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The mechanism for the maintenance of a color polymorphism in  
the myrmecophilous aphid, *Macrosiphoniella yomogicola*.

(アリ随伴型アブラムシである、ヨモギヒゲナガアブラムシ  
における色彩多型維持メカニズムの解明)

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## 要約

生態学において、最も重要な課題は、現実に見られる生物多様性の生成、維持機構の解明である。これまでに、既存の遺伝的多型維持メカニズムとして明らかになっているものは、1) 異なるタイプへの負の頻度依存選択、2) 平衡選択、3) 超優性、の3つである。1) の例としては、エンドウヒゲナガアブラムシ(*Acyrtosiphon pisum*)の色彩多型が挙げられる。*A. pisum* では赤型と緑型の2つの色彩多型が見られるが、これは赤型を好む捕食者(捕食性テントウムシ)と緑型を好む捕食者(寄生蜂)が、各色彩モルフに負の密度依存的捕食圧を与えることで、色彩多型が維持されていることが明らかになっている。つぎに、2) の平衡選択とは、異質性のあるパッチ状の環境で、異なる微環境では、それぞれ異なるタイプが有利になるが、生息域全体では、異なるタイプの平均適応度が等しくなる現象である。微環境での適応度が異なるため、平均適応度が等しくても、遺伝的浮動による多型の消失が起こらないので、全体集団中に遺伝的多型が維持される。さらに、3) 超優性とは、2つの対立遺伝子の間において、ホモの個体よりもヘテロの個体の方が適応度が高くなることである。そのため、複数の対立遺伝子が集団中に維持される。

ヨモギにつくヨモギヒゲナガアブラムシ(*Macrosiphoniella yomogicola*)はアリの随伴を受ける種であり、コロニー内で基本的に緑と赤の色彩多型があることが知られている。しかし、実際の野外では赤型と緑型の混合コロニーが普通に見られるのに対し、赤型または緑型の単色コロニーはほとんど見られず、本種には色彩多型を維持する何らかのメカニズムが存在していると考えられる。しかし本種は1) で挙げた *A. pisum* とは異なり、アリ随伴型のアブラムシ種であり、排泄物の甘露を介してアリの保護を受けるため、天敵からの捕食圧から解放されている。また、2つの体色型が同じヨモギの上にいる点に注意しなければならない。2番目の機構、多型維持が要求する環境異質性が起こりそうにないので、2つの対立する選択圧のバランスングセレクションでは、*M. yomogicola* の多型は説明できな

い。2つの体色型は、春夏の間は単為生殖で繁殖するので、超優性による多型もこの場合はありそうにない。以上のことから、*M. yomogicola*には既存の多型維持メカニズム1)～3)とは全く異なった色彩多型維持メカニズムが存在すると考えられる。また、他種のアブラムシではクローン間に増殖率の違いがあることによりクローン間競争が起こることが分かっており、さらに、ダーウィンの自然選択説によれば、このことから、増殖率の違う2つのクローンは、同一環境では一方が絶滅すると考えられるが、*M. yomogicola*ではほとんどのヨモギ上の集団(コロニー)で赤と緑が共存している。

そこで本研究では、アリの随伴が *M. yomogicola* の色彩多型維持に関わっており、赤型と緑型が混在したアブラムシコロニーはアリによく随伴され、より強く保護されるため、コロニーが有性虫の生産まで存続するのではないかと考え、野外実験および室内実験で検証してきた。その結果、赤型と緑型が混在したアブラムシコロニーはアリによく随伴され、コロニーは長く存続することが明らかになった(Watanabe et al 2016 参考文献 1)。ここで、赤と緑の間には増殖をめぐる競争があると考えて実験を行った結果、赤は緑よりも初期増殖率が高いことが明らかになった。これは、どのヨモギのクローンでも同じであったので、両者が増殖した場合、各ヨモギ上のアブラムシは赤だけになると予想されるが、実際の野外では赤と緑の両者は共存しているため、両者の増殖率に随伴アリが影響しているのではないかと考え、実験を行った。その結果、アリは赤よりも緑を好んで随伴することが明らかになり、さらに、随伴アリはアブラムシの増殖率を操作し、競争を中和すること両者を共存させていることが明らかになった。しかし、随伴するトビイロケアリ(*L. japonicus*)は他種のアブラムシとの共生では、甘露の出の悪いアブラムシ個体を捕食することが知られているため、甘露の質が低く、あまり好まない赤を、緑の高品質な甘露の収穫量を犠牲にして共存させておく意義が不明であった。そのため、有性虫産出までのコロニー存続にコロニー内の赤が貢献していると考え、実験を行った結果、有性虫生産までのコロニー存続には、ヨモギの花序の出芽と共に急減するコロニーサイズが、存続するコロ

ニーでのみ増加に転じる時点での赤の絶対数が正の効果を持つことが明らかになった。以上の結果から、*M. yomogicola*の色彩多型維持メカニズムの大筋は解明されたといえよう。つまり、アリは、直近の資源価値だけに注目すると緑だけにした方が良いが、来年以降の生涯資源獲得量を最大にするためには、赤を共存させる必要がある。また、アブラムシも、緑は生存に必須のアリ随伴を強く誘導し、赤は甘露コストを下げて増殖率を高めている。つまり、3者がみな、自己の適応度を最大化しようと適応した結果、永続的な共生系が成立し、全ての構成者の遺伝ラインが消滅しないような進化が起こったと考えられる。今後の課題は、三者(アリ、赤、緑)の組合せがどのようなとき、この共生系は最も永続しやすいのだろうか、ということである。これに関しては、随伴アリの種が3者の共生系の存続に重要な要因であることが明らかになっている。この他にも、環境の異なる個体群によって最適なコロニーの色比率や、アリ・赤・緑・ヨモギのクローン(または種)の組合せが存在する可能性が考えられる。今後は、実際の野外・室内で行う実証実験と、シミュレーションのようなモデルを組み合わせて、最も永続しやすい3者の組合せを検証し、それを実現する機構が進化しているかどうかを検討することが必要である。

## General introduction

One of the most important issues in ecology is the evolution of biodiversity in nature and the maintenance mechanism of it (Darwin 1859). There are three known mechanisms by which genetic polymorphisms are maintained in nature; 1) the negative frequency-dependent selection on each phenotype, 2) balancing selection between phenotypes, and 3) superdominance.

The example of the mechanism 1) is the color polymorphism observed in an aphid, *Acyrtosiphon pisum*. An example of the mechanism 1) is the case of an aphid, *Acyrtosiphon pisum*. This species has two color morphs (red and green). This polymorphism is maintained by different predators' preference on each morph (Losey et al. 1997). A lady bug (*Coccinella septempunctata*) more prefers the red morph as prey than the green morph, whereas, a parasitic wasp, (*Aphidius ervi* Haliday), more prefers the green morph as the host than the red morph (Libbrecht et al. 2007). Thus, the morph with a higher frequency than the other morph receives a stronger predation pressure than the less frequent morph. As a result, this negative dependent predation pressures hold the long lasting color polymorphism in populations (Losey et al. 1997; Libbrecht et al. 2007).

The mechanism 2) "balancing selection" means that two (or more) morphs with the same average fitness over the habitat will be maintained in the whole habitat that separated into many microhabitats for which the fitness of each type is different by the microhabitat. Thus, although the average fitness is the same, each type will not disappear by the random genetic drift (Kimura 1968) because the fitness of a type is

higher than the other type(s) in specific microhabitats and vice versa. For examples, a spider (*Nephila maculata*) has two color morphs. One is the black morph that is totally black in body color, and the other morph has standard color (green and black, white and yellow stripes on the abdomen). The standard morphs more reflect ultraviolet rays than the black morph, and this feature more attracts insect prays than the black morph (Tso et al. 2002). While, the black absorbs more sunlights than the standard morphs, and thus works as a cold resistance mechanism and a cryptic coloration (Tso et al. 2002). These different advantage of each the morph should maintain the polymorphism. Note that, probably this mechanism also requires a heterogenous patchily habitats as with the mechanism 1). Furthermore, 3) superdominance means that the fitness of a heterozygote is higher than a homozygote, and thus multiple alleles are maintained in a population.

A color polymorphism is known in the aphid *Macrosiphoniella yomogicola* that parasitizes on a mugwort, *Anochetus montana* (Agawa & Kawata 1995). This species receives ant attendance always, and the body colors are basically can classify into green or red. However, colonies consisted of a single color morph seldom observe in fields, i.e., most of colonies are polymorphic for aphid's color (Agawa & Kawata 1995). However, different from *A. pisum*, this aphid excretes honeydew to attract ants and always attended by ants. Attending ants protected the aphid colonies from predators. Therefore, there should be an unknown mechanism for the maintenance of the polymorphism because this aphid is free from the predation pressure of natural enemies. Note that, the two color morphs show sympatric cohabitations on a mugwort shoot. In addition, as the two morphs cohabit on the same mugwort's shoot, the environmental heterogeneity is unlikely in this aphid. Polymorphism by superdominance is also

unlikely in this case, because the two color morphs reproduce parthenogenetically during a spring-summer seasons. Thus, we can expect unknown new mechanism(s) for the maintenance of genetic polymorphism in *M. yomogicola*, which differ from the previously proposed ones.

In other aphid species, the competition among the different clones has occurred through difference in the reproductive rate among clones (Loaring & Hebert 1981). According to the natural selection theory (Kimura 1968), if two clones differ in the reproductive rate, one clone becomes dominate in the same environment (Gause 1934). However, in *M. yomogicola*, the red and green morphs coexist on almost mugworts in a population (colony). Because interclone differences in reproductive rate are likely, the coexist of the two morphs may include some unknown mechanisms to hold this cohabitation.

In this study, we investigate this ant-aphid symbiosis based on a hypothesis that the ant-attendance maintains the color polymorphism in *M. yomogicola*, and the aphid colonies in which the red and green morphs mixed is more attractive to ants, and thus easy to survive. By this mechanism, the polymorphism may be maintained in populations. We examine the above hypotheses by the field and laboratory experiments (Chapter 2). From the obtained results and new questions, we investigated the difference in reproductive rates between the red and green morphs, the preference of ants to the morph type, the effects of ant-attendance on aphid reproductive rate of each morph (Chapter 3). After the budding of inflorescences from the parasitized mugwort shoots in autumn, the most of aphid colonies become extinct rapidly. We investigated number of red morphs, green morphs and colony size and examined their effects on colony

survival until production of sexuparae in rate fall (Chapter 4). Then, as preliminary studies, we investigated whether parasitic rates by a parasitic wasp to aphid colonies those were attended by different ant species are different or not (Chapter 5). Furthermore, we investigated the effects of an aphid colony that survived and produced sexuparae on the growth of the host mugwort's shoot (Chapter 6). The differences in honeydew contents and inheritance mode of the red and green morphs are considered using preliminary data (Chapter 7). In the last chapter (Chapter 8) I made general discussions on the evolution of this symbiotic system.

# Chapter 1

## Life history of *Macrosiphoniella yomogicola*

### Study organisms

*Macrosiphoniella yomogicola* is an aphid distributed in Japan and inhabits a mugwort, *Artemisia montana*, in the Hokkaido area. This species shows a color polymorphism with several colors (Agawa & Kawata 1995). Although there are several color morphs in a population in Shizuoka, Japan, only two morphs (red and green) are found in our field in Hokkaido, Japan. At Sapporo, in early May, stem mother hatches from overwintered eggs those were laid by sexual reproduction in the previous autumn. The stem mothers produce clonal offspring by asexual reproduction, and her offspring continue asexual reproduction until Autumn. During this period, the body color was inherited. Several ant species were observed to attend the aphid colonies in the study area: *Lasius japonicus*, *Formica japonica*, *Formica sanguinea*, *Pheidole fervida*, *Myrmica kotokui*, *Camponotus japonicus*, and *Lasius nipponensis*. Most aphid colonies were attended by *L. japonicus* (Fig.1).

From early May to mid June, each colony increases in number with only non-winged morph. After mid June, the winged morphs appear in both the red and green morphs and they disperse to other mugwort shoots. From this season, the most of mugwort shoots was parasitized by color mixed colonies of *M. yomogicola*. At mid August, *A. montana* starts to bud inflorescences. From the start of the budding, numbers of the aphid colonies decreased rapidly (see Chapter 6), and the most of the aphid colony

become extinct. A few colonies survive to mid October at where the sexuparae are produced, and the females lay sexual eggs after mating with males. The laid eggs overwinter and hatch in the next Spring.

**Fig.1. Color polymorphism in *M yomogicola* and an attending ant, *Lasius japonicas*.** A small light green and a small red individuals are larvae of the green and the red morphs. A blackish green and a pinkish individual is each adult red and green morphs, respectively. The attending ant taking honeydew from the red larva.



## **Chapter2**

### **Color polymorphism in an aphid is maintained by attending ants**

#### **Introduction**

Polymorphisms are often found to persist in populations (Hedrick 2006, Leimer 2005, Forsman et al 2008, Subramaniam, Rausher 2000). The persistence of polymorphisms in populations may be explained by balancing selection. For genetic polymorphisms, two different nonmutually exclusive forms of balancing selection have been described: (i) negative frequency (or density) dependence of various morphs, and (ii) trade-off balance of two independent factors (Hedrick 2006, Leimer 2005, Forsman et al 2008, Subramaniam, Rausher 2000). For solely phenotypic polymorphisms (no genetic differences), adaptive coin flipping between different morphs is often the best solution for adapting to stochastic environments (Leimer 2005). In all cases, the balance between two different factors yields optimal polymorphisms (Hedrick 2006, Leimer 2005, Forsman et al 2008, Subramaniam, Rausher 2000).

In persistent genetic polymorphisms, a morph should become selectively advantageous when it is rare but disadvantageous when it is abundant (Rausher 2000). Negative frequency (or density)-dependent selection on various morphs is a prime example of this condition (Rausher 2000, Hori 1993, Takahashi et al 2010, Losey 1997, Hughes 2013). Scale-eating cichlids are known to exhibit frequency-dependent fluctuations in the frequency of mouth types (Hori 1993). A damselfly species is also known to show a

similar fluctuation between the gynomorphs and andromorphs of females due to frequency-dependent male disturbances of oviposition by attempted mating (Takahashi et al 2010). In some aphid species that are not attended by ants, color morphs are also maintained by negative frequency-dependent predation (Losey 1997). There is also evidence that rare color morphs in male guppies are more attractive to females (Hughes 2013). Thus, negative frequency dependence is a well-known mechanism for the maintenance of genetic polymorphisms.

Another mechanism has also been reported to explain the maintenance of genetic polymorphisms: fitness equalization between two (or more) morphs by a selective trade-off between two independent factors under environmental heterogeneity. For example, color morphs in a web spider (*Nephila maculata*) are explained by the selective trade-off between foraging efficiency and abiotic factors (for deterring predation). This spider exhibits two color morphs: (i) normal type (stripes of green, black, white, and yellow) and (ii) black type (Tso et al 2002). The colorful normal type is superior in attracting insect prey because the normal phenotype reflects more ultraviolet light than does the black phenotype. On the other hand, the black phenotype permits high tolerance to low temperatures because of its thermoregulatory advantages (black absorbs more light than does any other color). This phenotype is also considered a form of cryptic coloration. The balance between these opposing selective pressures would equalize the fitness of the two morphs (selectively neutral). However, if there were no negative frequency-dependent selection, this polymorphism would not be held by the effect of random genetic drift, causing a random walk leading to the eventual exclusion of one morph. Environmental heterogeneity is required to maintain the color

polymorphism in this web spider because each morph would have an advantage over the other in microscale habitats distributed in patches across the entire habitat. Thus, each morph does not disappear from the whole habitat area because of random genetic drift.

Another mechanism that maintains a genetic polymorphism is overdominance between two alleles on a locus, where the heterozygote has the highest fitness. In this case, the two alleles and consequent phenotypic polymorphism are maintained. In conclusion, as explained above, there are three major mechanisms known for the maintenance of genetic polymorphisms. Many species of aphids exhibit color polymorphisms (Forsman 2008). Polymorphic coloration originates from the color of cells, hemolymph, or symbionts (Miyazaki 1987). Coloration in aphid species is known to have a genetic basis (Caillaud & Losey 2010). Because color morphs are inherited through parthenogenetic reproduction, these morphs can be treated as a form of genetic polymorphism, even if coloration of morphs is controlled by symbionts. Few studies have investigated why these color polymorphisms are maintained in a population (Losey 1997, Libbrecht 2007, Balog, Schmitz 2013).

