# Evolutionary ecology in gall-forming aphids: extreme polyphenism and biased sex ratios

(虫こぶ形成アブラムシの進化生態学：極端な表現型可塑性と偏った性比)

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the Degree of Doctor of Agriculture (D.Agr.)

by

**XIN TONG**

Laboratory of Systematic Entomology, Graduate School of Agriculture
Hokkaido University 2021

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# Preface and Acknowledgements

The results in this book are from work that I have been doing during three years of my PhD studies. Along with the time I have invested into research, I have been inspired by many surprises. Thus, you may notice the contents in this book are all about gall-forming aphids but toward totally different aspects: from life history of gall-forming aphids, sex ratio variation, cuticular hydrocarbons, endoparasitic nematodes, to endosymbiotic bacteria of aphids. I have been trying to elucidate how a gall-forming aphid is involved in various symbioses interacting with other individuals or organisms, and surrounding environment. In the near future, I would like to explore more about the evolution of sex ratios, extreme polyphenism, and the mechanism of gall formation!

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# Introduction

## Gall-forming Aphids (Aphididae: Eriosomatinae)

Galling is one of the oldest, the most intimate and specialized interactions between plants and insects (Williams & Whitham 1986; Stern 1995; Schultz 2007). Redirecting plant developmental programs, insects are the intelligent engineers to elicit novel organs, insect galls on plants for their development and reproduction. Besides insects, various organisms can form galls including viruses, fungi, bacteria, parasitic plants, and many other invertebrates (Harris & Pitzschke 2019). Nevertheless, galls induced by insects especially by aphids and wasps usually have complicated structure and varieties. Galls can provide gall inducers with good nutrition, a shelter against natural enemies and extreme environment (Stone & Schönrogge 2003; Koyama *et al.* 2004; Suzuki *et al.* 2009).

I have been fascinated by galling phenomenon, and mainly work on gall-forming aphids (Hemiptera: Eriosomatinae) that are associated with host plants *Ulmus* and *Zelkova* trees (Sano & Akimoto 2011). These gall-forming aphids can generate a series of galls, from simple to complex galls: leaf curls, open pouch galls, and closed pouch galls. Another thrilling fact is that each gall-forming aphid can induce species-specific gall (Stern 1995; Sano & Akimoto 2011; see figure A). Besides the fascinating galling phenomenon, there are many unique biological

characters of gall-forming aphids: seasonal host-alternation, cyclical parthenogenesis, extreme polyphenism, biased sex ratios, and multiple symbioses of gall-forming aphids (Moran 1988; Akimoto & Yamaguchi 2004; Tong & Akimoto 2019; Tong *et al.* 2021).

## Polyphenism

In non-social insects, polyphenism can be associated with size, sex, or alternation of life stages (Simpson *et al.* 2011). Photoperiod, temperature, population density, and nutrition are usually key factors affecting aphid polyphenism, though so far gall-forming aphids are not yet examined for the mechanisms underlying polyphenism as the laboratory rearing system has not been established. Nevertheless, gall-forming aphids exhibit extreme polyphenism in response to their complex life cycles (Figure B. Complex life cycle of *Prociphilus oriens*). First-generation gall-forming aphids (foundresses) usually induce galls on host plants using specialized contents of salivary glands to manipulate host plant into gall development as this is the only generation related to gall inducing. Sexual dimorphism can often be observed in gall-forming aphids and can be related to maternal investment. Shifting between host plants, winged morphs are developed for migration. Further, root morphs of gall-forming aphids have intimate relationship with ants, though it is unknown whether this root morph is related to this mutualism in morphology. The

extreme polyphenism of gall-forming aphids can be a great success to adapt various environment. Nevertheless, each morph should interact with different optimum and inadequacy.

## Sex Ratio and Sex Allocation Theory

The sex ratio is the ratio of males to females in a population. In most sexually reproducing species, the ratio tends to be approximately 1:1 between males and females predicted by Fisher's principle (Fisher 1930). The sex allocation theories have explained deviations of population sex allocations from a 1:1 ratio as resulting from different maternal investment into individuals that compete or cooperate among siblings (West 2009).

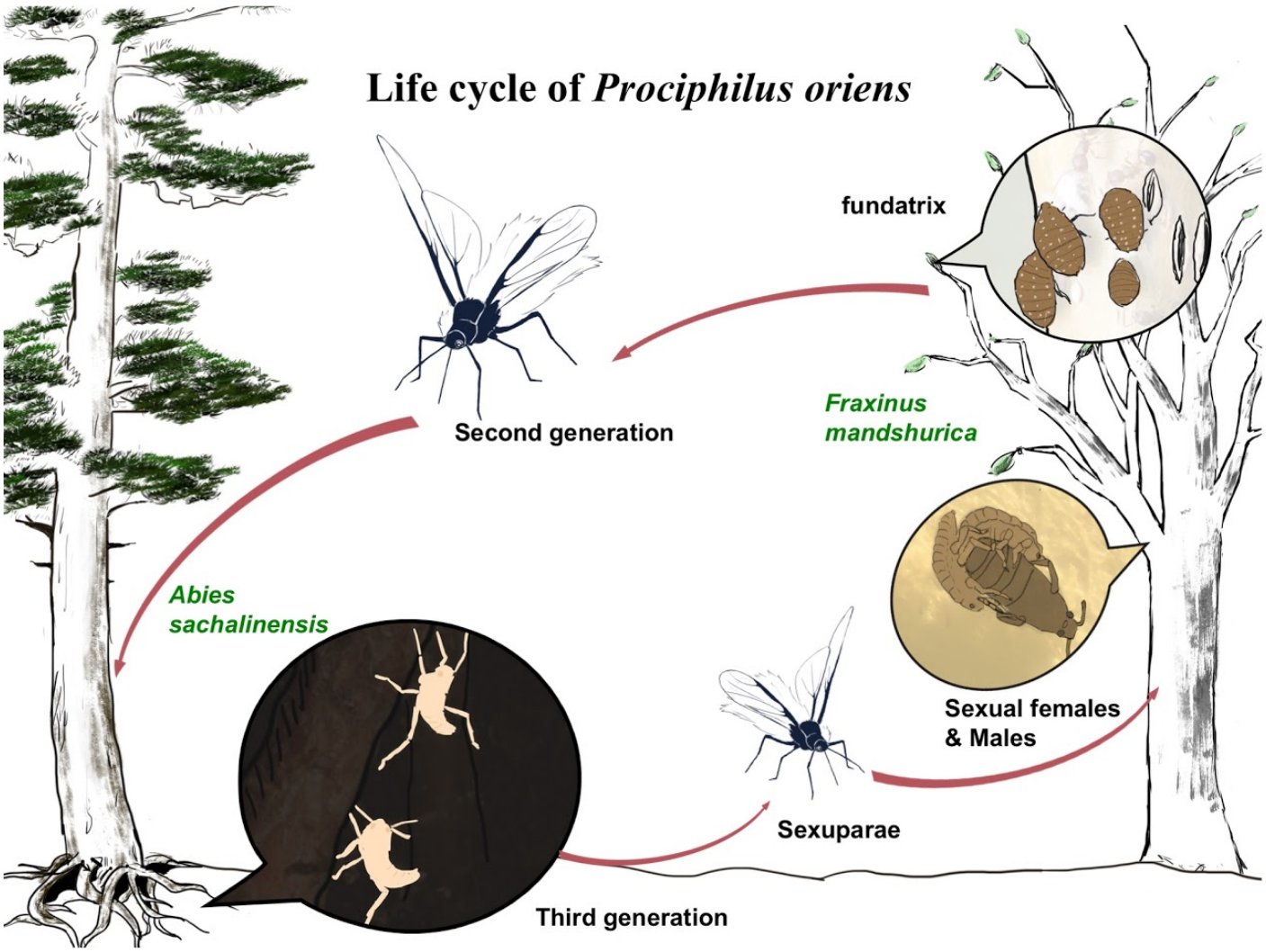
Sex allocation predict that if offspring of different sexes yield different reproductive returns per unit maternal investment, then equal allocation to the sexes will not be maintained at the population or the individual level (Charnov 1979; Frank 1987, 1990). Trivers and Willard hypothesized that if mothers differ in the amounts of reproductive resources to which they have access, then more fecund mothers should allocate more towards the sex with the greater reproductive return. This hypothesis has been applied to numerous vertebrates, such as ungulates, to evaluate the relationship between maternal reproductive status and offspring sex ratio

(Clutton-Brock *et al.* 1984; Clutton-Brock & Lason 1986; Kruuk *et al.* 1999; Myers 1987; Rose *et al.* 1998; Sheldon & West 2004).

**Figure A.** Galls induced by eriosomatine aphids.

**a.** gall of *Prociphilus* sp. on *Osmanthus* spp.; **b.** gall of *Tetraneura nigriabdominalis* on *Ulmus parvifolia*; **c.** gall of *Eriosoma harunire* on *Ulmus parvifolia*; d. gall of *Tetranera sorini* on *Ulmus davidiana*; **e.** gall of *Tetraneura yezoensis* on *Ulmus davidiana*; **f.** galls of *Tetraneura radicicola* (right) and *Tetraneura sorini* (left) on *Ulmus davidiana*.

**Figure B.** Complex life cycle of *Prociphilus oriens*.




* Illustrated by Su R & myself.

# Chapter 1. Endosymbiont *Buchnera aphidicola* in tripartite symbiosis

## 1.1 Introduction

Galls are abnormal plant organs manipulated by foreign agents (i.e. microbiota, parasitic plants, and many types of animals, including nematodes, insects, and mites) (Harris & Pitzschke 2019). Galls induced by insects are among the most representative models of plant manipulation by insect parasitism. Reprogrammed by different galling insects, the host plants present species-specific galls, which are finely-tuned plant tissues with nutrient accumulation such as the amino acids and sugars (Stone & Schönrogge 2003; Koyama *et al.* 2004; Suzuki *et al.* 2009). Meanwhile, many insects are known to have mutualistic symbiosis with microbial endosymbionts that can contribute to insect adaption by providing essential metabolites that are absent in the diet from the host plants or improving defense against unfavorable environmental conditions (Degnan & Moran 2008; Wilson *et al.* 2010; Hansen *et al.* 2012). In the galling system induced by insects, symbiosis can be a three-player game. However, the roles of endosymbionts, and the relationship between plants and endosymbionts in the galling system are still to be elucidated.

The bacterial symbiont *Buchnera aphidicola* is obligate for providing host aphids (Hemiptera: Aphididae) with essential nutrients that are limited in their diet

of plant-sap (Hansen & Moran 2011) and this symbiotic relationship with aphids began between 160 million and 280 million years ago (Baberjee *et al.* 2004). *Buchnera* is an intracellular and vertical-transmitted symbiont of aphids, and has lost many genes required for anaerobic respiration, the synthesis of amino acids, fatty acids, phospholipids, and complex carbohydrates due to strictly vertical transmission in the long association with aphids (Gil *et al.* 2002). Aphids have developed a bacteriome containing sixty to eighty bacteriocyte cells to independently enable the whole life cycle of *Buchnera* (Baumann 2005).

Gall-forming aphids (Aphididae: Eriosomatinae) are associated with the tree genera *Ulmus* and *Zelkova* as their primary host plant, on which foundresses (first-generation aphids) can induce species-specific leaf galls (Sano & Akimoto 2011). These species-specific galls are not only distinctive in gall morphology but also with totally different profiles of amino acids in gall tissues even on the same host plant (Suzuki *et al.* 2009). Since *Buchnera* is responsible for biosynthesis of amino acids in aphids, it remains unknown whether the species-specific galls with different profiles of amino acids are partly manipulated by *Buchnera*. The mechanism of gall formation is still not clear, though saliva secretion by galling insects might play an important role (Korgaonkar *et al.* 2021). It is also reported that some proteins found in aphid saliva are encoded by *Buchnera* genome (Chaudhary *et al.* 2014). Further,

gall-forming aphids can produce phytohormones (auxins and cytokinins) which are important in plant growth especially in gall development (Suzuki *et al.* 2014; Takei *et al.* 2015). If aphid galls are modulated intelligently by gall-forming aphids for their own nutrition supply and meanwhile obligate symbionts support this nutrition metabolism, I suppose that obligate endosymbiotic bacteria *Buchnera* might play a key role in gall formation as well as in complex tripartite symbioses in aphid-galling system. However, little is known about the function of *Buchnera* in galling system. Since previous studies on profiles of amino acids in galls induced by erisomatine aphids have been investigated by Suzuki *et al.* 2009, I performed genome sequencing of *Buchnera* of three gall-forming eriosomatine aphid species and hope to gain important genomic information for better understanding the complex tripartite symbioses in the aphid-galling phenomenon.

## 1.2 Methodology

### 1.2.1 Insect Samples & Genome DNA Extraction

Gall colonies of *Tetraneura sorini*, *Tetraneura nigriabdominalis* were collected in middle of June in 2020 at Shiroishi-ku, Sapporo, Hokkaido, Japan, and the gall colony induced by *Eriosoma harunire* was collected in middle of July in 2020 on Hokkaido University campus, Sapporo, Japan, on the host plant elm tree

*Ulmus davidiana*. All gall colonies were maintained in 99.95% ethanol at -20°C. Aphid species were identified by the morphology of galls and the gall formers (first-generation aphids).

Aphids isolated from a single gall of *T. sorini*, *T. nigriabdominalis*, and *E. harunire* aphids were respectively collected for genomic DNA extraction, in which samples included 6 individuals of the 2nd generation of *T. sorini*, 6 individuals of 2nd generation of *T. nigriabdominalis*, and 19 individuals of 2nd generation of *E. harunire*. As aphids of galling generation reproduce asexually by parthenogenesis, all individuals within a single gall colony are supposed to be genetically identical. DNA extraction was performed using QIAGEN Genomic-tip 20/G according to manufacturer's protocol.

### 1.2.2 Genome Sequencing, Assembly and Annotation

Before library preparation, gDNA quantity was checked by NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific), and Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Genome sequencing of *Buchnera* of three gall-forming aphids was conducted using methods of Nanopore long-read sequencing and corrected by shotgun data generated by Illumina TruSeq. The library preparation

was followed manufactures' instruction respectively. Libraries for Nanopore long reads were sequenced on GridION by Oxford Nanopore Technologies to generate long reads of genomes, and pooled libraries for Illumina TruSeq were sequenced on Illumina NextSeq 550 System to generate shotgun metagenomic sequence data of around 350 bp fragmented by Covaris Focused-ultrasonicator M220 at Functional Genomics Facility, National Institute for Basic Biology, Okazaki, Japan.

Nanopore linked long reads were mapped, assembled, and corrected by using Minimap2 (Li 2018), Miniasm (Li 2016), and Racon (Vaser *et al.* 2017), respectively. After correction by Racon, Nanopore linked long reads were polished using Pilon version 1.24 (Walker *et al.* 2014) with adapter-trimmed paired-reads generated by Illumina TruSeq after quality check by FastQC v0.11.9 (Andrews 2010). Assembled contigs were used to generate a BLAST database against reference bacterial genomes of *Buchnera* in *Acyrthosiphon pisum* (Shigenobu *et al.* 2000) and *Baizongia pistaciae* (van Ham *et al.* 2003) to detect *Buchnera* genomes and their plasmids. The complete circle genomes were confirmed by manually recircling the contigs and mapped by both raw Nanopore linked long reads using Minimap2 and polished again using Pilon v.1.24 by Illumina paired reads for the final scaffolds. The genomes of *Buchnera* in *T. sorini*, *T. nigriabdominlis*, and *E. harunire* aphids were annotated using Prokka v.1.14.6 (Seemann 2014).

## 1.3 Results & Discussion

### 1.3.1 Genome Features

*Buchnera* genomes isolated from *T. sorini*, *T. nigriabdominalis*, and *E. harunire* aphids by Nanopore linked long reads were assembled (Table 1), and annotated (Table 2). In total, genome size of 533,871, 530,863, and 627,315 bp were confirmed for *Buchnera* in *T. sorini*, *T. nigriabdominalis*, and *E. harunire* aphids. No plasmid is detected in *Buchnera* genomes of *T. sorini*, *T. nigriabdominalis*, and *E. harunire* aphids. Besides *Buchnera*, other endosymbiotic bacteria was also confirmed: in *T. sorini* aphids, there was bacteria of the family Orbaceae which was also found in the guts of honeybees and bumblebees (Kwong & Moran 2013); in *E. harunire*, *Arsenophonus* sp. was also detected.

### 1.3.2 Gene Loss of *Buchnera* in Tripartite Symbioses

When bacteria transits from free-living life cycle to permanent association with hosts, genome reduction and gene loss become common characters, such as in obligate endosymbiotic bacteria *Buchnera* of aphids (Moran 2002; McCutcheon & Moran

2011; Chong *et al.* 2019). Especially genes encoding key enzymes for the biosynthesis of amino acids are often translocated to plasmids in *Buchnera*. However, plasmids were not found in *Buchnera* of three gall-forming aphids in this study. Genes of *leu*ABCD that locate at the plasmid pLeu of *Buchnera* of *A. pisum* APS for biosynthesis of leucine are detected on the *Buchnera* chromosomes in three gall-forming aphids. Genes involved in leucine and tryptophan biosynthesis in *Buchnera* can be located on plasmids, or on the chromosome, and this varies in different lineages (Gil *et al.* 2006).

