RELATEDNESS AMONG GALLING APHIDS AS DETERMINED BY AFLP ANALYSIS

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Brian G. Taylor

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ABSTRACT

RELATEDNESS AMONG GALLING APHIDS AS DETERMINED

BY AFLP ANALYSIS

by

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Cooperative or eusocial behavior is not unknown among gall-inducing insects, but the ecological and evolutionary contexts which produce such behaviors vary in their details. In Northern California, the manzanita leaf-gall aphid *Tamalia coweni* has shown a tendency to communally occupy leaf galls, in contrast to solitary gall occupation, the mode more typical for galling aphids. One potential explanation for this behavior is that kin selection may make communal gall occupation a viable alternative to solitary gall occupation, due to a high degree of relatedness between gall co-occupants and increased numbers of offspring in communally occupied galls. To evaluate this, the "kin selection hypothesis," my study involved recording the frequency of communal behavior in a local population of *T. Coweni* on *Arctostaphylos patula,* and evaluated differences in reproductive rates between single and multiple-occupant galls and the degree of relatedness among gall co-occupants. Out of 375 galls examined, 11 percent were communally occupied, with double-foundress galls being the most common type of

communal gall. The maximum number of communal foundresses found in any single gall was five. The productivity of communal galls (measured by numbers of offspring per gall) was higher than for single-foundress galls on a per-gall basis but lower *per capita*. AFLP-PCR was used to gentotype individual aphids from communal galls. Analysis of genetic distance between individuals revealed that relatedness among gall cohabitants was higher, on average, than for non-gall cohabitants. Parsimony analysis of the AFLP profiles found a number of highly parsimonious pairs cohabiting in galls. These results support the kin selection hypothesis, and suggest that communal gall occupation in this species represents a form of cooperative behavior, which may facilitate elaboration of cooperation, and, potentially, the origins of caste and eusociality in this species.

CHAPTER I

INTRODUCTION

Natural selection is inextricably tied to the concept of fitness, which is defined in a number of ways, including possession of adaptations to the immediate environment (Darwin 1869), rates of survival (Spencer 1864), of reproduction (Austin & Short 1985), or even as a probability function (Smith 1989). The variable nature of fitness (and by extension, adaptation) must be remembered when attempting to interpret seemingly non-adaptive behaviors in an evolutionary context. In the social insects, for example, there are numerous instances of sterile worker or soldier castes whose presence benefits the colony as a whole but whose personal fitness as non-reproductives is effectively zero (Wilson 1971, Itô 1989, Stern & Foster 1996). The persistence of these organisms would appear anomalous by the standard definition of fitness via reproductive success; however, this phenomenon becomes less puzzling when the definition of fitness is expanded to include not only individual reproductive success but the success of relatives (sharing many of the same genes), a concept known as inclusive fitness (Hamilton 1963, 1964, Fletcher & Zwick 2006).

In the same way that Inclusive Fitness extends the definition of fitness beyond the level of the individual organism, a modified version of selection, referred to as kin selection (Smith 1964 Hamilton 1964, Miller 1998b), expands the concept of selection beyond the individual unit of selection to define it as acting at a family or population

level, where related individuals who have a large number of genes in common are selected for or against as a group. Kin selection is often invoked to explain occurrences where individuals behave in a way that enhances the fitness of other members of their family or population even at risk to their own fitness, known as "altruistic" behavior (Hamilton 1972, Abbot et al. 2001). Such behaviors are not adaptive at the individual level but kin selection may favor their evolution, and this is the most well-understood explanation for the prevalence of such behavior, which is common among social organisms. Thus, when similar behaviors arise in what are considered to be non-social species, it begs the question of whether such behaviors are merely anomalous or indicative of a degree of socialiity (and presumably, relatedness) within the species or population being observed.

One such example of apparently maladaptive behavior which may be an unrecognized form of altruism and is related to kin selection occurs in populations of the galling aphid *Tamalia coweni*. In northern California, this species induces leaf galls on the host plant *Arctostaphylos viscida* and both leaf and inflorescence galls on *Arctostaphylos patlua*. In the study population, communal gall occupation has been observed (multiple reproductives sharing a gall rather than each having their own). What is surprising about this behavior is that it appears to decrease reproductive success for all gall co-habitants by lowering numbers of offspring produced *per capita*, yet communal galling persists (Miller 1998a, b).

Tamalia coweni, like many other aphids, reproduces in part by parthenogenetic (asexual) reproduction, so one possible explanation for communal gall occupation is that the co-occupants are often genetically similar and thus are not

competing but rather cooperating with one another. Lending additional plausibility to this hypothesis is the fact that overall numbers of offspring produced are often higher in communally founded galls (meaning cohabitation could be beneficial if gall-mates are closely related) and that other galling aphid species which show communal galling behavior have evolved sociality (Abbot et al. 2001, Johnson 2002, Bryden & Jansen 2010).

Although kin selection may potentially explain communal gall occupation in *Tamalia coweni*, there is little direct support for this hypothesis. Therefore, this study was designed to assess: (I) the continued presence and frequency of gall cohabitation in a local population of this species, (II) the productivity of communally occupied galls, and (III) the relatedness of communal gall occupants. The first two aspects were evaluated by collection and dissection of galls in the study area; the third aspect was evaluated via the use of Amplified Fragment Length Polymorphism (AFLP) analysis, calculation of genetic distances and phylogenetic analysis using parsimony. I observed increased productivity in communally occupied galls, and a high level of relatedness among gall co-occupants. These results strongly support for the kin selection hypothesis.

CHAPTER II

LITERATURE REVIEW

Tamalia coweni – An Overview

Tamalia coweni was first described as *Phyllaphis coweni* by Cockerell (1905) although its earliest report was by Cowen (1895). This initial description was based solely on wingless individuals obtained from leaf galls. Both the oviparous and parthenogenic winged females were subsequently described by Gillette (1909). The genus *Tamalia* was described by A.C. Baker in 1920 in *Generic classification of the hemipterous family Aphididae* (the family Aphididae Latreille itself was established in *Histoire naturelle, générale et particulière des crustacés et des Insectes,* 1802). The Subfamily Tamaliinae was established within the Aphididae by Remaudière and Stroyan (1984). In addition to *Tamalia coweni* (Cockerell 1905) and its synonym *Cryptosiphon tahoense* (Davidson 1911) the genus includes the species *dicksoni* (Remaudière & Stroyan 1984), *keltoni* (Richards 1967), *pallidus* (Richards 1967) and *inquilinus* (Miller & Sharkey 2000).

A summary description of the genus by Gillette & Palmer (1931) reads as follows:

> *Characters* – Frontal tubercles absent. First tarsal joint triangular. Cornicles mere flanges on low, conical bases. Antennae 6-jointed, minutely setose and with narrow sensoria. Fore wings with media twice branched and arising proximad of stigmae, hind wings with media and cubitus. Cauda broader than long and rounded. Anal Plate entire in all female forms. Eyes in apterae of 3 facets only. Sexes both alate. Oviparae laying many eggs. Living concealed in pseudo-galls on leaves. (p. 883)

The galling behavior described in the final sentence is not is not unique to this genus, family, or order. Galling aphids, in general, tend to be affiliated with specific host plants, but although they seem to possess specific host preferences the relationships are not always obligatory, as some species have been observed using multiple host plants (Dixon 1985). In addition to alternating sexual and asexual generations, they also have complex life cycles which show high diversity and region-specific variations (Wool 2004).