A sympatric occurrence of a green and a red morph has been reported in the pea aphid, *Acyrtosiphon pisum*, which is not attended by ants. Previous studies have shown that frequency-dependent selection by two different predators on each morph maintains the color polymorphism in this species (Losey 1997). A ladybug (*Coccinella septempunctata*) preys on the red morph preferentially, whereas a parasitoid wasp (*Aphidius ervi*) preys on the green morph (Losey 1997). Thus, negative frequency-dependent selection acts on both morphs and maintains the coexistence of both morphs

(Libbrecht 2007, Balog, Schmitz 2013).

Here, we investigated a color polymorphic mugwort aphid (*Macrosiphoniella yomogicola*; Fig. 1) that is attended by ants. Because ants attend this species, *M. yomogicola* should not experience strong predator-mediated selection. Thus, the coexistence of the two color morphs in this species cannot be explained by frequency dependent predation. We should also note that the two color morphs cohabit sympatrically on a mugwort shoot (Fig. 1). This means that the balancing selection of two opposing selection pressures is not likely to explain this polymorphism, because the environmental heterogeneity that is required to maintain this type of polymorphism is unlikely to take place. Polymorphism by overdominance is also unlikely in this case, because the two color morphs reproduce parthenogenetically during the observed spring-summer seasons. Therefore, the former three hypotheses are not likely to explain the color polymorphism in *M. yomogicola*.

From on the *M. yomogicola*'s natural history (see Chapter 1), we hypothesized that the color polymorphism in *M. yomogicola* is maintained by the attending ants because they prefer aphid colonies with a specific ratio of the two morphs. To test this hypothesis, we measured the preference of the ants to the aphid colonies with different proportions of the two morphs. We initially evaluated the effects of attending ants on the survival of the aphid colonies by experimental removal of the ants. We show that the attending ants are necessary for the persistence of the colony. We then evaluated the preferences of ants for aphid colonies with different proportions of the two morphs.

## **METHODS**

### ***Experiment 1***

To examine the effect of ant attendance on the survival of aphid colonies, we selected three groups of eight, eight, and three mugwort shoots on which aphids colonized on 21 August 2015 at a field site at Hokkaido University, Sapporo, Hokkaido, Japan. On 21 August, the numbers of colonized aphids were counted for each colony. Ants were removed from the first eight colonies by painting Tanglefoot (sticky material) at the base of the mugwort shoots. Painting Tanglefoot prevents ants from moving up to the mugwort because of its stickiness, but most predators can reach the aphid colonies because they can fly. No Tanglefoot was applied to the eight colonies in the second group so that ants continued to attend the aphid colonies. To control for the effect of Tanglefoot application on aphid survival, Tanglefoot was painted at the surface of a leaf in the three colonies in the control group so that ants could continue to attend the aphid colonies. The numbers of colonized aphids were counted 9 days later. The number of predators found on each mugwort shoot was recorded three times during the experiment.

### ***Experiment 2***

#### ***Field collections and measurements.***

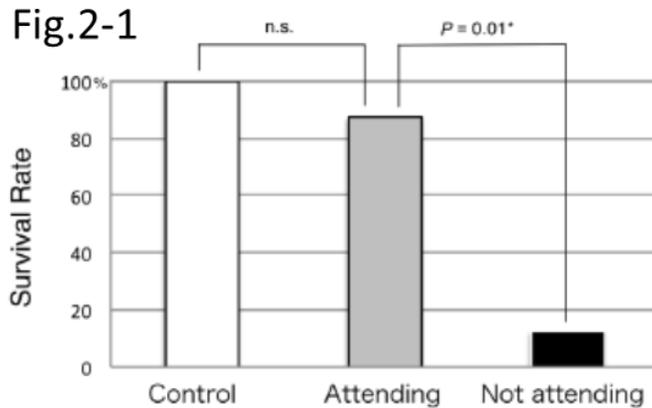
We collected a total of 85 *M. yomogicola* colonies from three sites and one site in Sapporo City in July 2012 and 2013, respectively. A colony on a mugwort shoot was collected and brought back to the laboratory in a vinyl bag with attending ants. To avoid multiple samplings from the same ant colony, we took a single shoot from the lumped shoots. The insects were frozen at  $-25^{\circ}\text{C}$  in a freezer for measurements. For each shoot,

the numbers of green morph, red morph, and attending ants were counted. The proportion of the green morph on the shoot was calculated using these data. The monomorphic colonies were removed from the analysis. For the rest of the colonies, APA was calculated. Because the body size of ant workers became larger as colony size increased (Hasegawa 1993), we used the average head width of the attending ants as an index of ant colony size. The head of an ant was separated and fixed on a glass plate and was measured to 0.01 mm by a micrometer on a microscope (Macromax SCORP-ON Direct USB, SCORPION). The head widths of all the attending ants on a shoot were averaged. This value was used as the index of ant colony size on the shoot. Statistical analyses three factors affect APA: the number of aphids on a shoot (aphid colony size), ant colony size, and the PG. Generally, the number of aphids is positively correlated with the number of attending ants. A large ant colony can mobilize more workers to an aphid colony than a small ant colony. We wanted to examine the effect of PG on APA without the effect of these other two variables. To remove the effects of both of these parameters, we first conducted a multivariate regression of the APA on both ant colony size and aphid colony size as independent variables. Second, the residuals of APA from the regression plane were calculated. Next, a simple regression of the residuals of APA on PG was conducted to examine the relationship between PG and APA. We used four types of regression functions in the simple regression of APA on PG: linear function, two-dimensional function, threedimensional function, and smoothing spline function. Each function was fitted on data, and AIC was calculated. The fitted function showing the lowest AIC was considered the regression that explained the data most effectively.

## RESULTS

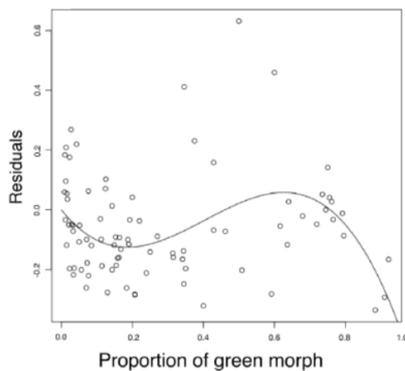
The ant removal experiment showed that ant attendance is necessary for the survival of a colony (Fig. 2-1). Most ant-attended colonies survived (seven of eight), whereas most colonies without ants became extinct (seven of eight) (Fig. 2-1; Fisher's exact test,  $P = 0.010$ ). Even in the survived colony with no ant attendance, only one aphid survived. Thus, ant attendance is necessary for the survival of aphid colonies in *M. yomogicola*. The total number of predators (larvae and adults of a ladybug, larvae of lacewing flies, and a parasitoid wasp) found on the mugwort shoots is marginally larger in the ant-removed treatments than in the ant-attended ones (t test,  $df = 12$ ,  $t = 1.782$ ,  $P = 0.087$ ), suggesting that the ants are likely to remove these predators from the aphid colonies. Note that all the above predators were observed in other locations around the aphid colonies, and we never found any predator within the aphid colonies attended by the ants. This observation suggests that the ants completely removed predators from the aphid colonies. The low survival rate of the aphid colonies with no ant attendance may stem from the effect of Tanglefoot (sticky insect repellent). However, the effect of Tanglefoot is likely negligible because the ratio of survived colonies is not significantly different between the control groups ( $n = 3$ ) and the ant-attended groups ( $n = 8$ ) (Fisher's exact test,  $P = 1$ ).

Fig.2-1 **The effect of ant attendance on the survival of aphid colonies.** The ratio of survived aphid colonies. Aphid colonies were attended by ants (Attending), not attended by ants (Not attending), or attended by ants but experienced the effects of Tanglefoot (Control). n.s., not significant.



The number of attending ants per aphid (APA) is highest when the green morph comprises 65% of the colony (Fig. 2-2).

Fig. 2-2. Effects of the proportion of the green morph in an aphid colony on degree of ant attendance. The plots represent the residuals from the two-dimensional regression plane, with ant colony size and aphid colony size as independent variables. The superimposed function (solid line) is a three-dimensional function that was selected as the best model from the four candidate functions based on Akaike's information criteria (AIC) comparisons.



The summary of the multiple regression analysis is presented in Table 2-1.

Table 2-1. The results of the multiple regressions of the two factors (aphid number is the number of aphids in a colony and ant head width is an index of ant colony size) on attending ants per aphid. Aphid numbers is negatively correlated with attending ants per aphid ( $p=0.00659$ ). Statistical significance is marginal for ant head width ( $p=0.06171$ ).  $F=5.449$ ;  $df=2, 82$ ;  $p=0.006$ ,  $R^2=0.1173$ .

<b>Factor</b>	<b>Estimate</b>	<b>SE</b>	<b>t</b>	<b>P</b>
Intercept	-0.0209	0.119257	-0.175	0.8613
Aphid number	-0.00031	0.000112	-2.788	0.00659
Ant head width ( $\propto$ ant colony size)	0.21410	0.113024	1.894	0.06171

The number of aphids was negatively correlated with APA ( $P = 0.0066$ ). There was a marginally significant positive relationship between ant colony size and APA ( $P = 0.0617$ ). Thus, we use a two-dimensional plane to control for the effects of both factors (ant colony size and aphid colony size) when examining the effect of the proportion of the green morph in an aphid colony (PG) on APA. In addition, the relatively low determinant coefficient ( $R^2 = 0.1173$ ) shows that these two factors explain only 11.7% of the variance in APA. The residuals of APA from the plane were calculated, and we tested for equality in variance of the residuals using the Breusch-Pagan test. The results showed that the null hypothesis that the variance of the residuals is distributed equally was not rejected ( $P = 0.3166$ ). Thus, the simple regression analysis of the residuals of APA on PG can be performed without statistical complications. Table 2-2 shows the results of the regression analyses of APA on PG. The linear regression was not significant ( $P = 0.8538$ ), and the parameters of the two-dimensional regression ( $Y = aX^2 + bX$ ) were not significant either ( $P = 0.1733$  for  $a$  and  $P = 0.0592$  for  $b$ ). Thus, these

two regressions did not adequately explain the distributions of the residuals of APA. The smoothing spline regression was statistically significant ( $P = 0.0015$ ), and AIC was  $-61.68$  (see Table 2-2). In the three-dimensional regression ( $Y = aX^3 + bX^2 + cX$ ), each coefficient was highly significant ( $P < 0.0001$  for  $a$ ,  $b$ , and  $c$ ), and AIC was  $-64.835$  (Table 2-2).

Table2-2. Regression coefficients and AIC values for the four regressed functions. Significance levels of the coefficients are indicated by asterisks (\*\*\*:  $p < 0.001$ ).

Function type	Equation	$a$	$b$	$c$	$P$	AIC
Linear	$aX$	$-0.0255$	—	—	$0.854$	$-50.931$
Second dimension	$aX^2 + bX$	$0.3304$	$-0.3312$	—		$-49.963$
Third dimension	$aX^3 + bX^2 + cX$	$-4.2858^{***}$	$5.2189^{***}$	$-1.4946^{***}$		$-64.835$
Smoothing spline	—	—	—	—	$0.0015$	$-61.681$

The above results indicate that the three-dimensional regression provided the best model for explaining the distribution of the residuals of APA. The results of the fit are shown in Fig. 2-2. The maximum value of the residual of APA is ca. 0.65 in PG. Thus, an intermediate ratio of both color morphs appears to attract ants maximally.

## DISCUSSION

Our analyses showed that an intermediate proportion (~65%) of the green morph in an aphid colony is favored by the attending ant species, *Lasius japonicus*. *M. yomogicola* is an aphid that is attended obligately by ants. In the field, initial colonies (a few larval aphids with the stem mother) are always attended by ants. Our ant removal experiment showed that ant attendance is essential for aphid survival (Fig. 2-1).

Agawa and Kawata (1995) have shown that more polymorphic colonies of *M. yomogicola* tend to decrease more slowly in populations. This trend may have occurred

because of the difference in the degree of ant attendance. Our second result demonstrated that the number of ants per aphid has a peak at ~65% of the green morph in an aphid colony (Fig. 2-2). This result suggests that these polymorphic colonies are protected more efficiently from predators by the attending ants than less polymorphic colonies. Thus, ant attendance may maintain the observed color polymorphism in *M. yomogicola*.

The present results suggest that attending ants select the optimal intermediate. This colony-level selection is a novel mechanism for balancing selection. The known traditional balancing selection includes frequency-dependent selection, balancing selection between opposing factors under heterogeneity, and overdominance. None of them fit in the current case. There is another interesting point to consider. Although our results suggest that selection favors an intermediate ratio of the two morphs at the level of the entire colony, the two morphs are likely to compete with each other within a colony. The body color of aphids reflects their condition (Tsuchida 2010). Therefore, there may be a difference in ecological characters (such as increasing rate and quality of honeydew) between the red and green morphs in *M. yomogicola*.

If this difference exists, an increasing rate of return per unit time may be higher in one morph than in the other because the former invests less energy into honeydew. If both morphs compete within a colony, one morph would soon become more common and most colonies would end up monomorphic. However, because the entire colony requires ant attendance for survival, a morph with a high reproductive rate producing presumably low-quality honeydew (red morph here) may obtain benefits from the

existence of the other more attractive morph (green morph) and hence increased ant attendance. If this is the case, individuals of the stronger morph under intermorph competition may behave cooperatively with the other morph to secure their own survival. This would be a very interesting hypothesis for future studies.

Why the ant, *L. japonicus*, prefers an intermediate ratio of both morphs would be an interesting question to examine in detail. In previous studies, *Lasius niger* ants have tended to prey on aphid individuals that produce less honeydew within an aphid colony (Sakata 1995). Ants prefer to attend and guard aphid species that produce condensed honeydew and maintain a ratio of such aphids (Völkl, et al 1999, Fischer et al 2001, Woodring et al 2004). *L. niger*, which is closely related to *L. japonicus* (previously, both have been classified as the same species), especially prefers trisaccharides in honeydew (Wäckers 2000). On the other hand, ants tend to prey on aphid individuals that produce less honeydew (Sakata 1994, 1995). Ants also attack and prey on aphid individuals from which ants have never collected honeydew within an aphid colony (Endo & Itino 2012). How these factors interact in our system is important for establishing the reasons for the symbiotic relationships between the ants and the two color morphs of *M. yomogicola*. We would require detailed chemical analyses of honeydew contents in both morphs.

In addition, how the ants control the morph ratio in an aphid colony to achieve the preferred ratio is an interesting question. More studies are needed to elucidate the proximate causes that achieve the observed symbiotic relationships among ants and aphids. Previous studies have shown that ants recognize prey or nestmates by cuticular hydrocarbons (Bonavita-Cougourdan et al 1987). *Stomaphis sp.* produces cuticular

hydrocarbons and thereby obviates predation by their host ants (Endo & Itino 2012). If there are differences in the cuticular hydrocarbons produced by the red and green morphs in *M. yomogicola*, the ants can discriminate between the color morphs. In addition, aphids invest in honeydew to induce ant attendance and thereby receive protection from predation (Völkl et al 1999, Fischer, Shingleton 2001). This fact leads to the following prediction about proximate mechanisms: a morph's investment in honeydew would be negatively correlated with the proportion of that morph in the subpopulation (Yao & Akimoto 2002).

For example, a morph (probably the red) increasing in relative proportion may produce honeydew with a lower quality and vice versa. If there are certain advantages for the ants to rear the morph producing the low-quality honeydew, the present symbiotic relationships among the two morphs and the ants would be long-lasting. One such possibility is that the red morph may bear a higher ability to suppress the budding of mugworts' inflorescences than the green morph. When a mugwort buds inflorescences in autumn, the aphid colony on that mugwort becomes extinct without sexual reproduction via overwintering eggs. A few aphid colonies surviving on the mugworts that do not bud inflorescences reproduce sexually to produce overwintering eggs. Thus, if the red morph bears a high ability to suppress the inflorescence budding of mugworts, the ants gain indirectly when rearing the red morph to exploit its honeydew resources in the following year. Thus, the ants should control the ratio of color morphs to achieve optimality as determined by the costbenefit ratio for the ants. This hypothesis could be further investigated in *M. yomogicola*.

