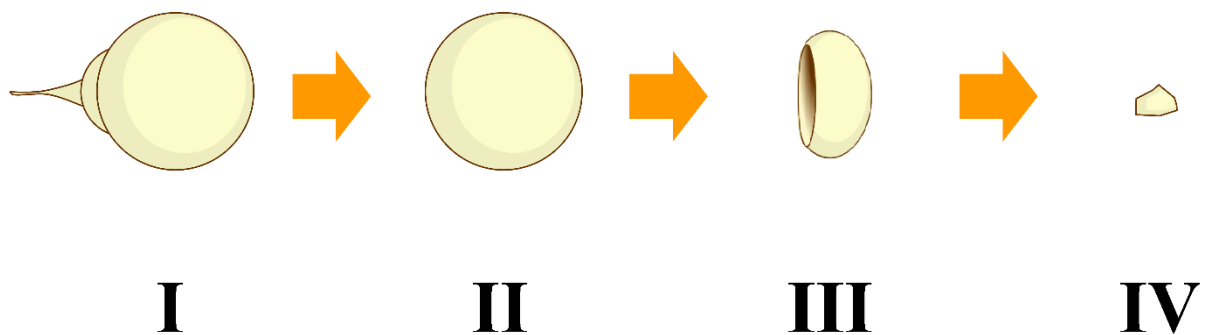
**Table 13** Forewing length and body mass (mean  $\pm$  SD) under short-day conditions (LD 10:14) at 25°C and 65% or higher R.H. in autumn-form female in *Eurema mandarina* in relation to their mating experience.

Diapause status	Mating experience	n	Forewing length (mm) *	Body mass (mg)			Increase **
				1st ***	2nd ***	3rd **	
Maintained	Mated	13	21.6 $\pm$ 1.6	63.40 $\pm$ 9.87	74.70 $\pm$ 9.37	72.40 $\pm$ 19.00	9.00 $\pm$ 18.25
	Unmated	15	21.9 $\pm$ 1.4	55.69 $\pm$ 10.93	63.67 $\pm$ 10.57	63.89 $\pm$ 12.01	8.20 $\pm$ 6.84
Terminated	Mated	3	21.1 $\pm$ 0.3	54.47 $\pm$ 2.26	54.02 $\pm$ 9.08	54.96 $\pm$ 11.97	0.49 $\pm$ 13.73
	Unmated	4	22.1 $\pm$ 0.9	58.68 $\pm$ 8.40	68.77 $\pm$ 4.89	68.18 $\pm$ 9.07	9.50 $\pm$ 5.60

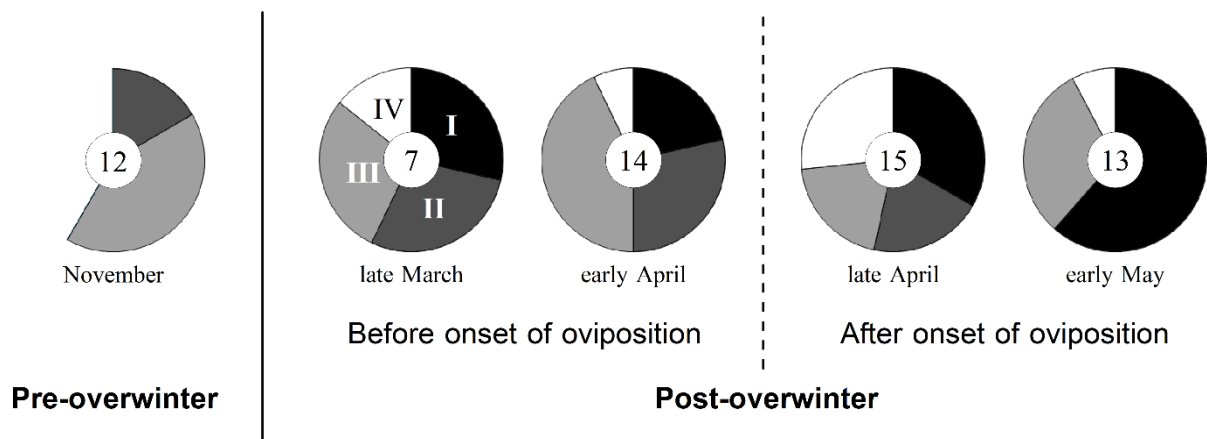
The body mass was measured once a week. Females containing mature eggs in the ovaries at the time of death were regarded as individuals in which diapause had been terminated. The asterisk (\*) indicates no significant relationships ( $P > 0.05$ ) to the diapause status and mating experience. The asterisks (\*\*) indicate a significant relationship ( $P < 0.05$ ) to the forewing length and no significant relationships ( $P > 0.05$ ) to the diapause status and mating experience. The asterisks (\*\*\*) represent significant relationships ( $P < 0.05$ ) to the forewing length and mating experience and no significant relationship ( $P > 0.05$ ) to the diapause status. The  $P$ -values were obtained from multiple regression analyses.

