Genetic Conflict and Sex Allocation in Scale Insects

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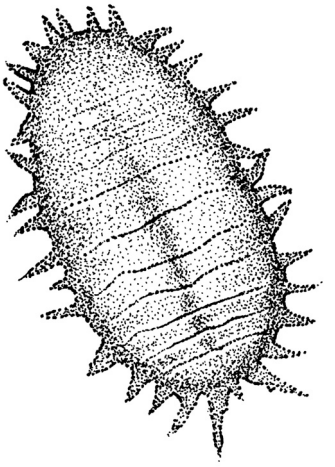


CHAPTER 1

Introduction

Laura Ross

One can recognize in the evolution of life several revolutions in the way genetic information is organized. In each of these revolutions, there has been a conflict between selection at several levels. The achievement of individuality at the higher level has required that the disruptive effects of selection at the lower level be suppressed. –

Maynard Smith (1988)

INTRODUCTIONS

Multi-cellular organisms can be regarded as nested hierarchies of cooperating entities; genes within chromosomes within cells. But under the surface of this apparent harmony is a hidden world of evolutionary conflict (Burt & Trivers, 2006). The integrity of higher levels is constantly being challenged by lower-level entities trying to transmit copies of themselves to the next generation, often at the expense of other lower-level entities or indeed the higher-level whole. A central question for evolutionary biology is how these conflicts are resolved, and how cooperation within and between levels evolves (Burt & Trivers, 2006; Leigh, 1971; Maynard-Smith & Szathmary, 1995).

One stage of a species' life history that might be particularly prone to these conflicts is reproduction. The benefits of conflict and cooperation hinge critically on aspects of reproduction, as only a limited number of genes are transmitted to the next generation (Burt & Trivers, 2006). Take for example meiosis. Normally, each gene will have an equal chance of entering a particular gamete. However, a gene able to outwit the fair gamble of meiosis will increase its transmission rate and evolutionary success (Leigh, 1977; Maynard-Smith & Szathmary, 1995). Another important component of sexual reproduction is sex determination, as some genes might only be transmitted by one sex. Examples include sex-linked genes or "cytoplasmic" genes such as the genomes of mitochondria. In these cases, the sex the genes find themselves in is crucial for their evolutionary future (Burt & Trivers, 2006; Cosmides & Tooby, 1981; Maynard-Smith & Szathmary, 1995). Genes that are able to affect reproduction and thereby enhance their own transmission relative to the rest of the genome are often called selfish genetic elements (Hurst, Atlan & Bengtsson, 1996).

Several aspects of reproduction are therefore expected to be challenged by the disruptive effects of genetic conflict and this might have given rise to the evolution of novel genetic and sex determination systems (Burt & Trivers, 2006). There is an extraordinary range of such systems among multi-cellular organisms (Bull, 1983; de Jong & Klinkhamer, 2005; Normark, 2003; Norton *et al.*, 1993), and the evolutionary significance of this diversity is poorly understood. Furthermore it is not clear why the diversity of genetic systems seems unequally distributed among taxonomic groups (Normark, 2003; White, 1973). The role of genetic conflict as a major force in the evolution of both sex determining mechanisms and genetic systems has gained considerable interest (Burt & Trivers, 2006; Hurst & Werren, 2001; Hurst, 1992, 1995; Hurst *et al*, 1996; Uller *et al*, 2007; Werren & Beukeboom, 1998) and the theoretical framework for these ideas is expanding (Haig, 1993a, b; Hamilton, 1967; Normark, 2004a; Van Doorn & Kirkpatrick, 2007). Here I first briefly review different selfish genetic elements and the effects they have on the organisms they are in. Then I will focus on their role in the evolution of genetic and sex determining systems and discuss both theoretical and empirical evidence. Then I will discuss what still needs to be done in order to test the importance of genetic conflict shaping the diversity of genetic systems across life. Finally I will give an overview of the work that is presented in this thesis.

CONFLICT BETWEEN NUCLEAR GENES

Genes can come into conflict during meiosis. For instance, in diploids alleles of nuclear genes normally have a fifty percent chance of being included in any particular gamete, but a gene that can increase this chance has an advantage and may spread if it is otherwise neutral, or even slightly deleterious for the individual it is in (Leigh, 1977). We now know of many examples of genes, often referred to as segregation distorters, that are able to do this (Burt & Trivers, 2006; Hurst & Werren, 2001; Lyttle, 1991). Segregation distorters can occur on all chromosomes (Lyttle, 1991), but their effect will be most noticeable when they occur on a sex chromosome, as in that case the drive will also lead to extremely biased sex ratios (Jaenike, 2001). Segregation distorters are not the only genetic elements able to increase their transmission chances during meiosis though. B chromosomes (or supernumerary chromosomes) are chromosomes that are not essential for an individual's survival and seem to exist solely because of their ability to enhance their own transmission (Jones & Rees, 1982). They differ from segregation distorters in that they do not drive at the cost of another locus and they are able to accumulate within a host. However, as segregation distorters parasitize the replication machinery of the host cells they occur in, their presence can incur a fitness cost to their "host" (Burt & Trivers, 2006; Camacho, Sharbel & Beukeboom, 2000; Nur, 1966a, b). An example of how extreme this cost to host fitness can be comes from a B chromosome found in the parasitic wasp *Nasonia vitripennis*. The existence of this B chromosome was discovered after it was noted that females mated to specific males only have sons, and that females mated to these sons had the same peculiar tendency (Werren, Nur & Eickbush, 1987). This was particularly odd as *N. vitripennis* is haplodiploid and therefore males develop from unfertilized eggs and are not expected to carry any genes of their father (or indeed have a father). It was discovered that this phenotype was caused by a B chromosome, named PSR (for paternal sex ratio). When a male carries PSR and mates with a female, all fertilized eggs that normally would develop into diploid females, become haploid males, as PSR destroys all paternal chromosomes except itself. So PSR spreads by each generation effectively destroying the genome of the individual they are in. This makes PSR probably one of the most selfish genetic element ever described (Nur *et al*, 1988; Werren *et al*, 1987).

CONFLICT BETWEEN NUCLEAR AND CYTOPLASMIC GENES

Conflict over transmission during meiosis is not the only aspect of reproduction where genes can come into conflict. In sexually reproducing organisms, most genes in an organism have a 50 percent change of having copies of them being passed on, but this is not true for all genes. For some of them, most notably those outside the nucleus such as mitochondrial genes, their transmission chances are affected by the sex of the individual they are in (Cosmides & Tooby, 1981). Cytoplasmic genes are

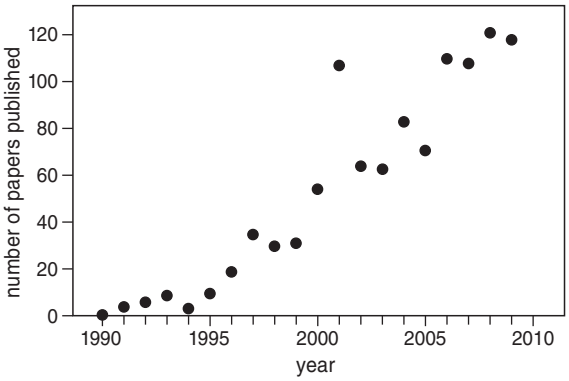


Figure 1.1 The number of papers published per year, that use the exact phrase "genetic conflict" anywhere in the paper. The results were obtained by using the advanced search option in Google Scholar.

generally only transmitted through females and not through males (although why this should be the case is probably also related to conflict over transmission (Partridge & Hurst, 1998) and some cases of paternal inheritance have also been observed (Skibinski, Gallagher & Beynon, 1994; Zouros *et al*, 1992). Therefore these genes have another option to enhance their own transmission, namely by affecting parental sex allocation to increase the number of daughters (Burt & Trivers, 2006). One of the best-known examples of cytoplasmic genes affecting sex allocation is cytoplasmic male sterility (CMS) in plants, where mitochondrial genes are able to suppress male function (pollen production) in otherwise hermaphroditic plants (Saumitou-Laprade, Cuguen & Vernet, 1994). CMS can spread rapidly through the population by reducing the investment in male function, and thereby increasing the number of female gametes through which it is transmitted. However, although there are some examples where genes in organelle genomes manipulate the reproduction of their host (Saumitou-Laprade *et al*, 1994), most examples come from endosymbionts.

Endosymbionts are microorganisms, such as bacteria and unicellular fungi, that live within the cells of their hosts. A large number of multi-cellular species have now been found to contain endosymbionts, but they are particularly common and well-studied in insects (O'Neill, Hoffmann & Werren, 1997) (Buchner, 1965; Moran & Baumann, 2000; Moran & Telang, 1998; Weeks, Velten & Stouthamer, 2003; Werren, 1997; Werren, Baldo & Clark, 2008). These endosymbionts have been shown to offer a wide range of benefits to their hosts, including nutritional benefits (Douglas, 1998; Moran & Baumann, 2000), adaptations to new host plants (Tsuchida, 2004) and parasite resistance (Oliver *et al*, 2003; Teixeira, Ferreira & Ashburner, 2008). However, many endosymbionts can themselves become parasites. Maternally inherited endosymbionts have evolved a wide variety of ways to manipulate their hosts so as to increase the number of female offspring. By doing so these endosymbionts are able to spread through the population even if they do not provide any benefits to their host or even if they are deleterious (Werren *et al*, 2008). Examples of these phenotypes include inducing asexual reproduction (Hurst, Godfray & Harvey, 1990; Koivisto & Braig, 2003; Stouthamer, Luck & Hamilton, 1990), the feminization of genetically male hosts (Negri *et al*, 2006; Terry, Dunn & Smith, 1997; Weeks, Marec & Breeuwer,

2001), and also "male-killing" (Werren *et al*, 2008). In the latter case, the endosymbionts do not directly affect sex determination or reproduction but instead kill the host they are in if it is a male. This can be beneficial to the bacteria if these males compete with female relatives, as these females carry bacteria related to those in the male and therefore by increasing their survival the bacteria gains an indirect fitness benefit (Hurst, 1991).

CONFLICT AS AN EVOLUTIONARY FORCE

The presence of these selfish genetic elements and the fitness cost they often incur to their "host" has been identified as a potentially important evolutionary force (Hurst & Werren, 2001; Partridge & Hurst, 1998, see figure 1.1), and these conflicts have been linked to diverse aspects of an organism's biology (Burt & Trivers, 2006; Hurst & Werren, 2001). These include the organization of the genome and the molecular machinery of meiosis, which have been suggested to have evolved in order to avoid conflict, since although segregation distorter and other selfish elements are common, meiosis is remarkably fair in most taxa (Leigh, 1971, 1977; Maynard-Smith & Szathmary, 1995). Genetic conflict has also been suggested to affect the way individuals interact with each other. For example, the evolution of new mating systems could be the result of selection against selfish elements: one such example is polyandry, where females mating with multiple males can evolve to avoid fertilization with sperm containing selfish genetic elements (Price *et al*, 2008; Zeh & Zeh, 1996). Finally, as well as interactions within species, speciation itself could be affected by the co-evolution between selfish elements and their host, and it has been suggested that such co-evolution might have led to the rapid divergence of lineages resulting in hybrid incompatibility (Johnson, 2010). Speciation can also be a direct result of conflict: a nice example of this comes from the *Nasonia* parasitoid wasp species complex, where the endosymbiont *Wolbachia* causes cytoplasmic incompatibility (Breeuwer & Werren, 1990), which prevents zygote formation from matings between mates that do not share the same strain of bacteria (this benefits *Wolbachia* because infected female wasps have a higher reproductive success than uninfected females as the latter's offspring are lost when they mate with a male infected with *Wolbachia* (Breeuwer & Werren, 1990)). As the different *Nasonia* species harbour different strains of *Wolbachia*, this has been suggested to have caused reproductive isolation (Bordenstein, O'Hara & Werren, 2001; Breeuwer & Werren, 1990, 1993), leading to host speciation.

CONFLICT AND THE CHANGE OF GENETIC SYSTEMS

Because genetic conflict is expected to be particularly strong during reproduction, it has been suggested that their disruptive effects might affect the way organisms transmit their genes and determine the sex of their offspring (Burt & Trivers, 2006; Uller

et al, 2007; Werren & Beukeboom, 1998). Therefore genetic conflict has been suggested to play an important role in shaping the observed diversity of genetic and reproductive systems (Burt & Trivers, 2006; Normark, 2003, 2006; Werren & Beukeboom, 1998). I will now discuss some of the empirical evidence for the role of genetic conflict driving transitions between different genetic systems and between different sex determining systems.

The first suggestion for the role of segregation distorters in the transition between genetic systems comes from Brown (1964), who suggested that paternal genome elimination (PGE), found in several taxa of insects and mites, could have evolved as the result of meiotic drive. In species with PGE, both sexes are diploid, however males only transmit copies of their mother's genes. Brown (1964) realized that once a mechanism for this "whole genome drive" evolved, it could easily spread, since males will then always exclusively transmit copies of their maternally-inherited genes and with them the genes encoding the drive. Since then, several hypotheses for the origin of this drive have been suggested. Haig (1993a) proposed that PGE might have originally been caused by an X-linked segregation distorter and that maternally derived chromosomes "hitchhiked" by becoming linked to the driving X, thereby increasing their own transmission probability. However, Normark (2004a) suggested a different origin; although PGE is characterized by nuclear drive, he suggested its origin might have been caused by conflict between the host and its endosymbiotic bacteria (see chapter 6). There is also some suggestion that selfish nuclear genes can influence the sex determination mechanisms (Kozielska *et al*, 2009; Van Doorn & Kirkpatrick, 2007): for example, it has been hypothesized that in some species the sex chromosomes might have originated from B chromosomes, and some support for this idea has been found for the Y chromosome of *Drosophila* (Carvalho, 2002; Carvalho, Koerich & Clark, 2009; Hackstein *et al*, 1996). Additionally, in the housefly (*Musca domestica*), where a large number of autosomal sex determining factors are known, the origin and spread of those factors has recently been attributed to genetic conflict (Kozielska, 2008; Kozielska *et al*, 2009).

While there is some suggestion for the role of nuclear selfish genes on the evolution of genetic and sex determination systems, the effect of cytoplasmic genes has been more thoroughly considered (Cosmides & Tooby, 1981; Engelstadter & Hurst, 2006; Kuijper & Pen, 2010; Normark, 2004a). Direct effects of endosymbionts on their host's reproduction are now well supported, and include induction of asexual reproduction (Hurst *et al*, 1990; Stouthamer *et al*, 1990) and overriding the host's sex determining mechanism (feminizing endosymbionts e.g. (Weeks *et al*, 2001)). However, indirect effects of host-endosymbiont conflict over reproductive control – where new genetic- or sex determining systems might emerge from a co-evolutionary arms race between host and –symbiont – have also been suggested (Normark, 2004a), but as yet little support is currently available.

In conclusion, although there are a lot of good and plausible stories regarding the role of genetic conflict in driving transitions between genetic and sex determining systems, the evidence is actually rather weak and taxonomically limited. It is current-

ly also unclear how important genetic conflict has been in shaping the observed diversity of genetic and reproductive systems. In part, this is probably due to the ancient origin of many genetic and reproductive systems, which may obscure the selective forces responsible for their evolution. Another reason though is that many evolutionary biologists work on a few, very well-studied model systems, which are remarkably uniform in terms of their genetics and sex determination (i.e. mostly diplodiploid with genetic sex determination and male heterogamety) and consequently might not give much insight into the effects of genetic conflict on the evolution of novel genetic and sex determination systems.

In order to rigorously investigate the role of genetic conflict we need to identify potential conflicts, attempt to quantify them in some way, and then generate predictions linking the extent of genetic conflict with transitions in genetic and sex determination systems. We know that the degree of variation in genetic and sex determination is not equal across taxonomic groups (White, 1973), and if genetic conflict has been important shaping this variation, we expect that high variation in genetic systems will coincide with a large scope for genetic conflict in particular taxonomic groups. Quantifying conflict is a central issue here. First of all, one could rather simply quantify how many different "genetic entities" are present that could potentially come into conflict. For example, in organisms with just a single chromosome and no organelles or endosymbionts, such as typical bacteria, there is little scope for conflict. Once the potential "players" have been identified, the next important step would be to identify traits under conflict (for example offspring sex ratio) and the strength of selection on the opposing actors to alter these traits. These factors together determine the strength of potential genetic conflict (Hurst *et al*, 1996). Finally, it is important to also consider the mechanism by which actors may manipulate the trait in order to assess their ability (power) to do so (Hurst *et al*, 1996).

Determining the strength of (potential) conflict and the costs, benefits and power of actors to manipulate traits under conflict can be difficult since for many actors the benefits of being "selfish" might hinge critically on various aspects of a species' biology (Partridge & Hurst, 1998). One important aspect is the amount of interaction between relatives. For example, inbreeding has been shown to generally reduce the spread of segregation distorters as it increases the chance that the distorter is paired with its homologue (Hurst & Werren, 2001; Smith, 2000). However, other interactions between related individuals might enhance conflict (i.e. selection increasingly favours different outcomes for different actors), especially when not all genes in an individual are equally related to those relatives (Hamilton, 1964). For example, male-killing endosymbionts only benefit from killing the male they are in if the resources freed up can be used by the female relatives of this dead male, which in turn carry the bacteria's relatives. If brothers and sisters did not compete, male-killing could not evolve (Hurst, 1991). Because so many aspect of a species' biology can affect the costs and benefits of selfishness, it is hard to devise general rules to predict the scope of conflict. However, as a rule of thumb, asexual reproduction and inbreeding limit the spread of selfish elements, while sexual reproduction and complex interactions

between relatives and mating partners seem to increase conflict. Finally, an important factor determining the strength of conflict is the degree of asymmetry in inheritance between different genetic entities. For example, in species with PGE, genes from paternal origin are not transmitted by males, while genes of maternal origin are, resulting in potential conflict (Ross, Pen & Shuker, 2010b). How genes are transmitted and the resulting biases in transmission are determined by a species genetic system. So while we are interested in the effect of conflict on the evolution of genetic system, some genetic systems themselves might increase the scope for conflict.

So, to determine if and when conflict can drive transitions between genetic systems, and how important it has been in shaping the observed variation across taxa. We will need to test the predictions that taxonomic groups with a high level of variation in genetic and sex determining systems have:

1) More genetic entities that could come into conflict.
2) A larger degree of asymmetry in inheritance of these different genetic entities.
3) Stronger (competitive) interactions between relatives.

A comparative approach might be useful in order to test these predictions and I have indeed used such approach chapter 6 of this thesis to test if endosymbiont presence correlates with the presence of haplodiploidy and PGE (according to prediction 1). Theoretical models might help to explore the selective optima of each genetic entity and thereby help to predict the strength of conflict under different conditions and for different taxa. Finally it will be important to use experiments in order to manipulate

Table 1.1 Glossary of important concepts used in this thesis

Concept	Definition
Genetic entity / element	Heritable unit (genes, linkage groups, organelles, endosymbionts) (Hurst *et al*., 1996).
Genetic (Genomic) conflict	Two or more genetic entities are said to be in conflict if their inclusive fitnesses are maximized for different trait values.
Conflict trait	Trait over which conflict exists (Chapman, 2006).
Selfish element	Heritable unit that act in a manner "useful" for themselves (i.e., competent to permit the invasion of the mutant when rare), but one that is not advantageous for other genes in the host (Hurst *et al*., 1996).
(Meiotic) drive Segregation distortion	Any process which causes some alleles to be over-represented in the gametes which are formed during meiosis.
Genetic system	Roughly speaking, the mode of organization and transmission of genetic material including ploidy level, sexual versus asexual reproduction and asymmetric inheritance of genes. Examples of genetic systems include haplodiploidy and diplodiploidy.
Sexual conflict	Special case of genetic conflict where the conflicting entities occur in different sexes (Partridge & Hurst, 1998).
Power	The relative ability of a given genetic entity to affect the conflict trait (Beekman, Komdeur & Ratnieks, 2003).

and test the power of different genetic entities in specific systems to investigate what role they could have played in the evolution of that system. This is the key challenge for the future.

WHY STUDY SCALE INSECTS?

In order to test these predictions, it is important to choose an appropriate study system. The most important criterion is a taxonomic group that contains enough variation in genetic and sex determination systems. However in order to use an experimental approach, it is also important to choose species with a genetic system that is either of reasonably recent origin, as this might make it easier to detect which forces are responsible for its evolution, or alternatively, a system where there might be clear ongoing conflict. Based on these criteria, I have chosen to focus my thesis work on scale insects, a superfamily of the Hemiptera, or "true bugs" (which I will further introduce in Box 1). This group contains about 8000 species and has the largest variation in genetic systems of any taxonomic group of similar size; indeed there is almost as much variation in genetic systems within scale insects as there is within the insects as a whole (Normark, 2003; Nur, 1980). Surprisingly however, scale insects have received little attention from evolutionary biologists. Apart from their variation in genetic systems, there might also be a large scope for genetic conflict as many scale insects harbour maternally transmitted endosymbionts (Buchner, 1965; Tremblay, 1989), many species have a genetic system that causes asymmetry in how and which genes are transmitted (Nur, 1980), and many scale insect have a life history that might lead to prolonged interactions between relatives (Gullan & Kosztarab, 1997; Normark, 2004a, 2006). This combination of variation in genetic systems, many of which have been suggested to have evolved as a result genetic conflict (Haig, 1993a; Normark, 2004a, 2006; Normark, 2009), and the other life history factors that might further promote conflict, makes scale insects perhaps the perfect group to test the predictions outlined in this introduction.

THESIS OVERVIEW:

Part 1: Genetic conflict in scale insects

In the first part of this thesis I use a combination of theoretical and comparative approaches to investigate the role of genetic conflict in causing transitions between genetic systems and thereby shaping the variety of genetic systems across life, and more specifically in scale insects.

To start, in **Box 1** I give a short introduction to scale insects, describing some of their unusual biology. Then, in **chapter 2** I try to establish the use of a genomic conflict framework to try and understand this unusual biology, especially focussing on their wide range of genetic systems. I do this first of all by using the wealth of data

on scale insects already available. The first half of the 20th century was the golden age of comparative cytogenetics and the many studies of chromosome structure and behaviour resulted in a wealth of data on bizarre chromosome systems (reviewed by White, 1973). However, much of this work was very descriptive and poorly understood in evolutionary terms, as the theoretical framework needed to understand the observations was not yet developed. Therefore re-interpreting data from that period can lead to valuable insights and also identify interesting cases worthy of further investigation. In addition, I also review the different hypotheses that have been published on the transitions between the different genetic systems, specifically focussing on the recent hypotheses that assume genetic conflict between different genetic entities can drive such transitions. I then discuss what conflicts might be present for different taxa and how this is affected by their genetic system. Finally, I try to link the empirical data with theoretical predictions in order to establish the importance of genetic conflict in shaping the variation of genetic systems observed in scale insects.

Conflict between males and females over several aspects of reproduction, such as the mating rate of females, has received considerable attention in recent years. However, one area of conflict that has until now received little attention is conflict between the sexes over their offspring's sex. In **chapter 3** I discuss under which conditions such conflict might be expected, what effects this could have, and empirical evidence for the importance of these conflicts.

In **Chapter 4** I present a theoretical analysis of conflict between genes of maternal and paternal origin in males of species with PGE. Paternally-derived genes in males do not have any direct fitness as they are discarded from the germline, but they might obtain indirect fitness through genes shared by their female relatives. Because of this, when siblings compete for resources, paternal genes in males could potentially benefit from killing the male they reside in, if the resources that become available as a result can be used by their sisters. In this chapter I use both an analytical and simulation approach to investigate: (1) if and under what conditions paternally expressed suicide genes can evolve; (2) how this is affected by inbreeding.

In **Chapter 5** I explore how sexual conflict could have been involved in the evolution of hermaphroditism from haplodiploidy. In haplodiploid species, mothers can influence the sex ratio of their brood by selectively fertilizing their eggs, but since her partner prefers a more female-biased sex ratio he might try to influence her control over fertilization. In this chapter I theoretically explore the evolution of an unusual type of hermaphroditism—where the sperm-producing tissue in a diploid hermaphrodite develops directly from haploid sperm-- that could be a result of this conflict.

In **Chapter 6**, I use a comparative analysis to test the hypothesis that endosymbionts have played an important role in the evolution of PGE and haplodiploidy in scale insects. In addition I also test if there is a correlation between asexuality and the presence and type of endosymbiont.

Part 2: Sex allocation in mealybugs

In this part I focus on patterns of sex allocation in one particular scale insect species

with PGE. I have chosen to work with the citrus mealybug *Planococcus citri* (Pseudococcidae), as this species is one of the best-studied scale insects and there is a substantial literature on its biology, life-history (see also Figure 2) and sex allocation.

In **chapter 7** I use an experimental approach to test how population density affects sex allocation in *P. citri* and how females change their offspring sex ratio during the ovipositing period. I then discuss the results in the light of current theoretical predictions. Finally I compare our results with those of earlier studies on sex allocation in *P. citri*.

In chapter 7 we predicted that males are overproduced under high densities because we assumed that they suffer less from high levels of competition, as they use less resources and are able to migrate as adults. However, in mealybugs the most important dispersal stage is during the first instar. First instar nymphs ("crawlers") are highly mobile and several factors have been identified that affect their dispersal behaviour. One aspect that has not yet been addressed however is the extent of any differences in dispersal behaviour between male and female crawlers. This is important as it would influence predictions concerning mealybug life-history, mating system and sex allocation evolution. Therefore in **chapter 8** I present data from 2 experiments testing for sex-specific crawler dispersal and how this is affected by both population density and sex ratio.

In **chapter 9** I use inbred lines from 3 different countries to test if the change in sex ratio over the oviposition period, as noted in earlier experiments (chapter 7) is 1) consistent across populations and 2) an adaptive response to synchronize the reproductive maturation of siblings. Additionally I also consider genetic variation in sex allocation behaviour and other life history traits.

Apart from density effects on sex allocation, other environmental conditions experienced by parents might also affect sex allocation as these factors could either affect the amount of resources parents can provide to their offspring, or predict the environment experienced by their offspring. In **chapter 10**, I therefore investigate the effect of three factors that might affect sex allocation: high temperature, delayed mating and food restriction.

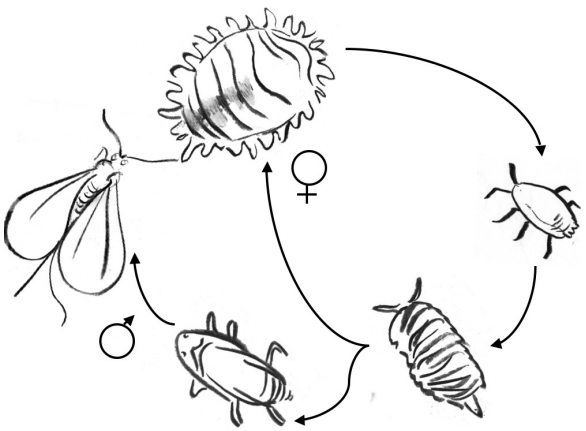


Figure 1.2 Cartoon of the life cycle of the citrus mealybug (*Planococcus citri*). Clock wise starting from the right: a first instar nymph (crawler), a second instar, male pupa, adult male and adult female.

BOX 1

Quick guide: Scale insects

Laura Ross and David M. Shuker

Current Biology 2009

What are scale insects? Scale insects (Hemiptera: Sternorrhyncha: Coccoidea) are a group of small plant feeding insects closely related to aphids and whiteflies. They are characterized by their unusual shapes, so much so that it is sometimes hard to recognize them as insects or even as animals! Adult females hardly ever move, lacking wings and often even legs. Instead of being able to run away they have evolved many other ways to protect themselves against danger, including a variety of protective secretions. For instance, the armoured scales hide under a toughened shield, whilst mealybugs cover themselves with white grains or strands of wax (Figure 1). Many felt scales on the other hand recruit the plants they infest to help protect them, forming galls on the host plant. All told, there are almost 8000 species of scales in about 32 different families, of which the mealybugs and the armoured scales are the most species rich. Scale insects are found everywhere in the world in almost all know biotopes and are know to feed on leaves, roots, trunks of a wide variety of host plants, and even under the bark of trees.

Males don't look much like females do they? That's right, adult males and females look so completely different that it is hard to believe they are the same species! Adult male are typically tiny (1–2 mm) and winged, while in most species adult females are much bigger (in some species up to 3 cm) with strongly reduced (or absent) legs and antennae and no wings. The specialised wax-secreting glands and structures are also typically only found in females. Males and females also have a very different pattern of development. As juveniles, the sexes are often indistinguishable, but after the second larval stage (or 'instar') males undergo a form of metamorphosis, emerging to live but a few days as adults. Females, on the other hand, retain their larval appearance throughout their lives (through several instars), living for up to several months. However, some species of scale insect lack males altogether and reproduce asexually.

What do scale insects do? Well, at first glance, not very much. The females form dense colonies on their host plants, attaching themselves firmly and feeding on the

plant juices. Adult females are usually completely sedentary and while adult males are winged, they do not disperse over great distances, because of their short lifespan and poor flight ability. In comparison to the adults, however, the young larvae ('crawlers') can be very mobile and do manage to get around, with a number of dispersal strategies, including behavioural and morphological adaptations for being carried by the wind. In an Australian species, young females even hitchhike on the back of their winged brothers. The female's sedentary lifestyle does mean that scales are incredibly fecund, with energy spent on little else apart from staying put and reproducing.

If they don't do very much, why are they important? This sitting around on plants causes millions of pounds worth of economic damage to crops and ornamental species the world over, making scale insects a major pest to both farmers and gardeners alike. They damage plants directly by feeding on the plant sap, but they can also indirectly cause damage by transmitting plant pathogens and by the production of large quantities of honeydew that can results in fungal growth on the plants, thereby reducing photosynthesis and promoting further pathogen attack.

They might be important, but they still just sit around — are they interesting? Yes! First, all scale insects harbour endosymbiotic bacteria (or fungi) that provide them with essential nutrients and allow them to live on the otherwise very poor diet of plant sap. Most scales have more than one species of endosymbiont, which often live together inside specialized host cells. In the case of mealybugs, these two bacteria even live inside each other — the only known case of bacterial – bacterial endosymbiosis!

Second, scale insects have a tremendous diversity of genetic systems, including haplodiploidy, six types of asexual reproduction and even hermaphroditism. One of the most puzzling genetic systems found in scales though is paternal genome elimination: both sexes are diploid but in males one of the parental chromosome sets is deactivated (via DNA heterochromatization) during early development. The deactivated set is always the set inherited from the father. Although the deactivated set divides faithfully in all somatic cell lines, it fails to end up in mature sperm because it is destroyed during meiosis. For an example see Figure Box 1.1.

How do they know whose chromosomes are whose? By genomic imprinting — both males and females 'tag' their genes and this affects which genes (from father or mother) are expressed in the offspring. In most taxa where imprinting is known to be important, only a small percentage of genes across the genome are involved. In scale insects, however, whole chromosomes are involved and in males only the mother's chromosomes are expressed, while the father's chromosomes are deactivated. Although the molecular mechanisms involved in the silencing of the paternal genes are fairly well understood, it is still a mystery why the silencing only happens in males and how the sex of the offspring is determined in the first place.

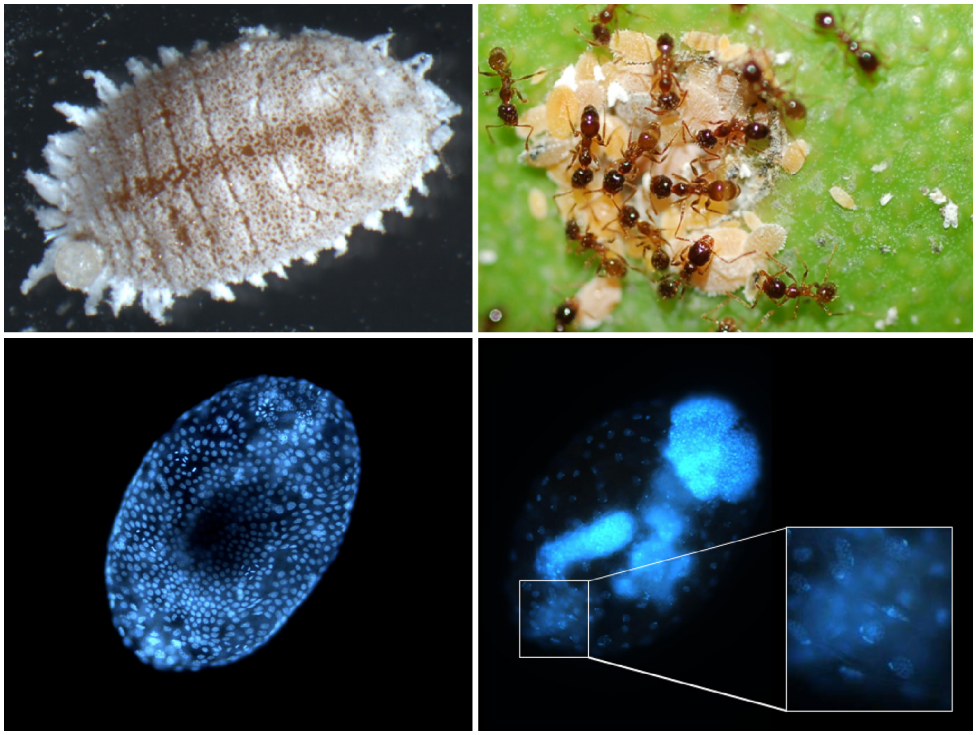


Figure Box 1.1 Mealybugs.
Clockwise from the top left hand corner: an adult female *Planococcus citri* mealybug; mealybugs being tended by mutualistic ants; a male *P. citri* DAPI-stained embryo, with the condensed paternal chromosomes visible within cell nuclei as dense dots (see inset); a female *P. citri* DAPI-stained embryo, showing normal nuclei. Pictures by Laura Ross and Alejandro Tena (mealybugs and ants).

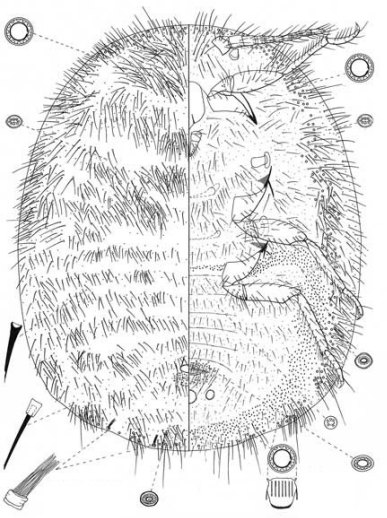
Are males completely helpless while their genes are destroyed? Probably not. Whilst the paternal genome is deactivated in nearly all male tissues, it is active in the cells making up the cysts in which spermatogenesis takes place. Thus, genes of paternal origin are silent except in the very place where they are prevented from gaining access to the sperm, suggesting that they might try to prevent their own destruction.

