FACTORS AFFECTING CASTE DEVELOPMENT, BROOD TYPE, AND SEX RATIOS OF A POLYEMBRYONIC WASP

by

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(Under the Direction of Michael R. Strand)

ABSTRACT

The polyembryonic parasitoid wasp, *Copidosoma floridanum*, has evolved a caste system, consisting of soldiers and reproductives. Prior studies suggest soldiers have evolved in response to two selective pressures: 1) defense against competitors, and 2) resolution of sex ratio conflict. Prior studies indicate that soldier development time critically influences the outcome of intraspecific competition, and may also be strongly influenced by allelic variants of the metabolic enzyme, glucose phosphate isomerase (*Gpi*). Other studies suggest sibling conflict exists in mixed sex broods due to the potential mating opportunities for males with both sisters and females from other hosts. My results indicate that *Gpi* is a gene of major effect in the timing of soldier emergence and outcome of competition. Other experiments indicate that brother-sister matings commonly occur in mixed broods but that mating opportunities for males after dispersing from the natal patch are low.

INDEX WORDS: *Copidosoma floridanum*, *Gpi*, soldiers, heterozygote advantage, partial local mate competition, life-history trade-offs

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CHAPTER ONE

INTRODUCTION

A distinguishing feature among social animals is the evolution of a caste system whereby some individuals in a group reproduce and others perform altruistic helper functions (Robinson 1992, Queller 2000). The greatest diversity of social species is found in the insect order Hymenoptera (bees, wasps, and ants). Also included within this order are polyembryonic parasitoids; a group of insects that combine the life history of a parasitoid with clonal development and evolution of a social system. These conditions have favored the evolution of a sterile soldier larvae caste and a reproductive larvae caste. The polyembryonic parasitoid wasp, *Copidosoma floridanum*, has been studied extensively for caste evolution mechanisms due to the wealth of information on its biology (Strand and Grbic 1997, Donnell et al. 2004, Giron et al. 2007b). Prior studies suggest polyembryonic wasps have evolved a soldier caste in response to two selective pressures: 1) defense against competitors (Cruz 1981, Harvey et al. 2000, Giron et al. 2007b) and 2) resolution of sex ratio conflict, created when the parent places both a male and female egg in the same host, resulting in mixed sex broods (Grbic et al. 1992, Gardner et al. 2007).

Experimental studies with *C. floridanum* lend support for both possibilities but several questions related to soldier development and function remains unclear. For example, recent results reported by Giron et al. (2007a) suggest the rate of soldier development is critical in resolution of intraspecific competition and that allelic variants of the metabolic enzyme glucose phosphate isomerase (*Gpi*) influences soldier emergence times. However, more detailed studies are required to determine whether *Gpi* functions as a gene of major effect in soldier development. Prior studies also circumstantially suggest sibling conflict exists in mixed sex broods produced by *C. floridanum* due to the potential mating opportunities for males with both sisters and females

from other hosts (Grbic et al. 1992, Gardner et al. 2007). Yet, we know too little about the mating behavior of *C. floridanum* to fully evaluate whether sibling conflict is a significant consideration in the life history of polyembryonic wasps. Thus, the three specific objectives of my thesis are as follows:

1. Establish multiple genetically independent lines homozygous for three different *Gpi* alleles (*Gpi*^{54}, *Gpi*^{100}, and *Gpi*^{120}) and assess if these *Gpi* alleles differentially affect soldier development times and the outcome of intraspecific competition.
2. Determine if *Gpi* allelic diversity is maintained through life-history trade-offs or balancing selection acting through a heterozygote advantage.
3. Gather empirical data on mating behaviors of males and females in mixed sex and single sex broods.

CHAPTER TWO

BACKGROUND AND LITERATURE REVIEW

Before addressing the objectives of my thesis in detail, a brief review of polyembryonic parasitoid biology, caste formation and function, and sex ratio theory will be given.

I. Polyembryony among hymenopteran parasitoids

Polyembryony is the asexual division of an egg into multiple embryos and is found throughout, although rarely, in the animal kingdom. Sporadic polyembryony commonly occurs among humans during a twinning event, while obligate polyembryony occurs in a few groups including certain insects, bryzoans, and armadillos (Craig et al. 1997, Loughry et al. 1998). The greatest diversity of obligate polyembryony occurs among insects known as parasitoids. Parasitoids develop by feeding on the bodies of other arthropods during their immature stages but are free-living as adults. The majority of parasitoids are either members of the order Hymenoptera or Diptera (true flies) and constitute about 8.5% of all insects (Godfray 1994). Parasitoids within the Hymenoptera, like other insects in the order, are haplo-diploid with unfertilized eggs developing into males and fertilized eggs developing into females. Polyembryony in parasitoids is found in four families of Hymenoptera, Braconidae, Platygastridae, Encyrtidae, and Dryinidae (Ivanova-Kasas 1972). Among some species of encyrtids, wasps have evolved a caste system containing sterile soldiers and reproductives. One such species, *Copidosoma floridanum*, exhibits one of the more extreme examples of polyembryony by forming up to 2000 reproductives and an average of thirty soldiers per egg within its host (Strand and Grbic 1997). Wasp development begins with a *C. floridanum* adult female ovipositing either a single male egg, a single female egg, or a male and female egg into the egg stage of its host, a plussine moth. Single laid eggs emerge as single sex broods, while

male and female eggs laid together will emerge as mixed sex broods from the same host. Caste formation only occurs in the larval stage, inside the body of the host.

II. Caste formation in *C. floridanum*

A host egg parasitized by *C. floridanum* will continue to develop into its final, fifth, instar over 14 days while the wasp egg also develops. Initially each wasp egg develops into a single embryo called a primary morula, followed by clonal development of up to an additional 2000 embryos that form an assemblage called a polygerm or polymorula (Ode and Strand 1995a, Strand and Grbic 1997, Grbic et al. 1998). From this assemblage emerge a morphologically distinct caste of soldier larvae with fighting mandibles and reproductive larvae, but the prevalence of each differs with the sex of the egg. Female eggs produce one to two sterile soldier larvae in the host's first instar with an additional two to ten soldiers developing throughout the host's second to fourth instars, resulting in an average of thirty by the host's last instar (Fig. 2.1, (Ode and Strand 1995b). Male eggs, on the other hand, produce almost no soldiers and only late in host development. In both sexes, the embryos that do not develop into soldier larvae become reproductives in the host's fifth instar, with smaller mandibles and a rounder body. Reproductive larvae consume the host, pupate, and emerge as adult wasps. Soldier larvae do not pupate and instead die from desiccation when the host is consumed by its reproductive siblings (Strand and Grbic 1997).

III. Soldier function and the evolution of a caste system

As previously noted, prior studies implicate two factors in the evolution of a soldier caste.

1.) Soldiers attack inter- and intraspecific competitors

Each host attacked by *C. floridanum* represents a nutrient-rich but limiting resource that can only support development of a finite number of wasps. Other parasitoid species seeking to develop in the same host leads to a decrease in the amount of host resources available to the brood (Ode and Strand 1995a). Moreover, only one species of parasitoid usually survives when hosts are parasitized by more than one species of wasp (Godfray 1994). Interspecific competitors

include other wasps like *Microplitis demolitor* that parasitizes a host during the larval stage (Browning and Oatman 1984, Strand et al. 1990). These larval parasitoids posses large fighting mandibles and engage in combat with wasps in the same host (Godfray 1994). The evolution of a sterile soldier caste has been regarded as a means to insure the survival of their reproductive siblings from attack by competitors (Browning and Oatman 1984). When exposed to such a competitor, *C. floridanum* can shift its caste ratio in favor of the production of more soldier larvae at the cost of producing less reproductives. As a result, laboratory studies reveal *C. floridanum* is an intrinsically superior competitor to *M. demolitor*, suggesting *C. floridanum* embryos perceive environmental cues associated with competitors (Harvey et al. 2000). In the absence of any competitor, caste ratios are biased toward reproductives.

In addition to interspecific competitors, *C. floridanum* soldiers defend their host against intraspecific competitors consisting of non-relative clones and brothers. In laboratory experiments, 96% of females with no previous oviposition experience oviposited into hosts that were parasitized by another wasp (superparasitism) (Giron et al. 2004). Examination of field *C. floridanum* broods indicate the majority of broods are mixed sex (Grbic et al. 1992). These results suggest *C. floridanum* soldiers defend a host against intraspecific competitors frequently in the field. Mixed sex broods lead to brothers and sisters developing in the same host and results in female soldiers attacking brothers much more frequently than sisters (Grbic et al. 1992). Non-relative clones and brothers were attacked more often than sisters even when host resources were severely limited, suggesting soldier aggression correlates with kinship but not resource competition (Giron et al. 2004). Giron and Strand (2004) have shown *C. floridanum* uses kin-recognition cues derived from the extraembryonic membrane, which is also essential in defense against the host's cellular immune response.

2.) Soldiers manipulate sex ratios

Field and laboratory reared *C. floridanum* produce a majority of mixed sex broods (Grbic et al. 1992). Within these broods, the sex ratio allocated by the mother is exactly 50:50 (one

female egg, one male egg) but female soldier aggression toward their brothers results in a strongly female-biased sex ratio upon emergence (Grbic et al. 1992, Ode and Strand 1995a). Female biased sex ratios are observed most frequently in gregarious parasitoid wasps with a high probability of sibmating. Hamilton (1964) explained these observations through a mechanism he coined as local mate competition (LMC). Female biased sex ratios are favored under LMC because a decrease in the number of sons means less competition for mates and an increase in the number of daughters means more potential mates for sons (Taylor 1993). Hamilton's model assumes that all mating is confined to the natal patch, all females are mated before dispersing, and mating within the patch occurs randomly among the offspring of all foundresses. Females may have a stronger selection favoring the reduction of brothers instead of males reducing the number of females due to the fact only enough males to mate all the females is needed (Grbic et al. 1992). If males obtain matings away from the natal patch, conflict exists between brothers and sisters over the sex ratio, with brothers favoring a male-biased sex ratio. Alternatively, if males do not seek mates away from the natal patch, no conflict over a female biased sex ratio would occur. The theory, coined conflict hypothesis, addresses the role of soldiers in resolving sex ratio conflict, should it exist. Female soldiers killing males would produce a female biased sex ratio, while male soldiers killing females would result in a male biased sex ratio (Gardner et al. 2007).

IV. Mechanisms regulating caste formation and function

Caste determination is usually regulated by environmental stimuli including diet, temperature density, and pheromones (Evans and Wheeler 2001, Shibao et al. 2004). These environmental cues stimulate changes in gene expression and physiological processes that result in development into one caste or another (Donnell and Strand 2006). In *C. floridanum*, however, caste members develop clonally from the same egg and are exposed to the same environmental conditions in the host. The caste fate of *C. floridanum* is instead mediated by differential allocation of germ cells to embryos (Donnell et al. 2004). Germ cells produce gametes and are the only cells that can undergo meiosis as well as mitosis (Cinalli et al. 2008). The first cells that

will give rise exclusively to germ cells by clonal mitotic divisions are called primordial germ cells (PGCs) (Extavour and Akam 2003). In similar insect species in the orders Diptera, Lepidoptera, and Hymenoptera, PGCs are specified early in embryogenesis via inheritance of maternal determinants (Extavour 2004). Unlike other insects, PGCs in *C. floridanum* proliferate substantially during the clonal phase of development and embryos that inherit the germ cells develop into reproductive larvae while embryos that do not develop into soldiers (Donnell et al. 2004). This suggests male and female *C. floridanum* differ in their parceling patterns of PGCs to embryos.