Gall-forming aphids induce species-specific galls especially with different profiles of amino acids (Suzuki *et al.* 2009). *Buchnera* of *E. harunire* has reduced genome and lost genes of *argCDE* encoding enzymes of biosynthesis of ornithine that is precursor to produce essential amino acid arginine while *Buchnera* of the other two gall-forming aphids *T. sorini* and *T. nigriabdominalis* maintains these genes. Nevertheless, the galls induced by *E. harunire* are rich in arginine while low in galls of *T. sorini* (Suzuki *et al.* 2009). Arginine is the only amino acid that was probably synthesized by aphids or endosymbionts (Wilson *et al.* 2010). With loss of these genes for arginine biosynthesis in pea aphids, *Buchnera* of pea aphid APS retains a complete pathway for arginine synthesis (Shigenobu *et al.* 2000; Wilson *et al.* 2010). Thus, even plant phloem sap is insufficient for providing arginine to pea

aphids. Pea aphids lack all the genes encoding enzymes of the urea cycle and unable to synthesize arginine but to reply on *Buchnera* for arginine biosynthesis. Though so far it is unknown whether *E. harunire* aphids have the genes that are responsible for ornithine biosynthesis, gall tissues induced by themselves provide good source of arginine and other essential amino acids.

Gall formation usually occurs with host alternation (Moran 1988). One hypothesis for this obligate host alternation is probably related to nutritional compensation. Though it is limited to explain how aphids form these species-specific galls with different profiles of amino acids, galling may provide aphids with exceptional nutritional supply. Further, there seems close tripartite symbiosis between aphids, host plants, and aphid endosymbionts, especially on nutritional transition. *Buchnera* can synthesize amino acids that are insufficient in plant phloem sap. When gall-forming aphids can consume enough nutrition from host plants by inducing their own galls, aphids or *Buchnera* may not synthesize or very much by themselves. In this study, I sequenced *Buchnera* genomes from three gall-forming aphids, *T. sorini*, *T. nigriabdominalis*, and *E. harunire* that share same host *plant U. davidiana,* but have different traits of arginine biosynthesis, which seems consistent with galling behaviors of aphids.

**Data availability.** The *Buchnera* genomes assembled from *T. sorini*, *T. nigriabdominalis*, and *E. harunire* in this chapter have not been deposited in any database but it is in preparation for a publication and the data soon will be uploaded after the manuscript is accepted for publication.

**Table 1**. Assembly statistics of Nanopore linked long reads.

| **Feature** | ***Buchnera* strains isolated from** | | |
|---|---|---|---|
| | *T. sorini* | *T. nigriabdominalis* | *E. harunire* |
| Total length (bp) | 30,482,001 | 1,572,268 | 1,787,508 |
| Estimated bases (Gb) | 4.79 | 2.08 | 3.64 |
| Reads generated (M) | 2.17 | 1.07 | 1.82 |
| Number of contigs | 1,133 | 92 | 137 |
| Largest length (bp) | 534,074 | 530,822 | 627,279 |
| Coverage (%) | 100 | 100 | 98 |
| N50 sequence length | 33,522 | 20,950 | 36,775 |

**Table 2**. Summary of the genome features of *Buchnera* isolated from three eriosomatine aphids and *A. pisum* (APS strain).

| **Feature** | ***Buchnera* strains isolated from** | | | |
|---|---|---|---|---|
| | *T. sorini* | *T. nigriabdominalis* | *E. harunire* | *A. pisum* (APS strain) |
| Genome size (bp) | 533,871 | 530,863 | 627,315 | 640,681 |
| G + C content (%) | 23.44 | 23.25 | 23.68 | 26.3 |
| CDS | 485 | 474 | 489 | 593 |
| tRNA | 32 | 32 | 32 | 32 |
| rRNA | 3 | 3 | 3 | 3 |
| tmRNA | 1 | 1 | 1 | 1 |
| Number of contigs | 1 | 1 | 1 | 3 |

# Chapter 2. Cuticular hydrocarbons in response to polyphenism

## 2.1 Introduction

In most terrestrial arthropods, the body surface is covered by cuticular hydrocarbons (CHCs), which function first as a desiccation barrier and second as chemical cues for potential nestmates, intracolonial communication or mate recognition (Wigglesworth 1945; Theresa 1998; Wagner *et al.* 2000; Greene & Gordon 2003; Thomas & Simmons 2010). Insect CHCs are composed of normal and branched saturated and unsaturated hydrocarbons, which are categorized by the presence or absence of double bonds or methyl-branches, as well as differences in chain length (Gibbs 1998; Blomquist & Bagneres 2010).

In sexual communication in insects, CHCs can serve as pheromone signals for potential mates (Chenoweth & Blows 2005; Peterson *et al.* 2007; Ruther *et al.* 2011), and for this reason CHCs are sexually dimorphic in dipterans, solitary bees and parasitic wasps (Pomonis 1989; Syvertsen *et al.* 1995; Sullivan 2002; Mant *et al.* 2005; Steiner *et al.* 2005). Furthermore, CHCs are well studied in social insects, which use CHCs to recognize sexes (Cuvillier-Hot *et al.* 2001; Thomas & Simmons 2008), nestmates/non-nestmates (Thomas *et al.* 1999; Sturgis & Gordon 2012; Smith

*et al.* 2013) and conspecifics/non-conspecifics (Martin *et al.* 2008). Differences in hydrocarbon compounds are also related to reproductive status and task groups in social insects (Sledge *et al.* 2001; Liebig *et al.* 2009) and burying beetles (Scott *et al.* 2008). Although CHC profiles are known to differ among body parts (Arsene *et al.* 2002; Wang *et al.* 2016), our knowledge of the relationships between CHC profiles and different developmental stages or distinct morphs is still limited, particularly in non-social insects.

Holometabolous insects have distinct developmental stages in a single generation, all of which are adapted to separate environments. The CHC profiles in such species reportedly differ between larvae and adults because of the distinct functions of CHCs among developmental stages (Baker *et al.* 1979; de Renobales & Blomquist 1983). In contrast, aphids, in particular, eriosomatine and hormaphidine aphids, pass through multiple generations annually, being characterized by distinct morphs adapting to different host plants or environments. Very few studies have reported whether polyphenism in such aphids results in differences in CHC profiles and functions among morphs. An exceptional and remarkable example is known from an eriosomatine species *Paracletus cimiciformis* (Fordini), which produces two apterous morphs on the secondary host; aphids of the round morph are attended by *Tetramorium* ants, whereas aphids of the flat morph are carried by ants into their

brood chamber where the aphids feed on the internal fluids of ant larvae using their stylet (Salazar *et al.* 2015). The two morphs differ in CHC profiles, with those of the flat morph resembling those of ant larvae. However, there have been no reports on the relationship between seasonal polyphenism and the CHC profiles in aphids.

The eriosomatine aphid *Prociphilus oriens* seasonally alternates host plants between the primary host *Fraxinus mandshurica* Ruprecht and the secondary host *Abies sachalinensis* Masters (Blackman & Eastop 1994; Akimoto 2006). *Prociphilus oriens* passes through five distinct morphs within a clonal line from spring to autumn (Li & Akimoto 2017, Fig. 1). In northern Japan, first-generation females (foundresses) hatch from overwintered eggs in late April and parthenogenetically produce nymphs on *F. mandshurica* shoots in mid-May. The second generation develop into winged females (spring migrants) inside leaf curls they induced and then migrate to *A. sachalinensis*, on which they parthenogenetically produce third-generation nymphs. These nymphs move to the roots of *A. sachalinensis* and develop into wingless females (root morphs), which continue to reproduce parthenogenetically across several generations until autumn by feeding on *A. sachalinensis* roots. In mid-October, winged females (autumnal migrants) appear on the roots, and migrate to *F. mandshurica* trunks, on which they produce the sexual generation (sexuals) consisting of dwarfish, wingless sexual

females and males. After mating, sexual females deposit single eggs in bark crevices where they overwinter and hatch early next spring.

The five morphs are not only associated with different host plants, but also distinct ecological settings and functions. Foundresses are sessile on host twigs andusually attended by *Lasius* ants (Akimoto, *unpubl. data*, 2016). Spring migrants are winged with waxy secretion on their abdomen and are also attended by ants during development. Root morphs live underground on host roots and are attended by *Lasius* ants. First instar nymphs produced by spring migrants (the third generation) are categorized into this morph because they are carried by *Lasius* workers to the roots after birth. Autumnal migrants are also winged, with a large amount of waxy secretion on their abdomen, and form aggregations on *Fraxinus* trunks before larviposition. Sexuals include both sexes, and the CHCs of sexual females could be different from those of males or asexual females to be used as a sexual attractant. Considering the diverse ecological conditions the morphs adapt to, I propose that the composition of CHCs could differ among morphs and between sexual and asexual females.

Here, my results present CHC profiles characteristic of each morph in *P. oriens* to address the dynamics of CHC components corresponding to polyphenic changes,

including the hydrocarbons of eggs and waxy substances. Both winged morphs (spring and autumnal migrants) have long, thick, and flocculent waxy fibers on the abdomen, which were separately analyzed from the CHCs. I suggest that polyphenic aphids can change their CHCs among the morphs depending on the environmental conditions they adapt to.

## 3.2 Methodology

### 3.2.1 Insect collection

Morphs of *P. oriens* were collected at the following three localities in Japan: (i) Hokkaido University Campus, Sapporo (43°04′09.5″N, 141°20′21.4″E); (ii) Hokkaido Shrine, Maruyama, Sapporo (43°03′18.0″N, 141°18′46.8″E); and (iii) Iwamizawa, Hokkaido (43°11′28″N, 141°46′53″E). Hydrocarbons were extracted from third or fourth nymphal instar foundresses, third or fourth nymphal instar spring migrants (the second generation), two samples of first instar root morphs (the third generation), adult autumnal migrants, adult males, adult sexual females, and eggs (Table 1). Additionally, waxy substances from the abdomen of spring and autumnal migrants were also collected. Hydrocarbon extraction was based on 47 to

318 individuals depending on aphid size (Table 1). Collected aphids were transferred alive to the laboratory and CHCs were extracted by submerging aphids in a shallow hexane bath for 60 s. The hexane extracts were used for analyses without concentration. For the analysis of waxy substances, winged females were submerged for 3 s. Finally, I used ten extracts from morphs and waxy substances for analysis. Because of the difficulty in bringing a number of samples into the laboratory alive, we were not able to prepare multiple replicates for each morph except for the third generation, but we have tried to use as many aphids as possible for each morph. All extracts were maintained at −20°C prior to chemical analyses.

### 3.2.2 Chemical assessment

The CHC extracts were analyzed using gas chromatography–mass spectrometry (GC-MS). The GC-MS system (Varian/CP-3800 and Varian/1200L, Varian Medical Systems, Inc., Palo Alto, California, USA) was equipped with the TC-5 column (30 m × 0.25 mm ID, 0.25 μm film; GL Sciences, Shinjuku, Tokyo, Japan). Temperature was kept at 100°C for 2 min, then increased by 40°C/min to 200°C, by 20°C/min to 260°C, by 10°C/min to 305°C, and finally by 5°C/min to 325°C. Helium was used as carrier gas with a constant flow of 1.8 mL/min. Analyses were run in a splitless mode with an injector temperature of 300°C. Electron

ionization mass spectra were recorded with an ionization voltage of 70 eV and an ion source temperature of 250°C. Components were identified by their characteristic mass spectral fragmentation patterns and retention times.

### 3.2.3 Statistical analysis

Peaks detected by GC-MS were identified and the amounts of hydrocarbons were analyzed. Several kinds of alcohol were detected but removed from the analysis. Of the 28 hydrocarbons detected, I removed three hydrocarbons that are possessed by single morphs only at a proportion of less than 1%, and subsequently the relative amounts of the remaining 25 hydrocarbons were normalized such that their total summed up to 100 (Table 2). I undertook principle component analysis based on the correlation matrix using the normalized relative proportions of hydrocarbons. All statistical analyses were carried out using JMP version 13 (SAS Institute, Cary, NC, USA).

## 2.3 Results

### 2.3.1 Profiles of CHCs in *P. oriens*

The cuticular extracts from each morph contained high amounts of straight-chain and branched-chain alkanes with chain lengths ranging from 23 to 35 carbons (Table 2). Cuticular hydrocarbon profiles were mainly comprised of a homologous series of n-alkanes (C25, C27 and C29) and monomethyl-branched alkanes (2-, 3-, 5-, 9-, 11- and 13-Me). Particularly, straight-chain n-alkanes were most abundant among all the morphs (Table 2). The total proportion of n-alkanes ranged from 74.6% (foundress) to 89.4% (root morph) among female morphs, but accounted for only 49.2% in waxy substances of autumnal migrants. In addition, small amounts of alcohols, octacosanol ($C_{28}H_{58}O$), triacontenol ($C_{30}H_{60}O$) and triacontanol ($C_{30}H_{62}O$), were detected in the foundresses, autumnal migrants, waxy substances of autumnal migrants and eggs.

### 2.3.2 CHC characters of different aphid generations

The principal component analysis indicated that the first and second principle components accounted for 33.2% and 22.0% of the total variance, respectively. The eigenvectors (loadings) showed that, in PC1, all straight-chain n-alkanes, from C23 to C31, had negative loadings, whereas all methyl-branched alkanes had positive loadings except for 2-MeC26, 13-MeC27, 3-MeC27 and 13-MeC29 (Table 3). This result suggests that morphs with negative PC1 values are characterized by high proportions of straight-chain n-alkanes, whereas morphs with positive PC1 values contain relatively high proportions of methyl-branched alkanes. In PC2, short-chain hydrocarbons ranging from C23 to C25 were characterized by positive loadings, whereas long-chain hydrocarbons ranging from C27 to C35 were characterized by negative loadings, irrespective of whether they were methyl-branched (Table 3). Therefore, negative PC2 values characterized relatively high proportions of long-chain hydrocarbons, and positive PC2 values characterized relatively high proportions of short-chain hydrocarbons.

In the PC1 axis, all female morphs including foundresses, nymphal spring migrants, root morphs (the third generation), autumnal migrants and sexual females had negative values; thus, they were characterized by relatively high proportions of n-alkanes (Fig. 1). In contrast, males, waxy substances and eggs were characterized by positive PC1 values, namely, higher proportions of methyl-branched alkanes.

Among female morphs, the foundresses, larval spring migrants and root morphs shared similar CHC profiles (Fig. 2, Table 2), in which the proportions of *n*-C25 (39.2–54.0%) were higher than those of *n*-C27 (26.7–31.6%). However, autumnal migrants and sexual females contained longer-chain *n*-alkanes and are characterized by low proportions of *n*-C25 (27.4–28.8%) relative to *n*-C27 (31.5–33.8%). These female morphs also contained high proportions (18.6–20.3%) of *n*-C29, which differed notably when compared with proportions found in males (6.1%), foundresses (2.4%), spring migrants (2.6%) and root morphs (0.6%) (Table 2).

The waxy substances analyzed were specifically characterized by methyl-branched alkanes, which occurred in a greater proportion in autumnal migrants than in spring migrants (Fig. 1, Table 2). Eggs contained the second highest proportions

of methyl-branched alkanes. Although the root morphs (first instars) were sampled from different localities, they showed very similar CHC profiles (Fig. 1, Table 2).

## 2.4 Discussion

I tested the possibility of polyphenic changes in CHC profiles using the host-alternating aphid *P. oriens*, which exhibits a complex life cycle with five morphs and multiple generations. Our observations confirmed this possibility, and I propose that polyphenic changes in CHC profiles can be explained by considering the ecological conditions each morph is confronted with.

The CHCs of all female morphs were characterized by high proportions of *n*-alkanes. It has been reported that n-alkanes are also typical constituents of leaf cuticular waxes (Eglinton & Hamilton 1967; Herbin & Robins 1969). This suggests that *n*-alkanes might have primarily evolved in female morphs as a chemical mimicry to the host leaves or twigs to evade predators and parasitoids. Similar adaptive camouflage to host plants is known in twig-like caterpillars of a geometrid moth (Akino *et al.* 2004). High proportions of *n*-alkanes are commonly found in the

CHCs of other aphids (Dillwith *et al.* 1993; Liepert & Dettner 1996; Lang & Menzel 2011).