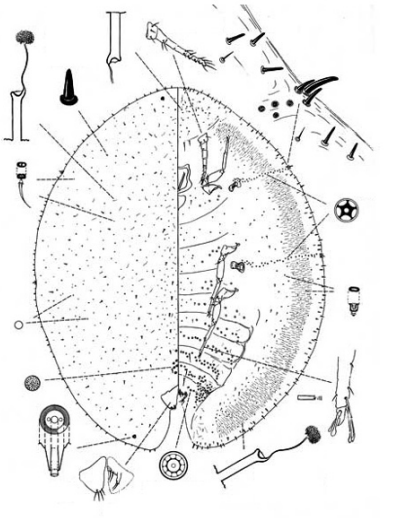
Why do scale insects have so many genetic systems? Good question! It might be because the male, female and the endosymbiotic bacteria value sons and daughters differently, leading to conflict over the control of sex allocation and sex determination. In species with paternal genome elimination, males do not pass on their genes through their sons (they do not make it to their sons' sperm) and might therefore favour daughters. The same is true for the bacteria, because these are only transmitted through the cytoplasm in scale insect eggs (by the maternal line of their hosts). Females however benefit from both male and female offspring. Therefore all three of them have an interest in controlling sex determination, which might have led to the

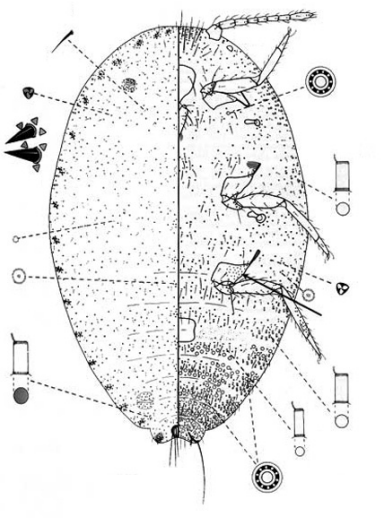
large diversity of unusual genetic systems in the group through evolutionary conflicts between female-expressed genes, male-expressed genes, and the symbiotic bacteria.

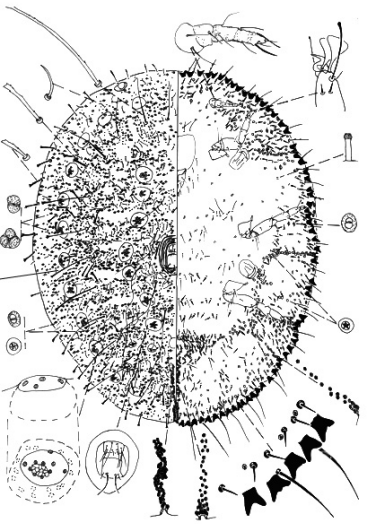
Males do seem to get a bit of a rough deal, don't they? This also might have to do with their bacteria. As the endosymbionts are only transmitted through females there is no selection pressure for them to do their job that well in males. Even worse, it might be beneficial for the bacteria to kill the males if this benefits their sisters, who carry the relatives of the bacteria. In order to avoid this, males appear to have been selected to depend less on their bacteria, which might explain their small size and short lifespan. There is even a scale insect where males don't have bacteria at all and do not feed. Instead they are fed by their mothers, who have evolved a placenta-like structure for this purpose.

Do scales interact with any other organisms? As with many other honeydew-producing insects (such as aphids), mealybugs and other scale insects are often visited by ants that collect the honeydew (Figure Box 1.1). This is obviously good for the ants, but also for the scales because ants can offer protection from predatory insects and spiders. Indeed, although many scale species are visited by ants, some have become completely dependent on them. For example, species of the genus *Stictococcus* would drown in their own honeydew if the ants didn't remove it! In addition, species of the mealybug genus *Hippeococcus* are used as cattle by the ants, which they transport with them to new host plants and there are observations of crawlers jumping on the back of the ants and being rescued in case of danger. In several ant species, the queen even takes one of her colony's mealybugs with her on her nuptial flight. The relationship can become so close that *Hippeococcus* species have lost their endosymbiotic bacteria and are dependent on direct feeding by their host ant.









CHAPTER 2

Genomic conflict in scale insects: the causes and consequences of bizarre genetic systems

Laura Ross, Ido Pen and David M. Shuker

ABSTRACT

It is now clear that mechanisms of sex determination are extraordinarily labile, with considerable variation across all taxonomic levels. This variation is often expressed through differences in the genetic system (XX-XY, XX-XO, haplodiploidy, and so on). Why there is so much variation in such a seemingly fundamental process has attracted much attention, with recent ideas concentrating on the possible role of genomic conflicts of interest. Here we consider the role of inter- and intra-genomic conflicts in one large insect taxon: the scale insects. Scale insects exhibit a dizzying array of genetic systems, and their biology promotes conflicts of interest over transmission and sex ratio between male- and female-expressed genes, parental- and offspring-expressed genes (both examples of intra-genomic conflict) and between scale insects and their endosymbionts (inter-genomic conflict). We first review the wide range of genetic systems found in scale insects and the possible evolutionary transitions between them. We then outline the theoretical opportunities for genomic conflicts in this group and how these might influence sex determination and sex ratio. We then consider the evidence for these conflicts in the evolution of sex determination in scale insects. Importantly, the evolution of novel genetic systems in scale insects has itself helped create new conflicts of interest, for instance over sex ratio. As a result, a major obstacle to our understanding of the role of conflict in the evolution of sex-determination and genetic systems will be the difficulty in identifying the direction of causal relationships. We conclude by outlining possible experimental and comparative approaches to test more effectively how important genomic conflicts have been.

Biological reviews, 2010

INTRODUCTION

Genetic systems vary widely, including the presence or absence of sex chromosomes, the number and sex specificity of those chromosomes, the developmental requirement for both parental sets of chromosomes, variation in levels of ploidy between the sexes and sometimes even the complete absence of sexual reproduction (Normark, 2003). The genetic system of an organism provides the context for the evolution of several fundamental biological processes, including sexual *versus* asexual reproduction, sex determination, and many aspects of genome evolution (Bull, 1983; Lynch, 2008; Maynard Smith, 1978). As such, we may expect genetic systems to be the firm foundations on which these other processes evolve. However, it is becoming abundantly clear that genetic systems themselves can be remarkably labile within and among closely related species (Bull, 1983; Normark, 2003). Similarly, determining which sex an organism develops into would appear, at first glance, to be a fundamental developmental process for sexually reproducing organisms. As such, the mechanisms underlying that developmental decision should perhaps be conserved across broad taxonomic groups. Again, however, it is now obvious that there is an extraordinary diversity of sex-determination systems across all levels of taxonomic diversity, including within single species such as the housefly *Musca domestica* (Dubendorfer *et al.*, 2002; Kozielska *et al.*, 2006; Uller *et al.*, 2007). This diversity is expressed at the molecular level as changes in the sex determination cascade (Evans, 2004; Marin & Baker, 1998; Van Doorn & Kirkpatrick, 2007), but is also reflected in genomic terms as variation in the genetic systems of closely related organisms (Normark, 2003).

Understanding the observed diversity of genetic systems across taxa has become an important avenue of research in evolutionary biology, with a central question being why do some groups of species vary tremendously in their genetic systems, whilst in other groups the genetic system is rather more conserved (e.g. female heterogamety across birds, or across Lepidoptera)? Currently, the idea that various forms of genomic conflict shape genetic systems is becoming increasingly influential (Burt & Trivers, 2006; Haig, 1993a; Normark, 2004a). Genomic conflict refers to conflicts of interest between different genetic entities over the state of a given trait, typically related to reproduction. Under conflict, selection favours different trait values for the different entities (Leigh, 1971). These different genetic entities may share a genome (intra-genomic conflict), with conflicts of interest between males and females (sexual conflict: Arnqvist & Rowe, 2005; Parker, 1979) and between parents and offspring (Godfray, 1995; Trivers, 1974) being the most familiar examples. Alternatively, the different genetic entities may be genomically isolated from each other, as with conflicts between hosts and endosymbiotic organisms (inter-genomic conflict). Whilst this separation is useful, conflicts with some reproductive parasites such as transposable elements could be viewed plausibly as either (Burt & Trivers, 2006).

In this review, we will consider whether genomic conflict can explain the observed patterns of the evolution of genetic systems. We will focus on two broad classes of conflict, those concerned with the genetic drive of selfish genetic elements of one sort

or another, which can result in the production of biased sex ratios, and those concerned with more direct conflicts over sex determination and sex ratio. As such, sex determination and biased sex ratios will form a common thread running through the article. Both types of genomic conflict can occur within genomes, as well as among genomes, and we will address both. The context for this review will be the extraordinary diversity of genetic systems and modes of reproduction in one group of insects: the scale insects.

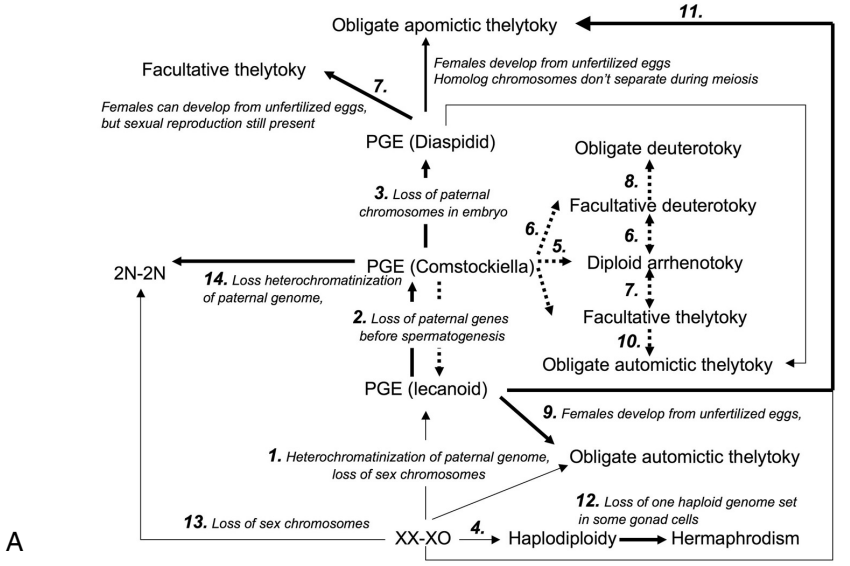
Scale insects (Hemiptera: Sternorrhyncha: Coccoidea) have one of the largest varieties of genetic and sex determination systems (Gullan & Kosztarab, 1997; Hughes-Schrader, 1948; Nur, 1980). Often, closely related species with very similar life histories differ in their genetic system. This makes them an ideal group to explore the evolution of different genetic/sex determining systems. Genomic conflict has been suggested to play an important role in the evolution of scale insects and their diverse genetics (Burt & Trivers, 2006; Normark, 2004a; Ross & Shuker, 2009). We will ask how well genetic drive and conflicts over sex determination and sex allocation can explain scale insect genetic systems, but we will also consider how genomic conflicts over transmission and reproduction feed back on each other, creating new opportunities for conflict to arise. These interactions make inference problematic, and whenever possible we highlight to what extent empirical work can unravel these complexities.

We begin by introducing the problem: the diversity of scale insect genetic systems. We then consider more traditional explanations for these patterns, before introducing and discussing intra- and inter-genomic conflicts over transmission and sex ratio. We finish by asking how best progress can be made in terms of both scale insects and patterns of genetic system evolution more generally.

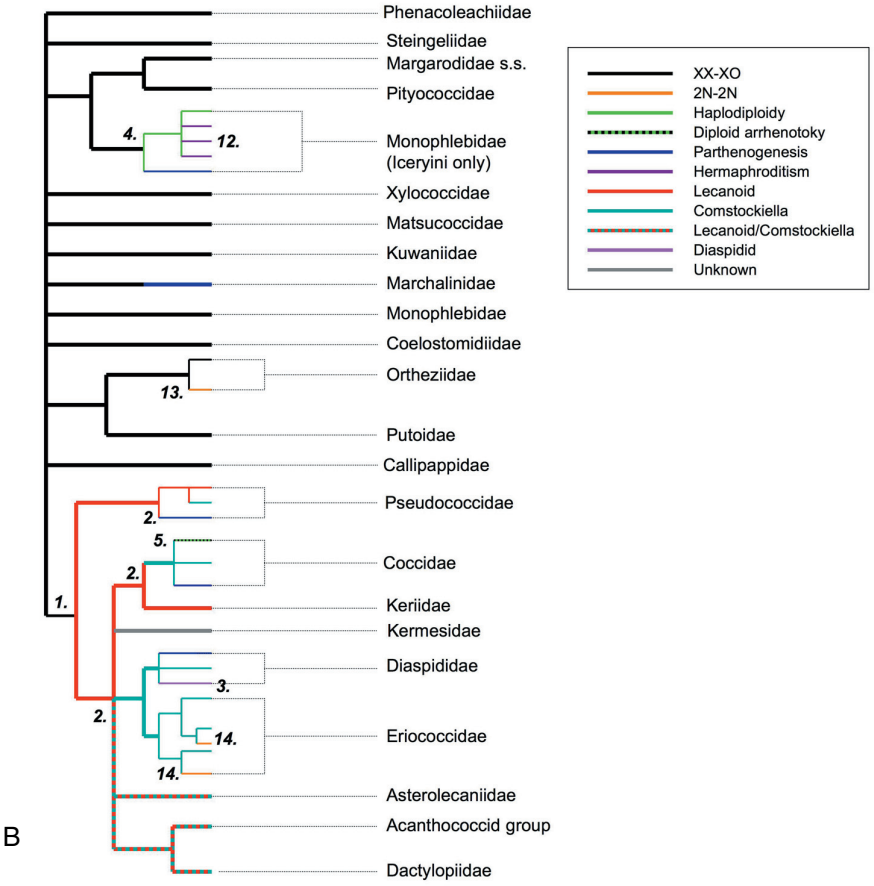
THE BIOLOGY OF SCALE INSECTS

Life history

Scale insects are small plant-feeding insects. They are a superfamily (Coccoidea) of the order Hemiptera, most closely related to the aphids and white-flies. There are more than 7000 species of scale insects described in approximately 28 families. Scale insects have in general a fairly similar (or uniform) life history. In most species, the sexes are indistinguishable as first-instar larvae, with both sexes having well-developed legs and antennae. It is generally assumed that the first-instar larvae ("crawlers") are the main dispersal stage. The development of males and females is similar until the end of the second instar, when the males pupate and stop feeding. As adults, males are typically fully winged, whilst females are typically wingless, with a mostly (and sometimes completely) sedentary lifestyle. Females have therefore evolved a wide variety of strategies to protect themselves against predators and other environmental influences. Most species form a waxy protective cover over their body (Gullan & Kosztarab, 1997) and species of several families have recruited the plants they infest to help protect them, forming galls on the host plant (Gullan, Miller & Cook,


Obligate apomictic thelytoky
11.
Facultative thelytoky
7.
Females develop from unfertilized eggs
Homolog chromosomes don't separate during meiosis
Females can develop from unfertilized eggs,
but sexual reproduction still present
PGE (Diaspidid)
Obligate deuterotoky
8.
3. Loss of paternal
chromosomes in embryo
Facultative deuterotoky
6.
6.
5.
2N-2N
14. Loss heterochromatinization
of paternal genome,
PGE (Comstockiella)
Diploid arrhenotoky
7.
2. Loss of paternal genes
before spermatogenesis
Facultative thelytoky
10.
Obligate automictic thelytoky
PGE (lecanoid)
9. Females develop from unfertilized eggs,
1. Heterochromatinization of paternal genome,
loss of sex chromosomes
Obligate automictic thelytoky
12. Loss of one haploid genome set
in some gonad cells
13. Loss of sex chromosomes
XX-XO
4.
Haplodiploidy
Hermaphrodism

A


Phenacoleachiidae
Steingeliidae
Margarodidae s.s.
Pityococcidae
4.
12.
Monophlebidae
(Iceryini only)
Xylococcidae
Matsucoccidae
Kuwaniidae
Marchalinidae
Monophlebidae
Coelostomidiidae
Ortheziidae
13.
Putoidae
Callipappidae
Pseudococcidae
2.
5.
2.
Coccidae
1.
Keriidae
Kermesidae
Diaspididae
3.
2.
14.
Eriococcidae
14.
Asterolecaniidae
Acanthococcid group
Dactylopiidae
XX-XO
2N-2N
Haplodiploidy
Diploid arrhenotoky
Parthenogenesis
Hermaphroditism
Lecanoid
Comstockiella
Lecanoid/Comstockiella
Diaspidid
Unknown

B

2005). Adult males locate females using pheromones, although due to their small size, short lifespan and limited flight ability it is not thought that males are able to migrate far (Gullan & Kosztarab, 1997).

Scale insects feed almost exclusively on the phloem of their host plant, forming dense colonies. This is problematic as phloem contains mainly carbohydrates and lacks certain essential amino acids. Scale insects have solved this problem by forming important symbioses with bacteria (and sometimes yeast-like fungi) (Buchner, 1965; Tremblay, 1989), which provide them with the essential nutrients lacking in their diet. We consider the significance of endosymbionts in more detail in Section V1.1. Most scale insects also produce vast amounts of honeydew in order to dispose of the excess sugar in their diet. The colonial habit means that many scale insects species are serious pests on crops and ornamental species the world over, causing severe economic damage (Ben-Dov, Miller & Gibson, 2009). They damage plants directly by feeding, but they can also indirectly cause damage by transmitting plant pathogens through injection or through the build-up of honeydew that can promote the attack of fungi and other plant pathogens.

The diversity of genetic systems in scale insects

Scale insects display a quite remarkable variety of genetic systems (Fig. 2.1). Several scale insects have a XX-XO system and this system has been assumed to be ancestral (Nur, 1980). However in many other taxa a variety of different genetic systems has evolved, often more than once. Nur (1980) reviewed the different genetic systems found in scale insects and reconstructed their possible evolutionary history. Fig. 2.1A presents the possible evolutionary pathways of the different genetic systems based on recent molecular phylogenies of scale insects and also includes recent data on the genetic systems of several scale insect taxa as reviewed by Gavrilov (2007). We review the major transitions in genetic system below, noting immediately the complex patterns of evolutionary loss and gain of different genetic systems. We number each transition as in Fig. 2.1A.

Figure 2.1 (left) (A) A schematic overview of the transitions between the different genetic systems across the Coccoidea. The schematic is adjusted from Nur (1980) based on the most recent molecular phylogeny and also including all the asexual systems. Thin arrows represent single transitions while thick arrows represent multiple transitions. Broken arrows represent uncertain or hypothetical transitions. The numbers correspond with the descriptions of the transitions in the text. Those transitions in italics are the most important transitions in the context of this article. PGE refers to paternal genome elimination, while 2N-2N refers to diplodiploidy systems that lack sex chromosomes. All genetic systems are described in more detail in Section II. (B) A family-level phylogenetic reconstruction of the scale insects based on Gullan & Cook (2007). The different coloured lines represent the different genetic systems found across the clade. If there are differences within families this is shown by including a schematic representation of the within-family relationships (the thinner lines, based on (Cook *et al.*, 2002; Downie & Gullan, 2004; Hardy, Gullan & Hodgson, 2008; Unruh & Gullan, 2008). Branch lengths are not to scale. The numbers again represent the transitions described in the text. Margarodidae s.s. refers to the Margarodidae *sensu stricto*.

Evolutionary transitions 1-3: paternal genome elimination

The most widespread and bizarre deviation from the XX-XO genetic system is that represented by paternal genome elimination (PGE) (Nur, 1980). PGE is found in 14 scale insect families, including the economically important mealybugs (Pseudococcidae) and armoured scale insects (Diaspididae) (see Fig. 2.1B). In this system both sexes develop from fertilized eggs, but during early development of the male offspring the paternal half of the genome is deactivated. Although the deactivated set divides faithfully in all somatic cell lines, it fails to end up in mature sperm because it is destroyed during meiosis and is not passed on to the offspring of the male (Schrader, 1921). Later studies showed that the deactivated genome set was of paternal origin (Brown & Nelson-Rees, 1961).

In the scale insects three different types of PGE are found, that differ in the timing of the loss of the paternal genome set (see Fig. 2.2). The ancestral system of PGE is the lecanoid system (Transition 1), found in the mealybugs (Pseudococcidae), the lac scale insects (Kerriidae) and some felt scales (Eriococcidae). In this system, although the paternal genome set is deactivated in early development it is only lost during spermatogenesis (see Fig. 2.2). In the more derived Comstockiella system (Transition 2, named after the genus of armoured scales it was first found in; (Brown, 1957)) the paternal genome is deactivated at the same time as in the lecanoid system and it is present in all somatic cells. The main difference between the lecanoid and the Comstockiella systems is that in the latter some of the heterochromatinized paternal chromosomes are lost just prior to spermatogenesis (Brown, 1963; Nur, 1980) (see Fig. 2.2). The remaining paternal chromosomes then undergo the same fate as in the lecanoid system, being separated from the euchromatic chromosomes and failing to end up in the sperm. The number of chromosomes that are lost before spermatogenesis can vary between species, between individuals of the same species and even between different germ line cells within a single individual (as in *Eriococcus araucariae* and *Eriococcus spurious* (Fig. 2.3F); Brown, 1967). In most species about 75% of the heterochromatic set is destroyed before spermatogenesis (Brown, 1967). The Comstockiella system seems to have evolved multiple times from the lecanoid system (see Fig. 2.1) and there is a suggestion that evolution has gone both ways and some taxa even seem to have a combination between the two systems within individuals (*Eriococcus araucariae* Brown, 1967). The third system of PGE is the Diaspidid system (Transition 3) found in the armoured scales (Diaspididae). In this system the paternal genome does not become heterochromatinized, instead it is lost during early development at about the same time that the paternal chromosome become heterochromatinized in the two other systems (Nur, 1980) (see Fig. 2.2). Elimination is accomplished by so-called anaphase lagging of the paternal set, whereby the chromosomes during the anaphase of mitosis do not move to the spindle quickly enough to be incorporated in a new nucleus. Males in the Diaspidid system therefore become completely haploid both in the somatic and germline cells, even though they develop from fertilized eggs. Recent molecular phylogenies of scale insects have confirmed Nur's (1980) original hypothesis that PGE only evolved once in scale insects (Cook,

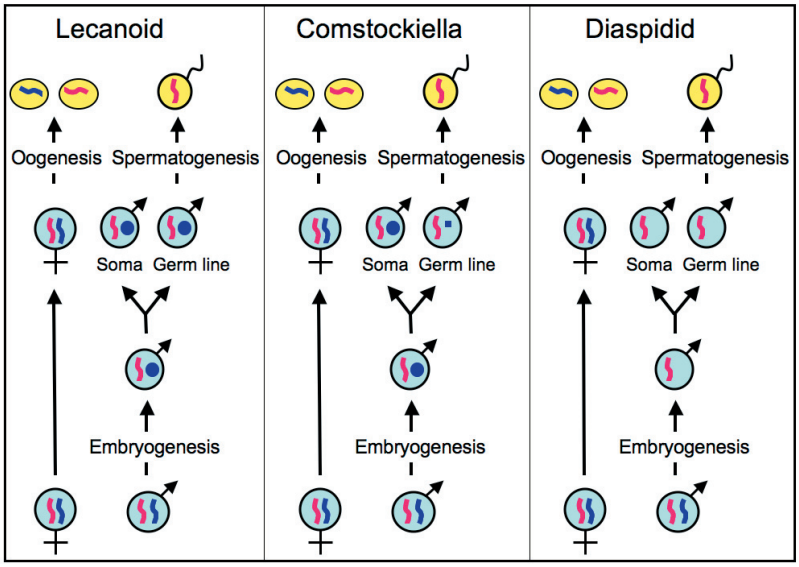


Figure 2.2 A schematic representation of the three different paternal genome elimination (PGE) systems found in scale insects: the lecanoid, Comstockiella and the Diaspidid systems. In each figure the pink line represents the maternally inherited chromosome set while the blue line represents the paternally inherited chromosomes. The blue circle represents the heterochromatinized state of the paternally derived genome, while the size of the circle indicates the number of paternal chromosomes.

Gullan & Trueman, 2002) and that the more derived Comstockiella and Diaspidid systems have evolved from the lecanoid system (see Fig. 2.1).

Evolutionary transitions 4 and 5: haplodiploidy and diploid arrhenotoky

True haplodiploidy is found in several species of the genus Icerya and seems to have evolved from a XX-XO system (transition 4). In this system females develop from fertilized eggs and are diploid, while males develop from unfertilised eggs and are haploid. In addition to true haplodiploidy, a very similar system has also been found in scale insects: diploid arrhenotoky (transition 5). This system differs from haplodiploidy in that, in the unfertilized eggs that develop into males, diploidy is restored by a fusion of the first haploid cleavage nuclei, so that both sexes are diploid. However, as if this was not complicated enough, shortly after diploidy has been restored in males, one of the chromosome sets becomes heterochromatinized, leaving males again with haploid gene expression. This curious system has so far only been found in one species of soft scale genus Parthenolecanium (previously Lecanium) (Nur, 1971, 1972). It probably evolved from the PGE system found in related species, although it might also have evolved from one of the parthenogenetic systems that are found in some species of the same genus (see below). What is fascinating about this species is that heterochromatinization happens to one set of chro-

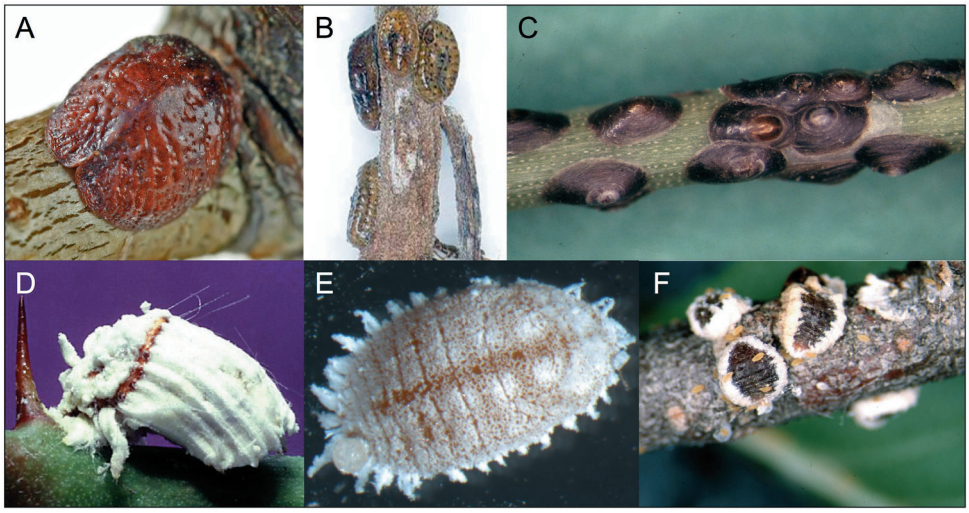


Figure 2.3 Scale insects from six different families. (A) Adult female of *Parthenolecanium corni,* a soft scale insect (Hemiptera: Coccidae) which reproduces by obligate automictic thelytoky © Entomart. (B) Adult females of *Stictococcus* sp. (Hemiptera: Stictococcidae). Photo by Alessandra Rung. (C) Adult females of the black pine scale *Dynaspidiotus californicus* on pine needle (Hemiptera: Diaspididae). Picture by Whitney Cranshaw, Colorado State University, Bugwood.org. (D) Adult female with ovisac of the cottony cushion scale *Icerya purchasi* (Hemiptera: Monophlebidae). (E) Adult female of the citrus mealybug *Planococcus citri*. Picture by Laura Ross (F) Adult females and first-instar larvae ("crawlers") of the European elm scale *Eriococcus spuria* (Hemiptera: Eriococcidae). Picture by Whitney Cranshaw, Colorado State University, Bugwood.org.

mosomes even though both sets of chromosomes in males presumably are identical (i.e. of maternal origin), both in terms of DNA sequence and any epigenetic marks such as genomic imprints (see Section IV for further discussion of how chromosomes are chosen for heterochromatinization).

Evolutionary transitions 6 - 11: parthenogenesis

There are six different parthenogenetic systems found in scale insects (extensively reviewed by Nur, 1971). These systems can be broadly divided with respect to three characteristics: (1) whether males are absent or occasionally present (obligate parthenogenesis or facultative parthenogenesis); (2) which sexes can develop from fertilized and unfertilized eggs; (3) how diploidy is restored in unfertilized eggs (see also Table 2.1).

In species with facultative deuterotoky (transition 6, also see Table 2.1), reproduction can be both sexual and asexual and both males and females can develop from either fertilized or unfertilized eggs (within the same species). Individuals that develop from unfertilized eggs restore diploidy by fusion of the first haploid cleavage nuclei, resulting in complete homozygosity. In species with facultative thelytoky

(transition 7), unfertilized eggs develop into females, fertilized eggs into both sexes. Meiosis is normal and diploidy is restored by fusion of the polar body with the haploid pronucleus. In obligate deuterotoky (transition 8), unfertilized eggs develop into both sexes but males are inviable or sterile and no sexual reproduction seems to take place. Three types of strictly obligate parthenogenetic systems (transitions 9, 10 and 11) are found in scale insects. In all systems females develop from unfertilized eggs and males are absent, the main difference being that in one system (obligate apomictic thelytoky, transition 11), the homologous chromosomes do not separate during meiosis and oogenesis produces diploid eggs. In the other two systems (obligate automictic thelytoky), meiosis is normal and eggs are haploid. Diploidy is restored either by the fusion of the pronucleus and one of the polar bodies (transition 9) or by fusion of the first haploid cleavage nuclei (transition 10). Table 2.1

Table 2.1 Overview of the different asexual reproductive modes found in the scale insects. For each system the characteristics of the particular form of asexuality and the taxonomic range are shown. Based on Nur (1971).

Genetic system	Reprod. asexual/ sexual	Females develop from	Males develop from	Diploidy restored by	Taxonomic range
Facultative deuterotoky	Both	Fertilized and unfertilized eggs	Fertilized and unfertilized eggs	Fusion of the first haploid cleavage nuclei	Coccidae: *Parthenolecanium cerasifex*
Obligate deuterotoky	Asexual	Fertilized and unfertilized eggs	Fertilized and unfertilized eggs	Fusion of the first haploid cleavage nuclei	Coccidae: *Pulvinaria hydrangeae*
Facultative thelytoky	Both	Fertilized and unfertilized eggs	Fertilized eggs	Fusion polar body with pronucleus	Coccidae: *Coccus hesperidum, Saissetia coffeae*
Obligate automictic thelytoky (type 1)	Asexual	Unfertilized eggs	Absent	Fusion polar body with pronucleus	Coccidae: *Parthenolecanium corni corni*, some populations of: *Coccus hesperidum, Saissetia coffeae* Pseudococcidae: *Phenacoccus solani*
Obligate automictic thelytoky (cleavage)	Asexual	Unfertilized eggs	Absent	Fusion of the first haploid cleavage nuclei	Coccidae: 2 species Monophlebidae: *Gueriniella serratulae*
Obligate apomictic thelytoky	Asexual	Unfertilized eggs	Absent	No meiosis	Coccidae: 2 species Diaspididae: eight species Marchalinidae: *Marchalina hellenica* Pseudococcidae: two species

summarises the characteristics of the different parthenogenetic systems and gives an overview of the taxa in which these systems are found.

Although parthenogenetic reproduction is common in scale insects and has been found to have evolved independently in many families, only two systems, obligate apomictic thelytoky and obligate automictic thelytoky, have been found outside the soft scales (Coccidae); all other systems described above are only found in a few related genera of soft scale (mainly in the genus *Parthenolecanium*, see Fig. 2.3A), with different systems often being found in closely related species (Nur, 1971, 1980). Currently very little is known about the evolution of parthenogenesis in scale insects. In order to understand better how the different parthenogenetic systems in scale insects have evolved and why parthenogenesis is both more common and more variable in soft scales than in any other scale insect family, more data are needed from the relevant genera, especially a detailed phylogeny of soft scales.

Evolutionary transition 12: hermaphroditism

Although hermaphroditism is common in many plants, vertebrates and crustaceans, it is extremely rare in insects (Jarne & Auld, 2006; Normark, 2003). The only insect taxa where hermaphroditism has been confirmed with certainty are three species of iceryine scale insects: *Icerya purchasi* (Fig. 2.3D), the African species *Gigantococcus bimaculata* (previously *Icerya*), and the Panamanian species *Crypticerya zeteki* (also previously *Icerya*) (Hughes-Schrader & Monahan, 1966). A recent molecular phylogeny of iceryine scales confirms that these three species constitute three independent origins of hermaphroditism (Unruh & Gullan, 2008). In all these species the hermaphroditic individuals develop from fertilized eggs and are diploid. However, certain cells in the gonad are haploid and these cells proliferate and eventually produce spermatozoa while the diploid cells in the gonad form the oocytes. Most oocytes are fertilized within the body of the hermaphrodite, whilst a small percentage (approximately 10% in *I. purchasi*) of the eggs do not get fertilized and develop into males. These haploid males are viable and are capable of copulating with the hermaphrodites, although it has not been established whether they are able to fertilize eggs (Hughes-Schrader & Monahan, 1966). When this process was first discovered it was assumed that the haploid cells in the gonad originate from diploid cells through the loss of one of the genome sets. However Royer (1975) suggested a different origin. He showed that contrary to what would be expected if the cell originated from genome loss, the haploid cells are present in a newly formed embryo from the moment of fertilization. He also showed that oocytes are often penetrated by multiple sperm (polysperm) and that although only one of these sperm cells fertilizes the oocyte, several form haploid pronuclei that persist in the embryo. Royer (1975) argued that these haploid sperm pronuclei form the haploid "male" germline. Normark (2009) discusses this remarkable finding as a male adaptation to ensure the fertilization of all oocytes a female carries as well as providing a means of fertilizing all her future female descendants by infecting females with what he calls "transmissible spermatogenic stem cells". Another interesting observation from this extraordinary

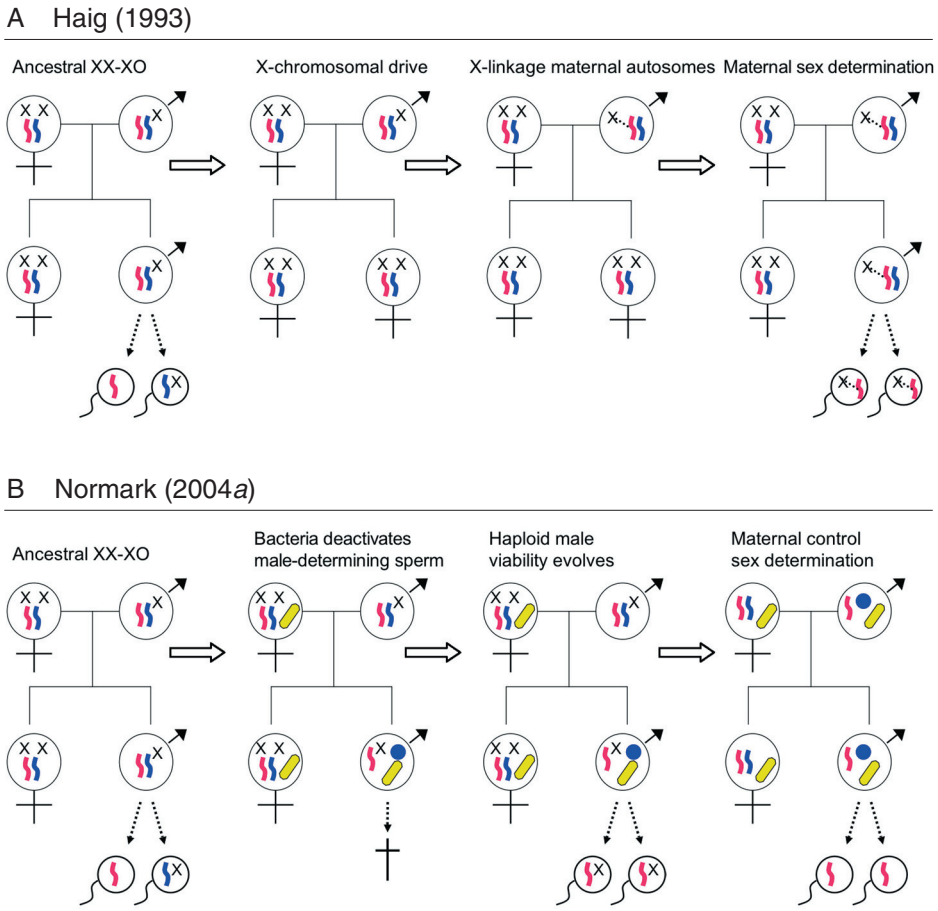


Figure 2.4 A schematic representation of two hypotheses for the evolution of paternal genome elimination (PGE). In each the pink line represents the maternally inherited chromosome set while the blue line represents the paternally inherited chromosomes. The blue circle represents the heterochromatinized state of the paternally derived genome. The "X" represents the X-chromosome and the yellow oval represents the endosymbionts. (A) The X-chromosome drive hypothesis based on Haig (1993). (B) The male-killing endosymbionts hypothesis based on Normark (2004a).

system is that the haploid sperm pronuclei tend to develop in close proximity to the host's endosymbiotic bacteria (Royer, 1975) (see Section VI.5). All other species in the genus *Icerya* that have been studied to date are strictly haplodiploid and no signs of hermaphroditism have been found (Nur, 1980).