**Table 14** List of insect species in which overwintering female adults mate both in autumn and spring with reference to the presence of non-diapause male adults in autumn.

Species	Mating with non-diapause males in autumn	Literature
<b>Orthoptera</b>		
<i>Anacridium aegyptium</i> (L.)	presence	Greenfield & Pener (1992)
<i>Stenocatantops splendens</i> (Thunberg)	unknown	Zhu <i>et al.</i> (2013)
<b>Heteroptera</b>		
<i>Menida scotti</i> (Puton)	unknown	Koshiyama <i>et al.</i> (1994)
<i>Megacopta cribraria</i> (F.)	unknown	Golec & Hu (2015)
<i>Nabis rugosus</i> (L.)	unknown	Roth & Reinhardt (2009)
<i>N. ericetorum</i> Scholtz	unknown	Roth & Reinhardt (2009)
<i>N. pseudoferus</i> Remane	unknown	Roth & Reinhardt (2009)
<i>Pyrrhocoris apterus</i> (L.)	unknown	Socha (2010)
<b>Coleoptera</b>		
<i>Coccinella 7-punctata</i> L.	unknown	Hodek & Čerkasov (1960)
<i>Plagioderma versicolora</i> (Laicharting)	unknown	Stevens & McCauley (1989)
<i>Epilacjma varivestis</i> Mulsant	unknown	Taylor (1984)
<i>Leptinotarsa decemlineata</i> (Say)	unknown	Baker <i>et al.</i> (2005)
<b>Lepidoptera</b>		
<i>Danaus plexippus</i> (L.)	presence	Leong <i>et al.</i> (2012)
<i>Eurema mandarina</i> (de l'Orza)	presence	Fukuda <i>et al.</i> (1982)
<i>Curetis acuta</i> Moore	unknown	Shirouzu (2006)
<b>Diptera</b>		
<i>Drosophila alboralis</i> Momma et Takada	unknown	Kimura (1980)
<i>D. pseudoobscura</i> Frolova	unknown	Collett & Jarman (2001)

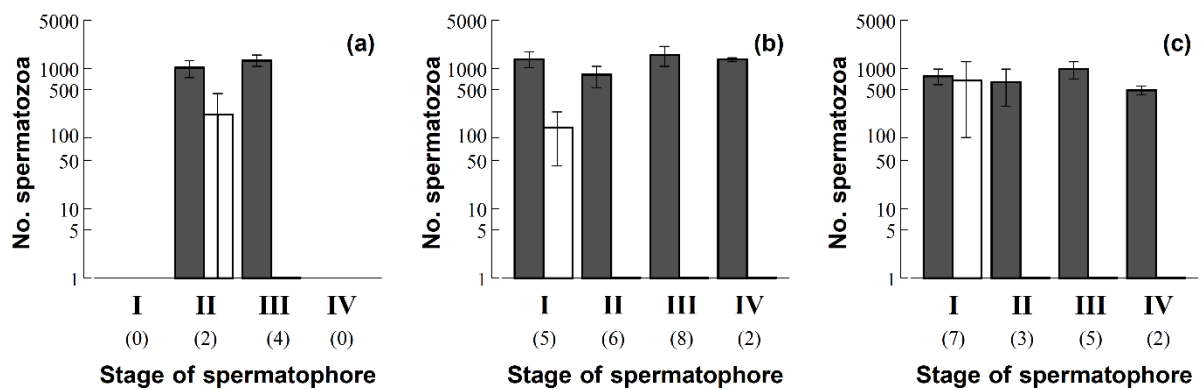


**Figure 1** A schematic representation of the process of spermatophore collapse in the bursa copulatrix of *Eurema mandarina* females. Stage I shows the intact spermatophore immediately after copulation. The spermatophore losing its neck is in stage II. Probably due to the activity of the bursa copulatrix, the spermatophore is squashed and the nutrients it contains are absorbed by the female (stage III). Consequently, only a tiny piece of the spermatophore capsule remains in the bursa copulatrix (stage IV).