The secreted wax is hydrophobic and can protect aphids from contamination of honeydew and provide some protection against enemies and unfavorable environmental conditions (Smith 1999; Pike *et al.* 2002; Moss *et al.* 2006; Ammar *et al.* 2013; Kasahara *et al.* 2019; Depa *et al.* 2020). However, there should be fitness costs to the production of external waxes (Pope 1983). Although every morph of *P. oriens* is equipped with waxy substances on the body surface, spring and autumnal migrants have especially long and thick wax filaments. High proportions of methyl-branched alkanes were detected in waxy substances of autumnal migrants, which migrate from Abies roots to *Fraxinus* trunks. After landing on *Fraxinus* trunks, autumnal migrants aggregate in bark crevices and start larviposition in the aggregate, as shown in an eriosomatine aphid, *Pemphigus spyrothecae* (Foster & Benton 1992). Possibly, the formation of aggregates by autumnal migrants is an adaptation for avoiding intraclonal mating (selfing) because larviposition by a single migrant likely leads to selfing (Akimoto 2006). During the formation of aggregates, autumnal migrants could recognize other members by using these methyl-branched alkanes, as they are generally known to mediate chemical communication in insects (Nelson 1978; Chung & Carroll 2015). This is further supported by the higher percentage of

methyl-branched alkanes in autumnal migrants (50.8%) when compared with spring migrants (27.2%), which do not form aggregates on *Abies* trunks like their autumnal counterparts.

Methyl-branched alkanes of insect waxes are often used by parasitoid wasps when they search for the target host insects (Nelson 1978). However, by the time autumnal migrants appear, parasitoids have ceased their reproductive activities because of low temperatures; thus, autumnal migrants and eggs are less likely to be parasitized. Interestingly, eggs also secrete waxy filaments, which contain a high proportion of methyl-branched alkanes. Although the function of waxy filaments in eggs is unknown, they could be adaptive in water repellency and freezing avoidance because this species is distributed in areas with heavy snowfall and severe winter.

Great differences in CHC profiles were found between three spring female morphs (foundresses, nymphal spring migrants and root morphs) and two autumnal female morphs (autumnal migrants and sexual females). The spring female morphs shared relatively high proportions of *n*-C25 to *n*-C27, whereas the autumnal female morphs were characterized by higher proportions of *n*-C27 and *n*-C29 than the spring morphs (Fig. 2). Notably, all spring female morphs are attended by *Lasius*

workers on the host plants, but autumnal female morphs are not. Lang and Menzel (2011) pointed out that the relative proportions of these n-alkanes in CHCs correspond to whether the aphid is ant-attended. In their observation, the CHCs of myrmecophilous aphids were characterized by high proportions of *n*-C25 relative to *n*-C27 and much lower proportions of *n*-C29, whereas those of non-myrmecophilous aphids contained *n*-C27 and *n*-C29 at higher proportions but very low proportions of *n*-C25. This result is consistent with our observation, suggesting that *P. oriens* has evolved CHC compositions such that spring morphs can secure attendance by *Lasius* ants. In a congeneric American species, *Prociphilus tessellatus*, a similar relationship between *n*-C25 and *n*-C27 was observed for the morph attended by ants (Lohman *et al.* 2006). Furthermore, ant attendance is critical for root morphs because the first instars laid on *Abies* trunks are carried by *Lasius* workers to the *Abies* roots, which is a critical move for their development (Akimoto, unpubl. data, 2016). Ant attendance for the spring morphs are facultative, rarely with colonies not ant-attended, whereas root morphs are obligatorily attended by *Lasius* ants. It has also been reported that *Lasius* ants carry root-morph nymphs of a closely related European species, *Prociphilus fraxini*, from one nesting site to another (Purkart *et al.* 2019).

Sexual females, as well as autumnal migrants, are peculiar in containing long-chain *n*-alkanes, which were definitely different from male CHCs. Insect species adapted to arid climates contain longer-chain *n*-alkanes, likely because of their role in desiccation resistance (Chung & Carroll 2015). As sexual females do not feed after birth (Akimoto 2006; Akimoto *et al.* 2012), it is expected that they would have a strong ability to resist desiccation. One reason for the presence of long-chain *n*-alkanes in sexual females might be attributed to desiccation resistance. However, this explanation is not likely, as males do not feed either and yet possess shorter-chain alkanes. Another possibility is that the long-chain *n*-alkanes of sexual females have evolved to be sexual attractants.

In most groups of aphids, sexual females have scent plaques on their hind tibiae, which secrete monoterpenoid sex pheromones (Patterson 1970; Harrington 1985; Murano *et al.* 2018). However, sexual females of Eriosomatinae, including *P. fraxini*, are reported to have no scent plaques (Harrington 1985), suggesting that they would use other organs for secreting sexual attractants. Our observation showed that sexual females of *P. oriens* do not have scent plaques either but large and conspicuous wax gland plates on the lateral sides of their abdominal tergites I–VI. In contrast, males have no wax gland plates and no special sensory organs in their antennae (Tong,

unpubl. data, 2019). Our observation suggests that males choose mates based on tactile stimuli. Therefore, evidence suggests that CHCs secreted from female wax gland plates function as a sex attractant.

In conclusion, the present study indicated that the CHC profiles of *P. oriens* vary among morphs probably through adaptation to the presence or absence of ant attendance or sexual reproduction. In the present study, as all samples were collected in the wild and brought alive to the laboratory, I was not able to prepare multiple replicates for each morph. As a result, this study did not address the question of geographical differences or clonal differences in CHCs. Nevertheless, the present study emphasized the importance of CHCs as a sex attractant. I expect that differences in CHCs are also found in gall-forming aphids that alternate between host plants (Wool 2004). In future studies, it is necessary to compare the CHCs of sexual females among closely related species to investigate whether CHC profiles have a critical role in reproductive isolation mechanisms.

**Note**: this chapter is a slightly modified version of "Seasonal changes in cuticular hydrocarbons in response to polyphenism in the host-alternating aphid *Prociphilus oriens*" published in Entomological Science and has been reproduced here with the permission of the copyright holder.

**Original publication:**

**Tong, X.**, Takata, Y. and Akimoto, S.-i. (2021), Seasonal changes in cuticular hydrocarbons in response to polyphenism in the host-alternating aphid *Prociphilus oriens*. Entomological Science, **24**: 18-26.

**Figure 1.** Results of principal component analysis for hydrocarbons of the five morphs, waxy filaments and eggs of *Prociphilus oriens*. The scores of principal component (PC)1 and PC2 axes were represented. Am, autumnal migrants; Egg, eggs; F, foundresses; Female, sexual females; Male, males; Rt-1 and -2, root morph; Sm, spring migrants; Wax-am, waxy filaments on autumnal migrants; Wax-sm, waxy filaments on spring migrants. Chemical characteristics of PC1 and PC2 axes are indicated.

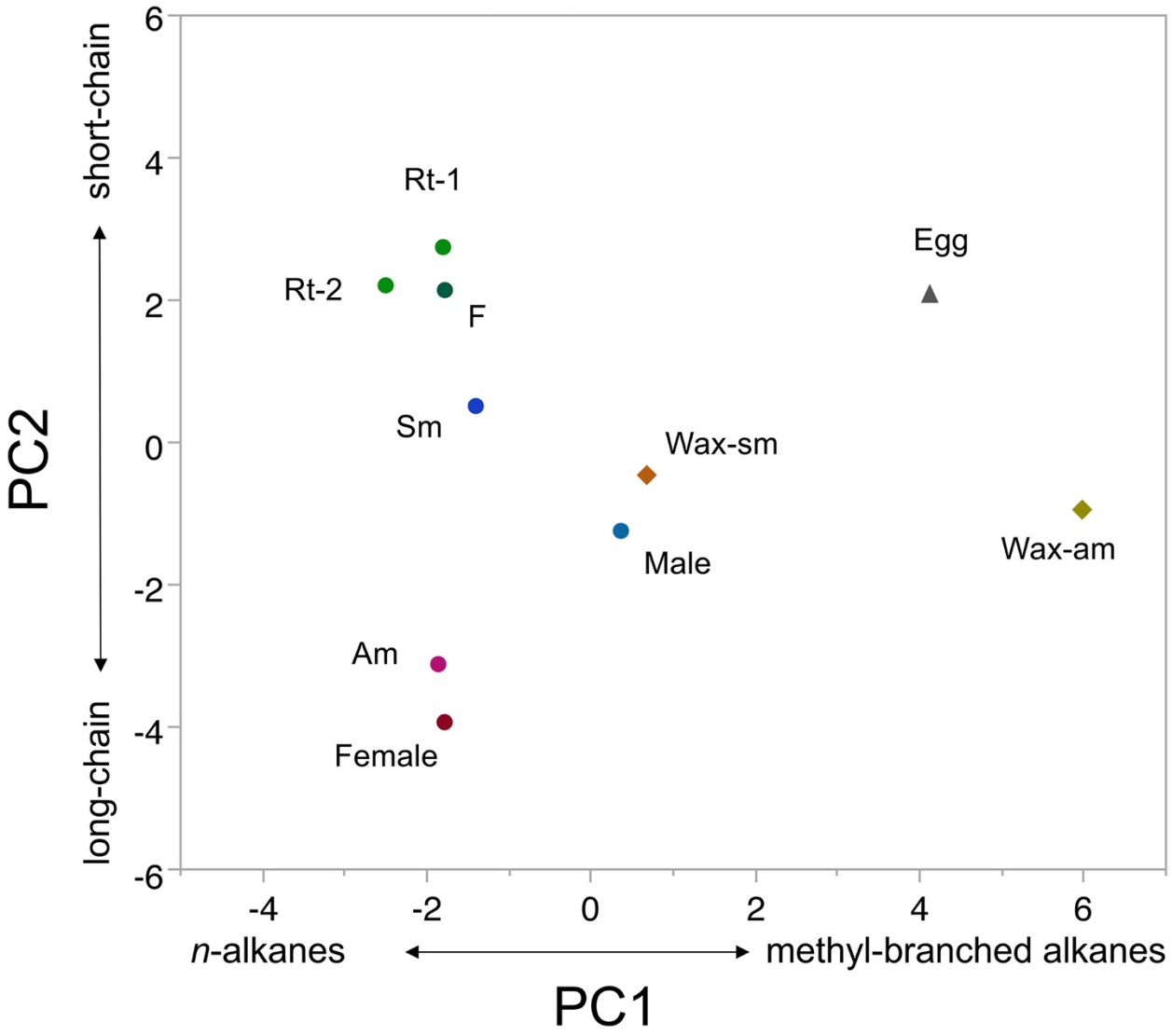


**Figure 2** Cuticular hydrocarbon profiles of five morphs, waxy substances and eggs of *Prociphilus oriens*. The positions of main *n*-alkanes and methyl-branched alkanes are indicated. Peaks indicated by A, B and C are alcohols, octacosanol ($C_{28}H_{58}O$), triacontenol ($C_{30}H_{60}O$) and triacontanol ($C_{30}H_{62}O$), respectively. IM, Iwamizawa, Hokkaido, Japan; MR, Maruyama, Sapporo, Japan.

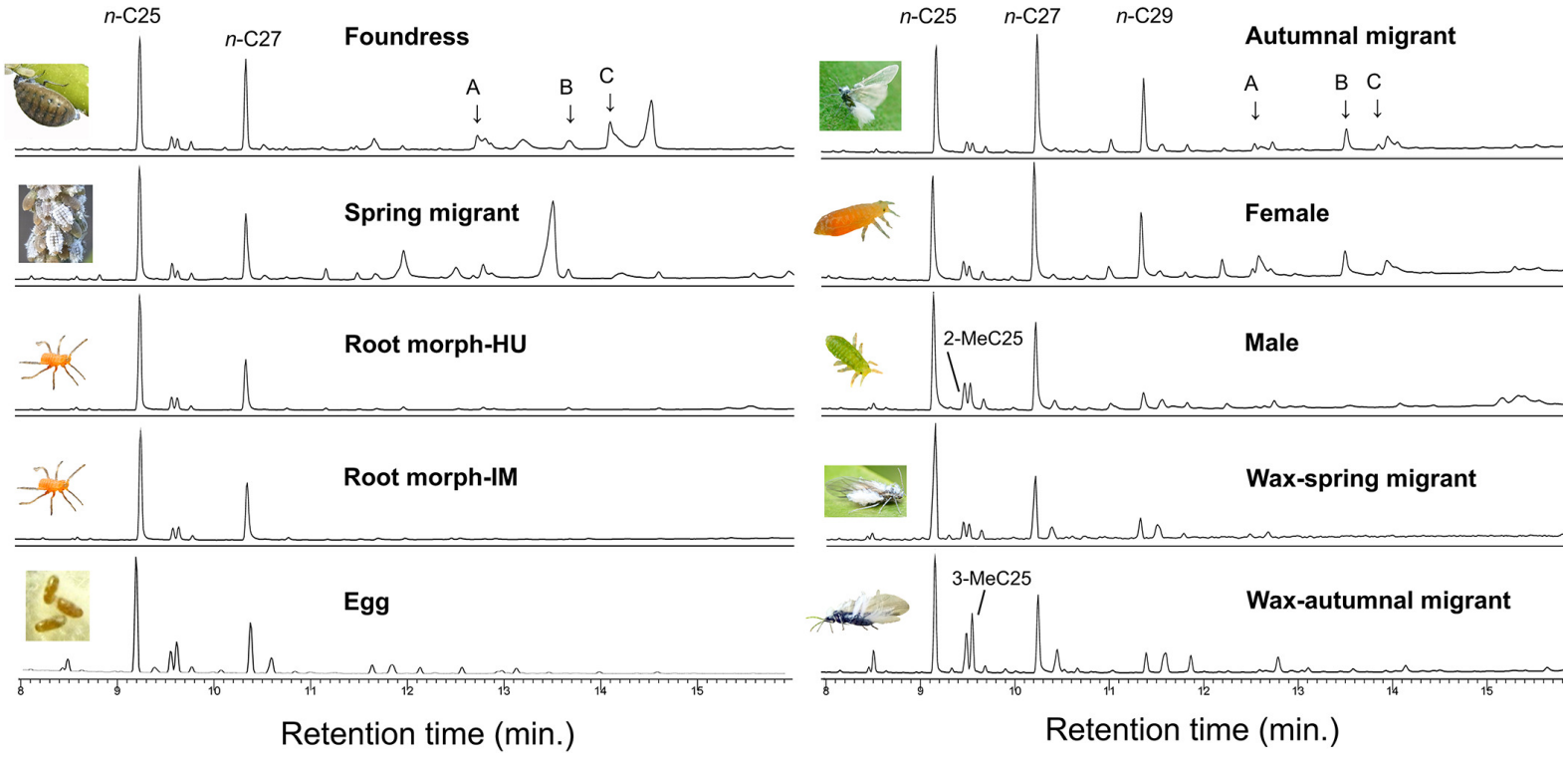


**Table 1** Collection data of *P. oriens* samples used in CHC analysis, including foundresses (the first generation), spring migrants (the second generation), root morphs (the third generation), autumnal migrants, sexual females/males (sexual morphs), eggs and wax substances. The CHCs of the attending ant *Lasius japonicus* were also analyzed.

| Sample name | Morph | Life stage | No. of individuals | Collection date | Collection location |
|---|---|---|---|---|---|
| F | Foundress | nymph[a] | 47 | May 22, 2017 | HU |
| Sm | Spring migrant | nymph[a] | 79 | May 25, 2017 | HU |
| Rt-1 | Root morph | nymph[b] | 234 | Jun 12, 2017 | Progeny of spring migrants, HU |
| Rt-2 | Root morph | nymph[b] | 318 | Jun 15, 2017 | Progeny of spring migrants, IM |
| Am | Autumnal migrant | adult | 80 | Oct 5, 2017 | MR |
| Wax-am | Autumnal migrant | Waxy filament | 69 | Oct 6, 2017 | HU |
| Female | Sexual female | adult | 102 | Oct 11, 2017 | Progeny of autumnal migrants, HU |
| Male | Male | adult | 90 | Oct 12, 2017 | Progeny of autumnal migrants, HU |
| Wax-sm | Spring migrant | Waxy filament | 88 | May 28, 2018 | HU |
| Egg | Egg | egg | 176 | Nov 29, 2018 | IM |
| Ant | Worker | adult | 43 | Jun 11, 2018 | from one artificial nest, IM |

**Table 2** Detailed data of each quantifiable peaks detected from CHC profile of *P. oriens* aphid samples and renormalized amounts used in PCA.