The specifics of *Tamalia coweni* biology have been elucidated most thoroughly by Miller, who has reviewed the species's ecology and life history (1998a), communal gall occupation and kin selection (1998b) and rates of evolution and host-plant specificity (2003), in addition to co-authoring a description of an inquiline species closely associated with *coweni*, *Tamalia inquilinus* (2000).

The terminology used to describe *Tamalia* biology is derived from Blackman's simplification of aphid terminology (Blackman 1994). Asexual (parthenogenetic) females are termed viviparous, while the mating females are oviparous. Any wingless morph which induces gall formation is referred to as a foundress, and a gall occupied by more than one foundress is a communal gall (Wilson 1971).

There are two distinct groups of gall producers in this life cycle, the first of which are the recombinant stem mothers, or fundatrices (Miyazaki 1987), which emerge between late April and early June from overwintering eggs laid at the base of the host plant (*Arctostaphylos patula* in the study area) by sexual winged females after mating. These stem mothers induce gall formation after migrating to the new leaves of the host plant, and undergo four molts while inside the gall, after which they produce a generation

of viviparous winged females by parthenogenesis (Miller 1998a). These asexual winged females also undergo several molts inside the gall before completing their final molt outside the gall and aerially dispersing. These second-generation winged females parthenogenetically produce winged males, which are also winged, winged mating females, and third-generation parthenogenetic winged and wingless females (Miller 1998a, Miller & Crespi 2003). The second group of gall inducers is the aforementioned third-generation wingless females (foundresses), which induce a second cohort of galls on inflorescences and leaves (Figure 2) of the host plants after emergence, where they produce a fourth generation of mating males and females. Males and mating females mate after dispersing and the overwintering eggs are deposited by the mating females between July and September. The cycle occurs a month later in *A. patula* than in the similar *A. viscida,* which is also used as a host plant in Northern California and it is not uncommon for additional gall-inducing generations to occur on *A. patula* (Miller & Crespi 2003).

In both cycles of gall induction, the early-phase gall is not fully enclosed, and thus allows entry by a variety of common aphid predators, includng hemipterans, dipterans and neuropterans (Hagen & VanDenBosch 1968, Moran 1993, Rojo & Marcos-García 1997). Although some Asian galling aphids have evolved soldier-like morphs (Ito 1989) which protect against predators, the only species known to do so in North America is *Pemphigus obesinymphae* (Moran 1993, Stern & Foster 1996, Foster & Rhoden 1998). However, not only do populations of *Tamalia coweni* lack the ability to exclude predators from their galls prior to enclosure (before the gall is fully formed and thus still open to invasion), they seem to show an unusual tolerance for other galling aphids. Thus,

foundresses can either initiate galls of their own or migrate to a site occupied by another foundress and communally occupy the gall formed there.

There is a well-documented history of competition and antagonistic behavior among sympatric galling aphid species and even conspecific foundresses, which can sometimes be severe enough to result in mortality (Whitham 1979, Aoki & Makino 1982, Stern & Foster 1996, Inbar 1998). In contrast, *T. coweni* foundresses readily tolerate the presence of other conspecific foundresses within their gall, as well as an inquiline species, *Tamalia inquilinus*. This behavior is puzzling from an adaptive perspective, because communal gall occupation appears to decrease *per capita* survival and productivity (Miller 1998b).

Miller (1998b) proposed that this is a density-dependent effect, and that "…communal gall occupation is foremost a function of foundress density in the absence of competitive displacement" (p. 100). Inbar (1998) arrived at a similar conclusion. Whether galling sites are truly limited is an open question; there are indications that the significance of gall location on reproductive success (and therefore the number of optimal galling sites) may be variable depending on the host and aphid species (Whitham 1978, Burstein & Wool 1993). An alternative hypothesis for the communal behavior is that a form of kin selection may be favoring the production of multi-foundress galls. This hypothesis is supported by the possibility that multiple asexual generations and low geographic dispersal distances create populations consisting of individuals who are closely related and despite losses in per capita fitness the overall productivity of multi-foundress galls is increased, thus potentially conferring an adaptive advantage at the group level (Hamilton 1987, Miller 1998b, Bryden & Jansen 2010).

In essence, this hypothesis would imply that these aphids are behaving cooperatively to enhance family-level fitness despite losses in personal fitness, that is, they are acting as primitively social or eusocial organisms. This implication is not unprecedented, as gall-making aphids in general are known to be one of the few groups of clonal and/or haplodiploid taxa to be considered eusocial, although that distinction is not applicable to all species or populations (Nowak et al. 2010). Here I review eusociality and the closely related concept of kin selection to provide the appropriate context for further examining the validity of this theory.

Eusociality and Kin Selection

The phenomenon of individual organisms exhibiting behavior which benefits others but is often harmful to their own fitness (for example, a sterile soldier ant defending a nest) was recognized by Darwin (1869) himself as a "special difficulty" for the theory of evolution. However, he proposed an explanation, "This difficulty, while appearing insuperable, is lessened, or as I believe, disappears, when it is remembered that selection may be applied to the family as well as the individual, and may thus gain the desired end" (Darwin 1869, p. 291).

Both Fisher (1914, 1930) and Haldane (1927, 1932, 1955) would later elaborate on altruism (acts beneficial to others that come at a cost to the individual) and the idea of inclusive fitness (where both the individual reproductive success and that of their relatives is considered in evaluating Darwinian fitness). Expanding on their work, Hamilton (1963, 1964) developed a mathematical model defining inclusive fitness, producing "Hamilton's rule" describing conditions favorable to cooperation which can be

expressed most simply as $r > c/b$, where c represents the cost (to the donor) of altruistic behavior, b is the benefit gained by the recipient of the behavior, and r is a coefficient of relatedness (Fletcher & Zwick 2006, Nowak et al. 2010).

In Hamilton's model, cooperative or eusocial behavior should be favored when r exceeds c/b, as the gain to the genotype as a whole outweighs the cost to the individual animal acting as a donor. These conditions are most likely to be met in populations of closely related individuals. Thus it is no surprise that the most frequent and most well-documented examples in animals have come from insects, in particular members of the order Hymenoptera which have haplodiploid sex determination, such as ants, bees and wasps (Michener 1964, 1974; Wilson 1971, 1975; Lin & Michener 1972, Chapman 1982, Holldobler & Wilson 1990, Ross & Matthews 1991, Itô 1993, Linksvayer & Wade 2005) as well as aphids, which can have multiple parthenogenetically produced generations within their populations (Itô 1984, Foster & Northcutt 1994, Abbot et al. 2001).

Given this context, it appears plausible that these aphids, like populations of other gall-forming species, cooperate to maximize group fitness. This would suggest that the productivity of communal galls and the relatedness among gall cohabitants should both be higher than average, which would promote such behavior. Productivity can be measured directly, but there are a number of methods for determining relatedness, discussed in the next section.