Evolutionary transitions 13 and 14: diplodiploidy (XX-XO/ 2N-2N)

There are two different types of diplodiploidy in scale insects. One of them, the XX-XO system, is found in basal scale insects and many other Sternorrhyncha and is

therefore assumed to be the ancestral genetic system in scale insects. The second system is a diplodiploid system that lacks the X chromosomes and has evolved an alternative sex determination system (2N-2N: transitions 13 and 14). It has evolved at least three times in scale insects, both directly from the ancestral XX-XO system in the genus *Orthezia* (transitions 13), and from two PGE lineages in the genera *Lachnodius* and *Stictococcus* (Fig. 2.3B) respectively (transitions 14) (Normark, 2003; Nur, 1980). Interestingly the loss of heterochromatinization in males coincides with the loss of endosymbionts in *Stictococcus* males (see Section V.2 and VI.5) (Buchner, 1965).

Explaining the diversity of genetic systems

How can we explain the remarkable diversity of genetic systems in scale insects highlighted above? A number of hypotheses have been formulated to account for the evolution of this diversity and in the next three sections we will consider the most important of them. First, several authors have discussed the possible influence of idiosyncrasies of scale insect biology, highlighting their unusual chromosomes and their life histories. Second, we will consider the more recent ideas concerning genomic conflict, first dealing with intra-genomic conflicts, before moving onto inter-genomic conflicts, and in particular conflicts with endosymbiotic bacteria. It will be important to remember that we seek to explain not only the diversity of genetic systems themselves, but also why we see such a richly dynamic set of transitions between them (Fig. 2.1). Different genetic systems have evolved multiple times, from different ancestral states, and apparently at different rates. As will become apparent, some hypotheses were formulated for rather specific transitions, while others are more general. Hypotheses specific to certain transitions may be correct, but may be harder to accommodate in a general theory of genetic system evolution that has to explain a diversity of outcomes from a diversity of starting points. Finally, it will also be important to remember that the dynamic pattern of transitions between different systems observed in scale insects makes inference of causation problematic, with selection for the maintenance of a given genetic system likely to differ from the selection underlying the origin of that system (if such selection existed at all).

THE POSSIBLE ROLES OF SCALE INSECT BIOLOGY IN THE EVOLUTION OF THEIR GENETIC SYSTEMS

Scale insects have a number of notable biological features that several authors have suggested may explain their rich array of genetic systems. For instance, as in other Hemiptera, scale insects have holokinetic chromosomes that lack a localized site for the attachment of spindle fibres (the centromere) (Hughes-Schrader, 1948). This means that even small, fragmented chromosomes can still regularly divide during cell division. Amongst other things, this might explain the instability in chromosome numbers observed in some scale insect families (e.g in the genus *Apiomorpha*) (Cook,

2000). In addition to their holokinetic chromosomes however, scale insects also have an inverse meiosis (Chandra, 1962; Hughes-Schrader, 1930; John, 1990). In inverse meiosis, the two sister chromatids disjoin first in meiosis I and only afterwards do the maternal-paternal homolog chromosomes become separated during meiosis II. The four haploid products that are formed by meiosis I and II do not separate but come to lie in a quadrinucleate spermatid. In taxa with the XX-XO system, all the haploid products form sperm, whilst in the species with PGE only the maternal chromosomes develop into sperm and the paternal chromosome products degenerate. Haig (1993a) therefore suggested that this system might predispose scale insects to exhibit genomic drive because the four haploid products of meiosis are contained in the spermatid, giving the opportunity for one set of the chromatids (either the X-bearing or the euchromatinized set) to produce a substance to harm the other set and prevent it from contributing to sperm production. The idea that the combination of holokinetic chromosomes and inverted meiosis might predispose the evolution of new genetic systems is also supported by the presence of PGE-like systems in mites, which are one of the few taxa that share these characteristics with scale insects (Wrensch, Kethley & Norton, 1994). Additionally, a factor that might not explain the evolution of PGE but that might have enabled its evolution is the high level of asynapsis (the failure of homologous chromosomes to pair during meiosis) found in many scale insects (Hughes-Schrader, 1955). This will reduce recombination between maternal and paternal chromosomes, which seems essential for the evolution of PGE (Haig, 1993a).

An alternative, but not mutually exclusive, suggestion for the diversity of genetic systems comes from scale insect life history. In all sexually reproducing scale insects there is a strong sexual dimorphism, with adult females being wingless, sessile and covered with protective secretions, while adult males are winged, do not feed as adults and are usually smaller and shorter-lived. Several authors have argued that this difference can lead to a shortage of males (because of their fragility and short lifespan) and that it would therefore be beneficial to evolve reproductive systems that do not depend on males (Hughes-Schrader, 1948). This theory could potentially explain the multiple evolution of parthenogenesis in several scale insects, and possibly also the evolution of true haplodiploidy in some iceryine scale insects. It is however hard to understand how PGE could have evolved and been stable for millions of years (Herrick & Seger, 1999) if scarcity of males was a strong selective pressure. First, the evolution of a reproductive system that depends less on males (like haplodiploidy or parthenogenesis) from PGE seems easy, but it is actually relatively rare (i.e. there are more PGE scale insects than haplodiploid scale insects and the particular transition has not been observed). Second, one might imagine that it is actually easier to evolve more robust males than to do without males all together. At the very least females could evolve facultative sex allocation such that extra males can be produced if environmental cues suggest that males are likely to be rare (see below for examples of facultative sex allocation). Moreover, it has been noted that the short lifespan and fragility of males in scale insects might themselves have

evolved as a response to conflicts between host and endosymbionts, making inferences about the direction of causality problematic (see host-endosymbionts conflicts below).

Finally, Bull (1983) suggested that the evolution of haplodiploidy might be explained by the life history of many of these species. He pointed out that their life history causes high levels of competition between offspring leading to the optimal sex ratios being biased (e.g. Charnov *et al.*, 1981; Hamilton, 1967). He therefore argued that this would lead to strong selection for females to be able to adjust their sex ratio accordingly and that haplodiploidy would allow females to do this, given the apparently straightforward sex determination mechanism of either fertilising eggs or not (making female and male offspring, respectively). He also argued that PGE has strong similarities with haplodiploidy (arrhenotoky) and that both systems are often found in closely related species. Therefore he considered PGE as an intermediate stage in the evolution of haplodiploidy from diplodiploid systems and that PGE systems will evolve towards haplodiplody (Bull, 1983). Although patterns consistent with this theory have been observed in mites (Cruickshank & Thomas, 1999), there is no evidence for this in scale insects, where haplodiploidy and PGE have both independently evolved from the ancestral XX-XO system (Nur, 1980). The recent finding of female sex ratio control in several species with a PGE system (Nagelkerke & Sabelis, 1998; Ross *et al.*, 2010a; Varndell & Godfray, 1996) does suggest however that Bull's (1983) hypothesis that selection for female control over sex allocation on the evolution of both PGE and haplodiploidy is plausible, even if the two systems evolved independently.

Aspects of scale insect biology may thus have influenced the evolution of their genetic systems, at least in terms of making certain transitions more attainable. However, such explanations are by their nature somewhat *post hoc*, and difficult to test in terms of predictions independent of the phenomena they set out to explain. Aspects of scale insect biology such as their inverse meiosis may therefore help us with some of the more proximate or mechanistic explanations for the evolution of the genetic systems, but we might have to look elsewhere for more ultimate, functional explanations.

INTRA-GENOMIC CONFLICTS OVER TRANSMISSION

Several hypotheses for the evolution of the variability of genetic systems in scale insects have been put forward that have gone beyond trying to pin their genetics down to particular aspects of their biology. As such, these theories may also be more generally applicable to species outside the Coccoidea. These hypotheses are based on the idea of evolutionary conflicts of interest between different genetic entities, such as males and females or hosts and endosymbionts (see Section VI). These conflicts of interest are fundamentally associated with how different genetic entities transmit copies of their genes into the next generation. Put another way, genes experience dif-

ferent patterns of selection depending on the context in which they find themselves (males or females, parents or offspring). As we will see, in many cases conflict over genetic transmission will lead to biases in the sex ratio (including the complete disappearance of males). In other cases, the sex ratio itself is a direct target of conflict. Some of these hypotheses concern the evolution of just one genetic system, while others try to explain the broad diversity of systems itself. In this section, we consider conflict arising within the scale insect genome over transmission, such that changes in the genetic system favour one genetic entity or another. For intra-genomic conflicts, these entities are either males and females, parents and offspring, or sex chromosomes and autosomes.

The first suggestion for an influence of genetic conflict on the evolution of haplodiploidy came from Brown (1963; 1964), who made some of the most important advances in understanding both the mechanisms and diversity of genetic systems in scale insects. He recognized that maternally inherited genes that cause the exclusive transmission of maternal chromosomes during spermatogenesis can have a selective advantage and therefore increase in frequency in the population (Brown, 1964). This idea was further advanced by Bull (1979), who rephrased Brown's (1964) ideas in terms of conflict between maternally and paternally inherited genes. Brown (1964) also proposed a model for the evolution of the different types of PGE, however at that time the Comstockiella system was misinterpreted and we therefore do not include his model in this review. Haig (1993a) more recently developed a model showing that X-chromosomal drive in combination with the evolution of maternal autosome X-chromosome linkage could lead to the evolution of PGE (Fig. 2.4A). This model states that if the evolution of X-chromosomal drive occurred in ancestral scale insects with an XX-XO system, a possible adaptive response of the maternal autosomal genes could have been to evolve a mechanism that would ensure the linkage between the driving X and the maternal autosomes. However this scenario would lead to a strongly female-biased sex ratio and therefore strong selection for an alternative (non-X linked) sex-determining mechanism. The evolution of these two factors, combined with the deactivation of the paternal genome in males, could have led to the evolution of a system similar to lecanoid PGE (see also Fig. 2.4A).

The previous models all focus on how PGE could have evolved from the ancestral XX-XO chromosome system (transition 1). However, the evolution of PGE across scale insects has involved repeated evolutionary events producing three different forms of PGE (see Fig. 2.1), that each developed a different mechanism for eliminating the paternal genomes (see Fig. 2.2). To address this, Herrick & Seger (1999) proposed that once PGE has evolved there would be strong selection on the paternally inherited genes to evolve a mechanism to resist being eliminated from the sperm. They argued that males could do this by either reversing the heterochromatinization of their chromosomes and having their chromosomes join the maternal euchromatised set, or by resisting the disintegration of their paternal chromosomes and forming paternally derived sperm. They also stressed that even a mutation that would allow a small number of paternal genes to escape elimination would rapidly spread though

the population and lead to a strong selection pressure for maternally inherited genes to suppress the "leak". They therefore suggested that this evolutionary arms race might have driven the evolution of earlier deactivation or elimination of the paternal genome in males and generated the variety of coccoid PGE systems (i.e. answering why there are several types of PGE and not just one).

Several other authors have proposed hypotheses for the variation in retention and loss of the paternal genome in species across the three different forms of PGE (Nur, 1980). Based on phylogenetic evidence, it has generally been assumed that the lecanoid system is the ancestral PGE system, with the Comstockiella and the Diaspidid systems being more derived (Fig. 2.1). Given this scenario, the earlier loss of the paternal genome has been explained by recourse to some (presumably metabolic) cost of retaining the inactive paternal genome (Nur, 1980). This hypothesis might be able to explain the evolution of the Diaspidid system where the paternal genome is lost during early development and therefore any cost would be largely avoided. It is however hard to see how this could explain the evolution of the Comstockiella system, by far the most common genetic system in scale insects and an evolutionary transition that has taken place several times (Fig. 2.1). In the Comstockiella system, some paternal chromosomes are eliminated just before spermatogenesis. It is therefore hard to see how this could reduce the cost of the retention of the paternal genome to any great extent because the paternal genes are present in all cells in the soma and are only eliminated from the germline at a very late stage.

Herrick & Seger (1999) therefore proposed their hypothesis for the loss (or elimination) of paternal genes in the Comstockiella system, based on the observation that in some taxa with the lecanoid system of PGE paternal genes are reactivated during spermatogenesis. They proposed that the timing of loss of paternal genes just before spermatogenesis is the result of antagonistic co-evolution between paternal and maternal genes over the extent of paternal gene expression during spermatogenesis, with maternal genes trying to avoid genetic conflict between the maternal and paternal genome over the elimination of the paternal genome during spermatogenesis. The transition between the lecanoid and Comstockiella systems seems to have evolved several times in both directions (Nur, 1980), which is suggestive (but not conclusive) evidence for co-evolutionary dynamics among paternal and maternal genes for control of spermatogenesis.

Additionally, Brown (1967) pointed out that the instability of the Eriococcidae (felt scales) chromosome systems, which have oscillated back and forth between Comstockiella and lecanoid systems for 80 million years, seems hard to reconcile with the great antiquity of the family. Again, co-evolution between the paternal and maternal genes over the early elimination or reactivation of the paternal genome might help explain these patterns. If the earlier elimination of paternal chromosomes can be explained by genetic conflict over the elimination of the paternal genome during spermatogenesis, we would expect that in more ancestral systems paternal chromosomes might occasionally manage to make it into the sperm. This could be very hard to observe though, both because it might be relatively rare and because of

a lack of helpful molecular tools for these species. However, Nur (1970) observed that if the paternal chromosomes in *Planococcus citri* manage to become euchromatinized during spermatogenesis, then they can escape destruction. He also observed in several mealybug species that occasionally sperm are produced that have one or more extra (presumably paternal) chromosomes (Herrick & Seger, 1999). Clearly there is scope therefore for paternal genome leakage, although the extent to which this leakage is "accidental" or the result of variation in paternal gene expression (and selectable) is unknown.

Although direct proof for the struggle for the elimination of the paternal genome between the sexes is lacking, data from several taxa are very suggestive and might indicate a co-evolutionary struggle for control over transmission between the maternal and paternal genome. Although they are deactivated in most tissues, Nur (1967) showed that in the mealybug *P. citri*, which has the lecanoid form of PGE, the male genome is reactivated in several tissues. Perhaps tellingly, one of these tissues is the cyst where spermatogenesis takes place and where the paternal genome gets eliminated from the gametes! This supports an earlier finding that males with a paternal genome set that had been damaged by radiation could survive, but were sterile (Nelson-Rees, 1962), suggesting a crucial role for the paternal genome in spermatogenesis. The exact function of the sperm cyst, which is a structure present in many insect taxa, remains unknown but it has been suggested to be involved in the imprinting of the genes in the gametes (Buglia & Ferraro, 2004b). These data are intriguing as there should be no selective advantage for the paternal genome set to assist spermatogenesis, except if in doing so it might be able to prevent its own elimination from the sperm.

The involvement of the paternal genome in spermatogenesis also seems to differ between closely related species. Although heterochromatinization in the sperm cyst cells is reversed in all mealybug species so far studied, the cells of the testis sheath (that contains the sperm bundles) lack a heterochromatinized set in *Pseudococcus obscurus* and *Phenacoccus gossypii*, but do contain a heterochromatinized set in *Planococcus citri*. Furthermore the testis sheath cells in *P. citri* are also characterized by endoreduplication of the euchromatinized (maternal) chromosome set, leading to cells that have multiple copies of the maternal set but only a single deactivated copy of the paternal chromosomes (Nur, 1966c). We can again speculate that this pattern might be caused by antagonistic co-evolution between the sexes, with the paternal genome trying to become activated to preserve its transmission and the maternal genome evolving suppression, possibly by using endoreduplication of maternal chromosomes in order to increase the expression of maternal genes.

More tangible evidence for direct suppression of paternal genes in males by the maternal chromosomes comes from the observation that in experimentally produced haploid male embryos or embryo's in which certain regions are made haploid the paternal chromosomes at first undergo normal heterochromatinization, but become euchromatinized later in development, suggesting that the presence of the maternal genome set is needed to "suppress" paternal chromosomes (Brown & Nur, 1964; Nur,

1962b). Most likely the deletion of the maternal genome set does not have an effect on the initial heterochromatinization because early in development there is not any gene expression in the embryo (Sabour, 1972) and development is regulated by maternally derived gene products, possibly the recently characterized histone protein HP1 (Bongiorni *et al.*, 2007). The suppression of the paternal genome set might also be closely linked (mechanistically) to the deletion of the paternal chromosomes during spermatogenesis. As alluded to above, Nur (1970) observed that in some mealybug species treated with high doses of radiation, occasionally the paternal genome set became decondensed in the spermatocytes and that these spermatocytes could give rise to diploid sperm containing the paternal chromosomes. This shows that euchromatinized paternal chromosomes do not disintegrate (i.e. they are viable). These findings indicate a possible mechanism for the elimination of the paternal chromosomes in which the euchromatic maternal chromosomes produce a substance that is harmful for chromosomes in a heterochromatinized state, but leaves euchromatic chromosomes unharmed. If paternally inherited chromosomes manage to decondense therefore, they are then left viable and able to enter the germ-line.

The previously described hypotheses mainly focus on the evolution of PGE. However Normark (2006) described a new theory to explain not just the evolution of PGE but also the whole variety of genetic systems in scale insects (and other taxa). He argued that if (1) there is prolonged association of kin groups (e.g. gregarious broods, maternal care and so on) and (2) a relatedness asymmetry within the kin group, with the offspring being more related through their maternal than their paternal genes (e.g. sharing the same mother but different fathers), then paternal genes are selected that make offspring more selfish (mediated, for instance, through patterns of genomic imprinting). Normark (2006) argued that the evolution of what he called asymmetric systems, in which only one parent transmits their genes, will suppress this possibly harmful conflict among offspring. This theory could account both for the evolution of several types of parthenogenesis and for all the systems in which only the female transmits her genes to the next generation and the offspring are clonal (promoting cooperation within the clutch). The evolution of both haplodiploidy and PGE will reduce the amount of conflict because males either lack, or have deactivated, paternal genes and therefore males are selected to be less selfish than in the ancestral XX-XO system. However, as we will discuss below, the evolution of these genetic systems by such a process may simply replace one set of genetic conflicts with another.

GENETIC CONFLICT, SEX DETERMINATION AND SEX ALLOCATION

Theoretical considerations

In the previous section we considered how maternally and paternally inherited genes may come into evolutionary conflict over transmission (i.e. getting into gametes), and that this conflict may select for the evolution of alternative genetic systems. A

possible side-effect of such conflicts could be the biasing of the population sex ratio. Here we turn our attention more directly to conflicts over sex ratio, and consider situations in which different genetic entities (males and females, parents and offspring) may be selected to favour different patterns of offspring sex allocation (including the numerical sex ratio). The rationale here is that if different entities come into conflict over sex allocation, then the sexes may each be selected to try to control sex allocation by taking over or influencing sex determination (Shuker, Moynihan & Ross, 2009). Conflict over sex determination could then lead to the evolution of alternative genetic mechanisms. We will begin by developing this rationale further, before reviewing what is known about sex determination in scale insects. However, central to considering the role of intra-genomic conflict over the sex ratio is determining what sex ratios are favoured by males and females (and their offspring), and whether or not sex allocation is fixed or facultative. We will therefore conclude with a review of sex allocation in scale insects, highlighting the potential scenarios where conflict may arise.

The scope for conflicts of interest over sex ratio has been long recognised [for instance by Burt & Trivers (2006) and Hamilton (1967)]. The conflicts may be rather direct, as is the case if there are selfish genetic elements actively influencing the sex of offspring, such as driving X- or Y-chromosomes, supernumerary B chromosomes, or parasitic endosymbiotic organisms such as male-killing bacteria (2006; Werren & Beukeboom, 1998). The conflicts initiated by such selfish elements may be intra-genomic (as with driving sex chromosomes) or inter-genomic (as with male-killers). Alternatively, the context for conflict over sex allocation may arise more indirectly. For instance, there can be conflict over the sex ratio between parents if one of the parents shares fewer copies of its genes with the offspring of one sex, as in asymmetric systems such as haplodiploid, where haploid male offspring develop from unfertilized eggs, while females develop from fertilized eggs. In this situation, fathers favour a more female-biased sex ratio than the mother because they only share genes with their daughters, while mothers share genes with all their offspring (Hawkes, 1992; Trivers & Hare, 1976).

There can also be conflict between parents and offspring over the sex ratio (Trivers, 1974). For instance, in cases where parents produce adaptively skewed sex ratios because of processes such as local resource competition or local mate competition (Clark, 1978; Hamilton, 1967), large asymmetries in reproductive value can be created between the two offspring sexes, with the rare sex having a much higher reproductive value. This means that if the offspring could influence which sex they developed into, they would prefer to be the rarer sex. This has the consequence that under certain conditions the offspring prefer a less biased sex ratio than the parents (Trivers, 1974; Werren & Hatcher, 2000), but see (Pen, 2006; Trivers & Hare, 1976). Finally, other apparently mutualistic associations may still form the context for conflicts over sex ratio, for instance if the mutualistic benefits are provided more by one sex than another. This could indeed be a possibility in species of aphids or scale insects tended by ants, in which the females of the plant-feeder provide the honeydew resource the ants receive in payment for protection from arthropod predators. In

this situation, the ants prefer a female bias in aphid or scale insect sex ratios, as they get greater benefits from larger symbiont herds.

Although the general theoretical framework for such genomic conflicts over sex ratio is reasonably well developed, there is a lack of direct evidence for their importance. Several models have shown that there should often be conflicts of interest over the sex ratio of the offspring, but outside of the social insects, there are currently few empirical tests of the assumptions of the models (thus confirming the conditions necessary for the conflict to be initiated) or specific tests of the models' outcomes (Shuker *et al.*, 2009). There might be several reasons for this lack of experimental confirmation. First, conflicts may be obscured, such that researchers have not really been encouraged to look for them. For example, it is possible that there are ongoing battles over the sex ratio but, because of coevolutionary processes in the past, the current conflict is hard to identify (Chapman, Pomiankowski & Fowler, 2005; Kozielska *et al.*, 2009). In addition, although there might be a conflict of interest over sex ratio between different parties (for example between the father and mother), not all parties may have the power to influence the sex ratio, as was assumed to be the case for haplodiploid Hymenoptera (Trivers & Hare, 1976; Werren & Beukeboom, 1998), but see (Shuker *et al.*, 2006). Conflict might also have happened in the past but have been resolved by the evolution of a new genetic or sex-determination system. For all of these reasons it may be difficult to observe past or present conflicts over sex allocation.

Sex determination in scale insects

What evidence is there that genomic conflict over sex allocation has led to the evolution of sex determination mechanisms, and that genetic systems evolved along with sex determination? First, let us consider patterns of sex determination in scale insects. Basal scale insects have a XX-XO genetic system (Fig. 2.1B), which suggests that they have genetic sex determination. This is supported by the observation that in species of the genus *Puto* (a genus that was placed with the mealybugs previously but is now considered to be part of a separate family, the Putoidae) during spermatogenesis sperm both with and without an X chromosome are formed in equal numbers (Brown & Cleveland, 1968). However, although the XX-XO system found in more basal scale insects suggests that genetic sex determination is the ancestral sex-determination system, data on the closely related aphids suggests differently. In aphids that seem to have an XX-XO genetic system, it has been discovered that all zygotes are initially XX females, but that some zygotes develop into males after one of the X chromosomes is randomly lost (Wilson, Sunnucks & Hales, 1997). Males only form viable sperm that carries the X chromosome and therefore autosomes that are not associated with the X chromosome are not transmitted to the next generation (Wilson *et al.*, 1997). This system shows some interesting similarities to the paternal genome loss system found in many more derived scale insects (Section II.3). However, although the cytology of many basal scale insects has been studied extensively (as reviewed by Hughes-Schrader, 1948) a system similar to that described in aphids has

never been found. It is therefore more likely that ancestral scale insects indeed had genetic sex determination and that the aphid system evolved independently in that lineage.

In other diploid scale insects there is the intriguing suggestion that endosymbiotic bacteria are associated directly with sex determination. For example, *Stictococcus* species lack both heterochromatinization and sex chromosomes and both sexes are diploid. However, the endosymbionts, although present in females, are absent in males. Buchner (1965) therefore suggested that in *Stictococcus* spp. sex is determined by the bacteria, with eggs containing bacteria developing into females, and eggs without bacteria developing into males. The diplodiploid system found in *Stictococcus* spp. has clearly evolved from a PGE lineage (Fig. 2.1). It might be significant in this instance that the loss of the endosymbionts in males coincides with the loss of heterochromatinization in males. However, definitive evidence for a role of the endosymbiotic bacteria as the sex-determining agent is still lacking.

In systems with paternal genome elimination the sex-determination mechanism is still poorly understood. No sex chromosomes have ever been observed in scale insects with PGE and the presence of autosomal genetic sex determination loci is also unlikely (Brown & Nur, 1964). Therefore it has been assumed that sex is determined either by facultative imprinting (offspring sex depends on the way the gametes are imprinted by the parents) or by maternal effect proteins that are added to the eggs. The latter hypothesis is supported by the observation that in several taxa with PGE the eggs containing male or female embryos differ in colour and that this colour difference is already present before fertilization (Nur, 1989). However, since maternal proteins may presumably influence methylation/other imprints as well, the two mechanisms could be hard to separate.

Despite our uncertainty over the mechanisms of sex determination themselves in PGE species, the molecular mechanism of how paternal chromosomes are eliminated has recently been unravelled, perhaps offering clues about the sex determination process. Central to the process is the difference in genomic imprinting between the paternally and maternally inherited chromosomes. The paternal genome is hypomethylated in comparison to the maternal genome and this difference is present in both sexes of the species (Bongiorni, Cintio & Prantera, 1999). Recently, a histone protein has been identified that is responsible for the heterochromatinization of the paternal genome in males. This protein, a HP1 homolog, is preferentially attached to the paternal genome and seems to recruit other histone proteins to the complex. Using RNA interference (RNAi), Bongiorni *et al.* (2007) knocked out expression of the HP1 homolog and this resulted in a reversal of heterochromatinization in males and also a lack of recruitment of two other histone proteins. One remaining question is whether this protein is of maternal origin or is expressed by the embryo itself. Bongiorni *et al.* (2007) showed that cleavage-stage embryos were particularly sensitive to the RNAi treatment while treatment of more advanced embryos had little effect. Sabour (1972) showed that, in mealybugs, gene expression only occurs after the 5th division; by that time the paternal genome is already condensed in males.

These findings suggest that the HP1 protein enabling heterochromatinization in males is indeed of maternal origin, suggesting both maternal influence over sex determination and providing a candidate for the maternal effect protein responsible for the control.

In a different vein however, Buglia & Ferraro (2004b) recently suggested a paternal sex-determination mechanism in the mealybug *Planococcus citri*. They observed that during spermatogenesis some sperm cells carry a higher concentration of two histone proteins, HP1 and H3 analogs, discussed above as being involved in the heterochromatinization process in *P. citri* (Bongiorni *et al.*, 2007). They argue that the percentage of sperm "tagged" with these proteins (approximately 50%) is similar to the offspring sex ratio observed in *P. citri*. They also hypothesised that the unusual mechanism of spermatogenesis found in coccoids, where sperm are formed in a sperm cyst, enables males to imprint their sperm in such a way as to influence the sex of the developing embryo. Although their findings are interesting they do not prove a direct relationship between the identified protein and sex determination, and it is also difficult to see how the paternal sex determination that they propose could have evolved as males are not expected to favour male production (see below).

Another suggestion for the sex-determination mechanism in PGE lineages comes from Buchner (1965) who, after observing the apparent involvement of endosymbionts in sex determination in *Stictococcus* spp. (see above), argued that endosymbionts might also be involved in PGE species. He envisaged bacterial titre being the key determinant, with eggs containing many bacteria developing into females, whilst eggs with low bacterial counts develop into males. This mechanism is supported by the observation that in the mealybug *P. citri* both high rearing temperatures and aging of females result in a reduction in endosymbionts and a more male-biased sex ratio (Brown & Bennett, 1957; Buchner, 1965; Kono *et al.*, 2008; Nelson-Rees, 1960). However, these data are circumstantial and do not exclude other correlated effects, either with respect to the bacteria or the scale insects themselves.

Sex allocation patterns in scale insects

The extent to which genes will experience conflicting selection pressures relating to the genetic context they find themselves in (mothers or fathers, parents or offspring etc.) may depend on the sex ratios produced. For instance, if sex ratio selection has favoured female-biased sex ratios (to reduce local mate competition for example), then there may be rather little conflict over sex ratio between mothers and fathers in haplodiploid or PGE species (Shuker *et al.*, 2006). If sex ratio selection favours a male bias, there may be considerable conflict over sex ratio, as genes in fathers would suffer extremely reduced transmission compared to genes in mothers. The same will be true for parent-offspring conflicts over sex ratio, where sex ratio selection helps define the difference in sex ratio optima (Pen, 2006; Uller *et al.*, 2007). Here we consider the known variation in sex allocation in scale insects in order to identify the possible scope for sex ratio conflict. Unfortunately, although sex allocation data are available for several scale insect species, well-controlled experiments are limited and

therefore reliable data are only available for a small number of species. We will give a short review of the sex ratio data available for coccoids, focussing especially on the few well-studied species and on those that seem to show a strong sex ratio bias.

Sex allocation in mealybugs has been studied most extensively in the mealybug *Planococcus citri* (Fig. 2.3E). Most studies show equal or slightly female-biased sex ratios (Nelson-Rees, 1960; Ross *et al.*, 2010a; Varndell & Godfray, 1996). Several factors have been identified that affect the sex allocation of *P. citri* (as reviewed by Ross *et al.*, 2010a). First of all, population density seems to affect the sex ratio, although the effects are complex (Ross *et al.*, 2010a; Varndell & Godfray, 1996). Second, temperature strongly affects sex ratio (James, 1937; Nelson-Rees, 1960) and finally the age of the female both at the time of mating and egg laying, affects sex allocation (Nelson-Rees, 1960; Ross *et al.*, 2010a).

Sex allocation of armoured scales (Diaspididae) has been reviewed by Nur (1989). In general, most taxa produce 50:50 sex ratios. Interestingly though, in some species significantly male-biased sex ratios are observed. One of the best-studied armoured scale insects is *Pseudaulacaspis pentagona*; in this species there is a sexual dimorphism in the eggs, with coral red eggs containing female embryos and white eggs containing male embryos. Brown & Bennett (1957) showed that the age of a female strongly affected the sex ratio of her offspring, with a female producing only female offspring in the first few days of oviposition before switching to producing only males. They also showed that females that were prevented from mating for 20 days produced a more male-biased sex ratio once allowed to mate (Brown & Bennett, 1957). A similar effect has also been observed in two other Diaspidid species (Nur, 1989).

The best data on sex allocation patterns in the field comes from the work of Alstad and Edmunds (1983; 1989) on the black pineleaf scale (*Dynaspidiotus californicus*, as *Nuculaspis californica*, Fig. 2.3C). They studied sex ratio patterns in the field over several years across several different locations. They initially observed an extremely female-biased sex ratio in the adult population (less than 10% males: Alstad & Edmunds, 1983). Further studies however established that the sex ratio at the crawler stage was only slightly female biased (40 % males); the primary sex ratio remains unknown (Alstad & Edmunds, 1989), suggesting male-biased mortality.

Several authors have attempted to understand the sex allocation patterns in scale insects based on sex allocation theory, for instance the sex ratios predicted by local resource competition (LRC) theory (Hamilton, 1967; Ross *et al.*, 2010a; Varndell & Godfray, 1996). The rationale here is that since female scale insects have a sedentary life style and form large colonies, related females (e.g. sisters) are likely to compete locally for resources, while males are able to disperse away from competition. LRC predicts male-biased sex ratios in order to reduce this local competition between related offspring. Recently it was shown that density affects sex allocation in *P. citri*, with females producing a more male-biased sex ratio under high densities, although density in that experiment reduced the extent of competitive interactions between kin relative to interactions among non-kin (Ross *et al.*, 2010a). These results therefore did not support LRC theory but instead suggest that competition between

unrelated individuals might affect sex allocation in this species (by reducing the reproductive value of daughters when "global" resource competition is high).

Many scale insects have a genetic system with asymmetric transmission and there is therefore a wide scope for conflicts over sex allocation in many taxa. Both in species with haplodiploidy and PGE, males "prefer" a more female-biased offspring sex ratio than their partner (as outlined above). The important question remains however as to whether or not males have the power to influence sex determination. Although female control of offspring sex ratio has been observed in several species, there is the suggestion of male involvement as well. It will be very important to test this formally both by using within-generation experimental crosses and across-generation quantitative genetics experiments to estimate the amount of variation in sex ratio that can be attributed to male mating partner/genotype and by directly manipulating the mechanism with which males are suggested to influence the sex ratio, for example by using RNAi techniques to block HP1, a technique that has already been successfully applied when studying heterochromatinization in the embryos (Section V.2).

INTER-GENOMIC CONFLICT: HOST-SYMBIONT CONFLICTS

In this section we will focus on genetic conflicts between scale insect hosts and their endosymbionts. We will both explore empirical data that suggest the possible involvement of the endosymbionts in the evolution of the different genetic systems and focus on conflict over sex determination and sex allocation between host and endosymbionts. We will begin by introducing the biology of the scale insect endosymbioses.

Endosymbiosis

Most scale insects have an obligate symbiotic relationship with one or more species of bacteria. Scale insects rely on their endosymbionts to synthesize and provide the essential amino acids and vitamins that are absent from their diet (Buchner, 1965; Fink, 1952). The endosymbiosis in scale insects and other plant-feeding insects has been well studied, especially by Buchner (1965), who summarised his findings in an extensive monograph. The relationships between scale insects and their symbionts are often ancient and many bacteria show strong patterns of co-speciation with their host (Baumann & Baumann, 2005; Gruwell, Morse & Normark, 2007). The endosymbiotic bacteria found in scale insects belong either to the Flavobacteria (Eriococcidae, Margarodidae, and Diaspididae) or to the Proteo-bacteria (Pseudococcidae, Putoidae, and possibly some Coccidae) (Gruwell *et al.*, 2004; von Dohlen *et al.*, 2001). However, the endosymbionts found in many soft scales (Coccidae) and in isolated members of other families are eukaryotes (fungi) (Buchner, 1965; Tremblay, 1989).

Endosymbiotic bacteria are typically confined to specialized organs (bacteriomes) that can make up to 30% of the body mass of the insect, although the structure and formation of these organs varies widely among different taxa (Tremblay, 1989). In

mealybugs and armoured scale insects the bacteriome is formed by the fusion of the maternal polar bodies with embryonic cells (Normark, 2004b; Nur, 1990; Tremblay & Caltagirone, 1973). This results in a polyploid organ, which contains both the embryo's genome, as well as the three (maternally derived) polar body genomes. The bacteria themselves are contained within the cytoplasm of the cells of the bacteriome (termed the bacteriocytes). In species of the family Putoidea the endosymbionts are actually transmitted within maternally derived bacteriocytes (Buchner, 1965). These bacteriocyte cells enter the oocytes, and during embryogenesis fuse with embryonic cells to form the bacteriocytes in the new embryo, transmitting both the endosymbionts as well as maternal genetic material. This results in a bacteriome of partly maternal origin and it also prevents the endosymbionts from ever coming into contact with offspring tissues. Soft scales, on the other hand, often lack a bacteriome and their endosymbionts float freely in the host haemolymph, and occasionally in modified polyploid fat cells. Curiously, as noted above, the endosymbionts in many soft scales are not bacteria but instead unicellular fungi (Buchner, 1965).