| Components | Foundress | Spring migrant | Root morph-a | Root morph-b | Autumnal migrant | Wax on Autumnal migrant | Sexual female | Male | Wax on spring migrant | Egg |
|---|---|---|---|---|---|---|---|---|---|---|
| Tricosane (nC23) | 0.471 | 0.844 | 1.189 | 0.595 | - | - | - | - | - | - |
| 3-Methyl tricosane (3-MeC23) | 0.314 | 0.633 | 1.405 | 1.351 | 0.743 | 4.936 | 0.000 | 1.368 | 1.776 | 3.812 |
| n-Tetracosane (nC24) | 0.353 | 0.380 | 0.594 | 0.432 | - | - | - | - | - | 0.587 |
| 2-Methyl tetracosane (2-MeC24) | 0.432 | - | - | - | - | - | - | - | - | 0.367 |
| 3-Methyl tetracosane (3-MeC24) | 0.079 | - | 0.054 | 0.108 | - | 0.196 | - | - | - | 0.293 |
| n-Pentacosane (nC25) | 39.262 | 42.194 | 54.025 | 54.054 | 28.794 | 24.558 | 27.384 | 34.211 | 42.283 | 36.657 |
| 11-Methyl pentacosane (11-MeC25) | 0.471 | 0.042 | - | - | - | 0.688 | - | - | 1.099 | 2.383 |
| 2-Methyl pentacosane (2-MeC25) | 3.965 | 5.359 | 4.808 | 0.432 | 2.805 | 7.195 | 4.596 | 7.527 | 5.201 | 7.001 |
| 3-Methyl pentacosane (3-MeC25) | 4.162 | 3.629 | 6.051 | 6.270 | 2.433 | 13.114 | 3.620 | 7.937 | 4.440 | 9.971 |
| n-Hexacosane (nC26) | 2.434 | 2.405 | 2.053 | 2.162 | 1.555 | 1.621 | 2.172 | 3.250 | 2.918 | 2.456 |
| 2-Methyl Hexacosane (2-MeC26) | 0.864 | 0.591 | 0.108 | - | - | 0.589 | 0.976 | - | - | - |
| Heptacosane (nC27) | 29.643 | 28.059 | 26.688 | 31.568 | 33.795 | 17.878 | 31.476 | 26.651 | 21.142 | 16.752 |
| 13-Methyl heptacosane (13-MeC27) | 2.591 | - | 0.162 | - | - | - | - | - | - | - |
| 9-Methyl heptacosane (9-MeC27) | - | 2.152 | - | - | - | 6.778 | 2.140 | 3.250 | 5.412 | 6.488 |
| 5-Methyl heptacosane (5-MeC27) | 0.510 | - | - | - | - | 0.933 | - | - | - | - |
| 3-Methyl heptacosane (3-MeC27) | 0.824 | 3.840 | 0.864 | 1.027 | - | 1.203 | - | - | - | 0.880 |
| n-Octacosane (nC28) | - | - | - | - | - | - | 1.259 | - | - | - |
| n-Nonacosane (nC29) | 2.395 | 2.616 | 0.594 | 0.595 | 20.345 | 4.641 | 18.634 | 6.124 | 6.427 | 3.372 |
| 13-methyl nonasosane (13-MeC29) | 8.009 | 0.000 | 1.080 | 1.405 | - | - | - | - | - | - |
| 9-Methyl nonasosane (9-MeC29) | - | 3.207 | - | - | 2.839 | 7.392 | 3.148 | 3.661 | 7.230 | 5.095 |
| n-Hentriacontane (nC31) | - | 0.759 | - | - | 1.453 | 0.540 | 1.920 | - | - | - |
| 13-Methyl hentriacontane (13-MeC31) | 3.219 | 3.291 | - | - | 2.230 | 4.028 | 2.675 | 2.566 | 2.072 | 2.199 |
| 14-Methyl dotriacontane (14-MeC32) | - | - | 0.324 | - | - | 0.761 | - | - | - | 0.513 |
| 13-Methyl tritriacontane (13-MeC33) | - | - | - | - | 3.008 | 1.719 | - | 3.455 | - | 0.806 |
| 13-Methyl pentatriacontane (13-MeC35) | - | - | - | - | - | 1.228 | - | - | - | 0.367 |

**Table 3.** Loadings of principal components (PC) 1 and 2 on the relative proportions of hydrocarbons for ten samples of *Prociphilus oriens.*

| Components | PC1 loading | PC2 loading |
|---|---|---|
| Tricosane (nC23) | -0.172 | 0.277 |
| 3-Methyl tricosane (3-MeC23) | 0.326 | 0.081 |
| n-Tetracosane (nC24) | -0.047 | 0.372 |
| 2-Methyl tetracosane (2-MeC24) | 0.059 | 0.202 |
| 3-Methyl tetracosane (3-MeC24) | 0.248 | 0.196 |
| n-Pentacosane (nC25) | -0.164 | 0.330 |
| 11-Methyl pentacosane (11-MeC25) | 0.239 | 0.127 |
| 2-Methyl pentacosane (2-MeC25) | 0.250 | -0.050 |
| 3-Methyl pentacosane (3-MeC25) | 0.303 | 0.097 |
| n-Hexacosane (nC26) | -0.016 | 0.065 |
| 2-Methyl Hexacosane (2-MeC26) | -0.026 | -0.099 |
| Heptacosane (nC27) | -0.314 | -0.113 |
| 13-Methyl heptacosane (13-MeC27) | -0.081 | 0.149 |
| 9-Methyl heptacosane (9-MeC27) | 0.317 | -0.049 |
| 5-Methyl heptacosane (5-MeC27) | 0.195 | 0.013 |
| 3-Methyl heptacosane (3-MeC27) | -0.002 | 0.159 |
| n-Octacosane (nC28) | -0.076 | -0.252 |
| n-Nonacosane (nC29) | -0.063 | -0.385 |
| 13-methyl nonasosane (13-MeC29) | -0.106 | 0.188 |
| 9-Methyl nonasosane (9-MeC29) | 0.272 | -0.175 |
| n-Hentriacontane (nC31) | -0.075 | -0.350 |
| 13-Methyl hentriacontane (13-MeC31) | 0.165 | -0.188 |
| 14-Methyl dotriacontane (14-MeC32) | 0.294 | 0.091 |
| 13-Methyl tritriacontane (13-MeC33) | 0.091 | -0.203 |
| 13-Methyl pentatriacontane (13-MeC35) | 0.302 | -0.020 |

# Chapter 3. Endoparasitic mermithid nematodes in gall-forming aphids

## 3.1 Introduction

Mermithid nematodes (Nematoda: Mermithidae) are obligate parasites that have been found in many invertebrates (Poinar 1975; Yeates & Buckley 2009; Kubo et al. 2016; Watanabe et al. 2021). As with other parasitic nematodes, free-living mermithid nematodes parasitize the hosts by actively penetrating the cuticles, or through ingestion of their eggs by host insects (Poinar 1979; Doi & Hasegawa 2006). During our biological survey of eriosomatine aphids, a species of an unidentified mermithid nematode was found in the abdomens of aphids collected in Hokkaido, Japan.

Aphids of Eriosomatinae (Insecta: Hemiptera: Aphididae) induce leaf galls on the primary host plants and parthenogenetically produce second-generation aphids within the gall from early May to mid-June in Hokkaido, Japan, a cool temperate zone. Second-generation aphids develop into winged adults, which migrate to the roots of secondary host plants to form colonies. In autumn, winged females

(sexuparae) appear on the roots and migrate back to the primary host plants to produce sexual offspring. These offspring (male and female embryos) develop inside the abdomens of the females at their nymphal stage and are viviparously born on the trunk of the primary host plant. Sexual offspring experience both underground and aboveground environments along with their mothers from the embryonic stage until they are delivered.

After colonizing the roots of the secondary host plants, eriosomatine aphids live in the soil environment from early summer to autumn, making them susceptible to infection by soil-living parasites, such as nematodes and microbes. In the present study, we examined the rate of parasitism of the unidentified mermithid and attempted to molecularly characterize the species by employing the sequences of two ribosomal RNA genes, 18S and 28S. Thereafter, the phylogenetic status of the species in the available mermithid sequences was inferred based on the rDNA sequences.

## 3.2 Methodology

### 3.2.1 Sample collection

On October 9, 2017, in Yoichi, Hokkaido, Japan (43°12′9″ N, 140°45′52″ E), autumnal winged females (sexuparae) were collected using forceps just after their alighting on the branches of *Ulmus davidiana* and maintained in 80% ethanol. Sexuparae were dissected and slide-mounted with their embryonic sexual offspring in Hoyer's mountant for morphological observation (Tong & Akimoto 2019). When a parasite was found inside the sexuparae, it was isolated for later morphological and molecular identification. Aphids were identified morphologically, and all specimens were deposited in the Laboratory of Systematic Entomology, Hokkaido University, Sapporo, Japan.

### 3.2.2 Measurement and photography

The wing lengths of sexuparae and the body area of sexual offspring (female and male embryos) after mountant were measured and used as an index of body size (Tong & Akimoto 2019). All images were captured using a microscope eyepiece camera (Dino-Eye, AnMo Electronic Corporation, Taipei) and measurement was

carried out using IMAGEJ software (http://rsbweb.nih.gov/ij/). Statistical analysis was performed using JMP software ver. Pro 14.

The isolated nematodes in mounted specimens with the host aphids were observed using light microscopy (Eclipse 80i, Nikon, Tokyo) with DIC optics and photographed with a digital camera system (MC170 HD, Leica, Wetzlar) attached to the microscope. The digital photographs were edited to enhance brightness and contrast in order to construct a micrographic figure (Fig. 1) using PhotoShop 2019 (Adobe).

### 3.2.3 DNA extraction, sequencing, and polygenetic analysis

One parasite found in an *Eriosoma auratum* sexupara was isolated, and its genomic DNA was extracted and purified using the DNeasy Blood and Tissue Kit (QIAGEN, Venlo, the Netherlands). The 18S ribosomal gene and the gene fragment of the large ribosomal subunit (LSU) 28S rDNA sequence were amplified and polymerase chain reaction (PCR) was performed according to Kobylinski *et al.* (2012) and Shih *et al.* (2019). The following primers were used: 18S, 18S-F: 5′-CAAGGAC GAAAGTTAGAGGTTC-3′ and 18S-R: 5′-GG AAACCTTGTTACGACTTTTA-3′, and for 28S, LSU-F: 50–

ACAAGTACCGTGAGGGAAAGTTG–30 and LSU-R: 50–TCGGAAGGAACCAGCTACTA–30 (Shih *et al.* 2019). The resulting templates were purified using a QIAquick PCR purification kit (QIAGEN Inc.) and sequenced in both directions using an ABI 3730xl Analyzer (Applied Biosystems). The resulting sequences were deposited in GenBank, and the BLASTn algorithm (Altschul *et al.* 1990) was applied to confirm the identity of the sequences.

The dataset of partial sequences of the nuclear 18S rDNA of mermithid nematodes in GenBank was searched and aligned using the MEGA X software package (Kumar *et al.* 2018). Host species were referenced to related publications and GenBank after obtaining 18S rDNA sequences of the parasitic mermithid nematodes (Table S1). Phylogenetic trees were constructed using Bayesian inference (BI) (Larget & Simon 1999) and maximum likelihood (ML) (Felsenstein 1981). The best-fit evolutionary model K2 + G + I was adopted by Mega X and used for all model-based methods (BI and ML). The Bayesian tree was constructed by MrBayes 3.2.7 (Ronquist et al. 2012) using a Markov chain Monte Carlo (MCMC) approach with 2 million generations, with tree sampling every 500 generations. The 1000 replicates were run for maximum likelihood (ML) bootstrap sampling using Mega X.

## 3.3 Results

### 3.3.1 Mermithid nematodes in aphids

In total, 418 eriosomatine sexuparae, consisting of eight species of two genera, *Tetraneura* and *Eriosoma*, were available for examination of parasitism. Five sexuparae of *E. auratum* and one of *T. radicicola* out of the 418 individuals were found to be parasitized by a slender worm (Table 1). One parasite coexisted with embryonic sexual offspring inside the abdomen of each parasitized aphid. One of the parasites was isolated for molecular identification. The others were individually maintained with the host sexuparae in the mounted specimens for morphological observation.

### 3.3.2 Sex ratios of aphids affected by the nematode parasitism

No significant difference was found in body size between adult mermithid-parasitized and uninfected *E. auratum* sexuparae (ANOVA, df = 1,32, F = 0.935, P = 0.34). However, the number and body size of sexual female embryos were significantly reduced in mermithid-parasitized sexuparae ( df = 1,32, F = 9.93, P = 0.0035; and df = 1,32, F = 16.87, P = 0.0003, respectively) compared to uninfected

sexuparae, whereas no such significant associations were found in male embryos ( df = 1,32, F = 0.15, P = 0.70; and df = 1,32, F = 0.26, P = 0.61, respectively).

### 3.3.3 Morphology and phylogeny of the mermithid nematodes

All isolated nematodes were juveniles without generic or species-specific characters, and some parts of the morphological structures were vague, likely because of the Hoyer fixation. Some morphological characteristics were confirmed in the specimens. The body was slender, approximately 1.5 cm long, with a smooth surface. One specimen that isolated from a *T. radicicola* sexupara was in relatively good condition and was examined under a stereo microscope for typological characters (Figure 1). Anterior end dome-shaped cephalic or labial papillae were not observed, possibly because of material conditions. The stoma was conspicuous, and its anterior end was forming a well-sclerotized an pointed piercing tooth; the pharyngeal tube possessed a conspicuous lumen, connecting the stoma and cardia, and at least two gland-like structures were observed on both sides of the stoma and the anterior part of the pharyngeal tube. The cardia was funnel-shaped. Genital anlage was not confirmed, possibly because of the material conditions. The posterior end of the intestine was inconspicuous, and the anus and rectum were not observed,

also likely due to the material condition. A short and bluntly pointed spike-like projection was observed at the tail tip.

The 18S rDNA and 28S rDNA gene fragments of the isolated parasite from *E. auratum* were successfully sequenced from one individual, and the sequences were deposited in GenBank under accession numbers MW649131 and MW653323. After alignment, 18S rDNA of 42 taxa and 563 base pairs were available for phylogenetic analysis. The BLAST search in GenBank indicated that the amplified sequence had the closest match and formed a clade with a previously sequenced mermithid juvenile 18S sequence (AY919185), which was collected from a grassland soil sample from Lincoln, Nebraska (Posers, pers. comm.).

Although GenBank reference sequences are limited for mermithid nematodes, here, the Bayesian-based phylogeny was constructed using currently available 18S rDNA sequences with information on the host range. Mermithid nematodes have broad host ranges, including 12 invertebrate genera, mainly Diptera and Hemiptera (Fig. 2). The mermithid sp., which was isolated from an aphid (Insecta: Hemiptera) in the present study, formed a clade with an environmental sample and was clearly separated from neighboring hemipteran associates (Fig. 2).

## 3.4 Discussion

For aphids and other herbivorous hemipteran insects that share a common arrangement of sucking mouthparts, mermithid nematodes cannot enter host bodies through mouthparts. In the present study, the unidentified mermithid nematode likely parasitized the aphid by penetrating the cuticle, or gaining entry through a natural opening such as the trachea and anus, or during embryonic development being parasitized through maternal transmission.

Mermithid parasitism of aphids is not commonly known and only three cases have been reported (Guercio 1899; Davis 1916; Poinar 2017), although this could be due to undersampling of the aphids for this condition. The most remarkable record is the parasitism of an extinct aphid, *Caulinus burmitis* (Hemiptera: Burmitaphididae) by a fossil mermithid, which was found in mid-Cretaceous Myanmar amber (Poinar 2017). This example implies that the parasitic association between aphids and mermithid nematodes has continued for more than 100-million years. In Italy, nymphs and winged adults of the root aphid *Trama radices Kaltenbach* were found to be parasitized by an unidentified mermithid in April and May 1899, which was dispersed and embedded in the winged aphid (Guercio 1899). Davis (1916) conducted fieldwork to collect mermithid-parasitized aphids in Indiana, USA between mid-September and October 1911, and found mermithid-parasitized

apterous viviparous and oviparous aphids of an *Anoecia* sp. on October 16th and 19th on the roots of *Muhlenbergia*. This is also the first record of mermithid parasitism in oviparous aphids.

The unidentified mermithid found in the present study was closest to a species collected from grassland soil around the root system of Leadplant, Amorpha canescens Pursh in the USA (Powers, pers. comm., also described in https://nematode.unl.edu/mermissp.htm), which possibly contained herbivorous insects, including aphids. However, the taxonomic status of the nematodes is unknown in both cases since the samples were juveniles not closely aligned to any identified species. In addition, although these two species formed a well-supported clade in the phylogenetic analysis (Fig. 2), they were clearly separated from each other considering the branch length between them. Therefore, they possibly represent separate, undersampled clades. Further collections followed by phylogenetic analyses are required to understand their relationships and taxonomic status.

The survival and performance of parasites can be largely affected by their hosts. Nematodes receive nutrition from the host tissues and hemolymph, competing with the host for nutrients that are important for its physiological development and

reproduction (Smith *et al.* 1985; Mcrae *et al.* 2015). Once mermithid nematodes parasitize host insects, they can manipulate host behavior for their own benefits. For example, Allahverdipour *et al.* (2019) reported that mermithid-parasitized female mosquitoes seek water three times more than a blood source, whereas uninfected females were twice as likely to seek blood than water. Moreover, parasitizing adult hosts could be a dispersal strategy for mermithid nematodes (Campos & Sy 2003; Di Battista *et al.* 2015). In the present study, obvious morphological or behavioral alterations were not confirmed in parasitized aphids and parasitism was not detected until dissection. Nevertheless, our study indicated that mermithid parasitism in sexuparae led to fewer and smaller female sexual embryos. It is not clear whether the parasites negatively affect offspring fitness by competing for nutritious resources directly or whether maternal investment changes in response to parasitism. Thus, it is necessary to increase the sample size to investigate host manipulation by mermithid nematodes in future studies.