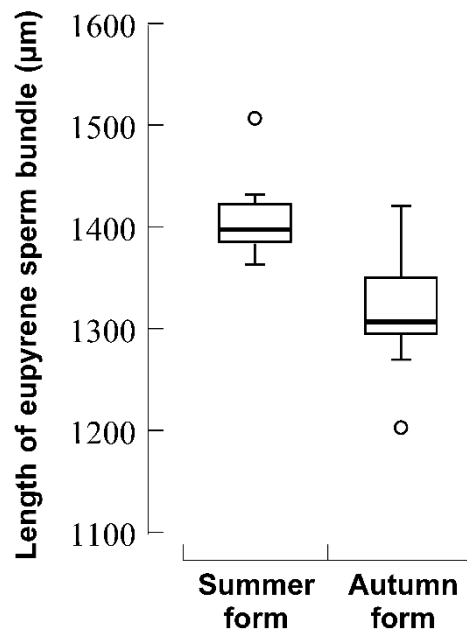


**Figure 2** Autumn-form females of *Eurema mandarina* classified by the stage of the largest spermatophore (I - IV) in the bursa copulatrix for each survey period. The onset of oviposition occurred in mid-April after overwintering. Out of 12 females captured in November, 7 had mated and 5 were virgins. The shape and the condition of spermatophores in each stage are shown in Figure 1. *Numerals* represent the sample size