The various endosymbionts need to transmit themselves from one scale insect generation to another. As is typical for endosymbionts more generally (Buchner, 1965), they are vertically transmitted through the hosts' maternal line, via the cytoplasm of the eggs. As such, many groups have very specialized mechanisms to ensure as many eggs are infected as possible (Buchner, 1965; Tremblay, 1989). For example, in *Planococcus citri* individual bacteriocytes break loose from the bacteriome and fuse with the ovaries releasing their bacteria, which then travel towards the developing oocyte and penetrate it. Initially the bacteria stay at the anterior pole of the egg. After fertilization the new bacteriocytes start to form in the embryo by the fusion of the polar bodies and embryonic cells, before also migrating in the direction of the endosymbionts. When the two meet the bacteriocytes absorb the endosymbionts, where they will effectively remain in culture before infecting the next generation of eggs (if in a female) or dying with or before the host (if in a male) (Schrader, 1922). Although the specialised bacteriocyte cells might allow the host to control the bacteria to some extent, during oogenesis there is a short period where the endosymbionts are not inside the cells and have free access to the hosts haemolymph and thus may be in a position to manipulate host physiology. This might be important in terms of whether or not endosymbionts have the opportunity (i.e. the power) to influence reproductive processes such as sex determination.

Although most Sternorrhyncha harbour their bacteria in bacteriocytes, the mechanism of the formation of the bacteriome seems to have evolved independently several times. For example, different cell types, sometime maternal, sometimes embryonic, or sometimes both, give rise to the bacteriome. However, there seems to be one common characteristic of many bacteriomes, especially those in taxa with PGE (Normark, 2001), and that is that they often consist of polyploid cells (Buchner, 1965; Tremblay & Caltagirone, 1973). In order to understand better the function and the evolution of bacteriomes, it might be crucial to understand the function of this polyploidy.

In addition to the strong, obligate relationships with their primary endosymbionts, which are generally phylogenetically conserved within families (Gruwell *et al.*, 2007; Thao, Gullan & Baumann, 2002), some scale insects also have a whole range of secondary symbionts (Buchner, 1965). In many cases the secondary symbionts are less strongly associated with a particular host, with closely related scale insects sometimes harbouring very different secondary endosymbionts (Thao *et al.*, 2002). Compared to the primary endosymbionts, the function of the secondary endosymbionts is much less well understood. It might be that they take over or complement some of the tasks of the primary symbiont, or provide their host with other advantages such as facilitating host adaptation or disease resistance, as found in various species of aphid (Scarborough, Ferrari & Godfray, 2005; Tsuchida, 2004). However, it might also be possible that they are purely reproductive parasites that make use of the transmission apparatus associated with the primary endosymbiont without providing any of the benefits. In many species, the primary and secondary endosymbionts live in close proximity, often within the same host cell, and in mealybugs the secondary endosymbionts actually live inside the primary endosymbionts (von Dohlen *et al.*, 2001). The fact that in mealybugs the two bacteria have never been observed independently suggests a strong mutualism between the two, although this has not been formally established. Interestingly, although most scale insects have more than one endosymbiont, in soft scales no additional bacterial endosymbionts have been observed co-infecting alongside their yeast-like primary endosymbionts (but see Gruwell *et al.*, 2004).

Conflicts over sex allocation

Host-endosymbiont conflict over sex allocation has been extensively studied in many taxa and is described in several reviews (Hurst, 1991; Werren, Nur & Wu, 1988). Until now however it has mainly focussed on a few well-known reproductive parasites mainly of the genera *Wolbachia* and *Cardinium* (Weeks, Tracy Reynolds & Hoffmann, 2002; Weeks *et al.*, 2003; Werren, 1997; Werren *et al.*, 2008). Very little is known however about conflict over sex allocation between hosts and their obligate mutualistic bacteria. Like many of the reproductive parasites, obligate mutualistic bacteria such as those found in most scale insects are strictly vertically transferred through the female line (Buchner, 1965). Therefore males do not transmit bacteria whilst females do. This results in the potential for conflict between the host and the bacteria over the sex ratio, with bacteria favouring a more female-biased sex ratio than the host, even to the point of the total eradication of males. Interestingly, such a conflict may mean that the interests of the endosymbionts are often aligned with the interests of genes in male scale insects under genetic systems such as PGE, which also favour female-biased offspring sex ratios as paternal genes are also only transmitted through female offspring.

The extent to which these conflicts should lead to or result in the evolution of alternative sex determination and genetic systems has been addressed to some extent. Reproductive parasites have evolved a whole array of mechanisms to affect their host's

reproduction (Charlat, Hurst & Merçot, 2003; Werren, 1997), including feminization of genetic males (Rigaud, 1997), male-killing (Hurst, 1991) and parthenogenesis induction (Stouthamer *et al.*, 1990). In doing so, these parasites have often subverted the existing mechanisms of sex determination and made aspects of the genetic system redundant (for instance *via* the elimination of males). More specifically in terms of scale insects, Normark (2004a) has combined many aspects of their biology (and indeed other taxa with PGE or haplodiploidy) to try to explain the evolution of PGE and haplodiploidy. He pointed out that a determining feature of species in which these genetic systems have evolved is that they typically: (1) all have endosymbiotic bacteria; (2) all have gregarious broods leading to high levels of competition between siblings. In his model, Normark (2004a) showed that under these conditions the endosymbiotic bacteria are selected to evolve male-killing and he proposed that they could accomplish this by the deactivation of male-determining sperm, haploidizing the male embryo and thereby killing it. This would initially be detrimental to the host and there would therefore be strong selection for the evolution of haploid embryo viability. Once this had evolved, females that produced these haploid males would have a selective advantage, as their sons would transmit their genes at a higher rate [the premise of Brown (1964) and Bull (1979), as discussed above] (see Fig. 2.4B). Normark's (2004a) original model has since been tested and adapted by several authors (Engelstadter & Hurst, 2006; Kuijper & Pen, 2010; Ubeda & Normark, 2006). In the latter case, Kuijper & Pen (2010) have recently shown that, although the stable evolution of PGE and haplodiploidy in the original model was rare, it can evolve more easily with a subdivided, highly inbred population and when the endosymbionts are mutualistic.

In order to consider the possible role of endosymbionts in the evolution of genetic systems in scale insects, we will start by discussing the possible presence of these bacterial-induced phenotypes in scale insects and then discuss which bacteria might be responsible.

Male-killing

Endosymbiotic bacteria are selected to have a male-killing phenotype when killing males will increase the fitness of related females, as this will benefit the fitness of the bacteria's relatives in those females (a kin selection benefit: Hurst, 1991). This will occur in situations where broods are gregarious and male and female offspring develop together and compete for resources; this situation is present in many scale insects where nymphs compete for space and resources both within the maternal structure they are raised in (e.g. ovisac, marsipium) and possibly also on their host plant (Normark, 2004a). However male-killing has not been observed in scale insects (but see below), although this might be caused by the fact that a shortage of males will often be hard to observe due to their small size and short lifespan. In most taxa where male-killing is observed the male-killing is active and usually occurs during early development (Hurst, 1991). In scale insects there could also be "passive" (or "incidental") male killing, where the bacteria simply do not function well in the male and thereby cause increased mortality indirectly. Interestingly, Gruwell *et al.* (2004)

found in their recent phylogeny of scale insect endosymbionts that the endosymbionts of several scale insect families are closely related to bacteria that are known to have a male-killing phenotype in ladybird beetles and cause parthenogenesis in other taxa. As such, the lack of male-killing in scale insects is perhaps unlikely to be due to phylogenetic constraint on the part of the endosymbionts.

Whilst there is no direct evidence for active early male-killing in scale insects, there are some suggestive data in the literature. In the black pineleaf scale (*Dynaspidiotus californicus*) extremely female-biased sex ratios were observed in the adult population (less than 10% males) (Alstad & Edmunds, 1983). As discussed above (Section V.3), it was later established that the sex ratio at the crawler stage was only slightly female biased (40% males), with the primary sex ratio remaining unknown (Alstad & Edmunds, 1989). These data therefore suggest massive male-biased mortality. Interestingly, the observed male mortality occurs relatively late in development compared to the male mortality observed in many species infected with male-killing bacteria, which would support the idea of more passive male-killing where the bacteria just do not work as effectively in males as they do in females. Unfortunately, nobody has tested directly the hypothesis that the extreme male mortality is caused by a bacterium with a male-killing phenotype. Another interesting observation is the behavioural difference between male and female nymphs of many armoured scale insects, where male crawlers feed on sites of the host plants that are both more nutritious, but also more exposed and dangerous than the sites chosen by their sisters (Normark, 2004a), thereby reducing competition with their sisters. This would be in the interest of the bacteria and it could be that males are forced to feed on these places as their bacteria do not provide them with enough nutrients. Unfortunately no experiments have yet been conducted to test this hypothesis.

Male-killing can have significant effects on host populations, influencing the evolution of mating systems and even leading to extinction (Dyson & Hurst, 2004). As such, co-evolutionary responses by the hosts are predicted. For instance, the rapid evolution of a zygotic male-killing suppressor has recently been observed in a species of butterfly (Hornett *et al.*, 2006). However, we suggest that scale insects might have evolved different ways to suppress male-killers. First of all, scale insect males might have evolved to compete as little as possible with their sisters, and as a consequence reduce the evolutionary benefit for the bacteria to express a male-killing phenotype. This might explain why males often stop feeding early in development and also why in some species males feed on different areas of the plants (see above). Second, male-killing can be avoided if the sex of the offspring is hidden from the bacteria. Normark (2004b) suggested that the peculiar formation of the bacteriome in armoured scales (the fusion of polar bodies with embryonic cells and some activity of the paternal genome) assures that the bacteria in both sexes are contained in similar tissues, this potentially being a mechanism to hide the sex of their host from the bacteria. A third mechanism that could stop male-killing is to avoid the transmission of the endosymbionts to males in the first place. This is exactly what has happened in the genus *Stictococcus*, where the absence of the endosymbionts in males is compensated for by

their mothers by the evolution of a placenta-like structure in order to feed their sons (Buchner, 1965). Decreasing the dependency of males on the endosymbionts might also help explain the evolution of the male life history of many scale insects, where males stop feeding and start losing their bacterial load at the same time as they start gonadal and somatic differentiation from females (Kono *et al.*, 2008) and therefore when they might be unable to "hide" their sex any longer.

Parthenogenesis induction

Parthenogenetic reproduction is common among scale insects and has evolved many times among the different families and there are several different parthenogenetic systems (Nur, 1971). Although the evolution of parthenogenesis in scale insects is poorly understood, there is evidence for the involvement of endosymbiotic bacteria in several taxa.

Nur (1972) found that in a species of soft scale (*Parthenolecanium cerasifex*) some females produced offspring by diploid arrhenotoky: females are produced sexually, but males develop from unfertilized eggs in which diploidy is restored, but have one chromosome set heterochromatinized (see transition 5, in Fig. 2.1), while others produced offspring by obligate automictic thelytoky (see transition 10). He observed that the asexually reproducing females contained needle-like bacteria in addition to the yeast-like endosymbionts normally found in soft scales. These bacteria were found in several tissues and not just in the fat cells like the fungi. He also observed that the bacteria had imperfect transmission, with transmission varying between females, such that between 20-90% of the embryos received the bacteria. The fact that this bacterium is not found in a specialized host structure, does not have perfect transmission, and is only found in part of the population, suggests that it is probably a reproductive parasite rather than an endosymbiont that benefits the host. It is also possible that it is in fact one of the known reproductive parasites previously identified in insects (such as a *Wolbachia* sp. or *Cardinium* sp.), although to date the identity of the bacterium has not been established. This was the first occasion that the presence of endosymbiotic bacteria was linked to asexual reproduction (Hurst *et al.*, 1990), but unfortunately the theoretical framework needed to understand this finding had not been developed, and so the observation remained relatively unnoticed.

Recently the presence of the endosymbiotic bacteria *Cardinium* spp. has been confirmed in a number of species of armoured scale insects and the presence of *Cardinium* spp. has also been shown to coincide with several incidences of parthenogenesis (Gruwell, Wu & Normark, 2009). However, not all cases of parthenogenesis in armoured scale insects could be attributed to *Cardinium* spp. and currently there are no experimental data available showing a direct relationship (e.g. by removing *Cardinium* spp. with antibiotics).

The role of host-endosymbiont conflict on the evolution of novel genetic systems

To date, empirical data supporting the role of host-endosymbiont conflict in the evolution of novel genetic systems is limited. Probably the best case for bacterial

involvement in PGE systems comes from the observation that species of the genus *Stictococcus* that have lost the endosymbionts from males also lack the heterochromatinization of paternal chromosomes in males (Buchner, 1965). However, another case has been suggested by Royer (1975) based on his observations on *Icerya purchasi*. This hermaphroditic species contains both diploid germline cells, which produce ocytes and haploid germline cells that produce spermatozoa (see Section II.6). Royer (1975) noticed that the haploid germline cells always develop in close association with the endosymbiotic bacteria which therefore might play a role in their development. The endosymbionts might perhaps therefore be involved in the origin of hermaphroditism in *I. purchasi*, which would be in their interest, as it results in a strongly female biased sex ratio. There is also a suggestion that endosymbionts influence host reproduction in three species of the genus *Hippeococcus* (*H. rappardi, H. wegneri* and *H. montanus*). These species, which hava an obligate relationship with ants, completely lack endosymbionts in both sexes. They do not seem to suffer from this lack of bacteria during their development, however adult females do not start forming oocytes while developing on the host plant, only doing so when the ants carry the adult females into their nest and feed them directly (Buchner, 1965). Perhaps some substances required for reproduction that are typically provided by the bacteria are in this case provided by the ant host. Several other taxa are known to have lost either their endosymbionts (in both, or just one sex) (Tremblay, 1989) or the heterochromatinization in males (Nur, 1980). It will be of great interest to focus on these taxa to see if there are more incidences of the absence of endosymbionts coinciding with a loss of heterochromatinization.

The role of endosymbionts in the evolution of parthenogenesis is well supported by several studies (see above), but all these examples are probably the result of reproductive parasites already discovered in other taxa. Moreover, only a small fraction of the known cases of parthenogenesis have been linked to endosymbionts. In particular, the presence of the wide variety of parthenogenetic systems in soft scale insects is not well understood. One possible hypothesis for the evolution of parthenogenesis in this group comes from Normark (2004b), given that the peculiar formation of the bacteriomes in many other scale insects (the fusion of polar body and embryo genomes) may serve as a way to hide the sex of the host from the bacteria. In many soft scale insects the endosymbionts are not contained in a specialized organ, but float freely in the host's haemolymph and even enter germ line cells (Buchner, 1965). It could be that this gives the host less control over the endosymbionts and that the actions of their endosymbionts could explain the evolution of the more extraordinary systems observed in this group (including parthenogenesis).

Although there are suggestions that conflict between the scale insect host and their obligate endosymbionts might have affected the evolution of many aspects of scale insect biology, much of the data supporting these ideas are often not more than anecdotal observations on single taxa. In order to better understand how important host-symbiont conflict has been in shaping the evolution of scales we need a proper comparative test of the hypotheses outlined above, based on data from a wide range of species and placed firmly in a phylogenetic context.

ANOTHER GENETIC CONFLICT IN COCCOIDS

Finally, we will briefly mention one last additional genetic element in scale insects that could potentially be an interested party in sex allocation: B chromosomes. B chromosomes are relatively common in Sternorrhyncha (Maryanska-Nadachowska, 2004), and in scale insects they are found to occur in at least three species, all of which have a PGE genetic system (Nur, 1962a; Nur, Brown & Beardsley, 1987). B chromosomes in scale insects were first observed by Nur (1962a) in the mealybug *Pseudococcus viburni* (previously *P. obscura*). He observed the presence of supernumerary chromosomes that behaved in a different way from the other chromosomes. *P. viburni* has a lecanoid PGE system, so in males the paternal chromosomes are silenced and not transmitted to the next generation. He observed, however, that certain supernumerary chromosomes when paternally derived behaved just like the other paternal chromosomes during development, but became euchromatic during spermatogenesis and segregated with the other maternally derived euchromatic chromosomes, thereby avoiding destruction (Nur, 1966b). Nur (1966b) also observed a strange behaviour of these chromosomes during oogenesis; in this circumstance they seemed to be preferentially excluded from the egg. This means that the B chromosomes are able to spread through the male line but are removed from the female line (in opposition to most reproductive parasites). Therefore B chromosomes in mealybugs are selected to favour male-biased sex ratios. In other families of Sternorrhyncha there is also the suggestion of involvement of B chromosomes in the evolution of genetic systems, particularly the evolution of sex chromosomes (Carvalho, 2002; Maryanska-Nadachowska, 2004). Apart from having a potential interest in sex allocation, the presence of B chromosomes and the mechanism by which they spread is extremely pertinent for PGE systems, as they represent the first evidence that it is possible for paternal chromosomes to avoid destruction during spermatogenesis. By studying the evolution of B chromosomes in species with PGE, we may get a more complete understanding about the conflict between the maternal and paternal genomes over the suppression and the deletion of the paternal chromosomes.

FURTHER DIRECTIONS

In this review we have attempted to highlight the extraordinary diversity of genetic systems in scale insects and outline the various different hypotheses put forward to explain them, with particular reference to the upsurge in interest in genomic conflict. However, as will have been apparent, we have been able to reveal lots of startling biology, and plenty of plausible hypotheses, but rather few robust attempts to link theory with empirical data, and little that could be called a compelling test of a given theory. We believe, though, that scale insects do have the potential to provide an exceptional resource for testing theories of genomic conflict, sex determination and the evolution of genetic systems. We would therefore like to finish with a short

overview of experimental and comparative approaches that we believe will help us to understand the role of genomic conflict in shaping the biology of scale insects.

Experimental and comparative approaches

Many of the hypotheses stated herein have not been formally tested. First it will be important to choose the right taxon to work with, depending on the particular question and hypothesis. To study the possible involvement of endosymbionts on the evolution of novel genetic systems and their role in sex determination, it will be best to focus on taxa that have lost or recently replaced their endosymbionts. An obvious choice here would be to focus on the African genus *Stictococcus*, in which only females have endosymbionts and in which there is a great deal of variation in endosymbiont status among closely related species (with some harbouring bacteria, others fungi and some both). To study sexual conflict over paternal genome elimination and sex allocation it will probably be best to focus on species with the ancestral lecanoid PGE system as paternal genes in this system might have more opportunity to influence transmission and sex allocation than in the other systems, as they are eliminated later in development. The obvious choice here would be the mealybugs, and especially the citrus mealybug *Planococcus citri*, as the pioneering work on both the genetic system and more recent work on the molecular mechanisms of PGE was carried out in this species.

One common pattern that is emerging is that the tissues that are expected to be the battlegrounds of both inter- and intra-genetic conflict (e.g. the bacteriome and parts of the testis) are characterized by both the reactivation of the paternal genome and by polyploidy. Furthermore, although this pattern seems widespread among species with PGE, there is a great deal of variation among closely related species in exactly which tissues the paternal genome is suppressed and in which the maternal genome becomes polyploid, suggesting the possible role of co-evolution between the sexes (in tissues involved in spermatogenesis) and between maternal, paternal and bacterial genes (in the bacteriome) as a driving force behind this variation. It might be possible, for instance, to test for maternal-paternal conflict over paternal genome elimination by making hybrid crosses between closely related species that differ in their patterns of paternal gene expression to see if the paternal genes might be able to escape.

Another testable prediction is the possible role of the endosymbiont in its host's sex determination. This could be done by manipulation of the bacterial titre or by studying sex allocation of old females that might not be able to transfer as much bacteria to their eggs. If the bacteriome has a function in avoiding conflict between the host and its bacteria then one would expect more influence of the bacteria on sex determination in taxa that lack a bacteriome. One would also expect to find a difference between taxa in which the bacteria have a "free" phase during transmission and those where the bacteria are transmitted within maternal bacteriome cells (e.g. Putoidae). It might be possible to address this by a comparative analysis, linking the variability of genetic systems with the amount of time the endosymbionts spend outside the bacteriocytes.

If the earlier deletion of paternal chromosomes in the Comstockiella system functions to prevent paternal chromosomes escaping destruction during spermatogenesis, then one might expect that B chromosomes, which manage to do exactly that, will be more prevalent and successful in species with a lecanoid PGE system or a Comstockiella system in which relatively few chromosomes are destroyed before spermatogenesis. Unfortunately data on the prevalence of B chromosomes in scale insects is limited. It would therefore be valuable to screen for B chromosomes, especially in families that have species with both lecanoid and Comstockiella systems.

Finally, it might be possible to address some of the hypotheses discussed herein via comprehensive comparative analyses across scale insect taxa, using data on endosymbiont status, genetic system and other life-history traits combined with recently available phylogenetic data. We are sure such attempts will prove fascinating.

CONCLUSIONS

(1) The broad array of diverse genetic systems in scale insects, and the multiple evolutionary transitions between them, provide an ideal opportunity to test theories regarding the evolution of genetic and sex-determination systems.
(2) Existing theories to explain this diversity in scale insects focus on either scale insect biology or the role of genetic conflict.
(3) Circumstantial evidence for the role of genetic conflict (within- and across-genomes) exists, but few compelling, independent tests of theory have been performed.
(4) With new phylogenetic information becoming available and increasing knowledge of some the mechanisms underpinning sex determination in scale insects, comparative analyses may provide the basis for these much-needed tests.
(5) Further study of scale insect biology is likely to yield fresh insight into the evolutionary significance of genetic conflict.

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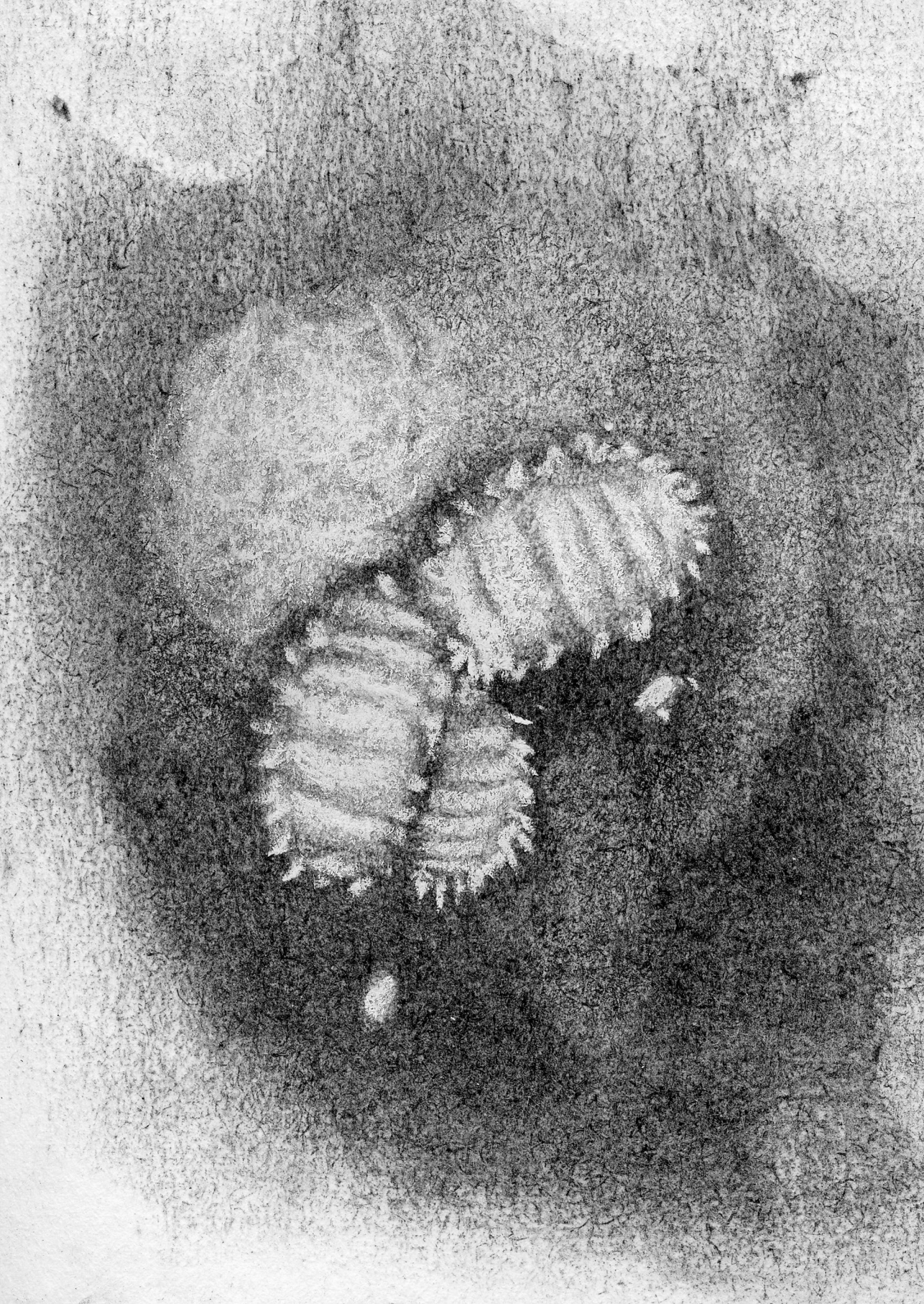

CHAPTER 3

Sexual conflict, sex allocation and the genetic system

David M. Shuker, Anna M. Moynihan and Laura Ross

ABSTRACT

Decisions over what sex ratio to produce can have far-reaching evolutionary consequences, for both offspring and parents. However, the extent to which males and females come into evolutionary conflict over aspects of sex allocation depends on the genetic system: when genes are passed to the next generation unequally by the two sexes (as in haplodiploidy for example), this biased transmission can facilitate a range of conflicts not seen in diploids. However, much less attention has been paid to these forms of sexual conflict, not least because it has not always been clear how the conflicts could be realised. Here we consider how biased gene transmission, as expressed in different genetic systems, enhances the opportunity for sex ratio conflict and give empirical examples that confirm that males as well as females have the opportunity to influence sex ratios.

Biology letters 2009

INTRODUCTION

Since Parker (1979), evolutionary biologists have identified numerous sexual conflicts over patterns of mating, parental care, and life history (Arnqvist & Rowe, 2005). Despite this interest, one reproductive decision has received less attention: sex allocation. Sex allocation describes how resources are partitioned between male and female offspring, including the proportion of each sex produced (the sex ratio, defined here as the proportion of male offspring). For brevity, we will equate sex allocation with sex ratio, although we acknowledge that they may differ, especially if parental investment extends beyond egg provisioning. In this article we consider the scope for sexual conflict over sex ratio, focusing on the role of different genetic systems, and giving empirical examples where both males and females can influence sex ratios.

SEXUAL CONFLICT OVER SEX RATIO

In the classic Düsing-Fisher scenario, frequency-dependent selection acts on sex allocation to equalise the marginal fitness returns of male and female offspring (Charnov, 1982; Edwards, 2000). In addition, in diploid species the average reproductive success of males and females has to be the same. Together these two facts have given the impression that sexual conflict over sex ratio may be rather limited in scope (Arnqvist & Rowe 2005). However, the Düsing-Fisher scenario fails in a number of important situations, with fitness returns through male and female offspring varying either due to genetics or the environment.

In terms of genetics, in the conventional diploid case at sex ratio equilibrium, mothers and fathers obtain equal fitness through both sons and daughters, and there is no sexual conflict. Under alternative genetic systems, however, the reproductive value of sons and daughters can differ markedly for mothers and fathers (Trivers and Hare 1976). For instance, in haplodiploids, genes in fathers are only transmitted through daughters, with sons being of no reproductive value to males. Females, on the other hand, gain fitness benefits through both sons and daughters, setting the scene for possible conflicts over sex ratio. Although the most familiar non-diploid organisms are the haplodiploid Hymenoptera (ants, bees and wasps), haplodiploid and related systems are found in more than 15% of animal species, including among thrips, beetles, scale insects and mites (Hedrick & Parker, 1997). These “asymmetric” genetic systems are therefore non-trivial.

The classic scenario can also fail because of environmental effects on offspring fitness, for instance if selection favours being a member of the rarer sex (Pen, 2006; Trivers, 1974), or if the fitnesses of sons and daughters are condition-dependent (Trivers & Willard, 1973). These environmental effects initiate conflicts between parents and offspring over sex allocation, and it is known that selection on sex ratio depends on whether parents or offspring are in “control” (Trivers & Hare, 1976). However, condition-dependent fitness differences between male and female offspring

can also create sexual conflict, and offspring that manipulate the sex ratio to their advantage will be selected (making sex ratio a conflict trait for brood-mates of the opposite sexes). In these situations, conflict can arise under diploidy as well as under other systems although the extent of the conflict (the difference in sex ratio optima) may be smaller in diploids (see Wild & West, 2009 for a thorough treatment). Genes and the environment can shape the conflict together of course, for instance if a species' ecology influences the mating system and pattern of inbreeding (potentially selecting for biased sex ratios Charnov, 1982; Hamilton, 1967). The degree of male-female conflict will then depend on the extent and direction of any sex ratio bias and what this means for maternal and paternal gene transmission.

To summarise, we can consider sexual conflict over sex ratio to occur in two broad categories. First, there may be male-female parental conflict over the sex ratio, with changes in sex ratio lead to changes in the transmission of paternal or maternal genes. This category will be intimately associated with the genetic system and typically occur outside of diploids. Second, male-female conflict may be a consequence of the sex an individual has been assigned and the sex ratio of the brood it is in. Changes in sex ratio lead to changes in the reproductive advantages of being male or female, and traits that lead to the manipulation of the sex ratio by offspring can be favoured. This means that there will often be an intimate relationship between sexual conflict and other genetic conflicts (parent-offspring and sibling rivalry) when it comes to sex allocation. Again, these conflicts are more likely to be apparent outside diploids.

However, for potential conflicts to become actual conflicts, both males and females must be able to influence sex ratio (Beekman & Ratnieks, 2003). Whilst this is often assumed to be true for females, the opportunities are less intuitively clear for males (Werren & Beukeboom, 1998). Our brief empirical sketch of the possible scope for sexual conflict over sex ratio will therefore focus on what evidence we have of male effects on sex ratio.

PARENTAL CONFLICT OVER SEX RATIO

Genetic conflict over sex ratio has been best studied in the social Hymenoptera. The relatedness asymmetries generated by haplodiploidy in social insect colonies are well-known to affect the optimal sex ratios for different colony members (Sundstrom & Boomsma, 2001). Since brood production is predominantly shaped by interactions between workers and queens, it has been assumed that males as fathers have little opportunity to influence sex ratio and increase daughter production. However, there is evidence that in multiply mated species males deliberately try to "clump" their sperm together. This sperm-clumping inside a queen means that cohorts of brood are singly-fathered, keeping the relatedness asymmetry high between male and female brood, which in turn favours the production of female-biased sex ratios (Boomsma, 1996). Males could also promote a high relatedness asymmetry in the brood by

avoiding non-virgin females, reinforcing any other selection for male choice of virgin queens. Mechanisms by which males influence sex ratio may be even more indirect however. For instance, in the mud-daubing wasp *Trypoxylon politum*, males that guard the nest end up with more daughters (Brockmann & Grafen, 1989). This is not due to direct manipulation, but rather nest-guarding enables females to invest more time in foraging and so provision more female brood (the costlier sex). Males guard nests in order to copulate, but they also indirectly decrease the sex ratio, increasing their genetic success. The costs to fathers of male production may not just be associated with the missed opportunity to sire a daughter: in autoparasitoid wasps such as *Encarsia*, females develop on "normal" (whitefly) hosts, whilst males can hyperparasitise the female larvae of their own species (Hunter & Woolley, 2001). This means that not only do male offspring fail to pass on paternal genes, but their development destroys paternal genes that manage to make it into daughters.

For Hymenoptera, since sex determination is via egg fertilisation (Heimpel & de Boer, 2008), the key process for sex ratio control is sperm usage during oviposition. Can males influence this? One possibility is via seminal proteins. While the effects of seminal proteins on female reproductive physiology are mostly known from *Drosophila* (Wolfner, 2002), there is some evidence for the action of seminal fluid on females in *Bombus* bumblebees (Baer, Morgan & Schmid-Hempel, 2001). Seminal fluid could affect the sex ratio by increasing the "leakiness" of the female's spermatheca, increasing the fertilisation rate. Suggestive (but by no means conclusive) evidence of a male effect on fertilisation rate comes from work by Shuker *et al.* (2006) on the parasitoid wasp *Nasonia vitripennis*. They showed that variation in sex ratio varied with genotype of the inseminating male, in a study that attempted to exclude effects of sperm limitation or gametic incompatibility. Moreover, sperm competition adaptations in haplodiploids that improve the likelihood that sperm is preferentially used could also be favoured if they generally increase sperm usage (and daughter production) regardless of sperm competition. In the parasitoid wasp *Dinarmus basalis*, Chevrier & Bressac (2002) showed that multiply mated females laid a greater proportion of daughters, which could result from male attempts to influence sperm usage (or, more prosaically, from multiply-mated females avoiding sperm limitation).

Resolving the extent to which selection has acted on males to influence sex ratio may not be straightforward in Hymenoptera, as variation among males in sperm quality or quantity may give the appearance of male control, since females need sperm to produce daughters (but not sons). However, a different group of insects may offer more opportunities to test the role of sexual conflict over sex ratio. Scale insects exhibit an array of genetic systems, including haplodiploidy and paternal genome elimination (Gullan & Kosztarab, 1997). The mealybug *Planococcus citri* exemplifies the scope for conflict. *P. citri* has paternal genome elimination (PGE) whereby in males the paternally-inherited chromosomes are condensed via DNA heterochromatinisation and, whilst present but untranscribed in somatic tissues, these chromosomes are lost during meiosis in the germ line (Nur, 1980). Male offspring are a dead-end for paternal chromosomes and selection would favour males that either

managed to subvert the destruction of their chromosomes in prospective sons or induced females into producing more daughters (as in haplodiploids). In terms of mechanisms, the role of genomic imprinting may be crucial. Scale insects boast widespread genomic imprinting and imprinting of the paternal chromosomes underpins PGE and sex determination (Buglia & Ferraro, 2004b; Normark, 2006). Paternal chromosomes that could hide their origin when transmitted would be at a selective advantage. Intriguingly, in *P. citri* the one place genomic silencing of paternal chromosome has failed is in the male germ tissue, suggesting that paternally-inherited genes may still have the ability to influence the fate of paternal chromosomes in the germ line.

That paternal chromosomes may sometimes "escape" destruction and be transmitted to the offspring forms the basis of Herrick and Seger's (1999) hypothesis for the evolution of the various forms of PGE in scale insects, with males and females selected to control the fate of chromosomes trying to enter the germ line. This hypothesis highlights that conflict may not only be facilitated by alternative genetic systems, but may drive the evolution of those genetic systems. For example, females able to exclude male gametes from some of their offspring gain an immediate transmission advantage, favouring the evolution of haplodiploidy and systems such as PGE (Bull, 1979).

CONFLICT BETWEEN MALE AND FEMALE BROOD

The relatedness asymmetry generated by haplodiploidy means that in social Hymenoptera colonies female workers are more related to their sisters than their brothers (the basis of the queen-worker conflict), and brood sex ratio manipulation by the destruction of male brood by their (worker) sisters has been well-documented (Ratnieks & Boomsma, 1995). Whilst this can be interpreted in terms of parent-offspring conflict over sex ratio, this is also a sexual conflict between the male and female brood themselves, arising as a consequence of the queen's pattern of sex allocation. A similar conflict arises in some species of polyembryonic wasp in which sex ratio is controlled by the offspring, both through embryonic proliferation and the production of (female) soldier larvae that preferentially kill males (Gardner *et al.*, 2007). Male larvae have also been known to kill female larvae though, for instance in the bee *Trigona postica* (Beig, 1972), and it is perhaps likely that more such conflicts will be uncovered, for instance among gregarious parasitoids with asymmetric larval competition (Sykes *et al.*, 2007).