Mermithid nematodes can infect a broad range of aquatic and terrestrial invertebrates. However, because nematodes are often collected as juveniles, their identification and host specificity are difficult to evaluate. *Mermis nigrescens*, a parasite of grasshoppers, is reported to be found in other insect orders, such as Dermaptera, Coleoptera, and Lepidoptera (Poinar 1979). However, because of the

difficulty in morphological identification, information on the host range needs to be confirmed by molecular barcoding analyses. In the present study, although the species status is still unknown, the molecular sequences can be regarded as a species-specific barcode for taxonomic identification and evaluation of the host range in future studies.

**Note**: this chapter is a slightly modified version of "First record of a mermithid nematode (Nematoda: Mermithidae) parasitizing winged females of gall-forming aphids (Hemiptera: Aphididae: Eriosomatinae)" under review in Entomological Science and the authors will soon apply for the permission of the copyright holder once the manuscript is accepted for publication.

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**Figure 1** Typological characters of a nematode isolated from *T. radicicola*. **A**: Whole body; **B**: Anterior region; **C**: Close-up of anterior end ("C" in subfigure B) in five different focal planes showing stoma and glands; **D**: Close-up of pharynx-intestional junction region ("D" in subfigure B) in six different focal planes showing funnel-shaped cardia and body surface structure; **E**: Posterior end of body; **F**: Close-up of tail tip ("F" in subfigure F) showing tail spike (appendage).

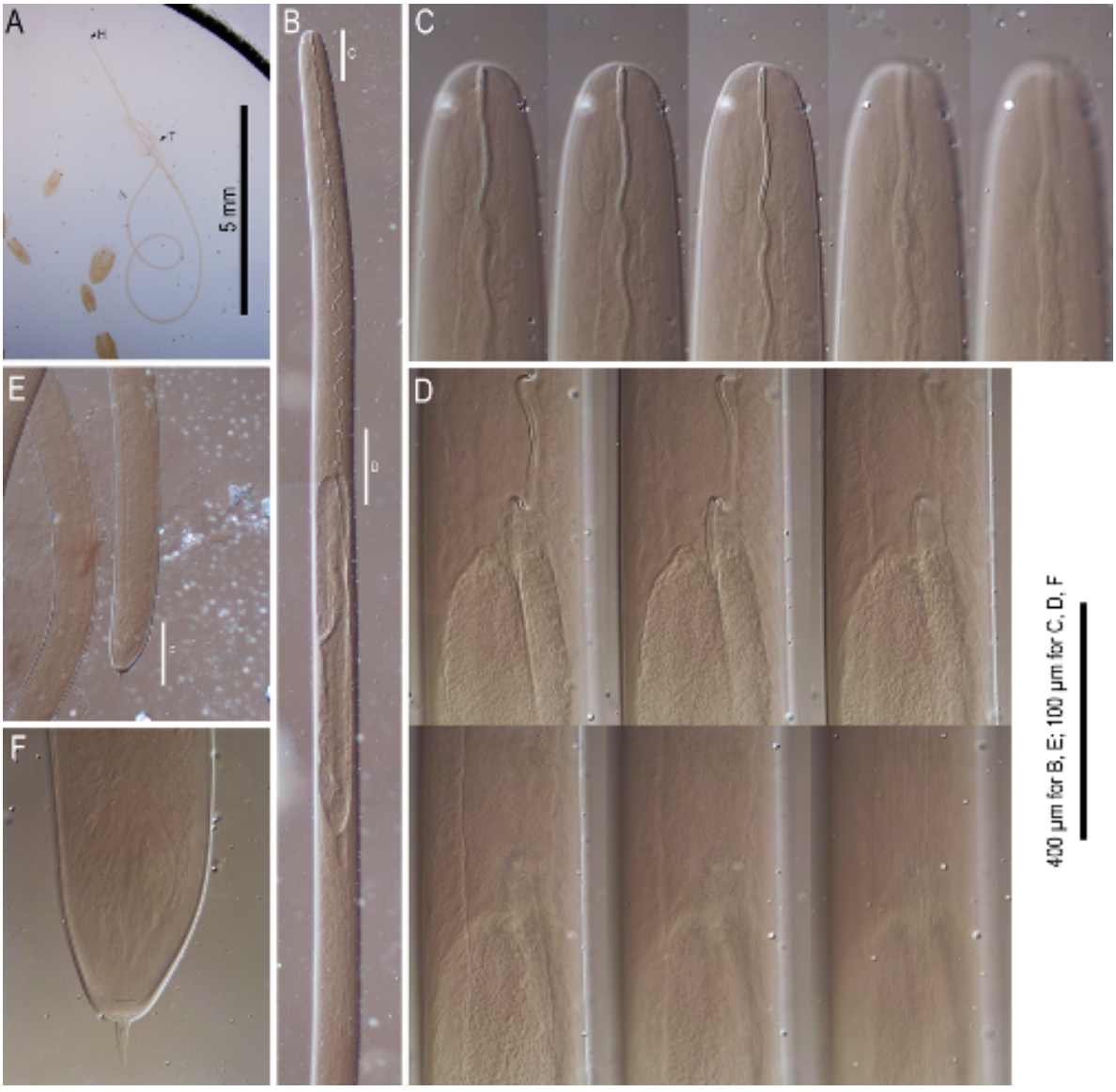

**Figure 2** Bayesian phylogenetic tree inferred from the 18S rDNA sequences of mermithid nematodes. Values on nodes represent posterior probabilities for Bayesian inference and bootstrap support for maximum likelihood, respectively. The orders of the hosts parasitized by mermithid nematodes are listed on the right of the tree in accordance with the record of parasites.

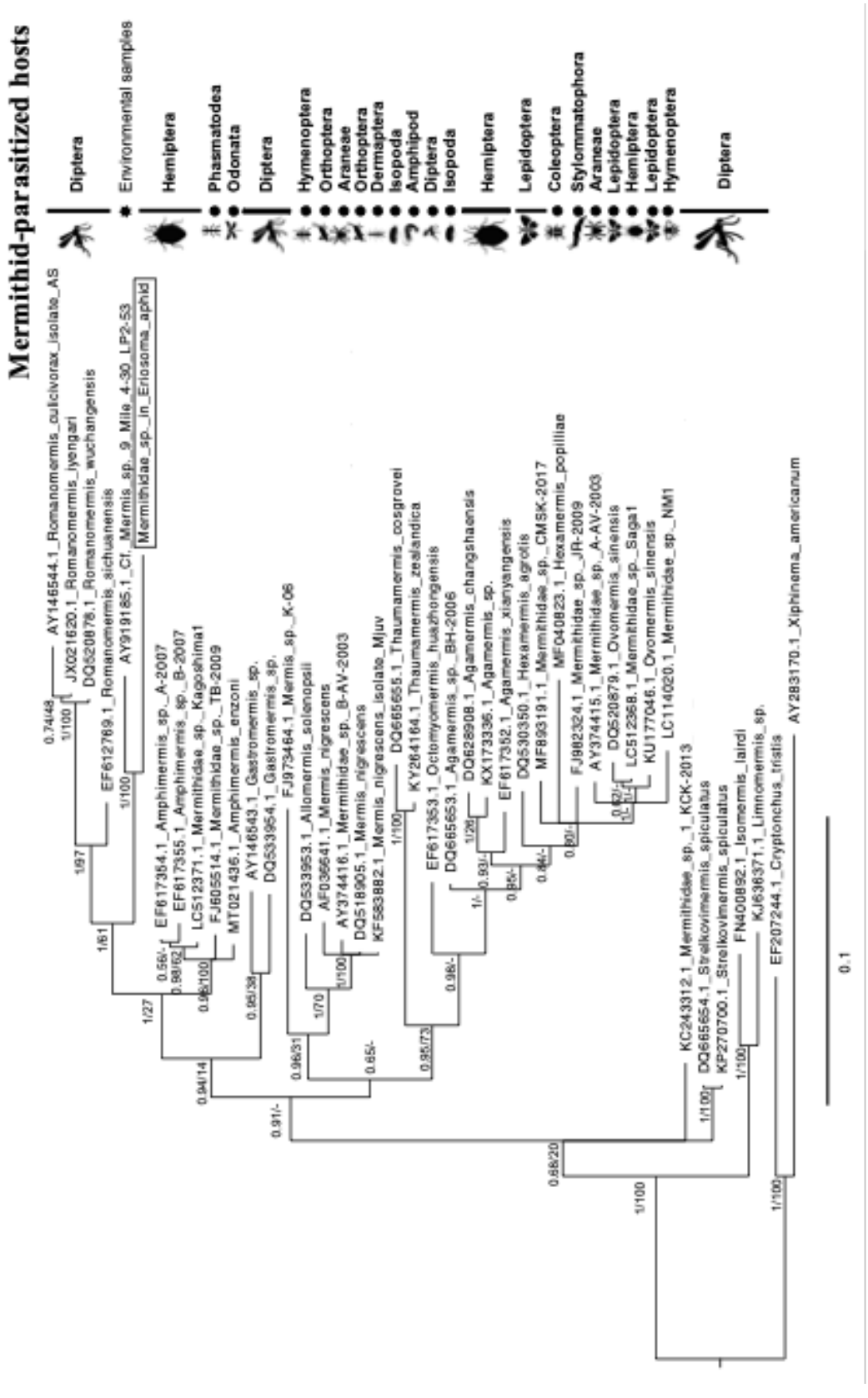

**Table 1** Proportion of mermithid parasitism in eriosomatine aphids collected in 2017.

| | *T. sorini* | *T. radicicola* | *T. triangula* | *T. nigriabdominalis* | *E. harunire* | *E. auratum* | *E. yangi* | *E. parasiticum* | Total |
|---|---|---|---|---|---|---|---|---|---|
| No. examined | 273 | 49 | 15 | 8 | 29 | 41 | 2 | 15 | 432 |
| No. parasitized | 0 | 1 | 0 | 0 | 0 | 5 | 0 | 0 | 6 |
| Parasitized rate (%) | 0 | 2.04 | 0 | 0 | 0 | 12.20 | 0 | 0 | 1.39 |

**Table S1.** List of GenBank accession numbers, sample species, and references for host information included in the phylogenetic tree. DS: direct submission to GenBank.

| GenBank accession no. | Sample species | References for host information |
|---|---|---|
| AY146544.1 | *Romanomermis culicivorax* | Shamseldean & Platzer 1989 |
| JX021620.1 | *Romanomermis iyengari* | Suman *et al.* 2012 |
| DQ520878.1 | *Romanomermis wuchangensis* | Duan *et al.* 2016 |
| EF612769.1 | *Romanomermis sichuanensis* | Peng *et al.* 2002 |
| AY919185.1 | *Mermithidae* sp. | DS |
| EF617354.1 | *Amphimermis* sp. | Xu *et al*. 2005 |
| EF617355.1 | *Amphimermis* sp. | Xu *et al*. 2005 |
| LC512371.1 | *Mermithidae* sp. | Iryu *et al.* 2020 |
| FJ605514.1 | *Mermithidae* sp. | Yeates & Buckley 2009 |
| MT021436.1 | *Amphimermis enzoni* | Rusconi *et al.* 2020 |
| AY146543.1 | *Gastromermis* sp. | Nickle 1972 |
| DQ533954.1 | *Gastromermis* sp. | Poinar *et al.* 2007 |
| FJ973464.1 | *Mermis* sp. | DS |
| DQ533953.1 | *Allomermis solenopsii* | Poinar *et al.* 2007 |
| AF036641.1 | *Mermis nigrescens* | Gordon & Webster 1971 |
| AY374416.1 | *Mermithidae* sp. | Vandergast & Roderick 2003 |
| DQ518905.1 | *Mermis nigrescens* | Gordon & Webster 1971 |
| KF583882.1 | *Mermis nigrescens* | Presswell *et al.* 2015 |
| DQ665655 | *Thaumamermis cosgrovei* | Tang & Hyman 2007 |
| KY264164.1 | *Thaumamermis zealandica* | Poinar *et al.* 2002 |
| EF617353.1 | *Octomyomermis huazhongensis* | Wang *et al.* 2007 |
| DQ665653.1 | *Agamermis* sp. | DS |
| DQ628908.1 | *Agamermis changshaensis* | Xu *et al.* 2005 |
| KX173336.1 | *Agamermis* sp. | Stubbins *et al.* 2016 |
| EF617352.1 | *Agamermis xianyangensis* | Kubo *et al.* 2016 |
| DQ530350.1 | *Hexamermis agrotis* | Li *et al.* 1993 |
| MF893191.1 | *Mermithidae* sp. | Kumar *et al.* 2018 |
| MF040823.1 | *Hexamermis popilliae* | Mazza *et al.* 2017 |
| FJ982324.1 | *Mermithidae* sp. | Ross *et al.* 2010 |
| AY374415.1 | *Mermithidae* sp. | Vandergast & Roderick 2003 |
| DQ520879.1 | *Ovomermis sinensis* | Sun *et al.* 2020 |
| LC512368.1 | *Mermithidae* sp. | Iryu *et al.* 2020 |
| KU177046.1 | *Ovomermis sinensis* | Sun *et al,* 2020 |
| LC14020.1 | *Mermithidae* sp. | Kubo *et al.* 2016 |
| KC243312.1 | *Mermithidae* sp. | Kobylinski *et al.* 2012 |
| DQ665654.1 | *Strelkovimermis spiculatus* | Poinar & Camino 1986 |
| KP270700.1 | *Strelkovimermis spiculatus* | Poinar & Camino 1986 |
| FN400892.1 | *Isomermis lairdi* | Gradinarov D 2014 |
| KJ636371.1 | *Limnomermis* sp. | Villemant *et al.* 2015 |
| EF207244.1 | *Cryptonchus tristis* | Holterman *et al.* 2008 |
| AY283170.1 | *Xiphinema americanum* | Neilson *et al.* 2004 |

# Chapter 4 Biased sex ratios in a gall-forming aphid

## 4.1 Introduction

Animals with female- or male-biased sex allocation have widely been observed (Charnov 1982; Hardy 2002; West 2009; Wrensch & Ebbert 1993). Current sex allocation theories have explained deviations of population sex allocations from a 1:1 ratio as resulting from competition and cooperation among siblings (West 2009). Local mate competition (LMC) among male offspring (Hamilton 1967; Taylor & Bulmer, 1980; Werren 1980) and local resource competition (LRC) among female offspring (Clark 1978; Silk 1984) are assumed to result in female-biased and male-biased population sex allocation, respectively. Similarly, local resource enhancement (LRE) in cooperatively breeding species is predicted to produce biases in population sex allocation (Schwarz 1988; Silk & Brown 2008). Sex allocation theories based on nonlinear models (Frank 1990) predict that if offspring of different sexes yield different reproductive returns per unit maternal investment, then equal allocation to the sexes will not be maintained at the population or the individual level (Charnov 1979; Frank 1987, 1990; Maynard 1980). Particularly for mammals, Trivers and Willard (1973) hypothesized that if mothers differ in the amounts of

reproductive resources to which they have access, then more fecund mothers should allocate more towards males with the greater reproductive return.

This hypothesis has been applied to numerous vertebrates, such as ungulates, to evaluate the relationship between maternal reproductive status and offspring sex ratio (Clutton-Brock *et al.* 1984; Clutton-Brock & Lason, 1986; Kruuket *et al.* 1999; Myers, 1978; Rose *et al.* 1998; Sheldon & West, 2004). For example, female reproductive condition in red deer, *Cervus elaphus*, is determined by her social rank, such that mothers with a higher rank can invest more resources in their offspring. The breeding success of sons depends on their fighting ability and body size, which are determined by the nutrition they receive during the first 18 months of life (Clutton-Brock *et al.*, 1984). However, the reproductive success of daughters is less strongly affected by maternal rank. Thus, mothers in good condition can greatly increase their sons' breeding success by providing them with a large amount of resources, thereby leading to male-biased sex allocation in their offspring. The Trivers–Willard hypothesis and nonlinear models of sex allocation can be tested if an animal has the opposite biological characteristics to those of the red deer. Namely, if female–female competition is frequent in a species, and larger females are more likely to win competitive interactions, and the mother can control their investment in sons and daughters, then we can expect that mothers in good resource condition

should have female-biased sex allocation. In this study, we test the Trivers–Willard hypothesis and nonlinear models by using a gall-forming aphid in which gall foundresses frequently fight with one another. In aphids (Insecta: Aphididae), mothers can control the sex of each of their offspring by either retaining two X chromosomes or eliminating one of them from the oocyte (Blackman 1987). Oocytes retaining two X chromosomes develop into females, whereas those from which an X chromosome is eliminated are destined to develop into males. In aphid males with XO sex determination, spermatocytes have one X chromosome or none. Because spermatocytes without an X chromosome degenerate, all sperm carry one X chromosome. For this reason, the next generation produced after mating, the foundress generation, is composed entirely of females. In aphids belonging to the Eriosomatinae (wooly aphids), autumnal winged females ("mothers" in Figure 1) parthenogenetically produce female and male embryos (the sexual generation) in their abdomen and deposit them all at once viviparously on the trunk of one of several deciduous trees. Because mothers have already completed their sex allocation at the time of eclosion, this aphid group is well suited for evaluating maternal sex allocation (Akimoto *et al.* 2012; Foster, 2002; Kurosu & Aoki 1991; Moran 1993; Yamaguchi 1985).