Molecular Genotyping Methodologies

One of the earliest methods for assessing genetic diversity utilized variable number tandem repeats, or 'minisatellites,' 60-100 base pair regions of high variability throughout the genome whose combinations are unique to each individual **(**Jeffreys et al. 1984, 1985, Nakamura 1987). The technique was enhanced by the discovery that enzymatic amplification of these regions was possible using DNA polymerase and specific oligonucleotide primers which allow a degree of selectivity in the amplification, a technique called polymerase chain reaction or PCR (Jeffreys et al. 1998).

Refinements of these techniques have yielded a variety of similar methods being developed, such as analysis of simple sequence repeats (SSR); "microsatellites," highly polymorphic 2-6 base pair DNA sequences (Goldstein 1995, Blouin et al. 1996, Dakin & Avise 2004, Varela & Amos 2010), analysis of restriction fragment length polymorphisms (RLFP), fragments of variable length produced by enzymatic cleavage of DNA at specific sites in the genome which are also used in linkage and mapping studies (Botstein *et al*. 1980, Paterson et al. 1988, Borgo et al. 1996, Wolf et al. 2000), random amplified polymorphic DNA markers (RAPD), arbitrarily amplified sequences produced by PCR using nonspecific primers (Williams et al. 1990, MacGowan 1993, Barbanera *et al*. 2010), and amplified fragment length polymorphisms (AFLP), restriction-digested DNA amplified using selective primer combinations (Vos et al. 1995, Vos & Kuiper 1997, Ren & Timpko 2001, Vuylsteke et al. 2007).

Most of these techniques have been successfully applied to analyses of genetic variation in aphid populations (Carvalho et al. 1991, Fukatsu & Ishikawa 1994, Abbot 2001, Johnson et al. 2002). The AFLP technique has become increasingly popular

because it is fast, accurate, requires no prior knowledge of the sequences being amplified and can be performed with small quantities of sample DNA. These advantages became apparent quickly; and the AFLP method has become more commonplace. It has been utilized for aphid populations in particular by Hawthorne and Via (2001) Braendele (2005), Timm et al. (2005), Ritter *et al*. and Vorwerk & Forneck (2007), among others. Because of its advantages over other methods and its previous use in similar contexts, the AFLP method was chosen for this study.

The AFLP process consists of three principal components: enzymatic digestion of genomic DNA using a combination of restriction enzymes, ligation of PCR adaptors to the digest products, and amplification of the ligated products using DNA polymerase and carefully chosen primers. The enzymes used in the first step are typically EcoR1 and Mse1, a combination which produces fragments of optimal size while simultaneously limiting the number of potential amplification products (Vos et al. 1995). Because the sequences cut by these enzymes are known, the adaptor pairs are designed to correspond to these sequences. The primers used in the amplification reactions extend into the fragments themselves and only complementary regions are primed for amplification. Typically there are two rounds of amplification, one (the "preslelective" amplification) using a single-base extenstion into the fragment and the second (the "slelective" amplification) using a two to four-base extenstion (Muller & Wolfenbarger 1999). The use of selective primers reduces the number of fragments amplified. Amplification products were traditionally separated by gel electrophoresis, although the use of fluorescently-labeled primers and capillary electrophoresis using automated scoring software is becoming more common (Meudt & Clark 2007).

For this research, I used fluorescently labeled primers and capillary electrophoresis. The specifics of the AFLP-PCR protocol are given in more detail in chapter III.

CHAPTER III

MATERIALS AND METHODS

Sample Acquisition

Samples were taken on multiple dates in June-September 2007 from an undeveloped area of northern California accessed via a logging road (N 40°, 2’24’’, W, 121° 36’25”, elevation 1350m, Figure 1). This locality has an abundance of *Arctostaphylos patula*, which exhibit the late-blooming inflorescences characteristic of higher-elevation populations, and also seem to be particularly susceptible to gall induction on both leaves and inflorescences into late August and even September.

Galls were sampled haphazardly along atransect at approximately 5-meter intervals. Galls were inspected to determine if they appeared intact (fully eclosed without obvious apertures or indications of herbivory); galls which appeared to have been vacated or damaged were not collected. Once a viable gall had been obtained on a particular plant, no further samples were collected from the same plant. Galls were bagged and transported to the Biology department at CSU, Chico, where they were stored at 4° C.

Collected galls were opened and examined individually under a dissecting microscope, and numbers of foundresses and total offspring per gall were recorded. The presence of inquilines and predators was also noted. Foundresses were rinsed in deionized water and individually stored in salt buffer at 4°C. Foundresses from communally occupied galls were assigned a prefix indicating the number of foundresses

Figure 1. Map of the sampling location. The enlarged area is Butte County."X" indicates the sampling area.

(2X, 3X, etc.) numbered sequentially, assigned a suffix demarcating individual foundresses (F1, F2, etc.), and set aside for use as samples in the ALFP-PCR reactions. Ultimately, fifteen double-foundress galls (2X01-2X15) and one triple-foundress gall (3X01) were profiled using the AFLP-PCR protocol described below.

Extraction of Genomic DNA

An Ultra UltraClean® Tissue & Cells DNA Isolation Kit was purchased from MoBio laboratories, Inc. (http://www.mobio.com/tissue-cells-dna-isolation/ultraclean-

tissue-cells-dna-isolation-kit.html). Individual aphids were ground with sterile pestles in a microcentrifuge tube, and then homogenized using a bead solution and vortex mixing at high speed for 10 minutes. Supernatant containing suspended genominc DNA was separated by centrifugation and an approximate volume of 400 µl was recovered. DNA extracts were stored at -20°C until they could be used in AFLP-PCR reactions.

Restriction-Ligation Protocol

A small-genome AFLP kit including primers, AFLP core mix and adapter pairs was purchased from Applied Biosystems (http://www.appliedbiosystems.com/absite/us/en/home.html), and the manufacturer's protocol was followed to optimize products for subsequent analysis using an ABI PRISM® 310 Genetic Analyzer.

An enzyme master mix was prepared using 1 µl 10X T4 DNA ligase buffer with ATP, 1.0 µl 0.5 M NaCl, 5 µl Bovine serum albumin (1 mg/ml), 10 units T4 DNA ligase (.5ul), 1µl sterile water. This solution was stored on ice until it could be distributed into individual reaction tubes. Five µl of extracted DNA was combined with 1 µl 10X t4 dna ligase ligase buffer, 1.0 µl 0.5 M NaCl, 0.5 µl Bovine serum albumin (1 mg/ml), 1 µl of enzyme master mix, and 1.0 µl each of Mse1 and EcoR1 adaptor pairs. Prior to use, adaptor pairs were annealed by heating at 95 °C for 5 minutes and allowed to cool to room temperature. The mixture was then maintained at 37 °C for two hours using a thermal cycler with a heated lid to allow the reaction to proceed. After the two hours 189 µl of 0.1X TE buffer was added. The restiriction-ligation products were stored at -20° C for later use in amplification reactions.