CONCLUSIONS

Some genetic systems may be more predisposed to sexual conflict over sex ratio than others, given the links between sex ratio and gene transmission. Males cannot be

assumed to be passive players in sex allocation, although as yet we have little more than an idiosyncratic collection of interesting items of biology, rather than a compelling body of empirical work. One of the main challenges will be that the boundaries between traditional conflicts such as parent-offspring, sibling, and sexual conflicts may often be blurred. However, resolving how these forces interact will give us a much clearer picture of the evolutionary importance of genetic conflict, both in terms of sex allocation and more generally.

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CHAPTER 4

The evolution and suppression of male suicide under paternal genome elimination

Laura Ross, David M. Shuker, Ido Pen

ABSTRACT

Different genetic systems can be both the cause and the consequence of genetic conflict over the transmission of genes, obscuring their evolutionary origin. For instance, with paternal genome elimination (PGE), found in some insects and mites, both sexes develop from fertilized eggs, but in males the paternally derived chromosomes are either lost (embryonic PGE) or deactivated (germ line PGE) during embryogenesis and not transmitted to the next generation. Evolution of germ line PGE requires two transitions: (1) elimination of the paternal genome during spermatogenesis; (2) deactivation of the paternal genome early in development. Hypotheses for the evolution of PGE have mainly focused on the first transition. However, maternal genes seem to be responsible for the deactivation and here we investigate if maternal suppression could have evolved in response to paternally expressed male suicide genes. We show that sibling competition can cause such genes to spread quickly and that inbreeding is necessary to prevent fixation of male suicide, and subsequent population extinction. Once male-suicide has evolved, maternally expressed suppressor genes can invade in the population. Our results highlight the rich opportunity for genetic conflict in asymmetric genetic systems and the counter-intuitive phenotypes that can evolve as a result.

Evolution, in press

INTRODUCTION

It is now known that there is a great diversity of genetic and sex determining systems across taxa, resulting in differences in reproductive mode, ploidy levels between the sexes and the mechanisms of sex determination (Normark, 2003; Norton *et al.*, 1993; Uller *et al.*, 2007). Furthermore, these differences can occur between closely related taxa (such as scale insects: Ross *et al.* 2010). However, the evolutionary significance of this variation is poorly understood. Recently the role of conflict between different genetic entities on the evolution of novel genetic and sex determination systems has gained widespread attention (Hurst, 1995; Normark, 2004a, 2006; Ross *et al.*, 2010b; Uller *et al.*, 2007). These genetic conflicts can arise both within genomes (for instance between driving sex chromosomes and autosomes: (Burt & Trivers, 2006)) or between genomes (for instance between hosts and symbionts: (Wernegreen, 2004; Werren *et al.*, 2008)). In this paper, we consider the role of intra-genomic conflict on the evolution of one particular system: paternal genome elimination (PGE).

PGE is found in several taxa among insects and mites (Normark, 2003; Norton *et al.*, 1993; Nur, 1980). PGE can be roughly divided into two classes. The first is *embryonic* PGE, in which the paternal genome is eliminated early during male embryonic development, rendering males haploid (Brown, 1965; Normark, 2003; Nur, 1980). This system is found in some armored scale insects (Hemiptera: Diaspididae) (Nur, 1980) and in some Pytoseeid mites (Acari: Phytoseiidae) (Cruickshank & Thomas, 1999). The second is *germ line* PGE, in which the paternal genome remains present in males, but is eliminated from the germ line during or just before spermatogenesis and is therefore not transmitted, making males effectively haploid in terms of their transmission genetics (Brown & Nelson-Rees, 1961; Normark, 2003; Nur, 1980; Schrader, 1921). This system in found in most scale insects (Hemiptera: Coccoidea) (Nur, 1980), in sciarid flies (Diptera: Sciaridae) (Goday & Esteban, 2001) and in the coffee berry borer beetle, *Hypothenemus hampei* (Coleoptera: Scolytidae) (Borsa & Kjellberg, 1996).

Although the evolutionary relationship between the two systems is unresolved in some taxa, it is clear at least in scale insects that embryonic PGE has evolved from germ line PGE (Morse & Normark, 2006; Nur, 1980; Ross *et al.*, 2010b). Interestingly, in species with germ line PGE, even though the paternal genome is present in all tissues, it is deactivated in most. In one scale insect (the mealybug *Planococcus citri*) this deactivation has been shown to be induced by the maternal genome (Brown & Nur, 1964; Chandra, 1962; Nur, 1962b). Therefore, the evolution of germ line PGE consists of two important evolutionary transitions: (1) the elimination of the paternal genome from the germ line; (2) the deactivation of the paternal genome early in development. Explanations for the evolution of PGE have in general focused only on the first of the two transitions. The hypotheses of Brown (1964) and Bull (1979) assume that maternal chromosome drive has led to the evolution of PGE and therefore focus only on the first transition. Similarly, the hypothesis of Haig (1993a) considers the role of X-chromosomal drive in the evolution of PGE and again focuses

exclusively on the first evolutionary transition. These three models all consider *intra*-genomic conflicts. In contrast, the fourth hypothesis, formulated by Normark (2004a), assumes the involvement of male-killing endosymbionts. He argued that in order to kill males (which do not transmit the endosymbionts) the endosymbionts destroy male-determining sperm when they fertilize the oocytes. However once the host evolves haploid male viability, this leads to a similar type of maternal chromosome drive as in the models of Brown, Bull and Haig.

Herrick and Seger (1999) were the first to note that once the elimination of the paternal genome from the male germ line has evolved, this leads to other evolutionary conflicts of interest between paternal and maternal genes in males. Specifically, they argued that there would be selection on the paternal genome to evolve mechanisms to prevent this elimination. The paternal genome might have several options for doing so. For instance, it could completely block PGE, by restoring a fair meiosis and resisting the elimination during spermatogenesis. Alternatively individual chromosomes might occasionally be able to swap place with a maternal homologue and thereby gain access to the sperm. Herrick and Seger (1999) also argued that these attempts by paternally inherited genes to regain transmission will select for a counter response by the maternal genes. They argue that one way for the maternal genome to prevent counter adaptation by the paternal genome is to deactivate the paternal genome. In a verbal model they propose that continuing co-evolution between the maternal and paternal genes in males might have lead to the gradual deactivation of the paternal genome, starting with genes or chromosomes in germline cells, as these might be more "powerful" in affecting their own transmission, but gradually spreading to the soma as well. They also argued that this maternal-paternal co-evolution might have caused the evolution of the different types of PGE in which the paternal genome is eliminated from the germ line progressively earlier (reviewed by Ross *et al.*, 2010b).

However, although there will be strong selection on the paternal genes to regain access to the germline and thereby gain direct fitness, this might be hard to achieve. In species with PGE, meiosis and spermatogenesis are modified so that even if paternal chromosomes avoid elimination this might not necessary lead to successful transmission, as it will often lead to diploid or non-functional sperm. Furthermore, "normal" meiosis and spermatogenesis might not have taken place in PGE species for millions of generations and the resulting loss of necessary genes might hinder the restoration of normal diplodiploidy (Herrick & Seger, 1999; Nur, 1970).

There might however be another way in which paternal genes can increase their fitness. Although males do not transmit their paternal genes to the next generation and therefore the paternal genome in males does not have any direct fitness, paternal genes can obtain indirect fitness by enhancing survival or reproduction of sisters or other relatives. This leads to a situation within a sib-group where paternal genes in males can favor their sister's reproduction at the expense of their own (Normark, 2001). Specifically, we argue that paternal genes may be selected to commit suicide, if the surviving sisters can use the newly-available resources and increase their

fitness. This is then an intra-genomic version of the well-known argument for male-killing by maternally-transmitted endosymbionts (Hurst, 1991).

The first aim of this paper is to investigate theoretically under what conditions a paternally expressed suicide gene could invade a population. We will test how population sub-structure and resulting levels of sib-mating will affect (1) if a suicide gene can invade and (2) what level of male-killing is expected under different levels of inbreeding. Once a male suicide gene has invaded in the population, this will have strong effects on the population sex ratio. We therefore also explore if the presence of a paternally expressed male suicide gene selects for biased primary sex ratios. Finally the invasion of a paternally expressed male suicide gene is expected to impose a strong selection pressure on the maternal genes in males to suppress the suicide phenotype. We therefore also model the spread of a maternally expressed suppressor gene, once a male-suicide gene is present, and discuss if this could have led to the deactivation of the paternal genome in males.

Inclusive fitness model for suicide evolution

In order to understand if paternal suicide genes could evolve in taxa with PGE, we need to consider the life history of those taxa. Normark (2004a) pointed out that most taxa with PGE not only have strong levels of sib-competition (which would increase the selection pressure for male suicide) but also high levels of sib-mating and inbreeding. At first glance, one might expect inbreeding to counteract the spread of paternal male suicide as it can lead to increased relatedness between the maternal and paternal genome of individual. However, inbreeding also increases relatedness between sibs, which might promote male suicide. To make matters even more complicated, a life history with inbreeding and sib-competition may select for female-biased sex ratios, thus increasing the reproductive value of individual males, which might be an additional obstacle to the evolution of male suicide. Clearly, a formal model is required to investigate the balance of these opposing effects.

We consider the fate of a partially suicidal gene that is expressed in males by the paternally inherited half of their genome. We allow for some degree of inbreeding by assuming that the population is subdivided in standard-sized patches of n mated females whose offspring mate randomly on their natal patch followed by dispersal of newly mated females to random patches according to a standard "island model" of dispersal.

Offspring mortality occurs in two subsequent "rounds". In round one - the male suicide round - some males may die during early development as a result of the action of a paternally inherited gene. The resources accumulated by (or not exploited by) dead males can be partially recycled and enhance the survival of their sibs during the second round of offspring mortality. Specifically, we assume that a focal male commits suicide with probability x, while x_b is the average suicide probability among all males in the focal brood and x_p is the patch-level suicide probability of males during round one. In the second round, individual male and female survivors of round one will survive an additional round with (non-sex specific) probability

$$y_b = y_0 + (1 - y_0)bsx_b \,. \tag{1}$$

Here $0 < y_0 \leq 1$ is a baseline level of survival in case no male sibs were killed during round one, and the second term on the right represents the (linear) increase in survival with the amount of resources made available by deceased male sibs. Parameter $0 \leq b \leq 1$ is a measure of recycling efficiency and $0 \leq s \leq 1$ is the brood sex ratio (proportion males). Thus, minimal survival in phase two equals y_0, while survival approaches unity in case the brood consists almost entirely of suicidal males that are recycled with maximal efficiency ($x_b \cup 1$, $b \cup 1$ and $s \cup 1$. In what follows, for the easy interpretation of the derived formulas, we assume $y_0 = 1/2$, but this has no qualitative effect on the conclusions.

We want to calculate the inclusive fitness effect of a small change in the suicidal tendencies of the focal gene, and for this we need to consider how the fitness of females and males depend on x, x_b and x_p. We assume the fitness of a female depends only on her brood-level x_b (i.e. the mean suicide rate of her brothers) mediated by its effect on round two survival of females:

$$W_f = y_b \,. \tag{2}$$

The fitness of a focal male is his probability of survival $(1 - x_b)$ y_b across both rounds times his expected number of mates $(1 - s) / [(1 - x_p)s]$:

$$W_m = (1 - x)y_b \frac{1 - s}{(1 - x_p)s}. \tag{3}$$

The inclusive fitness effect of a small change in x can then be calculated according to a standard method (Pen, 2006; Taylor & Frank, 1996) as

$$\Delta W_{IF} = s \frac{\partial W_m}{\partial x} r + 2(1 - s) \frac{\partial W_f}{\partial x_b} r_f + s \frac{\partial W_m}{\partial x_b} r_{m_b} + s \frac{\partial W_m}{\partial x_p} r_{m_p} \,. \tag{4}$$

The right-hand side is evaluated at $x = x_b = x_p$. The marginal fitness effects (the partial derivatives) for each sex are multiplied by the frequency of each sex, as dictated by the sex ratio s. Female fitness is additionally multiplied by 2 since in haplodiploids the reproductive value of a daughter is twice that of a son in terms of passing on genes to future generations (Bulmer, 1994; Hamilton, 1979). The various r-parameters are different coefficients of relatedness from the viewpoint of the controlling gene, in this case the paternally inherited x-gene in a focal male. Specifically, the coefficient r is the relatedness of the maternal genome to the paternal genome in the focal male, and it equals the inbreeding coefficient f, since f is by definition the probability that an individual's maternally and paternally inherited genes are identical by descent. The coefficient r_f is the relatedness of a sister to the controlling gene in the focal male, and this equals $r_f = \frac{1}{2} + \frac{1}{2}f$, the mean of the relatedness of the sister's paternal genes to the controlling gene (a relatedness of 1, since fathers are effectively haploid) and the relatedness of her maternal genes to the controlling gene (by

definition, f). Similarly, $r_{m_p} = f$ is the relatedness of a brother's maternal genome to the paternal genome of the focal male, and $r_{m_p} = (1/n)f$ is the relatedness of a random male competitor from the focal patch to the paternal genome of the focal male.

Replacing the coefficients of relatedness in (4) with the derived expressions in terms of inbreeding coefficients gives

$$\Delta W_{IF} = s\frac{\partial W_m}{\partial x}f + (1-s)\frac{\partial W_f}{\partial x_b}(1+f) + s\frac{\partial W_m}{\partial x_b}f + s\frac{\partial W_m}{\partial x_p}f/n\,. \qquad (5)$$

From inspecting the definitions of W_m and W_f, it is clear that all partial derivatives on the right-hand side of (5) are positive except for the first one $\partial W_m / \partial x$. Therefore, if there is no inbreeding $f = 0$, only a single positive term remains, and suicide (x) of males will evolve to its maximal value (i.e. all males commit suicide). Therefore some minimum level of inbreeding (i.e. $f > 0$)is required for selection against 100% male suicide.

The equilibrium suicide rate is found by calculating the derivatives in (5), evaluating them at $x = x_b = x_p = x^*$, setting the right-hand side equal to zero and solving for x^*:

$$x^* = \frac{n(1+2f) - (n-1)f/(bs)}{n + (3n-1)f}\,. \qquad (6)$$

or $x^* = 0$ if the right-hand side is negative (i.e. there is no male suicide). Note that $x^* = 1$ when $f = 0$, i.e. in the absence of inbreeding selection favors 100% male suicide, which would cause population extinction.

The inbreeding coefficient f depends on patch size n, and can be considered a "fast variable" relative to the speed of evolution, whose quasi-equilibrium value can be calculated from a standard recursion equation (see Taylor, 1988):

$$f = 1/(4n-3)\,. \qquad (7)$$

Plugging the resulting f into (6) gives the main result

$$x^* = \frac{n(4n-1) - (n-1)/(bs)}{4n^2 - 1}\,. \qquad (8)$$

or $x^* = 0$, whichever is larger. From inspection, it is clear that - all else being equal - for sufficiently small b-values there will be no selection for suicide. A female-biased sex ratio (small s) also leads to lower suicide rates, and finally, x^* increases with n.

Some examples of x^* for varying values of b and n are shown in figure 4.1. For the brood sex ratio s we took the equilibrium value under maternal control, and we show in appendix 1 how this is calculated. In addition to the analytical solutions, we also show results of individual-based simulations in order to verify the stability of the equilibria (see appendix 2; C++ code is available on request). It is clear from Figure 4.1 that male suicide is straightforward to evolve. It is also interesting that primary

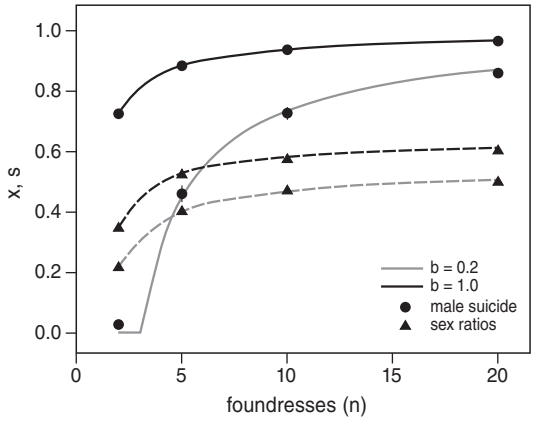


Figure 4.1 Male suicide can evolve and generate male biased equilibrium sex ratios. Equilibrium levels of male suicide rates x and brood sex ratios s (proportion male), as a function of number of females (foundresses, n) per local patch. Solid curves represent male suicide as predicted by the analytical model for two values of b, the efficiency of recycling killed males into resources for sibs. Dashed curves represent co-evolved sex ratios as predicted by the analytical model. Note that male-biased sex ratios arise for some parameter combinations. The individual-based simulation results are presented by symbols representing averages 1 standard deviation) of 10 replicates (circles: male survival; squares: sex ratios). The simulations fit the analytical predictions quite closely.

sex ratios can be male-biased, in contrast to the sex ratios in standard LMC models (West, 2009).

In order to confirm our prediction that under no inbreeding the evolution of male suicide can lead to population extinction in figure 4.2 we show simulation results where we assume a single large random-mating population (n = 10000) and show that male suicide quickly evolves to 100% and that this drives the population extinct. Further details on this simulation can be found in Appendix 2.

Counter-evolution of maternally inherited suicide-suppressors

In the previous section we have shown that under PGE, a paternally expressed gene is able to evolve male suicide, as long as sibs can benefit sufficiently from recycled resources. Here we explore if suppression expressed by maternally inherited genes can evolve, once male suicide is present. We use an individual based simulation approach, where we allow a maternally expressed suppressor gene z to evolve simultaneously with x. This locus determines the probability of expression of x. We would first like to see if a maternal suppressor (z) is able to invade, under what conditions it will invade, and if it will lead to partial or complete suppression. We would also like to see how fast such a maternally-expressed suppression gene will spread and if it will go to fixation. Finally we explore how the efficiency (b) with which the resources that become available after male-killing can be used by the male's siblings affects the evolution of maternal suicide suppression. Simulation results are shown for a local

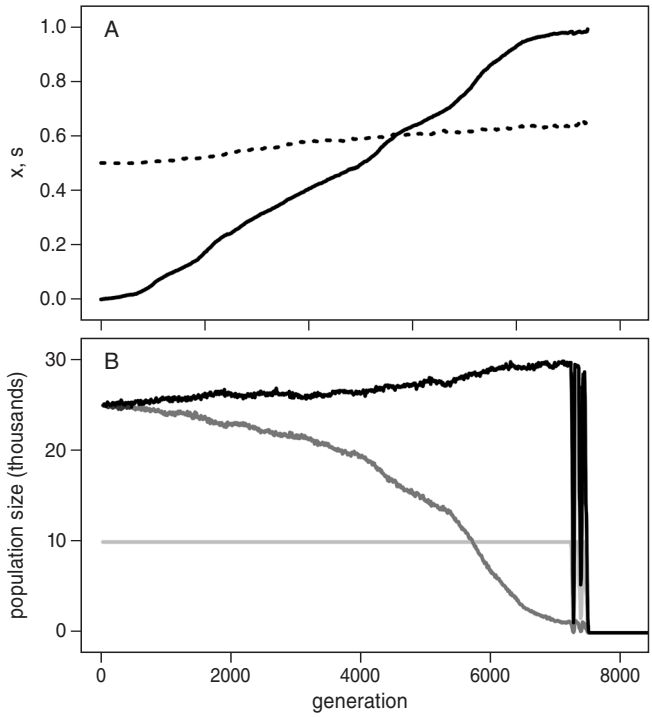


Figure 4.2 Under the absence of inbreeding, male suicide can lead to population extinction due to the resulting lack of males. Simulation results for the evolution of male suicide in a large undivided population. The top panel shows the value of the suicide gene x (solid line) and the sex allocation gene s (dashed line). The bottom panel shows the number of reproducing females (light grey) and the number of surviving sons (dark grey) and daughters (black). Further parameter values are given in Appendix 2.

mate competition scenario with 4 foundresses per patch (figure 4.3; see Appendix 2 for details) and four different recycling efficiencies (b). These results first of all show that a maternally-expressed suppression gene can invade under all the conditions that were considered and that it leads to complete suppression of the paternally expressed suicide gene. Secondly, they show that although the suppression gene spreads to fixation under all conditions, the recycling efficiency rate b affects how fast z spreads and becomes fixed, with a faster spread at higher recycling efficiencies.

DISCUSSION

Asymmetric genetic systems, in which transmission is unequal for different genetic entities or elements, are a rich evolutionary playground for strange and seemingly counter-intuitive phenotypes (Burt & Trivers, 2006; Normark, 2006). We have shown that in species with one such asymmetric system, paternal genome elimination, if

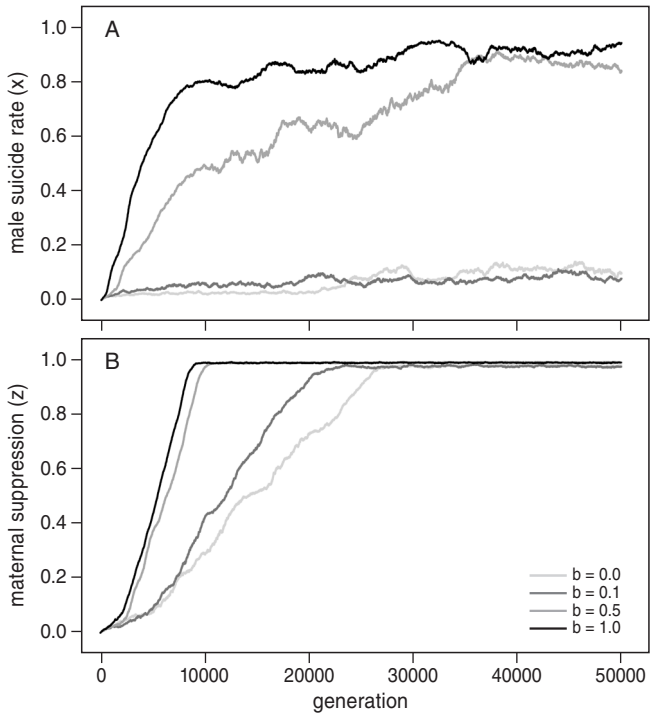


Figure 4.3 Maternal suppression of male suicide can evolve, even when there is rather little suicide. Simulation results for the evolution of a maternally expressed suicide-suppressor. The top panel shows the value z of the suicide suppressor. The bottom panel shows the value of x, when x and z are allowed to evolve simultaneously (and the expression of x is determined by z). In both panels results are shown for different levels of the recycling efficiency (b = 0, 0.1, 0.5, 1.0). Further parameter values are given in Appendix 2.

paternal genes are expressed in males then the evolution of genes causing male suicide is possible, as long as sibs can profit from the recycled resources of killed males. In the absence of inbreeding, our model predicts the evolution of a rate of 100% male suicide, which will lead to population extinction (figure 4.2), while increasing levels of inbreeding limits the extent of male suicide or may even prevent it altogether. As male suicide evolves, co-evolution of the sex ratio may occur, and this can lead to male-biased primary sex ratios, as males may benefit their sibs when they commit suicide. This is surprising as predictions of male-biased population sex ratios are rare under the standard sex ratio models, and the population structure modeled here, would normally predict strongly female biased sex ratio (according to local mate competition theory (Hamilton, 1967). We have also shown that once these male suicide genes have evolved, a maternally expressed suppressor can evolve and that this results in a complete suppression of the paternally-derived suicide genes.

As discussed earlier, the evolution of PGE will lead to conflict between maternally and paternally inherited genes in males. It has previously been noted that PGE results

in selection on the paternal genes to resist their elimination from the germline in males. However, only two cases of reversal from PGE back to normal diplodiploidy have been observed (in the scale insect genera *Lachnodius* and *Stictococcus*) (Nur, 1980) and both evolved from germline PGE. So although this shows that reversal is possible, it is rare. Our results show that in cases where paternal genes cannot – for whatever reason - defeat PGE, they may still obtain indirect fitness benefits by evolving a male-killing phenotype.

Our results also suggest that the evolution of paternally-expressed suicide genes could trigger the evolution of maternal suppression of the paternal genome set in order to silence suicide genes. Although (partial) paternal genome deactivation in males has been shown in all taxa with germ line PGE, the mechanism of suppression has been mainly studied in mealybugs. In these species it has been shown that DNA methylation plays an important role in the deactivation. The paternal genome is found to be hypo-methylated in both sexes and several histone proteins have been shown to be involved in the deactivation (Bongiorni *et al.*, 1999; Bongiorni *et al.*, 2007). When the expression of these histone proteins is blocked, this results in the reactivation of the paternal genome (Bongiorni *et al.*, 2007). These results agree with earlier observations of individuals with artificially constructed haploid embryos that lacked the maternal genome in which the paternal genome became active (Brown & Nur, 1964), suggesting maternally expressed suppression.

It has been argued earlier that conflict over transmission through sperm could have led to the evolution of maternal deactivation of the paternal genes to stop paternal attempts to regain transmission (i.e. "policing" PGE itself; Herrick and Seger 1999). However although the deactivation of the paternal genome in males would indeed prevent those attempts it will presumably come with a considerable fitness cost for the male. Furthermore it is hard to reconcile with the observation that in mealybugs although the paternal genome is deactivated in most tissues it is active in the testis, the very place where it is eliminated. If the paternal genes are deactivated to prevent them from fighting their elimination, we would expect them to be repressed most strongly in tissue where they might have most power to affect their transmission.

The alternative explanations for the deactivation of the paternal genome will be difficult to distinguish, and currently little has been done to experimentally manipulate maternal deactivation of paternal chromosomes in these species, and so the phenotypes that would result are unknown. If maternal deactivation is preventing paternally-driven male suicide, then male death (including failed embryos) may be the result of such manipulations. However, such phenotypes are inherently hard to study, especially in terms of confirming the cause of the embryonic (or later stage) mortality. In order to test if paternally-expressed suicide genes have indeed evolved and that the suppression of the paternal genome has evolved in response it may be helpful to focus on systems where the suppression is incomplete, or where the extent of male suicide is incomplete in the absence of maternal suppression.

In addition to wrestling over control of paternal gene expression in males, there are other possible outcomes or ways to avoid male suicide. In sciarid flies only certain

paternal chromosomes are lost during embryogenesis, while the others remain active in the soma (Haig, 1993b). This might make *Sciara* particularly susceptible to the evolution of paternally-expressed male suicide genes. However, many species of sciarid flies are completely monogenic (i.e. females produce broods of one offspring sex only, thus exhibiting "split sex ratios" (Haig, 1993b)) or have monogenic strains. This will presumably eliminate selection in favor of male suicide as males do not have sisters to channel indirect benefits. Simulations confirm (results not shown) that a monogenic population cannot be invaded by paternally inherited alleles that cause male suicide. Whether the converse also holds true – that monogeny is an adaptation to male suicide – remains an interesting speculation. Monogeny appears to be quite rare, having been found mostly in dipteran species with PGE: Sciarids and Cecidomyids (Dorchin & Freidberg, 2004; Haig, 1993b).

Currently no direct evidence for paternally expressed male suicide is available for species with PGE. However many species are poorly studied and male-suicide will be hard to observe as it might it might only reveal itself as female-biased sex ratios, which could be easily overlooked or interpreted as facultative sex ratio adjustment. Furthermore, observing male-suicide might be difficult as once such a phenotype evolves there will be strong selection on maternal genes, for example by the suppression of the paternal genome, or by producing split sex ratios. Additionally, if such suppression does not evolve quickly enough it might lead to population extinction. Comparative approaches to testing the correlates of PGE might help us make progress though. Interestingly, one such study has recently shown that each of the two origins of embryonic PGE in scale insects is associated with an increase in net diversification rate, possibly indicating a reduced extinction rate as a result of suppressing paternal gene expression (Andersen, 2009).

The evolution of suicidal phenotypes might seem counter-intuitive, but there are ample examples in other contexts. Perhaps best known are those induced by endosymbiotic bacteria that either kill their male host (and thereby themselves) to benefit related endosymbionts in females: "male-killing" (Hurst, 1991, 1995) or that kill early embryos resulting from crosses between an infected male and uninfected female: "cytoplasmic-incompatibility" (Wade & Stevens, 1985; Werren *et al.*, 2008). Similar transmission genetics impose similar selection on mitochondria. Although mitochondria have not been found to induce male suicide, they have been linked to reduced male fitness, especially reducing sperm function in a number of taxa (Wade & Brandvain, 2009). Additionally mitochondria have been found to induce the sterility of male function in hermaphroditic plants (Saumitou-Laprade *et al.*, 1994). Finally mitochondria have recently been found to play a crucial role in apoptosis (programmed cell death: (Blackstone & Green, 1999)), although the evolutionary significance of this finding is not well understood. Wade and Brandvain (2009) recently showed that although mitochondria cannot obtain any direct fitness through males, either under inbreeding or in situations where males help their sisters, they can obtain indirect fitness. This might explain why there is selection against mitochondrial mutations that have a deleterious effect on male fitness under these conditions.

However, as our model shows, under conditions of sib competition, such a mutation might spread.

Other genetic entities that under certain conditions could be selected to induce suicide are the polar bodies. These cells form during meiosis and contain the three haploid genome sets that do not form the final germ cell. In most species these cells quickly degenerate although in some taxa they persist, for instance forming the endosperm in plants (Haig, 1986). Similarly, in some scale insects the maternally-derived polar bodies fuse with an embryonic cell to form the organ in which the endosymbiotic bacteria reside (Brown, 1965; Normark, 2001, 2004b; Tremblay & Caltagirone, 1973). This inclusion of the maternally-derived polar bodies in an embryo might increase genomic conflicts within the individual as it creates tissue which contains both maternal and embryonic genes (Burt & Trivers, 2006; Normark, 2001, 2004b). With sibling-competition, the interests of the embryo- and polar body-derived genes might not coincide as some polar body genes might be absent from the embryo but present in its siblings and so in line with the previous argument for the evolution of paternally-expressed male-killing, the genes derived from the maternal polar bodies might also be selected to evolve suicide (Normark, 2001). Therefore some of the variation in bacteriome formation found in mealybugs and armored scale insects might have evolved through selection on chromosomes outside the bacteriome to limit the expression of suicidal genes. For example, Brown (1965) showed that in some armored scale insect species the bacteriome contains three condensed haploid genomes. He suggested that these are the chromosomes from the polar-bodies that, although present, have been deactivated (Normark, 2001). If this is indeed the case it shows an interesting similarity with the fate of the paternal genome in the soma of males with PGE.

An important assumption underpinning our models is that there is competition among siblings and that the resources that become available through the death of a male can be used by its sisters. There is evidence of sibling competition in a species of mite with PGE (Nagelkerke & Sabelis, 1998), while scale insects (where PGE is the most common genetic system) have evolved several reproductive adaptations that lead to intensive and prolonged contact between siblings. For example vivipary and ovoviviparity are common among scale insects and many taxa have evolved an ovisac or a marsipium in which their offspring develop (Gullan & Kosztarab, 1997). Moreover, scale insects are also often sedentary and settle close to the place they were born, typically forming large colonies on host plants. Due to these factors strong sibling-competition might be expected (Normark, 2001, 2004a) .

However, the flip-side of an ecology that promotes sibling competition is that it might also promote sib-mating. Recently it has in fact been noted that paternal genome elimination often evolves in species with mating systems that lead to high levels of sib-mating (Hamilton, 1993; Normark, 2004a). Our results show that whilst under PGE paternal suicide genes can invade, inbreeding leads to a lower level of suicide. It is therefore tempting to suggest that inbreeding might be required to prevent population extinction (due to fixation of paternally expressed suicide genes)

and perhaps this is why PGE is observed primarily in species with high levels of sib-mating. However, it will be difficult to disentangle the opposing effects of sib-competition and sib-mating in promoting or preventing male suicide.

In this paper we have presented the possibility that in species with paternal genome elimination intra-genomic male killing can evolve. The conditions that are required for the evolution of intra-genomic male killing to evolve are similar to those required for inter-genomic, endosymbiont induced male killing (Hurst, 1991). Furthermore, most taxa with PGE harbor endosymbiotic bacteria (Normark, 2004a), with which they often have an intimate and obligate association. This suggests that in many of these taxa both the endosymbiont and the paternal genome in males could be selected to induce male killing and this therefore raises the tantalizing possibility that inter- and intra-genomic suicidal interests may interact to facilitate male-killing.

Acknowledgements

We would like to thank Benjamin Normark and one anonymous reviewer for their careful comments on this manuscript. We were supported by the Natural Environment Research Council and the University of Groningen.

APPENDIX 1: sex ratio co-evolution

Here we derive an inclusive fitness model for the co-evolution of brood ratios under maternal control in a subdivided population of patches with n females each.

A focal mother produces a brood sex ratio s_b (proportion sons), while the patch-level mean sex ratio is s_p. Her fitness through daughters is then given by

$$W_f = (1 - s_b) y_b \,. \tag{A1}$$

Note that $y_b = y_0 + (1 - y_0) b s_b x_b$ depends on the brood sex ratio, and this is where our model differs from the standard models of sex ratio evolution in subdivided populations (West 2009). Also note that for $x_b = 0$ our model reduces to the standard models.

A focal mother's fitness (number of mated females) through sons is given by

$$W_f = s_b (1 - x_b) y_b \frac{1 - s_p}{s_p} \,. \tag{A2}$$

The inclusive fitness effect of a small change in the mother's sex ratio is then obtained according a standard direct fitness method (Taylor and Frank 1996):

$$\Delta W_{IF} = 2 \frac{\partial W_f}{\partial s_b} r_{f_b} + \frac{\partial W_m}{\partial s_b} r_{m_p} + \frac{\partial W_m}{\partial s_b} r_{m_p} \,. \tag{A3}$$

Note that female fitness is multiplied by two to account for their double reproductive value compared to males in haplodiploids. The relatedness coefficients are as follows. The relatedness of daughters to their mother is given by

$$r_{f_b} = \frac{1 + 3f}{2 + 2f} \tag{A4}$$

Relatedness of sons to their mother: $r_{m_p} = 1$; relatedness of random male to mother:

$$r_{m_p} = 1/n \tag{A5}$$

Analytical solutions of (A3) are easily available but rather uninformative. In the case of $x_b = 0$ they reduce to well-known results (Hamilton 1979, Taylor and Bulmer 1980, West 2009).

In the scenario of co-evolving suicide rates and sex ratios, equations (5) and (A3) must be solved simultaneously. Note that (8) is no longer an explicit solution of (5), since the s in (5) now depends on x. We did not analytically check for stability of solutions but relied on the individual-based simulations to verify stability properties.

APPENDIX 2: Details of individual-based simulation models

(1) Paternally expressed male suicide

The simulations work with a population of diploid individuals, sub-divided into n_P standard-sized patches, each founded by n mated females. Each female lays a clutch of $k = 50$ offspring with a binomial sex ratio determined by a single additive gene locus. The early survival of male offspring is determined by an additional unlinked single gene locus x which is paternally expressed. The survival of the remaining offspring is influenced by (1) the number of male sibs that have died; and (2) the efficiency b of re-allocation of dead sibs. Specifically, survival y_b follows:

$$y_b = 0.5 + 0.5b\,\frac{k - k'}{k - 1}$$

where k' is the number of surviving siblings after male suicide. Note that $0.5 \leq y_b \leq 1$.

The surviving offspring mate with a random individual from the same patch. When there are no males in a patch all females are unable to mate and the patch will go extinct. After mating females disperse with probability d. The dispersing females are randomly assigned to a patch until the n breeding positions on a patch are occupied.

Alleles were mutated with a rate of 0.01 per generation, and given that a mutation occurred, the mutation step size was drawn from a normal distribution with mean zero and standard deviation 0.01 (see table A.1). More realistic lower mutation rates (e.g. 10^{-6}) did not affect the evolutionary trajectories, but did slow down the simulations considerably.