Rate of soldier development affects outcome of competition

The study by Giron et al (2007a) used three common alleles (*54, 100, 120)* of a genetic marker, glucose-phosphate isomerase, (EC number 5.3.1.9 [Gpi]) and manipulative laboratory experiments to assay the effect soldier behavior, *in vivo*, had on the outcome of competition. The behavioral experiments indicate early-emerging soldiers attack intraspecific competitors while later-emerging soldiers attack interspecific competitors. Their results also reveal the resident larvae are more successful at monopolizing the host because its soldiers can attack the later-laid competitor while it's still in its embryonic phase. Repeating the experiment with a host parasitized simultaneously by two *C. floridanum* eggs revealed the soldier eclosing (emerging) the soonest had the advantage. These results strongly suggest timing of first soldier emergence influences the outcome of intraspecific competition. Analyses of the results in relation to genotype revealed $Gpi^{100/100}$ and $Gpi^{120/120}$ clones produce first soldiers significantly earlier than $Gpi^{54/54}$ clones and that clones with the former two genotypes outcompete those with the latter genotype. This finding suggests soldier developmental rates may have a strong genetic basis, with *Gpi* being a gene of major effect. Although this result was unexpected, much research has been done on *Gpi* allelic variation and its role in metabolic activities, including development.

Gpi affects metabolic processes because it is a dimeric enzyme that catalyzes the reversible conversion of glucose-6-phosphate into fructose-6-phosphate in the second step of

glycolysis, for a review see Achari (1981). Its use has varied from a molecular marker among populations to addressing questions of allozyme adaptation. Due to this there is considerable background information on the structure, kinetic properties, and metabolic roles of *Gpi* from a variety of organisms, including fungi (Bidochka et al. 2002), plants (Chojecki and Gale 1982), nematodes (Vilas et al. 2000), and insects (Dahlhoff and Rank 2000, Rank et al. 2007). Whether allelic enzyme polymorphisms in natural populations have any adaptive significance has long been debated, with evidence supporting both the appearance of some neutral allozymes (Eanes 1999) and some with fitness affects (Avise 1994). Research on *Colias* butterflies (Watt 1983, Watt et al. 1983, Carter and Watt 1988, Watt et al. 1996), water striders (Zera 1987), and montane willow beetles (Dahlhoff and Rank 2000, Rank et al. 2007) indicate the latter. Findings on *Gpi* allelic variation in *Colias* butterflies show several fold differences in kinetics, thermal stability and flux response during flight between the ten genotypes of four wild alleles. Survival, male mating success, and female fecundity are dependent on flight and were correctly predicted to differ congruently in fitness. In the montane willow beetle, results indicate *Gpi* is under temperature selection due to the differences among *Gpi* genotypes in running speed, a crucial character for survival and fitness (Rank et al. 2007). Due to the involvement of *Gpi* in metabolic processes, it is reasonable to assume it's more than a molecular marker, but a gene of major effect in the development, metabolism, and flight of insects.

V. Conclusions from literature and direction of thesis

Key features of the biology of *C. floridanum* is characterized by several previous studies (Grbic et al. 1996, Grbic et al. 1997, Strand and Grbic 1997, Grbic and Strand 1998, Harvey et al. 2000, Giron et al. 2004, Giron et al. 2007a). From these studies we know *C. floridanum* embryos clonally divide and differentiate into either soldier or reproductive larvae based on asymmetrical inheritance of germ cells. Female soldiers are aggressive toward interspecific and intraspecific competitors, including brothers, while males rarely produce soldiers and are only effective against interspecific competitors. Timing is an important feature in determining the outcome of

competition among soldiers, including timing of oviposition and emergence. *C. floridanum* lays either a single male egg, a single female egg, or both a male and female egg into a host. Single laid eggs emerge as single sex broods, while male and female eggs laid together will emerge as mixed sex broods from the same host. As predicted under conflict hypothesis theory, the sex ratio from mixed sex broods is strongly female biased.

Direction of thesis

The study by Giron (2007a) determined the importance of timing in the outcome of competition within soldiers and implicated a metabolic enzyme-encoding gene, *Gpi*, as a possible gene of major effect in soldier developmental rates. However, this finding was unexpected and as a result the design of the experiment lacked statistical power. To determine a gene of major effect, larger sample sizes are required with genetically independent wasps. Genetic independence is necessary to ensure the results gathered aren't due to a correlation of another trait found among wasps with similar genetic backgrounds.

The ability of *C. floridanum* broods to be either single sex or mixed sex complicates current theories on sex ratios in polyembryonic parasitoids, mainly due to the lack of direct knowledge on their mating structures (Hardy 2002). Observations of matings between brothers and sisters at the natal site have been recorded, but empirical data are lacking. If the majority of females are mated by their brothers prior to dispersing and males rarely mate away from the natal site, then a female biased sex ratio is preferred. As a result, there would be a predominance of mixed sex broods among *C. floridanum* populations. Therefore gathering data on mating behaviors of males and females in mixed sex broods will provide baseline predictions to sex ratio theories of polyembryonic parasitoids.

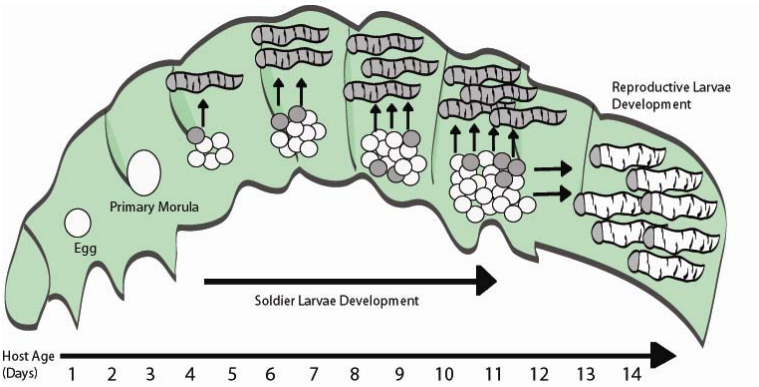

Fig 2.1: Life cycle of *C. floridanum* in its host *T. ni*. The figure illustrates a host larva and the developmental stages of *C. floridanum*. Below the schematic is the host's age (days) in relation to each developmental stage of *C. floridanum* at 27°C.

CHAPTER THREE

GPI GENOTYPE AFFECTS TIMING OF SOLDIER EMERGENCE AND THE OUTCOME OF COMPETITION

Introduction

As previously noted, *Gpi* is a metabolic enzyme involved in glycolysis that exhibits high allelic diversity in insects, and in several species strongly affects fitness under different environmental conditions (Eanes 1999). *Gpi* was first used in *C. floridanum* as a neutral molecular marker to assess soldier behavior *in vivo*, but revealed itself as a gene of possible importance in controlling soldier development time (Giron et al. 2007a). To further test this hypothesis genetically independent lines exhibiting different *Gpi* allelic variations had to be established. These lines could then be used to determine when the first soldier larva hatched (eclosed) and how this affected the outcome of intraspecific competition. My results confirm *Gpi* is a candidate gene of major effect in soldier developmental rates and *Gpi* allelic differences determine the outcome of intraspecific competition.

Materials and Methods

Survey of Gpi alleles from field collected C. floridanum populations

Parasitized hosts of *C. floridanum* were collected on soybeans and cotton in six counties across Georgia (Tift, Sumter, Decatur, Toombs, Bacon, Mitchell) in the fall of 2005, 2006, and 2007 (Table 3.1). Emerged wasps were immediately collected and frozen in a -80°C freezer and a survey of *Gpi* alleles was conducted. Fifteen female wasps from single sex and mixed sex broods were homogenized together using 25µL of a 0.05M Tris/HCl allozyme extraction buffer. Electrophoresis was conducted in 14% horizontal starch gels for 3.5 hours at 75mA with banding phenotypes visualized by histochemical staining as outlined by Shoemaker, et al (1992). *Gpi* is a dimeric enzyme and heterozygotes display a three-banded gel profile with the middle band about

twice the intensity of the flanking bands. Measurements of relative electromobility for homodimeric bands identified *Gpi* alleles found in the survey.

Timing of soldier emergence based on Gpi genotype

Using *C. floridanum* broods collected in 2007, six inbred isofemale lines were created for each of the three common alleles (Gpi^{54}, Gpi^{100}, and Gpi^{120}) by mating female wasps homozygous for each allele to hemizygous brothers bearing an identical allele. Once established the lines were designated as 54A-F, 100A-F, and 120A-F and maintained using *Trichoplusia ni* as previously described by Strand (1989a) and held in a 27°C incubator. To directly assay the effect of *Gpi* genotype on timing of soldier emergence, female wasps from each line were allowed to parasitize newly laid *T. ni* eggs and cohorts of these eggs were dissected at hourly intervals for first soldier emergence.

Outcome of intraspecific competitions based on Gpi genotype

To determine whether *Gpi* genotype affects the outcome of intraspecific competition, a host egg was simultaneously parasitized by a female wasp homozygous for one of the *Gpi* alleles (e.g. Gpi^{54}A) with another female wasp homozygous for a different *Gpi* allele (e.g. Gpi^{100}A). After the superparasitism event, hosts were reared on artificial diet and the emerging wasps were immediately frozen at -80°C. A total of twenty female wasps, ten individuals per gel lane, were sampled from each emerged brood and analyzed for *Gpi* composition using starch gel electrophoresis as indicated previously.

Results

Survey of Gpi alleles from field collected C. floridanum populations

Field collected *C. floridanum* broods over three years (2005-2007) from various counties across Georgia conform to proportions expected under Hardy-Weinberg equilibrium for *Gpi* genotypes (Fisher's exact test, P=0.1720). A significant amount of variation was seen across counties and years. Of the 143 broods genotyped, eight alleles were found, designated as *54*, *55*,

69, *77*, *100*, *109*, *120*, and *124* (Table 3.1). When all years and counties were combined the three most common genotypes were Gpi^{54}, Gpi^{100}, and Gpi^{120}, exhibiting allele frequencies of 0.169, 0.655, and 0.116 (GENEPOP 4.0), respectively. A sample starch gel of the *Gpi* variation observed in field populations is given in Figure 3.1.

Timing of soldier emergence based on Gpi genotype

A total of 18 assays were conducted to determine the effects of *Gpi* genotype on timing of soldier emergence. Soldiers homozygous for Gpi^{100} and Gpi^{120} emerged an average of 65 hours post-parasitism, while soldiers homozygous for Gpi^{54} emerged 67 hours post-parasitism (Figure 3.2). A Kruskal-Wallis test indicated that timing of first soldier emergence differed significantly with *Gpi* genotype (n=18; p=0.0132). To establish which genotype differed significantly, a Bonferroni-Dunn test compared each *Gpi* genotype to one another. This analysis indicated soldiers of the Gpi^{100} and Gpi^{120} clones emerged at similar times (p=1), but Gpi^{54} soldier clones emerged significantly later (p=0.003 for each pairing). The only exception to this trend was the Gpi^{54}F line whose soldiers eclosed on average earlier than the other Gpi^{54} lines.

