UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Development and use of microsatellites to quantify the mating system of the pollinating fig wasp, *Platyscapa awekei*

By

Gert Johannes Jansen van Vuuren

Submitted in partial fulfilment of the requirements for the degree MSc (Agric) Genetics

In the Faculty of Natural & Agricultural Science

University of Pretoria

Pretoria

March 2005

© University of Pretoria

Summary

Mating system, mating behavior and the evolution thereof is the foundation of this study. More specifically the effect of inbreeding on the evolution of mating behavior is investigated. To this end the pollinating fig wasp, *Platyscapa awekei,* lends itself to inquiry about inbreeding and the effect on its behavior.

A pollinating fig wasp female will lay her eggs inside a syconium, and all offspring will mate with each other. Interestingly the abovementioned pollinating wasp exhibits male dispersal, not commonly expected to occur in a haplodiploid species observed to inbreed frequently.

Several theories attempt to explain the evolution of male dispersal in this case, but very little work has been done on the effect of inbreeding on the choice to disperse. In order to study the effects of inbreeding it was necessary to be able to measure the inbredness of individuals. For this reason I developed microsatellite markers both to determine the inbredness of individuals but also to derive parentage from offspring genotypes. With the inbreeding status in hand I had to correlate this with fitness measures in order to derive the effect of inbreeding on this species.

Interestingly I found both inbreeding and outbreeding depression, with optimal fitness at some point between fully inbred and fully outbred status. I give some explanations for the occurrence of dispersal in this species but come to the conclusion that dispersal is merely part of a mixed mating system and that more detailed work need to be done to derive what the specific effect of dispersal is on fitness.

Acknowledgements

So many people have contributed to make this study happen. I would like to thank a few whose exceptional support and guidance were both irreplaceable and indispensable.

To my wife, Wynina, thank you for you never-failing optimism and constant support. You were prepared to start our life together with me and my thesis, I applaud your devotion.

I am greatly indebted to my parents, Frans and Babie. It was your support and encouragement from my first day of school, that helped me make it this far. Thank you for your love, inspiration and financial support, without which none of this could have been possible.

To my brother, Heinrich, a special word of thanks. You have always been a faithful companion throughout my life, forever willing to arguments about life, divinity and, of course, biology. May we never stop ploughing the field of knowledge.

Pieter and Martinette, my in-laws, thank you for your support, encouragement and advice. You allowed me to write part of this thesis in your home while trying to win the hand of your daughter. It seems I have succeeded in both these feats.

I must mention one person for his brilliance and understanding of evolution, Jaco Greeff. It was a privilege to work with such a talented person and academic. Thank you for guiding me to appreciate evolution. I am still learning.

Thanks to all the lab mates, Glen, Jason, Jamie, Christoff, Ronnie, Aret, Vinet and Emanuelle, for a pleasant work environment, field trips and many evenings of philosophy.

Finally my gratitude owed to Pioneer Hi-Bred International, in particular Brooks Coetzee, for allowing me to finish my thesis wile being employed by you. Also my appreciation extended to the University of Pretoria and the NRF for facilities and funding during 3 years of the Masters degree.

Table of Contents

List of tables and figures

1. Introduction

In order to appreciate this investigation into mating systems, it is necessary to give an overview. In the present chapter I will discuss some aspects of sexual reproduction and how it relates to haplodiploidy. I will shortly discuss the proposed evolution of haplodiploidy in order to give an appreciation of how this work fits into the bigger picture. I will then discuss the subject species chosen for this study, accentuating the particular deviations in behaviour from related species, and give possible explanations for the evolution of these deviations.

Mating system

Sexuality and social behaviour are issues that address our most basic functions and obsessions. This statement holds true for all organisms that partake in sexual reproduction. Interestingly sexuality and social behaviour are both influenced by the same set of co-operating yet conflicting parties, males and females. Both have to contribute to make sex work, yet in most cases parents have opposing interests, depending on the investment of each parent and the probability of an unfaithful partner (Diamond, 1997). With all this competition between the sexes for control over reproduction it is not surprising that sexual reproduction has developed many different forms of co-operative breeding strategies, which include diverse genetic systems and various behavioural adaptations (Thornhill and Alcock, 1983; Choe and Crespi, 1997).

Genetic systems can be classified into obligate amphimixis (sex), thelytoky (all-female systems, uniparental reproduction and parthenogenesis) or a mixed mating system. Obligate amphimixis describes a system where every female inherit one haploid genome from her mother and one haploid genome from her father. This group include diplodiploidy and haplodiploidy. Normark (2003) describes diplodiploidy as the system where every male inherits a haploid genome from its father and one from its mother, and both these genomes have equal probability of transmission through his sperm. Haplodiploidy can be defined as a uniparental-male system, where the male transmits only his mother's genome. This can be achieved through arrhenotokous haplodiploidy, where males develop from unfertilised eggs, or from paternal genome elimination (PGE). During PGE a male develops from a zygote consisting of both maternal and paternal genomes, but only the maternal genome is transmitted through his sperm.

Thelytoky is defined in the broad sense as genetic systems in which females transmit only maternal genes and produce only daughters. This term encompasses many systems including such systems where no males are needed, or where males of a related species is needed to initiate development. Normark (2003) summarises the major features of thelytoky with the following: a) reproductive efficiency where little or no energy is wasted on "all this silly rigmarole of sex" (Hamilton, 1975), and b) there is a lack of recombination between the genomes of different individuals. (For a detailed classification of the major genetic systems of insects see Normark, 2003)

Sex is a widespread phenomenon and thus seems to be very common despite the costs involved. For example, it is not clear that the heritable variance in fitness is significantly increased by sex (Barton and Charlesworth, 1998) as is commonly assumed. Additionally recombination can break up favourable sets of genes selected for

by the environment, this is called recombinational load (Barton and Charlesworth, 1998). Segregational load can also occur by the breaking up of single locus heterozygosity. The cost of sex lies in the fact that an asexual female will produce double the number of females each generation, than a sexual female (Barton and Charlesworth, 1998).

Sexual systems have some clear advantages, for example, the reshuffling of genes during recombination to increase fitness and variation to adapt (Barton and Charlesworth, 1998). Some argue that sex is a handy way of overcoming genetic damage (Bernstein *et al.*, 1984), even though not all agree (Barton and Charlesworth, 1998). I will explain some of the arguments for the origin of sex, and the critique on these arguments, to explain the existence of haplodiploidy.

In order to understand the factors influencing the evolution of haplodiploidy, as is the sexual system of *Platyscapa awekei*, we should consider the argument for the origin of sex. Bernstein (1984) gives the following argument to explain the role of repair mechanisms in the evolution of diploidy. He argues that an ancestral RNA replicator would have been prone to both single and double stranded RNA damage caused by environmental conditions in the form of radiation from the sun and harsh chemicals. Single stranded mutations in a double stranded organism would have been easy to repair with the help of exonucleases and the correct sequence on the complimentary strand. However, double stranded nicks or Tymidine-Tymidine dimers would have caused reduction in fitness and subsequent death of the replicator. A replicator with an extra copy of the genetic material will be able to repair the damaged area through homologous recombination and have a selective advantage. In this way replicators could occasionally fuse their capsules and have sex for the sole purpose of repairing

their genetic material. Various strategies could have evolved to acquire such an extra copy of genetic material. This is not only a possible explanation for the evolution of sex, but also of diploidy. In the case of diploidy, an organism could use the redundancy of an extra copy's genetic material to overcome genetic damage. The change from haploid to diploid is an easy one, considering that in the diploid phase, lethal recessives that arise by mutations will not be detrimental to the carrier. In contrast no lethal alleles can persist in a haploid population, and a mutation will instantly eliminate the individual in which it arises. Thus a diploid will have a selective advantage over a haploid, all be it for a short while. Over time, mutations will accumulate and genetic load will be te same for a haploid and a diploid system. Again it becomes necessary for both haploid and diploid to either purge disadvantageous alleles or to adopt sex as a repair mechanism (Bernstein *et al.*, 1984).

Note that the abovementioned argument for the evolution of sex is not widely accepted. To this end, Barton and Charlesworth (1998) argues that a mechanism to overcome double stranded nicks in DNA will at least not be a system that uses double stranded nicks to repair damaged DNA. They approached the problem from the population genetic perspective by acknowledging the interaction between selection and variation. Barton and Charlesworth (1998) argue that the effect of sex and recombination in breaking down the negative correlations between favourable variants at different loci is the likely factor that sustains sex and recombination, seeing as the breakdown of the negative correlations will increase fitness.

Returning to the evolution of haplodiploidy, how does it fit into this debate? Hamilton (1978, 1993) noted that in insects, haplodiploidy arises in lineages that use woody plant stems as food, a nutritionally poor source; also these lineages rely on

maternally inherited bacteria. It seemed unclear what the connection was between maternally inherited bacteria and haplodiploidy, but Hamilton (1993) speculated that haplodiploidy might be the outcome of a history of conflict between intercellular bacteria and their hosts over sex determination. Feminising the host will ensure that the maternally inherited endosymbionts survive. Hamilton (1993) considered the situation where the endosymbionts attack and eliminate the male determining chromosomes; the host responds by moving the sex-determining elements across the genome. The conflict pursues with the bacteria attacking more targets, until finally all surviving autosomes behave like X chromosomes and sex determination rests on chromosome dosage alone. In this way males are haploid, originating from unfertilised eggs, and females are diploid stemming from fertilised eggs.