(2) Extinction under random mating

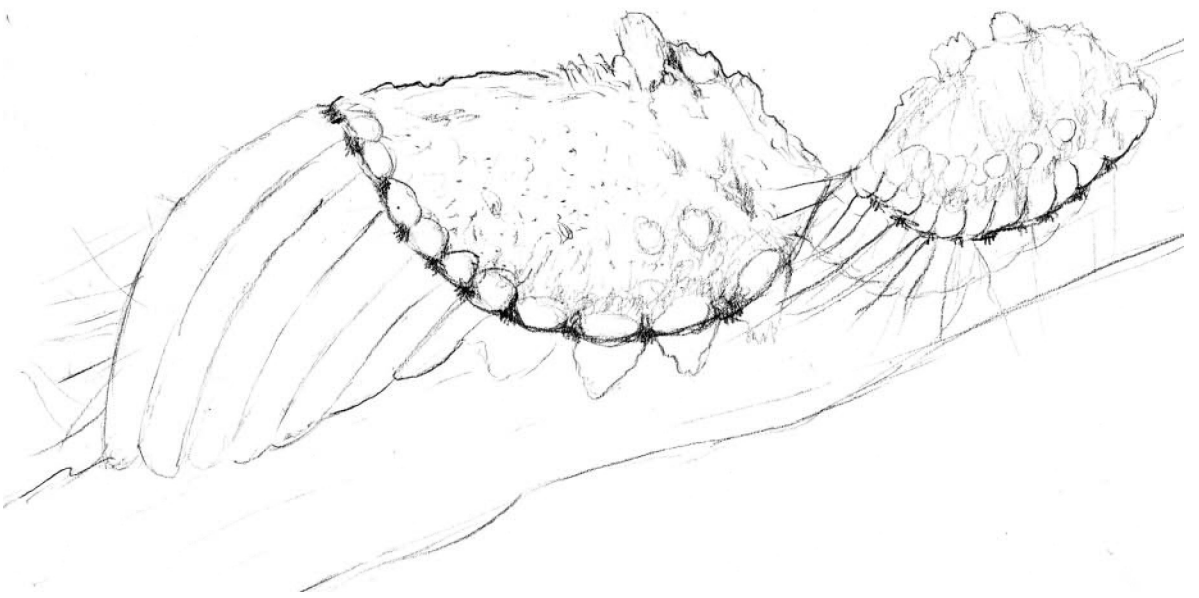
In this simulation we test if male suicide can lead to population extinction when there is no inbreeding (under random mating). The simulation is similar to the one described above but with two important differences. First of all in this simulation we assume one large random-mating population (instead of a sub-divided population as previously assumed). Secondly here we make an additional assumption on the number of females a male can successfully inseminate, with a maximum of 20 females per male. Each female in the population is randomly assigned a mate, however when her mate has already had 100 previous mating, the female remain uninseminated and will fail to produce offspring. See table A1 for the parameter values used in this simulation.

(3) Maternal suppression

This simulation explores the evolution of a gene that suppresses the paternally inherited suicide genes. The simulation is identical to described above, except an additional independently segregating gene coding for maternally inherited suppression, that determines the probability of expression of x.

Table A.1 Overview, description and values of the parameters used in the simulations. The numbers in brackets in the third column show which parameter values have been used in each simulation and correspond with those in Appendix 2 (simulation 1: Paternally-expressed male suicide, results shown in figure 4.1, simulation 2: Maternal suppression, results shown in figure 4.2 and simulation 3: Polar body induced male suicide, results shown in figure 4.3)

Parameter	Description	Value used in simulation
n_P	Number of patches	2500 (1,3), 1 (2)
n	Number of mated females per patch	4 (1,3), 10000 (2)
k	Clutch size	10 (1,2,3)
s	Sex ratio	evolving (1,2,3)
b	Efficiency re-allocation of dead sons	1.0 (1)
		0, 0.1, 0.5, 1.0 (2)
		0.5 (3)
x	Male suicide rates	evolving (1,2,3)
z	Suppressor gene (maternally expressed)	evolving (2)
μ	Mutation probability	0.01 (1,2,3)
σ	Standard deviation mutation size	0.01 (1,2,3)



CHAPTER 5

The evolution of hermaphroditism by an infectious male-derived cell lineage: an inclusive fitness analysis

Andy Gardner & Laura Ross

ABSTRACT

There has been much recent interest in the role for genetic conflicts to drive the evolution of genetic systems. Here we consider the evolution of hermaphroditism in the scale insect tribe Iceryini, and the suggestion that this has been driven by conflict between a female and an infectious male tissue derived from her father. We perform an inclusive fitness analysis to show that, owing to genetic relatedness between father and daughter, there is scope for collaboration as well as conflict over the establishment of the infectious tissue. We also consider the evolutionary interests of a maternally-inherited bacterial symbiont, that has been implicated in mediating the tissue's establishment. More generally, our analysis reveals that genetic conflicts can drive the evolution of hermaphroditism.

INTRODUCTION

There exists a wide diversity of reproductive strategies among multicellular organisms, and understanding the evolutionary significance of this variation remains an important challenge for evolutionary biologists (Policansky 1982; Heller 1993; Barrett 2002; Normark 2003; de Jong & Klinkhamer 2005; Avise & Mank 2009). The first and most fundamental difference in the way that organisms reproduce is the distinction between sexual and asexual reproduction (Cuellar 1977; Judson & Normark 1996; Vrijenhoek 1998; Otto 2009). A second important difference, among sexual organisms, is between those species with separate sexes (gonochorism) and those in which the same individual produces both male and female gametes (hermaphroditism; (Charnov, Smith & Bull, 1976; Ghiselin, 1969). Hermaphroditism is found in a large number of taxa, across a wide taxonomic range (Ghiselin 1969; Charnov *et al.* 1976; Barrett 2002; Jarne & Auld 2006). Although hermaphroditism is very common in some taxonomic groups, it is rare or absent from others. For example, whilst only 5–6% of all animal species are estimated to be hermaphroditic, the estimate rises to ~30% if insects are excluded (Schärer, 2009). The reasons for the rarity of hermaphroditism among insects, a speciose group characterized by its wide diversity of genetic systems, remain obscure.

The traditional paradigm for understanding the evolution of genetic systems has been to seek adaptive explanations at the level of the individual organism (Bull, 1983; Darlington, 1958). Thus: a separation of the sexes is expected when there are efficiency benefits for individuals specializing in a single reproductive mode (Charnov, 1982; Charnov *et al.*, 1976); sequential hermaphroditism is expected when one sex benefits from a size difference more than the other (Ghiselin, 1969); and simultaneous hermaphroditism is expected to evolve when finding a partner or investing in specific sexual function is expensive (Charnov *et al.* 1976; Puurtinen & Kaitala 2002). Such explanations have focused upon ecological and demographic factors. For example, both low population density and impaired mobility has been suggested to drive the evolution of simultaneous hermaphroditism, owing to scarcity of mating opportunities (Ghiselin 1969; Puurtinen & Kaitala 2002; Eppley & Jesson 2008).

In contrast to this traditional approach, recent years have seen growing interest in the role for conflicts between genes to mediate the evolution of novel genetic, reproductive and sex-determination systems (Haig 1993; Hurst 1995; Hurst *et al.* 1996; Werren & Beukeboom 1998; Hurst & Werren 2001; Normark 2004; Burt & Trivers 2006; Uller *et al.* 2007; Van Doorn & Kirkpatrick 2007). One source of conflict that has been especially well documented is that between nuclear and cytoplasmic genes (Cosmides & Tooby 1981; Hurst 1992; Werren & Beukeboom 1998; Charlat *et al.* 2003; Wernegreen 2004; Burt & Trivers 2006). Many insects harbour intracellular bacteria that are transmitted only via daughters (Buchner, 1965); Moran & Telang 1998; Moran & Baumann 2000; Moran 2002), and hence have an interest in biasing their host's sex allocation towards females (Cosmides & Tooby 1981; Stouthamer

et al. 1990; Werren *et al.* 2008). Another source of conflict is that between females and males in species with sex-asymmetric transmission. In haplodiploid species – where females develop from fertilized (i.e. diploid) eggs and males develop from unfertilized (i.e. haploid) eggs – males pass on their genes only through daughters, whereas females can achieve fitness through both offspring sexes, leading to a potential for conflict over sex allocation (Normark 2009; Shuker *et al.* 2009). Females typically control sex allocation by deciding the fraction of eggs to be fertilized, and any male adaptation to increase this fraction would be strongly favoured.

Such conflict over fertilization rate has been suggested to have driven the evolution of an unusual form of hermaphroditism, found in three species of the scale insect tribe Iceryini (Hemiptera: Coccoidea; (Normark, 2003; Nur, 1980) – the only known instance of hermaphroditism in insects (Hughes-Schrader 1925; Hughes-Schrader 1930; Royer 1975). Scale insects are small, plant-feeding insects (Gullan & Kosztarab, 1997; Ross & Shuker, 2009), that exhibit a remarkable variety of genetic systems – a diversity that has been suggested to reflect the operation of extensive genetic conflicts (Ross *et al.* 2010). Hermaphroditism in scale insects has evolved in an otherwise haplodiploid clade (Hughes-Schrader & Monahan 1966; Nur 1980; Ross *et al.* 2010), and molecular phylogeny suggests that it has evolved independently in each of the three species for which it has been described (Unruh & Gullan 2008). In the hermaphroditic species of *Icerya*, males are rare and females – who contain an ovitestis, capable of producing sperm and oocytes – can internally self-fertilize and hence produce offspring in the absence of a mating partner (Hughes-Schrader, 1925). The sperm-producing gonads of the ovitestis are haploid (Hughes-Schrader, 1963), and this tissue appears to derive from excess sperm that penetrated the oocyst when the female was conceived (Royer, 1975). Normark (2009) has suggested that this peculiar reproductive mode has been driven by conflict between males and females over genetic transmission: by infecting his daughters with cells that form male gametes inside their bodies, a father is able to fertilize the eggs of his daughters as well as those of their mother.

Here we perform an inclusive fitness analysis to examine the evolutionary origin and subsequent spread of infectious male tissue. Whilst Normark (2009) has suggested that the infectious tissue is always parasitic upon the female, and will always spread owing to the transmission advantage that it provides for the male, we consider the possibility for collaboration as well as conflict between the female and her infectious tissue. Some overlap of interests is possible, owing to genetic relatedness between father and daughter; the former perhaps showing some restraint, and the latter perhaps showing some shared interest in allowing the infectious tissue to establish. In addition, we consider the interests of a maternally-inherited bacterial symbiont, which has been implicated in facilitating the establishment of the infectious tissue (Ross *et al.*, 2010b; Royer, 1975). More generally, our analysis confirms that genetic conflicts may have driven the evolution of this unusual form of hermaphroditism.

MODEL AND ANALYSIS

Basic model

We build upon the familiar model of haplodiploidy, in which the family unit is made up of an adult female (F), an adult male (M), a juvenile daughter (D) and a juvenile son (S). Females are diploid, with one maternal and one paternal genome, and males are haploid, with one maternal genome. We extend this model by additionally assigning every female a haploid infectious tissue (T), and we allow this tissue to father some of the female's daughters (and hence also their infectious tissues). We thus discriminate five classes of juvenile individual: α-sons, regular males derived from unfertilized eggs in the usual way; β-daughters, regular females fathered by regular males in the usual way; γ-daughters, females that are fathered by their mother's infectious tissues; δ-sons, infectious tissues that are fathered by regular males, and incorporated into the bodies of β-daughters; and ε-sons, infectious tissues fathered by infectious tissues, and incorporated into the bodies of γ-daughters. For simplicity, we assume that females are unrelated to regular males with which they mate. An illustration of the model is given in Figure 5.1.

The behaviour of an adult female and her infectious tissue impacts upon the allocation of reproductive resources to each of her five types of offspring. With probability 1-a the infectious tissue fails to establish in the focal female's body, and in this event the female fertilizes a proportion x of her eggs using sperm derived from a regular male and a proportion 1-x of her eggs remain unfertilized. With probability a the infectious tissue successfully establishes, which incurs a relative fecundity cost k for the female, and in this event she fertilizes a proportion x' of her eggs using sperm derived from a regular male, and her infectious tissue fertilizes a proportion y of the remaining eggs. Hence, denoting the number of eggs produced by an uninfected female by n, the expected numbers of offspring of each class produced by the focal female are: $n_\alpha = n((1-a)(1-x)+a(1-k)(1-x')(1-y))$ α-sons; $n_\beta = n((1-a)x+a(1-k)x')$ β-daughters; $n_\gamma = na(1-k)(1-x')y$ γ-daughters; $n_\delta = n((1-a)x+a(1-k)x')$ δ-sons; and $n_\varepsilon = na(1-k)(1-x')y$ ε-sons (Table 5.1). Thus, the expected numbers of male, female and tissue offspring produced by the focal female are $n_m = n_\alpha$, $n_f = n_\beta + n_\gamma$ and $n_t = n_\delta + n_\varepsilon$, respectively. We denote population averages (for example, of x) with an overbar (for example, $\overline{x}$). We also denote the sex ratio (proportion of regular individuals who are male) by $z = \overline{n}_m/(\overline{n}_m+\overline{n}_f)$ and the proportion of females that are of type β by $\phi = \overline{n}_\beta/\overline{n}_f$.

Inclusive fitness

A focal actor is expected to value her social partners according to how well they transmit copies of her genes to future generations (Frank, 1998; Hamilton, 1964). This is product of two quantities: first, the social partners' ability to transmit copies of their own genes to future generations (reproductive value, v: Fisher, 1930; Frank, 1998); and second, the extent to which genes transmitted by the social partners are the same as those carried by the actor (relatedness, r: Frank, 1998; Hamilton, 1964).

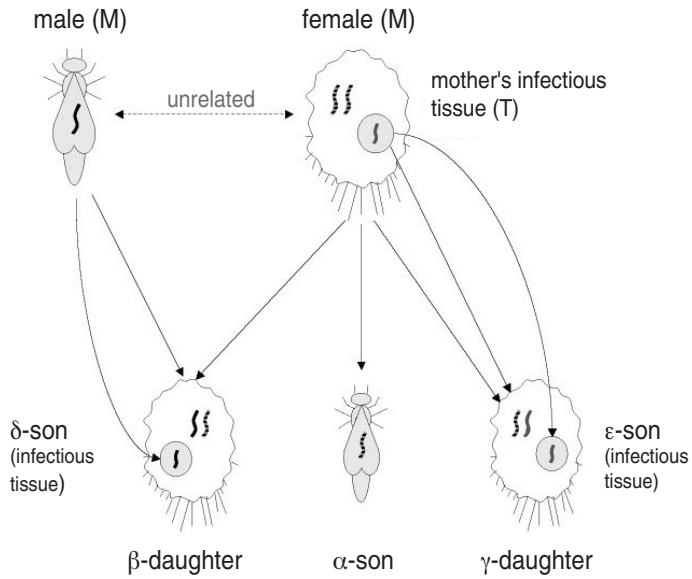


Figure 5.1 THE FAMILY UNIT. Our model is based upon standard haplodiploid inheritance, with only the mother (M) contributing a genome to her haploid son (α-son) and with the mother and father (F) each contributing a genome to their diploid daughter (β-daughter). In addition, the father contributes a genome to infectious tissue that grows in his daughters (δ-son), and the mother's infectious tissue (T) can fertilize her eggs to produce daughters (γ-daughter) and also further infectious tissues (ε-son).

Table 5.1 Offspring type, number, reproductive value and relatedness to mother and infectious tissue. The proportion of females who are β-daughters is $\phi = \overline{n}_\beta/(\overline{n}_\beta + \overline{n}_\gamma)$, and the average number of offspring of each sex is $\overline{n}_m = \overline{n}_\alpha$ males, $\overline{n}_f = \overline{n}_\beta + \overline{n}_\gamma$ females and $\overline{n}_t = \overline{n}_\delta + \overline{n}_\varepsilon$ infectious tissues.

Type (X)	Number (n_X)	Reproductive value (v_X)	Relatedness to mother (r_{FX})	Relatedness to infectious tissue (r_{TX})
α	$(1-x)(1-a(1-(1-k)(1-y)))$	$\frac{\phi}{\overline{n}_m}$	1	$\frac{1}{1+\phi}$
β	$x(1-ak)$	$\frac{2\phi}{\overline{n}_f}$	1/2	$\frac{1}{2+2\phi}$
γ	$a(1-k)(1-x)y$	$\frac{2\phi}{\overline{n}_f}$	1	$\frac{2+\phi}{2+2\phi}$
δ	$x(1-ak)$	$\frac{1-\phi}{\overline{n}_t}$	0	0
ε	$a(1-k)(1-x)y$	$\frac{1-\phi}{\overline{n}_t}$	1	1

We assume all genetic similarity owes to shared genealogy, e.g. we exclude greenbeard effects (Gardner & West, 2010). Thus, in the context of the present model, the inclusive fitness H_{A} of an actor A is defined as:

$$H_{\mathrm{A}} = n_{\alpha} v_m r_{\mathrm{A}\alpha} + n_{\beta} v_f r_{\mathrm{A}\beta} + n_{\gamma} v_f r_{\mathrm{A}\gamma} + n_{\delta} v_t r_{\mathrm{A}\delta} + n_{\varepsilon} v_t r_{\mathrm{A}\varepsilon}, \quad (1)$$

where: v_m, v_f and v_t are the reproductive values of a juvenile male, a juvenile female, and an infectious tissue residing in a juvenile female, respectively (expressions for these coefficients are provided in Table 5.1; see Appendix for derivation); and rAX is the genetic relatedness of a type X offspring to the actor A, from the perspective of the actor (expressions for these coefficients are provided in Table 5.1; see Appendix for derivation). The condition for natural selection to favour an increase any character is that this increases the inclusive fitness of the actor (Hamilton, 1964).

Female fertilization strategies

We first consider the fertilization strategies of the female. In the event that her infectious tissue does establish, she fertilizes a proportion x' of her eggs using sperm derived from a regular male. The condition for natural selection to favour an increase in the value of this character is that this increases her inclusive fitness. Assuming vanishing genetic variation, this condition is $\partial H_{\mathrm{F}}/\partial x' > 0$, i.e.:

$$\frac{\partial n_{\alpha}}{\partial x'} v_m r_{\mathrm{F}\alpha} + \frac{\partial n_{\beta}}{\partial x'} v_f r_{\mathrm{F}\beta} + \frac{\partial n_{\gamma}}{\partial x'} v_f r_{\mathrm{F}\gamma} + \frac{\partial n_{\delta}}{\partial x'} v_t r_{\mathrm{F}\delta} + \frac{\partial n_{\varepsilon}}{\partial x'} v_t r_{\mathrm{F}\varepsilon} > 0 \,, \quad (2)$$

where all derivatives are evaluated in a monomorphic population ($x = \bar{x}$, $x' = \bar{x}'$, $y = \bar{y}$, $a = \bar{a}$). Using the information provided in Table 5.1, and assuming that $\bar{y} = 1$ (justified in the next section), we find that condition (2) is never satisfied, hence the population is expected to converge upon the strategy value $x'^{*} = 0$.

In the event that the infectious tissue does not establish itself, the female fertilizes a proportion x of her eggs using sperm derived from a regular male. The condition for natural selection to favour an increase in this character is $\partial H_{\mathrm{F}}/\partial x > 0$, i.e.:

$$\frac{\partial n_{\alpha}}{\partial x} v_m r_{\mathrm{F}\alpha} + \frac{\partial n_{\beta}}{\partial x} v_f r_{\mathrm{F}\beta} + \frac{\partial n_{\gamma}}{\partial x} v_f r_{\mathrm{F}\gamma} + \frac{\partial n_{\delta}}{\partial x} v_t r_{\mathrm{F}\delta} + \frac{\partial n_{\varepsilon}}{\partial x} v_t r_{\mathrm{F}\varepsilon} > 0 \,, \quad (3)$$

where all derivatives are evaluated in a monomorphic population ($x = \bar{x}$, $x' = \bar{x}' = 0$, $y = \bar{y} = 1$, $a = \bar{a}$). Using the information provided in Table 5.1, condition (3) can be rewritten as $\bar{x} < (1 - \bar{a}(2 - \mathrm{k}))/2(1 - \bar{a})$. Hence, the population is expected to converge upon the strategy value x^{*}, given by:

$$x^{*} = \begin{cases} \dfrac{(1 - \bar{a}(2 - k)}{2(1 - \bar{a})} & \text{if} \quad \bar{a} < 1/\,(2 - k) \\ 0 & \quad\;\; \bar{a} \geq 1/\,(2 - k) \end{cases}$$

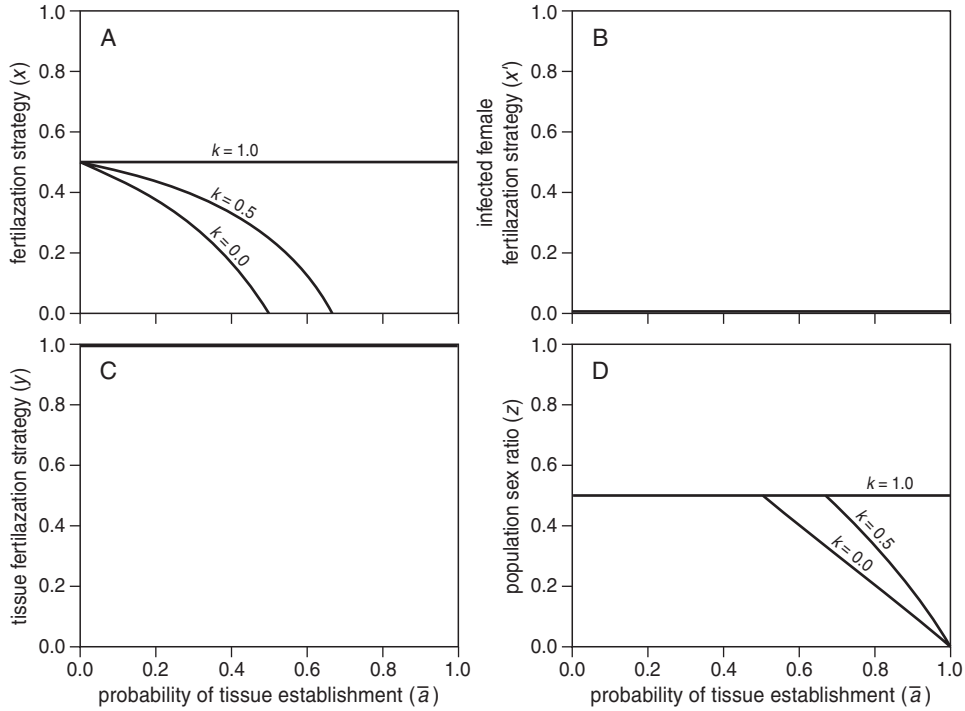


Figure 5.2 EVOLUTION OF FERTILIZATION STRATEGIES. (A) Uninfected females are favoured to fertilize a proportion of their eggs ($x^* = (1 - \bar{a}(2 - k))/2(1 - \bar{a})$) with sperm from regular males, that decreases as the probability of tissue establishment ($\bar{a}$) increases and increases as the cost of tissue establishment (k) increases. (B) Infected females are favoured to fertilize none of their eggs ($x'^* = 0$) with sperm from regular males. (C) The infectious male tissue is favoured to fertilize all of an infected female's eggs ($y^* = 1$). (D) The sex ratio ($z = \min(1/2, (1 - \bar{a})/(1 - \bar{a}k))$; proportion of regular individuals who are male) remains fixed at one half when the probability of tissue establishment is low ($\bar{a} < 1/(2 - k)$) and falls to zero as the probability of tissue establishment approaches unity ($z \to 0$ as $\bar{a} \to 1$).

Thus, the female fertilizes some or none of her eggs with sperm derived from a regular male when her infectious tissue does not establish ($x^* \geq 0$; this is $x^* = 1/2$ when $\bar{a} = 0$; Figure 5.2A), and she fertilizes none of her eggs with sperm derived from a regular male when her infectious tissue does establish ($x'^* = 0$; Figure 5.2B). As a consequence, the population sex ratio is given by $z = 1/2$ if $\bar{a} < 1/(2 - k)$ and by $z = (1 - \bar{a})/(1 - \bar{a}k)$ if $\bar{a} < 1/(2 - k)$, which decreases to $z \to 0$ as $\bar{a} \to 1$ (Figure 5.2D).

Tissue fertilization strategy

Next, we consider the fertilization strategy of the infectious tissue. In the event that the tissue does establish in the body of a female, it fertilizes a proportion y of any eggs that she has failed to fertilize using sperm from a regular male. Above, we assumed $\bar{y} = 1$; i.e. a successfully establishing tissue fertilizes all of the female's eggs.

Here we will show that this fertilization strategy is indeed the one that maximizes the tissue's inclusive fitness. The condition for natural selection to favour an increase in the tissue's fertilization strategy is $\partial H_T/\partial y > 0$, i.e.:

$$\frac{\partial n_\alpha}{\partial y} v_m r_{T\alpha} + \frac{\partial n_\beta}{\partial y} v_f r_{T\beta} + \frac{\partial n_\gamma}{\partial y} v_f r_{T\gamma} + \frac{\partial n_\delta}{\partial y} v_t r_{T\delta} + \frac{\partial n_\varepsilon}{\partial y} v_t r_{T\varepsilon} > 0 \,, \tag{5}$$

where all derivatives are evaluated in a monomorphic population ($x = \bar{x}$, $x' = \bar{x}' = 0$, $y = \bar{y}$, $a = \bar{a}$). Using the information provided in Table 5.1, condition (5) can be rewritten as $(r_{T\gamma} - r_{T\beta})v_f + r_{T\varepsilon}v_t > 0$ which (owing to $r_{T\gamma} > r_{T\beta}$) is always satisfied, hence the population will converge upon the strategy value $y^* = 1$. Thus, the infectious tissue is always favoured to fertilize all of the female's eggs (Figure 5.2C).

Tissue establishment

We now examine the evolution of the probability of tissue establishment, a. We begin by considering the interests of the female, by assigning her full control of the probability of establishment, and determining when she is favoured to increase or decrease this quantity. The condition for natural selection to favour an increase in the probability of tissue establishment is that this increases her inclusive fitness. Assuming vanishing genetic variation, this condition is $\partial H_F/\partial a > 0$, i.e.:

$$\frac{\partial n_\alpha}{\partial a} v_m r_{F\alpha} + \frac{\partial n_\beta}{\partial a} v_f r_{F\beta} + \frac{\partial n_\gamma}{\partial a} v_f r_{F\gamma} + \frac{\partial n_\delta}{\partial a} v_t r_{F\delta} + \frac{\partial n_\varepsilon}{\partial a} v_t r_{F\varepsilon} > 0 \,, \tag{6}$$

where all derivatives are evaluated in a monomorphic population ($x = \bar{x} = x^*$, $x' = \bar{x}' = 0$, $y = \bar{y} = 1$, $a = \bar{a}$). Using the information provided in Table 5.1, and assuming $\bar{a} < 1/(2-k)$ (and hence $x^* = (1-\bar{a})(2-k))/2(1-\bar{a})$), condition (6) can be rewritten as $k < 1/((2-\bar{a}))$. If instead $\bar{a} > 1/(2-k)$ (and hence $x^* = 0$), then condition (6) is always satisfied. Hence, when tissue establishment is relatively uncommon ($\bar{a} < 1/(2-k)$) the female is favoured to promote the establishment of her infectious tissue when the fecundity cost of establishment is low ($k < 1/(2-\bar{a})$) and is favoured to suppress the establishment of her infectious tissue when the fecundity cost is high ($k > 1/(2-\bar{a})$). In the special case of vanishingly rare establishment of tissues ($\bar{a} \to 0$) the maximum cost the female will endure without being favoured to suppress tissue establishment is the loss of half of her fecundity ($k = 1/2$), and as tissue establishment becomes more common (higher $\bar{a}$) the female is favoured to promote establishment for even higher fecundity costs (Figure 5.3).

Next, we consider the interests of the infectious tissue, by assigning it full control of the probability of its own establishment, and determining when it is favoured to promote or suppress its own establishment. Natural selection favours an increase in the probability of establishment when $\partial H_T/\partial a > 0$, i.e.:

$$\frac{\partial n_\alpha}{\partial a} v_m r_{T\alpha} + \frac{\partial n_\beta}{\partial a} v_f r_{T\beta} + \frac{\partial n_\gamma}{\partial a} v_f r_{T\gamma} + \frac{\partial n_\delta}{\partial a} v_t r_{F\delta} + \frac{\partial n_\varepsilon}{\partial a} v_t r_{T\varepsilon} > 0 \,, \tag{7}$$

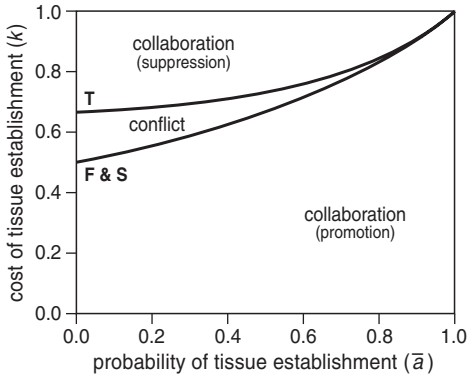


Figure 5.3 EVOLUTION OF INFECTIOUS TISSUE ESTABLISHMENT. Females (F), infectious tissues (T) and maternally-inherited symbionts (S) are all favoured to promote tissue establishment when this is sufficiently common (higher $\bar{a}$) and when the cost of tissue establishment is sufficiently low (lower k). For uncommon tissue establishment (low $\bar{a}$) and intermediate cost of establishment (intermediate k), females and maternally-inherited symbionts are favoured to suppress establishment whilst infectious tissues are favoured to promote establishment, giving rise to an evolutionary conflict. Elsewhere, all parties are favoured to either promote tissue establishment (when the cost is low; small k) or suppress tissue establishment (when the cost is high; large k), giving rise to an evolutionary collaboration. Note that the interests of females and maternally-inherited symbionts are exactly aligned for this trait.

where all derivatives are evaluated in a monomorphic population ($x = \bar{x} = x^*$, $x' = \bar{x}' = 0$, $y = \bar{y} = 1$, $a = \bar{a}$). Using the information provided in Table 5.1, and assuming $\bar{a} < 1/(2-k)$ (and hence $x^* = (1-\bar{a})(2-k))/2(1-\bar{a})$), condition (7) can be rewritten as $\bar{a}k^2 + (3–4\,\bar{a})k - 2(1-\bar{a}) < 0$. If instead $\bar{a} > 1/(2-k)$ (and hence $x^* = 0$), then condition (7) is always satisfied. Hence, when tissue establishment is uncommon ($\bar{a} < 1/(2-k)$) the tissue is favoured to promote its establishment when the fecundity cost is low ($k < (4\,\bar{a} - 3 + \sqrt{(9 - 8\,\bar{a}(2-\bar{a}))})/(2\,\bar{a})$) and is favoured to suppress its establishment when the fecundity cost is high ($k > (4\,\bar{a} - 3 + \sqrt{(9 - 8\,\bar{a}(2-\bar{a}))})/(2\,\bar{a})$). In the special case of vanishingly low frequency of tissue establishment ($\bar{a} \to 0$), the maximum fecundity cost to the female that the tissue will endure without being favoured to suppress its own establishment is corresponds to her fecundity being reduced by two thirds ($k = 2/3$), and as the tissue establishment becomes more common the tissue is prepared to accept even higher collateral damage to the female (Figure 5.3).

Notice that, when the probability of tissue establishment is low ($\bar{a} < 1/(2-k)$), both the infectious tissue and the female can be favoured to promote or inhibit the establishment of the former, depending upon the fecundity cost incurred by the latter. Moreover, the critical cost value from the perspective of the infectious tissue is always equal to or greater than the critical cost value from the perspective of the female ($0 < 1/(2-\bar{a}) < (4\,\bar{a} - 3 + \sqrt{(9 - 8\,\bar{a}(2-\bar{a}))})/(2\,\bar{a}) < 1$). Hence: when the fecundity cost is low ($k < 1/(2-\bar{a})$) both parties are favoured to promote the establishment of

the infectious tissue (collaboration); when the fecundity cost is high ($k > (4\,\bar{a} - 3 + \sqrt{(9 - 8\,\bar{a}(2 - \bar{a}))})/(2\,\bar{a})$) both parties are favoured to suppress the establishment of the infectious tissue (collaboration); and when the fecundity cost is intermediate ($1/(2 - \bar{a}) < k < (4\,\bar{a} - 3 + \sqrt{(9 - 8\,\bar{a}(2 - \bar{a}))})/(2\,\bar{a})$), the tissue is favoured to promote, and the female to suppress, the establishment of the infectious tissue (conflict). The scope for conflict narrows as the establishment of infectious tissue becomes increasingly common in the population, with both parties becoming more inclined to promote establishment (Figure 5.3).

Finally, we consider the interests of a maternally-inherited symbiont carried by the female, by assigning it control of the probability of infectious tissue establishment, and seeing how it is favoured to adjust this. The condition for natural selection to favour an increased probability of tissue establishment is $\partial H_S/\partial a > 0$, i.e.:

$$\frac{\partial n_\alpha}{\partial a} v_{m|S} r_{S\alpha} + \frac{\partial n_\beta}{\partial a} v_{f|S} r_{S\beta} + \frac{\partial n_\gamma}{\partial a} v_{f|S} r_{S\gamma} + \frac{\partial n_\delta}{\partial a} v_{t|S} r_{S\delta} + \frac{\partial n_\varepsilon}{\partial a} v_{t|S} r_{S\varepsilon} > 0\,, \qquad (8)$$

where: reproductive values are in terms of transmission of symbionts, rather than autosomal genes (i.e. $v_{m|S} = v_{t|S} = 0$, $v_{f|S} = 1$); relatedness coefficients are in terms of presence or absence of a descendant symbiont (i.e. $r_{S\alpha} = r_{S\beta} = r_{S\gamma} = 1$, $r_{S\delta} = r_{S\varepsilon} = 0$); and where all derivatives are evaluated in a monomorphic population ($x = \bar{x} = x^*$, $x' = \bar{x}' = 0$, $y = \bar{y} = 1$, $a = \bar{a}$). Using the information provided in Table 5.1, and assuming $\bar{a} < 1/(2 - k)$ (and hence $x^* = (1 - \bar{a})(2 - k))/2(1 - \bar{a})$), condition (8) can be rewritten as $k < 1/(2 - \bar{a})$. If instead $\bar{a} > 1/(2 - k)$ (and hence $x^* = 0$), then condition (8) is always satisfied. Notice that these are precisely the conditions derived under the assumption of female control of tissue establishment. Hence, the interests of the maternally-inherited symbiont and the female are exactly aligned in this respect (Figure 5.3).

DISCUSSION

We have considered the evolution of hermaphroditism, driven by genetic conflicts between the sexes in an ancestrally-haplodiploid population. This hypothesis, proposed by Normark (2009), suggests that by infecting females with sperm-producing tissue, males may fertilize not only their partners, but also their future daughters. We have performed an inclusive fitness analysis of this evolutionary model, confirming the potential for a genetic conflict of interests to have driven this unusual form of hermaphroditism. However, whilst Normark (2009) assumed that the infectious male tissue would always be parasitic – harmful to the interests of females, and favoured solely on the basis of a selfish transmission advantage – we have shown that there is scope for collaboration as well as conflict between females and their infectious male tissues in the evolution of this novel reproductive system.

In particular, we have found that, owing to relatedness between father and daughter and hence between a female and her infectious male tissue, the infectious

tissue can be favoured to suppress its own establishment if the fecundity costs incurred by the host female are too great and, conversely the female may be favoured to promote the establishment of the tissue if the fecundity costs are sufficiently low. Thus, whilst each party may disagree over the critical values of these fecundity costs (the male accepting a greater collateral damage to the female's fecundity than the female is prepared to accept for herself), giving rise to a zone of conflict in the parameter space defined by the evolutionary model, there is also scope for both parties to collaborate in establishing the infectious tissue and thereby promoting the evolution of hermaphroditism (Figure 5.3).