Outcome of intraspecific competitions based on Gpi genotype

The two hour difference in soldier emergence times significantly affected the outcome of intraspecific competition when a host was simultaneously parasitized by two different *Gpi* genotypes (Figure 3.3). To minimize non-independence of replicates at least ten assays were conducted for each of the 18 intraspecific competitions. All emerged wasps exhibited one *Gpi* genotype or the other, never was there an instance of mixed genotypes emerging from a superparasitized host. A binomial test for each pairing of hosts parasitized with a Gpi^{100} and a Gpi^{54} egg indicated a significant disadvantage for the Gpi^{54} genotype ($p<0.001$ for A-E pairings), with the majority of emerged wasps being Gpi^{100}. The same results were seen when Gpi^{120} and Gpi^{54} were parasitized together ($p<0.05$ for all pairings), with emerging wasps bearing the *120* allele, and no adults with the *54* allele. Wasps of Gpi^{100} or Gpi^{120} had an equally likely chance of outcompeting the other, with emerged adult wasps composed entirely of *120* alleles or *100*

alleles. Again 54F behaved differently from other Gpi^{54} lines by emerging with equal frequency from hosts simultaneously parasitized with the Gpi^{100} line, 100F.

Discussion

Collectively, these data provide additional genetic support for the conclusion that *Gpi* is a gene of major effect on development time of *C. floridanum* soldiers. Statistical analysis confirmed development times among lines for each *Gpi* allele were relatively uniform, whereas the difference in development time between Gpi^{54} soldiers versus Gpi^{100} and Gpi^{120} soldiers was highly significant. My results also indicate this difference in development times contributes to Gpi^{100} and Gpi^{120} wasps outcompeting Gpi^{54} wasps due to the earlier emergence of their soldier larvae, which immediately attack and kill Gpi^{54} embryos.

It is interesting to note a host originally parasitized by wasps of two different *Gpi* genotypes resulted in progeny of only one genotype. The ability of Gpi^{100} and Gpi^{120} soldier clones to monopolize a host against Gpi^{54} clones when given only a two hour advantage is remarkable. However, this ability supports previous laboratory experiments and related observations from the field. The host, a plusiine moth, lays its eggs singly on the leaves of plants and the age of the egg affects oviposition decisions of *C. floridanum*. Females will lay more female eggs in younger hosts (<12 hours old) and more mixed broods in older hosts (Ode and Strand 1995a). As a result, the window of opportunity for parasitizing suitable eggs is quite small, less than twelve hours. Therefore, it is important, from the perspective of a soldier clone, to be able to monopolize a host as quickly as possible.

It is still unclear in this system how, mechanistically, *Gpi* genotype differentially affects soldier development rates. A continuing central issue in evolutionary biology is understanding the mechanisms by which genetic variation might lead to fitness differences among individuals (Feder et al. 2003). Among metabolic genes, natural selection is expected to target enzymes that control flux, or the rate of flow of metabolites along a pathway (Eanes 1999, Watt and Dean 2000). Under the principles of metabolic control analysis, the regulation of flux is distributed

among enzymes throughout the pathway, with branch point enzymes disproportionately regulating or controlling the expression of biochemical phenotypes (Eanes et al. 2006). Therefore the overall flux of a system can be independent of variations in activity at each step and most enzyme polymorphisms would have little effect. One criticism of control theory, however, is its disregard to the complexities of branched pathways (Eanes 1999). Under metabolic network theory variation in a single gene can affect complex metabolic pathways and therefore entire physiologies, as supported by recent reconstructions of biochemical reaction networks from sequenced genomes (Herrgård et al. 2008). From previous studies conducted on *Colias* butterflies, *Gpi* alleles differing in kinetic behavior (V_{max}/K_m ratios) differed in metabolic performance by altering the resupply of adenosine triphosphate needed for flight muscle function via glycolysis and downstream pathways (Watt et al. 2003). Sequence and structural analysis of *Gpi* indicates the most consistent differences among alleles are at the charge-changing amino acid sites in the catalytic center, thereby altering the enzyme's substrate specificity and being the proposed site for selection (Wheat et al. 2006).

Within my results the Gpi^{54} line, 54F, phenotypically behaved as either a *100* or *120* allele based on soldier developmental rates and the outcome of intraspecific competition (Figs. 3.2, .3.3). Polymorphisms among allozymes is thought to be attributable to nucleotide substitutions causing replacements of charged amino acids (Avise 1994). One criticism of allozyme electrophoresis is the inability to see high levels of variation in the bands. Therefore the 54F behavioral difference could result from the presence of a "cryptic", functionally *100/100*-like allele, that encodes a protein bearing the net charge of, and thus electrophoretically indistinguishable from, a *54/54* allele product. The 54F exception could also lend support to the complexities of metabolic pathways by providing evidence that an amino acid substitution with the same charge is enough to alter an enzyme's activity and affect the overall phenotypic outcome of the pathway. Alternatively, *Gpi* could simply be a marker for a tightly linked gene that has the major functional effect, and the 54F allele is simply a recombinant haplotype. Extending studies

of sequence and structure of *Gpi* variants in *Colias* butterflies to populations of *C. floridanum* will help to understand the underlying causes of *Gpi* genotype influence on soldier development rates.

Table 3.1: Frequencies of *Gpi* alleles found in various counties across Georgia in the fall of 2005, 2006, and 2007 from females of single sex and mixed sex broods. Allele frequencies calculated using GENEPOP 4.0. The three most common alleles were *54, 100,* and *120*, exhibiting frequencies of 0.169, 0.655, and 0.116 for all counties and years combined.

County	***Gpi***	**Allele Frequency**		
		2005 (n=29)	**2006 (n=6)**	**2007 (n=8)**
Tift	*54*	0.190	0.250	0.188
	55	------	0.167	------
	77	0.017	------	------
	100	0.655	0.333	0.688
	109	0.017	------	------
	120	0.121	0.250	0.125
		2005 (n=14)	**2006 (n=3)**	**2007 (n=13)**
Sumter	*54*	0.179	------	0.167
	55	------	0.167	------
	69	------	------	0.042
	100	0.679	0.833	0.625
	109	------	------	0.125
	120	0.143	------	0.042
		2005 (n=17)	**2006 (n=4)**	**2007 (n=10)**
Decatur	*54*	0.118	0.250	0.250
	55	0.029	------	------
	100	0.765	0.500	0.650
	109	------	0.250	------
	120	0.088	------	0.100
			2006 (n=15)	
Toombs	*54*		0.107	
	55		0.071	
	77		0.036	
	100		0.571	
	120		0.143	
	124		0.071	
			2006 (n=12)	
Bacon	*54*		0.167	
	100		0.708	
	120		0.125	
				2007 (n=12)
Mitchell	*54*			0.125
	55			0.042
	100			0.667
	120			0.167

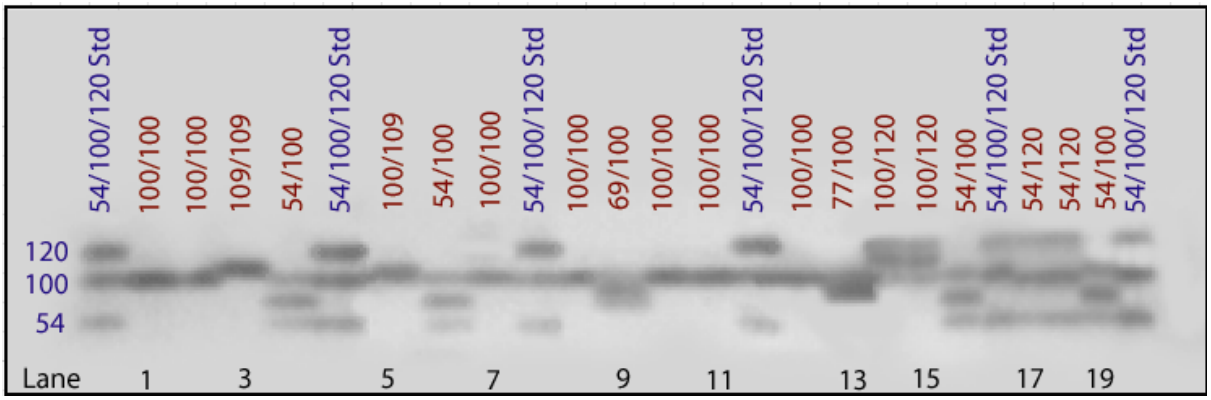

Fig. 3.1: A sample starch gel displaying the allelic diversity of *Gpi* found among *C. floridanum* samples collected in Georgia. Each lane represents 15 clonal females pooled from a single brood. *Gpi* is a dimeric enzyme and heterozygotes display a three-banded gel profile with the middle band about twice the intensity of the flanking bands. Measurements of relative electromobility for homodimeric bands and comparisons to that of a *54, 100,* and *120* allele standard helped identify rare *Gpi* alleles.

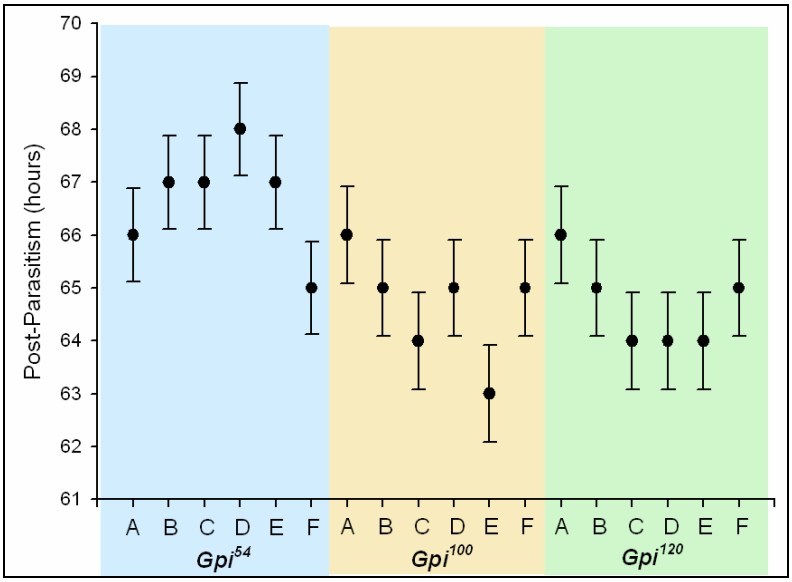

Fig 3.2: Time of soldier emergence based on *Gpi* genotype. Wasps from six lines (A-F) homozygous for the *54* allele, the *100* allele, and the *120* allele oviposited into newly laid host eggs. Hourly dissections of hosts (n=10) were conducted starting at 61 hours post-parasitism. Data points represent the hour in which all hosts contained an eclosed soldier. Soldier clones of Gpi^{100} and Gpi^{120} had similar emergence times, while Gpi^{54} soldiers emerged significantly later (n=18; Kruskal-Wallis; p=0.0132).