Pre-selective Amplification Protocol

Four µl of the restriction-ligation product were combined with 0.5 µl each of EcoR1 and Mse1 preselective primers and 15.0 µl AFLP core mix. Samples were loaded into a thermal cycler and run through a cycle of 2 minutes at 72°C, 20 cycles of (20 seconds at 94°C, 30 seconds at 56°C, two minutes at 72°C) and were then held at 60°C for 30 minutes.

Selective Amplification Protocol

Preliminary tests on single-foundress galls using varying primer combinations (Miller, unpublished) determined that the primer combination Mse1-CAC and EcoR1-ACA yielded the highest variability, and this was the combination used in the selective amplifications.

A preparatory dilution was prepared with 10.0 µl of pre-selective amplification product combined with 190.0 µl of 0.1X TE Buffer in a sterile 0.5 ml PCR reaction tube. Dilutions were used immediately after preparation. The selective amplification mix consisted of 3.0 µl diluted preselective amplification product, 1.0 µl Mse1-CAC primer 1.0 µl fluorescently labeled EcoR1-ACA primer and 15.0 µl AFLP core mix, which were combined in a 0.2 ml PCR reaction tube and run through a PCR cycle of 2 minutes at 94°C, 10 decreasing temperature cycles of (20 seconds at 94°C, 30 seconds at 66-57°C, and 2 minutes at 72°C) 20 cycles of (20 seconds at 94°C, 30 seconds at 56°C, and 2 minutes at 72°C) and a 30 minute hold cycle at 60°C using a thermal cycler with a heated lid. Products were stored at - 20°C.

To ensure products were free of extraneous primers, nucleotides, or other impurities, a PCR purification kit was purchased from Qiagen (http://www.qiagen.com). A binding buffer was used to cause DNA adsorbtion to a silica-gel membrane contained in a spin column while impurities were separated by centrifugation. The DNA was then eluted into sterile buffer solution. The final elution volume was 50 ul.

Product Analysis

Separation and sizing of the purified selective amplification products using capillary electrophoresis was performed at the CSUPERB Microchemical Core Facility at San Diego State University (http://www.sci.sdsu.edu/dnacore/sdsu_dnacore.html). Samples were processed using an ABI PRISM® 310 Genetic Analyzer using POP-4™ polymer, and ROX-500 as a size standard.

Output from the genetic analyzer was processed using Peakscanner® software, (appliedbiosystems.com). For all samples, ROX-500 was the size standard and the sizing default analysis method was used. In accordance with the analyzer's capabilities (single base pair detection below 250 bp, 2bp detection above 250) fragments from 100-250 base pairs were scored at 1 bp intervals and fragments from 250-500 bp were recorded at 2 bp intervals. Fragment sizes were recorded at their nearest integer value, and a binary matrix was constructed with products scored as either present (1) or absent (0) for each individual.

Data Analysis

To determine the similarity between the AFLP profiles produced by different individuals, genetic distance (GD) was calculated according to the formula:

$$D_{xy} = 1 - [2N_{xy}/(N_x + N_y)]$$

where Nx is the number of loci detected for individual *x*, N*y* is the number for individual *y,* and N*xy* is the number that individuals x and y have in common (Nei & Lei 1979, Ren & Timko 2001). This formula produces values from 1 to 0, where 1 represents the maximum possible distance between individuals (N*xy*=0) and 0 represents the maximum homology between individuals, where 2N*xy* = (N*x* + N*y*). GD values were calculated for intra-gall pairs (individuals from the same gall) and all possible inter-gall pairs (individuals from different galls).

The program PAUP (Phylogenetic Analysis Using parsimony, Version 4.0) was used to compare the fragment profiles from all individuals and GD values were compared using the program R, version 2.14.1 (http://www.r-project.org).

CHAPTER IV

RESULTS AND DISCUSSION

Frequency of Gall Cohabitation and Gall Productivity

A total of 375 galls were examined, of which 43 were found to be communally occupied. Among the communally occupied galls, double-foundress galls were the most common, followed by triple-foundress galls. An equal number of quadruple- and quintuple-foundress galls were found. No galls were found with more than five foundresses. Results from the gall dissections are summarized in Table 1.

Table 1. Numbers of single and multi-foundress galls and numbers of offspring per gall.

Gall type (# of foundresses)	# of galls	Percentage	Offspring per gall, mean
Total Galls Examined	375	-	-
Single (1X)	332	89%	20.33 ± 11.4
Double (2X)	24	6%	25.58 ± 14.9
Triple (3X)	11	3%	34.00 ± 14.9
Quadruple (4X)	4	1%	32.33 ± 12.2
Quintuple (5X)	4	1%	31.00 ± 6.0
Total communal galls	43	11%	27.18 ± 15.0

*Note: galls with zero offspring or signs of predation were excluded from this calculation.

The number of offspring per gall was highly variable, with a number of galls having one or multiple foundresses present with no offspring, and the maximum number for any gall being 79, in one of the quintuple-foundress galls. A number of galls were found to contain predators, and because it could not be determined how many of the

occupants were consumed prior to dissection, these galls were not included in the analysis of productivity. Single-foundress galls had an average of roughly 20 offspring per gall, while communally occupied galls had an average of approximately 31. Triple-foundress galls had the highest overall productivity with an average of 34 offspring per gall (Figure 2).

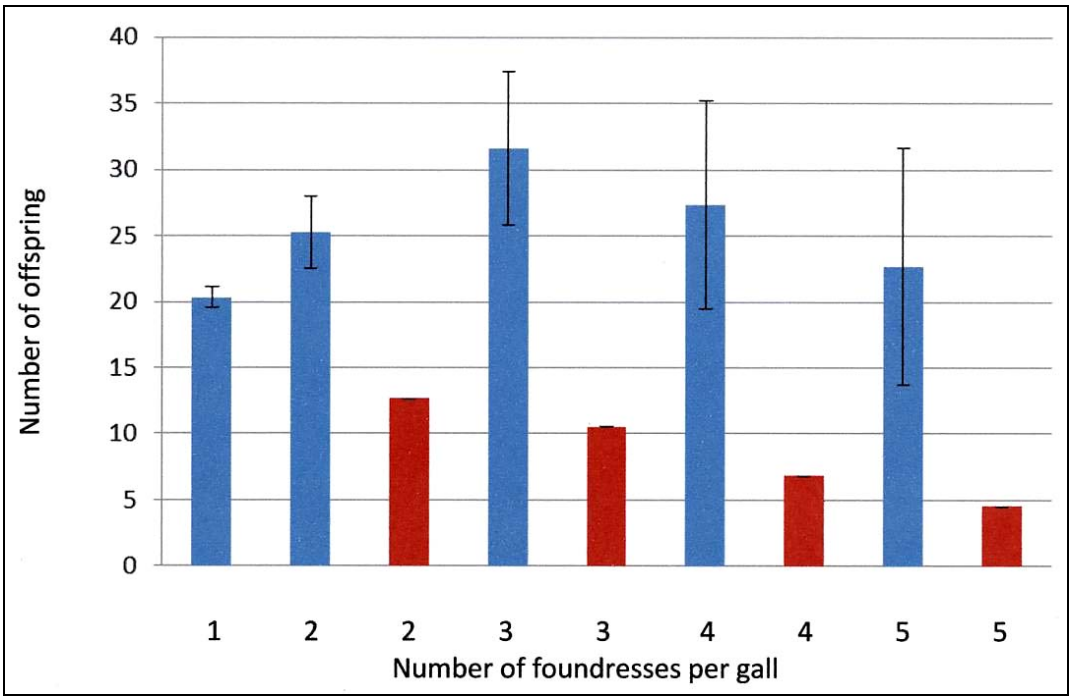

Figure 2. Average offspring per gall vs. number of foundresses per gall. Blue = per gall. Red= per capita (estimated). Brackets represent standard error.