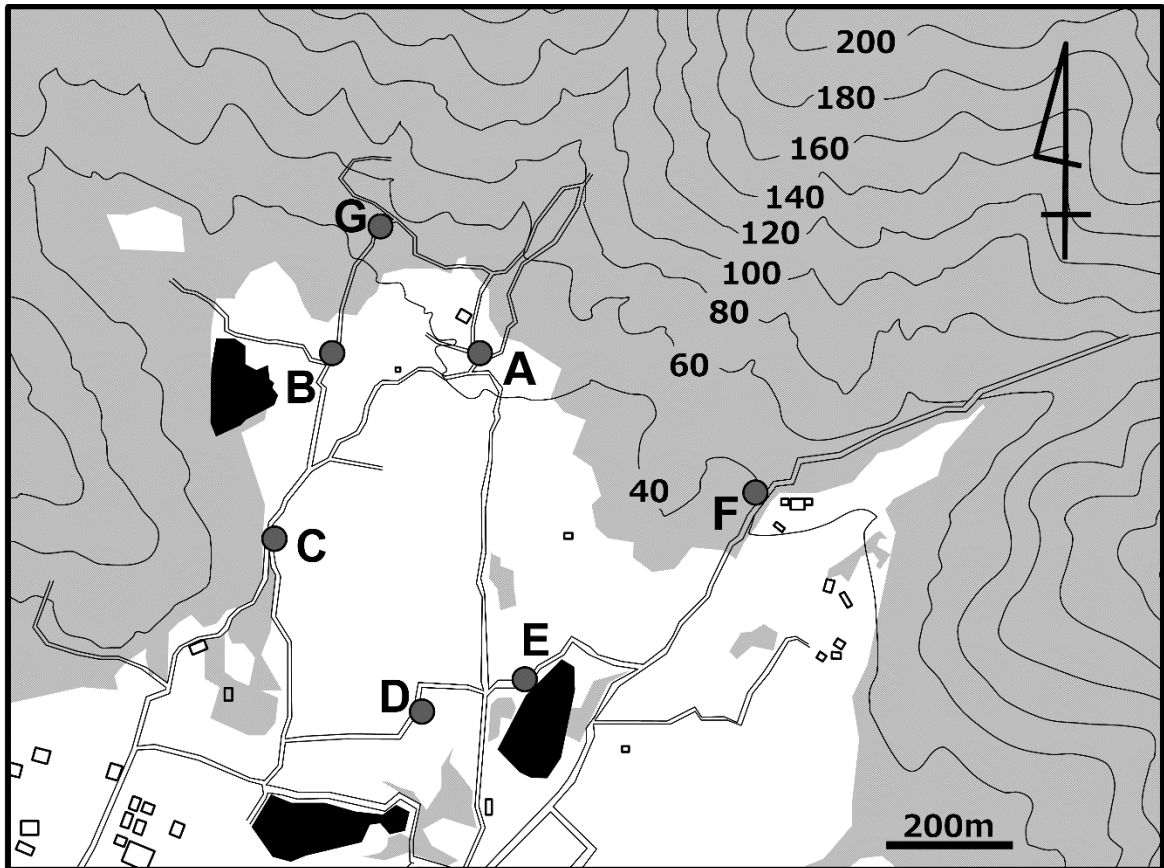




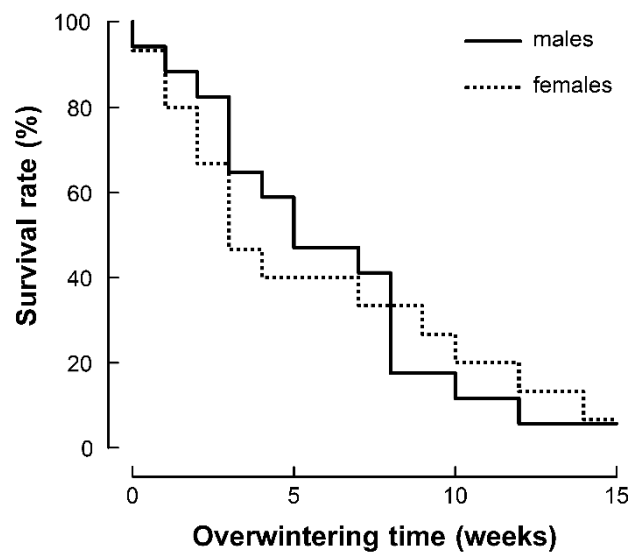
**Figure 3** The number of eupyrene (*closed*) and apyrene (*open*) spermatozoa stored in the spermatheca of females of *Eurema mandarina* collected in the pre-overwintering period (a) and in those collected before (b) and after (c) the onset of oviposition in the post-overwintering period, classified by the stage of the largest spermatophore (I–IV) in the bursa copulatrix ( $\pm$  SE). Numerals in parentheses represent the sample size.



**Figure 4** Length of eupyrene sperm bundle in the duplex of summer-form males ( $n = 7$ ) and autumn-form males ( $n = 9$ ) in *Eurema mandarina*. Median  $\pm$  the first and third quartiles (Mann-Whitney  $U$ -test,  $U = 10$ ,  $P < 0.01$ ). Bars represent the longest and shortest bundles, and the *circle* represents an outlier

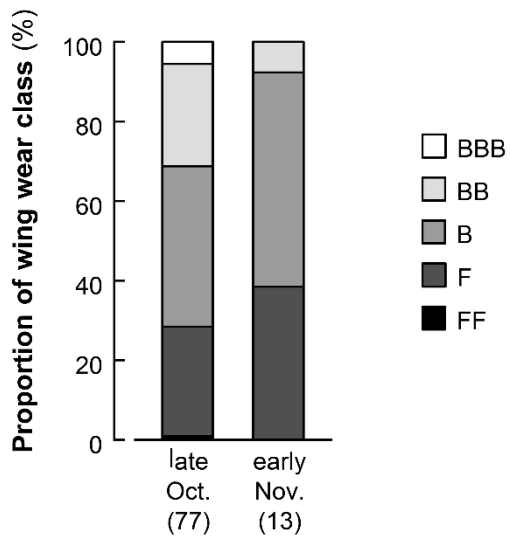


**Figure 5** Survey area of the mark-release-recapture experiment. White represents open grasslands and rice paddy fields, gray represents deciduous forest, and black represents ponds. Roads, residential houses, and contour lines are also indicated. A to G represent the survey sites. Except for the areas surrounding F and G, most of the forests were not suitable for *Eurema mandarina*, because bamboo grass grew at a high density.

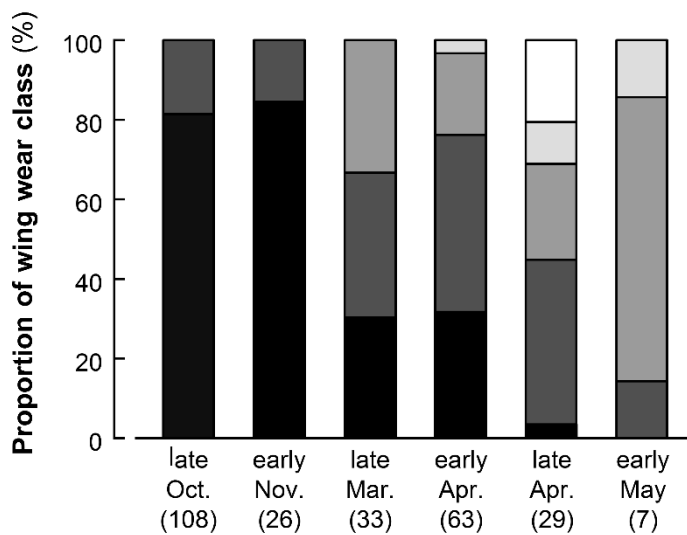


**Figure 6** Survival curves of overwintering autumn-form *Eurema mandarina* adults under semi-natural conditions that were caught on November 17 in Kyoto, Japan. The solid line indicates autumn-form males ( $n = 15$ ), and the dashed line indicates autumn-form females ( $n = 17$ ).