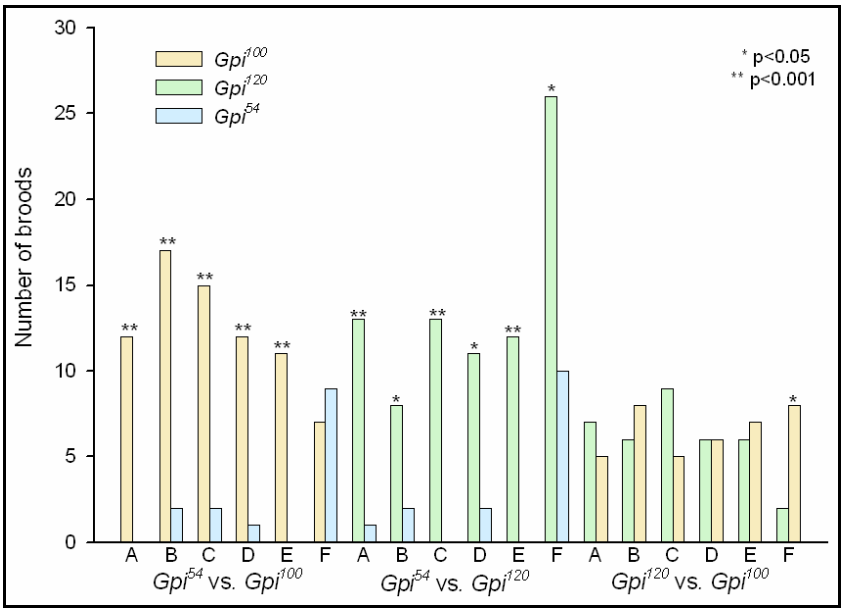

Fig 3.3: The outcome of intraspecific competition of a superparasitized host. Letters along the x-axis correspond to the two homozygous lines used for each superparasitism assay. For example, Gpi^{54A} was parasitized with Gpi^{100A}, Gpi^{54B} was parasitized with Gpi^{100B}, and so on for all three genotypes and six A-F lines (see Methods). All progeny emerging from a host were of a single genotype and the number of broods emerging from each assay is plotted on the y-axis. Gpi^{100} and Gpi^{120} wasps significantly emerged more when parasitized with Gpi^{54} (Binomial test; $p<0.001$ for A-E pairings). However wasps of Gpi^{100} and Gpi^{120} equally emerged from a host superparasitized with the two genotypes.

CHAPTER FOUR

LIFE-HISTORY TRADE-OFFS AFFECT GPI ALLELE FREQUENCIES, NOT HETEROZYGOTE ADVANTAGE

Introduction

Based on the results from chapter three, *Gpi* is a gene of major effect in soldier development time and the outcome of intraspecific competition. In this regard wasps homozygous for the *54* allele are at a disadvantage relative to wasps homozygous for the *100* and *120* alleles. However, the field survey conducted in chapter three indicates the *54* allele is the second most common allele in a population maintained in Hardy-Weinberg equilibrium. The *120* allele is the third most common allele, but has the same soldier development rates as the most frequent *100* allele.

Studies in other organisms suggest two possible explanations for maintenance of disadvantageous alleles: a life-history trade-off and/or balancing selection acting through a heterozygote advantage (Zera and Harshman 2001, Kneitel 2004, Sgro and Hoffmann 2004). Trade-offs represent costs associated with fitness when a beneficial change in one trait is linked to a detrimental change in another (Stearns 1989). In the case of *Gpi* allelic variation in *C. floridanum*, the *54* homozygous allele prolongs soldier emergence, but it could result in an advantage in another aspect of development or survival that encourages its common occurrence in the field. Reciprocally, the *120* allele provides wasps the benefit of earlier emergence of soldiers, but potentially at the cost of being disadvantageous in another trait. Life-history trade-offs associated in allelic diversity have been established in numerous systems ranging from marine gastropods (Cotton et al. 2004), to insects (Trumbo and Robinson 2004, Zera 2005) and birds (Lack 1947, Lindén and Møller 1989, Godfray et al. 1991). Taken together with observations of fitness traits being maintained well within the limits otherwise imposed by history and design

indicate selection does act through life-history trade-offs (Stearns 1989). Physiological studies suggest life-history trade-offs are the result of competition among different functions for limited internal resources (Ricklefs and Wikelski 2002). Since *Gpi* is a metabolic enzyme and is known to affect development of soldier larvae, two additional developmental traits were investigated for differential fitness affects based on *Gpi* genotype.

Based on other studies of *Gpi* polymorphisms in insect systems and the results reported thus far for *C. floridanum* population, *Gpi* frequencies could also be under a form of balancing selection (Katz and Harrison 1997, Watt and Dean 2000, Haag et al. 2005, Hanski and Saccheri 2006). In the survey results of *Gpi* alleles within *C. floridanum* from Chapter Three, the populations maintain stable allele frequencies of two or more phenotypic forms, the classical definition of balancing selection. The two most well studied mechanisms of balancing selection include heterozygote advantage (overdominance) and frequency dependent selection. In heterozygote advantage, an individual who is heterozygous at a particular locus has a greater fitness than a homozygous individual. For example, in *Colias* species complexes studied so far, there is widespread *Gpi* heterozygote advantage in kinetics, expressed in high values of V_{max}/K_m (Watt 1977). Further, all *Gpi* homozygotes display consistent trade-offs of kinetic performance versus enzyme stability (Wheat et al. 2006).

In field collected *C. floridanum* broods, populations conformed to proportions expected under Hardy-Weinberg equilibrium for *Gpi* genotypes, with more *54/100* heterozygotes present (22%) than *54* homozygotes (4%). This suggests *Gpi* heterozygotes could have an advantage compared to homozygous wasps in regard to soldier developmental rates. In response, the *54/100* genotype will emerge from a host superparasitized with either a Gpi^{100} or Gpi^{54} homozygote. Testing this prediction required repeating the timing of soldier development assays and the intraspecific competitions of objective one with wasps heterozygous for Gpi^{54} and Gpi^{100}.

I examined two developmental traits for possible life-history trade-offs relative to soldier development: total development time and total brood size. Of the two traits, there was a

significant difference of brood size among the three *Gpi* allele variations. For assays investigating the possibility of heterozygote advantage in maintaining Gpi frequencies, my results reveal heterozygotes are intermediate to homozygotes. Overall, the data suggests life-history trade-offs are the reason for *Gpi* frequencies within *C. floridanum* and not a heterozygote advantage leading to balancing selection.

Materials and Methods

Life-history trade-off assays

Newly emerged, mated female wasps from each *Gpi* line established from chapter three (Gpi^{54} A-F, Gpi^{100} A-F, Gpi^{120} A-F) were allowed to parasitize newly laid cabbage looper eggs (<24 hours old). Upon oviposition, hosts continued development on a pinto bean diet in a 27°C incubator with a 16 hour light/8 hour dark cycle. Date of emergence and brood type were recorded for each sample and total development time was taken as the number of days from oviposition to emergence of adult wasps. Subsamples of these broods were immediately frozen in a -80°C freezer upon emergence and used for the assays of brood size. Brood sizes were established by counting the number of individuals from single sex and mixed sex broods under a microscope.

Heterozygote advantage assays

It has been established from Chapter Three that Gpi^{100} and Gpi^{120} homozygous wasps exhibit similarities of soldier emergence times and the outcome of intraspecific competition (Figs. 3.2, 3.3). Therefore only Gpi^{100} wasps were used with Gpi^{54} to assay heterozygote advantage in soldier development times and the outcome of intraspecific competition. Soldier development times were assessed as described in Chapter Three, but with four heterozygous lines. Heterozygotes were created by mating a female wasp homozygous for the *54* allele (ie: 54B, 54D, 54E, and 54F) with a male wasp hemizygous for the *100* allele (ie: 100B, 100D, 100E, and 100F). Therefore the four heterozygotes used for the assay were $Gpi^{54B/100B}$, $Gpi^{54D/100D}$, $Gpi^{54E/100E}$ and $Gpi^{54F/100F}$. Intraspecific competition assays were conducted as described in chapter three, but

host eggs were simultaneously parasitized by a homozygous female wasp and a heterozygous female wasp. The heterozygotes used were the same four created for the soldier development assays, and the homozygotes used were Gpi^{100E}, Gpi^{100C}, Gpi^{100B}, Gpi^{100A}, Gpi^{54C}, and Gpi^{54A}. The intraspecific competition assay pairings are shown in Table 4.1.

Results

Total development time of the reproductives is not affected by Gpi genotype

Of the 18 lines established from Chapter Three, 296 broods homozygous for the *54* allele, 137 broods for the *100* allele, and 132 broods for the *120* allele with all 18 lines represented were assayed for total development time. For all three genotypes, the average number of days from oviposition to emergence of adult wasps was 28 (Fig. 4.1). Analysis of covariance was conducted on total development time with brood type (single sex, mixed sex) serving as the covariates to investigate factors contributing to variance in total development time. This test revealed *Gpi* genotype did not affect development time (JMP software version 8.0, F=2.8055, p=0.0613).

Total brood size is affected by Gpi genotype

The number of individuals from single sex and mixed sex broods were counted for each *Gpi* line established from Chapter Three for a total of 54 assays (Fig. 4.2). Analysis of covariance was conducted on brood size with brood type serving as the covariate to investigate factors contributing to variance in brood size. This test revealed *Gpi* genotype affects brood size, with Gpi^{100} broods producing fewer females than either Gpi^{54} or Gpi^{120} broods (F=3.4805, p=0.014).

Heterozygous soldiers development times are intermediate to Gpi^{54} and Gpi^{100} homozygote

A total of 4 assays, one for each *Gpi* heterozygote line created, were conducted to determine the existence of a heterozygote advantage. The heterozygous soldiers emerged between 64 and 67 hours post-parasitism for an average of 65.5 hours post-parasitism. My results indicated that heterozygous soldiers emerged at intermediate levels in comparison to the homozygotes lines used to create them, except 54F (Fig. 4.3).

Heterozygosity lessens the 54 allele disadvantage

A total of six different intraspecific competition experiments were generated for heterozygotes and homozygotes (Table 4.1, Fig. 4.4). A binomial test revealed Gpi^{100} significantly emerged in two of the assays (1 and 2; $p<0.0001$), while $Gpi^{54/100}$ significantly emerged in two other ($p<0.0001$ for assay 3; $p<0.05$ for assay 4). Of the two assays competing homozygous *54* wasps against heterozygous *54/100* wasps, Gpi^{54} wasps emerged significantly more often from assay 5 ($p<0.0001$) while $Gpi^{54/100}$ significantly emerged in assay 6 ($p<0.05$). The seemingly unpredictable outcome of intraspecific competitions can be explained with the soldier developmental rates collected for heterozygotes and homozygotes. In assay one ($Gpi^{54B/100B}$ vs. Gpi^{100E}) heterozygote soldier emerge at 66 hours (Fig. 4.3), homozygote *100E* soldiers emerge at 63 hours (Table 4.2, Fig. 3.2). This difference in soldier emergence times explains the ability of homozygotes to outcompete heterozygotes in assay one. This scenario holds true for the majority of intraspecific competitions and Table 4.2 relates each intraspecific competition with soldier developmental rates. Overall, faster developing homozygote soldiers can outcompete slower developing heterozygote soldiers, and therefore homozygotes emerge significantly more frequently from a superparasitized host. The opposite is also true, with faster developing heterozygote soldiers affecting the outcome of intraspecific competition in their favor when simultaneously parasitized with slower developing homozygous soldiers.