Genetic Distance Values

The Genetic Distance (GD) values calculated for all individual pairs are shown in Table 2. Mean GD between all foundresses within the sample group was calculated to be 0.49. This value was consistent whether evaluated on a per-gall or per-

Table 2. Pairwise genetic distance values. Individuals were numbered sequentially by foundress pairs in order of gall number (i.e., 1=2X01F1, 2=2X02F2, 3=2X02F1, etc.). Individuals 31-33 represent sample numbers 3X01F1-F3.

Specimen Number																															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**
1		0.29	0.55	0.45	0.53	0.49	0.44	0.49	0.39	0.59	0.34	0.33	0.43	0.29	0.38	0.48	0.48	0.61	0.41	0.48	0.44	0.62	0.52	0.43	0.68	0.45	0.42	0.33	0.48	0.52	0.56	0.4	0.49
2	0.29		0.57	0.64	0.58	0.69	0.6	0.54	0.46	0.69	0.45	0.53	0.61	0.5	0.53	0.55	0.45	0.72	0.48	0.66	0.44	0.77	0.45	0.43	0.59	0.62	0.55	0.54	0.69	0.67	0.71	0.6	0.59
3	0.55	0.57		0.63	0.62	0.69	0.59	0.6	0.65	0.65	0.6	0.65	0.51	0.58	0.6	0.65	0.58	0.68	0.63	0.62	0.44	0.77	0.68	0.58	0.79	0.68	0.61	0.56	0.56	0.66	0.68	0.63	0.75
4	0.45	0.64	0.63		0.66	0.48	0.64	0.61	0.69	0.62	0.61	0.66	0.62	0.52	0.65	0.69	0.69	0.51	0.58	0.57	0.44	0.58	0.61	0.63	0.82	0.53	0.59	0.55	0.58	0.64	0.63	0.61	0.51
5	0.53	0.58	0.62	0.66		0.31	0.32	0.44	0.4	0.52	0.39	0.45	0.28	0.39	0.39	0.42	0.42	0.49	0.39	0.38	0.44	0.53	0.48	0.32	0.72	0.42	0.34	0.61	0.42	0.41	0.48	0.41	0.47
6	0.49	0.69	0.69	0.48	0.31		0.44	0.43	0.39	0.42	0.38	0.44	0.3	0.31	0.48	0.44	0.58	0.46	0.41	0.37	0.44	0.48	0.54	0.46	0.68	0.39	0.44	0.37	0.42	0.43	0.48	0.32	0.44
7	0.44	0.6	0.59	0.64	0.32	0.44		0.41	0.34	0.47	0.33	0.39	0.34	0.39	0.4	0.46	0.46	0.58	0.4	0.38	0.44	0.57	0.56	0.45	0.41	0.31	0.43	0.38	0.46	0.53	0.46	0.42	0.5
8	0.49	0.54	0.6	0.61	0.44	0.43	0.41		0.37	0.46	0.28	0.48	0.44	0.45	0.42	0.42	0.42	0.44	0.32	0.4	0.44	0.53	0.54	0.35	0.55	0.42	0.37	0.32	0.42	0.49	0.48	0.41	0.41
9	0.39	0.46	0.65	0.69	0.4	0.39	0.34	0.37		0.24	0.26	0.41	0.33	0.38	0.42	0.44	0.36	0.62	0.36	0.45	0.44	0.58	0.48	0.43	0.52	0.47	0.39	0.37	0.48	0.51	0.5	0.41	0.56
10	0.59	0.69	0.65	0.62	0.52	0.42	0.47	0.46	0.24		0.42	0.48	0.43	0.45	0.54	0.53	0.5	0.59	0.41	0.48	0.44	0.53	0.63	0.58	0.62	0.47	0.44	0.46	0.45	0.46	0.48	0.46	0.56
11	0.34	0.45	0.6	0.61	0.39	0.38	0.33	0.28	0.26	0.42		0.3	0.32	0.33	0.31	0.35	0.41	0.53	0.32	0.36	0.44	0.52	0.53	0.39	0.58	0.49	0.35	0.3	0.41	0.45	0.45	0.48	0.48
12	0.33	0.53	0.65	0.66	0.45	0.44	0.39	0.48	0.41	0.48	0.3		0.38	0.28	0.47	0.49	0.49	0.62	0.46	0.53	0.44	0.58	0.53	0.52	0.55	0.46	0.46	0.42	0.5	0.45	0.57	0.39	0.56
13	0.43	0.61	0.51	0.62	0.28	0.3	0.34	0.44	0.33	0.43	0.32	0.38		0.31	0.36	0.39	0.52	0.53	0.39	0.37	0.44	0.49	0.63	0.41	0.72	0.36	0.36	0.31	0.29	0.41	0.49	0.38	0.53
14	0.29	0.5	0.58	0.52	0.39	0.31	0.39	0.45	0.38	0.45	0.33	0.28	0.31		0.4	0.47	0.41	0.57	0.34	0.42	0.44	0.53	0.47	0.42	0.71	0.41	0.44	0.32	0.37	0.36	0.45	0.33	0.46
15	0.38	0.53	0.6	0.65	0.39	0.48	0.4	0.42	0.42	0.54	0.31	0.47	0.36	0.4		0.44	0.52	0.48	0.46	0.42	0.44	0.62	0.59	0.45	0.68	0.52	0.41	0.36	0.44	0.56	0.49	0.4	0.51
16	0.48	0.55	0.65	0.69	0.42	0.44	0.46	0.42	0.44	0.53	0.35	0.49	0.36	0.47	0.44		0.46	0.5	0.36	0.42	0.44	0.52	0.61	0.42	0.57	0.44	0.36	0.32	0.44	0.48	0.53	0.43	0.6
17	0.48	0.45	0.58	0.69	0.42	0.58	0.46	0.42	0.36	0.5	0.41	0.49	0.48	0.41	0.52	0.46		0.57	0.28	0.39	0.44	0.6	0.38	0.37	0.59	0.49	0.41	0.45	0.49	0.55	0.53	0.5	0.45
18	0.61	0.72	0.68	0.51	0.49	0.46	0.58	0.44	0.62	0.59	0.53	0.62	0.53	0.57	0.48	0.5	0.57		0.45	0.41	0.44	0.48	0.64	0.49	0.68	0.5	0.48	0.41	0.48	0.53	0.46	0.44	0.42
19	0.41	0.48	0.63	0.58	0.39	0.41	0.4	0.32	0.36	0.41	0.32	0.46	0.39	0.34	0.46	0.36	0.28	0.45		0.2	0.44	0.51	0.46	0.34	0.53	0.36	0.33	0.32	0.38	0.42	0.44	0.37	0.38
20	0.48	0.66	0.62	0.57	0.38	0.37	0.38	0.4	0.45	0.48	0.36	0.53	0.37	0.42	0.42	0.42	0.39	0.41	0.2		0.44	0.52	0.5	0.41	0.66	0.42	0.37	0.35	0.36	0.43	0.45	0.36	0.39
21	0.58	0.72	0.69	0.72	0.43	0.44	0.51	0.42	0.47	0.49	0.44	0.51	0.42	0.52	0.46	0.44	0.58	0.47	0.48	0.44		0.24	0.67	0.5	0.68	0.41	0.44	0.42	0.38	0.43	0.48	0.52	0.58
22	0.62	0.77	0.77	0.58	0.53	0.48	0.57	0.53	0.58	0.53	0.52	0.58	0.49	0.53	0.62	0.52	0.6	0.48	0.51	0.52	0.24		0.67	0.6	0.73	0.41	0.47	0.51	0.48	0.38	0.5	0.61	0.55
23	0.52	0.45	0.68	0.61	0.48	0.54	0.56	0.54	0.48	0.63	0.53	0.53	0.63	0.47	0.59	0.61	0.38	0.64	0.46	0.5	0.67	0.67		0.3	0.68	0.61	0.55	0.54	0.63	0.59	0.64	0.53	0.48
24	0.43	0.43	0.58	0.63	0.32	0.46	0.45	0.35	0.43	0.58	0.39	0.52	0.41	0.42	0.45	0.42	0.37	0.49	0.34	0.41	0.5	0.6	0.3		0.63	0.53	0.37	0.35	0.42	0.52	0.53	0.41	0.44
25	0.68	0.59	0.79	0.82	0.72	0.68	0.41	0.55	0.52	0.62	0.58	0.55	0.72	0.71	0.68	0.57	0.59	0.68	0.53	0.66	0.68	0.73	0.68	0.63		0.48	0.65	0.58	0.71	0.69	0.68	0.63	0.71
26	0.45	0.62	0.68	0.53	0.42	0.39	0.31	0.42	0.47	0.47	0.49	0.46	0.36	0.41	0.52	0.44	0.49	0.5	0.36	0.42	0.41	0.41	0.61	0.53	0.48		0.46	0.39	0.44	0.48	0.42	0.45	0.52
27	0.42	0.55	0.61	0.59	0.29	0.44	0.43	0.37	0.39	0.44	0.35	0.46	0.36	0.44	0.41	0.36	0.41	0.48	0.33	0.37	0.44	0.47	0.55	0.37	0.65	0.46		0.24	0.32	0.45	0.4	0.45	0.48
28	0.33	0.54	0.56	0.55	0.41	0.37	0.38	0.32	0.37	0.46	0.3	0.42	0.34	0.32	0.36	0.32	0.45	0.41	0.32	0.35	0.42	0.51	0.54	0.35	0.58	0.39	0.24		0.22	0.38	0.39	0.28	0.46
29	0.48	0.69	0.56	0.58	0.42	0.42	0.46	0.42	0.48	0.45	0.41	0.5	0.29	0.37	0.44	0.44	0.49	0.48	0.38	0.36	0.38	0.48	0.63	0.42	0.71	0.44	0.32	0.22		0.29	0.4	0.4	0.51
30	0.52	0.67	0.66	0.64	0.41	0.43	0.53	0.49	0.51	0.46	0.45	0.45	0.41	0.36	0.56	0.48	0.55	0.53	0.42	0.43	0.43	0.38	0.59	0.52	0.69	0.48	0.45	0.38	0.29		0.43	0.37	0.51
31	0.56	0.71	0.68	0.63	0.48	0.48	0.46	0.48	0.5	0.48	0.45	0.57	0.44	0.42	0.49	0.53	0.53	0.46	0.44	0.45	0.48	0.5	0.64	0.53	0.68	0.42	0.4	0.39	0.4	0.43		0.35	0.48
32	0.4	0.6	0.63	0.61	0.41	0.32	0.42	0.41	0.41	0.46	0.37	0.45	0.38	0.33	0.4	0.43	0.5	0.44	0.37	0.36	0.52	0.61	0.53	0.41	0.63	0.45	0.45	0.28	0.4	0.41	0.35		0.28
33	0.49	0.59	0.75	0.51	0.47	0.44	0.5	0.41	0.56	0.56	0.48	0.56	0.53	0.46	0.51	0.6	0.45	0.42	0.38	0.39	0.58	0.55	0.48	0.44	0.71	0.52	0.48	0.46	0.51	0.51	0.48	0.28	