Body colors in *A. pisum* are determined by carotenoid pigments (Moran & Jarvik 2010, Valmalette et al 2012). The ability to produce body colors may be determined genetically or environmentally (Valmalette et al 2012). In addition, the removal of internal symbionts in aphids has changed body color in *A. pisum* (Tsuchida et al 2010). In *Myzus persicae*, body colors are fixed for syngensis generation (parthenogenetic reproduction) but are determined by one pair of allelomorphs for sexual generation (sexual reproduction) (Takada 1981). Unfortunately, a mechanism for the inheritance of body color in *M. yomogicola* is not known, although its body colors are parthenogenetically inherited. Investigations of the inheritance manner of body colors are important to elucidate the origin of color polymorphism in this aphid. In summary, we detected an effect of the proportion of aphid color morphs on the degree of ant attendance—even when the effects of both aphid colony size and ant colony size were statistically removed. We found that there is an optimal proportion of the two morphs in a colony that maximizes the attractiveness to ants. Our results suggest that color polymorphism is maintained by the optimality of the intermediate combinations of the two morphs. This mechanism may be quite different from previously known mechanisms via balancing selection.

## **Chapter 3**

### **Ants improve the reproduction of inferior morphs to maintain polymorphism in symbiont aphids**

#### **Introduction**

Many natural ecosystems exhibit fairly high biodiversity. Such high biodiversity in nature is the most fundamental question in ecology and evolutionary biology (Darwin 1859) because interspecific competition excludes competing species (Gause 1934). However, natural communities consist of many competing species. A heritable genetic polymorphism is one such biodiversity where persistence is difficult to explain. In this sense, persistence of a polymorphism is one of the fundamentally important issues in ecology and evolution because it helps us to understand observed biodiversity in nature.

Why does a polymorphism persist in a population? Previously, three major mechanisms were known for a polymorphism: (1) negative frequency-dependent selection, (2) balancing selection of two opposing factors, and (3) superdominance. Under negative frequency-dependence, any morphs become advantageous when rare but disadvantageous when abundant (Losey 1997). Polymorphisms therefore are very stable because each morph is protected from extirpation. In contrast, polymorphisms by two opposing factors is rather unstable because random walk (genetic drift) can easily lead the extinction of any one morph. Therefore, some averaging mechanisms that protect from random walk is necessary in these polymorphisms, e.g., spatial heterogeneity yielding patchy microhabitats (Tso et al 2002).

Recently, we found another mechanism for polymorphism in an ant-aphid symbiotic system. An aphid, *Macrosiphoniella yomogicola*, exhibits a heritable color polymorphism with green and red morphs (Fig. 3-1, Watanabe et al 2016). We found that an attending ant, *Lasius japonicus* (Fig. 3-1), is attracted by and guards most strongly a mixed aphid colony (approximately 65% green morph) to obtain honeydew from the aphid colony (Watanabe et al 2016). Because *M. yomogicola* colonies are soon extirpated by predation when the attending ants are excluded (Watanabe et al 2016), the color polymorphism in the aphid is protected by the attending ants. However, stable polymorphism is not guaranteed in this symbiotic system. For example, if competitive ability or growth rates differ between morphs, any aphid colony will soon lose the inferior morph. Here, we use the term “competitive ability” in a broad sense, including the difference in the rates of growth and reproduction, even if no actual competitive interactions occur between the morphs. Note that any such difference between morphs leads to the exclusion of the inferior morphs. In the field, surprisingly nearly all colonies consist of both green and red morphs. How are these two morphs maintained in a colony by the attending ants?

Ant-attended aphids are known to excrete a high quality of honeydew when ants are present (Yao & Akimoto 2001, Yao & Akimoto 2002). There are trade-offs between the quality of honeydew and the growth and fecundity of aphid individuals (Yao & Akimoto 2000). Thus, such trade-offs and the resulting competitive interactions are expected between the color morphs in *M. yomogicola*.

The morph excreting high-quality honeydew is known to be less productive (have slower reproduction) compared with the other morphs (Yao & Akimoto 2000, Stadler & Dixon 1998). These facts imply that, if the attending ants prefer one morph, that morph is expected to excrete high-quality honeydew. Note that the qualities of honey dew are known to be different between preferred and non-preferred morphs (Völkl et al 1999). Therefore, the preferred morph is expected to decrease in numbers over the season and eventually be eliminated from a colony, even if ants prefer them. However, in our field, the coexistence of the two color morphs on every hostplant continues from June (the appearances of alates) to November (the end of the season). How can the two color morphs achieve long-lasting coexistence on the same hostplant when the competitive exclusion of the inferior morphs is expected?

This study aims to resolve the reason for the long-lasting coexistence of both color morphs in every colony of *M. yomogicola* on hostplants. In this study, we investigated the reproductive (i.e., population growth or parthenogenetic reproduction) rate of both morphs of *M. yomogicola* under several experimental settings. First, we compared the reproductive rate of each monoclonal red and green morph on several cloned hostplants. Second, we examined the difference in the reproductive rates between the color morphs using a single cloned hostplant. Then, we measured the reproductive rate of each morph in mixed colonies free from predators with or without attending ants. Finally, we tested the ants' preference to the color morphs. The results show that the attending ants improve the reproductive rate of their preferred green morph, even though the green morphs have inferior reproductive rates compared with the red morphs without attending ants. Because of ants' manipulation, the reproductive rate of the green morphs

is equalized with that of the red morphs. This manipulation enables an aphid colony to maintain a long-lasting coexistence of both morphs in a single colony on a hostplant.

## **Methods**

### *Experiment I*

I-1. In May 2014, we reared one red and one green adult on separate mugwort shoots of the same clone plant without ants in the greenhouse of our facility. Each shoot was covered by a nylon mesh (30 x 20 cm) to protect the colonies from predators. The number of aphids was recorded once every 2 or 3 days. This process was repeated for five different clones for both red and green morphs on a single hostplant clone, and the results were averaged for each morph (Fig 3-1a).

I-2. From June to September 2014, five red and five green clones were reared independently on 10 shoots each of the same hostplant clone without ants at the same location as experiment I-1. Predators were removed by the same method as in I-1, and the number of aphids was recorded once every 3 days (Fig. 3-1b).

I-3. From May to August 2016, a red and a green clone were each reared on a shoot of the same hostplant clone with ants, resulting in a large clonal colony. From the clone pool of each morph, 3 adult aphids were transferred to one of 5 shoots of 5 hostplant clones. We recorded the number of aphids on each shoot without ants once every 3 days (Fig. 3-1c). Predator removal was conducted in the same way as in the above two experiments.

### *Experiment II*

In June 2014, fifteen hostplants were parasitized by mixed-aphid colonies (containing both morphs) and attended by ants that were selected randomly in the field. One pair of shoots was also selected randomly. One shoot remained as it was (with ant attendance), and the other shoot was rubbed with a sticky liquid (Tanglefoot®) at its base to remove attending ants. The latter shoot was covered with nylon mesh to prevent predators. Before the start of the experiment, the number of each morph was recorded for all shoots and was recounted again 3 days later (Fig. 3-2).

### *Experiment III*

From June to September, 19 clonal shoots were prepared in a plastic pod (15 x 20 cm) and a clone of a color morph was parasitized on each shoot. In addition, 19 pairs of two pods each were prepared with monoclonal colonies of each color morph. Nineteen wild hostplants were selected randomly, on which aphid colonies were parasitized with ants, and a pair pods with different color morphs was connected to a wild hostplant using a long, thin bamboo stick to enable the attending ants of the wild hostplant to come to the connected reared monoclonal colonies. The number of aphids and attending ants was counted for each shoot 4 days later. In a pair, a green morph had been extirpated for 4 days; thus, data for this pair was removed from the analysis. In addition, in another pair, a green colony attracted too few ants and did not have a normal distribution of symbiont ants and aphids ( $p=0.0001$ ); data for this pair was also removed from the analysis as abnormal data. As a result, we examined the ants' preference using 17 pairs of pods (Fig.3-3).

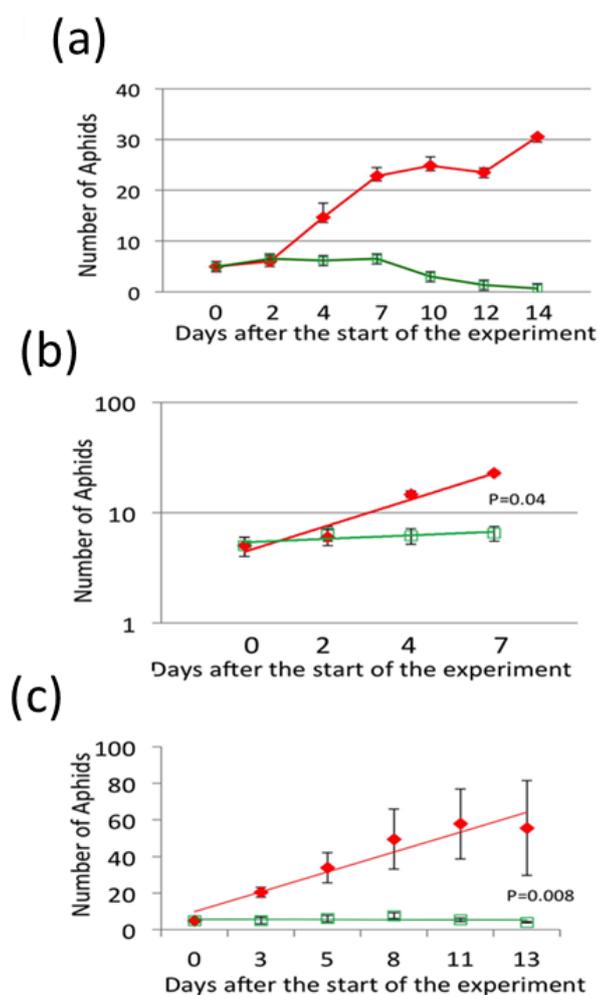
### *Statistics*

We compared the slopes of the linear regressions using ANCOVA. R (ver. 3.3.1) to test significant differences among the slopes.

### **Results**

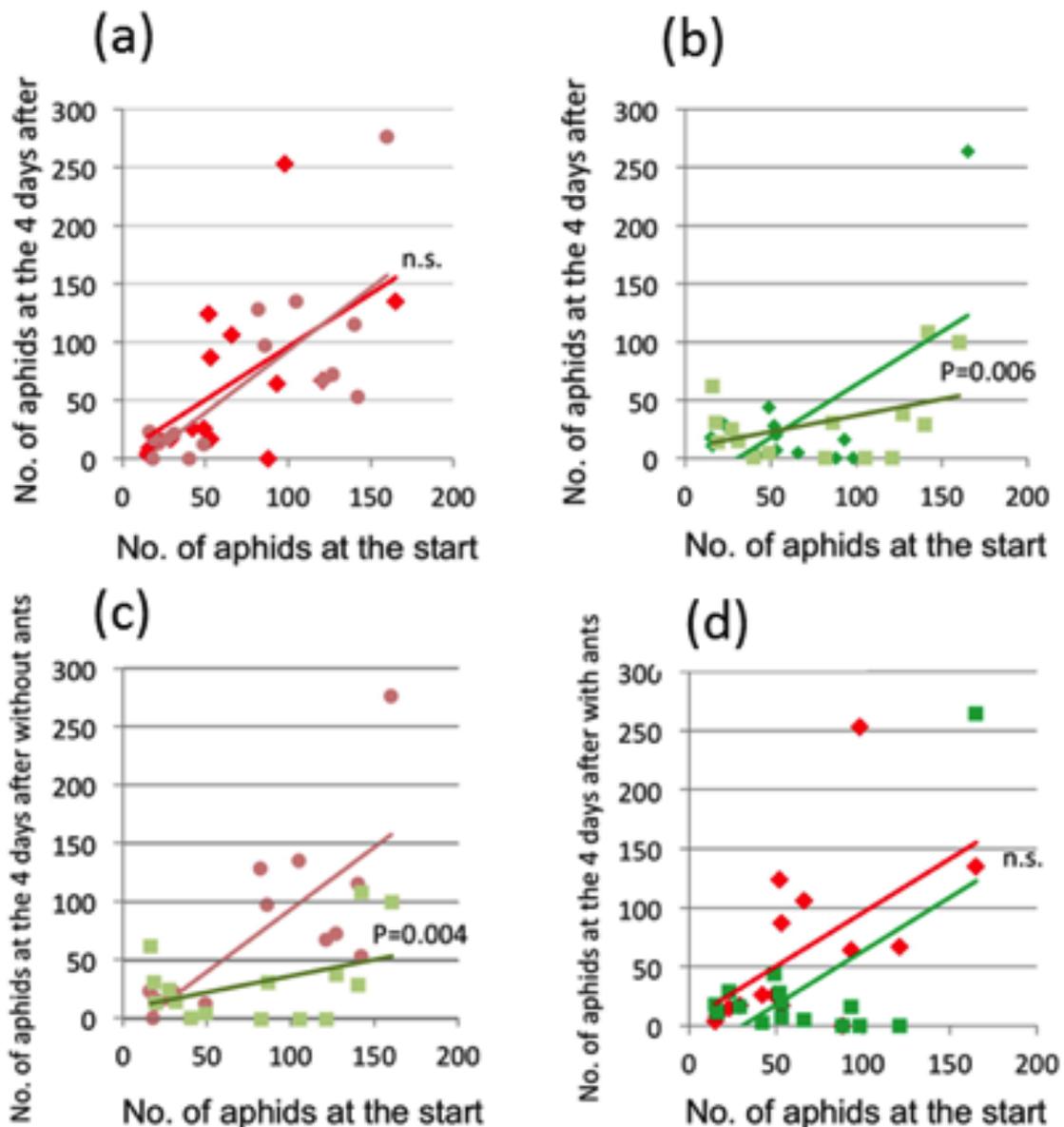
Five clones each of both morphs were reared on each one clonal shoot of the single hostplant (a mugwort: *Artemisia vulgaris*). Without attending ants, the number of aphids increased quickly (7 days) but soon saturated and then decreased because of the environmental deterioration caused by their own excreted honeydew (Fig. 3-1a). This trend was more striking in the green morphs (Fig. 3-1a). We then compared the reproductive rates of both morphs for the first 4 records (7 days) that showed no deterioration. A model selection using AIC provided a simple linear regression with log-transformed aphid numbers as the best model. The regressions showed that the reproductive rate of red clones was significantly higher than that of green clones (Fig. 3-1b). Then, we compared the reproductive rates of a red and a green clone on 5 different clones of the hostplant. Again, the reproductive rate of the red morphs was significantly higher than that of the green morphs on the average of the five hostplant clones (Fig. 3-1c). Thus, the reproductive rates of the red morphs were significantly higher than those of the green morphs, irrespective of the difference in aphid clones (Figs. 3-1a and 3-1b) or hostplant clones (Fig. 3-1c).

Fig. 3-1. **Reproductive rates of both red and green morphs of *M. yomogicola* without ant attendance.** (a) The reproductive rates of five different clones of green and red morphs reared on a single clone of the hostplant. After seven days, both morphs decreased because of deterioration of the environment caused by their own excreted honeydew. (b) The linear regressions of the log-transformed aphid numbers on days for each morph (the marks are the average of the 5 clones) on a single hostplant clone for the first 4 records (7 days) in Fig. 3-2a. The slope is steeper in the red morph than it is in the green one (ANCOVA,  $t=2.733$ ,  $n=6$ ,  $P=0.009$ ), indicating that the red morph increased more rapidly than did the green morph on the same hostplant. (c) The linear regression of aphid numbers on days for each clone in both color morphs on five different clones of the hostplant. The slope is significantly steeper in the red morph than it is in the green one (ANCOVA,  $t=-2.741$ ,  $n=5$ ,  $P=0.008$ ), showing that the red clone increased more rapidly than did the green one, irrespective of variations in hostplant clones.