Two additional intraspecific competition experiments were conducted with two additional heterozygous lines; $Gpi^{54A/100A}$ vs. Gpi^{100F} and $Gpi^{54F/100F}$ vs. Gpi^{54A}. Data for soldier development time for these heterozygotes is lacking, and therefore the results were not included in Tables 4.1, 4.2 or Figs. 4.3, 4.4. However the results of the intraspecific competition experiments are as follows. A binomial test of the $Gpi^{54A/100A}$ vs. Gpi^{100F} assay revealed Gpi^{100F} broods emerged significantly more ($p<0.001$). A binomial test of the $Gpi^{54F/100F}$ vs. Gpi^{54A} revealed heterozygotes and homozygotes emerged equally.

Discussion

My results suggest a life-history trade-off may exist for the *54* allele in regard to soldier development time and female brood size. The field survey conducted in Chapter Three indicated that the *120* allele is the third most common allele, even though its soldier development rates are similar to the most common *100* allele. According to my results the *120* allele also produces large female broods, a trait that I conclude maintains the *54* allele in field populations. This suggests the possibility of other life-history trait that could have an effect on the *120* allele. Previous work on differential fitness effects of *Gpi* polymorphisms in other insect systems provide some clues for potential life-history traits to examine for the existence of trade-offs (Zera and Harshman 2001). Glycolysis supplies energy to insect flight, and the fitness of many insect species is dependent on flight capacity. In adult *Colias* butterflies *Gpi* polymorphisms differ in enzyme kinetics and thermal stability (Watt et al. 1983). This difference can accurately predict flight performance among the allelic variations in the wild (Watt 1992). As a result, survival, in terms of male mating success and female fecundity are affected (Watt et al. 2003). Within Glanville fritillary butterflies the genetic variation of *Gpi* on flight metabolism, results in differential dispersal rates and thereby metapopulation dynamics (Haag et al. 2005). In montane leaf beetles variation at the *Gpi* locus is consistently related to traits that allow individuals to cope with elevated and subzero temperatures in nature (Dahlhoff et al. 2008). Investigating the advantages/disadvantages of any one of these traits for a wasp bearing the *54, 100* or *120* allele could provide a more complete answer to the *Gpi* allele frequencies seen in *C. floridanum* populations.

My results do not indicate a heterozygote advantage, and therefore suggests *Gpi* is not being maintained through balancing selection. However, *Gpi* seems to be co-dominant with heterozygotes emerging intermediate to homozygotes and as a result, the two hour soldier development difference seen in *54* homozygotes is diminished. Originally, I thought *Gpi* heterozygotes would be at an advantage in the outcome of intraspecific competition because of previous results in *Colias* butterflies. However, the intermediacy of *Gpi* heterozygotes has been

documented in other systems (Hoffmann 1981, Dahlhoff and Rank 2000). In the montane willow beetle, *C. aeneicollis*, the fastest migrating *Gpi* allele (designated as 1-1) had greater K_m values and measures of thermal stability of enzymatic activity than the heterozygote (1-4). In turn the heterozygote had greater K_m and lower thermal stability temperatures than the slower migrating allele, designated as 4-4 (Dahlhoff and Rank 2000). Overall the data suggests the 1-4 heterozygotes have intermediate functional properties between the 4-4 and 1-1 homozygotes. These results and the fact that certain *Gpi* alleles were more common in different geographic regions, implies that directional selection is maintaining allelic diversity and not balancing selection within *C. aeneicollis* (Dahlhoff et al. 2008). However, my survey of *Gpi* alleles did not indicate certain alleles were favored in different regions, but samples were all taken from soybean and cotton fields, and not diverse geographic regions. Future studies with *C. floridanum* sampled from more diverse geographic regions will help establish if certain *Gpi* alleles are more common in different locations and if directional selection is maintaining allelic diversity.

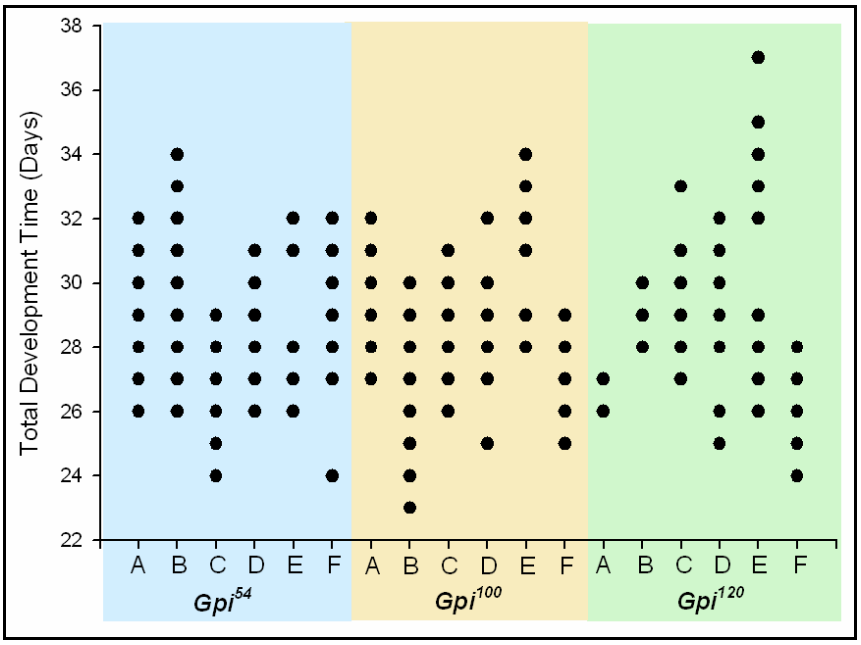

Fig. 4.1: Total development times based on *Gpi* genotype. The number of days from oviposition to emergence of adult wasps were counted for each of the 18 *Gpi* lines (Gpi^{54} A-F, Gpi^{100} A-F, Gpi^{120} A-F) established from Chapter Three (n=296 for Gpi^{54}, n=137 for Gpi^{100}, n=132 for Gpi^{120}). Analysis of covariance indicated no significant difference between the *Gpi* genotypes for total development time (JUMP 8; F=2.8055, p=0.0613)

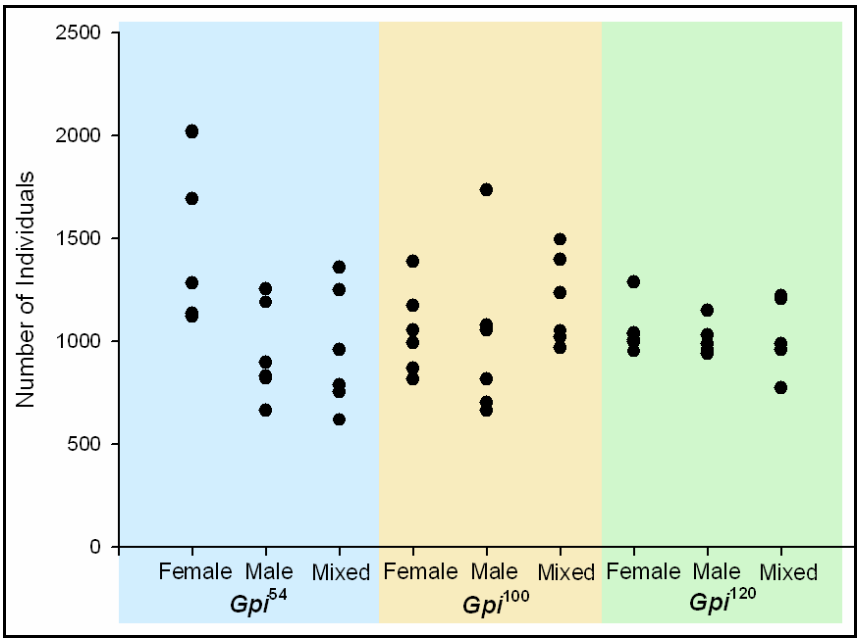

Fig. 4.2: Brood size of single sex and mixed sex broods based on *Gpi* genotype. One female brood, one male brood, and one mixed sex brood from each *Gpi* line established in chapter three were counted for the number of individuals present. Analysis of covariance indicated a significant difference among the number of Gpi^{100} females from single sex broods (F=3.4805, p=0.014).

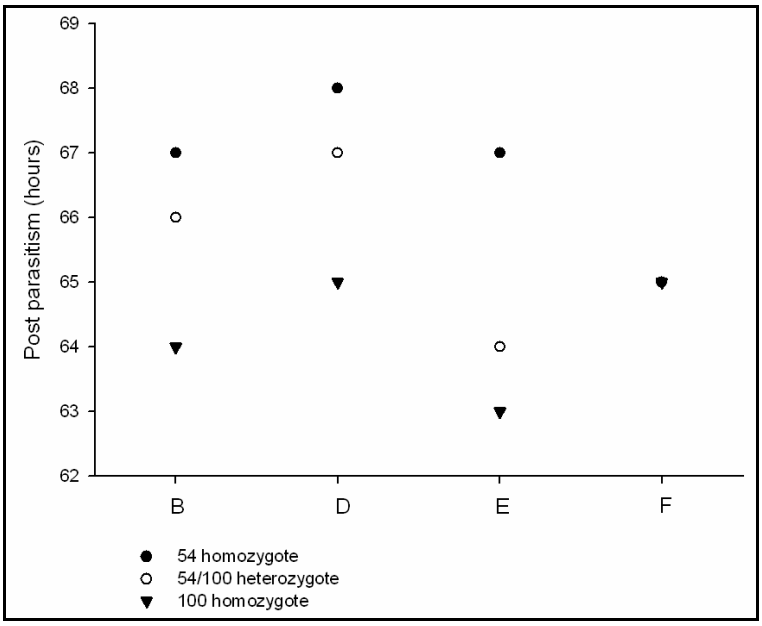

Fig. 4.3: Soldier development times for heterozygotes and homozygotes. The letters along the x-axis correspond to the family line of homozygotes used to create the heterozygotes. For example, letter B: line 54B (closed circle) was crossed with line 100B (closed triangle) to create the Gpi^{54B} x Gpi^{100B} heterozygote (open circle). This data indicates *Gpi* heterozygotes have intermediate soldier development times compared to homozygotes when kept at a constant temperature of 27°C.

Table 4.1: Intraspecific competition assays conducted with heterozygous crosses superparasitized with homozygous lines. Heterozygotes were created by mating a female wasp homozygous for a *54* allele with a male wasp homozygous for a *100* allele. Offspring (F1) of this crossing were superparasitized along with a different homozygote line of either the *54* or the *100* allele.