foundress basis (Table 3). The mean GD between pairs of co-habiting foundresses was found to be 0.35. The difference in GD values between the population average (extra-gall pairs) and the average for gall cohabitants was statistically significant (Paired *t*-test, $t = 5.77$, df = 17, $p \leq .001$). The GD data for extra- and inter-gall pairs are shown in Figure 3.

Table 3. Average GD values per gall. F1 and F2 represent the mean value for all pairwise comparisons *(n*=17) for foundress1 and foundress 2, respectively. Gall Average is the mean of F1 and F2, and F1-F2 GD is the value calculated between foundress 1 and foundress 2 from the same gall. For the triple-foundress gall (3X01), each possible pair (F1-F2, F1-F3 and F2-F3) was evaluated separately.

Gall	F1	F2	Gall Average	F1-F2 GD
2X01	0.4729	0.5868	0.5298	0.2889
2X02	0.6340	0.6097	0.6218	0.6279
2X03	0.4506	0.4556	0.4531	0.3134
2X04	0.4501	0.4423	0.4462	0.4118
2X05	0.4485	0.5123	0.4804	0.2424
2X06	0.4126	0.4872	0.4499	0.3000
2X07	0.4312	0.4278	0.4296	0.3077
2X08	0.4746	0.4727	0.4736	0.4367
2X09	0.4780	0.5253	0.5017	0.5714
2X10	0.4123	0.4441	0.4282	0.1951
2X11	0.5079	0.5561	0.5320	0.2449
2X12	0.5611	0.4587	0.5010	0.3030
2X13	0.6458	0.4627	0.5542	0.4783
2X14	0.4388	0.4059	0.4223	0.2368
2X15	0.4516	0.4917	0.4717	0.2877
3X01(F1-2)	0.5063	0.4468	0.4766	0.3478
3X01(F1-3)	0.5063	0.5110	0.5086	0.4792
3X01(F2-3)	0.4468	0.5109	0.4789	0.2791
Average	0.4850	0.4893	0.4871	0.3529

Fragment Profiles and Parsimony Analysis

A total of 152 AFLP-PCR products were detected in the 100-500 bp range (Figure 4). Two products were present throughout the entire population, the first in the 102 bp range and the second in the 350-bp range. Maxiumum likelihood parsimony analysis using PAUP produced a total of 31 unrooted best-fit trees using this data. All characters were equally weighted and there were a total of 29 non-informative characters.