Considering the evolutionary origin of the infectious tissue, our model predicts that the tissue itself would be favoured to pursue this unusual mode of transmission only when the relative fecundity cost to the infected female was less than two thirds. Before having been honed by natural selection, to become adapted to its new environment within the female's body, the infection can be expected to have caused disruption to normal female function, and hence incurred substantial fecundity costs. It seems very likely, then, that the early stages of the evolution of this reproductive mode occurred within the zone of conflict between the female and her infectious tissue (i.e. $1/2 < k < 2/3$; Figure 5.3). Hence, the females would initially have been favoured to suppress the establishment of the infection, before eventually their interests aligned and conflict gave way to collaboration. We might therefore expect to find remnants of this historical conflict in the biology of contemporary infections.

Although lack of adaptedness to the internal environment of the female would have presented a barrier to the initial evolution of the infectious tissue, this barrier need not have been insurmountable. Indeed, very little structural adaptation appears to have been necessary, as the ovitestis strongly resembles the original female ovaries, and the testis portion serves the dual role of sperm production and sperm transport (becoming hollow as the sperm mature, and forming a duct by which they reach the maturing oocytes (Hughes-Schrader, 1925). Also, the male and female function of the ovitestis is separated in space and time, with sperm developing first and in the central portions of the ovitestis and the oocytes developing later and on the periphery of the common gonad (Hughes-Schrader, 1925).

A curious aspect of the developmental biology of the infectious male tissue is the interaction this appears to have with endosymbiotic bacteria, inherited from the mother, during early embryonic development. Although there is no conclusive evidence that the endosymbiont – which *Icerya* harbours for nutritional reasons – is involved in the establishment of the infectious tissue, Royer (1975) observed that there was a strong physical association between the developing haploid cells and the bacteria, with the bacteria surrounding the haploid cells. Royer (1975) suggested that the bacteria may protect the haploid cells from degeneration, and hence play a crucial role in the evolution of their host's hermaphroditism. In order to assess the likelihood of this suggestion, we investigated the evolutionary interests of a maternally-inherited symbiont with regards to the establishment of the infectious tissue. The symbiont is expected to promote tissue establishment when this increases the expected

number of daughters produced by its host. In the context of our model, we found that the interests of the symbiont are exactly aligned with those of the female host: although ultimately the inclusive fitness objectives of the two parties are not the same, they are in perfect agreement more proximately, in terms of how large a fecundity cost should be endured before suppression of the infectious tissue is favoured (Figure 5.3). Thus, the endosymbiont does have a stake in mediating the establishment of the infectious male tissue. Endosymbiotic bacteria in other taxa have proven capable of manipulating their host's reproduction in numerous ways: if this role of endosymbionts in *Icerya* were to be confirmed, it would provide the first known example of endosymbiont-induced hermaphroditism.

Our model accounts for the rarity of males among the hermaphroditic species of *Icerya*. Although all three species can reproduce by "selfing", regular males have been observed in each of these species, where they develop from unfertilized eggs. The reported frequencies of males vary between studies and species (roughly 0-10% Hughes-Schrader 1925, 1930, 1963; Hughes-Schrader & Monahan 1966). We have shown that, for populations in which it is the norm for females to carry the infectious tissue ($\overline{a} < 1/(2 - k)$), those females for which the male tissue has failed to establish are predicted to fertilize none of their eggs ($x^* = 0$; Figure 5.2A). Hence, regular males are expected to be produced whenever there is a less than perfect rate of infection, even though they have essentially zero reproductive value: the behaviour of the females that leads to regular males being produced (failure to fertilize eggs with sperm from regular males; Figure 5.2A) means that there is essentially no prospect for regular males to achieve reproductive success!

Why are uninfected females favoured to invest resources into the production of sons, when they could use sperm from such regular males to fertilize their eggs, and hence produce daughters who could go on to have their own offspring? The reason is that daughters have similarly bleak prospects, in terms of longer-term reproductive value. Whilst these daughters can reproduce, essentially all of the genetic ancestry of the population belongs to the infectious tissues. As the proportion of daughters fathered by regular males falls to zero, so to does the reproductive value of females, and hence so too does the inclination of the female to fertilize her eggs with sperm from regular males. Factoring in the higher relatedness to sons than to daughters, uninfected females maximize their miniscule inclusive fitness by fertilizing none of their eggs. More generally, once all daughters are fathered by infectious tissues, the only prospect for a female to achieve inclusive fitness is by producing daughters to serve as vehicles that carry the male infection into future generations.

There is growing interest in the role for genetic conflicts to explain the evolutionary transitions between several genetic systems, including the evolution of well-known and widespread systems such as haplodiploidy and parthenogenesis (Bull 1979; Hurst *et al.* 1990; Normark 2004). The hypothesis considered in this paper constitutes the first suggestion that the evolution of hermaphroditism can been driven by such conflicts (Normark, 2009). In other taxa, genetic conflicts have been implicated in evolutionary transitions in the opposite direction: e.g. cytoplasmic

sterility as an adaptation of mitochondria to induce loss of male function in hermaphroditic plants, to give rise to a system of gynodioecy (Saumitou-Laprade *et al.*, 1994). More generally, whilst the ecological dominance of one reproductive mode over another may be determined by such factors as mate availability and the costs and benefits of specializing in different sexes, the evolutionary transitions between such systems may be driven by rather different pressures, including conflict between genes over their transmission.

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APPENDIX

Reproductive value

The reproductive value of a class is the expected asymptotic contribution of genes made by individuals of that class to future generations (see Taylor & Frank, 1996 for an accessible account). This can be calculated recursively: the reproductive value of a focal class is equal to the total reproductive value of all classes in the next generation, each being weighted by the proportion of its genes donated by the focal class in the current generation. We will consider three classes: males (m; comprising α-males), females (f; comprising β-females and γ-females), and infectious tissues (t; comprising d-tissue and e-tissues). The reproductive value of the male class is $c_m = \sum_X g_{X \leftarrow m} c_X$, where $g_{X \leftarrow m}$ is the proportion of class-X genes contributed by males (i.e. $g_{m \leftarrow m} = 0$, $g_{f \leftarrow m} = \phi/2$ and $g_{t \leftarrow m} = \phi$). We can write corresponding equations for each of the three classes, and summarize these in linear algebraic form:

$$(c_m \;\; c_f \;\; c_t) = (c_m \;\; c_f \;\; c_t) \begin{pmatrix} 0 & 1 & 0 \\ \phi/2 & 1/2(1-\phi)/2 \\ \phi & 0 & 1-\phi \end{pmatrix}, \qquad \text{(A1)}$$

where: $\phi = \overline{n}_\beta / \overline{n}_f$ is the proportion of females who are of type β (see main text); and each element of the gene-flow matrix specifies the proportion of genes in the recipient class (row) that derive from the donor class (column). The class reproductive values are found by solving equation (A1). (Formally, they are given by the left eigenvector of the gene-flow matrix; Taylor 1996) They are $c_m = \phi/(1+2\phi)$, $c_f = 2\phi/(1+2\phi)$ and $c_t = (1-\phi)/(1+2\phi)$. Note that, for the classical haplodiploidy scenario ($\overline{\phi} = 0$), all reproductive value belongs to males and females ($c_m + c_f = 1$, $c_t = 0$), and the class reproductive values are in the usual ratios ($c_m = 1/3$, $c_f = 2/3$). Conversely, if all females are fathered by infectious tissue ($\overline{\phi} = 1$) then all reproductive value belongs to the infectious tissues ($c_m + c_f = 0$, $c_t = 1$).

In a monomorphic population, the reproductive value of a class is shared equally over all individuals in that class. Since we may scale reproductive values by any constant of proportionality K, we can write the reproductive value of an individual male as $v_m = K\, c_m \,/\, T\, \overline{n}_m$, where T is the total number of adult females in the population. Setting $K = T(1+2\phi)$ obtains $v_m = \phi \,/\, \overline{n}_m$, and similarly the reproductive value of an individual female is $v_f = 2\phi \,/\, \overline{n}_f$, and the reproductive value of an individual infectious tissue is $v_t = (1-\phi) \,/\, \overline{n}_t$. These expressions are listed in Table 5.1.

Relatedness

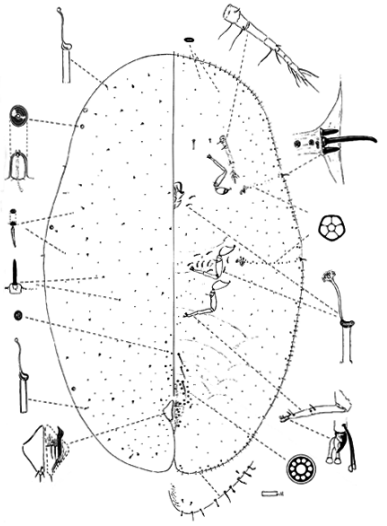
Analysis of kin selection in our model requires calculation of probabilities for social partners to share genes that are identical by descent: these are termed coefficients of consanguinity (Bulmer, 1994). The consanguinity between an actor A and a social partner X will be denoted p_{AX}. The actor will be either the adult female (F) who is mother to the brood, or her infectious tissue (T), or a maternally-inherited symbiont

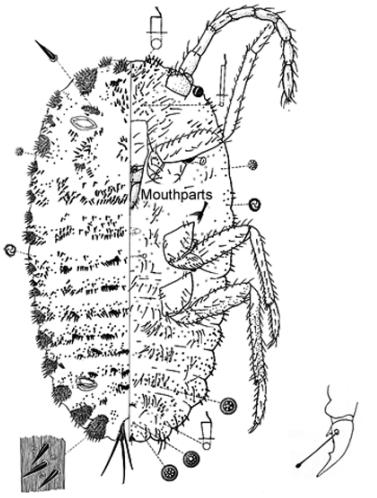
also carried by the mother (S). The recipient is an individual of one of the five types of offspring (α–ε).

We begin by denoting the consanguinity of an adult female to her infectious tissue by p; this is the probability that two genes picked at random from the same locus from these two individuals are identical by descent. Note that, because the female's infectious tissue is genetically identical to her paternal genome (both deriving from her haploid father), the consanguinity of the female to herself is also p. This is the probability that two genes picked at random, with replacement, from any one of her loci, are identical by descent, and is given by $p = (1+f)/2$, where f is the consanguinity of her parents. With probability ϕ she is a β-female (her father was a regular male), in which case her parents were unrelated; otherwise, with probability $1-\phi$, she is a γ-female (her father was her mother's infectious tissue), in which case the consanguinity of her parents is p. Thus $f = (1-\phi)p$, and hence $p = 1/(1+\phi)$.

The consanguinity of the female to her: α-son is $p_{F\alpha} = p$ (she supplies her son's genome); β-daughter is $p_{F\beta} = p/2$ (she supplies one of her daughter's genomes, and an unrelated male supplies the other); γ-daughter is $p_{F\gamma} = p$ (she supplies one of her daughter's genomes, and her infectious tissue supplies the other); δ-tissue is $p_{F\delta} = 0$ (an unrelated male supplies this genome); ε-tissue is $p_{F\varepsilon} = p$ (her infectious tissue supplies this genome). The consanguinity of the female's infectious tissue to the: α-son is $p_{T\alpha} = p$ (the female supplies the son's genome); β-daughter is $p_{T\beta} = p/2$ (the female supplies one of the daughter's genomes, and an unrelated male supplies the other); γ-daughter is $p_{T\gamma} = p/2 + 1/2$ (the female supplies one of the daughter's genomes, and her infectious tissue supplies the other); δ-son is $p_{T\delta} = 0$ (an unrelated male supplies this genome); ε-son is $p_{T\varepsilon} = 1$ (the haploid tissue supplies this genome).

Coefficients of relatedness are obtained by dividing the coefficient of consanguinity between actor and social partner by the consanguinity of the actor to herself ($r_{AX} = p_{AX}/p_{AA}$; Bulmer 1994). This scaling is not necessary for a kin selection analysis, but is adopted in this article simply because coefficients of relatedness are more familiar than coefficients of consanguinity. The consanguinity of the female to herself is p, so her relatedness to each of her offspring is: $r_{F\alpha} = p_{F\alpha}/p = 1$ to her α-son; $r_{F\beta} = p_{F\beta}/p = 1/2$ to her β-daughter; $r_{F\gamma} = p_{F\gamma}/p = 1$ to her γ-daughter; $r_{F\delta} = p_{F\delta}/p = 0$ to her δ-son; and $r_{F\varepsilon} = p_{F\varepsilon}/p = 1$ to her ε-son. The consanguinity of the tissue to itself is 1, so its relatedness to each of the female's offspring is: $r_{T\alpha} = p_{T\alpha} = p$ to her α-son; $r_{T\beta} = p_{T\beta} = p/2$ to her β-daughter; $r_{T\gamma} = p_{T\gamma} = (1+p)/2$ to her γ-daughter; $r_{T\delta} = p_{T\delta} = 0$ to her δ-son; and $r_{T\varepsilon} = p_{T\varepsilon} = 1$ to her ε-son. Making the substitution $p = 1/(1+\phi)$, all coefficients of relatedness are listed in Table 5.1.

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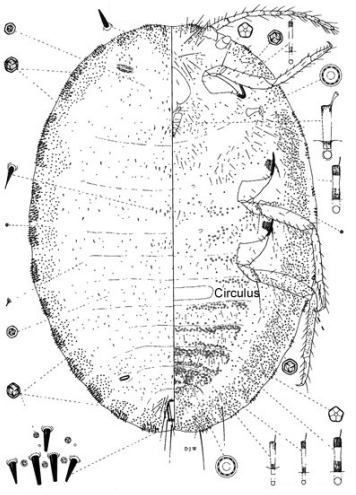
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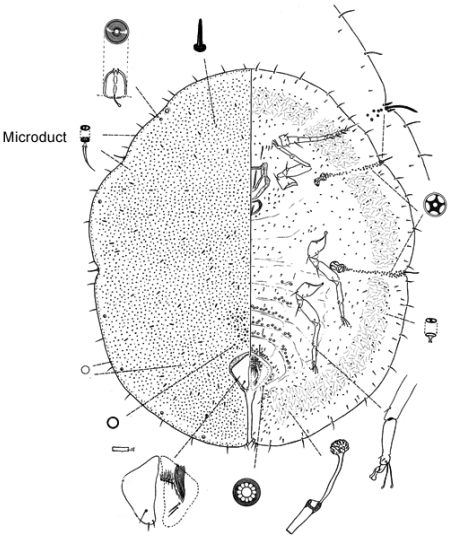

Microduct

CHAPTER 6

The role of endosymbionts in the evolution of haploid-male genetic systems in scale insects (Coccoidea)

Laura Ross, David M. Shuker, Benjamin B. Normark & Ido Pen

ABSTRACT

There is an extraordinary diversity in genetic systems across species, but this variation remains poorly understood. In part this is because the mechanisms responsible for transitions between systems are often unknown. A recent hypothesis has suggested that conflict between hosts and endosymbiotic micro-organisms over transmission could drive the transition from diplodiploidy to systems with male-haploidy (haplodiploidy, including arrhenotoky and paternal genome elimination). Here we present the first formal test of this idea with a comparative analysis across scale insects (Hemiptera:Coccoidea). Scale insects are renowned for their large variation in genetic systems, and multiple transitions between diplodiploidy and haplodiploidy have taken place within this group. Additionally most species rely on endosymbiotic micro-organisms to provide them with essential nutrients lacking in their diet. We show that species harbouring endosymbionts are indeed more likely to have a genetic system with male-haploidy, which supports the hypothesis that endosymbionts might have played a role in the transition to haplodiploidy. We also extended our analysis to consider the relationship between endosymbiont presence and transitions to parthenogenesis. Although in scale insects there is no such overall association, species harbouring eukaryote endosymbionts were more likely to be parthenogenetic than those with bacterial symbionts.

Unpublished manuscript

INTRODUCTION

There is an extraordinary diversity in genetic systems across species, which includes variation in ploidy level, presence or absence of sexual reproduction and difference in sex determination mechanisms (Normark, 2003; Norton et al., 1993; White, 1973). However this variation is not spread equally across the tree of life: within some higher taxa there is no variation at all while in others variation exists between closely related species or indeed within a single species. One group that stands out for the extraordinary diversity of its genetic systems is the scale insects (Nur, 1980; Ross *et al.*, 2010b). Scale insects comprise one superfamily within the order Hemiptera, yet there is almost as much variation in genetic systems within scale insects (see Table 6.1) as there is across insects as a whole (Normark, 2003). Recently ideas that intergenomic conflict can shape patterns of genetic system evolution have come to prominence (Bull, 1983; Burt & Trivers, 2006; Hamilton, 1993; Uller *et al.*, 2007; Werren & Beukeboom, 1998), including in scale insects (Brown, 1964; Normark, 2004a, 2006; Ross *et al.*, 2010b; Shuker *et al.*, 2009).

In order to understand the variation in genetic systems we need to understand the transitions between the different systems. In scale insects it has generally been assumed that diplodiploidy with a genetic sex determination system (in this case XX-XO) is the ancestral genetic system (Nur, 1980). This system is found in most of the families within the relatively species-poor paraphyletic assemblage traditionally referred to as the Archaeococcoidea. However, the majority of scale insect species have a remarkable genetic system called paternal genome elimination (PGE). In this system both sexes develop from fertilized eggs and are diploid. However in males the chromosomes inherited from the father are deactivated during early development and subsequently lost from the germline during spermatogenesis (Brown & Nelson-Rees, 1961; Schrader, 1921). PGE is a synapomorphy of a major clade of scale insects, informally termed Neococcoidea. It has previously been suggested that since this remarkable system shows interesting similarities with arrhenotoky (where females develop from fertilized and males from unfertilized eggs) it might constitute an intermediate stage between diplodiploidy and arrhenotoky (Bull, 1979; Bull, 1983; Schrader & Hughes-Schrader, 1931). There is some evidence for this in mites (Cruickshank & Thomas, 1999), but in scale insects extant PGE and arrhenotokous clades have clearly evolved independently from diplodiploidy (Cook *et al.*, 2002). Although diplodiploidy has been assumed to be ancestral, in a few taxa diplodiploidy seems to be a derived feature resulting from a reversion from PGE to diplodiploidy (Nur, 1980). These taxa, which generally lack sex chromosomes, are of particular interest when trying to understand the evolution of the variety of genetic systems in scale insects (Herrick & Seger, 1999; Ross *et al.*, 2010b). Another important genetic system found in scale insects is hermaphroditism, a system found in no other insects (Hughes-Schrader, 1925; Normark, 2003; Royer, 1975). Apart from a variety of sexual reproductive systems, asexual reproduction is also common in scale insects, found in members of both the Archaeococcoidea and in the Neococcoidea. Again

there is a lot of variation in the form of asexuality, with up to six different systems described (Table 1: Nur, 1971; Ross *et al.*, 2010b).

Several hypotheses have been brought forward to explain the transition between genetic systems and the resulting diversity in scale insects (as reviewed by Ross *et al.*, 2010b). Although some of these hypotheses are plausible none of them have yet been formally tested. One recent hypothesis focuses on the transition from diplodiploidy to systems with haplodiploidy (arrhenotoky and PGE) and considers endosymbiotic bacteria the key driver of this transition through conflicts between hosts and endosymbionts over transmission (Normark, 2004a). We will first briefly review the presence and significance of endosymbionts in scale insects before detailing Normark's hypothesis.

Table 6.1 The genetic systems observed in scale insects (Normark, 2003; Nur, 1980; Ross *et al.*, 2010b).

Genetic system	Description
SEXUAL SYSTEMS	
Diplodiploidy (XX-XO)	Both sexes develop from fertilized eggs and are diploid. Females are XX, males XO.
Diplodiploidy (2N-2N)	Both sexes develop from fertilized eggs and are diploid. No sex chromosomes have been observed
Arrhenotoky	Females develop from fertilized eggs and are diploid, males develop from unfertilized eggs and are haploid
Hermaphroditsm	Diploid hermaphroditic individuals have a diploid female reproductive system producing oocytes and haploid testis cells producing sperm
Germline paternal genome elimination (lecanoid, Comstockiella,)	Both sexes develop from fertilized eggs and are diploid but in males paternal genes are deactivated during early development and subsequently not transmitted.
Embryonic paternal genome elimination (Diaspidid)	Both sexes develop from fertilized eggs and are diploid but in males paternal genes are lost during early development rendering males haploid.
Diploid arrhenotoky	Females develop from fertilized eggs and are diploid, males develop from unfertilized eggs, become diploid due to fusion of the haploid cleavage nuclei, but have haploid gene expression as one of the two genome sets is deactivated.
ASEXUAL SYSTEMS	
Deuteroky	Reproduction can be both sexual and asexual and both males and females can develop from either fertilized or unfertilized eggs. Individuals that develop from unfertilized eggs restore diploidy by fusion of the first haploid cleavage nuclei.
Automictic Thelytoky	Females develop from unfertilized eggs, males are absent. Meiosis is normal and diploidy is restored either by the fusion one polar body with the pronucleus, or by the fusion of the first haploid cleavage nuclei.
Apomictic Thelytoky	Females develop from unfertilized eggs, males are absent. Meiosis does not take place

Scale insects, like many Hemiptera, feed almost exclusively on phloem of their host plant. This constitutes a problem, as phloem is very rich in sugars but poor in other nutrients, most notably in essential amino acids. In order to compensate for the imbalance in their diet, many phloem-feeding insect have engaged in a symbiotic relationship with micro-organisms (Buchner, 1965; Moran & Telang, 1998). It has even been suggested that this evolutionary invention has allowed them to colonize a niche that would have otherwise been out of reach and has allowed the rapid diversification of phloem feeders (Gullan & Kosztarab, 1997). Most scale insect species have an obligate relationship with one or several micro-organisms, which live inside the host cells (Buchner, 1965; Tremblay, 1989; Tremblay, 1997). The relationship between host and endosymbiont is often close, and several endosymbiont taxa have been found to have phylogenies that parallel those of their hosts, indicating strict vertical transmission (Baumann & Baumann, 2005; Downie & Gullan, 2005; Gruwell *et al.*, 2007). Scale insect endosymbionts are transmitted through the female line and a variety of mechanisms have evolved to ensure successful transmission of symbionts from a mother to offspring (Buchner, 1965; Tremblay, 1989). Another feature which indicates the close association between host and endosymbiont is that in many species endosymbionts are kept in specialized cells (bacteriocytes) or even in a specialized organ (the bacteriome: Buchner, 1965).

Although obligate endosymbiosis is found across many Hemiptera, in scale insects the absence of endosymbionts is relatively common (at least compared to aphids and whiteflies: Buchner, 1965; Tremblay, 1989). There are several possible explanations for this. In a number of cases changes in diet or feeding behaviour are associated with the loss of endosymbionts. These include species that have switched to feeding on parenchyma tissues (Gullan & Kosztarab, 1997), species that form galls (Cook, in prep.) and species that are obligately associated with ants (three mealybugs of the genus Hippeococcus) (Buchner, 1965). Additionally in the Stictococcidae, males are fed by mothers via a placenta-like structure, do not feed independently, and lack endosymbionts during all life stages, though Stictococcid females do harbor endosymbionts. Within taxa that posses endosymbionts there is also variation in the type of endosymbiont (Buchner, 1965; Gruwell *et al.*, 2004; Tremblay, 1989). The endosymbionts of most species are bacteria, but in several species the endosymbionts are unicellular fungi.

Normark's (2004a) suggestion that the endosymbionts of scale insects might have played a role in the evolution of the observed variation in their genetic systems is based on the fact that endosymbionts are vertically transmitted, but only through the female line. This creates conflict between host and endosymbiont, as males constitute an evolutionary dead end for the symbionts. Endosymbionts are therefore selected to try to manipulate their host's reproduction towards producing more female offspring. Several endosymbiotic bacteria have been found to manipulate host reproduction, most notably by inducing asexual reproduction, which removes the need for males altogether, thereby resolving the conflict between host and endosymbiont (Hurst *et al.*, 1990; Stouthamer *et al.*, 1990). Instead of manipulating host reproduction

directly, another way for endosymbionts to increase their inclusive fitness is by killing their host when they find themselves in a male. This might benefit the bacteria if there is competition between siblings and the resources that become available though the dead of a male can be utilized by its sisters, which carry bacteria that are related to those in killed males (Hurst, 1991). Male-killing phenotypes have indeed been observed in several endosymbionts (Hurst, 1991). Normark's (2004a) hypothesis takes advantage of the fact that the life history of many species with haplodiploidy and PGE leads to strong and prolonged interactions between kin and that most of them contain endosymbiotic bacteria. Under these conditions male-killing may evolve, for instance with the endosymbionts destroying or deactivating incoming male-determining sperm. This would haplodize male offspring, and generally kill them. As a result, there would be strong selection for haploid viability of males, with any mutation responsible spreading rapidly as haploid males will always pass on this mutation (haploid transmission advantage).

One problem with Normark's hypothesis is that male-killing phenotypes have only been observed for reproductive parasites that do not provide their hosts with any benefits, while the endosymbionts present in many haplodiploid and PGE species are obligate mutualists. Furthermore, whilst several additions to Normarks original model have since been published, confirming the plausibility of Normark's original hypothesis (Engelstadter & Hurst, 2006; Kuijper & Pen, 2010; Ubeda & Normark, 2006), these studies also point out that the scenario is more likely when the transmission efficiency is high, which would be expected to be the case for mutualistic endosymbionts but not necessarily for reproductive parasites.

Although Normark's idea has received theoretical attention, no formal attempts have yet been made to try to test this hypothesis. Under Normark's hypothesis we would expect that species that have endosymbionts are more likely to have male-haploid genetic systems compared to those that do not contain endosymbionts. Here we present results from a comparative analysis based on data from 582 scale insect species in 27 families. We first test if there is a relationship between the presence or absence of bacteria and their genetic system (diplodiploidy vs. male-haploid systems). Then we extend our treatment of the evolutionary significance of the association between endosymbionts and genetic system by considering a possible relationship between endosymbiont presence and asexual reproduction, including in this a role of endosymbiont identity (bacterial versus eukaryote). Finally we consider the importance of the intimacy of the relationship between host and the endosymbiont (i.e. specialised cells or tissues for the symbiont), as differences in how the host and endosymbiont interface inside the host may predict the extent to which endosymbionts can manipulate the host.

METHODS

Data collection

The data used for this analysis were collected between April 2007 and August 2009. We used a variety of sources. The main source for information on the genetic systems of scale insects was a recent review by Gavrilov (2007), which lists published information on genetic systems in scale insects. For information on endosymbiont status and identity the primary source was Buchner's (1965) extensive monograph on endosymbiosis and the references therein. Another important source was ScaleNet, an online database that collects an extensive amount of data on scale insect biology and is also an important source for literature on scale insects. In addition, new references were identified via Web of Science and Google Scholar as well as by inspecting the references of all papers of interest and by searching for citations of key papers. The taxonomy used in the analysis was based on the standard arrangement of families given in ScaleNet (Ben-Dov, Miller & Gibson, 2010), except that Margarodidae has been broken into several families per a recent revision (Hodgson & Foldi, 2006). This reflects the current standard classification used for example in Gullan and Cook (2007). Figure 1 shows the relationships between the scale insect families for which data were available in this analysis. For a few families no data were available for any of the considered factors. These are the Carayonemidae, Coelostomidiidae, Pityococcidae and Stigmacoccidae, (Archaeococcoidea) and the Micrococcidae (Neococcoidea). In species of the family Stictococcidae, only females harbor endosymbionts. Following the rationale of Normark -- that it is only the presence of endosymbionts in males that selects for male-killing -- we included the Stictococcidae as "endosymbionts absent".

Analysis

Phylogenetic inertia can cause statistical problems, as closely related taxa are more similar to one-another than more distantly related taxa are, thus violating the assumption of independence (Felsenstein, 1985). Therefore, in order to obtain reliable estimates from a comparative analysis, it is important to include information on the evolutionary relationships between the taxa included in the analysis. This information can come from taxonomy or from (molecular) phylogenetic inferences (Harvey & Pagel, 1991).

Several molecular phylogenies describing the relationships between scale insects have recently been published. Some of these studies focus on relationship between species within the different families: Diaspididae (Morse & Normark, 2006), Planococcidae (Downie & Gullan, 2004; Hardy *et al.*, 2008), Eriococcidae (Cook & Gullan, 2004) and Monophlebiae (Unruh & Gullan, 2008). Other studies explore the relationships between families (Cook *et al.*, 2002; Gullan & Cook, 2007). Although sequence data is available for more than 250 species, many of the relationships remain poorly resolved, especially at higher taxonomic levels.

The method adopted in this paper is to use a generalized linear mixed model

(GLMM) approach where the relationship between taxa can be fitted as a random effect. For a taxonomic GLMM, taxonomic classification (for example, order, family, genus) can be fitted to include species relationships while in a phylogenetic GLMM the phylogenetic relationship between species can be fitted as a random effect (based on branch lengths between nodes) (Hadfield & Nakagawa, 2010). The latter would be the preferred method, but reliable phylogenies are not always available. We chose not to use a species-level phylogeny partly because of the issues described above and partly because although sequence data is available for a reasonable number of scale insect species, there is little overlap with the species for which relevant data on genetic systems and endosymbionts are available. We included family as a random effect, but chose not to include genus, as there is little variation in genetic system or endosymbiont status within families (see results). As such, our analysis assumes a largely polytomic relationship between families. However, in order to include some information on the higher relationships between families, we divided the families into two groups, the Archaeococcoidea and the Neococcoidea. Although Archaeococcoidea is almost certainly paraphyletic, the Neococcoidea is one of the few well-supported supra-family groupings within scale insects (Gullan & Cook, 2007)(Figure 6.1).

We tested for a relationship between our characters of interest -- genetic system, reproductive mode (sexual vs. asexual reproduction), endosymbiont presence and identity and bacteriome presence (see Figure 6.2)-- using a bivariate binary mixed model approach. The models were fitted using the R package MCMCglmm (Hadfield, 2010a), which provides a Bayesian framework for generalized mixed model analysis. We used a multivariate normal prior for the fixed effects with a null mean vector and a diagonal covariance matrix with variances of $10+\pi 2/3$, which is approximately flat on the probability scale when the sum of the variance components is 10 (roughly the posterior mode). An inverse Wishart prior was used for the family covariance matrix, with the covariance matrix at the limit set to an identity matrix, and degree of belief parameter 1.002. This is equivalent to having marginal inverse gamma priors for each variance, with the scale and shape parameters set to 0.001 (Spiegelhalter *et al.*, 2003). We also used a prior with the covariance matrix at the limit set to a diagonal matrix with the variances set to $10+\pi 2/3$, and degree of belief parameter set to 3. This is an approximately flat prior for the correlation coefficient. The residual variances were fixed at one because they are not identifiable in binary models, and the residual correlation was set to zero. The residual correlation is generally estimable in bivariate binary models, but in each of the different analyses performed very few families were variable for both traits considered, thus there is little information to estimate the within-family correlation. An additional model in which the residual correlation was estimated (with a weak prior; Barnard's (2000) prior with degree of belief parameter equal to 5) was run for each analysis and gave qualitatively (and quantitatively) similar answers. The models were all run for 13 million iterations and the first 3 million iterations were discarded to ensure that the models had converged.

The analysis calculates the correlation coefficient between two response variables on the link scale at the family level. For simplicity, we will denote this correlation

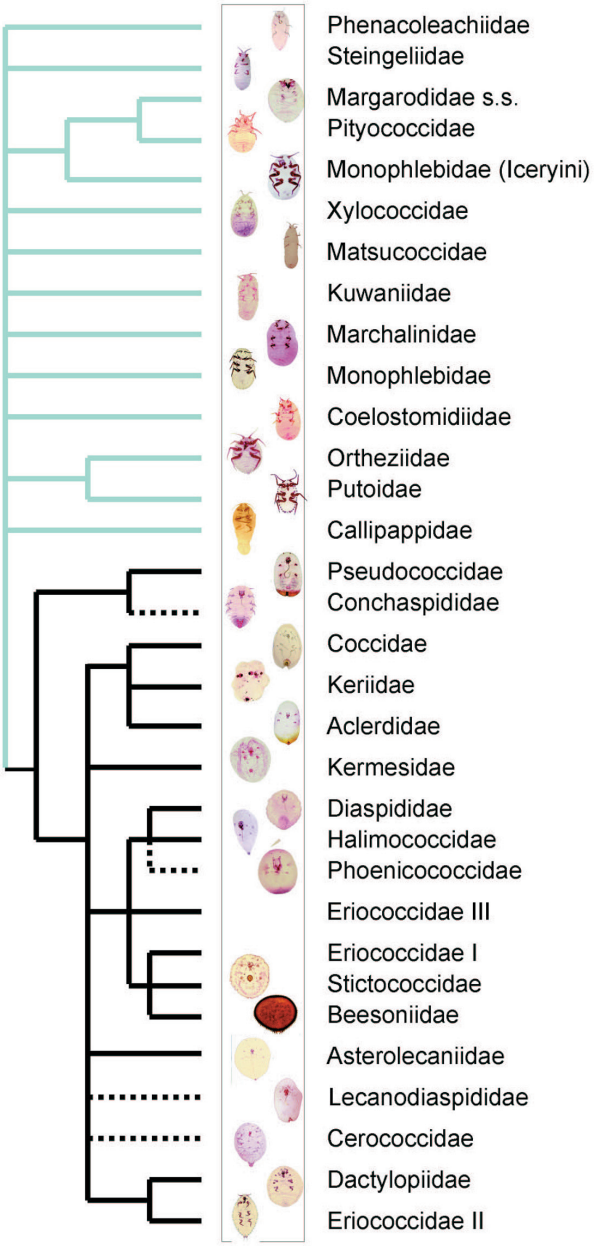


Figure 6.1 Schematic representation of the phylogenetic relationships between the Coccoid families included in our analysis. The solid lines are based on published phylogenetic studies (Cook & Gullan, 2004; Cook *et al.*, 2002; Gullan & Cook, 2007)(Andersen in prep.; Normark, unpublished data), while the dashed lines show the hypothetical relationships of families for which no published sequence data are available, based mostly on a recent review of their taxonomic status (Gullan & Cook, 2007). Turquoise lines show the relationships between Archaeococcoids, while the black lines show the relationships between the Neococcoids. The pictures show slide-mounted specimens of representative species of each family.

coefficient as *r*. For each estimate we also calculate the 95% credibility interval (a Bayesian analogue to the confidence interval), which we will refer to as a 95% CI. We consider the correlation between two factors to be statistically significant if the 95% credibility estimates do not include zero. In order to test how much of the variation of a given response is explained by "family" we calculated the intraclass correlation (correlation between the estimated phenotype of two species within the same family) given by (Hadfield, 2010a), as a measure of phylogenetic signal:

$$\frac{\sigma^2 family}{\sigma^2 family + \sigma^2 residual + \pi^2/3}$$

In order to test if taxonomic group (Archaeococcoidea vs. Neococcoidea) had a significant effect on the different factors considered in this analysis we estimated the fixed effects (taxonomic group was fitted as a fixed effect, as it only has two levels), presented as the posterior mode and 95% CI.

RESULTS

The presence of diplodiplody was strongly associated with the absence of endosymbionts $r = -0.96$ (95% CI = –1.00 – –0.61), n = 432 (Figure 6.2B). Family explained most of the variation for both the presence of endosymbionts and that of diplodiploidy. The intra-class correlation was $r = 0.72$ (95% CI = 0.36 – 0.95) for the presence of diplodiploidy and $r = 0.97$ (95% CI = 0.83 – 0.99) for presence of endosymbionts. There was also a significant difference between the Neococcoids and Archaeococcoids in the presence of diplodiploidy (fraction of species with diplodiploidy, Archaeococcoidea = 0.60, Neococcoidea = 0.008, posterior mode: –8.89, 95% CI = –12.72 – –5.74). However there was no significant difference between the two scale insect taxonomic groups in terms of the occurrence of endosymbionts (fraction of species with endosymbionts, Archaeococcoidea = 0.76, Neococcoidea = 0.89, posterior mode: 0.36, 95% CI = –3.91 – 5.96).