Assay	**Heterozygote**		**Homozygote**
1	$Gpi^{54B/\ 100B}$	vs.	Gpi^{100E}
2	$Gpi^{54D/\ 100D}$	vs.	Gpi^{100C}
3	$Gpi^{54E/\ 100E}$	vs.	Gpi^{100B}
4	$Gpi^{54F/100F}$	vs.	Gpi^{100A}
5	$Gpi^{54D/\ 100D}$	vs.	Gpi^{54C}
6	$Gpi^{54F\ /100F}$	vs.	Gpi^{54A}

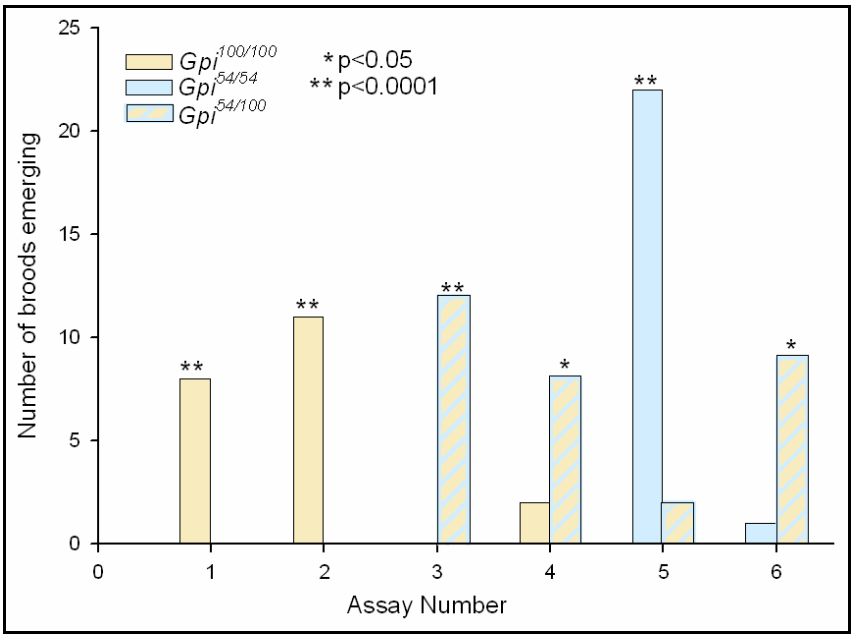

Fig. 4.4: Brood *Gpi* composition of emerged wasps from a host superparasitized by two female wasps exhibiting a homozygous *Gpi* and a heterozygous *Gpi*. The outcome of intraspecific competition was dependent on the soldier developmental times in Table 4.2. Faster developing homozygote soldiers emerged significantly more than slower *54/100* heterozygotes (Binomial test; $p<0.001$ for assay 1, 2, 5). Faster developing heterozygote soldiers emerged significantly more than slower homozygote soldiers ($p<0.001$ for assay 3; $p<0.05$ for assay 4, 6). Numbers along the x-axis correspond to the assay number in Table 4.1.

Table 4.2: Intraspecific competition assays conducted with heterozygotes and homozygotes with soldier development times. Homozygote development times were established from chapter three. In comparison to the outcome of intraspecific competitions represented in Fig. 4.4, the *Gpi* genotype with the faster soldier emergence time emerged significantly more from a host.

Assay	***Gpi* Genotype**	**Time to Soldier Emergence (hours post-parasitism)**
1	$Gpi^{54B/100B}$	66
	Gpi^{100E}	63
2	$Gpi^{54D/100D}$	67
	Gpi^{100C}	64
3	$Gpi^{54E/100E}$	64
	Gpi^{100B}	65
4	$Gpi^{54F/100F}$	65
	Gpi^{100A}	66
5	$Gpi^{54D/100D}$	67
	Gpi^{54C}	67
6	$Gpi^{54F/100F}$	65
	Gpi^{54A}	66

CHAPTER FIVE

MATING BEHAVIORS OF MALES AND FEMALES

Introduction

The diversity of life histories among parasitoid wasps is associated with similarly wide variations in mating strategies. These mating systems include complete inbreeding, partial inbreeding, outcrossing, and production of all-male broods by unmated females (Godfray 1994). As addressed in the background to this thesis, *Copidosoma floridanum* produces mixed sex and single sex broods, indicating the possibility for both outbreeding and inbreeding. Recent studies have tried to predict mating systems based on the sex ratios of a population (Taylor 1993, West et al. 2000, Greeff 2002). In *C. floridanum*, previous studies have established the presence of a female biased sex ratio among field populations (Grbic et al. 1992, Ode and Strand 1995a). In haplodiploid organisms two factors are known to select for a female biased sex ratio; local mate competition (LMC) and inbreeding. Under strict LMC, males mate exclusively on their emergence patch and brothers compete for a limited number of females to mate (Hamilton 1967). However, among many organisms, including *C. floridanum*, males have the ability to disperse from their natal site and find other patches where they also compete for access to females. Nunney and Luck (1988) considered this scenario and developed a partial LMC theory to explain male dispersal decreasing the competitive asymmetry between sons and daughters and therefore diminishing the sex ratio bias toward females.

The goal of this objective was to characterize the mating behavior of *C. floridanum* and how this might contribute to the brood type and sex ratios observed in the fields. Theory predicts male wasps emerging from a mixed sex brood will mate with their sisters before dispersing. Testing this prediction requires collecting dispersed females from mixed sex broods and recording mating status, as indicated by the presence or absence of sperm in the spermatheca.

However, male mating success after dispersal can also determine the extent of local mate competition and inbreeding. Thus, I also examined male mating behavior after dispersal from a natal site. Overall my results indicate most females are mated by their brothers before dispersing. Females will multiply mate but almost all female offspring were sired by the first male she copulated with. To replicate conditions in the field of mating behaviors, males were offered a varying number of virgin, mated, young, old, and previously oviposited females. My results suggest once males disperse from a mixed sex brood, their chances for successful matings decreases.

Materials and Methods

C. floridanum and its host, *Trichoplusia ni*, were reared at 27°C as described by Strand (1989b) from a lab culture originally collected from field populations in Wisconsin and Georgia. Analyses of Gpi allelic composition of the lab culture indicate the population is fixed at the *100* allele, and therefore any *Gpi* affect on mating was controlled. Assays using brothers and sisters were conducted on females and males emerging from mixed sex broods immediately upon emergence and collected in individual test tubes to ensure individuals were virgins.

Mating proportions of females from mixed sex broods

To assess the proportions of females in mixed broods that mate with brothers before dispersing, I constructed a 2'x 2' using ½" PVC (polyvinyl chloride) pipe surrounded on five sides with white organza material was used. Prior observations revealed wasps will continue to walk along a surface if given the opportunity instead of flying. Therefore the top of the dispersal arena was left open and any wasps reaching a set "dispersal" line at 1'8" from the floor were collected into test tubes. A mummy containing a known mixed sex brood was placed in the center of the arena upon emergence. An aspirator containing a physiological saline solution in the collecting chamber was connected to a vacuum. At thirty minute intervals, wasps reaching the dispersal line were collected and later sexed. Subsamples of ten females from each thirty

minute interval were dissected in Pringle's solution for the presence or absence of sperm in the spermatheca to determine mating status. Brood sex ratios, the time required for all progeny to emerge from a mummy, and the percentage of females that mated before dispersing from the arena were determined.

Sperm use after multiple mating

To establish which sperm a female used to sire offspring when mated by a relative and a non-relative, the father of the offspring was determined. Using the *Gpi* lines created for objective one in my thesis, females of one *Gpi* genotype (e.g. Gpi^{54}) were sequentially mated with a brother, then with a non-relative of a different *Gpi* genotype (e.g. Gpi^{100}). The same assay was repeated with another female, but with a non-relative male used first then a brother. To expedite the multiple mating events males and females were isolated inside the tip of a 1mL clear pipette tip. After the multiple mating events, females were allowed to oviposit their eggs into a host and reared following the protocol in chapter three. Upon emergence, female broods were collected, immediately frozen and genotyped for their *Gpi* allele composition as discussed previously in chapter three. The resulting *Gpi* genotype of the female offspring revealed which male the female used to sire her offspring.

Factors affecting time to first mating

For all assays on factors affecting time to first mating, wasps were placed under a Falcon 60x15mm disposable polystyrene petri dish top, with a white square piece of paper underneath. Mating behaviors were observed using a dissecting microscope. Prior observations indicated if mating occurred, it happened within the first five minutes, and rarely at a later time. Except for assays studying the effect of age on mating, males and females used were newly emerged (less than twelve hours old). Wasps were fed a 10% sugar water solution before each assay. A stopwatch was used to determine a mating event, consisting of a male on top of a female while extending his aedeagus.

Density of females

Males from a mixed sex brood are in close and repeated contact with numerous females and as a result intense mating occurs at the natal site. Males from single sex broods, however, are not exposed to females upon emergence and must mate away from the natal site. Therefore assays were conducted to determine if the number of females affects the time to first mating. Males from a single sex brood were individually placed with either a single, virgin female or multiple females (2-30). New males and females were used for each assay, and time to first mating event was recorded.

Mating status of females and males

Previous experience with females may improve a male's ability to locate and mate with a female. Therefore the effect of male mating status on time to mating was investigated. Ten males from a single sex brood were forced to mate with single females. Once mated, males were separated into individual test tubes and fed. As a control, males from the same single sex brood were individually separated into test tubes and fed. After six hours the previously mated males were put with a single, virgin, newly emerged female and time to first mating was recorded. The same assays were repeated on the control males. To determine the effect of female mating status on time to first mating, mated females and virgin females were paired with single virgin males. Females from a single sex brood were forced to mate with a male. Once mated the females were separated into individual test tubes, fed, and held for six hours. Twenty of these mated females were then put with an individual male. The time to first mating as well as the number of matings in three minutes was recorded. Twenty virgin females from the same single sex brood went through the same conditions and were used as a control.

Oviposition status of females

To establish the effect of female oviposition status on a male's ability to initiate a mating event, virgin females (control) and post-oviposition females were used. Individuals and groups of twenty females were allowed to oviposit once in a host and immediately placed with an

individual male. The same assay was conducted on virgin females, no oviposition (control) with time to first mating event recorded.

Age of females and males

There is evidence from some parasitoids that if females fail to find a mate early in life (in some cases just after one day) they are subsequently unreceptive to males and thus only produce male eggs (Godfray 1994). To establish if the same is true for *C. floridanum*, females from a single sex brood were separated into individual test tubes, fed, and held for 24 hours. For each assay, a day-old female (n=20) or a newly emerged female (n=20) was placed with a single, newly emerged male and the time to the first mating event was recorded.

Models of age-related mate choice predict that females should prefer older males as mates because they have proven survival ability (Kokko and Lindstrom 1996, Proulx et al. 2002). Therefore by mating with older males, females gain indirect benefits through production of higher-quality offspring. However, female discrimination against older males has been reported in a few species (Ritchie et al. 1995, Jones et al. 2000) To determine how male age affects mating in *C. floridanum,* the time to first mating event with old and young males were recorded. Males from a single sex brood were held in individual test tubes for 24 hours, and fed prior to each assay. Assays consisted of a single, newly emerged female with either a single newly emerged male (n=20) or a single, 24-hour old male (n=20).

Results

High levels of sib-mating among mixed sex broods

Ten observations of mixed sex mummies placed within the dispersal arena indicated progeny emerged from hosts over a period of 1.5 to 4 hours. During the assays, there was a large density of wasps remaining within a five inch radius of the host, while others were dispersing quickly. To determine if these individuals were male or female a subsample was collected and sexed. The majority of individuals collected were male, indicating males stayed within close proximity of the host and dispersed when all progeny from the mummy emerged. Further support

for this scenario comes from the sex ratio of the dispersed individuals gathered every thirty minutes. In the majority of assays, the number of dispersed males is low toward the beginning and middle of emergence, but increases dramatically within the last hour of emergence (Fig. 5.1). One observation was omitted from Figure 5.1 as an outlier, but is reported in Fig. 5.2. Males from mixed sex broods are clone mates and are genetically identical, therefore no male competition occurs at the natal site. Dissections of dispersed females from each thirty minute interval (n=10) revealed that two-thirds to all were mated upon dispersal, even when the brood sex ratio (male:female) was as low as 7% (Fig. 5.2). Mating at the emergence site and protandry (males emerging before females) is very common among parasitoids. The data collected suggest protandry may allow polygynous males to maximize their opportunities to mate with females before dispersal.