Hamilton (1967) also found an interesting association between haplodiploidy and extreme inbreeding. He predicted a set of characteristics correlated with extreme inbreeding and haplodiploidy, and these were found to be increasingly common (Brun *et al.*, 1995; Hamilton, 1967). These include extremely female-biased sex ratios and small flightless males.

Taking into account Hamilton's observation of maternal inheritance and inbreeding associated with haplodiploidy, two hypotheses for the evolution of haplodiploidy have been suggested. The first is known as the maternal transmission hypothesis, and is derived from the relatedness asymmetries of haplodiploids (Smith, 2000). The relatedness of a haplodiploid son to his mother is one, whereas the relatedness of a diploid son to his mother is ½. Thus one can say that if a haplodiploid modifier would arise in a diploid population, it would spread easily because of its superior transmission through the maternal germ line. A haplodiploidy modifier will ensure that all sons from

a carrier female will be haploid, and subsequently all female offspring from haploid sons will produce haploid males. The theory predicts that the modifier will spread in an outbred population as long as haploid male fitness does not drop below half that of diploid male fitness. Smith (2000) took this hypothesis further by testing if the modifier affects the whole genome and not only a single chromosome at a time as predicted by Hamilton (1993). Interestingly Smith (2000) found that the evolution of haplodiploidy is constrained by inbreeding in the maternal transmission theory by inbreeding reducing the proportion of heterozygotes, and the spread of the haplodiploidy modifier needing the heterozygous state of the ploidy modifier locus to spread in the population (Bull, 1979). The haplodiploidy modifier acts as a selfish gene, and can only spread by virtue of its improved transmission relative to the diplontic modifier.

The second idea discussed by Smith (2000) is known as the mutation hypothesis. This hypothesis utilises the fact that purifying selection against deleterious mutations differ between haploids and diploids. In a haploid population the selection pressure against recessive mutations are greater than in diploid populations because of the shielding effect of dominant alleles over detrimental recessives in diploids. As a result in an outbred population with no sex ratio bias, the mutational load for a haplodiploid population is ¾ that of a diploid population (Smith, 2000). Thus, because of the inherent fitness advantage, the haplodiploidy modifier will spread in the population (Goldstein, 1994). In this hypothesis Goldstein (1994) stated that the effectiveness of the spread of the modifier depends on the strength of selection against deleterious mutations, penetrance of deleterious mutations in diploids, and the level of recombination. The outcome is selection that generates linkage disequilibria in which the haplodiploidy modifier associates with a higher fitness viability allele, and the diploidy modifier associates with a lower fitness viability allele. Adding recombination

to the argument, it suggests that haplodiploidy is favoured over diploidy in the absence of recombination. Thus when recombination takes place, linkage disequilibria are broken up and the cost of diploidy associating with a lower fitness allele is reduced. When the recombination rate becomes high enough, diploidy can be favoured over haplodiploidy due to incomplete penetrance, because a diploid is less affected by single deleterious mutations than a haploid.

Inbreeding, as shown by Hamilton (1967), is closely associated with haplodiploidy. It remains however debatable if this association is a result of a causal relationship rather than the confounding effect of another variable. According to Werren (1993) haplodiploids will suffer less inbreeding depression than diploid species, and will thus make the transition from outbreeding to inbreeding more readily. Borgia (1980) has stressed the alternative view that inbreeding eases the transition from diploidy to haplodiploidy. Smith (2000) argued that inbreeding will favour haplodiploidy under the deleterious mutation hypothesis in two ways, first, inbreeding reduces the difference between mutation load in the diploid and haploid phases reducing the cost of the transition from diploidy to haplodiploidy. Second he argues that inbreeding reduces the effectiveness of recombination, and as argued above reduced recombination favours haplodiploidy because recombination favours diploidy by breaking up the association between the haplodiploidy modifier and the higher fitness viability allele.

The point made thus far must not be mistaken as an absolute situation. Even though the transition form outbreeding to inbreeding is easier for haplodiploids than for diploids (Werren, 1993), haplodiploidy does not necessarily lead to inbreeding. It is imperative to note that mating systems in haplodiploids vary from strict brother-sister mating (e.g., the genus *Mellitobia*, eumenid wasps: Cowan, 1979; Chapman and

Stewart 1996, thrips: Chapman and Crespi, 1998) to population wide random mating (Hardy, 1994; Godfray and Cook, 1997). Also inbreeding depression varies among haplodiploids, but is still less than the trend among diploids (Henter, 2003). Henter (2003) approached the problem by studying the effect of artificial inbreeding on an outbreeding haplodiploid wasp and found inbreeding depression in various traits. But more interesting were the traits unaffected by inbreeding and traits affected by inbreeding in males that is not obviously sex limited. In this study she could distinguish selection regimes on different traits, possible modes of inbreeding depression and the effectiveness of purging in certain traits. This all paints a picture to present a wide variety of influences on the mating system. It might still be far from describing the origin of haplodiploidy, but with the knowledge of how it may have evolved we could start guessing.

There is an advantage to sibmating, a female will pass more copies of her genome to more offspring, but it is restricted by the reduced fitness of these offspring (Bengtsson, 1978). Thus, one can say that if the cost of inbreeding is low, increased sibmating will be favoured. High levels of inbreeding depression will force an individual to avoid inbreeding, to increase its fitness. It is important to note that inbreeding depression also evolves, due to the purging or accumulation of fitness lowering alleles. The effect thereof is that even though the cost of inbreeding may initially be great, the cost of inbreeding will decrease as more deleterious alleles are purged (Goldstein, 1994) and vice versa. Ever so often one stumbles upon an example where a polymorphism of inbreeding and outbreeding occurs. The question now is whether the combined effects of a sibmating advantage and inbreeding depression are able to maintain a mixed mating system with a stable polymorphism between outbreeding and sibmating behaviour. Taylor and Getz (1994) studied models of inclusive fitness to define the

parameters of a stable polymorphic sexual system. Their model predicted that under diploidy, a stable balance between outbreeding and sibmating are never found. But under haplodiploidy, for a very narrow range of inbreeding depression levels, a stable polymorphism can occur.

It should now be clear that the mating systems and inbreeding depression should be viewed, not as separate entities, but as two factors influencing each other's evolution (Antolin, 1999). Antolin (1999) also suggests that sex ratio be included in this reasoning, but the influence of sex ratio will be discussed later.

The aim of this study is to investigate the co-evolution of mating systems and inbreeding depression. I will consider a known haplodiploid, and study the effects of its inbreeding habits on fitness. The rationale behind this is that inbreeding will have an effect on fitness (inbreeding depression), and the mating system will have a certain ability to cope with inbreeding depression. With the inbreeding depression derived, I will be able to explain the ability of the mating system to adjust to the mutational load. Selection should adjust the amount of sibmating to compensate for the inbreeding depression. In this way I will try to explain the evolutionary path taken to arrive at this optimal mating system.

Platyscapa awekei – Pollinator with a difference

Platyscapa awekei provide us with the ideal opportunity to study the mating system of close inbreeding organisms, with local mate competition. The genetic system of this insect is known and also the social behaviour has been studied thoroughly (Moore and

Greeff, 2003; Greeff *et al.*, 2003). Various theoretical models on haplodiploid behaviour have been constructed (Taylor and Getz, 1994). According to behavioural studies, the mating behaviour of *P. awekei* seems to contradict these predictions in one important aspect in that a fraction of males disperse before mating leading to a mixed mating system.

The pollinator starts life as an egg laid inside a galled uniovulate flower within the syconium of *Ficus salicifolia*. The sex of the eggs depends on the fertilization status, as in other haplodiploids, the unfertilised eggs are haploid and develop into males. When the wasp is fully matured it will chew a hole in its own gall. Males will exit from their galls while females will only exit after having been mated by a male. Males will first start chewing an exit hole from the fig and then proceed to mate with females (R.M. Nelson, pres. comm.). This exit hole will be used as an escape tunnel for females to leave the fig. The female will crawl out of the fig and fly to a receptive fig on a different tree, where it will crawl into a syconium through the ostiole. Once inside the fig, the foundress will lay her eggs singly in flowers that are also galls, and the cycle repeats.

An interesting aspect that influences the mating system of *Platyscapa awekei* is the relatedness of wasps inside the fig. In 79% of cases only one female enters the fig (Greeff *et al.*, 2003). This will lead to sib mating within the fig cavity. The result of sib mating and local mate competition is a female biased sex ratio (Hamilton 1967, Taylor, 1981). But the foundress number is not constant (Herre *et al.*, 1997), thus the relatedness within the fig is not always constant and local mate competition varies (Frank, 1985; Herre, 1985). We expect that the sex ratio will be adjusted to accommodate a lower level of LMC (Frank, 1985; Herre, 1985; Herre *et al.*, 1997). To this end Greeff *et al.* (2003) found foundress numbers to vary from 1 to 3 per fig with 79% of figs having a single foundress (Greeff *et al.*, 2003). The sex ratio laid by the

female also varies, even though it is mostly female biased. In my own data generated from introduction experiments, where only single foundresses were allowed to enter figs, sex ratio varied from 0.03 to 0.41 with a mean of 0.15, but never occurred above 0.5.

Another aspect in the mating behavior of *P.* awekei that is able to change the relatedness in the fig is dispersal of male wasps (Greeff *et al.*, 2003). If male dispersal leads to successful mating outside the natal fig, it will lower the relatedness in the fig where it procured extra natal mating. This means that LMC and inbreeding will be lowered. Females mated by an unrelated male should produce a different sex ratio than sib-mated females (Greeff, 1995).