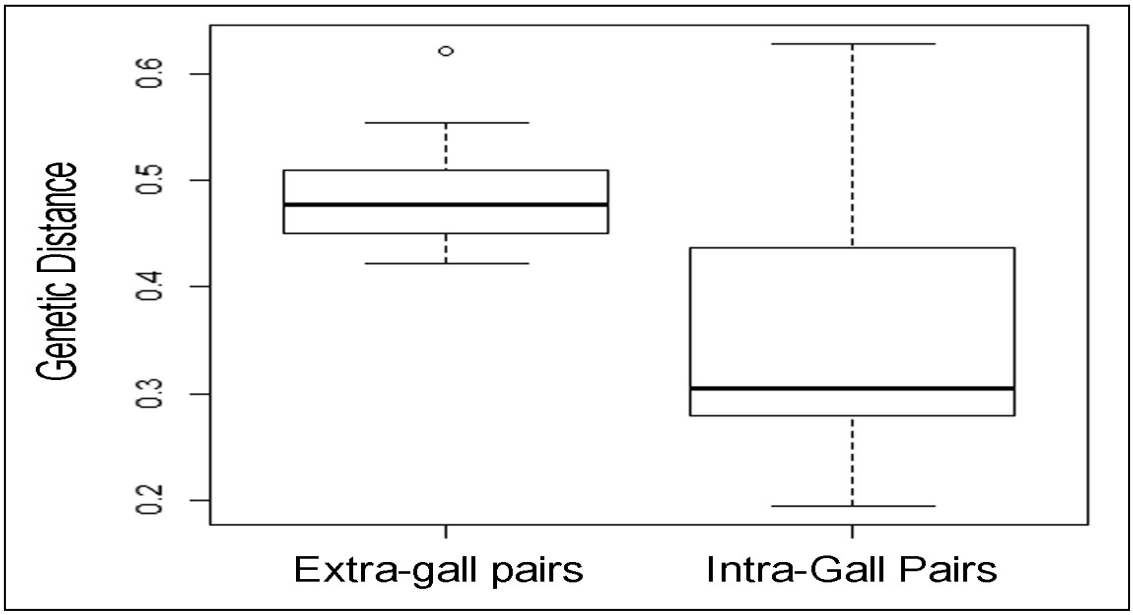

Figure 3. Boxplot representing mean GD gall; intra-Gall values. Extra-gall pairs = pairs not sharing the same pairs = pairs from the same gall. The dark central line represents the mean, the box is the upper and lower quartile, the whiskers represent minimum and maximum values and the circle indicates a statistical outlier.

For 9 out of the 16 communal galls examined, the cohabitants were determined to have the greatest amount of genetic similarity with their gall mate (Figure 5).

Discussion

The results of the genetic distance analysis indicate that relatedness among gall cohabitants is highly variable on a per-gall basis but is significantly higher overall than between non-cohabitants. Parsimony analysis of the data matrix produced by the AFLP-PCR profiles revealed that in roughly half of the communal galls studied the foundresses with the most similar profiles were found within the same gall. Hence in the remaining galls combinations of two or more genotypes occur, implying no necessary effect of relatedness on galling cohabitation. Other studies on galling aphids have also

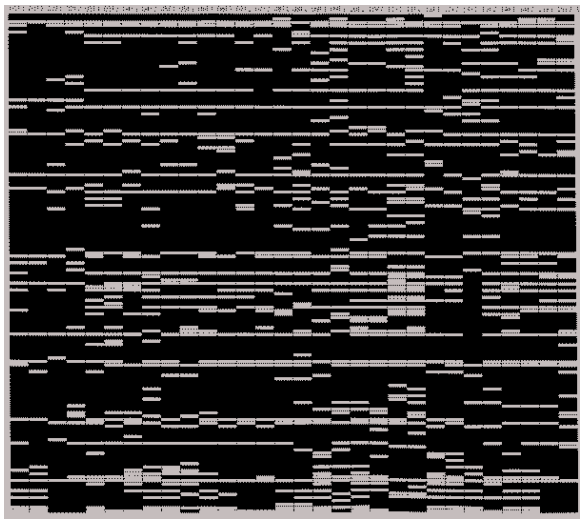

Figure 4. Fragment profiles collected for all individuals (*n*=33) from AFLP-PCR reactions. White bands indicate the presence of a product. Products are arranged in descending order from 100-500bp.

mixing of genotypes in similar proportions, resulting from both intra- and inter-species cohabitation (Abbot et al. 2001, Wang et al. 2008).

Because only one species was examined in this study, and because only the final gall-inducing generation is parthenogenetically produced, the highly synonymous intra-gall pairs may represent clones deposited by a second-generation female. The resulting larvae either co-founded the gall or joined occupants in existing, early-phase galls. The less-parsimonius pairs would then represent mixing of two different clones. The fact that even the highly parsimonious pairs showed some genetic variation does not necessarily refute this hypothesis, as intraclonal genetic variation has been known to occur in aphids and high-resolution molecular markers such as AFLPs are able to detect these differences, which can be attributed to both random mutations and the presence of

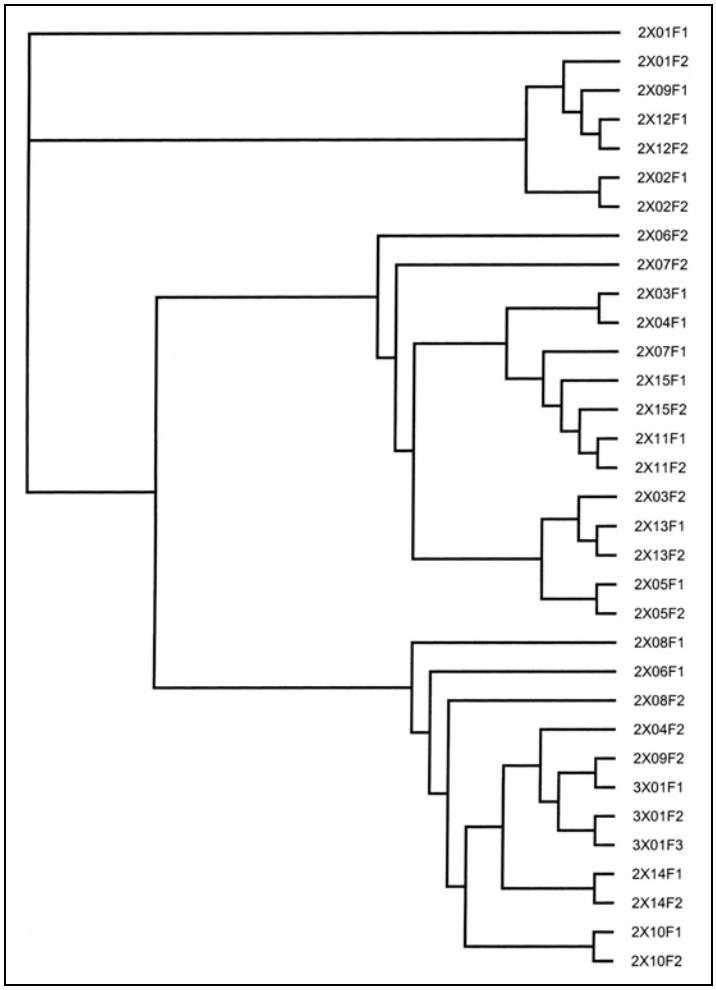

Figure 5. Maximum parsimony tree for foundresses from communally occupied galls at the study site.

symbiotic bacteria (Vorwerk 2007, Vorwerk & Forneck 2007, Loxdale 2008, Monti et al. 2012).