Sperm use after multiple mating

To determine if females mated by their brothers and with non-relatives will use sperm from the latter to sire her offspring to avoid inbreeding, assays of sperm precedence were conducted. Six assays revealed the first male to mate with a female sired all of her offspring, independent of the relatedness of the first male (Fig. 5.3). For all replicates (n=69) there was no occurrence of mixed genotypes emerging from a host. A binomial test conducted on each of the 6 assays, revealed a statistical significance existed for the first male's *Gpi* allele occurring among the offspring ($p<0.001$ for five assays, $p<0.05$ for one assay). Assays to determine the propensity of males to mate with a previously mated female were also conducted and the results are reported later in this thesis.

Factors affecting time to first mating event

Mating is female-density dependent

One hundred replications with single, virgin males and varying numbers of virgin females (one to thirty) were conducted and the time to mating was recorded. Spearman's correlation revealed a significant negative correlation between the number of females present and

the time to mating (Spearman's ρ= -0.5619, $p<0.0001$, Fig. 5.4). To confirm that mating was sex-density dependent, and not due to a male's affinity to mate with any sex present in great numbers, a single male was placed with thirty other males (n=10). In the five minute observations, no mating event was recorded. Close observations of some of the assays indicate that a male's dependence on density is not correlated with the frequency of encounter rates, but the number of encounters with different females. The assays were conducted in a relatively small arena (60x15mm) with males usually more active than females. During one observation, a single male and single female came within contact of each other 17 times in twenty-three minutes. On each occasion the male would drum his antennae over the female, and over half the time the male would break contact and move away. In another observation a single male and a single female came within contact of each other 8 times in five minutes. The same male was then placed with 20 virgin females and mating occurred with the fifth female he encountered after 41 seconds.

Neither mating status of male nor female affects time to first mating

Assays leading to the result that mating is density dependent were conducted on virgin males. To determine if "experienced" males were able to recognize and mate with females quicker than "inexperienced" males, single females were placed with virgin and non-virgin males. A Spearman's correlation test revealed there is no significant correlation with the time to first mating and the mating status of the male (Spearman's ρ= -0.3243, $p=0.1631$; Fig. 5.5). This suggests the results seen in the density-dependence assays were not due to a lack of male mating experience.

For the sperm precedence assays females and males were confined to a small area to increase the likelihood of a mating event. To determine if, in more natural circumstances, males choose not to mate with non-virgin females; previously mated females (n=14) and virgin females (n=14) were offered to single males. Since males are more likely to mate when more than one female is present, groups of twenty females were used. A Spearman's correlation test revealed no correlation between the time to a mating event and the mating status of females (Spearman's

ρ= -0.008, p=0.9644, Fig. 5.6). The fact that females were previously mated did not affect the number of mating events either. In three minutes there were on average 6 mating events among virgins and with females previously mated. In the experimental setup, the duration between first and second mating was less than ten minutes. Combined with observations of repeated mating during rearing of the lab culture, females do not enter a refractory period upon emergence, contrary to other insects (Arnqvist and Nilsson 2000).

Oviposition status of females does not affect time to first mating

To determine the affect of oviposition on time to first mating, single males were offered single, post-oviposited females (n=10) and single females with no oviposition experience (n=10). The same assays were conducted with groups of twenty females to exclude any density dependence factors. A Spearman's correlation test revealed no correlation between the time to a mating event and the oviposition status with either a single female (ρ=0.1018, p=0.6693, Fig. 5.7), or twenty females (ρ=-0.2603, p=0.2676, Fig. 5.7).

Neither age of female nor male affects time to first mating

Newly emerged (n=20) and 24-hour old (n=20) females were placed with single, virgin males (Fig. 5.8). The results indicate that males mate with day old females and there is no correlation with time to first mating and age of the female (Spearman's ρ=-0.1218, p=0.4542). The results suggest that females are still receptive to males, even after 24-hours from emergence and there are no restrictions of mating to first day of adult life.

Reciprocally assays were conducted to determine if the time to first mating is affected by the age of the male, by using newly emerged males (n=20) and 24-hour old males (n=20; Fig. 5.9). A Spearman's test revealed there is no significant correlation between male age and time to first mating (ρ=-0.1713, p=0.2906). The results indicate females are receptive to either young and old males, suggesting neither group had significant costs or benefits.

Discussion

My results confirm predictions that the majority of females are mated upon dispersal from a mixed sex brood by their brothers. Also if females multiply mate, the sperm from the first male will fertilize her broods, independent on whether the first male is a relative or non-relative. Of the factors investigated for affecting time to first mating event (number of females, mating status, oviposition status of female, age) only the number of females revealed a significant correlation.

Although a majority of females mate before dispersing from a mixed sex brood, many females disperse without mating. Combined with that fact that females from single sex broods likely must also disperse to mate, suggests *C. floridanum* is under partial local mate competition and therefore has partial inbreeding. This is comparable to other parasitoids, such as *Trichogramma papilionis* and *T. pretiosum* in which 10-15% of females left the host without mating (Antolin 1999). The key element in partial LMC is off-patch mate attraction. The frequency and timing of mating events by a male was highest with the high densities of females (Fig. 5.4). However, males do not discriminate between age, mating status, or oviposition status of females when it comes to mating (Figs. 5.5-5.9). In addition females are receptive to males, independent of age, mating status, or oviposition status. This indicates wasps have the potential to mate with any individual they encounter, with the highest possibility occurring when females are in high numbers. The results supporting the negative correlation of female numbers and time to first mating could be indicative of swarming behavior. In one species of *Copidosoma* in California male swarms were observed, and are believed to be used by an Australian *Copidosoma sp.* (Nadel 1987, Walter and Clarke 1992). Little information is available about swarming in parasitoids, but a few are known to form all-male swarms that are visited by virgin females, including braconids, dryinids, and chalcids (Godfray 1994). Future studies investigating swarming behavior in *C. floridanum* will help explain a male's disinclination to initiate mating with a single female.

Multiple mating of females (polyandry) occurs frequently among insect species (Arnqvist and Nilsson 2000) and data has shown *C. floridanum* males mate multiple virgin females and previously mated females (Fig. 5.6). For these reasons one could argue the high rate of mated females from mixed sex broods does not constitute inbreeding because sperm from a relative might not be used to fertilize eggs. In other parasitoid species the evolution of dispersal away from the natal site has been hypothesized as an inbreeding avoidance mechanism (Nadel and Luck 1992, Loch and Walter 2002). However the finding that a female will use the sperm from the first mating event to fertilize her egg suggests females mated before dispersing will fertilize eggs with sperm from a relative (Fig. 5.3) and therefore not employ any inbreeding avoidance mechanisms. It is still unclear why a male will mate with a non-virgin female when sperm precedence goes to the first male. Working with *C. floridanum* observations reveal that if a female is mated too many times she is unable to fertilize her eggs. It appears that the spermathecal ducts become blocked with excessive ejaculates. This could simply indicate that males are unable to recognize a mated female from a virgin female. In species where females gain immediate benefits from males, such as nuptial gifts, nest sites, or male protection, the evolution of polyandry is understandable. However, in *C. floridanum* and other species where females gain no immediate benefits and mating incurs costs associated with time, energy and increased mortality, it is not evident for the occurrence of polyandry (Arnqvist and Nilsson 2000). Understanding all the costs and benefits associated with multiple mating in *C. floridanum* will help provide an explanation for its prevalence in the field and the laboratory.

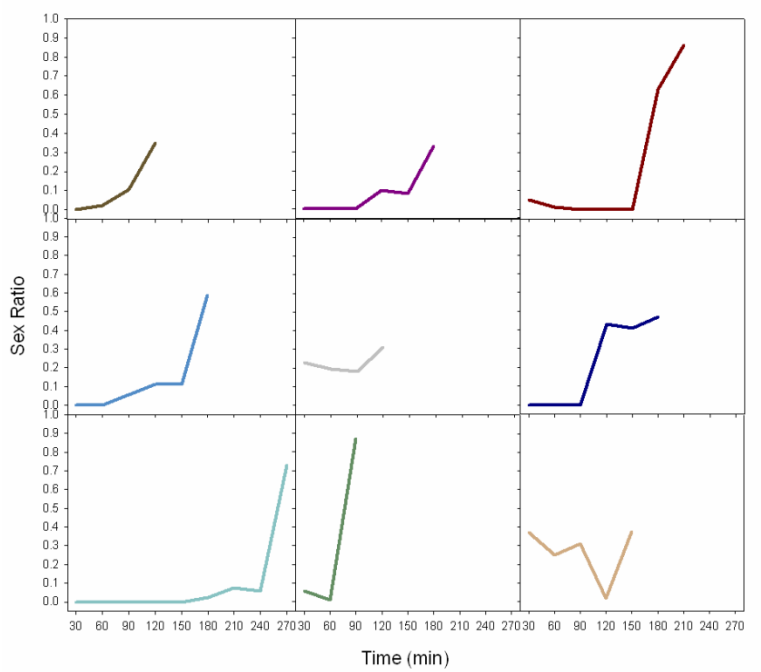

Fig. 5.1: Sex ratios of dispersed wasps from a mixed sex brood. During emergence, dispersed wasps were collected at thirty minute intervals and sexed (n=9). Complete emergence lasted between 1.5 to 4 hours, and the number of collection intervals varied between 3 to 8. The sex ratio corresponds to the number of males to females that dispersed in each thirty minute collection interval. The color of the lines corresponds to the color of the data points in Fig. 5.2, to match the sex ratio of dispersing individuals to the sex ratio of the brood.

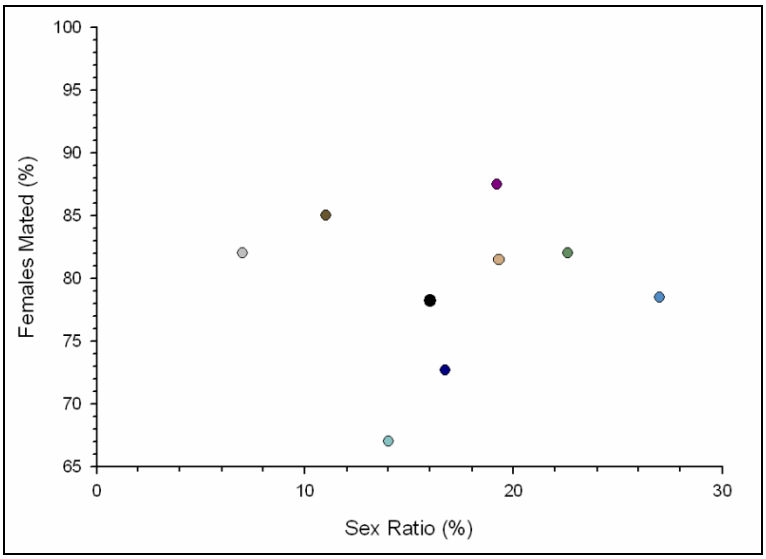

Fig. 5.2: Sex ratio and percentage of females mated, pre-dispersal, from a mixed sex brood. Dispersed individuals were collected every thirty minute interval (n=9), sexed, and females were dissected to determine mating status (see Methods). The percentage of total mated females is recorded on the y-axis in relation to the sex ratio of the brood from which they emerged. The color of the data points correspond to the color of the lines in Fig. 5.1, to match the sex ratio of dispersing individuals to the sex ratio of the brood.