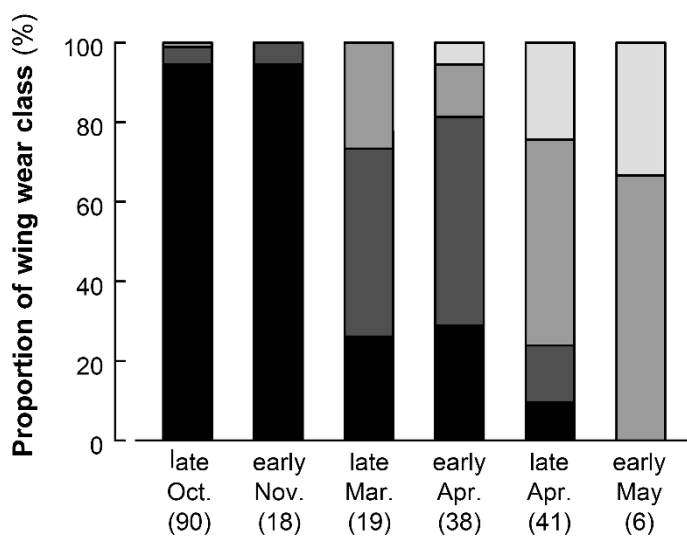
**(a) summer-form males**



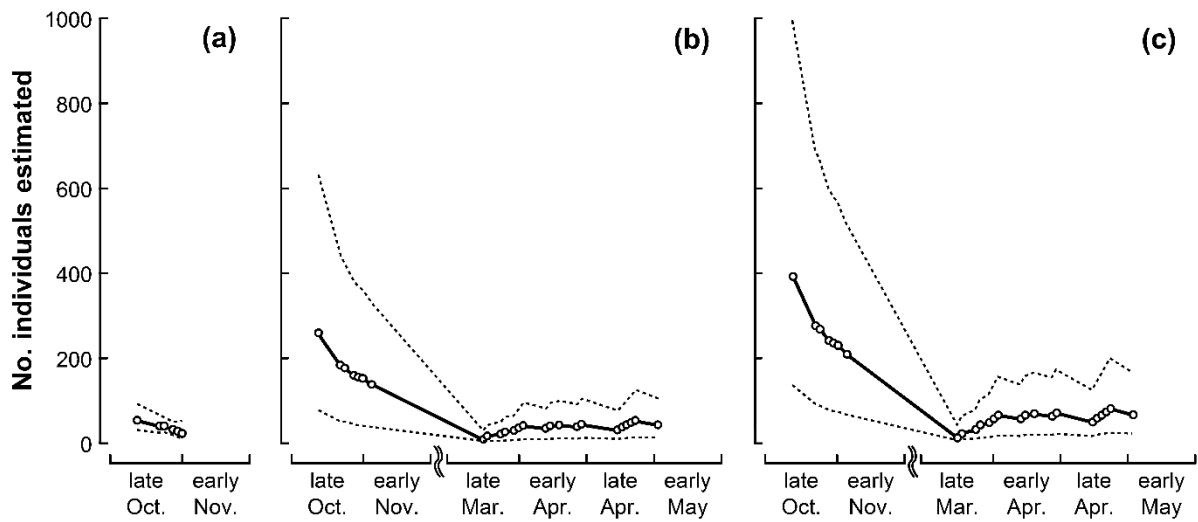
**(b) autumn-form males**



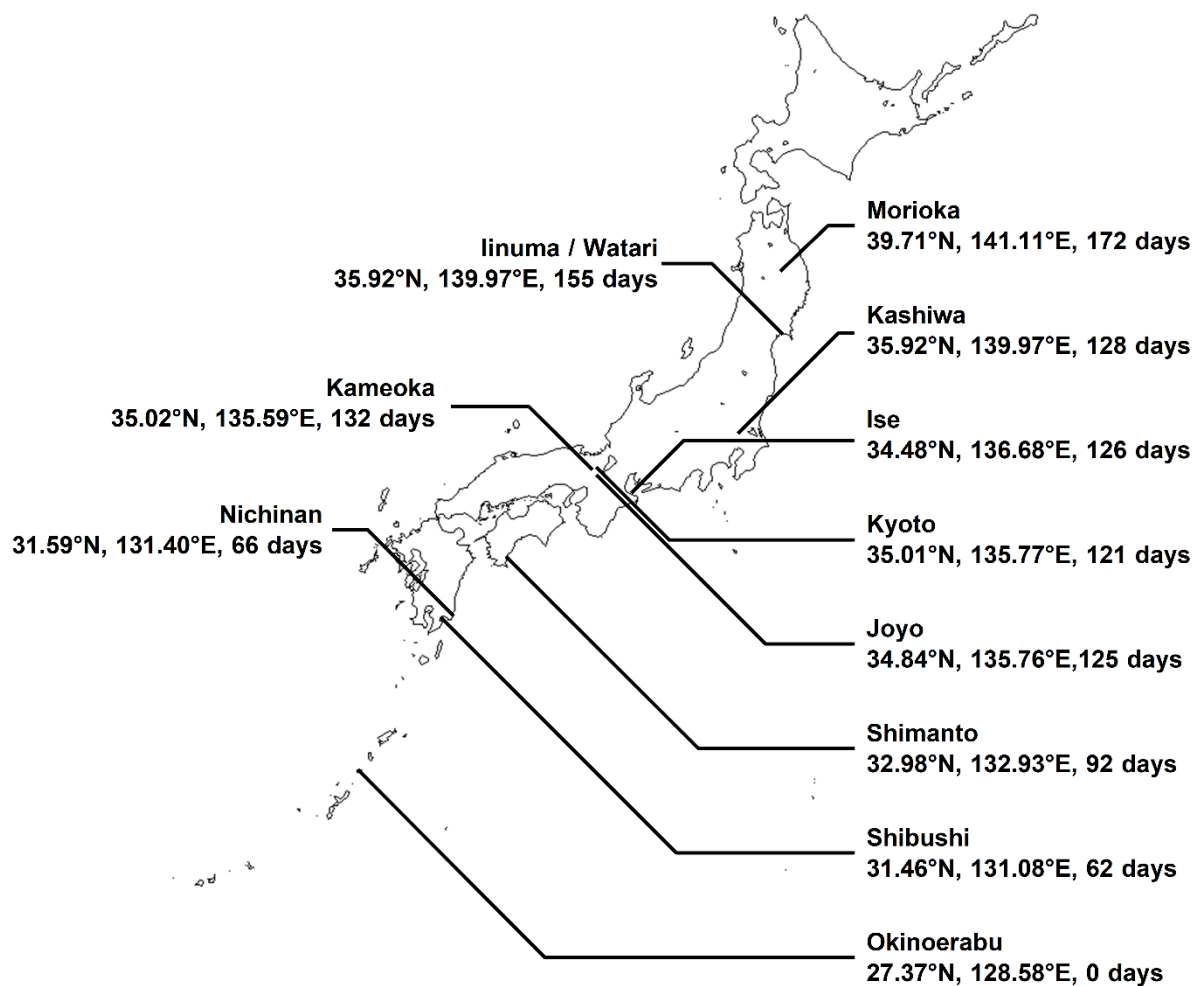
**(c) autumn-form females**



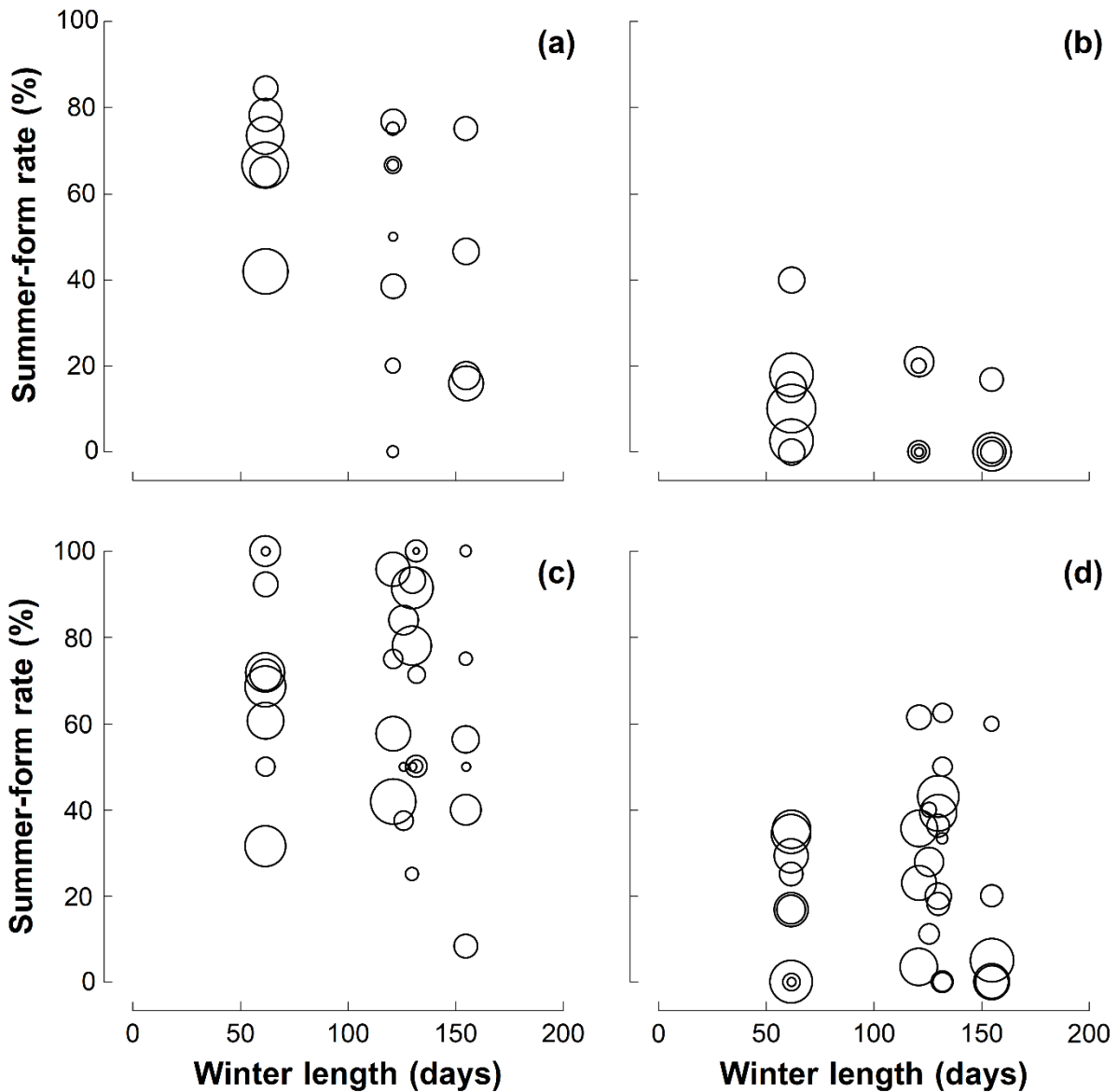
**Figure 7** Proportions of *Eurema mandarina* individuals in different wing-wear classes. FF, intact wings; F, fresh wings with fine tears but less lustrous scales; B, some wing tears and scale loss; BB, notched tears and many scales