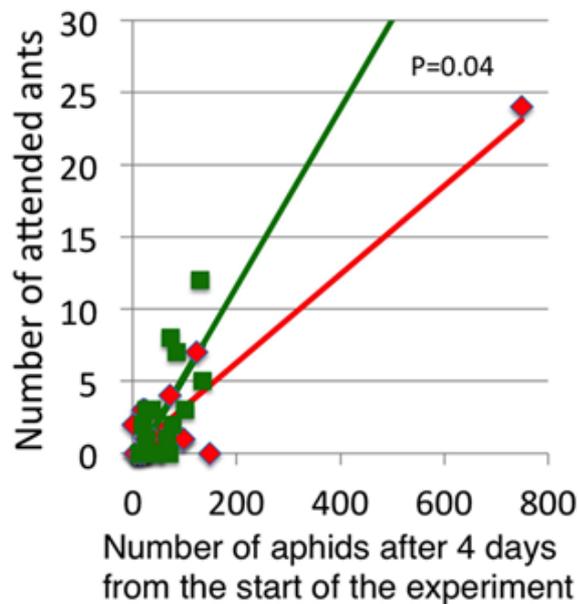
In mixed aphid-colonies, the reproductive rates of each morph were affected differently by ant attendance (Figs. 3-2a-d). The reproductive rate of the red morph did not vary, irrespective of the presence or absence of ants (Fig. 3-2a), but that of the green morph significantly increased when ants were in attendance (Fig. 3-2b). Under the ant-excluded conditions, the red morphs increased more rapidly than did the green morphs (Fig. 3-2c), confirming the competitive superiority of the red morphs. However, this difference disappeared when ants were in attendance (Fig. 3-2d). In conclusion, the attending ants improved the reproductive rate of the green morph, such that the green morph became equally competitive with the red morph.

Fig. 3-3. Patterns in reproductive rates of both green and red morphs in mixed-color colonies in the field. (a) The reproductive rates of the red morphs with or without attending ants. No significant differences in the reproductive rates are detected with or without the ants (ANCOVA,  $t=0.453$ ,  $n=15$ ,  $P=0.65$ ). (b) The reproductive rates of the green morphs with or without attending ants. The green morphs with the ants increased significantly faster than did those without the ants (ANCOVA,  $t=-2.995$ ,  $n=15$ ,  $P=0.006$ ). (c) Under the ant-absent conditions, the red morph showed a significantly faster increase than did the green morph (ANCOVA,  $t=3.094$ ,  $n=15$ ,  $P=0.004$ ), (d) but this difference disappeared when the ants were present (ANCOVA,  $t=0.002$ ,  $n=15$ ,  $P=0.998$ ). Note that each morph is likely to include multiple clones.



When wild ants were given a chance to select a red or a green monoclonal aphid-colony; more ants per aphid selected the green colonies (Fig. 3-4). This result indicates that an ant (*L. japonicus*) prefers the green morph over the red morph.

Fig. 3-4. The relationships between the number of attending ants and the number of monoclonal aphids 4 days after the start of the experiment. The slope of the regression line is significantly larger in the green morph than it is in the red morph (ANCOVA,  $t=2.111$ ,  $n=17$ ,  $P=0.0432$ ), indicating more ants were attending to the green morph when the number of aphids was equal.



## Discussion

This study demonstrates that a mutualistic species actively maintains a polymorphism in their symbiont partners. The population-growth experiments of aphids show that the red morph increases faster than the green morph, irrespective of the variations (different clones) in aphids or hostplants (Fig. 3-3) and that the red morph is superior to the green

morph in reproductive rates. Accordingly, the red morph should dominate within a mixed colony on a shoot of the hostplant. However, in the field, most aphid colonies are mixed, with both red and green morphs (Watanabe et al 2016). Our results show that attending ants positively manipulate the reproductive rates of the green morph to neutralize the competitive superiority of the red morph (Fig. 3-3).

In ant-attending aphids, there is a trade-off between the quality of excreting honeydew and the fecundity of individuals (Yao & Akimoto 2000). This may be true in *M. yomogicola* because the ants preferred the green morph over the red one (Fig. 3-4), but the former showed lower reproductive rates than the latter without ant-attendance (Fig. 3-3a,c). The attending ants improved the reproductive rate of the preferred green morph (Fig. 3-3b), probably to obtain a large amount of high-quality honeydew. Improvement in the reproductive rate of ant-attending aphids have been reported (El-Zaydy 1960, Stadler & Dixon 1999). In the current ant-aphid system, ants prefer the approximately 65% green mixed colonies (Watanabe et al 2016) and neutralize the competitive (clonal reproduction) inferiority of the green morph to maintain constant proportions of each morph (current results).

An interesting question is why the ants do not remove the red morph from this symbiotic system. For attending ants, the increase in the less-valuable red morphs (with low quality honeydew) should not be a preferable condition. In fact, *L. niger* (a sister species of *L. japonicus*) selectively predate aphid individuals who excrete less honeydew in symbioses with other aphid species (Sakata 1995). Ant workers (*L. japonicas*) may be able to discriminate the morphs of aphids (*M. yomogicola*) because,

when they were given an opportunity to select between the two monoclonal morphs, they preferred only the green morphs (Fig. 3-3). The workers also prefer a mixed colony with more green morphs (Watanabe et al 2016). In a mixed colony, they manipulate the reproductive rate of green morphs. Ants are known to recognize opponents by the cuticular hydrocarbons of their body surface (Lahav et al 1999, Thomas et al 1999, Wagner et al 2000, Salazar et al 2015). Therefore, in the current system, the recognition of green and red morphs by ants is suspected to be based on the differences in the cuticular hydrocarbons on their body surface, because ants selectively manipulate the reproductive rates of green morphs. This discrimination is also supported by the fact that they improved the reproductive rate of only the green morph in mixed colonies (Fig. 3-3a,b). *L. japonicus* workers must have manipulated the aphid morphs to maintain the long-lasting coexistence of both morphs in every aphid colony, in addition to their preference for the mixed colonies containing approximately 65% green morphs (Watanabe et al 2016). Therefore, reasons must exist for the ants to maintain the red morph in their attended aphid colonies.

One possibility for maintaining the red morph is for the sexual reproduction of overwintering eggs (stem mothers) for the next year. Most of the *M. yomogicola* colonies disappear after inflorescence budding in hostplants, before the aphid colonies have been able to sexually reproduce the overwintered eggs. The red morphs may have a higher ability for suppressing the development of flower budding in hostplants, such that their colony can survive and reproduce sexupara in late autumn. The pure green colony may fail to reproduce sexupara to lay the overwintering eggs. The red morph may be important for the attending ants to maintain the available aphid colonies as a

honeydew resource for the next year. This interesting hypothesis is being tested now, and if supported, each of the three participants in this symbiotic system would be receiving benefits from long-lasting coexistence with genetically heterogeneous contributors.

There are still few studies on the effects of mutualism in community ecology (Morin 2011). Mutualism may contribute to the origination and maintenance of biodiversity (Tokita & Yasutomi 2003, Iwata, et al 2011). For example, the extreme biodiversity of trees in tropical rainforests may be mediated by repeated speciations of tree-symbiont (animal seed dispersers) systems during glacial periods (Kakishima 2015). In this report, we discover another case, where symbiosis actively maintains polymorphic diversity in aphids.

## **Chapter 4**

### **Effects of the color morphs on the colony survival of the aphids until the production of sexuparae.**

#### **Introduction**

In Chapter 3, I expected that each mugwort would be occupied by only the red morph under a natural selection (Darwin 1859), because the reproductive rate per time was higher in the red morph than that of the green morph (see Chapter 3). However, the attended ants neutralized the competition between the red and green morphs by increasing the reproductive rate of only the green morph in a colony. As a result, the red and green morphs could coexist on every the mugwort shoots (see Chapter 3). However, why didn't the ants remove (predate) the red morph to which the ants shows a lesser preference than to the green morph? From the results shown in the previous chapters, it was unclear that why the attended ants maintain the red morph in a colony in tripartite symbiosis system that consists of the red and green morphs, and the attended-ants.

When inflorescences start to bud at mid-August, many aphid colonies rapidly decrease (see Fig. 4-1, in Chapter 4). This decrease is probably due to an inadequate physiological change of the host plant with the buddings of inflorescences for the parasitized aphids. The sexuparae were produced in the survived aphid colonies from mid-October. Thus, only the aphid colonies those have survived the risk of extinction at mid-August can produce the sexuparae and can sustain to the next year. On the course of the studies for this thesis, I found that almost of the survived colonies produced

sexupare tend to have large colony sizes and many red individuals (Murakami and Watanabe, Personal observations). Therefore, I hypothesized that the red morphs in a colony may contribute to the survival of a colony until mid-October and investigated effects of colony compositions of aphid colonies on the survival of those in the field. In Chapter 1-4, it was unclear that why did *L. japonicus* coexist the red morph with the greens in a colony. Irrespective of a report that *L. niger* (the present *L. japonicus*) predates a symbiotic aphid individuals those excreted a less amount of honeydew than the survived individuals in the symbiosis with *Myzocallis kuricola* (Sakata 1995).

As I mentioned in Chapter (Chapter 1), most aphid colonies on the mugworts decreased rapidly in number, and many of them become extinct (see Chapter 4 Fig4-1) after the budding of inflorescences of the mugworts until the end of August. A few colonies survive until mid-October, and they produce the sexuparae. The sexuparae oviposit eggs for overwintering. Therefore, the aphid individuals in the colonies extincted until October cannot obtain any fitness. I hypothesized that "the red morph in a colony has a low ability to attract ants because of low quality of honeydew (see Chapter 7), but they may sustain their colony to mid-October at when a colony produced the sexuparae. I examined this hypothesis by the following field investigations.

## Methods

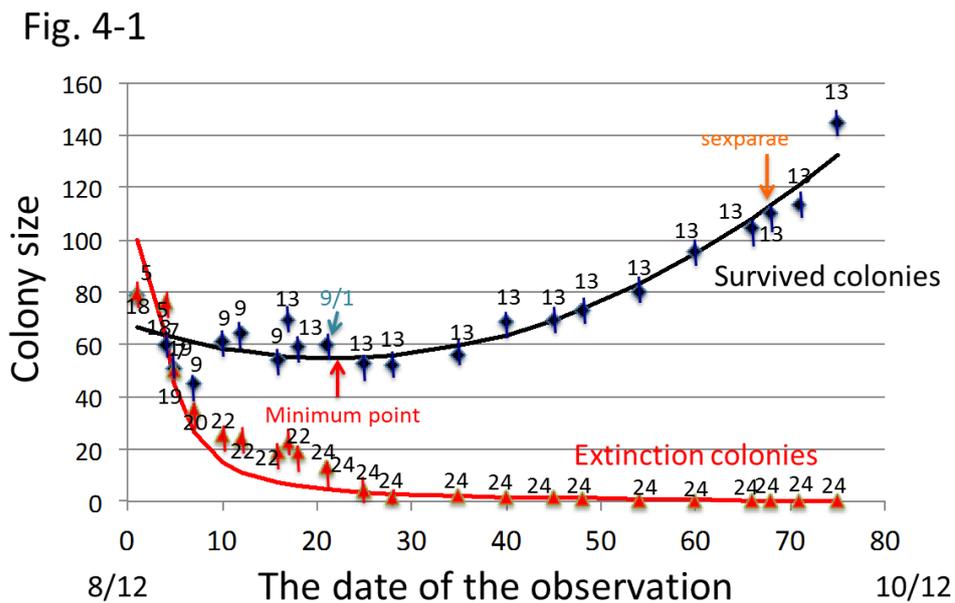
I counted and recorded numbers of the red and green individuals in randomly selected 40 mugwort shoots with aphid colonies with intervals of 2-3 days from mid-August to mid-October until the appearance of sexuparae. The investigation was conducted in the property of Hokkaido Univ. from 12 August to 12 October. Then, I compared the pattern of the change in numbers of aphids between the survived colonies and the colonies those had become extinct before the production of sexuparae. I regressed numbers of aphids in both the types of colonies on the total observed days. As a result, the aphids in the survived colonies decreased once but increased again (see Fig. 4-1), and the nearest sampling day to the smallest colony size is 1 September 2016. Therefore, we examined the effects of colony composition and the colony size at this day on the colony survival by using a multivariate generalized linear model (GLM).

The independent variables of GLM were the number of reds (R), the number of greens (G) and the residuals of the colony size from the linear regression of colony size on the number of the reds (CS). This treatment is to test the effect of colony size on the colony survival as the colony size is  $R+G$ , using  $R+G$  as an explanation variable has no information in a GLM model. The explanation variable is the survival (1) or extinction (0) of the colony. I made multivariate GLM models using R, G, CS, and with or without interaction terms as independent variables and calculated Akaike's information criterion (AIC; Akaike 1973) for all the possible combination of the explained variables. The model with the least AIC was adopted as the best model.

## Results

Among all the investigated colonies (n=40) investigated, only 13 have survived until October 12 at when the first sexupara had been confirmed. The sexuparae have been produced in all the these 13 colonies, and the rest 27 colonies had become extinct. In Fig. 4-1, I plotted the mean number of the survived colony (blue circles) and those of the colonies had become extinct (red triangles).

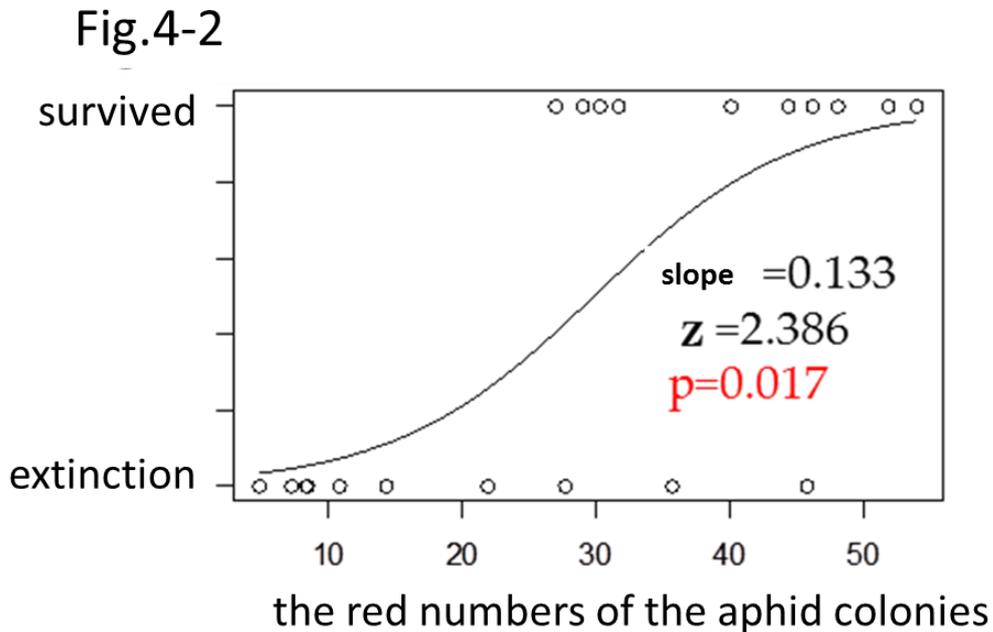
Fig. 4-1. **The change in the mean number of the extinct colonies and the survived colonies.** The red and blue lines represent the regression line for each data. The survived colonies decreased in the number of aphids once but increased again. The least point is the nearest to a sampling date (1 September).