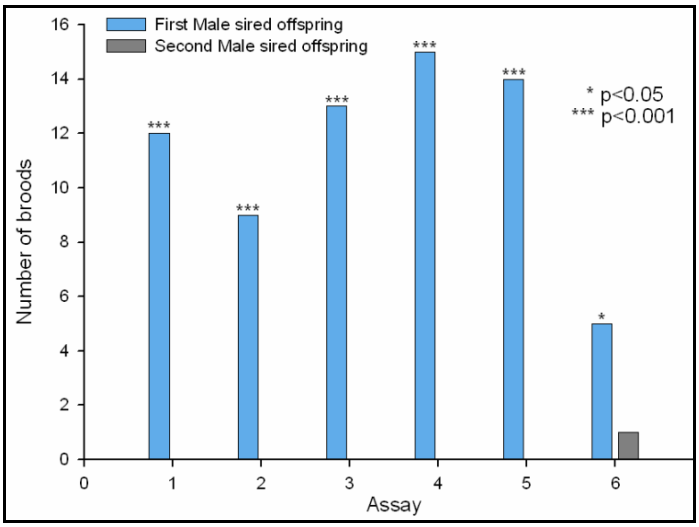

Fig. 5.3: Sperm use of multiply mated females. Females (n=8 per assay) were multiply mated by two males and the sperm used to sire her brood was determined (see Methods). In all six assays, the female used the sperm from the first male significantly more than the sperm from the second male to sire her brood (Binomial Test; $p<0.05$).

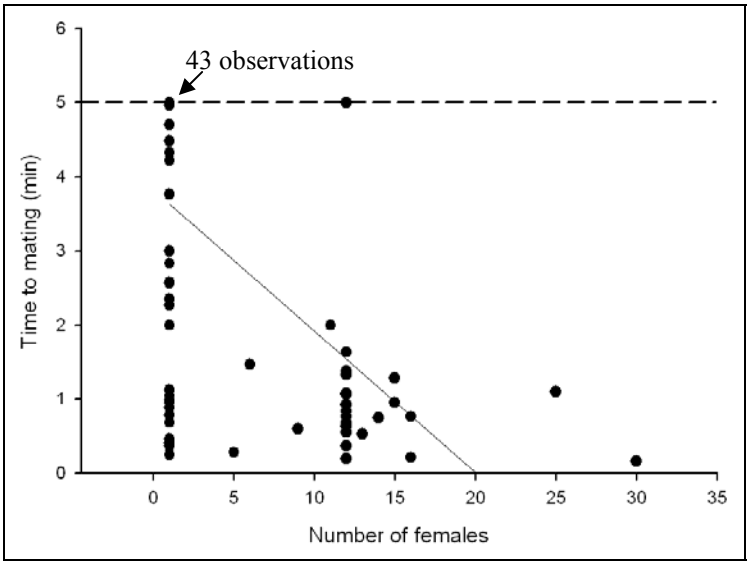

Fig. 5.4: Number of females and the time to first mating. A varying number of virgin females (1-30) were offered to single virgin males and the time to the first mating event was recorded (n=100, see Methods). The number of females is negatively correlated with the time to first mating event as represented by a linear regression line (Spearman's $\rho= -0.5619$, $p<0.0001$). The single data point at five minutes for x=1 represents 43 observations. The dashed line represents the end of the experiment at 5 minutes.

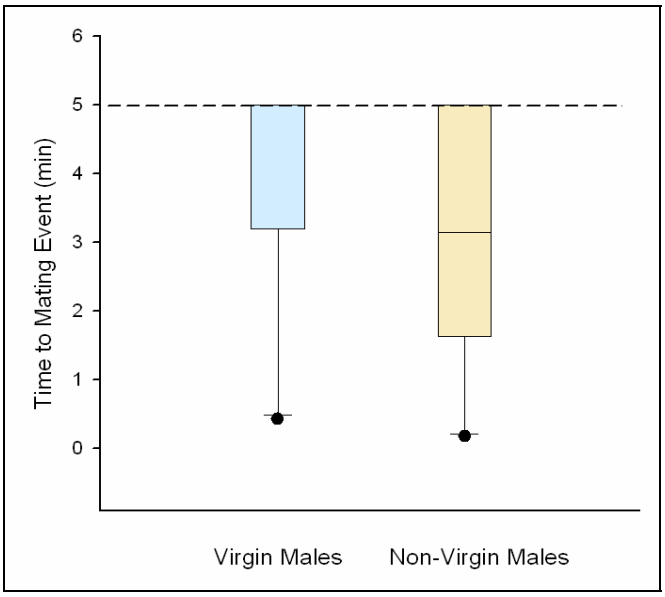

Fig. 5.5: Mating status of males and time to first mating. Single virgin females were offered to virgin males (control, n=10) and non-virgin males (n=10) and time to the first mating event was recorded (see Methods). There is no correlation between the mating status of the male and the time to first mating (Spearman's $\rho = -0.3243$, $p=0.1631$). Data points were collapsed into a box plot to show mean (middle line), standard deviation (whiskers), and outliers (dots). Dashed line represents the end of the experiment at 5 minutes.

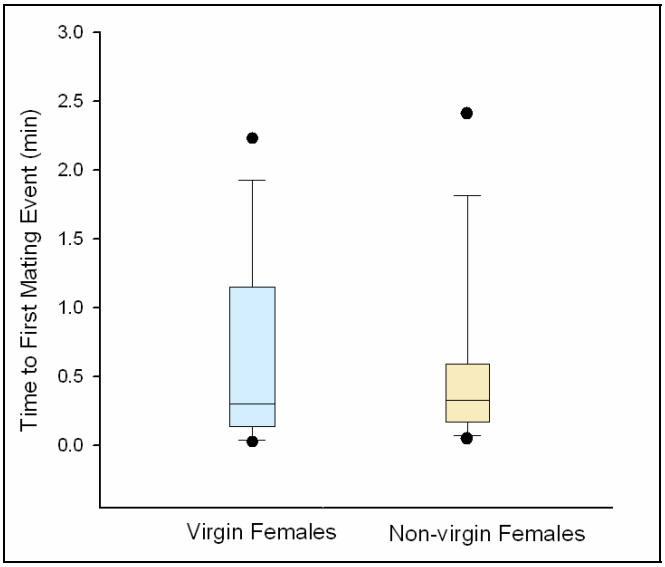

Fig. 5.6: Mating status of females and time to first mating. Single virgin males were offered twenty virgin (n=14), or non-virgin females (n=14) and time to first mating event was recorded. There is no correlation between the time to a mating event and the mating status of females (Spearman's $\rho = -0.008$, $p=0.9644$).

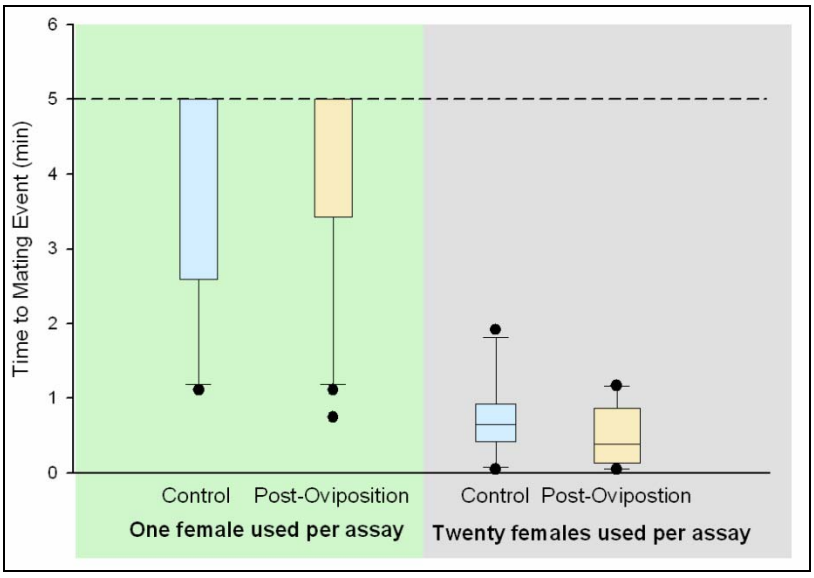

Fig. 5.7: Time to first mating based on oviposition status of females. Single and twenty females pre-oviposition (control) and females post-oviposition were used and time to first mating was recorded (10 assays per group). There is no correlation between the time to a mating event and the oviposition status of either a single female (Spearman's ρ=0.1018, p=0.6693), or twenty females (Spearman's ρ=-0.2603, p=0.2676).

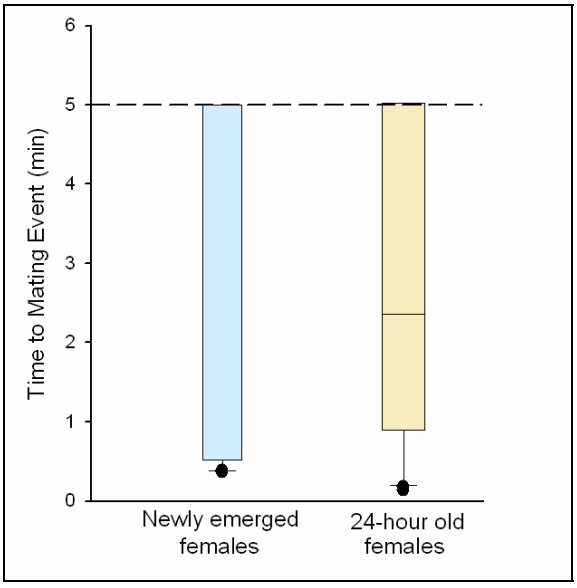

Fig 5.8: Age of females and time to first mating. Single, newly emerged (<12 hours old, n=20) females and single 24-hour old virgin females (n=20) were offered to newly emerged virgin males and time to first mating event was recorded. There is no correlation with the age of female and time to first mating (Spearman's ρ=-0.1218, p=0.4542).

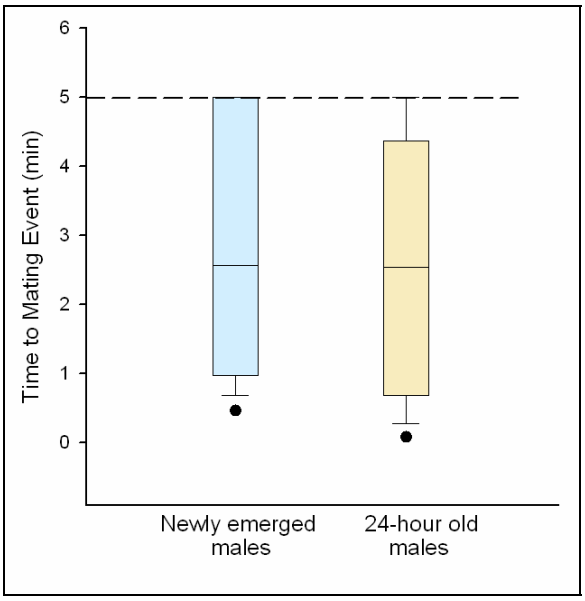

Fig. 5.9: Age of males and time to first mating. Single, newly emerged (<12 hours old, n=20) males and single 24-hour old virgin males (n=20) were offered newly emerged virgin females and time to first mating event was recorded. There is no correlation with the age of male and time to first mating (Spearman's ρ=-0.1713, p=0.2906).

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