In the Eriosomatinae, the sexual females and males do not feed after birth, moult four times and mature sexually using resources provided by the mother (Heie

1980; Miyazaki 1987). Each female produces only a single egg, which occupies a large part of her body cavity (Figure 1). After eggs overwinter on the host trunk, foundresses hatch from the eggs in the following spring and induce the formation of galls on the developing leaves of their host tree. Thus, the body size of a foundress is strongly correlated with the mother's size ("female" in Figure 1), which was determined by the grandmother's investment ("mother" in Figure 1). Sexual females are larger than males in all eriosomatine species examined so far (Akimoto & Yamaguchi 2004; Foster & Benton 1992; Lampel 1968; Yamaguchi 1985). Thus, if the production costs of males and sexual females can be estimated, then the exact sex allocation of a mother to her sons and daughters can be evaluated (Akimoto *et al.* 2012). By sampling autumnal winged females randomly from a population, one can also determine the population-level sex allocation. *Tetraneura sorini* is a gall-forming eriosomatine aphid, which induces a gall on developing host leaves (Blackman & Eastop 1994). Foundresses (gall formers) frequently fight with one another over the possession of incipient galls (Akimoto & Yamaguchi 1997). Larger foundresses are more likely to win these fights (Muramatsu & Akimoto 2016). Therefore, one can assume that mothers can achieve greater reproductive returns by investing a larger proportion of resources in individual sexual females and producing larger foundresses (Figure 1). Thus, *T. sorini* provides an interesting opportunity to test the Trivers–Willard hypothesis under the opposite conditions to those in red deer.

Another remarkable feature of this species is occurrence of dimorphic broods. *Tetraneura sorini* mothers produce either an all-female brood or a mixed-sex brood (including males and sexual females); the former type accounted for approximately half of the winged females in a population (Akimoto & Yamaguchi 2004). Although this dimorphism has been found in other eriosomatine species (Akimoto & Yamaguchi 2004; Li & Akimoto 2017; Moran 1993), previous studies based on the LMC hypothesis have failed to adequately explain the origin of this dimorphism. In another eriosomatine species, *Prociphilus oriens*, long-term observations indicated that the sex allocation was strongly biased towards females (71%–73%) at the population level, which was attributed mainly to competition among unrelated foundresses (Li & Akimoto 2017). In cyclically parthenogenetic animals, in which the bisexual generation is followed by unisexual generations, exploitative competition is expected to be severe throughout the unisexual generations (Williams, 1975). Thus, we postulated that mothers in good condition could achieve higher fitness returns by producing more females than males because these mothers could then have more granddaughters in the first unisexual generation (the foundress generation). Because a male can inseminate more than 10 sexual females (Li & Akimoto 2017), it is not disadvantageous for mothers to produce female-biased sex ratios. However, evidence of competition among foundresses has not been obtained in *P. oriens*. Since *T. sorini* foundresses fight intensively with one another, this

species is a suitable study organism for confirming this hypothesis. In the present study, we used an aphid species with unique biological characteristics to describe the maternal and clonal sex allocation patterns, evaluate the cost of producing a female relative to a male and test our own and previous hypotheses on the optimal sex allocation. In particular, we address three main questions: (1) whether mothers in good condition bias the sex ratios of their broods towards females; and (2) whether mothers in better condition invest more in individual females (Veller *et al.* 2016); and (3) what factors lead to biased sex allocation at the population level. The present study focuses on autumnal winged females (mothers), males and females (sons and daughters), and foundresses (granddaughters) (Figure 1). To distinguish these, autumnal winged females are referred to as "mothers," and sexual females are hereafter referred to as "females." Males and females are collectively called "sexuals."

## 4.2 Methodology

### 4.2.1 Study organisms

*Tetraneura sorini* seasonally alternates host plants between the Japanese elm, *Ulmus davidiana*, and the grass *Miscanthus sinensis*. First-instar larvae hatching from overwintered eggs induce or usurp pouch galls on developing leaves of *U. davidiana* in early May in Hokkaido, northern Japan. In the galls, they mature and parthenogenetically produce second-generation larvae, which develop into winged females (emigrants) and migrate to *M. sinensis*. On the roots of this plant, several wingless generations reproduce parthenogenetically throughout the summer and autumn. During mid- to late October, a mass of winged females ("mothers" in Figure 1) emerges from the roots and flies back to *U. davidiana* to produce sexuals. Sexuals are wingless and dwarfed; they mature without consuming food. *Tetraneura sorini* foundresses are more likely to fail to induce galls than those of other *Tetraneura* species, but can act as a "parasite" and usurp incipient galls induced by other *Tetraneura* species (Akimoto & Yamaguchi 1997; Muramatsu & Akimoto 2016). The *T. sorini* foundress is the largest in body size of the *Tetraneura* species, with well-developed hind legs, and thus, it has an overwhelming advantage in aggressive interactions. Muramatsu and Akimoto (2016) showed that where the density of other *Tetraneura* species (host species) is lower, *T. sorini* foundresses more frequently

fight with conspecifics, such that foundresses are subject to stronger directional selection for larger body sizes.

### 4.2.2 Insect collection

In mid-October, autumnal winged females (hereafter “mothers”) were collected using forceps just after alighting on the branches of *U. davidiana* and preserved in 80% ethanol. Two samples of mothers were used for analyses: (a) the “2001 sample” was collected in Iwamizawa, Hokkaido, Japan (43°11′28″N, 141°46′53″E), on 8 October 2001; and (b) the “2017 sample” was collected in Yoichi, Hokkaido, Japan (43°12′9″N, 140°45′52″E), on 9 October 2017. For the 2001 sample, all winged mothers were dissected and the numbers of males and females in the brood were recorded. The wing lengths of all mothers were measured after the forewings were slide-mounted using Hoyer's mountant. The forewing length was correlated with the length of hind femur ($r = 0.84$, $n = 20$, $p < 0.0001$) and was used as an index of body size. For the 2017 sample, all males and females in a brood and the forewings of the mother were mounted on a slide glass. All images were captured in a computer through a microscope eyepiece camera (Dino-Eye, AnMo Electronics Corporation, Taipei), and male and female body areas and forewing lengths were measured by using ImageJ software (http://rsbweb.nih.gov/ij/). To investigate the

sex allocation patterns of *T. sorini* clones, we reared clones individually and obtained winged females from each clone. *Miscanthus sinensis* plants were collected from the wild in mid-May and transplanted into pots. Mature galls of *T. sorini* were collected from *U. davidiana* leaves in Iwamizawa, Hokkaido, Japan, in mid-June and placed in small plastic cages (30 mm width × 10 mm height × 30 mm depth) lined with dampened filter paper. After winged females (emigrants) appeared from the cleaved galls and parthenogenetically produced third-generation larvae, we transferred the larvae to the potted *M. sinensis* plants. Larvae from one gall were transferred to a single plant, and the plants were netted to prevent the entrance of different clones and predators. In mid-October, all winged mothers that emerged from the roots were collected. We used only 9 of the 10 clonal colonies for analyses because one of the clones produced fewer than 20 winged mothers. The other 9 clones produced from 21 to 120 mothers. All the mothers were dissected and the numbers of males and females in a brood and the wing length of the mother were recorded.

### 4.2.3 Measurement of costs of producing females

The cost of producing females was evaluated as described in Akimoto *et al.* (2012). This method postulates that mothers with the same wing length have the same amount of reproductive resources. We chose two groups of mothers: those that

produced all females (abbreviated as all-F mothers), and those that produced six males and any number of females (a mother produces a maximum of six males). The number of females was compared between the two groups of mothers. Six-male-producing mothers produced fewer females than all-F mothers (see Section 3). The difference in female numbers (i.e., K females) was attributed to the cost of producing six males. Therefore, if mothers are equivalent in body size, then the production cost of one female could be concluded to be equal to 6/K times the production cost of one male. Because the wing length varies among mothers, the number of females produced needed to be adjusted for this. We conducted regression analyses of the relationship between maternal wing length and the number of females produced for the two mother groups and then assessed whether regression lines with the same slope could be applied to the two mother groups using ANCOVA. In the model, the maternal wing length, mother groups and their interaction were treated as explanatory variables. If the interaction was not statistically significant, then regression lines with the same slope could be applied to both mother groups. In this case, the difference between the intercepts of the regression lines (K) was calculated using the least-square means. The 2001 and 2017 samples were used for computing the relative production cost of a female (F = 6/K). Investment in females was estimated to be (female number * F). The total maternal investment was calculated

as (male number + female number * F). The relationship between the investment in females and the total maternal investment was analysed by a simple linear regression.

### 4.2.4 Statistical analysis on sex allocation

The correlation between the maternal wing length, an index of body size and the total maternal investment was calculated. Subsequently, we tested whether brood sex ratios (female ratios) were affected by the maternal wing length or not using a generalized linear mixed model (GLMM). In the model, the ratio of females to males in a brood was treated as the response variable, whereas the maternal wing length was treated as the explanatory variable. The mother identification (ID) number was also included in the model and treated as a random effect. The glmmML function in the R package "glmmML" was used for this analysis, with a binomial error structure. Variation in female body area among broods was tested with a GLMM. In the model, the body area of a female was treated as the response variable, and the numbers of females and males in that brood and the maternal wing length were treated as explanatory variables. The mother ID number was also included in the model and specified as a random effect. Variation in male body area was also tested using the same statistical model. In this analysis, the lmer function in the r package "lme4"

was used, assuming a Gaussian error structure. R version 3.4.2 was used for these statistical analyses. Other calculations were performed using JMP ver. 13.2.

## 4.3 Results

### 4.3.1 Wild populations and reared clones

Dimorphic broods were detected in wild populations of *T. sorini*. In both the 2001 and 2017 samples, two peaks were found, corresponding to (a) all-F mothers producing two to four females and (b) mothers producing five or six males plus females (Figure 2; higher peaks are indicated by redder areas). The proportion of females in the population was 41.8% in the 2001 sample and 45.2% in the 2017 sample (Table 1).

The rearing of clones indicated that female proportions varied widely among the nine clones, ranging from 16.7% in clone T4 to 97.5% in clone B. Almost all the clones produced both all-F mothers and six-male-producing mothers (Table 1). The proportion of all-F mothers also varied greatly among the clones, ranging from 4.2% in clone T4 to 92.5% in clone B. Across the nine clones, female proportions were

not significantly correlated with the mean wing lengths of mothers ($n = 9$, $r = 0.27$, $p = 0.48$).

### 4.3.2 Costs of producing females

In both all-F mothers and 6-male-producing mothers, the number of females increased as the maternal wing length increased (Figure 3; ANCOVA for wing length: $F_{1,42} = 52.8$, $p < 0.0001$ in 2001; $F_{1,153} = 88.6$, $p < 0.0001$ in 2017). There was no significant difference in the regression slopes between all-F mothers and six-male-producing mothers (ANCOVA for the interaction between wing length and mother groups: $F_{1,42} = 0.62$, $p = 0.44$ in 2001; $F_{1,153} = 0.002$, $p = 0.96$ in 2017). However, a significant difference was detected in the intercepts of the regression lines between the two groups of mothers (ANCOVA for mother groups: $F_{1,43} = 88.5$, $p < 0.0001$ in 2001; $F_{1,154} = 181.1$, $p < 0.0001$ in 2017). In the 2001 and 2017 samples, six-male-producing mothers produced, on average, 2.016 fewer females and 1.899 fewer females, respectively, than did all-F mothers. The cost of producing six males was estimated to be equal to the cost of producing 2.016 females (in 2001) or 1.899 females (in 2017). Therefore, the cost of producing one female was calculated as 2.976 times and 3.159 times that of producing one male in 2001 and 2017, respectively. Using this production cost for females, the relationship between

the total maternal investment and investment in females was evaluated (Figure 4); this showed that more fecund mothers invested more in females (simple regression: $F_{1,116} = 250.4$, $p < 0.0001$ in 2001; $F_{1,268} = 496.7$, $p < 0.0001$ in 2017). The per cent allocation to females at the population level was 68.1% in 2001 and 72.3% in 2017.

### 4.3.3 Maternal investment, sex ratios, and female size

The total maternal investment was strongly correlated with the maternal wing length (in 2001: $n = 83$, $r = 0.701$, $p < 0.0001$; in 2017: $n = 268$, $r = 0.647$, $p < 0.0001$), suggesting that wing length is a suitable index of maternal investment. Analysis with a GLMM indicated that the proportion of females in a brood was significantly and positively affected by the maternal wing length (in 2001: $z = 2.88$, $p = 0.004$; in 2017: $z = 2.08$, $p = 0.038$). This result suggests that larger mothers tend to bias the sex ratios of their broods more towards females. Female body area was positively affected by the maternal wing length, but negatively affected by the numbers of females and males in that brood (Table 2). In addition, all-F mothers produced, on average, larger females than did mothers with a mixed-sex brood (ANOVA: $F_{1,158} = 6.64$, $p = 0.011$). Male body area was negatively affected by the

number of females in that brood, but not by the maternal wing length or the number of males (Table 2).

## 4.4 Discussion

### 4.4.1 Sex allocation in *T. sorini*

The Trivers–Willard hypothesis predicts that mothers in better condition should bias the progeny sex ratios more towards males and invest more in individual males if they yield an increasing rate of reproductive returns on investment, but if females have a constant rate of reproductive returns (Frank 1987; Veller *et al.* 2016). This hypothesis has mainly been applied to mammals and birds (Clutton-Brock 1986; Clutton-Brock & Lason 1986; Cockburn *et al.* 2002; Frank 1990; Krackow 1995); however, it can also be applied to *T. sorini*, although the sex roles are reversed. Larger foundresses have an advantage in fighting, and they hatch from larger eggs. In eriosomatine aphids, the female lays only one egg, which is almost as long as herself (Heie 1980). Therefore, high maternal investment in individual females can consequently produce large foundresses (granddaughters). Our analyses of *T. sorini* mothers supported the Trivers–Willard hypothesis, indicating that more fecund mothers invested more in females in total (Figure 4); that is, they produced more female-biased broods and larger females (Table 2). However, there was no tendency

for more fecund mothers to produce larger males. Veller *et al.* (2016) pointed out that two predictions are included in the Trivers–Willard hypothesis (a prediction for progeny sex ratios and that for investment in individual sons and daughters) and examined the conditions under which two predictions are established. They indicated that the prediction for progeny sex ratios is generally applicable for several forms of return functions for sons and daughters. However, the second prediction that when a mother has a mixed brood, mothers in good condition and in poor condition, respectively, should bias their parental care towards sons and daughters holds true under restrictive conditions. One of the necessary conditions for the second prediction is that the slope of male fitness function is much steeper than that of female fitness function when the offspring have received large investment, but much flatter when they have received small investment (Veller *et al.* 2016). In *T. sorini*, we confirmed that both of the predictions from the Trivers–Willard hypothesis are true, though the sex roles are reversed. Therefore, our result suggests that given additional maternal investment in offspring, increase in female fitness is larger than increase in male fitness if the offspring have received large investment, but smaller if they have received small resources. This prediction is consistent with our observation that larger foundresses, which are produced by larger sexual females, have greater advantages in fighting, whereas males do not fight intensively. In conclusion, the pattern of maternal investment in *T. sorini* was consistent with the

predictions from the Trivers–Willard hypothesis. The Trivers–Willard hypothesis focuses on mothers who produce a single son or daughter at a time. However, *T. sorini* mothers produce varying numbers of males and females (1–10 offspring, of which 0 to 5 (2001) or 0–6 (2017) are female, and 0–6 are male).

Theoretical studies have pointed out that predicting the population-level sex allocation is not straightforward because it varies depending on (a) the shapes of return curves for sons and daughters, (b) the distribution of maternal resources, and (c) trade-offs between the numbers and sizes of sons and daughters (Frank 1987, 1990; Frank & Swingland 1988; West 2009; Wild & West 2007). If return curves of sons differ from those of daughters, population sex ratios are predicted to be biased towards the sex produced by parents with relatively low levels of resources, but the population sex allocation ratio may be biased towards either sex (Frank 1990). These predictions were corroborated by *T. sorini*, which showed a male-biased sex ratio but a female-biased sex allocation at the population level. Provided that returns from investment in females increase linearly and maternal resources vary, population sex allocation is predicted to be biased towards females if returns from investment in males can be described by a positive exponential curve (Frank 1987, Figures 2 and 4, $t = 1$, $s > 1$) or an S-shaped curve ($t = 2$, $s \geqslant 3$). Since the sex roles are reversed in *T. sorini*, this theory predicts a larger allocation to males at the population level.