The number of both the types of colonies decreased from August 12, but increased again in the survived colony from the 22 days after the start of the observation (the minimum

point of the 2<sup>nd</sup> dimension regression). Whereas, the extinct colonies continued to decrease until the extinctions. I analyzed the colony composition at the nearest sampling day (1 September) to the 22 days, and I examined which factor(s) affected the survival or extinction of the colonies. I compared R, G and CS between the survived colonies (n=13) and extinction colonies (n=16) at 1 September. R is larger significantly in the survived colonies (Welch's two samples t-test,  $t = 3.0965$ ,  $df = 12.538$ ,  $p = 0.008831$ ), but there is no significant difference in G (Welch's two samples t-test,  $t = 0.67505$ ,  $df = 17.805$ ,  $p = 0.5083$ ) and CS (Welch's two samples t-test,  $t = 2.0965$  that there was not significant difference to colony size,  $df = 17.183$ ,  $p = 0.05114$ ). Then, I examined the factor(s) that affected the survival or extinction of the colonies by the multivariate GLM. The model, Survival/Extinction = R+ the intercept, was selected as the best model based on AIC. The slope of R in the best model was significant (slope = 0.1334,  $n=19$ ,  $Z=2.386$ ,  $p=0.017$ ). AICs for all the possible models are presented in Table 4-1. G and CS were not included in the best model, meaning that the number of greens or colony size have no effect on the survival of a colony. Fig. 4-2 represents the logistic regression line from the best model.

Fig. 4-2. The logistic regression line for the colony survival on the red numbers (final extinction = 0, survival = 1) of the aphid colonies.



Colonies with many red individuals (ca. more than 30) tend to survive until the production of sexuparae. Thus, the red morph was assumed to have an ability to resist the change of physiological condition of the mugworts with budding of inflorescences, and the red morph was the specialized morph to sustain the colony to the next year.

### Discussion

The results indicate that the number of the red individuals in a colony at the critical point (the days from decreasing to increasing in the colony size; see Fig. 4-1) persists them until the production of the sexuparae. From this fact, the red morph is assumed to controls the budding of inflorescences of the mugwort by some mechanism (for the

suppression of inflorescence budding, see Chapter 6), and can sustain the colony to the sexuparae production. Note that, the sexuparae have been produced nearly the same day (12 October) in all the investigated colonies. This fact suggests that the seasonal changes in the daylight length and temperature (low-temperature and short-daytime) would be key factors of sexuparae production in *M. yomogicola*.

At the present, the way of the red morph to suppress the budding of mugwort's inflorescences is unknown. However, the survived colonies have certainly suppressed the budding (see Chapter 6). Thus, there should be some mechanisms of the red morph to manipulate the developmental mechanisms of the host plant. A possibility is that the red morph inject plant hormone like chemicals into the host shoot. Several parasitic insects of plants, including aphids, induce structures such as galls by manipulating the developmental pathways of the host plant (Sopow et al. 2003, Hovanitz 1959). The red morph of *M. yomogicola* might manipulate the mugwort's physiological system by similar ways to be advantageous to own survival. In my opinion, the green morph of *M. yomogicola* produces high quality honeydews (for a preliminary analysis, see Chapter 7) to attract ants more strongly than the red morph (see Chapter 3). The attending ants are necessary for the survival of aphids (see Fig 2-1 in Chapter 2). Although the red morph produces low quality honeydew (see Chapter 7), they contribute to sustain the colony to the next year. In addition, it has been known that there is a trade off between the reproductive rate and the quality of honeydew in an ant attending aphid, *Tuberculatus quercicola* (Yao & Akomoto 2001, Yao & Akomoto 2002). Thus, the ants can gain a high quality honeydew more by rising the reproductive rate of the green morph and, simultaneously, can sustain the aphid colonies (a resource) to the next year

by keeping the red morph with green one (see Chapter 4). Both the G and CS showed no effect on the survival of the aphid colonies. Therefore, the ants secure a resources in the next year by sacrificing the quality of the resources in this year. As ants are a perennial organism, this current sacrifice would produce more alates throughout the colony's life than in the case in which attending ants culled the red morph with inferior honeydew.

In conclusion, all the three participants evolved to maximize their own fitness, the sustainable symbiotic system has been established. There is a view that an ant-aphid symbiosis is a host-parasite relationship (Katayama 2007) because the aphids invest own resources to honeydew to attract the ants and thus, ant-attendance was costly for the aphids (Yao et al 2000, Yao & Akomoto 2001, Yao & Akimoto 2002). However, from the results of this study, the ant-aphid symbiosis found in *M. yomogicola* and ants seems to be a true symbiosis in which all the contributors increase fitness in the case of lacking any of the contributors. This symbiosis should select the color polymorphism of *M. yomogicola* for. *L. japonicus* has shown the strongest preference to the colony with ca. 65% of the green morph (Watanabe et al 2016). This ratio might be the best value to balance to resources acquisition and the long-term colony persistency. In further studies to examine this hypothesis, it is necessary to quantify the ability of the red, the green and the attending ants, and estimate whether the 65% of the green optimize the balance between the persistency of the symbiotic relationship and the efficiency of resource acquirement in each year. In addition, the previous view for the maintenance of polymorphism has thought that as results of competition among each morph, the fitness of each allele is equalized, and the genetic polymorphism maintains by several

mechanism mentioned in Chapter 1 (Losey et al 1997, Tso et al 2002, Takahata & Nei 1990). However, unlike the previous theories, the above true symbiosis in which all the participants gain a fitness benefit resulting from the attempts to maximize their own fitness. It may be a new mechanism to maintain the bio diversity in nature. An ultimate aim of ecology is elucidation of the maintenance mechanism of biodiversity in nature from the viewpoints of how and why. The maintenance of polymorphism by symbiosis may bring many new insights into the evolution of biodiversity and will be an important issue to study in evolutionary ecological field.

## Chapter 5

### **Effects of attending ant species on the fate of colonies of an aphid, *Macrosiphoniella yomogicola* (Matsumura) (Homoptera: Aphididae), in an ant-aphid symbiosis.**

#### **Introduction**

The ant-aphid symbiosis is one of the representatives of mutually beneficial relationships in nature. Ants receive honeydews from aphids, and aphids are protected from predations by ants (Buckley, 1987). Both the contributors gain fitness benefits from this symbiotic system (Way, 1963). In some cases, aphids adjust contents of the honeydew to follow ant' preferences to obtain their attendance (Stadler and Dixon, 1998) by sacrificing their own reproduction (Yao et al, 2000, Yao and Akimoto, 2002) because this cost is necessary for survival of an aphid colony (Banks 1962, Fischer, 2001, Watanabe et al. 2016).

An aphid, *Macrosiphoniella yomogicola* (Matsumura), establishes an obligate symbiosis with ants. The aphids cannot survive without the attended ants because predators predate aphids exhaustively when ants have been removed (Watanabe et al 2016). Thus, the protection by ants is necessary for reproductive successes of the aphids. There are several species of ants those attend to this aphid in our study area. *Lasius* (*Lasius*) *japonicus* Santchi is the most abundant attender for this aphid, but *Lasius* (*Dendrolasius*) *fuji* Radchenko, *Pheidole fervida* Smith, *Tetramorium tsushimae* (Emery), *Formica japonica* Motscholsky, and *Myrmica kotokui* Forel are found as rare

attenders of this aphid in this area.

If there are differences in abilities of the care of aphid colonies among these species, the fitness of an aphid individual is affected by the attending ant species. A previous study has demonstrated that *L. japonicas* repels the most of natural enemies of this aphid (Watanabe et. al. 2016). However, it is unknown that there is any difference among attending ant species for maintenance ability of aphid colonies in this ant-aphid symbiotic systems. In addition, we have seldom observed that some aphids became “mummy” from which parasitic wasps will be appeared. Parasitic wasps are assumed to be the most difficult enemies to prevent the attending ants from their parasitism on the cared aphids because they can fly and lay eggs to aphids within a very short time. Thus, differences in the caring ability of ants would be reflected more strongly to such enemies. The aim of this study is comparing rates of mummies in *M. yomogicola* colonies those are attended by different ant species. In addition, we investigated the final fate of the aphid colonies those are attended by the different ant species.

## **Methods**

We selected a total of 9 *M. yomogicola* colonies those parasitized on shoots of a host plant (a mugwort; *Artemisia montana*) community in the property of Hokkaido University at Sapporo, Hokkaido, Japan. In these colonies, three colonies were attended by *L. japonicas*, three ones were reared by *P. fervida*, and the rest three were cared by *T. tsushima*. In this area, we could find each only 3 shoots those are attended by different species from *L. japonicus*. From 27 August to 14 September 2017, we counted numbers of live aphids and mummies in each colony. The counts were conducted 4 times (27

August, 4, 9, 14 September) during the observation period.

For each colony in each counted day, we calculated the proportion of mummies in colonies attending different ants, and these values were compared among the ant species. Furthermore, change in the parasitism rates were compared among attended ants for each counted day. Differences in the changing pattern in aphid numbers was examined by comparing the slopes of liner regression lines of aphid's numbers on days for each ant species.

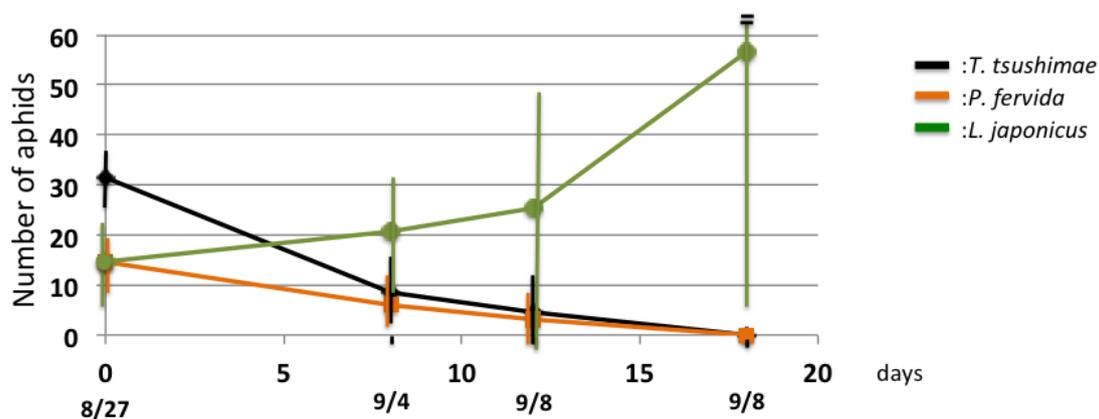
### **Statistics**

The ratio data were compared by the Fisher's exact probability test. Differences in the regression slopes were examined by using ANCOVA. When multiple comparisons were conducted, the significance levels were corrected by the Bonferroni correction for multiple comparisons (Sokal & Roff 1981). All statistic tests were conducted by using R ver. 3.2.1 (R Core Team 2016).

### **Results**

Fig. 5-1 shows change in the average aphid number during the observation period by attended ant species. Only colonies with *L. japonicas* (Lj) survived at the end of the observation period. All the colonies attended by *T. tsushimae* (Tt) and *P. fervida* (Pf) had become extinct by 14 September.

Fig. 5-1. **Change in average numbers of aphids in each 3 colonies attended by each 3 ant species.** For the 4 observed days, mean  $\pm$  S.D. are shown. Black diamonds, orange squares and green circle show the data for *T. tsushimae*, *P. fervida* and *L. japonicus*, respectively.



Figs. 5-2a-c represent the linear regressions of aphid numbers on total days by each attended ant species. For Tt and Pf, the slopes are significantly negative, and for Lj, the slope is marginally significant positive (Fig. 5-2a, Tt;  $Y=-1.748X+27.778$ ,  $F=42.25$ ,  $df=1,10$ ,  $p=0.000069$ ; 2b, Pf;  $Y=-0.824X+13.741$ ,  $F=15.61$ ,  $df=1,10$ ,  $p=0.002728$ ; 2c, Lj;  $Y=2.191X+8.519$ ,  $F=3.90$ ,  $df=1,10$ ,  $p=0.07651$ ).

Fig. 5-2.

**Changes in numbers of aphids in each 3 colonies those were attended by each a different ant species.** (a) The change in *T. tsushimae* attendance. (b) The change in *P. fervida* attendance. (c) The change in *L. japonicus* attendance. All the colonies attended by *T. tsushimae* and *P. fervida* have been extinct before the end of the observation period, whereas all the colonies with *L. japonicus* have survived until the end of the observation.

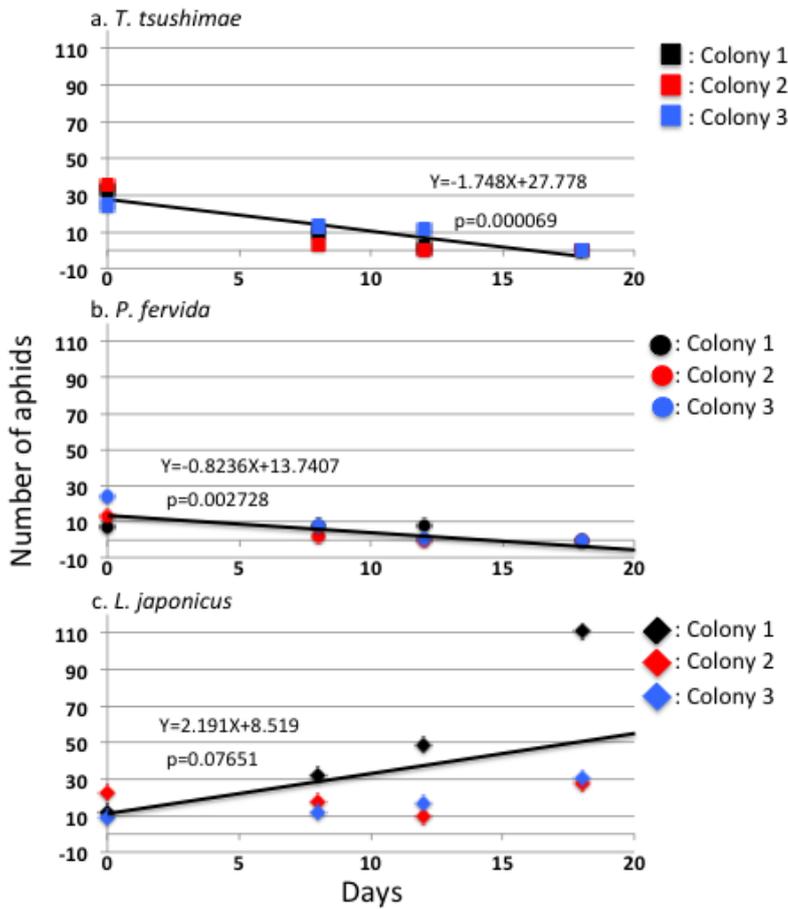
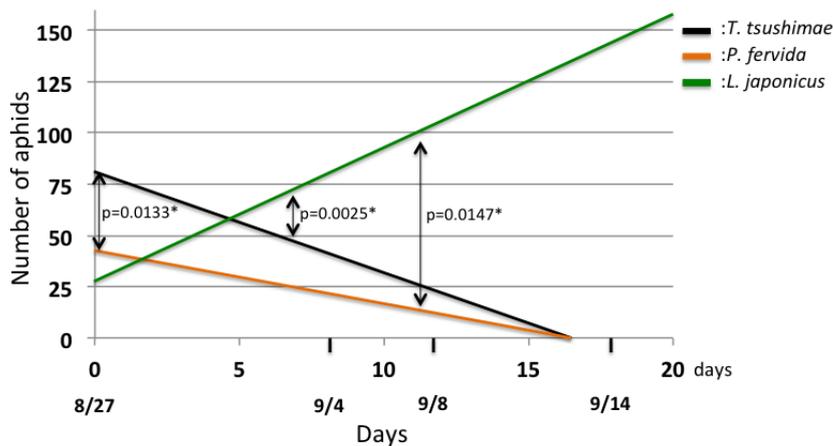


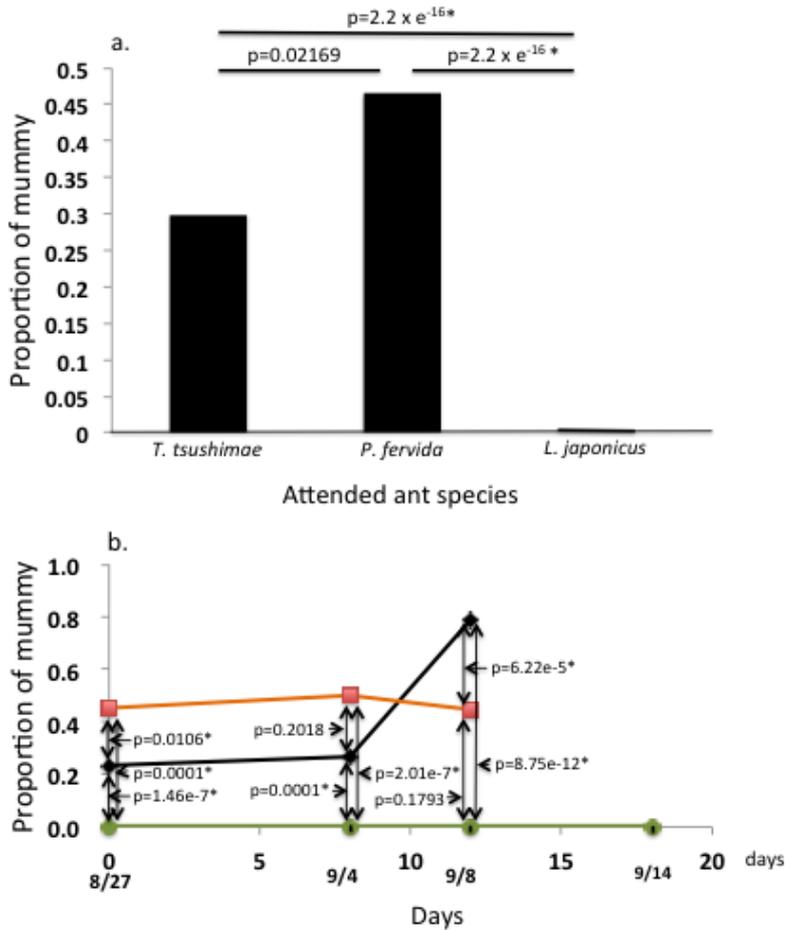
Fig. 5-3 shows statistical differences among the slopes. All the pairs of the slopes were significantly different (Tt vs. Pf;  $F=7.3865$ ,  $df=1, 20$ ,  $p=0.0133$ ; Tt vs Lj;  $F=11.9098$ ,  $df=1, 20$ ,  $p=0.0025$ ; Lj vs. Pf;  $F=7.1320$ ,  $df=1, 20$ ,  $p=0.0147$ ; all the p value are significant after the Bonferroni correction for multiple comparisons). Therefore, number of aphids changed differently by the attended ant species.