Taylor and Getz (1994) found that although it is possible in haplodiploids that both inbreeding and outbreeding can occur within the same mating system, they found that this polymorphism in breeding occurs under very strict conditions and are not expected to be common (Taylor and Getz, 1994). Yet, many haplodiploid populations, including *P. awekei*, have evolved polymorphic breeding strategies.

Male dispersal

The specific behavioural trait that begs an explanation, given the discussed ideas is male dispersal. Dispersal and fighting by pollinator males are two aspects that are not expected (Hamilton, 1979). These two behavioural factors are possibly linked in a number of ways. Greeff *et al.* (2003) noted that the morphological adaptations to fight in males also enabled them to be more mobile on flat surfaces. Longer, thinner legs, a broader, shortened thorax and the capacity to retract the gaster, so it becomes less

cumbersome, allow the fighter male to move faster. In stark contrast the non-fighting male falls over when on a flat surface, and are unable to disperse (Greeff *et al.*, 2003). The morphological adaptation to accommodate both these behaviours are so interlinked that no specific morphological aspect can be used to position either dispersal or fighting as the primary evolved behaviour.

Several ideas can explain the occurrence of dispersal. In short these are (1) Operational sex ratio, (2) Kin selection and (3) Inbreeding avoidance.

Operational sex ratio: This idea relates to the sex ratio variation within the species. Different figs have different sex ratios and dispersal may simply take place from low female biased figs to high biased figs. The motivation behind dispersal in this case is simply the availability of females; males will disperse to those figs that have more mating opportunities.

Kin selection: Males might disperse to decrease the competition between relatives (Hamilton and May, 1977 and Perrin and Mazalov, 2000). In this case the dispersing males can reduce their own fitness, but this reduction can be outweighed by the improvement in fitness of those males staying behind.

Inbreeding avoidance: It is likely that the pollinator male of *P. awekei* is avoiding inbreeding when it disperses. In general it is expected that haplodiploids are not as susceptible to the effects of inbreeding as are diploids. This is due to the purging of fitness decreasing alleles in the haploid males. It is argued by some that inbreeding depression is in fact possible in haplodiploids, as the males being purged only express male characteristics (Werren, 1993). In this case it is possible that female expressed characters could be in danger of inbreeding depression. In the same way that inbreeding

avoidance motivates dispersal, heterozygote advantage can also encourage dispersal. Heterozygote advantage differs slightly from inbreeding avoidance in that inbreeding depression does not necessarily exist in the haplodiploid, but that the heterozygote has a higher fitness than the homozygote. We expect that haplodiploids have limited inbreeding depression, and specifically none in the haploid phase, thus males would most likely disperse because the heterozygote offspring will have a fitness advantage.

It can be said that pollinating fig wasps play a vital role in both the propagation of *Ficus* and the nourishment of the eco-system where they occur. Studying these small insects becomes even more important if one considers the impact that human development has on *Ficus* populations in the wild. Fragmentation of habitat can limit gene flow of both the pollinator, and the pollen they carry. Pollinators can be considered as the most fragile aspect of *Ficus* procreation, and resource provision in their habitats. It is thus very important to study wasps, not only because of their academic importance in terms of mating system, but also of their environmental importance. In order to understand the role of fig wasps in the ecosystem, we need to understand their dispersal.

In the following chapters I will investigate the mating system of Platyscapa awekei. First, I describe the development of 6 polymorphic microsatellite loci. I then use these markers to study the level of inbreeding within a population of P. awekei and the level of male wasp dispersal between figs. In addition, I determined the gene flow between two geographically distant populations. In the second part of the thesis the focus shifts to fitness-genotype correlations, still using the microsatellite loci. In this chapter the occurrence of inbreeding depression is quantified to test an explanation for the dispersal of male P. awekei.

2. Isolation of microsatellite loci and quantifying the population structure of the pollinating fig wasp, *Platyscapa awekei.*

Abstract

In order to estimate inbreeding levels, maternity and population diversification we developed 6 variable microsatellite loci for the pollinating fig wasp *Platyscapa awekei* (Hymenoptera: Agaonidae). By following an enrichment protocol we found 11 microsatellite loci in 48 transformed *E. coli* colonies containing SSR enriched fragments. From these 11 colonies 7 loci were unique and of these 6 loci proved to be reliable and variable. Behavioural studies have shown that these pollinating fig wasps often mate with sisters in their natal fig, but also that males disperse to mate with females from other figs. In fig wasps the level of inbreeding is estimated by assuming that the probability of sibmating is equal to the inverse of the number of foundresses. Since the number of effective dispersing males is unknown, an alternative approach to estimate the level of inbreeding is required. If one assumes that no dispersal occurs, FIS is expected to be less than 0.66. However FIS was estimated to be 0.5 using microsatellite analyses. Therefore dispersing males may account for as many as 14% of matings. We compared two populations from different localities in South Africa. Despite the large geographical distance and the small size of the wasps, no significant genetic structure was found. A lack of linkage disequilibrium suggest that unlike some Panamanian fig wasps, where sister species co-occur in one species of fig, *Ficus salicifolia* has only one species of pollinator.

Introduction

Pollinating fig wasps have interesting mating systems that include close inbreeding and mixed mating systems, variation in sex ratio and diverse breeding behaviour (e.g. fighting and dispersal) (Godfray, 1994; Godfray and Cook, 1997; Bean and Cook, 2001; Greeff *et al.*, 2003). In order to study the evolutionary factors that shape these diverse mating systems in pollinators we need genetic tools with high resolution — microsatellites provide just that.

More specifically it has become crucial to develop genetic markers to test models inferring mating systems from sex ratios (Read *et al.,* 1995; Pickering *et al.,* 2000; West *et al., 2000a, b*; Greeff, 2002), and to determine individual sex allocation strategies (Orzack, 2002 p.389) in pollinating fig wasps. The pollinating fig wasp *Platyscapa awekei* shows male dispersal that is not common in this group of wasps (Hamilton, 1979; Greeff *et al.*, 2003). Various hypotheses for the evolution of male dispersal exist, but in order to prove or disprove these hypotheses, we need a tool that is able to distinguish between individuals and estimate the level of inbreeding.

Molecular markers can greatly assist behavioural, ecological and population genetic studies. Microsatellites are popular genetic markers due to their high informative value. The high number of alleles and genome wide distribution makes this marker very useful to type individuals, genome regions or linked genes (Strachan and Read, 1996). Microsatellites are frequently used to differentiate between populations (Goldstein and Pollock, 1997; Jarne and Lagoda, 1996; Banks, 1999), infer demographic history (Schug *et al.*, 1998), identify specific regions of the genome affected by natural

selection (Schlötterer, 2000) and infer relatedness and levels of inbreeding (Kemeyama *et al.*, 2002)

We developed 6 microsatellite loci for the pollinator of *Ficus salicifolia*, *Platyscapa awekei*. Here we quantify the variation in the number of alleles at the microsatellites by genotyping 48 individuals from 2 populations. All loci have in excess of 10 alleles. Estimates of FIS and FST are interpreted in the light of *P. awekei*'s breeding system. Results show that the pollinator is indeed inbred, but that the levels of inbreeding are less than predicted. Using the microsatellites we could confirm that male dispersal accounts for as much as 14% of matings, and that populations show very little differentiation.

Materials and Methods

Microsatellite development

Ten female wasps from different figs, collected in the National Botanical Gardens in Pretoria, South Africa, were used as starting material to develop a microsatellite library. The protocol used in all procedures is based on an adaptation of Fleischer and Loew (1995). DNA extractions were done with DNeasy Tissue kit (Qiagen) following the manufacturer's protocol. Success of extraction was established by visualising extraction on a 1.2% agarose gel. This genomic DNA was digested to completion with Sau 3A1 (Roche) using manufacturer conditions and buffer. Fragments of 300-800 bp were isolated on a 1.2 % agarose gel, and extracted from the gel using High pure PCR product purification kit (Roche). Fragment concentration was determined and self-designed linkers (Forward: 5'-GAT CCC AAG CTT CCC GGG TAC CGC-3',

Reverse: 5'-GCG GTA CCC GGG AAG CTT GG-3') were ligated to fragment ends. Six repeat dinucleotides containing di-, tri- and tetranucleotide repeats and biotin labelled ends were used to enrich the genomic fragments (Table 2.1). Streptavadin coated magnetic beads (Dynabeads ® Dynal, Lake Success, NY, USA) that bind to biotin were used to separate fragments containing repeats from unwanted fragments.