Although the above-mentioned models evaluate the total reproductive returns on total male or female investment per investment period (e.g., one offspring at a time), an increasing number of offspring per period make the return curves more linear, bringing the predicted allocation closer to Fisher's equal allocation theory (Frank 1987, 1990). Therefore, theories predict male-biased or approximately equal population allocation for the case of *T. sorini*. However, our observation (an allocation of 68%–72% to females) is contradictory to this prediction, which requires an explanation. Three factors could have affected the evolution of population sex allocation in *T. sorini* (Figure 1). The first is LMC among related males, and the second is LRC among related females. These factors are linked to the low population density and immobility of males and females after birth. If a few winged mothers found a breeding population, and if males of a brood do not disperse away from one another, then they may compete for mates. However, on elm trunks a vast number of *T. sorini* winged females congregate to produce sexuals, and the breeding populations consist of a large number of mothers. In addition, our observations showed that males keep walking to search for mates after the last moulting, as was observed by Foster and Benton (1992) for *Pemphigus spyrothecae*. These conditions render LMC among males of the same brood unlikely. No empirical studies have confirmed LMC in aphids although it has often been postulated (Dagg & Vidal 2004; Foster 2002; Moran 1993; Yamaguchi 1985). We observed weak male–male

competition similar to that observed in P. spyrothecae (Foster & Benton 1992); males sometimes push one another on the back of a female. However, no morphological specialization for fighting is observed in males. In contrast, fighting among first-instar foundresses is very severe and sometimes fatal, as foundresses occasionally kill their competitor using their stylet (Akimoto & Yamaguchi 1997).

Foundresses are adapted for fighting in their morphological specializations, including large body size, strongly sclerotized dorsum and stout elongated hind legs. If fighting occurs among relatives, from the viewpoint of inclusive fitness one would not expect the evolution of such a lethal fighting (but see West *et al.* 2001). The third factor is competition between unrelated foundresses. Several foundresses start galling on the same leaf and foundresses who failed to induce their own galls attack those who are inducing an incipient gall (Akimoto & Yamaguchi 1997). Muramatsu and Akimoto (2016) examined the body sizes of foundresses from nine populations, and in all of these, they detected significant directional selection for larger body size. Mothers who produce a larger number of bigger females could have more numerous granddaughters that survive the galling process and reproduce because of their better fighting ability. Thus, mothers' genetic returns would increase rapidly as they invest more in individual females. Of the three factors described above, competition among unrelated foundresses appears to have had the most significant effect on the

evolution of female-biased sex allocation in *T. sorini* because its effects are stable and accompanied by the evolution of weapon morphology in foundresses. Conversely, LMC would occur only in very limited conditions such as those of extremely low density, since males can disperse freely. LRC would also occur rarely and mainly result in male-biased population sex allocation. Our analyses indicated that mothers have the potential to control the sizes of female and male embryos. Mothers producing a few males produced larger-sized females than those producing four to six males (Figure 5, Table 2). In addition, when mothers produced more females, males of the same brood were smaller in size (Figure 5, Table 2). Therefore, complex trade-offs existed among the sizes and numbers of females and males. These trade-offs would have led to a dichotomy in brood sex composition to maximize maternal returns. In particular, to produce larger foundresses, mothers have to reduce the production of males, thus resulting in a high proportion of all-F mothers (28% in 2001 and 42% in 2017). In contrast, mothers who produce several males can obtain high returns through their sons' reproductive success. Therefore, these trade-offs between the need for specializations for female fighting ability or male function could explain the observed dichotomy in brood sex composition. In contrast, LMC models do not predict the occurrence of all-F mothers (Stubblefield & Seger 1990; Yamaguchi 1985) or variation in female size associated with the maternal nutritive status. Consequently, we postulate that competition among

foundresses could have led to variation in maternal investment in individual daughters, as predicted by the Trivers–Willard hypothesis.

Sexual dimorphism was remarkable in *T. sorini* sexuals. The relative size of females to males was larger than that in any other *Tetraneura* species (Akimoto & Yamaguchi 2004). Furthermore, the relative costs of female production to males, 3.0–3.2, were greater than those in *Prociphilus oriens* (1.85, Akimoto *et al.* 2012). The gigantic size of *T. sorini* females may have evolved due to their gall-usurping behaviour and fighting. Sheldon and West (2004) pointed out that the Trivers–Willard effect is stronger in mammal species in which greater sexual dimorphism is found. The rearing of clones showed that sex allocation and brood sex composition varied widely among clones. Although all the clones produced both sexes, clone B produced a high percentage of all-F mothers, whereas clone A produced a high percentage of 6-male-producing mothers. Overall, the proportion of females produced across all the clones was 44.1%, which was close to that observed in wild populations (41.8% and 45.2%). The differences in the sex ratio among clones were not related with the mean wing length of the mothers, an index of the mean resources acquired by the clones. Thus, this result suggests that nutritional quality on the host plant is not the only determinant of clonal sex allocation. Although we did not design experiments to separate genetic and environmental elements, this variation may

partly have resulted from a genetic factor. In the bird cherry-oat aphid, *Rhopalosiphum padi*, two types of clones were observed: one producing male-biased offspring, and the other producing female-biased offspring, and there was a genetic basis to the two different phenotypes (Rispe *et al.* 1999).

### 4.4.2 Sex ratios in other aphids

Although *T. sorini* is peculiar in the fatal fighting among females, the competition hypothesis can generally be applied to other aphid species and other organisms that alternate between bisexual and unisexual generations. The sex allocation of these species is predicted to be affected by competition in the unisexual generation, including exploitative and interference competition. Williams (1975) emphasized clonal competition as the main factor selecting for the locally best genotype when clonal reproduction continues for several generations within the same habitat. In the wild, aphid clones compete severely, such that the extinction of rare clones and the prevalence of generalist clones have both been documented (Haack *et al.* 2003; Vorburger 2006). In species where exploitative competition is common, mothers could take genetic advantage by producing a larger number of granddaughters in the first unisexual generation (Li & Akimoto 2017). A larger number of granddaughters could provide the mother and her offspring with a head

start for competition continuing through unisexual generations. The tendency for more fecund mothers or clones to exhibit female-biased sex allocation has widely been observed in aphids (Akimoto *et al.* 2012; Akimoto & Murakami 2012; Dagg & Vidal 2004; Miller & Aviles 2000). In some species of Aphidinae, the winged males grown on the secondary hosts migrate to the primary hosts for mating, so that LMC is unlikely. Nevertheless, female-biased sex ratios have been reported in such species (Hales *et al.* 1997). In aphid foundresses, the extent of competition will vary depending on the population density and plant forms. In gall-forming aphids in particular, fighting among foundresses over galling sites has been reported (Akimoto 1988; Aoki & Makino 1982; Inbar 1998; Kurosu & Aoki 1990; Whitham 1979). Based on our results, we predict that as competition in the first unisexual generation becomes stronger, the best strategy for aphid clones would change from female-biased sex allocation to the production of parthenogenetic females only. Further studies are needed to evaluate the relationship between the extent of competition and allocation to both sexes and parthenogenetic females.

**Note**: this chapter is a slightly modified version of "Female-female competition leads to female-biased sex allocation and dimorphism in brood sex composition in a gall-forming aphid" published in Functional Ecology and has been reproduced here with the permission of the copyright holder.

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**Figure 1** Production of the sexual generation (males and sexual females) and the foundress generation in *Tetraneura sorini*. The sexual generation is fully grown in the mother's abdomen and does not feed after birth. Each sexual female has a single egg, which occupies the majority of her body cavity, and deposits it after mating. After hatching from eggs, first-instar foundresses induce galls, or usurp galls induced by conspecifics or heterospecifics. Occurrences of potential competition are indicated as follows: local mate competition (LMC), local resource competition (LRC) and competition against unrelated foundresses.

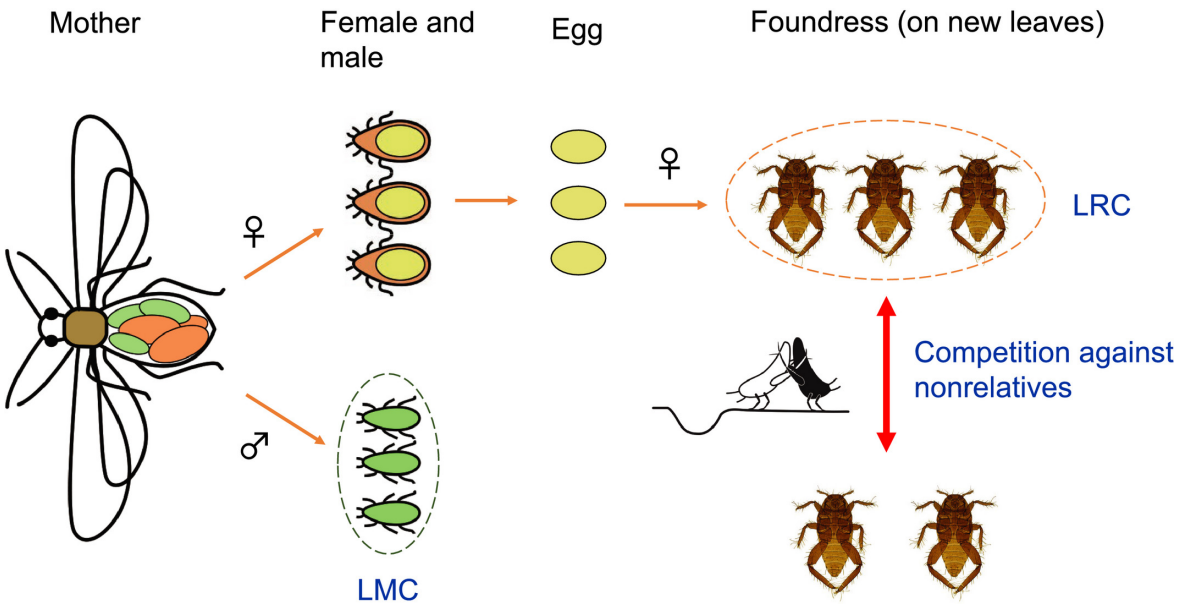

**Figure 2** Contour representation of the distribution of numbers of males and females in a brood in wild populations of *Tetraneura sorini*. The numbers of mothers are indicated in different colours.

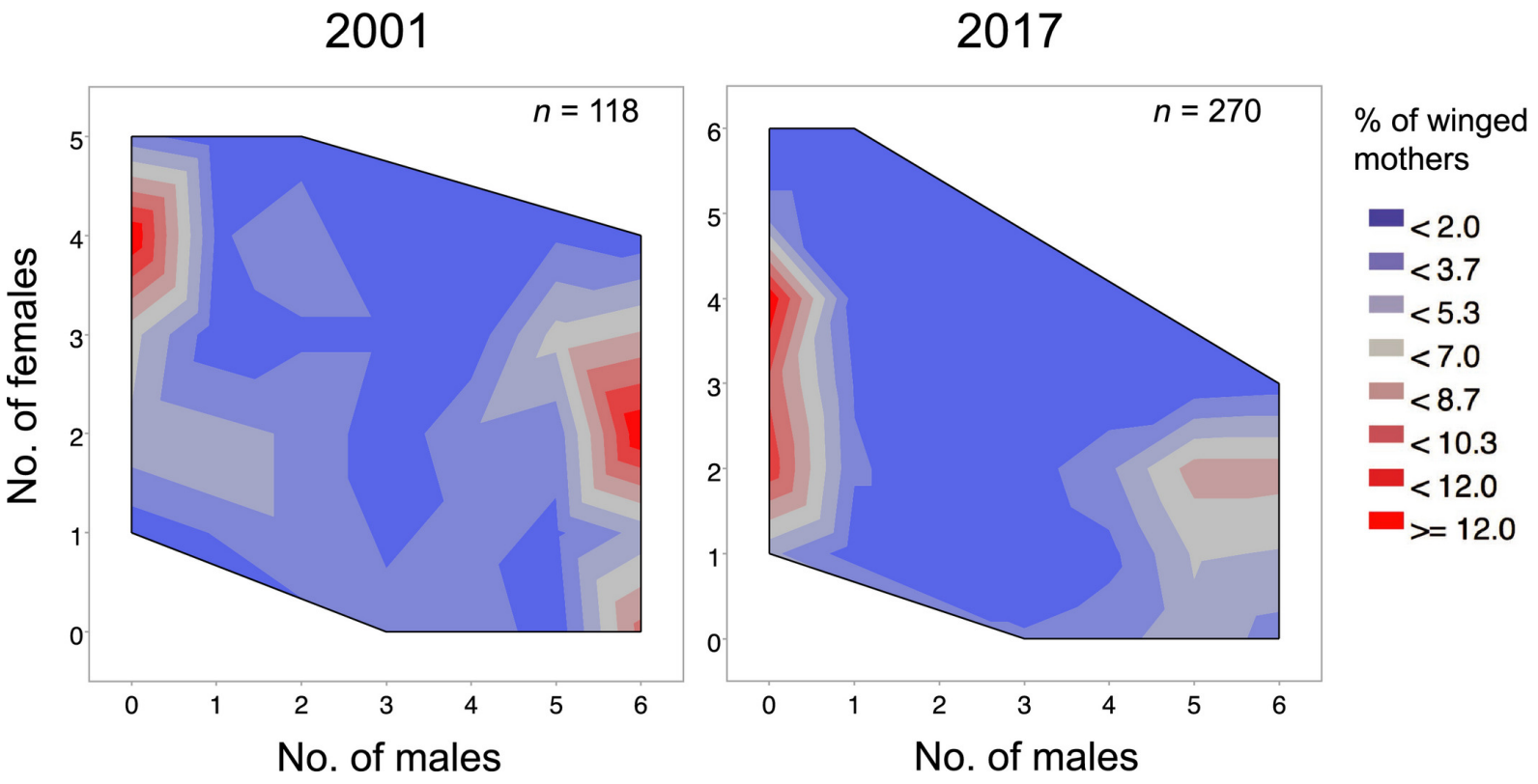

**Figure 3** Regression of numbers of females produced against the maternal wing length for mothers producing either six males or no males. Regression lines with the same slope were applied to these two mother groups.

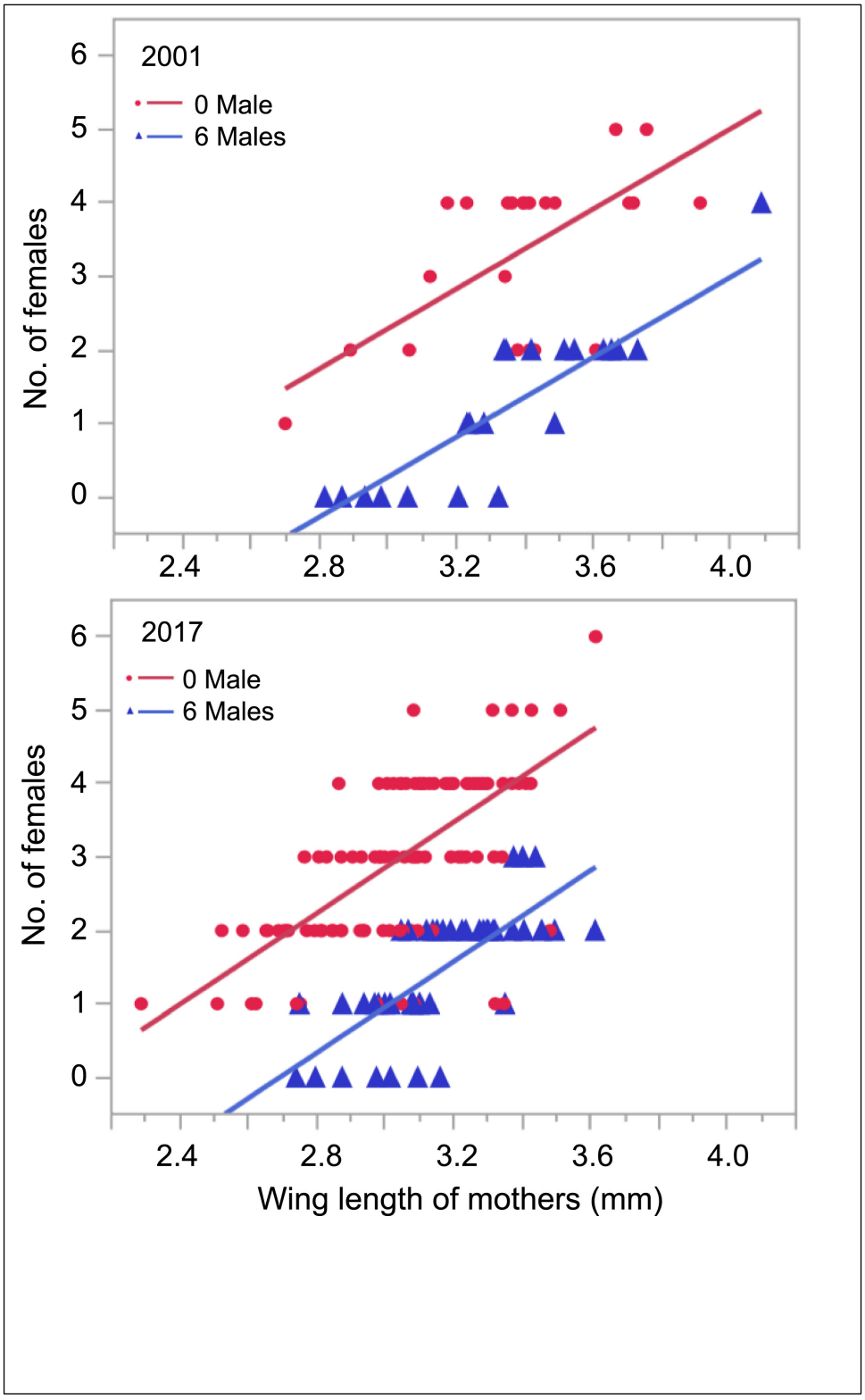

**Figure 4** Regression of the investment in females against the total maternal investment. The amount of investment is converted into the number of males, even if the mother had a mixed-sex or all-female brood.