lost; BBB, broken or extensive tears and many scales lost. Values in parentheses indicate sample sizes. The time intervals were 2 weeks.



**Figure 8** Estimated changes in the daily population size of *Eurema mandarina* from late October to early May for (a) summer-form males, (b) autumn-form males, and (c) autumn-form females. Solid lines with open circles represent the daily population size, and dashed lines represent the 95% CI.

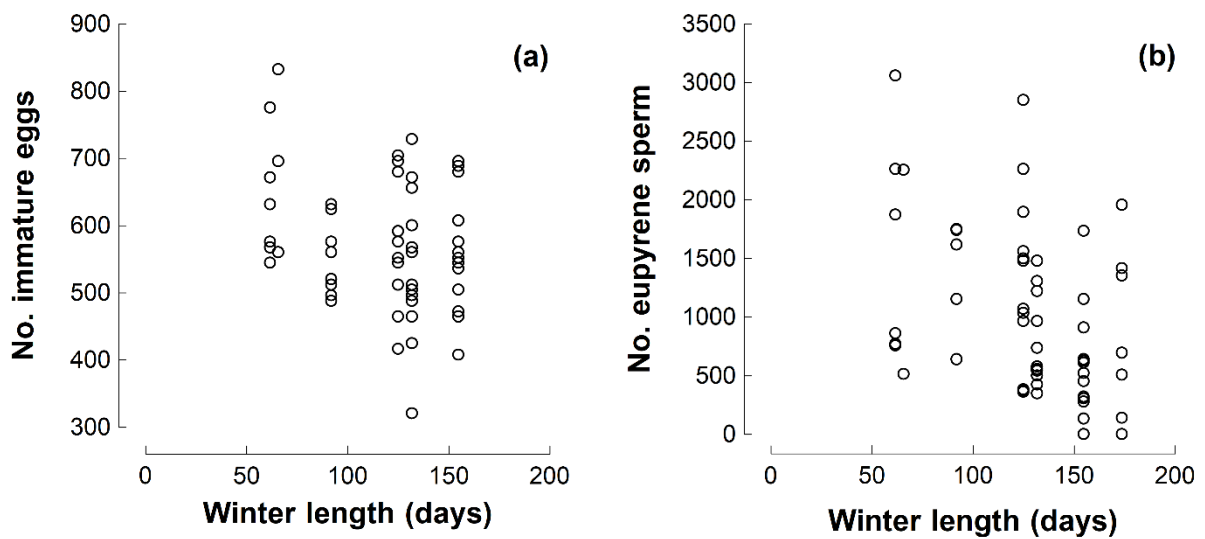


**Figure 9** The latitude, longitude and winter length in survey sites. Winter length was defined as the number of days when the average maximum temperature in 1981-2010 was below 15°C. The average maximum temperature was obtained from Japan Meteorological Agency (2017). Because there is no temperature data of Kashiwa City, Kameoka City, and Joyo City, the data of Ryugasaki City (35.53°N, 140.13°E), Nantan City (35.07°N, 135.27°E) and Kyotanabe City (34.50°N, 135.46°E) were used instead respectively.