Figure 5-3. **Comparisons between the regression slopes between two groups those were attended by each a different ant species.** The differences were tested by ANCOVA. \* represents the significant difference between the two slopes after the Bonferroni correction for multiple comparisons.



For the parasitism rates by parasitoid wasps, there is no mummy in the aphid colonies attended by Lj, whereas, mummies were found in the aphid colonies attended by the other 2 ant species. Fig. 5-4a shows the proportion of the mummies observed during whole the period by the attended ant species. There is no difference in the parasitism rates between the Tt and Pf colonies. However, both types of the colonies contained highly significant proportion of the mummies than the colonies attended by Lj. This trend is the same the data was analyzed separately by the observed days (Fig. 5-4b; p values were calculated by the Fisher's exact probability tests).

**Fig. 5-4. The differences in rates of the mummies between the aphid colonies with each a different ant species.** (a) Total proportions over the observation period. (b) Comparisons for each the observation day. \* represents the significant difference between the two ratios after the Bonferroni correction for multiple comparisons.



These results indicate that *L. japonicus* is a better partner than the other 2 ant species for *M. yomogicola* because the aphid colonies with *L. japonicus* are more long-lasting and are more protected from the parasitism than those with the other two ant species.

## Discussion

Our results demonstrated that there are differences in the ability of ant species to care aphid colonies. *L. japonicus* perfectly protected their aphids from the parasitism by parasitic wasps (the rate of mummies is 0 for all the aphid colonies at all the observation days; Fig. 5-4), and the aphid colonies attended by this ant were more long-lasting than those attended by the other two ant species (Fig. 5-1). Whereas, the aphid colonies attended by *T. tsushimae* and *P. fervida* have been suffered from the parasitism by parasitic wasps, and have been extinct before the end of the observation period. As *M. yomogicola* produce sexuparae at mid October in this area, an aphid colony would be difficult to reproduce when they are attended by the other ants from *L. japonicus*. Why does this difference arise? One possibility is that the ant species belonging to the genus *Lasius* depend mostly on honeydews of aphids and scale insects for foods (Itioka and Inoue, 1999, Pekas et al, 2011, Endo and Itino, 2012). This fact suggests that they are specialists for rearing the host symbionts. Whereas, *T. tsushimae* and *P. fervida* is each omnivorous species those gather various foods such as dead insects, plant seeds and honeydews (Hölldobler and Wilson 1990; Wilson 2003). The later two species would not crucially depend on honeydews of aphids, and thus they would pay less cost for maintaining aphid colonies. This difference in habits of attended ant species might be the cause of the observed differences.

The aim of ants to attend the aphids is exploiting honeydews from them. Ants get honeydews from aphids, and the aphids are protected from predators by ant-attendance (Watanabe et al 2016). Thus, this is a kind of mutually beneficial symbiosis. However, *L. japonicus* has been known to selectively predate aphid individuals those excrete less

honeydew in a symbiosis with another aphid species (Sakata 1995). In addition, symbiotic aphids have been known to change contents of honeydews when ants attended (Yao, 2002). Thus, attending ants would be sensitive to the quality of hosts' honeydew. *L. japoniscus* (previously *L. niger*) prefers special kinds of sugar than other sugars (Detrain et al, 2010). Especially they showed a strong preference to Melezitose (Völkl 1999). Our preliminary experiments showed that the sugars in the honeydew of *M. yomogicola* consists of only Melezitose and Sucrose (Watanabe et al, unpubl.). As shown in this study, when they are attended by the other ants of *L. japonicus* the final fate of them are likely to be extinction. Thus, there is a possibility that *M. yomogicola* excretes honeydews to which *L. japonicus* only shows a strong preference. The most of wild colonies of *M. yomogicola* are attended by *L. japonicus* (Watanabe et al 2016). In fact, we found only each 3 shoots with different ant species (*P. fervida* and *T. tsushimae*) of *L. japonicas* in the investigated host plant community. Chemical analyses of honeydew contents and bioassays for preferences of each ant species to each substance in the honeydew are required to examine this hypothesis.

The cause of differences in the change patterns in number of aphids is ambiguous. *M. yomogicola* colonies are abundant in summer, but rapidly decrease after budding of inflorescences of the host plants at mid August. Perhaps, the physiological condition of the host plant change with the budding of inflorescences and becomes inadequate for the aphid's reproduction. Only a few colonies survived to mid October and produce sexuparae those lay overwintered eggs after copulations. The observation period is just the period at when the fate of a colony will separate into either extinction or survival. All the 6 colonies those have been attended by other ants of *L. japonicus* had been

extinct within the observation period. However, whether the cause of this extinction is attributed to the species of attended ants or not is unclear by the obtained data in this study. However, it is a definite fact that all the 3 colonies those has been attended by *L. japonicus* survived until the end of the observation. Because an ant colony is perennial and can hold a source of resources in future if the current aphid colony will persist to the next year. As mentioned above, *L. japonicus* seems to be a specialist feeder of honeydews of insects. Holding a source of honeydews in future would be more important for *L. japonicus* than for the other omnivorous ants. Therefore, this species might have been evolved to persist the attended aphid colonies to the next year. Further studies will bring interesting insights into the evolution of symbiosis in nature.

## Chapter 6

### Effects of aphid parasitism on the fitness of the host plant in an aphid-host relationship.

#### Introduction

Arthropod-plant interactions are one of the important issues from several viewpoints in basic or applied ecology, e.g., the evolution of adaptations in arm-races (Krebs & Davies 1993), improvements in amounts of agricultural products (Oerke 2006) and the evolution of the host-parasite relationships. Aphids depend absolutely on the phloem sap of the host plant, and therefore, one of the serious pests of the plant cultures (Emden & Harrington 2017). Therefore, elucidating how the host plant does resist to the parasitism, and how an aphid species does away from the resistance by the host are important issues for understanding the evolution of adaptations under arm races (Bernays & Graham 1988). As aphids exploit the phloem sap of the host plant, their parasitism would have negative effects on the fitness components of the host plant. However, there is little empirical evidence that have measured the impact of the parasites on the fitness components of the hosts.

An aphid, *Macrosiphoniella yomogicola*, parasitizes on a mugwort, *Astemisia montana* in cold regions in Japan (high elevation areas in Honshu and lowlands in Hokkaido). In Hokkaido, stem mothers of *M. yomogicola* hatch from overwintered eggs and produce offspring asexually on the mugworts. From mid June to early July, winged individuals are produced asexually and disperse to the distant mugworts. During a summer, the

aphid generations cycle by asexual reproductions, and most mugworts are parasitized by the aphid colonies. However, the most aphid colonies become extinction after the budding of inflorescences of the host plant at the mid August, probably by a physiological change in the host plant with the budding. Only the aphid colonies survived until mid October can produce sexuparae, and female sexuparae lay overwintered eggs after mating with males. Therefore, the aphid cannot get fitness unless overcoming the resistance (the budding of inflorescences) of the host plant, i.e., the existence of an arm race between them.

Some aphids have been known to manipulate the host's developmental system to make a gall within which the aphids can take the phloem sap with a protection from predations (Larson & Whitham 1991). Thus, *M. yomogicolla* may manipulate the host plant to suppress the budding of inflorescences by similar ways with the above examples to sustain their genetic lines to the next year.

In this study, in order to examine the above hypothesis, we investigated the relationship between the aphid parasitism and the fitness components of the host plant at the time to sexual reproduction of the parasite. The effects of the aphid parasitism on the growth of the host plants, number of inflorescences and numbers and weights of the floral pads. We will discuss on the evolution of adaptations of both the host and the parasite under this arm race.

## **Methods**

After the budding of the mugwort's inflorescences, number of aphids decrease rapidly and many colonies become extinct by early autumn. A few colonies survive to mid October, and sexuparae are produced eggs for overwinter by sexual reproduction. The rapid decreases of aphid colonies are probably due to a change in physiological conditions of the host plant with the budding of inflorescences. The aphid cannot get any fitness unless surviving this critical period. Thus, what is the cause of the survival is a very important to consider the aphid's adaptive strategy. Contrarily, if buddings of inflorescences is the cause of extinctions of the aphid colonies, the aphid might had evolved to suppress the budding in order to survive. Thus, it is expected that there is an arm race between the aphid and the mugwort.

### ***Data sampling***

From 12 August to 12 October, 2016, we investigated a total of 40 mugworts those were parasitized by *M. yomogicola*. The mugworts were selected randomly in the property of the Sapporo campus of Hokkaido University. During the study period, we recorded numbers of green and red on each mugwort with ca. 3 days interval (several rainfalls prevent us to count with the same intervals during the study periods).

For these data, we examined colony extinctions after the budding of the inflorescences. In each investigated days from 12 August to 1 September, we calculated the survival rate of the colonies. As several parasitized mugworts were added to the samples on the course of the investigation, we used the survival rate instead of numbers of aphids because we want to know how many colonies become extinct in this period. The

survival rates were regressed on the days after the start of the investigation, and the significance of the slope of the linear regression was tested statistically.

Next, we compared several characteristics of the host plants with and without *M. yomogicola* at the period of sexuparae production. From 9 to 15 October 2017, we measured several characteristics of the host plants at two sites (the back yard of the Hokkaido Univ. Museum (Site 1) and the side of the Faculty of Engineering of Hokkaido Univ. (Site 2)). For Site 1 and 2, five vs. 11 and 5 vs. 11 mugworts with and without the aphid colony were measured, respectively. The measured characteristics are as follows, height of the plant (H; the nearest to cm by using a tape measure), stem width at the 5 cm from the ground (W; the nearest to 0.01 mm by using a digital caliper (Digimatic caliper<sup>TM</sup>, Mitutoyo, Kanagawa, Japan), number of inflorescences within the top 5 cm of the plant (Inf), number of floral buds on these inflorescences (fb), the total dry weight (measured to the nearest to 0.001 g by using a digital balance (UX220H, SHIMADZU, Tokyo, Japan) after 24 hrs drying in 80°C of a drying oven (SK401, YAMATO, Tokyo, Japan). Then, we calculated H/W(HW) as an index of the plant condition.

### ***Analyses***

In order to estimate the most strongly affected factor for the number of inflorescences, by using only the mugworts without the aphids, we conducted a multivariate generalized linear model (GLM) analysis with a normal distribution in setting Inf as the dependent variable and H, W and their interaction term as the independent variables. The best model was determined by comparing the Akaike's information criterion (AIC)

for each model. The model with the lowest AIC was selected as the best model. Then, we regressed the number of inflorescences on the best estimator (detected by the above process) of Inf separately by the site. Differences in the slopes and the intercepts of the two regression lines were compared by ANCOVA, and then we judged that whether the data can be combined or not. Then, the measured characters were compared between the mugworts with and without the aphids.

Finally, we compared fitness of the mugworts with and without aphids. The fitness index is set as the total weight of floral buds because this value reflects the number and the weight of a floral bud.

### ***Statistics***

H, W, HW and the average fb weight between mugworts with and without the aphids were compared by Wekch's *t*-test. The differences in Inf was tested by Mann-Whitney's U-test. All the statistical analyses were conducted by R (ver. 3.2.1; R development core team).

### **Results**

Fig.6-1 shows the change in the survival rate of the investigated aphid colonies in each the counted day. There is a significant negative relationship ( $\text{Rate} = -2.0092 * \text{Days} + 97.877$ ; for the slope,  $df=8$ ,  $t=-3.42$ ,  $p=0.0091$ ). As the first budding of the hosts' inflorescences had been confirmed 12 August, many of the aphid colonies become extinct after the budding of the inflorescences. The GLM analysis indicated that a model with HW only as a dependent variable is the best model to estimate of the number of inflorescences (see Supplementary Table S1). Fig. 6-2 represented two

regression lines of the HW on Inf in each study site. The slopes of two lines are not different statistically (for Site 1; 0.3602, for Site 2; 0.3522,  $F=0.0007$ ,  $df=44$ ,  $p=0.9795$ ) but the intercepts were significantly different between the sites (for Site 1; 0.7996, for Site 2; -2.7839,  $df=44$ ,  $t= -2.789$ ,  $p=0.0088$ ). Thus, we analyzed data from the two sites separately.

Figs. 6-3a-h represent the means of the measured characters between the mugworts with and without aphids at both the investigated sites. H and W are not different significantly in relation to the aphid parasitism, but there is a significant difference in HW at Site 1. Number of inflorescences is significantly different between the mugworts with and without aphids, and p values are small. However, as there is a significant difference in HW at Site1, these differences might be due to this HW difference. Thus, we calculated residuals of Inf values to the regression of Inf on HW (the lines in Fig.6-2). Then, the mean residuals were compared between the mugworts with and without aphids for both the sites. Note that, we used the regression lines those calculated for the mugworts without aphid only because we intend to know the aphid parasitism has how many degree of negative effect on the plants's fitness. Thus, the regression of healthy mugworts on Inf should be used as a reference. Figs. 6-4a, b show the effects of the aphid parasitism on the number and mean weight of floral buds. Both the number and the mean weight were affected negatively by the existence of aphids. The mean weight at Site 2 was impossible to test because, in this site, none of the parasitized shoot buds any inflorescence, and thus weight data for floral buds could not measure at this site. Figs. 6-5 shows the mean residuals of Inf at the two sites. In both the sites, the mean residuals are negative for mugworts with the aphid parasitism, and there are significant

differences from those of the healthy mugworts. Therefore, it can be concluded that the aphid suppressed the budding of inflorescences of the host plant and has a serious negative effect on fitness of the host plant. Fig. 6-4b compares the mean obtained fitness between the mugworts with or without aphids. Again, at both the sites, the fitness of the host plants were decreased largely by the aphid parasitism.