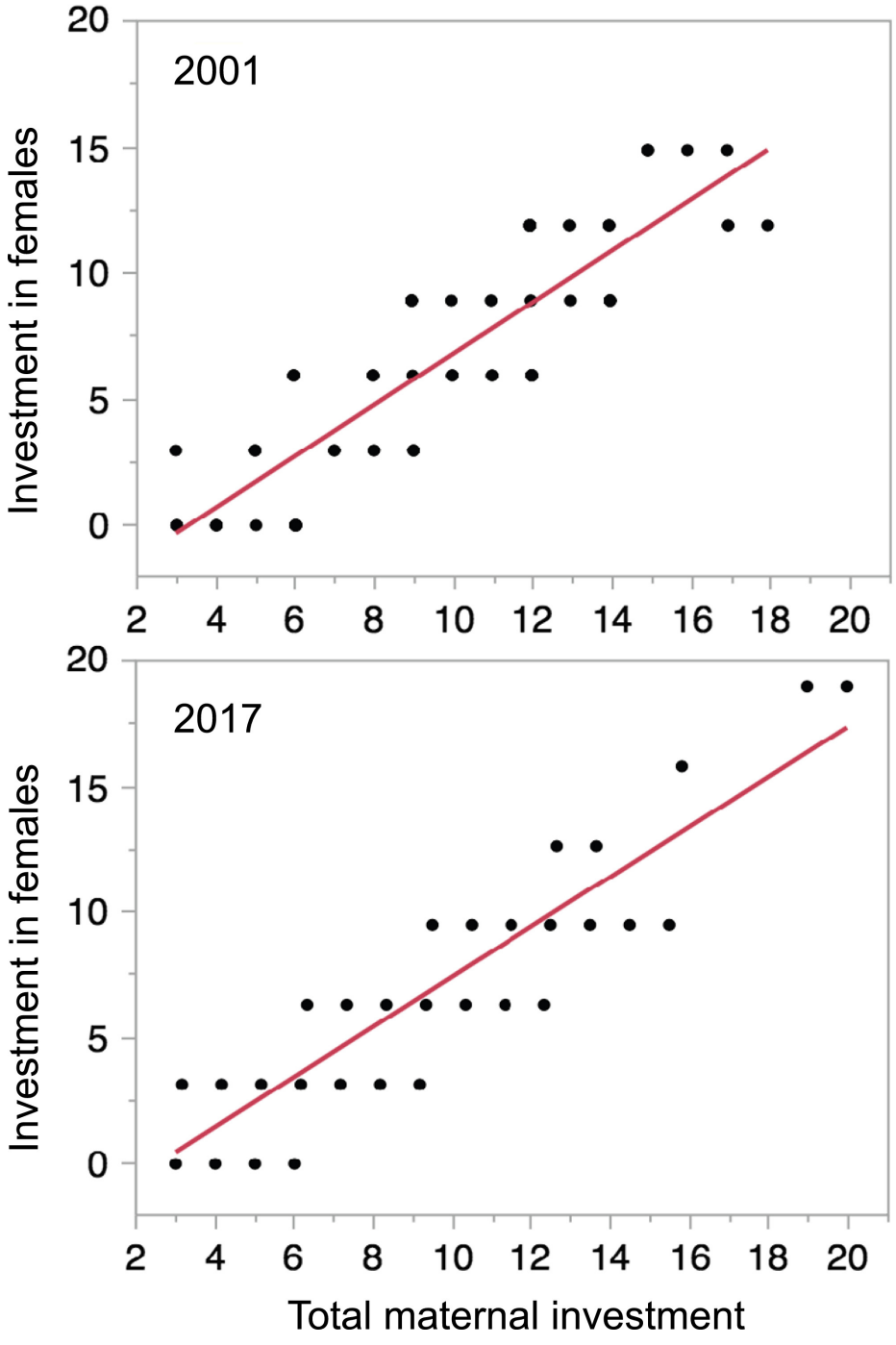

**Figure 5** Contour representation of the distribution of male and female sizes (area) in a wild population. Male and female areas were averaged for each combination of brood sex composition. Redder areas indicate larger body size.

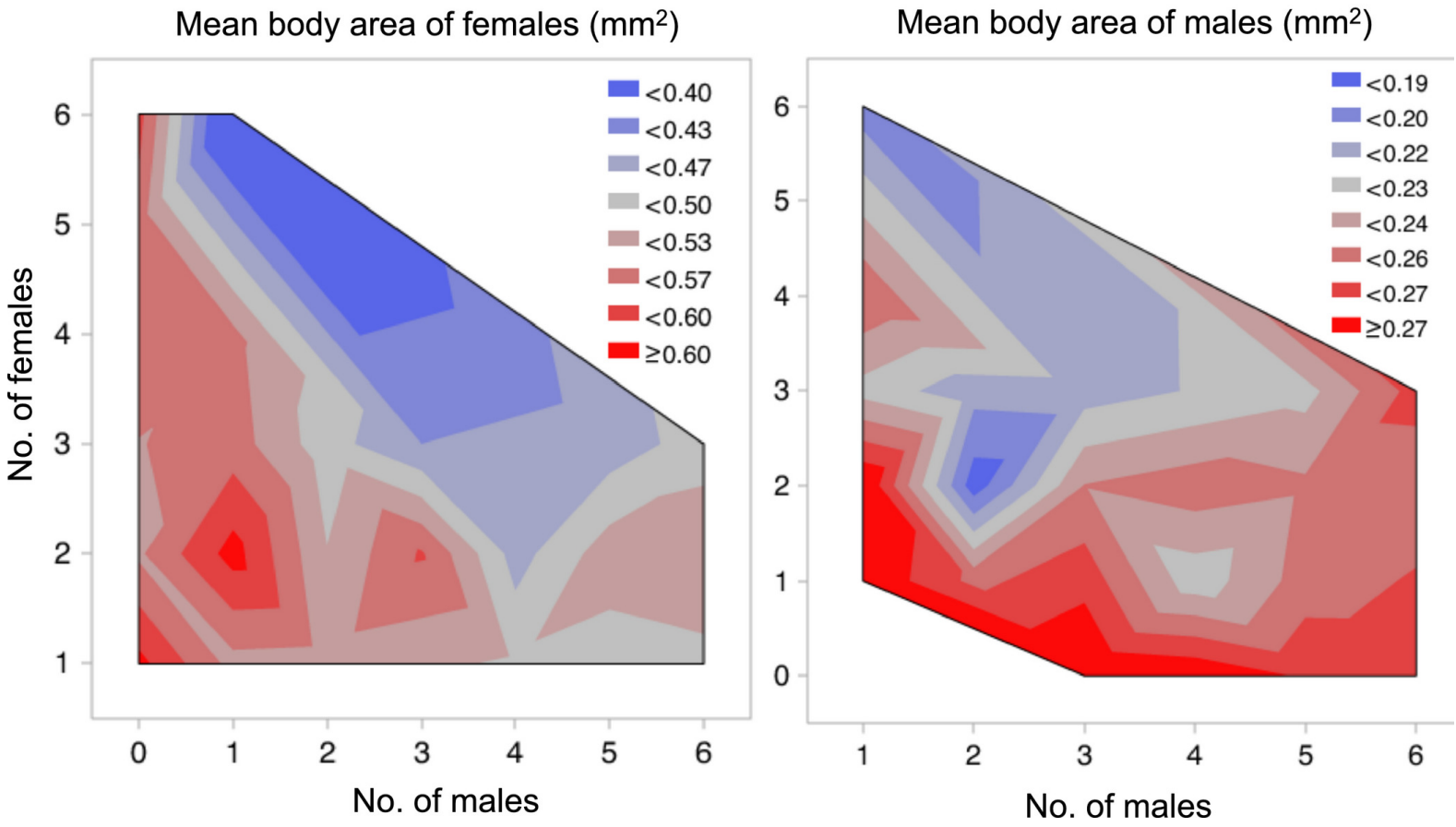

**Table 1.** The sample size, female proportion and the mean maternal wing length in wild populations and reared clones. % females, the percentage of females in sexuals; % M0, the proportion of mothers producing an all-female brood; % M1-5, the proportion of mothers producing 1–5 males and females; and % M6, the proportion of mothers producing six males and females. Mean wing length (in mm); different letters following the figures indicate significant difference in Tukey–Kramer method at a 5% significance level.

| Source of mothers | No. of mothers | % females | % M0 | % M1–5 | % M6 | Mean wing length |
|---|---|---|---|---|---|---|
| Wild population | | | | | | |
| 2001 | 118 | 41.8 | 28 | 44.1 | 28 | 3.41 |
| 2017 | 270 | 45.2 | 41.9 | 41.1 | 17 | 3.07 |
| Clone | | | | | | |
| A | 80 | 24.6 | 5.0 | 41.3 | 53.8 | 2.96 d |
| B | 40 | 97.5 | 92.5 | 7.5 | 0.0 | 3.00 c,d |
| C | 52 | 68.9 | 53.9 | 44.2 | 1.9 | 3.05 c,d |
| D | 52 | 64.9 | 57.7 | 30.8 | 11.5 | 3.37 a |
| T0 | 105 | 63.2 | 58.1 | 40.0 | 1.9 | 3.19 b |
| T1 | 21 | 28.4 | 42.9 | 28.6 | 28.6 | 2.76 e |
| T4 | 119 | 16.7 | 4.2 | 61.3 | 34.5 | 3.18 b |
| T6 | 90 | 22.7 | 12.2 | 82.2 | 5.6 | 2.99 d |
| T7 | 120 | 48.6 | 53.3 | 20.8 | 25.8 | 3.11 b,c |

**Table 2.** Analysis for variation in female body area or male body area using generalized linear mixed models. Female numbers and male numbers in the same brood and the maternal wing length were used as explanatory variables. The mother ID number was specified as a random effect.

| | Female body area | | | | Male body area | | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **Coeff.** | ***df*** | ***F*** | ***p*** | **Coeff.** | ***df*** | ***F*** | ***p*** |
| Female number | −0.0312 | 1,151.3 | 19.88 | <0.0001 | −0.0152 | 1,100.0 | 15.54 | 0.0001 |
| Male number | −0.0178 | 1,156.5 | 34.48 | <0.0001 | −0.0038 | 1,106.3 | 3.07 | 0.082 |
| Maternal wing length | 0.1627 | 1,156.8 | 28.94 | <0.0001 | 0.0285 | 1,100.4 | 2.78 | 0.099 |

# General Discussion

In this book, endosymbiotic bacteria *Buchnera aphidicola*, cuticular hydrocarbons, endoparasitic nematodes, and population sex ratios of gall-forming aphids (Aphididae: Eriosomatinae) are studied and indicating complex biological characters and multiple symbioses of gall-forming aphids.

## *Nutritional compensation in aphid-galling system*

During the coevolution of aphids and *Buchnera*, regulating the biosynthesis of amino acids to host aphids' need becomes advantageous (Latorre *et al.* 2005). Nevertheless, the gall-forming aphids are unique as they can induce species-specific galls that can provide different nutritional contents to aphids to meet different biological requirements (Suzuki *et al.* 2009). Here in chapter 1, the *Buchnera* of *E. harunire* is found losing biosynthesis pathway of ornithine which is precursor of biosynthesis of essential amino acid arginine. Meanwhile, the gall induced by *E. harunire* are rich in arginine compared to galls induced by *T. sorini* and *T. nigriabdominalis* in which *Buchnera* contains ornithine biosynthesis pathway. Nevertheless, arginine is exceptional because it is the only amino acid that was probably synthesized by both the insect and bacterial ancestor of the aphid-*Buchnera* symbiosis and subsequently lost by some aphids (Wilson *et al.* 2010). Though it is unknown whether ornithine can be synthesized by *E. harunire* and its production level, there is close tripartite symbiosis among host plants, gall-forming aphids, and aphid endosymbionts. In addition, gall development is initiated by effectors in aphid saliva which may also contain proteins originated from *Buchnera* (Chaudhary *et al.*

2014). Further, the question remains unanswered whether endosymbionts are involved in gall development towards this close tripartite symbiosis.

To maintain good nutritional source and microhabitats, fighting is commonly observed in gall-forming aphids (Whitham 1979; Aoki & Makino 1982; Kurosu & Aoki 1990a; Akimoto & Yamaguchi 1997; Tong & Akimoto 2019). Competition for incipient galls or galling sites occur among foundresses by kicking and pushing competitors. The fighting is usually fatal for one side as once any competitors fail in gall usurpation, they lose all fitness. In chapter 4 in *T. sorini*, larger foundresses have an advantage in fighting, and they hatch from larger eggs. In eriosomatine aphids, the female lays only one egg, which is almost as long as herself (Heie 1980). Therefore, high maternal investment in individual females can consequently produce large foundresses (granddaughters). The analysis of *T. sorini* mothers supported the Trivers–Willard hypothesis with reversed sex roles, indicating that more fecund mothers invested more in females in total; that is, they produced more female-biased broods and larger females. However, there was no tendency for more fecund mothers to produce larger males. The tendency for more fecund mothers or clones to exhibit female-biased sex allocation has widely been observed in aphids (Akimoto *et al.* 2012; Akimoto & Murakami 2012; Dagg & Vidal 2004; Miller & Aviles 2000). Although *T. sorini* is peculiar in the fatal fighting among females, the competition hypothesis can generally be applied to other aphid species and other organisms that alternate between bisexual and unisexual generations. In some species of Aphidinae, the winged males grown on the secondary hosts migrate to the primary hosts for mating, so that LMC is unlikely. Nevertheless, female-biased sex ratios have been reported in such species (Hales, Tomiuk, Wohrmann, & Sunnucks, 1997).

## *Polyphenism and multiple symbioses of gall-forming aphids*

Gall-forming aphids exhibit seasonal polyphenism to adapt various environment and polyphenism undoubtedly enhance the formation of multiple symbioses. In galling generation, there is close tripartite symbiosis among plants, gall-forming aphids, and aphid endosymbionts. Further, endosymbiotic bacteria *Buchnera* is strictly vertically transmitted among all generations. Along with host alternation and polyphenism, unexpected symbiotic relationship may come as well. My record in chapter 3 is reporting the first case of a mermithid parasitizing eriosomatine aphids and the fourth record with respect to Aphididae. Mermithid parasitism of aphids is not commonly known and only three cases have been reported (Guercio 1899; Davis 1916; Poinar 2017), although this could be due to undersampling of the aphids for this condition. Mermithid nematodes parasitized autumnal females that emerge after generations underground. Juvenile mermithid nematodes were found to parasitize autumnal females of *E. auratum* and *T. radicicola* and this parasitism in autumnal females led to fewer and smaller female sexual embryos. It is not clear whether the parasites negatively affect offspring fitness by competing for nutritious resources directly or whether maternal investment changes in response to parasitism. Thus, it is necessary to increase the sample size to investigate host manipulation by mermithid nematodes in future studies. Moreover, parasitizing adult hosts could be a dispersal strategy for mermithid nematodes (Campos & Sy 2003; Di Battista *et al.* 2015).

To confront with different ecological conditions during seasonal polyphenism and host alternation, functions and components of CHCs vary among generations. The CHCs of all female morphs were characterized by high proportions of n-alkanes. It has been reported that n-alkanes are also typical constituents of leaf cuticular

waxes (Eglinton & Hamilton 1967; Herbin & Robins 1969). This suggests that n-alkanes might have primarily evolved in female morphs as a chemical mimicry to the host leaves or twigs to evade predators and parasitoids. Although every morph of *P. oriens* is equipped with waxy substances on the body surface, spring and autumnal migrants have especially long and thick wax filaments. High proportions of methyl-branched alkanes were detected in waxy substances of autumnal migrants, which migrate from *Abies* roots to *Fraxinus* trunks. During the formation of aggregates, autumnal migrants could recognize other members by using these methyl-branched alkanes, as they are generally known to mediate chemical communication in insects (Nelson 1978; Chung & Carroll 2015). Sexual females, as well as autumnal migrants, are peculiar in containing long-chain n-alkanes, which were definitely different from male CHCs. Sexual females of *P. oriens* do not have scent plaques either but large and conspicuous wax gland plates on the lateral sides of their abdominal tergites I–VI. In contrast, males have no wax gland plates and no special sensory organs in their antennae (Tong, unpubl. data, 2019). Males choose mates based on tactile stimuli. Therefore, evidence suggests that CHCs secreted from female wax gland plates function as a sex attractant.

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