Table 2.1 Biotinylated enrichment probes

NAME	SEQUENCE (5'-3')
Dinucleotide-A	Biotin-GAGAGAGAGAGAGAGAGA
Dinucleotide-B	Biotin-CACACACACACACACACA
Trinucleotide-A	Biotin-CAACAACAACAACAACAACAA
Trinucleotide-B	Biotin-AATAATAATAATAATAATAAT
Tertanucleotide-A	Biotin-GATAGATAGATAGATAGATAG
Tertanucleotide-B	Biotin-GACAGACAGACAGACAGA

Enriched fragments were amplified in 50 µl reactions. Reaction mixtures contained 5 µl 1x buffer (Promega), 1 mM MgCl2, 0.16 mM of each dNTP, 0.8 pM of Primer and 1.25 U of *Taq* polymerase (Promega). The reaction was carried out on an Eppendorf Mastercycler Gradient with denaturing incubation at 95oC for 2 min. Amplification consisted of 30 cycles of denaturing at 95oC for 40s, annealing for 1min at 60oC, extension for 2min at

72oC, and a final extension cycle of 1 min 40s at 72oC. PCR product was purified using columns QIAquick ® PCR purification kit (Qiagen), eluted in 100 µl UHQ. To ensure 3 | A overhangs needed for cloning with Invitrogen cloning vector, 50 µl DNA was incubated with 0.5 unit of *Taq* polymerase (Promega). Cloning of fragments were carried out with the use of TOPO TA Cloning ® Kit for Sequencing (Invitrogen) following the manufacturers protocol. TOP10 chemically competent *E. coli* cells, supplied by Invitrogen as part of the cloning kit, were used to transform the cloned vectors. Thirty micro litres of transformed cells were grown on two selective plates for white/blue screening. One hundred transformed colonies were picked and 48 clones were sequenced using an ABI3100 automated sequencer (ABI). Twenty-two sequences contained microsatellite repeats and 11 primer sets were subsequently designed (Table 2.2). Primers were designed with the help of an interactive primer design website. Seven of the tested primers amplified correct sequences, these were further analysed by genotyping of 46 unrelated individuals and 6 loci proved variable.

DNA extractions from 46 individuals collected below were done using DNeasy Tissue extraction (Qiagen) following the manufacturer's protocol. PCR reactions using the newly designed primers were set up in 10 µl reaction volumes containing 1x PCR buffer with $MgCl_2$ (Roche), 800 nM of each dNTP, 700 nM of Primer and 0.7 units (3.5U/ul) High Fidelity Expand *Taq* polymerase (Roche). The cycling program consisted of hotstart denaturing incubation at 95oC for 2 min. Amplification consisted of 30 cycles of denaturing at 95oC for 40s, annealing for 1min at 60oC, extension for 2min at 72oC, ending with a one cycle of 1 min 40s at 72oC.

Table 2.2 Microsatellite primer sequences

	Primer sequence
Pa 1	F: GTAGCGCCGTATCAAATTGCAA
	R: GGGAAGCTTGGGATCTTTAACGA
Pa 4	F: GGGTGTTGTCGGTTTGTGAGA
	R: GGCAAACATCCATCGGAGTGA
Pa 7	F: CTGCCGGTCAGAGGAGGAA
	R: TATGACGTCATCGGTTTGGCA
Pa 8	F: GAGGAAGTCCGATGAATGAACGA
	R: GCGAACAGGAGACAAAGACAG
Pa 21	F: GCTGTCGAGGCGAAACACA
	R: GCGCGAGGCATTGGCAA
Pa 32	F: CGGTGTTCAATTGCCAAGTGA
	R: TCGTGTTCTTCGTAATCGCGTA

Population analysis

Two locations, about 500km apart, were chosen with the expectation that restricted gene flow would occur: a tree in Olifants in Mpumalanga South Africa, and another in Pretoria, Gauteng, South Africa. Figs were collected from Pretoria and Olifants and wasps were taken from the galls of each fig and preserved in 95% EtOH. A single female from each fig was used for analysis to ensure independence of samples. Twenty-

two wasps from Olifants and twenty-four from Pretoria were analysed. DNA extractions were done using Chelex extraction protocol (Estoup *et al.*, 1996). PCR amplification with microsatellite loci were done according to the optimised reactions mentioned above, and amplification results checked in 1.5% agarose gel. Genescan reactions were carried out on an ABI 3100, and alleles scored using GENEMAPPER software (ABI). Linkage disequilibrium and F statistics (Weir and Cockerham 1984), observed and expected heterozygosities were obtained from the program Genepop (Raymond M. and Rousset F, 1995). A 2-Dimentional factorial analysis of correspondence was done using Genetix software to determine any population structure.

Results

Microsatellite development

The microsatellite primers developed (Table 2.2) are highly variable, with 10 to 24 alleles per locus (Table 2.3). After optimisation of the PCR protocol the loci amplified easily and proved to be informative in the analysis that followed. All loci were designed to produce product lengths that do not overlap, however some unique alleles did cause some overlapping (Table 2.4). Different coloured primers provided the distinction between overlapping loci when using GeneMapper (ABI). The observed heterozygosity, ranging from 0.33 to 0.67 with an average of 0.52, was lower than the expected 0.90 across all loci.

Table 2.3 Characteristics of 6 microsatellite loci for *Platyscapa awekei*, pollinator of *Ficus salicifolia*. T_a, working annealing temperature; H_O, observed heterozygosity; $H_{E(TOTAL)}$, unbiased average heterozygosity expected (Nei 1978). F_{IS} and F_{ST} derived according to Weir and Cockerham (1984) using the program GENEPOP (Raymond and Rousset, 1995).

Locus	T_a (°C)	Repeat motif	H_O	$H_{E(TOTAL)}$	F_{IS}	F_{ST}	Product length ranges (number of observed alleles)
Pa 1	58	$(CT)_3CA(CT)_{15}$	0.48	0.90	0.45	0.04	229-268 (15)
Pa 4	58	$(CT)_{23}$	0.67	0.89	0.26	0.00	194-237 (20)
Pa 7	58	$(CT)_2TT(CT)_2TT(CT)_{15}$	0.61	0.94	0.38	-0.01	291-341 (24)
Pa 8	58	$(CT)_6CC(CT)_5CG(CT)_3$	0.33	0.76	0.56	0.02	193-219 (10)
Pa 21	58	$(TC)_2CC(TC)_{16}TT(TC)_3$	0.51	0.91	0.43	0.00	164-196 (17)
Pa 32	58	$(AG)_{11}TG(AG)_6$	0.48	0.91	0.48	0.01	110-165 (17)
		Average over populations	0.52 ± 0.0305	0.90 ± 0.0251	0.42	0.01	

Mating structure

Analysis showed no significant linkage disequilibrium, suggesting that the population is not composed of a mixture of sister species. FIS averaged 0.42 across all loci, and proved highly significant when tested with a randomisation test (GENETIX). Using the equation $s=4F/1+3F$ (Suzuki and Iwasa, 1980), the proportion of sib mating is calculated to be 0.75.

Table 2.4 Allele size and frequency.

Pa 1 Green	Allele Frequency	Pa 4 Yellow	Allele Frequency	Pa 7 Blue	Allele Frequency	Pa 8 Green	Allele Frequency	Pa 21 Yellow	Allele Frequency	Pa32 Blue	Allele Frequency
225	8.14	192	2.17	289	1.11	191	4.55	160	20.45	108	3.26
229	2.33	194	1.09	291	1.11	193	1.14	164	2.27	110	1.09
231	4.65	196	2.17	293	2.22	195	3.41	165	3.41	116	2.17
233	12.79	201	4.35	295	5.56	197	3.41	168	7.95	120	3.26
235	10.47	203	1.09	297	1.11	199	11.36	170	10.23	122	5.43
237	2.33	205	18.48	301	3.33	201	19.32	172	9.09	124	15.22
239	6.98	207	7.61	303	5.56	203	5.68	174	2.27	126	4.35
241	5.81	209	15.22	305	5.56	205	7.95	176	1.14	128	13.04
243	11.63	210	1.09	308	3.33	207	40.91	178	3.41	130	6.52
245	4.65	211	1.09	310	6.67	217	1.14	180	3.41	132	11.96
247	19.77	212	1.09	312	7.78	219	1.14	182	6.82	134	6.52
249	3.49	213	15.22	314	1.11			184	2.27	136	5.43
251	1.16	215	7.61	315	2.22			186	4.55	138	4.35
255	1.16	217	9.78	316	4.44			188	5.68	142	9.78
257	3.49	219	3.26	318	10.00			190	4.55	147	1.09
268	1.16	221	2.17	320	2.22			192	9.09	149	3.26
		223	1.09	322	6.67			194	1.14	155	2.17
		225	2.17	324	3.33			196	2.27	165	1.09
		229	1.09	325	1.11						
		231	1.09	326	10.00						
		237	1.09	329	2.22						
				331	4.44						
				333	3.33						
				339	2.22						
				341	3.33						

Population structure

F_{ST} values across all loci were expected to be high, but analysis averaged 0.01. Significance were tested with a randomisation test (GENETIX) and found the F_{ST} value to be well within the randomised normal distribution, suggesting that the two populations cannot be differentiated. Further tests using a randomised allocation of individuals to populations (GETENIX), showed no difference between individuals from

either locality. Using a private alleles model, GENEPOP estimated the number of migrants to be 2.73 individuals per generation.

Discussion

Microsatellites

The protocol used showed good results with a 12½% success rate. The protocol increases the success of cloning SSR containing fragments by the use of short repeat sequences. These fragments are synthesized to contain the SSR repeat that is mostly expected in the species. This expected sequence could be derived from other microsatellites developed for related species. In our case we used repeats of tetra- tri- and dinucleotides, but found only loci containing dinucleotide repeats. It is difficult to explain the reason for the predominance of dinucleotide repeats, but it might be due to stringent PCR conditions. The six loci obtained from this procedure proved to be variable and reliable for this study.