## **Discussion**

Our results demonstrated that the aphid parasitism gives a serious negative effect on the fitness of the host mugwort shoot. The fitness (estimated as a total dry-weights of the floral buds) decreased to 26.73% and 0% at Site 1 and Site 2, respectively (Fig 6-4b). This decrease came from both the decrease in number of inflorescences and in the average weight of floral buds (the later was found in Site 1 only). Fig. 6-4a showed that the parasitized aphids suppressed the budding of inflorescences of the host shoot compared with the shoots without the aphids. Although the average HW was significantly smaller in the shoots with aphids than those without aphids, the results in Fig. 6-4a controlled statistically for this difference in HW by using a residual analysis. Thus, the aphids controlled the host's physiology by some ways. Gall-making aphids manipulate the developmental systems of the host plant in order to induce the development of the galls (Katherine et al, 1991). *M. yomogicolla* might make a similar manipulation to *A. montana*.

The reason of this manipulation by the aphids seems to be an inconvenient physiological change of the host plant with the budding of inflorescences. Fig. 6-1 showed that after the budding of inflorescences, many colonies of the *M. yomogicola*

will be extinct. Within a few weeks, numbers of aphid colony were decreased to ca. 60%. Note that, if a colony has become extinct several such shoots parasitized by a few aphids, probably moved from living colonies or were transported by attending ants. Thus, the number of survived colonies re-increased in some cases. Anyway, there should be an arm-race condition between *A. montana* and *M. yomogicola* because both parties can get more fitness when could suppress the opponent's tactic that leads own fitness drastically. *M. yomogicola* will loss all the fitness without suppressing the budding of host's inflorescence, and an *A. montana* shoot that allowed will loss from 74.27 to 100% of fitness in a year (see above). These serious negative effects on the opponent's fitness would lead an arm-race evolution between the two parties (Roy and Kirchner 2000).

An interesting viewpoint is that *A. montana* is a perennial plant making a clonal plant community (Luijtenet al 2000). The shoots in a community are connected to each other by roots. This feature of *A. montana* enables them to evolve their traits via kin selection (Hamilton 1964a, b). Self-sacrificing traits of a shoot can evolve if these sacrifices increase the fitness components of the rest shoots in a clonal community. Kin-selected traits of plants have been reported (Queller 1983). If the parasitism of *M. yomogicola* gives some benefits to a clonal community of *A. montana*, a clonal community may sacrifice a part of fitness components from a part of the shoots. Are there such assumable benefits to *A. montana* from the parasitism by *M. yomogicola*. A possibility is that *M. yomogicola* is an aphid with absolutely ant attendance (Watanabe et al 2016). Attending ants repel most of the predators of *M. yomogicola* (Watanabe et al 2016). Similarly, they may repel herbivorous insects those eat *A. montana*. If a clonal

community of *A. montana* gain fitness benefits from the attending ants on *M. yomogicola*, sacrificing a part of shoots in order to survive the aphids may evolve through kin selection. Aphid attendance ants have been known to protect the host plants from the herbivorous insects those are not predators of the aphids (Buckley 1987).

Evolution of a kin selected trait can be explained by the well-known Hamilton rule ( $br-c > 0$ ;  $b$ =the fitness benefit of a recipient by altruistic traits of the doner,  $c$ =the fitness cost of a doner,  $r$ =genetic relatedness between the doner and the recipient). In the case of *A. montana*, as  $r=1$  among clonal shoots, and thus if  $b > c$ , altruistic traits of a clonal shoot can evolve. We can examine this possibility by investigating the fitness cost of a “clonal community” (not of a shoot) and the benefit of *M. yomogicola* parasitism on a clonal community. We can estimate the later by removing aphids by rubbing a sticky liquid at the base of shoots to remove attending ants from an aphid colony because the aphids will be predated and become extinct soon after removing of ants (Watanabe et al 2016). The former part (cost of the aphids’ parasitism for a shoot) was estimated in this study. Note that, the abundance of *M. yomogicola* parasitism on the most mugworts may suggest the existence of merits to allow the parasitism during the growth period of *A. montana*. The host plant may be better to remove the aphids only after the start of reproductive season (after the budding of inflorescences. In fact, the aphid colonies rapidly decreased during the reproductive season (Fig.6-1). However, it may be better for the host plant to allow to sustain a part of aphids in order to receive the merits from them in the next year sacrificing a part of fitness from the parasitized shoot. Therefore, we can challenge to this very interesting hypothesis. The results of such studies bring us to many new insights into plant-insect interactions. If the above is the case, this

aphids-plant relationship is a symbiosis rather than an one-sided effect. This interesting viewpoint awaits to clarify by further studies.

### Figure legends

Figure 6-1. **Decreasing pattern of *M. yomogicola* colonies on the host mugwort shoots in Autumn 2016.** The parasitized colonies rapidly decreased with the buddings of inflorescences of the host plant (the first budding has been confirmed at 12 August). During the investigated period, the survival rate has risen sometimes. One may feel that this is strange but a colony that has been extinct frequently re-parasitized by a few aphids at the next observation day, probably due to moving of aphids from the other colony or transporting of aphids form the other colonies by attending ants.

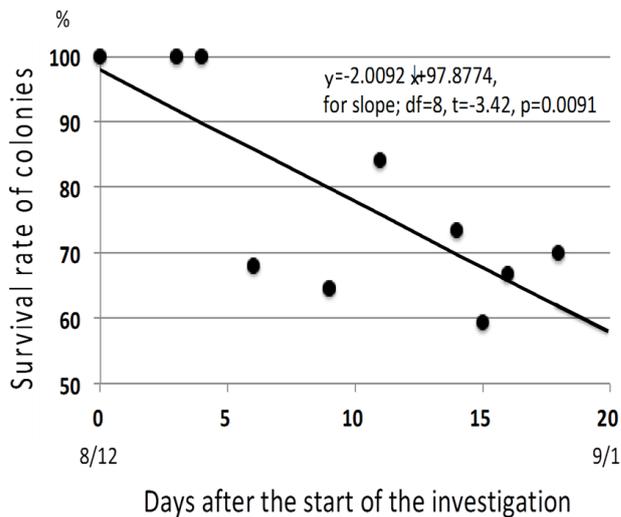


Figure 6-2. **The regression lines of no. of inflorescences within the top 5 cm of each shoot on the height/the basal width of each shoot.** The slopes of the two study sites are not different statistically (for Site 1; 0.3602, for Site 2; 0.3522, ANCOVA,  $F=0.0007$ ,  $df=44$ ,  $p=0.9795$ ), but the intercepts are statistically different (for Site 1; 0.7996, for Site 2; -2.7839,  $df=44$ ,  $t= -2.789$ ,  $p=0.0088$ ). Thus, we cannot combine data.

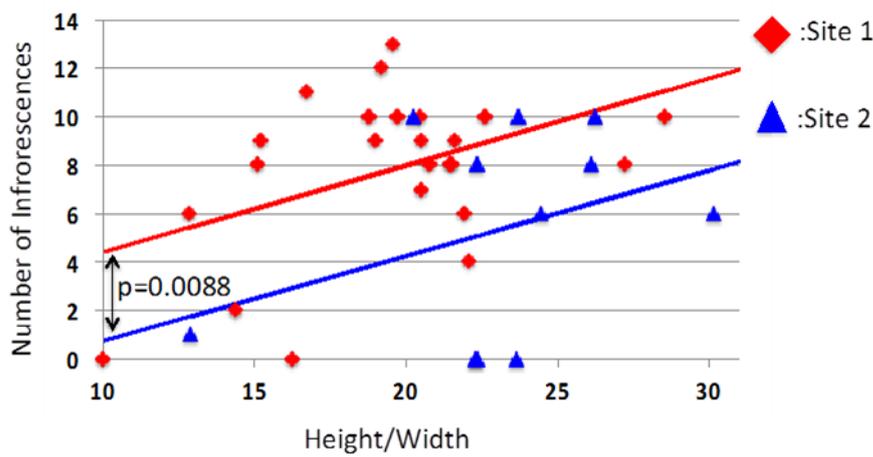


Figure 6-3. Comparisons of the characters measured between the shoots with (green bars) and without (black bars) the aphids. The whiskers represent 1 S.E. The height and the basal width are not different in both the site (3a-d). However, height/width (an index of the physical condition of the host plant) is different statistically in Site 1 (3e, f). No. of inflorescences are highly significantly different in both the sites (3g, h).

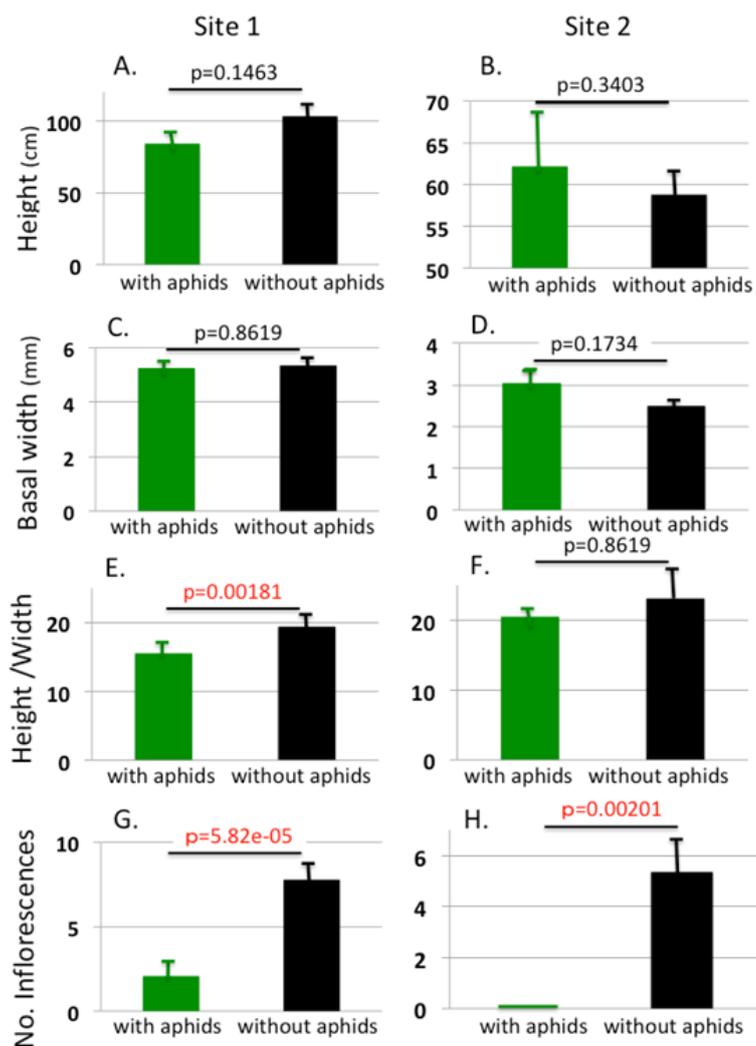


Figure 6-4. **Comparisons of the floral characters between the shoots with (green bars) and without (black bars) the aphids.** In both the sites, no. of floral buds and the mean weight of a floral bud were affected negatively with the parasitism of *M. yomogicola* (3a-d). For Site 2, we could not test statistical difference for the mean weight of a floral bud because all the parasitized shoots have not bud any inflorescence (3d).

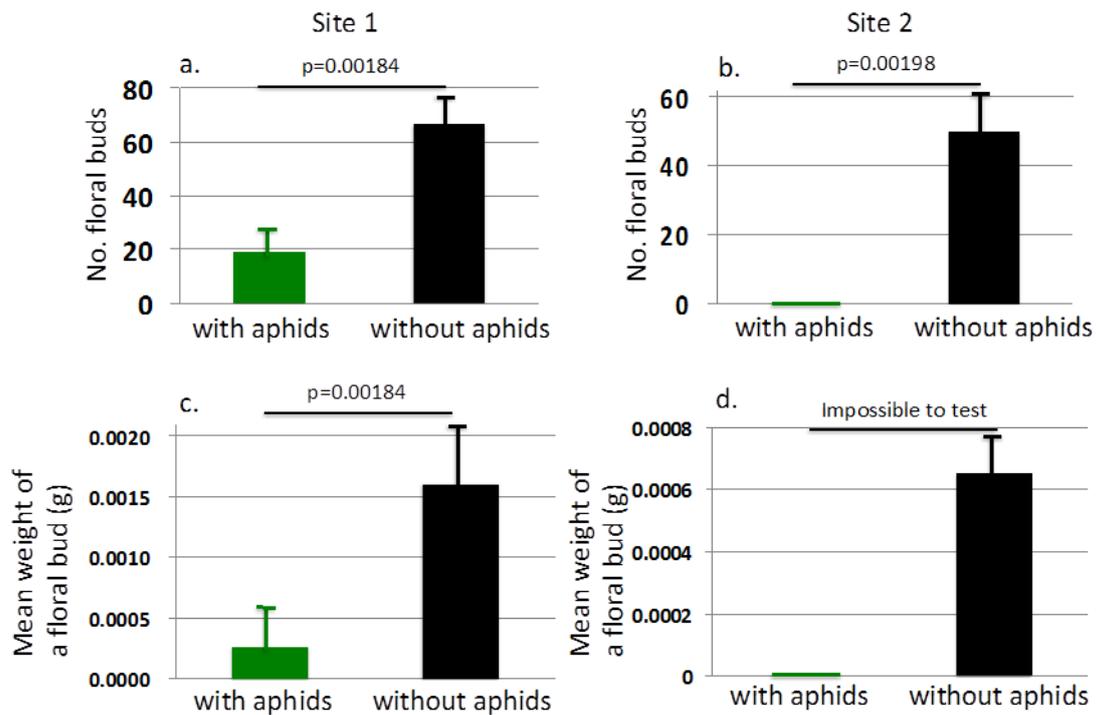
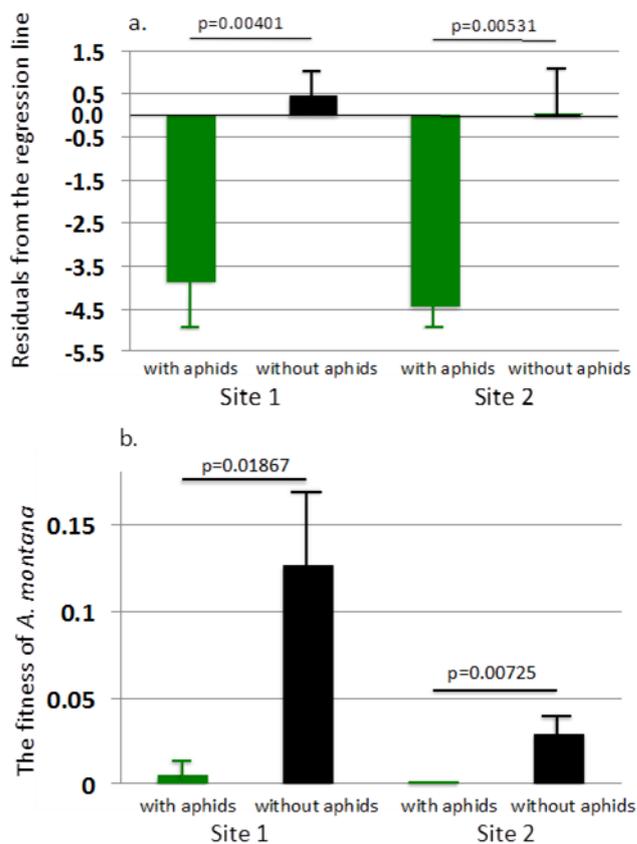


Figure 6-5. **Comparisons of fitness of *A. montana* between the shoot with (green bars) and without (black bars) the parasitism.** Fig. 6-5a shows the comparisons of the mean residuals of the no. of inflorescences of the shoots with and without the aphids. The residuals were calculated as a value from the regression line in Fig. 6-2 (the regression of no. of inflorescences on HW among the shoots without the aphids). The residuals of the parasitized shoots were calculated for these lines because we intended to know the effect of aphid parasitism from the normal shoots.



## Chapter 7

### **The mode of inheritance of body colors and a preliminary analysis of sugar composition of honeydews between the red and green morphs.**

#### **Introduction**

It is unclear that there is any difference in the quality of honeydew between the two color morphs. In addition, how inherit the body color to the next generation is also unknown. Answering to these questions is important to clarify the further proximate mechanism to maintain the polymorphism in *M. yomogicola*.

At present, the elucidations of these questions are insufficient, but, in this chapter, I show preliminary data and will discuss on how can I answer to these questions as further issue.

Hypotheses to be examined.