The high degree of relatedness between these putatively-clonal-pairs contributed to the significant difference in GD values for intra-gall pairs and extra-gall pairs (Table 3, Figure 3). It is noteworthy that the difference was so pronounced despite the inclusion of a relatively equal number of non-parsimonious pairs. This is important because it suggests that despite the relatively high frequency of apparently unrelated

foundresses sharing galls, the similarity between the closely rated individuals is high enough that overall, the average relatedness between gall co-habitants is significantly greater than relatedness between non-cohabiting individuals.

The productivity of communally occupied galls was of also of particular significance in this study. Communally occupied galls with no increase in productivity relative to single-foundress galls would suggest a net loss in reproductive fitness regardless of relatedness between gall-mates, because the numbers of offspring are likely to represent a combination of offspring from multiple foundresses rather than just a single foundress. Our findings indicate that this is not the case, and that communally occupied galls are more productive than single-foundress galls. However, the increase in numbers of offspring produced are not linear multiples of foundress number (in other words, a double-foundress gall does not double the productivity of a single-foundress gall), and so *per capita* reproduction is still reduced. These results are in accordance with Miller's experimental data for this species (1998b).

It is possible that this reduction in *per capita* fitness selects against cohabitation in larger numbers. For example, double-foundress galls contained, on average, 6 more offspring per gall than single-foundress galls, a 30% increase. Triple-foundress galls, contained, on average, 14 more offspring per gall than single-foundress galls, a 70% increase. But the number of offspring per individual, assuming equal reproduction among all foundresses, would decrease from 20 to 13 to 11 in 1X, 2X and 3X galls, respectively. As the average productivity for 4X and 5X galls was no higher than that of 3X galls (Fig. 5), it appears that *per capita* fitness diminishes with each additional foundress (it should be noted that because of the low number of 4X and 5X

galls found, these estimates may be less reliable than those for the other groups). These diminishing returns may explain why double-foundress galls, though less productive than triple, quadruple or quintuple-foundress galls, were far more common, totaling more than half of the total communal galls found.

The fact that per capita losses seem to outpace group gains was not unexpected in light of Miller's (1998b) previous results. It should be remembered that in assessing cooperative behavior, a high degree of relatedness may mitigate imbalances in cost/benefit ratio at the individual level. As noted earlier in the discussion, the relatedness among communal gall occupants is higher than the population average, which is largely due to the occurrence of a number of closely related pairs of gall occupants. In these pairs, the genetic similarity between occupants is so high that, from a genotypic perspective, the decrease in *per capita* productivity is likely to be less relevant than the overall increased productivity per gall.

These results are in accordance with what would be expected if kin selection is truly occurring (i.e., there should be increased productivity in communally occupied galls, and a high level of relatedness among gall co-occupants). The combination of increased overall productivity and higher than average genetic relatedness in communal galls strongly supports the hypothesis that some form of kin selection is occurring in this population, although it is difficult to say whether this behavior is highly adaptive or simply a viable enough alternative to non-collaborative behavior that it is not selected against. The relatively low occurrence of communal galls (11% of the sample population) suggests that the latter may be the case. As noted By Miller (1998b) and Inbar (1998), a limited number of ideal galling sites may precipitate communal galling even in the

absence of kin selection. Nevertheless, the fact that this behavior has shown to be potentially beneficial at a group level means there is little selective pressure against cohabitation, and thus the non-aggressive interactions among *Tamalia* foundresses can be explained: as long as they are cohabiting with their own clone-mates or close kin in sufficient numbers, communal galling is at least somewhat beneficial and thus engaging in costly defensive or territorial behavior would ultimately be counterproductive. A consequence of this is that the presence of intraspecific "invaders" of different genotypes is tolerated.

Suggestions for Future Studies

This mixing of various lineages within the same gall may be relevant to the production of soldiers, which are truly altruistic (i.e., non-reproductives who act to enhance the fitness of their colony rather than produce offspring of their own) and are indicative of eusociality in aphid colonies. In describing the evolution of altruism in another North American subfamily, the Pemphiginae, Abbot (2009), noted that "the factors that favored intraspecific competition and clonal admixture…may have also acted to predispose lineages to soldier production" (p. 2688). The . . . recent discovery of soldier-like morphs in other northern California *Tamalia* populations (Donald G. Miller, personal communication; unreferenced.) may indicate that a similar process is occurring in this species as well, and the population observed in this study represents a transitional period from solitary to cooperative, and eventually eusocial, behavior.

This would represent an opportunity for further studies, as it remains largely unknown why some aphid species evolve higher levels of sociality while otherwise

similar species do not. The *status quo* in current populations may favor some degree of cohabitation, but a trend towards higher gall invasion rates by competing lineages or species, or the presence of soldier castes, which have been seen to deter both predation and/or cohabitation, depending on the circumstance, has the potential to alter this dynamic. It is difficult to predict these developments or their consequences, but knowledge of the current population dynamics provided by these results can provide a frame of reference to allow a comparison between present and future studies, and thus if social behavior does evolve in this species the ecological factors which helped produce it would be more easily determined.

Along those lines, future studies should be designed with a careful eye towards any significant morphological or behavioral deviations in foundresses or their offspring. For example, Uematsu et al. (2007) found that they could induce defensive behavior in first-instar offspring of the Japanese galling aphid *Quardratus yoshinomiyai* by exposing them to predaceous caterpillars in test arenas. A similar study could be conducted using *Tamalia* soldier-like morphs to observe if they are capable (or inclined) to display defensive behavior. Changes in communal galling rates or fecundity would also be a significant development. These could be easily observed by random sampling and gall dissection. An assessment of parsimony among gall-mates in galls with higher numbers of foundresses using methodology similar to this study is feasible, although given the relatively rare occurrence of galls with more than two foundresses, a large number of galls would likely need to be processed in order to attain a reasonable sample size. Even a simple repetition of the current study would be informative, as there is little information about the year-to year fluctuations in galling behavior.

Conclusions

As I found a higher than average degree of relatedness among communal gall occupants produced in large part by a number of highly parsimonious intra-gall pairs, and an increase in productivity in communally inhabited galls as measured by numbers of viable offspring, the results of this study strongly suggest that kin selection is a plausible explanation for the occurrence of communal gall occupation in this species. Despite the apparent benefits of communal occupation, however, there also appear to be factors limiting the overall frequency of cohabitation, namely a high rate of invasion by individuals of differing genotypes and diminishing reproductive efficiency in galls inhabited by three or more foundresses. The circumstances of communal gall occupation in this species are similar to those found in other aphid species which have evolved eusociality, and thus future studies should emphasize the potential for social interactions in these aphids, specifically the origins of altruistic soldier castes, precursors of which have already been observed in similar populations in California. This species may present an opportunity to study the transition from solitary to social behavior as it occurs, and thus answer several unresolved questions regarding the complex nature of the evolution of altruism and sociality in these insects.

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