Mating structure

Mating systems can be quantified by estimating the inbreeding coefficient that reflects the degree of sib mating. In fig wasps a maximum estimate of sib mating can be derived from the number of foundress females in natural populations. If it is assumed that each fig contributes the same number of females regardless of the number of foundresses and that foundresses sharing a fig produce identical clutches, then *s*, the proportion of sib mating, is equal to the inverse of the harmonic mean number of foundresses (Herre, 1985). But, *s* is equal to the inverse of the arithmetic mean when one assumes that all females, regardless of the number of foundresses, produce the same

number of daughters (Greeff, 2002). Due to the limitation on brood size in a single fig and sex ratio adjustment, the truth may tend toward the former. The average number of foundresses in *Platyscapa awekei* is 1.126 (Greeff, pers comm.). The proportion of sib mating without dispersal will then be 0.89, and FIS is expected to have a maximum of 0.66. Thus the estimate of 75% from the microsatellite analysis suggests that 14% of matings are procured by dispersing males. A study on a different species, using a single morphological marker, estimated that dispersers of that species account for 6% of matings (Greeff, 2002). The implication would be that a less female bias sex ratio could be expected. Null alleles could also elevate FIS, but none of these were found after screening individual males in chapter 3.

Population structure

The two locations where sampling took place are about 500 km apart. The intervening area is scarcely populated with fig trees, and we expected this to restrict geneflow between the two localities. Previous studies found that dispersal of female wasps in arid regions along riverbeds with abundant fig trees can be up to 150km (S.G. Compton, pers. comm.).

It seems that these wasps migrate across vast distances (more than 500km). Due to their size in relation to the geographical distance, it is not expected to be direct migrants. There is however small pockets of fig trees scattered between the two locations. It is thus possible that migrants can be carried by wind from one fig pocket to another, in a stepwise manner completing their journey between these two populations.

3. Causes and consequences of the mating system of *Platyscapa awekei*.

Abstract

Male fig wasps of some pollinating species disperse from their natal figs and procure matings outside their natal fig. The reason for the evolution of male dispersal is investigated here. We evaluate three fitness traits and correlate them to the number of homozygotic loci in order to test four possible explanations for the evolution of dispersal. These traits are female body size, egg number within the female and brood size. Homozygosity is estimated from genotyping individuals at 6 microsatellite loci. The number of homozygotic loci does not influence female size and egg number, while variation in egg number is explained largely by body size. Brood size is significantly related to homozygosity and both inbreeding as well as outbreeding depression are detected. Data suggests that the evolution of dispersal might be influenced by optimal fitness being at intermediate levels of inbreeding and outbreeding.

Introduction

The evolution of the level of inbreeding (mating system), inbreeding depression and sex ratio are intertwined (Antolin 1999). Each of these factors influences each other in such a way that none of these can be studied without some reference to the other. The mating system determines the sex ratio (Taylor; 1993; Greeff, 1995); whether being complete inbreeding, partial inbreeding or inbreeding avoidance. In return the level of inbreeding depression will allow certain levels of inbreeding (Taylor and Getz, 1994) and thus again define the sex ratio. Studies should thus strive to address all of these aspects in the same species.

Many studies have investigated the effect of the mating system on sex ratio. In fact Hamilton (1967) noted that a population's mating structure could affect the sex allocation of parents. To this end he demonstrated that if a female were to lay her eggs in a secluded patch where mating opportunities are limited to offspring of the same mother, it would benefit the mother to lay more females than males. The explanation is that fewer males will decrease local mate competition (LMC) between males and increase the mating success of each son (Hamilton, 1967; Taylor, 1981). Greeff (2002) investigated the relationship between mating system and sex ratio in the light of the fact that recent studies have assumed the relationship between sex ratio and mating system to be so rigorous, that mating system could be derived from sex ratio (Read *et al.,* 1995; Pickering *et al.,* 2000; West *et al., 2000a, b*). He found that in a male dispersing fig wasp, it was inaccurate to derive mating system from sex ratio. He also stressed that before sex ratios can be used as a proxy for the mating system, it is necessary to investigate all other factors that can influence sex ratio variation (Greeff, 2002).

Greeff and Taylor (1997), and also Greeff and Compton (1996), predict that inbreeding depression has an influence on sex ratio. Inbreeding depression has a small effect on sex ratio when few females sib mate. With frequent sib mating, the effect of inbreeding depression on sex ratio is overshadowed by the effect of high sib mating. It is important to note that sex ratio, mating system and inbreeding depression could form a dynamic triad (Antolin, 1999). The level of inbreeding depression will evidently allow a certain level of inbreeding. In return the level of inbreeding can change the inbreeding depression by purging disadvantageous alleles (Werren 1993). The effect of increased inbreeding and the lowering of inbreeding depression can result in a more female biased sex ratio (Greeff and Taylor 1997, Antolin 1999). Thus it is clear that when considering the mating system of a population, inbreeding depression should be quantified

The fig wasp *Platyscapa awekei* has a female biased sex ratio, and routinely sibmate (Greeff *et al.*, 2003). Jansen van Vuuren (Chapter 2) confirmed this highly sibmating habit with the proportion of sibmating estimated at 0.75. Due to this mating pattern and haplodiploidy, *P. awekei* is expected to have low inbreeding depression (Smith and Shaw, 1980; Crozier, 1985; Werren, 1993). It is therefore thought that male dispersal in this species (Greeff *et al.*, 2003), which account for 14% of matings (Chapter 2), is not the result of incest avoidance.

Several possible hypotheses could explain the presence of male dispersal. First, males may experience above average operational sex ratios in their natal fig and disperse to figs with more favorable ratios of females to males.

Second, kin selection can explain male dispersal. Kin competition reduces inclusive fitness, and one way to reduce kin competition, and hence, increase inclusive fitness, is

by dispersal (Perrin and Mazalov, 2000; Hamilton and May 1977; Motro, 1982a, b; Frank, 1986; Taylor, 1988; Gandon and Michalakis, 1999).

Third, inbreeding depression can lead to the evolution of male dispersal behavior (Bengsston, 1978; Waser *et al.*, 1986; Motro, 1991; Perrin and Mazalov, 1999; Gandon 1999). It is not expected to find much inbreeding depression in haplodiploids as the mutational and recessive loads are selected against in the haploid phase (Smith and Shaw, 1980; Crozier, 1985; Werren, 1993). Some argue that traits not associated with the haploid phase will be susceptible to inbreeding depression (Werren, 1993). Traits that are expressed only in the diploid phase and not the haploid phase will be under less purging selection than the traits expressed in the haploid phase. In this instance, female traits such as fecundity, host searching, and sex ratio might have a genetic load in contrast to the rest of the genome expressed in both sexes (Werren, 1993). Additionally, inbreeding would reduce heterozygote advantage.

This study was designed to determine if inbreeding depression affects the evolution of male dispersal. In order to detect inbreeding depression within a haplodiploid, we need to examine traits associated with the diploid phase of the genetic system, i.e. the female. To this end we decided on brood size, egg load and female body size. Brood size, the number of offspring to reach the imagine stage; will give an estimate of the fitness of the female. Egg load determines the number of eggs that can be laid. A larger body size would enhance fitness during resource defense (Moore and Greeff, 2003) and possibly dispersal, but it is not known if body size will affect the fitness of a female with no competition within a fig. In order to detect inbreeding depression, it is necessary to derive the inbreeding status of the individuals tested. We used homozygosity as a measure for inbredness. The six microsatellite loci developed in

chapter 2 were used to genotype the females under investigation. For each female the number of loci that are heterozygous or homozygous were determined. The proportion of homozygous loci will give an estimate of inbreeding for the individual. The homozygosity of the females is then correlated with the three measurements. We found that brood size is indeed prone to inbreeding depression, but curiously outbreeding depression also occurred.

Materials and Methods

Introduction experiments

In order to obtain the brood size for a single female we did single foundress introductions into virgin figs under a controlled environment. A single female introduction would also ensure that the mother of the brood could be reconstructed from the genetic data of the male offspring.

We collected figs ready to release from the botanical garden in Pretoria during 3 sessions, April 2002, November 2002 and January 2003 (Table 3.1). On average 50 to 100 figs were placed in a 200 ml plastic bottle, with a total of about five bottles per collection. Collections were made from different trees, usually three to five trees, with figs from one tree put into the same bottle. The figs were kept in bottles to release the wasps. Female wasps were collected with a brush and placed upon a receptive fig. Figs to be used in the introduction experiments were bagged 2-3 weeks in advance with breathable mesh material bags, allowing sunlight to penetrate, before they became receptive. This ensured that introductions were made into virgin figs (figs with no wasps inside), and that we had absolute control over the number of foundress wasps we

introduce into the fig. After the wasp entered the fig, and ensuring that the female went in completely, we closed the ostiole with "Pratley putty", sticky putty that closes off the ostiole of the fig. This ensured that no other females could enter the fig. After the introductions the figs were bagged again and two weeks thereafter the putty was removed from the ostiole to ensure unhampered fig development. Figs were left until ready to release; the time varied depending on the season. The figs were picked and placed individually into plastic containers, wholes were drilled into the tops of the holders and netting was fastened to the inside. This ensured that figs could release in the individual pots and the wasps could not suffocate or escape. The wasps were collected from each pot under a dissection microscope and placed into 1.5 ml eppendorf tubes with 95% EtOH. The wasps were collected while still alive, since initial microsatellite analysis showed that dead wasps did not yield good quality template DNA. Brood size and sex ratio were then counted from the wasps from each fig.

Table 3.1 Population sources of *Platyscapa awekei*, where females were collected from for introduction experiments. The key features of the sampled population are indicated.