In past investigations, the sugar concentrations in honeydew are higher in the green morph than that of the red one (2007. Aoyama, personal communication). As the attended ants more prefer the green morph than the red morph (Fig. 3-4 in Chapter 3), it is expected that the honeydew of the green morph has a higher quality for ants than that of the red morph (the hypothesis 1).

*L. japonicas* (previously, *L. niger*) is known to show a strong preference to a sugar (melezitose) than to other sugars (Woordring et al. 2004). In addition, several aphids increase amounts of amino acid in honeydew when attended by ants (Yao & Akimoto

2002). Thus, it is expected that the honeydew of the green morph contains more sugars or amino acids than that of the red morph.

Although the body color was inherited in asexual generations (from Spring to early Autumn). The mode of inheritance of body colors at sexual reproduction is unknown. Preliminary observations tell us that the sexuparae of both the colors did not seem to choose the color of mates, i.e., there were all possible pairs of two color morphs in the observed mating pairs (S. Watanabe, personal observations). The mode of inheritance has a much effect on the evolution of the color polymorphism in *M. yomogicola*. Therefore, to clarify the inheritance mode at the sexual reproduction is important for further examinations.

### **Experiment 7-1.**

#### **Difference in sugar composition in honeydew between the red and green morphs.**

### **Methods**

#### Experiment 6-1.

Using various clones of *A. montana* reared in plant pots, I established several aphid colonies each of which is a monoclonal group. Using a ultra-free micro capillary (0.1  $\mu\text{m}$  in diameter, 30mm in length), I collected honeydew of monoclonal groups from the reared colonies in 6 September 2017. In total, I obtained 5 samples for the red clones and 2 samples for the green clones. The obtained honeydews were prepared to a biochemical analysis using UPLC-MS as follows. First, the honeydews collected were weighed and

were recorded the length in the microcapillary in mm. Second, I added 200ul of 99.9% methanol and 40ul of distilled water (ratio of 5:1) to each honeydew. Third, in this mixture, the capillary including honeydew was crushed well by a sterilized plastic pestle, and this liquid was filtered by using a glass Pasteur pipette staffed with a cotton ball. Then, I analyzed the sugar composition in the liquid by using a UPLC-MS (Waters ACQUITY UPLC system, Waters, Milford, MA) in (Laboratory of natural product chemistry, Hokkaido University), Graduate school of Agriculture, Hokkaido University. Then, I weighed each glass vial for measurements and poured the filtered liquid. The samples were weighed again to determine the weight of the liquid. When the liquid clouded, I centrifuged the liquid at 15000rpm in 1 min by a microcentrifuge machine (CF15RX II、HITACHI, Tokyo, Japan), then 100ul of the supernatant was analyzed.

In this analysis, I did not use a standard to quantify the amount of sugars, I compare the amount of detected sugars by comparing the area of the focal peaks among the samples.

Experiment 6-2.

The transplant of the sexuparae of this aphid to another clone of the host plants is very difficult. Therefore, it is nearly impossible to obtain fertilized eggs from arbitrary pairs. Therefore, in this study, I removed the aphids of one color morph from several colonies just before the production of sexuparae in October 2016 and made monocolour colonies (constructed by only the red or the green morph). The manipulated colonies were separately covered by a mesh cloth to prevent escaping of the aphids from the shoot. The appeared sexuparae allowed to mate free in the net. This treatment allowed matings between the same color. I retrieved the laid eggs at early December, and the eggs were

placed in a petri dish (114 mm in diameter and 20 mm in depth). The dishes were maintained at 5°C and checked for hatchings at Spring of 2017. If the different colored stem mothers hatched from the eggs from a netted shoot, some Mendelian inheritance is assumed. As the aphids colonies become extinct soon by predations without ant-attendance (Watanabe et al 2016, see Chapter 1), I set a vinyl tube (5cm in length) with a slit to the base of stem of each shoot to allow ant-attendance through the gap between the tube and stem. This treatment allow the aphid colonies to attend by ant but prevent them from predations. Then, all the colonies were collected with the nets in early December at when the sexuparae had finished ovipositions. Then, I counted the number of eggs and the sexuparae (males and females) for each colony. The eggs collected from each colony were moved on a filter paper that has been moisten, and this paper put into a petri dish (114 mm in diameter and 20 mm in depth) with sealing by a vinyl film (PARAFILM<sup>®</sup>, Bemis, Chicago, USA) The dishes were maintained in an incubator at 5°C until the next Spring. The dishes were taken out from the incubator in early May at when the aphid eggs hatches in the field, and kept at room temperatures to induce hatchings. I recorded the number of the hatched stem mothers and transplanted each of them to the potted mugworts. I reared the aphids until second inster at when I could distinguish clearly their body colors and recorded the body colors of the asexual daughters produced by each stem mother.

By this experimental process, the number of the stem mothers hatched from a colony and the number of sexuparae, and the color ratio among offspring are obtained. From the color ratio in the offspring from a mating between the same colors, I estimated the inheritance mode of body colors in this aphid.

## Results

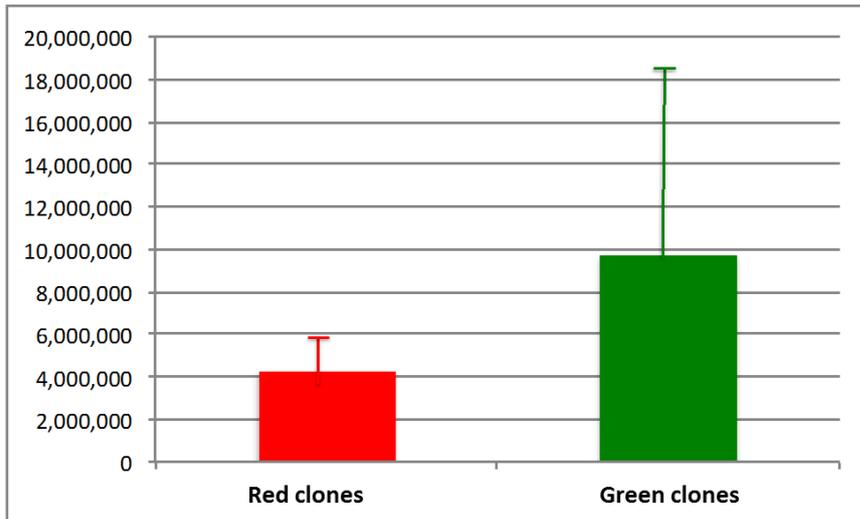
Sucrose and melezitose were detected from the honeydew of both the red and green morphs. In addition, the tendency the difference was seen in red and sucrose in green honeydew, melezitose density, and in which sugar green was higher than red (Fig 7-1).

**Table 7-1. The honeydew samples used in this analysis.**

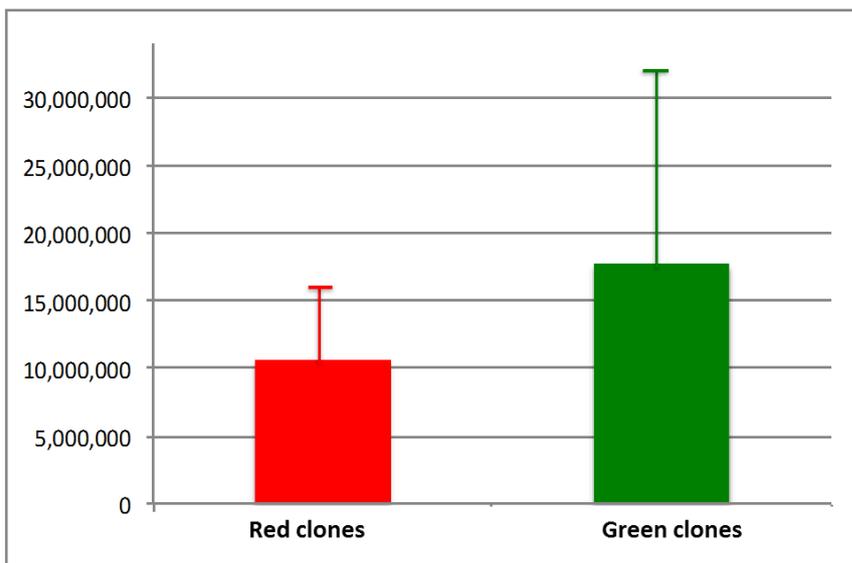
No.		colony	color	Honeydew ( $\mu$ l)	Honeydew (mg)	Sol. Wt(mg)
1	R1	A	red	0.0314843750	1.3105	569.30
2	R2	B	red	0.1599062500	6.6557	242.10
3	R3	B	red	0.0670312500	2.7900	110.00
4	R4	B	red	0.1702500000	7.0862	258.00
5	G1	A	green	0.0121875000	0.5073	155.70
6	G2	B	green	0.0877031250	3.6504	116.60

**Figure 7-1. The amounts (areas) of sugars (a: sucrose; b: melezitose) in honeydew from the green clones (the green bar) and the red clones (the red bar).**

**a. Sucrose**



**b. Melezitose**



## **Experiment 7-2.**

### **The mode of inheritance of the red and green body color at sexual reproductions.**

As mentioned above, in outdoor observations from 2013 through 2017, males and females sexuparae mated with an opposite sex individual with any color (S. Watanabe, personal observations). From the Green:Red ratio in the sexuparae in a year and that of stem mothers in the next spring during the above period, I guess the mode of color inheritance may follow a Mendelian manner (S. Watanabe, personal observations).

## **Discussion**

For both the sugars, honeydew from the green clones tend to contain a larger amount of the sugar than that from the red clones, although the difference is statistically insignificant due to small sample size (red=5, green=2).

If the difference becomes statistically significant when the sample size is increased, the difference will become the cause of the difference in ants' preference to the red and the green morph shown in Chapter 3. In addition, as a further issue, it is necessary to examine amino-acid/sugar compositions and concentrations of the honeydew based on an enough number of samples to which a robust statistical test is possible to apply.

### *Honeydew compositions*

The honeydew of the green clones seems to contain more sugars, and this result corresponds to ants' preference to the green morph (Fig.3-4). Although the robust statistical test was impossible due to too small sample size, I can confirm this difference

when increase the sample size. I will try to elucidate this in 2018.

Previous studies have shown that the honeydew of other ant-attended aphids contain several kind of sugars (Wäckers 2000). However, the honeydew of *M. yomogicola* contained only melezitose and sucrose both of which are especially preferred by *Lasius* ants (Wäckers 2000). These facts suggest that *M. yomogicola* is apECIALIZED to attract ants by excreting the honeydew to which ants are attracted strongly. In fact, I observed very frequently searching ants for aphids on young mugwort shoots in early Spring (S. Watanabe, personal observations). When I found a stem mother with a few offspring on a shoot in early Spring, there are always attending ants (S. Watanabe, personal observations). These observations suggest that ants (especially *L. japonicas*) positively search aphid colonies to utilize them as a source of foods.

As shown in Chapter 5, *Lasius* ants are better attenders for *M. yomogicola* than the other ants. Thus, *M. yomogicola* might have evolved to attract *L. japonicas* positively. The composition of sugars in their honeydew may reflect the strategy of this aphid. Meanwhile, honeydews of aphid have been known to include much amino acids (Yao 2002, Woodring et al 2004). In a preliminary analysis, the honeydew of *M. yomogicola* contained many kinds of amino acids (T. Aoyama, personal communications). To understand this ant-aphid symbiosis in more deeply I need to analyze the amino acid compositions in the red and green morph more detail.

*The inheritance mode of body color.*

Unfortunately, the eggs had dried up during the holding in the incubator and I obtained no stem mother in Spring 2017. Thus, the mode of inheritance of the body colors at sexual reproductions could not elucidate in this time. The experiment is continued in 2017 by taking care for preventing drying the eggs. Thus, the mode of body color inheritance in *M. yomogicola* will be clarified until Summer of 2018. If I know the inheritance mode of body colors in this aphid, it will bring us to many new insights into the evolution of this absolute symbiosis, and its roles to generate biodiversity in nature. Further studies are required to achieve this important issue in ecology.

## Chapter 8

### General Discussion

In this study, a hypothesis that the attendance of the ants maintains the color polymorphism of *M. yomogicola*. Because the colonies with an intermediate ratio (ca. 65% of green) of red and green morph was attended and protected by ants most strongly (Watanabe et al 2016, see Chapter 2). The mixed colony in this ratio might easy to survive until the production of the sexuparae, I examined this hypothesis by the field and laboratory experiments. The results showed that the mixed aphid colonies were attended by the ant, *L. japonicas* more strongly, resulting in a long lasting colonial life (Chapter 2). This result is corresponded to the results in a previous study. Agawa & Kawata (1995) has reported that the more diverse in color composition the less decreasing rate of colonies in *M. yomogicola* in a Shizuoka population. If the attended ants prefer more color diverse colonies in the Shizuoka population as with the Sapporo population, their results are convincible. As a result, a remained issue is how is the color polymorphism maintained on each shoot although a competition for increasing rates among aphid clones.

My experiments showed that the early reproductive rate was higher in the red than that in the green morph irrespective of clones of the host plant and the aphid (see Chapter 3). Thus, a natural selection should toward a colony to consist of red only. However, the red and green morphs coexisted always in the wild colonies. The attending ants may manipulate the reproductive rate of both morphs and coexist them. The experiments

indicated that the ants preferred and attended more to the greens than the reds (Chapter 3). The experiments revealed that the attending-ants increased only the reproductive rate of the greens and neutralized the reproductive competition between the two morphs and induced a coexisting of both the morph on a shoot. (Chapter 3).

However, *L. japonicus* predate the aphid individuals those secreted less amounts of honeydew in the symbiosis with another aphid species (Sakata 1994, 1995). The honeydew of the red morph was seemed to be low quality (Chapter 7), and the ants more prefer the green than the red (Chapter 3). Although the data shown the ants allow to coexist both the morphs on a shoot, it was unclear why the ants don't predate the reds and exploit high quality honey dew only from only the greens.

The experiments in Chapter 4 indicated that only the colonies with a certain number of the red morphs at the critical period can survive until the period of the production of sexuparae. The experiments in Chapter 4 shown that aphid parasitism suppress the buddings of inflorescence at early Autumn from when most of the aphid colonies become extinct. Therefore, the red morph is a specialized one to sustain the aphid colonies to the next year. As ant is a perennial and sedentary organism, if they can keep aphid colonies near the nest in the next year, they will hold a good source of foods. This might be the cause of the ant's manipulation on the reproductive neutralization between the two morphs.

From the above results in Chapter 2 to 4, the maintenance mechanism of the color polymorphism in *M. yomogicola* is likely to be elucidated.

If the ants consider the current benefits (the high quality honeydew of the green morph),

they will not hold food resources in the next year. Thus, the observed sacrifice of the current benefits (keeping the red morphs in a colony by decreasing the number of the greens) would maximize the life-time reproduction of alates in the attending ant colony by holding a food resource in every years.

In addition, the green attracts strongly the attended ants for their own survival, and the red lowers the cost of honeydew production and increases the reproductive rate (Chapter 3, Chapter 7). Both the morphs seem to maximize their own fitness. In other words, as a result of trying to maximize own fitness in all the three contributors (ants, reds and greens), a sustainable symbiosis system is established, and it is considered that this symbiotic system is hard to extinct. This feature of this symbiotic system would be beneficial for all the contributors from evolutionary viewpoint even if each participant lost a part of reproductive fitness instead of a raising the probability of persistency. A remained issue is what combination of which type of the three contributors (ants, greens and reds) optimize the balance between the persistency to future and the current benefits for each party. From the results of Chapter 5, it was clear that the species of attended ants was the important factor to sustain the aphid colonies. Like with this difference, there may be differences in aphid's ability among clones in each morph. If so, there are various combinations of the contributors that has a different balance between the persistency of the system and the current benefits for each contributor by system by system. Other than the above viewpoints, different environments may have different optimal combinations of contributors. In addition, the feature of host mugwort would also affect the fate of this symbiotic system.

In further studies, I need to combine field experiments with theoretical works to confirm the evolution of persistent symbiosis and whether the contributors evolved traits to realize the optimal combination of them depends on the environment. When I obtained the answers to these issues, we will take many new insights into the evolution of symbiotic systems, and its contributions to the generation and maintenance of the fascinate biodiversity in nature.

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