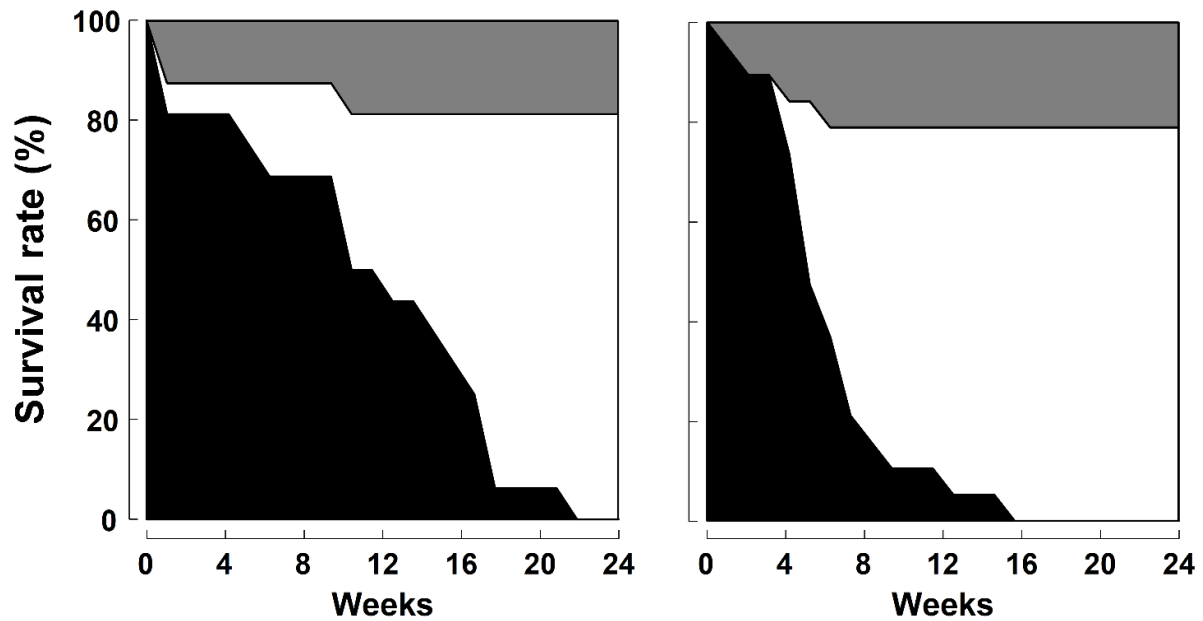


**Figure 10** The proportion of summer-form adults of various geographic populations (20°C: Iinuma/Watari, Kyoto and Shibushi, 25°C: Iinuma/Watari, Kashiwa, Ise, Kyoto and Shibushi) in *Eurema mandarin* at 20°C (males (a), females (b)) and 25°C (males (c), females (d)). One plot indicates one lineage, and the plot size is proportional to the sample size of lineage. Winter length was defined as the number of days when the average maximum temperature in 1981-2010 is below 15°C for each investigation site (Japan Meteorological Agency 2017).





**Figure 11** The number of immature eggs (a) and the number of eupyrene sperm stored in the spermatheca (b) in relation to the winter length of autumn-form females in seven local populations (Morioka, Iinuma/Watari, Kameoka, Joyo, Shimanto, Nichinan and Shibushi) of *Eureka mandarina*. Winter length was defined as the number of dates that the average maximum temperature in 1981-2010 is below 15°C for each investigation site (Japan Meteorological Agency 2017).



**Figure 12** Survival curves of mated (left,  $n = 16$ ) and unmated (right,  $n = 19$ ) autumn-form female adults of *Eurema mandarina* under short-day conditions (LD 10:14) at  $10^{\circ}\text{C}$  and 90% R.H. Black area, surviving insects; gray area, insects that have died with mature eggs; white area, insects that have died without mature eggs. These insects were reared under short-day conditions (LD 12:12) at  $25^{\circ}\text{C}$  at the egg, larval, pupal stages and the first 15-21 days in the adult stage. Adults were fed 10% sucrose solution at  $25^{\circ}\text{C}$ , but not allowed to feed at  $10^{\circ}\text{C}$ .