Date	Source Population	# Introductions	Family Number	Average Brood size	Sex Ratio	Number Genotyped
April 2002	Pretoria	20	#70-90	50.7	0.23	17
November 2002	Pretoria	10	#91-100	53.3	0.12	10
January 2003	Pretoria	70	#1-70	38.8	0.18	30

Since each male gets its allele from its mother, the chance that a maternal allele will be missed if the mother is heterozygous is $(1/2)^N$, where N is the number of males typed. Therefore when at least six males have been genotyped, the chance that a

heterozygous locus is classed as homozygous is less than 1.7%. In some cases where no males were present, or where too little males were available, we used the same amount of females as we would have used males. The genotype data from the offspring were then used to deduce the mother's genotype. Alleles at each locus from each clutch were compared to find the alleles from the mother. After visual inspection of the data a general linear model was fitted to brood size, with time as a categorical variable and number of homozygous loci as well as the square of number of homozygous loci as continuous variables (SPSS 12.0)

Egg count and female size

Female wasps collected from the National Botanical gardens in Pretoria and surrounding areas were collected for this study. Females from different figs were used to ensure independence of samples. Wasps were dissected under microscope and the ovaries taken out and placed on slides. Eggs of the two ovaries were counted separately. Tibia length was also measured under a dissecting microscope. The remainder of the wasps' thorax were then used for DNA extraction and genotyped. The genotype data were correlated with egg number and body size. First, a general linear model was fitted to explain body size with number of homozygous loci as a continuous variable. Then, a general linear model was fitted to explain egg number as a function of body size (continuous) and number of homozygous loci (continuous, SPSS 12.0).

Genotyping

All samples were genotyped on ABI 3100 automated sequencer and genemapper software. All DNA extractions were carried out using a standard Chelex 100 sodium form (SIGMA) protocol (Estoup *et al.*, 1996). The wasps were prepared for extraction by placing the eppendorf containing the wasp into liquid nitrogen. The frozen wasps

were then ground up using a sterile eppendorf pestle. 500ul Chelex solution, consisting of 10% Chelex in UHQ, was added to the ground wasp. Care was taken to ensure that the Chelex solution was at 60°C and a wide bore pipette tip was used. All samples were incubated for 15 min at 100°C and immediately thereafter Proteinase K (Fermantas, 7.5ul of 20mg/ul) was added. Protein digestion commenced at 55°C for 3 hours, shaking the solution at regular intervals. The proteinase reaction was halted by incubation at 100°C for 15 min. These solutions were used directly in PCR reactions, with care taken to settle Chelex from the rest of the solution by centrifuging for 15 to 20 seconds. Chelex should never be separated from the rest of the solution; otherwise the degradation of DNA will occur. Extractions should also be stored at -20°C, and never left at room temperature for too long.

Amplification of wasp primers was done using the following reaction mixtures. Concentrations are displayed as final concentrations of each reagent, including final concentration for forward and reverse primers. 4ul of template from the Chelex extraction were used in each reaction.

The PCR conditions consisted of initial denaturation, 2 min at 95°C with 30 cycles of 40 sec at 95°C, 1 min at 60°C and 2 min at 72°C. No final elongation step was used as to avoid stutter peaks on genescan results. Reactions were cooled to 4°C and stored at this temperature until diluted for genescan.

All amplifications were diluted 20:1 with UHQ; different primer amplifications were mixed using 2:2:1:1 volume additions of mixture 1, 2, 3 and 4 (Table 3.2). Amplifications were genotyped and the results analysed with genemapper software.

Table 3.2 Reaction mixtures showing different primer combinations amplified together.

	Primer	dNTP's	Taq Polymerase	Mixture volume
Mixture 1 Primer: Pa 1	1 000nM	800nM	0.7 units	10 µl
Mixture 2 Primer: Pa 7,Pa 8,Pa 21	583nM	666nM	0.7 units	12 µl
Mixture 3 Primer Pa 32	700nM	800nM	0.7 units	10 µl
Mixture 4 Primer Pa 4	700nM	800nM	0.7 units	10 µl

Results

Fitness measures and genotype

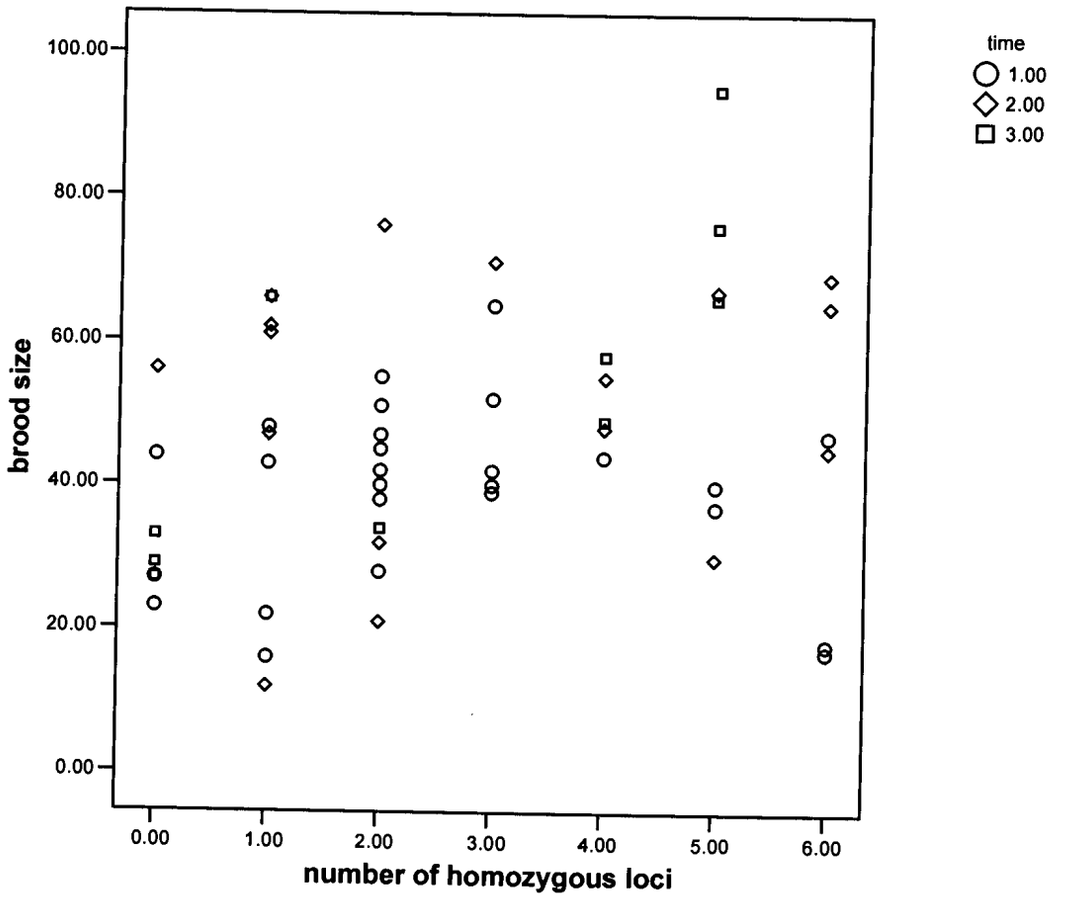
Brood size

From the introduction experiments there were 57 cases where the female genotype could be assigned from the offspring genotype. DNA extractions were done from 96 samples, and subsequently 39 samples yielded low quality DNA that did not amplify adequately for genotyping. In many cases there seemed to be evidence of multiple paternal alleles within a brood, but this requires further investigation to confirm. The general linear model showed that the number of homozygous loci has a significant effect on the brood size (Table 3.3, Figure 3.1). Note that only 23.5% of variation is explained by the model.

Table 3.3 The results of a general linear model explaining brood size as a function of number of homozygous loci, its square, and the time period. The adjusted $R^2 = 0.235$ and the error degrees of freedom = 52.

Source	df	F	P	coefficient estimates
Intercept	1	49.119	.000	41.226
# of homozygous loci	1	6.628	.013	10.354
(# of homozygous loci)^2	1	4.217	.045	-1.326
time				2003/01: -16.884
	2	6.591	.003	2002/04: -2.895
				2002/11: 0

Figure 3.1 Brood sizes as a function of number of homozygous loci. Circles indicate January 2003, square November 2002 and diamonds April 2002. Note that individuals of intermediate homozygosity have the highest fitness.



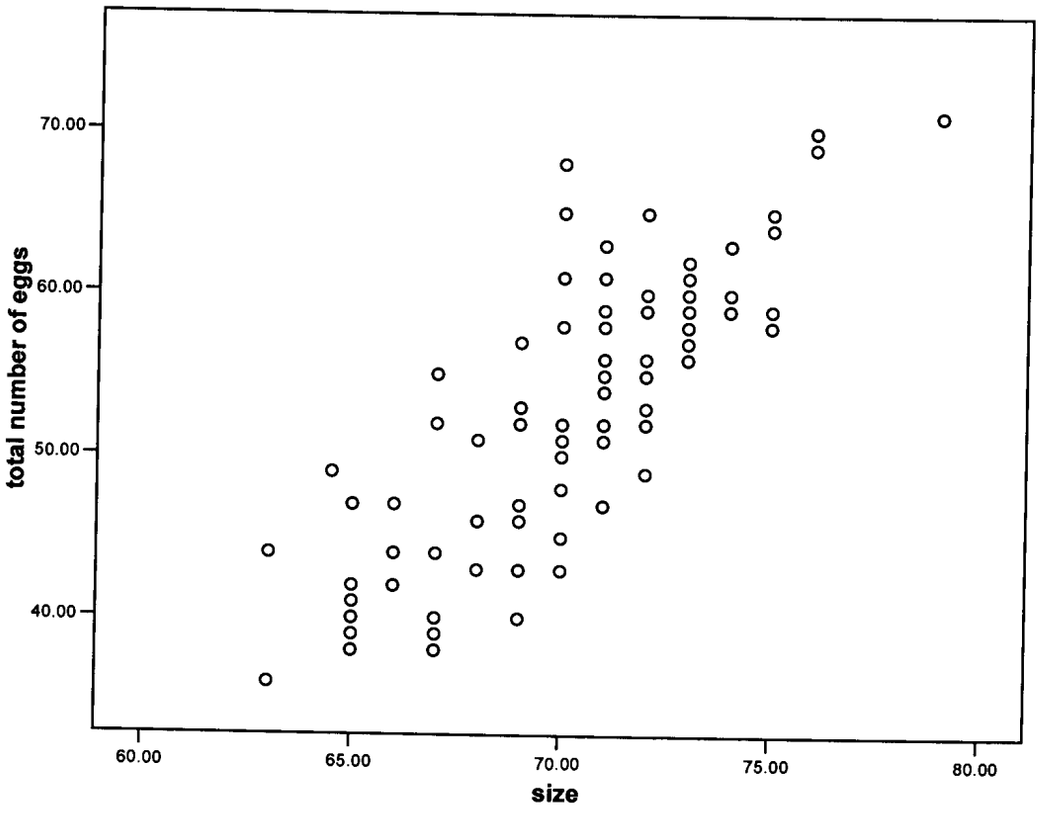
Female size and egg count

From the analysis it seems that the level of inbreeding has no effect on the size of the wasp (Table 3.4), nor on the number of eggs (Table 3.4). Confirming previous findings that wasp size had a significant effect on the number of eggs (Table 3.4, Figure 3.2).

Table 3.4: The results of a general linear model explaining total egg number as a function of number of homozygous loci and female size. The adjusted $R^2 = 0.686$ and the error degrees of freedom = 85.

Source	df	F	P	coefficient estimates
Intercept	1	78.596	.000	-95.968
size	1	191.591	.000	2.112
homozygosity	1	.401	.528	0.181

Figure 3.2 Total number of eggs as a function of wasp size.



Discussion

Egg number and female size were unaffected by the level of homozygosity. This result is not surprising given that fig wasps are haplodiploid chalcids that routinely inbreed (Smith and Shaw, 1980; Crozier, 1985; Werren, 1993). This is because any recessive genetic variant that decreases fitness will be unshielded in the haploid phase and immediately selected against. Thus, there is theoretically little chance of fitness decreasing alleles accumulating within the population of haplodiploids.

On the other hand, there is some evidence that both inbreeding and outbreeding depression exist when considering brood size. Note that inbredness explains only a small fraction of the variation in clutch size, and it maybe biologically unimportant and hard for selection to act upon. One explanation for the occurrence of inbreeding depression might be that recessives are maintained by the diploid phase and not purged successfully. Recessives can also be maintained by infrequent and uncontrolled outbreeding in multi-foundress figs. Another reason for inbreeding depression could be the lack of heterozygosity at loci showing heterozygote advantage.

Most surprising is the occurrence of outbreeding depression in brood size. Outbreeding depression is most likely due to the break up of epistatic interactions selected for under close inbreeding within families. Clearly more work is required to determine how important this effect of inbreeding and outbreeding depression is.

One has to keep in mind that for the individual male of *Platyscapa awekei* there are only two choices, inbreeding or outbreeding (dispersal). There is no breeding strategy or immediate choice that will ensure that the male's offspring will stay within the range

of highest fitness. Added to this, multiple foundresses will also produce uncontrollable outbreeding events. Thus, it seems that an optimal mating system is impossible, and possibly the best strategy is a mixed system.

4. Conclusion

The aim of this study was to infer and understand the mating system of a haplodiploid wasp. More to the point the focus laid on the occurrence of dispersal within a mating system where inbreeding is favored. The question about how this "abnormal" breeding behavior evolved seems to dominate the outlook of this study. But in actual fact the reference of this study can be better understood if it is considered in the context of the interaction between fig trees and their pollinators, and the use of molecular technology in behavioral ecology.

In part, this study also explored the possibility to use molecular techniques in order to answer evolutionary and behavioral questions. I developed six microsatellite loci and found it to be very informative and variable. Using these markers I was able to define population parameters previously only speculated about. Two populations speculated to be isolated, was shown to be undifferentiated. I could establish that these wasps are able to travel, albeit indirectly, over great distances. One can speculate that conditions are conducive to distant traveling by frequent pockets of habitat sustaining small populations of fig trees. Still, this fact was only speculated about previously. Low linkage disequilibrium established that only one species of wasp occupy the figs of *Ficus salicifolia* in Southern Africa. In some Panamanian species of pollinating wasps sister species will live side by side within the same fig.

Population analysis made it clear that these wasps are inbred, even with the observation of multiple females in figs and dispersal of males. The mating success of dispersing males was also quantified with 15% of mating occurring by dispersing males. This showed that these wasps adopt a mixed mating system.

Fitness analyses were done to evaluate one of three theories behind the evolution of dispersal. These are the theories of inbreeding depression, operational sex ratio and kin selection. Fitness traits that were measured and compared to 6 microsatellite loci data were female body size, egg number within females and brood size. Data suggests that inbreeding does not influence female body size or egg number. These two traits showed some correlation to each other but this is expected, as the number of eggs carried by a female will be limited by her size. The third fitness trait, brood size, showed that inbred individuals as well as outbred individuals suffered from lowered fitness. The explanation for inbreeding depression seems to be that within haplodiploidy, purifying selection on fitness lowering alleles are not as stringent in traits expressed exclusively in the diploid phase. Traits that are expressed in the haploid phase are under direct purifying selection because alleles are expressed unshielded by dominant alleles. Purifying selection may also be hampered by infrequent and uncontrolled outbreeding events in multi foundress figs. Added to this inbreeding can occur due to a lack of heterozygote advantage. Outbreeding depression is most likely caused by the breakdown of favorable interactions between alleles at different loci that were built up during successive generations of inbreeding.

The evolution of dispersal seems to be influenced by the effects of inbreeding. Inbreeding depression lowers fitness to such an extent that males need to disperse. Dispersal evidently causes even more fitness loss, but within a few generations of inbreeding fitness will reach a maximum again. Further inbreeding will lead to a decrease in fitness and the scenario will play itself out again. More to the core of the problem it seems that dispersal is due to an inability to adapt to a fully inbred mating system. Due to the less stringent purifying selection in the diploid phase and multiple foundress figs, genetic load is carried, and thus will always lower fitness under

inbreeding. In this species it seems that neither stringent inbreeding nor outbreeding is possible and thus a mixed mating system is needed.

Despite all the work that has been done on the evolution of inbreeding and mating systems, we are still at the beginning of a journey in understanding its essence. In particular mixed mating systems pose many options for its origins. This study gives evidence on only one species of a small wasp, utilizing a very small piece of genetic information, 6 microsatellite loci. And in the words of Hamilton (1993), "it seems to me that notwithstanding all the facts and theories in this book (including mine), we hardly begin to know answers to any of these questions."

Literature Cited

ANTOLIN, M.F. 1999. A genetic perspective on mating system and sex ratios of parasitoid wasps. *Researches in Population Ecology*. 41:29-37.

BANKS, M.A., BLOUIN, M.S., BALDWIN, B.A., RASHBROOK, V.K., FITZGERALD, H.A., BLANKENSHIP, S.N. & HEDGECOCK, D. 1999. Isolation and inheritance of novel microsatellites in Chinook salmon (*Oncorhynchus tschawytscha*). *Heredity*. 90:281-288.

BARTON, N. H. & CHARLESWORTH, B. 1998. Why sex and recombination? *Science*. 281:1986-1989.

BEAN, D. & COOK, J.N. 2001. Male mating tactics and lethal combat in the non-pollinating fig wasp *Sycoscapter australis*. *Animal Behaviour*. 62:535-542.

BENGTSSON, B.O. 1978. Avoiding inbreeding: at what cost? *Journal of Theoretical Biology*. 73: 439-444.

BERNSTEIN, H., BYERLY, H. C., HOPF, F. A. & MICHOD, R. E. 1984. Origin of sex. *Journal of Theoretical Biology*. 110:323-351.

BORGIA, G. 1980. Evolution of haplodiploidy: Models for inbred and outbred systems. *Theoretical Population Biology*. 17:103-128.

BRUN, L.O., BORSA, P. GAUDICHON, V., STUART, J.J., ARONSTEIN, K., *et al.* 1995. Functional haplodiploidy. *Nature*. 374: 506.

BULL, J. J. 1979. An advantage for the evolution of male haploidy and systems with similar genetic transmission. *Heredity.* 43:361-381.

CHAPMAN, T. W. & CRESPI, B. 1998. High related and inbreeding in two species of haplodiploid eusocial thrips (Insecta: Thysanoptera) revealed by microsatellite analysis. *Springer – Verslag,* 43:301-306.

CHAPMAN, T.W. & STEWART, S.C. 1996. Extremely high levels of inbreeding in a natural population of the free-living wasp *Ancistrocerus antilope* (Hymenoptera: Vespidae: Eumeninae). *Heredity.* 76:65-69.

CHOE, J.C. & CRESPI, B.J. 1997. *Mating systems in insects and arachnids*. Cambridge University College.

COWAN, D. P. 1979. Sibling mating in a hunting wasp: adaptive inbreeding? *Science.* 205:1403-1405.

CROZIER, R.H. 1985. Alternative consequences of male haploidy. In: *Spider mites: Their biology, natural enemies and control.* Helle, W. & Sabelis, M.W. (eds), p. 201-222. Elsivier: Amsterdam.

DIAMOND, J. 1997. *Why is sex fun? The evolution of human sexuality.* Weidenfield & Nicolson, London.

ESTOUP, A., RODOLFO, C., PERROT, E. & CHOURROUT, D. 1996. Rapid one tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. *Molecular Marine Biotechnology*. 5:295-298.

FLEISCHER, R.C. & LOEW, S. 1995. Construction and screening of microsatellite-enriched genomic libraries. In: *Molecular zoology: Advances, strategies and protocols.* Ferraris, J. & Palumbi, S. (eds.), Wiley-Liss, New York.

FRANK, S.A. 1985. Hierarchical selection theory and sex ratios. II. On applying the theory, and a test with fig wasps. *Evolution*. 39:949-964.

FRANK, S.A. 1986. Dispersal polymorphisms in subdivided populations. *Journal of Theoretical Biology*. 122:303-309.

GANDON, S. & MICHALAKIS, Y. 1999. The evolution of dispersal in a metapopulation with extinction and kin competition. *Journal of Theoretical Biology*. 199: 275-290.

GANDON, S. 1999. Kin competition, the cost of inbreeding and the evolution of dispersal. *Journal of Theoretical Biology*. 200:345-364.

GODFRAY, H. C. J. & COOK, J. M. 1997. Mating systems of parasitoid wasps. p.

211-225. In: *The evolution of mating systems in insects and arachnids*. J. C. Choe & B.J. Crespi, (eds.) Cambridge University Press, Cambridge, U.K.

GODFRAY, H. C. J. 1994. *Parasitoids behavioural and evolutionary ecology*. Princeton Univ. Press. Princeton, NJ.

GOLDSTEIN, D. B. 1994. Deleterious mutations and the evolution of male haploidy. *American Naturalist.* 144:176-183.

GOLDSTEIN, D. B. & POLLOCK, D. D. 1997. Launching Microsatellites: A Review of mutation processes and methods of phylogenetic inference. *Journal of Heredity*. 88:335-342 (83).

GREEFF, J. M. 1995. Offspring allocation in structured populations with dimorphic males. *Evolutionary Ecology*. 9:550-558.

GREEFF, J. M. 2002. Mating system and sex ratios of a pollinating fig wasp with dispersing males. *The Royal Society,* 269:2317-2323.

GREEFF, J.M. & COMPTON, S.G. 1996. Sequential oviposition in pollinating fig wasps. *Ecological Entomology*. 21:300-302.

GREEFF, J.M. & TAYLOR, P.D. 1997. Effects of inbreeding depression on relatedness and optimal sex ratios. *Evolutionary Ecology*. 11: 245-247.

GREEFF, J.M., VAN NOORDT, S., RASPLUS, J. & KJELLBERG, F. 2003. Dispersal and fighting in male pollinating fig wasps. *C. R. Biologies.* 326:121-130.

HAMILTON, W. 1993. Inbreeding in Egypt and in this book: a childish view. In: *The natural history of inbreeding and outbreeding.* Thornhill, N. W. & Shields, W. M. (eds.), p.429-450. University of Chicago Press, Chicago.

HAMILTON, W. D. & MAY, R. M. 1977. Dispersal in stable habitats. *Nature*. 269:578-581.

HAMILTON, W. D. 1967. Extraordinary sex ratios. *Science.* 156:477-488.

HAMILTON, W. D. 1975. Innate social aptitudes of man: An approach from evolutionary biology. In: *Biosocial Anthropology.* R. Fox (ed.), p. 133-155. John Wiley & Sons, New York, NY, USA.

HAMILTON, W. D. 1978. Evolution and diversity under bark. In: *Diversity of insect faunas.* L. A. Mound and N Waloff (eds.), Blackwell, London.

HAMILTON, W. D. 1979. Wingless and fighting in males in fig wasps and other insects. In: *Sexual selection and reproductive competition in insects.* Blum, M. S. & A. Blum, N. (eds.), p. 167-220, Academic press, New York.

HARDY, I. C. W. 1994. Sex ratios and mating structure in the parasitoid hymenoptera. *Oiks.* 69:3-20.

HENTER, H. J. 2003. Inbreeding depression and haplodiploidy: experimental measures in a parasitoid and comparisons across diploid and haplodiploid insect taxa. *Evolution.* 57(8): 1793-1803.

HERRE, E. A. 1985. Sex ratio adjustment in fig wasps. *Science.* 228:896-898.

HERRE, E. A., WEST, S.A., COOK, J.M., COMPTON, S.G. & KJELLBERG, F. 1997. Fig-associated wasps: pollinators and parasites, sex ratio adjustment and male polymorphism, population structure and its consequences. In: *Mating systems in insects and arachnids.* Choe, J.C. & Crespi, B.J. (eds), p.226-238. Cambridge University Press.

JARDNE, P. & LAGODA, P. J. L. 1996. Microsatellites: from molecules to populations and back. *Trends in Ecology and Evolution.* 11:424-429.

KAMEYAMA, Y., ISAGI, Y. & NAKAGOSHI, N. 2002. Relatedness structure in *Rhododendron metternichii* var. *hondoense* revealed by microsatellite analysis. *Molecular Ecology.* 11:519-527.

MOORE, J.C. & GREEFF, J.M. 2003. Resource defence in female pollinating fig wasps: two's a contest and three's a crowd. *Animal Behaviour.* 66:1101-1107.

MOTRO, U. 1982a. Optimal rates of dispersal. I. Haploid populations. *Theoretical Population Biology.* 21:349-411.

MOTRO, U. 1982b. Optimal rates of dispersal. II. Diploid populations. *Theoretical Population Biology*. 21:412-429.

MOTRO, U. 1991. Avoiding inbreeding and sibling competition: the evolution of sexual dimorphism for dispersal. *American Naturalist.* 137: 108-115.

NEI. M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89:583-590.

NORMARK, B.B. 2003. The evolution of alternative genetic systems in insects. *Annual review of Entomology.* 48:397-423.

ORZACK, S. H. 2002. Using sex ratios: the past and the future. In: *Sex ratios: concepts and research methods.* I. C. W. Hardy (ed.), p. 383-398. Cambridge University Press.

PERRIN, N. & MAZALOV, V. 1999. Dispersal and inbreeding avoidance. *American Naturalist*. 154:282-292.

PERRIN, N. & MAZALOV, V. 2000. Local competition, inbreeding and the evolution of sex- biased dispersal. *American Naturalist.* 155:116-127.

PICKERING, J., READ. A.F., GUERRERO, S. & WEST, S.A. 2000. Sex ratio and virulence in two species of lizard malaria parasites. *Evolutionary Ecology Research.* 2:171-184.

RAYMOND, M. & ROUSSET, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumericism. *Journal of Heredity*. 86:248-249.

READ, A.F., ANWAR, M., SHUTLER, D. & NEE, S. 1995. Sex allocation and population structure in malarial and related parasite protozoa. *Proceedings of the Royal Society of London.* B. 260:359-363.

SCHLÖTTERER, C. 2000. Evolutionary dynamics of microsatellite DNA. *Chromosoma*. 109:365-371.

SCHUG, M. D., HUTTER, C. M., WETTERSTRAND, K. A., *et al.* 1998. The mutation rates of di-, tri-, and tetranucleotide repeats in *Drosophila melanogaster*. *Molecular Biology and Evolution.* 15:1751-1760. (334,337).

SMITH, N. G. C. 2000. The evolution of haplodiploidy under inbreeding. *Heredity.* 84:186-192.

SMITH, R.H., & SHAW, M.R. 1980. Haplodiploid sex ratios and the mutation rate. *Nature*. 287:728-729.

SUZUKI, Y. & IWASA, I. 1980. A sex ratio theory for gregarious parasitoids. *Researches in Population Ecology*. 22:366-382.

TAYLOR, P. D. & GETZ, W. M. 1994. An inclusive fitness model for the evolutionary advantage of sibmating. *Evolutionary Ecology,* 8:61-69.

TAYLOR, P.D. 1981. Intra-sex and inter-sex sibling interactions as sex ratio determinants. *Nature*. 291:64-66.

TAYLOR, P.D. 1988. An inclusive model for dispersal of offspring. *Journal of Theoretical Biology*. 130: 363-378.

TAYLOR, P.D. 1993. Female-biased sex ratios under local mate competition: an experimental confirmation. *Evolutionary Ecology*. 7:306-308.

THORNHILL, R. & ALCOCK, J. 1997. (eds) *The evolution of insect mating systems.* Harvard college, USA.

WASER, P.M., AUSTAD, S.N. & KEANE, B. 1986. When should animals tolerate inbreeding? *American Naturalist.* 128: 529-537.

WEIR, B.S. & COCKERHAM, C.C. 1984. Estimating F-Statistics for the analysis of population structure. *Evolution*. 38:1358-1370.

WERREN, J. H. 1993. The evolution of inbreeding in haplodiploid organisms. In; *The natural history of inbreeding and outbreeding.* Thornhill, N. W. & Shields, W. M. (eds.) *pp.42*-59. University of Chicago Press, Chicago.

WEST, S.A., HERRE, E.A. & SHELDON, B.C. 2000a. The benefits of allocating sex. *Science*. 290:288-290.

WEST, S.A., SMITH, T.G. & READ, A.F. 2000b. Sex allocation in apicomplexa (protozoa) parasites. *Proceedings of the Royal Society of London.* B. 267:257-263.