We also tested for a possible relationship between endosymbiont presence and reproductive mode (asexual versus sexual reproduction). First of all, there was much more within-family variation in reproductive mode than in the occurrence of diplodiploidy (the intraclass correlation for reproductive mode: $r = 0.35$, 95% CI = 0.10 – 0.55). There was a negative correlation between endosymbiont presence and reproductive mode, however the effect is not significant ($r = -0.89$, 95% CI = –0.98 – 0.17, n= 475, Figure 6.2C). This suggests that endosymbiont presence does not significantly correlate with the presence of asexual reproduction. We also tested if the reproductive mode (sexual vs. asexual reproduction) was related to the identity of the endosymbiont. We found that there was a correlation between reproductive mode and bacteria identity, with asexuality being more common in species that contain eukaryote endosymbionts and this relationship was significant (È = –0.95, 95% CI= –0.99 – –0.23, n= 447, Figure 5.2D).

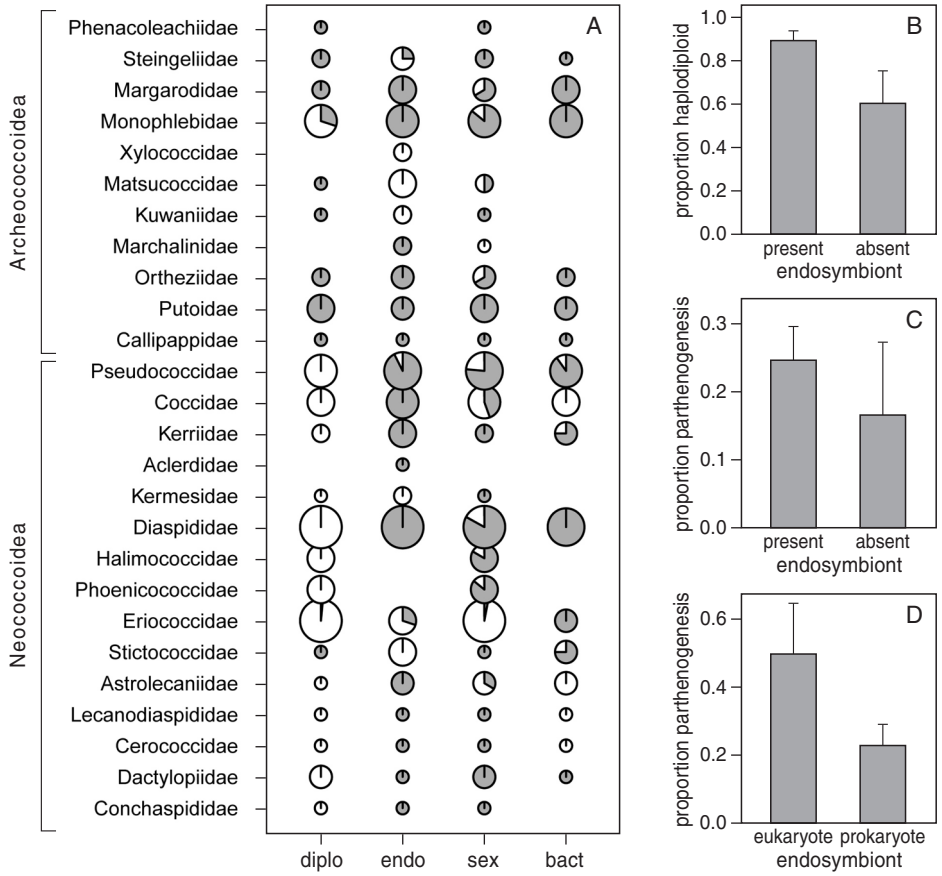


Figure 6.2 (A) Summary of the data used for the analysis. Pie-charts show the proportion per family for each of the four characters: proportion of species with diplodiploidy (Diplo), proportion of species that reproduce sexually (Sex), endosymbiont presence (Endo) and proportion of endosymbionts that are prokaryote (Bact). Size of the pie reflects sample size for each family. (B) Proportion of species with haplodiploidy for species with and without endosymbiont. Error bars show the binomial standard errors. The graph only includes species for which data was available for both characters. (C) Proportion of species with asexual reproduction for species with and without endosymbiont. Error bars show the binomial standard errors. (D) Proportion of species with asexual reproduction for species with prokaryote and eukaryote endosymbionts. Error bars show the binomial standard errors.

Finally, we tested if the identity of endosymbionts was associated with how closely integrated the endosymbionts are by the host (i.e. in a specialized organ or not). We found that eukaryote endosymbionts were less likely to be housed within a bacteriome (r = –0.97, 95% CI= –0.99 – –0.27). The intra-class correlation of both factors was high, suggesting that family explains a large part of the variation in these factors (endosymbiont id: r = 0.86, 95% CI = 0.59 – 0.99, bacteriome: r = 0.85, 95% CI = 0.60 – 0.97, n = 238).

DISCUSSION

Scale insects that possess endosymbionts are more likely to have a haplodiploid genetic system. This supports the hypothesis postulated by Normark (2004a) who considered that coevolution between host and endosymbionts with a male-killing phenotype could have led to the evolution of male-haploid genetic systems. The results presented here are the first formal analysis that shows support for this hypothesis. Male-haploid genetic systems are found in a large number of taxa of both insects and mites. It will be of great interest to see if a similar role of endosymbionts can be observed in these systems.

Normark's (2004a) hypothesis considers coevolution between male-killing endosymbionts and their host as the driving force behind the transition to male-haploid genetic systems. However, recently it has been shown that another phenotype commonly induced by endosymbionts, cytoplasmic incompatibility (CI), might also lead to conflict between host and symbiont resulting in the evolution of male-haploid genetic systems (Engelstadter & Hurst, 2006). Both mechanisms are consistent with our findings.

Although we find a relationship between genetic system and endosymbiont presence, we do not find a relationship with reproductive mode (i.e. sexual versus asexual). However the type of endosymbiont (bacteria or unicellular fungi) does correlate with reproductive mode, with asexual reproduction more often found in species with eukaryote endosymbionts. This could be because the latter are generally distributed freely in the haemolymph and are even found to be able to penetrate a variety of cells (including germline cells) (Tremblay, 1989). This means that they might be less tightly controlled by their host than bacterial endosymbionts, which generally are restricted to specialized cells, giving them more opportunity to influence their host's reproduction (Ross *et al.*, 2010b).

The role of endosymbionts in the evolution of asexual reproduction has been well established in many insects (Duron *et al.*, 2008; Hurst *et al.*, 1990; Koivisto & Braig, 2003; Stouthamer *et al.*, 1990; Weeks *et al.*, 2002; Weeks *et al.*, 2003; Werren, 1997; Werren *et al.*, 2008; Zchori-Fein & Perlman, 2004) and the endosymbiotic bacterium *Cardinium* is associated with parthenogenesis in a species of armoured scale insect (Provencher *et al.*, 2005). The fact that in our analysis we do not find strong support for a generalised role of endosymbionts on the presence of asexual reproduction in scale insects might be due to the fact that in our analysis we simply used presence or absence, and were unable to distinguish between primary and secondary symbionts, as the role of the symbionts described in scale insects are mostly unknown. However, previous analyses have mainly found that the effect of secondary (often purely parasitic) bacteria is associated with asexual reproduction. More work on scale insect endosymbionts is clearly merited.

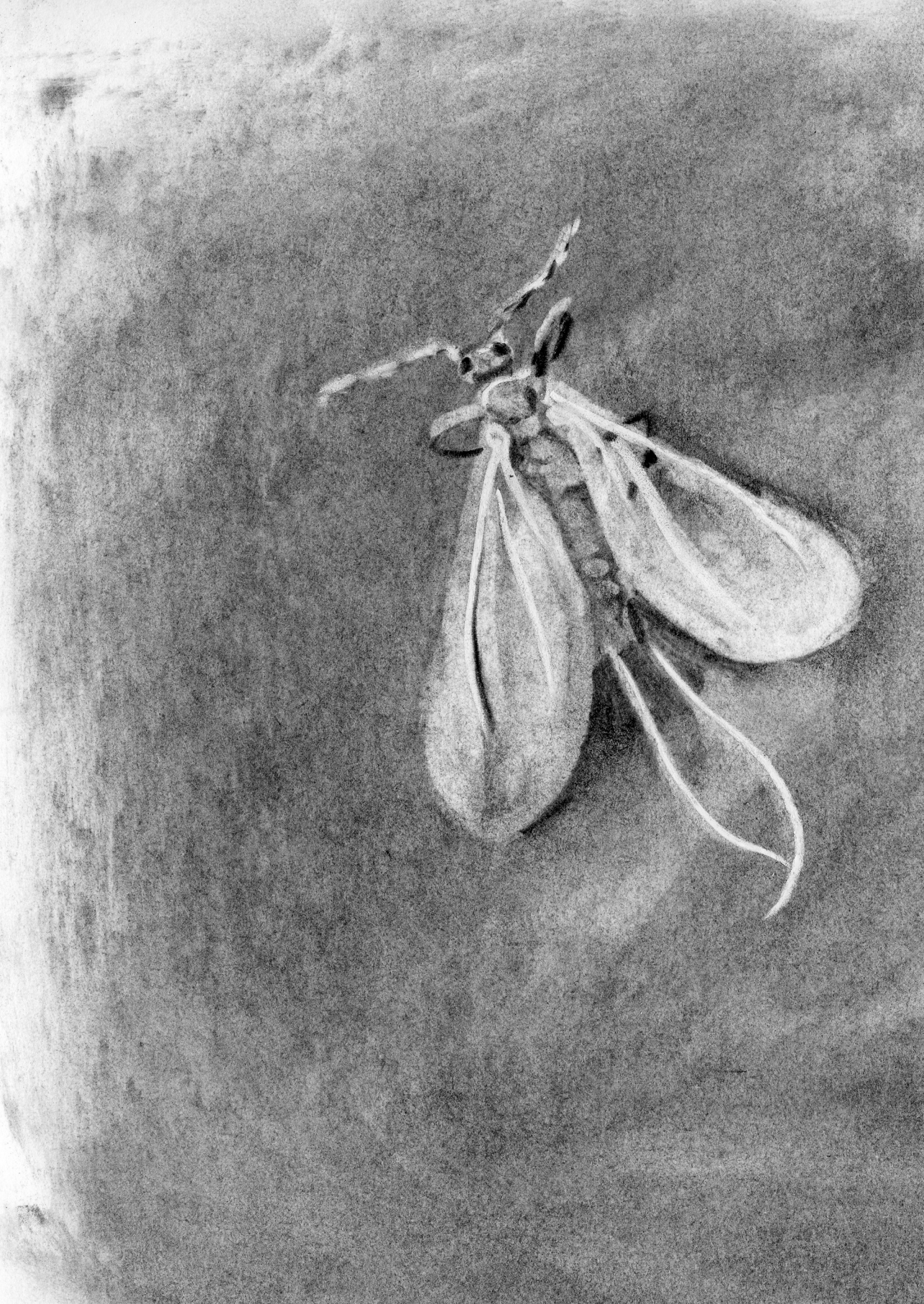
Apart from the presence of endosymbionts, population structures leading to high levels of kin competition is an important assumption of Normark's model, as selection on the endosymbiont for a male-killing phenotype will only be strong under such

conditions. Scale insects usually have gregarious clutches and have evolved a variety of ways in which eggs and larvae are protected by the mother (in a marsupium, or ovisac for example). Additionally, crawlers often settle close to their mothers (Gullan & Kosztarab, 1997). All these factors lead to prolonged associations between kin and could therefore lead to high levels of kin competition (Normark, 2004a, 2006; Ross *et al.*, 2010b). This might help to explain how endosymbiosis in scale insects could have led to conflict between endosymbiont and host, and resulted in a change in the host's genetic system. Reduced sib-competition might help to explain the few cases where endosymbiosis has not resulted in the evolution of haplodiploidy, although sib-competition is hard to quantify (data might be available on factors that could correlate with the level of sib-competition e.g. the presence of gregarious clutches, the clutch size and how mothers protected their eggs). Such data might also in the future be able to distinguish between the importance of male killing vs. cytoplasmic incompatibility, as sib- competition is only a requirement for the former.

Recently the importance of genomic conflict in shaping defining characteristics of genomic organisation and key aspects of biology such as reproductive mode and genetic system has become apparent and received considerable attention (extensively reviewed in Burt & Trivers, 2006). However, few studies have actually made an attempt to test these hypotheses formally. Further comparative analyses such as the one undertaken here will help to increase our understanding of the evolutionary importance of genetic conflict.

Acknowledgements

We would like to thank Marcel Buczkiewicz and Bram Kuijper for useful discussions about the approach used in this manuscript, Albert Phillimore for comments on the manuscript, Jarrod Hadfield for his help with the statistical analysis and Lyn Cook for providing advice and phylogenetic data. We were supported by the Natural Environment Research Council, the National Science Foundation, the Royal Society and the University of Groningen.



CHAPTER 7

Sex allocation in a species with Paternal Genome Elimination: the role of crowding and female age in the mealybug *Planococcus citri*.

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ABSTRACT

BACKGROUND: In species with paternal genome elimination, both sexes are diploid. However, in males the chromosomes inherited from the father are deactivated during early development and eliminated from the germ line. Sex allocation theory predicts that, all else being equal, females should bias their offspring sex ratio towards the sex that competes least with relatives.
ORGANISM: The mealybug *Planococcus citri*, a cosmopolitan pest on a wide range of agricultural and ornamental plant species.
HYPOTHESIS: In mealybugs, females compete locally for resources. In order to avoid competition among daughters, females should therefore produce a male-biased sex ratio when alone, but a more equal sex ratio when together with other unrelated females. This will result in a rise of the number of female offspring with density. However, competition associated with population density might have different fitness effects for male and female offspring respectively, because females need more resources and have less opportunity to migrate compared to males, selecting for the opposite pattern of sex allocation.
METHODS: Measuring sex ratios in an experiment to manipulate the density a female experiences during two life stages.
RESULTS: Females that experienced high density as adults produced more male-biased sex ratios. Additionally the sex ratio females produced was strongly dependent on their age.
CONCLUSION: Female mealybugs facultatively adjust their sex ratio, but in the direction opposite to that predicted by local resource competition, suggesting that sex-specific fitness consequences of density determine sex allocation in mealybugs.

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INTRODUCTION

How individuals allocate resources to their male and female offspring is an important reproductive decision that can have significant fitness implications (Charnov, 1982; Fisher, 1930; Hamilton, 1967; West, 2009). For instance, females have been suggested to facultatively adjust their sex ratio to avoid competition among their offspring (Charnov *et al.*, 1981; Hamilton, 1967) and in response to environmental factors influencing the relative fitness of male and female offspring (Trivers & Willard, 1973). There is an extensive body of empirical data testing sex allocation theory, especially for the haplodiploid Hymenoptera and the data often fit the theoretical predictions well (West, Herre & Sheldon, 2000; West, Shuker & Sheldon, 2005). However, there are fewer data from other taxa. This is an important omission, as it limits our understanding of sex allocation, especially in terms of the role of different sex determination mechanisms on the evolution of sex allocation. Scale insects (Hemiptera: Coccoidea) vary considerably in terms of their genetic and sex determination systems (Nur, 1980; Ross *et al.*, 2010b), yet most scale insects have very similar life histories (Gullan & Kosztarab, 1997). Sex allocation in scale insects is poorly understood; in most species either unbiased or female-biased sex ratios are observed, but very little is known about the factors that influence sex allocation and the extent to which sex allocation is facultative (Brown & Bennett, 1957; Nelson-Rees, 1960).

Among the scale insects, probably the best studied is the mealybug *Planococcus citri* (Coccoidea: Pseudococcidae). Like most mealybugs, *P. citri* has a form of paternal genome elimination (PGE) (Nur, 1980; Schrader, 1921). Both sexes are diploid but in males one of the parental chromosome sets is deactivated (via heterochromatinization) during early development. The deactivated set is always the set inherited from the father (Brown & Nelson-Rees, 1961). Although the deactivated set divides faithfully in all somatic cell lines, it fails to end up in mature sperm because it is destroyed during meiosis. As a result, males can only pass on the genes they inherited from their mother, making *P. citri* effectively haplodiploid in terms of their transmission genetics (Brown & Nur, 1964). This process is presumably partly under control of the mother who can "tag" paternal genomes destined for destruction by genomic imprinting (Khosla, Mendiratta & Brahmachari, 2006), but paternal effects have also been observed (Buglia & Ferraro, 2004b).

An important argument that is often used to explain the evolution of haplodiploid genetic systems is that it enables sex ratio control by the mother (Bull, 1983). Although sex ratio control is well established in many truly haplodiploid species (such as the Hymenoptera), it is less obvious in taxa with paternal genome elimination if and how parents control their offspring sex ratio. This is especially important because PGE has been previously considered to be an intermediate system in the evolution of haplodiploidy from diplodiploidy (Bull, 1983). Two previous studies have found evidence of sex ratio adjustment in species with PGE. First, Nagelkerke and Sabelis (1998) found sex ratio adjustment in accordance with Hamilton's (1967)

theory of local mate competition (LMC) in two mite species with PGE, but failed to repeat the results in other populations of the same species (Sabelis, personal communication). Second, Varndell and Godfray (1996) tested the effect of population density on sex allocation in *Planococcus citri* mealybugs. In mealybugs, adult males are winged and dispersive while adult females are sedentary and seldom move once adult (Gullan & Kosztarab, 1997). Females often settle close to where they hatched creating competition between related females for food and space. This competition could become intense since mealybugs, like many phloem-feeding Hemiptera, often form very dense colonies on their host plant. Varndell and Godfray (1996) therefore expected females to produce relatively more sons under high density, from the specific assumption that increasing density would increase competition between related females. Competition among relatives for resources, termed local resource competition (LRC), is expected to favor females that limit competition among offspring of the competing sex by producing more of the sex that avoids competition (in this case males: Charnov *et al.*, 1981; Clark, 1978). They tested this hypothesis by varying both juvenile and adult densities. Although they observed the expected effect of juvenile density, the opposite effect of adult density was found with females producing more female-biased clutches at higher densities. Additionally, the sex ratio was female biased for all treatments.

However, colony density could actually affect sex allocation in several ways. First of all, from the point of view of local mate competition theory (LMC: Hamilton, 1967), females could be selected to try and avoid competition between siblings for mates by adjusting the sex ratio towards the sex that competes less for mates (i.e. favoring female production, and reducing LMC amongst sons). Since the extent of LMC declines when a female's sons also compete with the sons of other females on a patch, a female ovipositing alone is predicted to produce a more female biased sex ratio than when she oviposits together with other females. Although LMC could feasibly influence sex allocation in *P. citri*, the mating system of *P. citri* (with males being the dispersive sex) does not fit the classic LMC scenario. Moreover, the sex ratios observed by Varndell and Godfray in their adult density treatment (assuming density is correlated with an increase in the number of mothers contributing eggs to a resource patch) was opposite to that predicted by LMC theory. .

Second, following the logic of Varndell and Godfray (1996) outlined above, increasing local population density might increase competition between related females for resources, leading to heightened LRC and a decrease in female offspring production (as outlined above and tested by Varndell and Godfray, 1996). This requires that population density exacerbates the interactions between related female offspring. However, if the number of mealybugs (especially non-dispersing female mealybugs) increases because of oviposition by an increasing number of unrelated mothers, colony density may actually lead to a reduction of LRC as competition will occur more frequently between non-relatives. This will select for a greater production of daughters with increasing density. This latter scenario is perhaps closer to the "classic" model of local resource competition.

The effects of resource competition resulting from local population density could also influence sex allocation in a third way. Very dense colonies, whilst ameliorating local competition amongst related female offspring, may end up reducing the value of daughters compared to sons. In mealybugs, males are winged and able to disperse as adults; they also stop feeding after the second instar and might therefore have a better chance of survival under high resource competition. Female mealybugs could therefore be selected to bias their sex ratio towards more males under high-density conditions. This means that population density should influence sex allocation in opposing directions, depending on the importance of local competition among relatives (LRC) versus competition more generally for a female offspring's fitness ("global" competition). A crucial factor underlying this dichotomy will be how density influences patterns of relatedness among interacting female offspring. For instance, an increase in the number of related females in a patch will not reduce LRC, whereas an increase in unrelated females will. One aspect of the experiment of Varndell and Godfray (1996) experiment that makes interpretation difficult is that in some of their treatments all individuals were full-sibs, while in other treatments the relatedness among individuals was lower. This means that density and relatedness changed in different ways across their experimental design.

Finally, on a completely different tack, dense colonies could even enhance female offspring fitness via local resource enhancement (LRE), with greater numbers of mealybugs facilitating the access and acquisition of resources from the host plant (for instance by debilitating host defenses). This was the suggestion Varndell and Godfray (1996) put forward to best explain their data. Moreover, each of these patterns of selection on sex ratio could interact (West, 2009).

Given these numerous alternatives and the difficulty of interpreting Varndell and Godfray's results, here we report a renewed attempt to understand the sex allocation behaviour of *P. citri*. We focus first of all on understanding how the sex ratio is affected by population density and secondly on the ability of females to facultatively adjust their sex ratios. We repeated the experiment of Varndell and Godfray (1996), manipulating both juvenile and adult female densities. However, in our experiment all treatments contain a mixture of kin and non-kin (in contrast to Varndell and Godfray, where the juvenile low density treatment consisted solely of full-sibs), thereby controlling to some extent the effect of high relatedness between ovipositing females. From our experimental design, increasing population density in both juvenile and adult treatments will lead to reduced interactions among kin and a reduction of LRC, and so we predict that sex ratios will be more male-biased at low density and more female-biased at high density if classic LRC theory explains sex ratio in *P. citri*.

If female mealybugs can assess density as both juveniles and adults, then the density information may either be used equally (thus yielding similar sex ratio changes for females reared at similar juvenile and adult densities), or females may assess or use the information differently. For instance, adult density may be a better predictor of the density a female's offspring will actually experience and thus be the most important cue. This could mean that adult density is used for sex ratio decision-

making whilst juvenile density is not, or that there is an interaction between the two such that the effect of juvenile density depends on what adult density is experienced. From work in other organisms on information use and information processing with respect to sex ratio decisions (e.g. Boomsma *et al.*, 2003; Shuker & West, 2004) we predict that adult density will be a more reliable cue and may therefore be the most important for facultative sex ratio changes.

Finally, in addition to trying to better control relatedness and density, we also increased the density in both the juvenile and adult high density treatments to better mimic the natural population structure (Nestel *et al.*, 1995). Given that *P. citri* sex ratios have been observed to vary across the female lifespan (Nelson-Rees, 1960; Varndell & Godfray, 1996), we considered both the total sex ratio produced and sex ratio with respect to female age.

METHODS

Study organism

Mealybugs (Pseudococcidae) are the second largest family of scale insects, represented by approximately 2000 species (Hardy *et al.*, 2008). They have strong sexual dimorphism, with adult females being wingless and covered with wax, while adult males are winged and usually smaller. An important aspect of scale insect biology is their requirement for endosymbionts (typically bacteria), which provide essential amino acids absent from their diet of plant fluids (Buchner, 1965). Here we consider the citrus mealybug *Planococcus citri*, a cosmopolitan pest species previously studied in terms of both its sex allocation and sex determination mechanisms. In *P. citri*, adult males are short-lived (a few days at most), not feeding past the second instar. Females on the other hard are usually long-lived (up to several months), forming large colonies and producing gregarious clutches. The first instar nymphs ("crawlers") disperse to new feeding sites when the population is overcrowded (Gullan & Kosztarab, 1997). Adult females become immobile and often settle close to where they hatched whilst adult males are winged and disperse. *P. citri* has an obligate endosymbiosis with two bacteria: the primary endosymbiont is the β-proteo-bacteria *Tremblaya princeps* (Tremblay, 1989; von Dohlen *et al.*, 2001); the secondary endosymbiont is a γ-proteo-bacteria that lives inside the primary endosymbionts (von Dohlen *et al.*, 2001). Reproduction is strictly sexual (James, 1937; Schrader, 1922). Females lay their eggs in an ovisac that consists of fibers that protect the eggs and newly emerged crawlers. Females can produce in excess of 500 eggs during their reproductive lifetime. An advantage of studying sex allocation in mealybugs is that it is possible to determine the primary sex ratio, by examining embryos for the presence of the condensed paternal genome in males. The mealybug culture that was used for the experiment was obtained from Mike Copland at Wye College, London, U.K.

Culture conditions

All experimental mealybugs were cultured on sprouting potatoes (cultivar Desiree) in large rectangular plastic boxes (3.2 litre in volume) covered with fine mesh. Cultures were kept at 25°C and 70% relative humidity under a 12 h light, 12 h dark regime. Under these conditions they have a generation time (time from oviposition until sexual maturity) of approximately 30 days. The cultures were maintained by transferring an infected potato sprout to a new box with fresh potatoes each month. For the experiment, individual mealybugs were transferred to smaller boxes and raised on a single potato. Potatoes used for the experiment had a single sprout that was cropped to 15 cm and all potatoes were weighed (in grams) at the start of the experiment to control for the effect of food quantity.

Experiment

The experiment consisted of two juvenile and two adult female density treatments, in a fully-factorial design, where the density each female experiences was manipulated at both stages of her life. This allowed us to measure the sex allocation response to density during two different life-stages independently. Additionally we measured the effect of density on other life history factors, most importantly female lifespan and reproductive success.

To generate the two juvenile density treatments, females were randomly selected from the mass culture and transferred to fresh potatoes, where they were allowed to lay eggs for one day. After this day the egg masses were removed and placed either in groups of 3 or in groups of 15 in the treatment boxes (rectangular plastic boxes 1.1

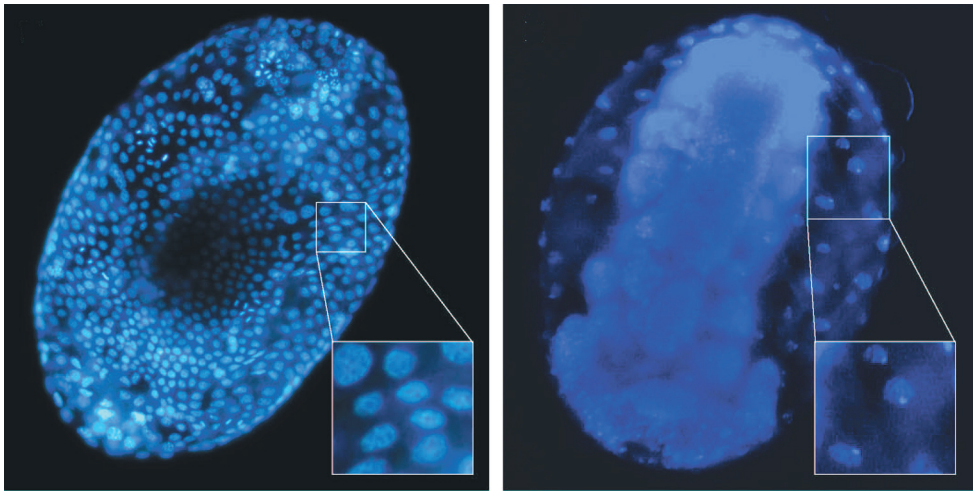

Figure 7.1 Mealybug eggs stained with DAPI, examined under a fluorescence microscope at 200x magnification. (A) A female embryo, in which the nuclei are uniformly coloured and lack the brightly stained body (see insert). (B) A male embryo in which the condensed paternal genome is visible as a brightly stained body in every nucleus (see insert).

litre in volume) to form the low and high juvenile density treatments respectively. The eggs were allowed to hatch and the crawlers to develop until the third instar, when the sexes of the nymphs become distinguishable (20 days after the juvenile treatments were set up). From every juvenile treatment box, third instar females were randomly selected, removed and transferred either singly or in groups of 50 females to new boxes to form the solitary and crowded adult treatments, so that every juvenile treatment box gave rise to two adult treatment boxes. In addition, third instar males were similarly collected and isolated in glass tubes (males do not feed after the second instar). Both sexes were allowed to develop further until 10 days after the adult treatment was set up. By this time both sexes had reached sexual maturity. Males were randomly selected and introduced in groups of three to the female boxes to allow mating. After the males were introduced females were checked every day for the presence of an ovisac. In the high density treatment one female was randomly chosen to be the experimental female and she was marked with a marker pen and only this female was used in the experiment to avoid pseudoreplication. As soon as females started ovipositing, eggs were removed with a small bristle, making sure not to disturb the female (because of the ovisac, egg masses could be attributed to individual females in the high density treatment). Collected eggs were then fixed and stained to determine the primary sex ratio of each clutch (see methods below). On the first day of oviposition the size of the female was measured with a calliper (to the nearest 0.1 mm). Females were checked and eggs collected every day until death.

Egg staining

The egg masses were collected and placed on a glass slide. The protecting fibres around the eggs were removed with a small brush and the eggs were transferred to an eppendorf tube with fixative (Carnoy's fluid, 4:3:1, chloroform: ethanol: glacial acetic acid). Eggs were kept in the fixative in a fridge at 4°C for 4 days before they were stained. If eggs had to be stored for longer period, eggs were transferred into 90% ethanol and again stored at 4°C. In order to determine the sex of each embryo, eggs were transferred to a glass slide, stained with DAPI (Sigma D9564, diluted 1:1000 in PBS) and examined under a fluorescence microscope (200×). The differences between male and female eggs can easily be observed because of the condensed paternal chromosomes that form a brightly stained body in the nuclei of male embryos, which is absent in females (see figure 7.1)

Data analysis

All data analyses were performed using the statistical program R (R Development Core Team, 2008) and for linear mixed models the R package NLME was used (Pinheiro *et al.*, 2007). The relationships between the several life history traits were explored using generalised linear models with Gaussian error structures. The lifetime sex ratios were analysed using generalized linear models with quasibinomial error structure to correct for overdispersion. Due to difficulties with model fitting using generalized linear mixed models, the sex ratio data per day were analysed by using a

linear mixed model approach with arcsine-square root transformed sex ratios and female identity fitted as a random effect. Throughout, sex ratios are considered as the proportion of offspring that are male. Age effects on clutch size were analyzed with a linear mixed effect model with female identity again as a random effect. Models were simplified by removing non-significant interaction terms first and then non-significant main effects to generate the minimal adequate model (for procedure see Crawley, 2007).

RESULTS

Life history

In total 48 females commenced ovipositing, producing a total of 22425 eggs. 97.2% of the examined eggs could be sexed successfully. Females lived on average 17.55 (s.e. = 0.98) days following the commencement of oviposition, and they laid eggs for on average 11.5 (s.e. = 0.74) days. During their lifetime females laid a mean of 467 eggs (s.e. = 33.96) with a sex ratio of 0.51 (s.e. = 0.014). Female body size was positively associated with lifetime egg production ($F_{1,40} = 50.2$, $P < 0.001$) as was oviposition period ($F_{1,39} = 76.4$, $P < 0.001$). Adult density treatments influenced lifetime egg production with females producing more eggs when housed at higher density ($F_{1,43} = 5.19$, $P = 0.028$; Figure 7.2). Juvenile density however did not have a significant effect on lifetime egg production ($F_{1,42} = 0.95$, $P = 0.34$), nor did the size of the potato during the two different life stages (potato weight adult treatment: $F_{1,40} = 0.002$, $P = 0.97$, potato weight juvenile treatment: $F_{1,41} = 2.62$, $P = 0.11$). There was however a significant interaction between juvenile treatment and potato weight during juvenile treatment on the lifetime egg production, with females at high densities producing more eggs when raised on smaller potatoes ($F1,38 = 9.04$, $P = 0.005$). Female body size was not affected by either treatment (adult density: $F_{1,42} = 0.01$, $P = 0.90$, juvenile density: $F_{1,43} = 0.01$, $P = 0.93$) or potato weight (potato weight adult treatment: $F_{1,40} = 0.72$, $P = 0.40$, potato weight juvenile treatment: $F1,41 = 0.79$, $P = 0.38$). There was no significant effect of female size ($F_{1,41} = 1.46$, $P = 0.24$) or potato weight during juvenile development ($F_{1,41} = 0.06$, $P = 0.82$) on the length of the oviposition period, but there was a significant interaction between the two ($F_{1,41} = 4.76$, $P = 0.035$) with a stronger positive effect of potato size in the high density treatment. There was no effect of the juvenile and adult density treatments on the oviposition period however (adult density: $F_{1,39} = 0.53$, $P = 0.47$, juvenile density: $F_{1,40} = 0.13$, $P = 0.73$).

Sex ratio

The effect of juvenile and adult density treatments on sex ratio was analysed in two different ways. First, we considered the total sex ratio that a female produced during her lifetime using a GLM with a quasibinomial error structure. There was a significant effect of adult treatment ($F_{1,46} = 8.51$, $P = 0.005$) on the overall sex ratio, with

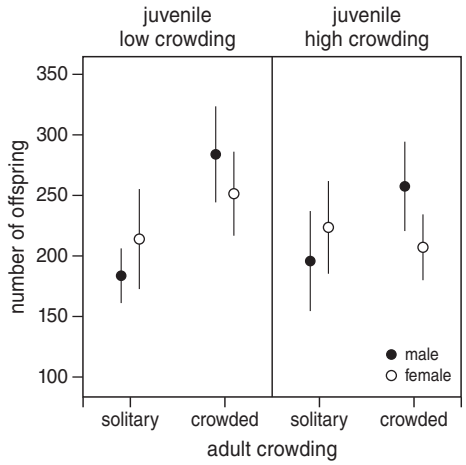

Figure 7.2 The average number of male and female offspring produced per female for each treatment. The error bars are the standard errors. The adult crowding is shown on the x-axis, while the two juvenile treatments are shown in the two vertical panels.

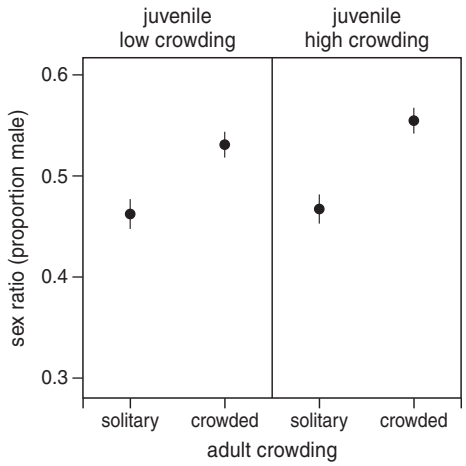

Figure 7.3 The average lifetime sex ratio (proportion male) per treatment. The error bars are the binomial standard errors. The adult crowding is shown on the x-axis, while the two juvenile treatments are shown in the two vertical panels.

females at high adult densities producing male-biased sex ratios (Figure 7.3). There was also a significant effect of potato weight during juvenile development ($F_{1,46}$ = 15.62, $P << 0.001$) on sex ratio, with females raised on bigger potatoes producing a more female biased sex ratio. There was however no effect of juvenile density ($F_{1,44} = 0.20$, $P = 0.66$), the length of the oviposition period ($F_{1,44} = 3.70$, $P = 0.06$), female body size ($F_{1,44} = 0.55$, $P = 0.46$) or potato weight during the adult development ($F_{1,45} = 3.50$, $P = 0.07$). There were also no significant interactions between these factors (all $P > 0.25$).

Second, we considered sex ratios over time. There was a strong effect of the age of a female on the sex ratio she produced (Figure 7.4). Females initially produced very male biased clutches, with their sex ratios becoming more female biased for approximately 5 days, before the sex ratio started to rise again. Older females produced generally male biased sex ratios. Therefore our second analysis considered the

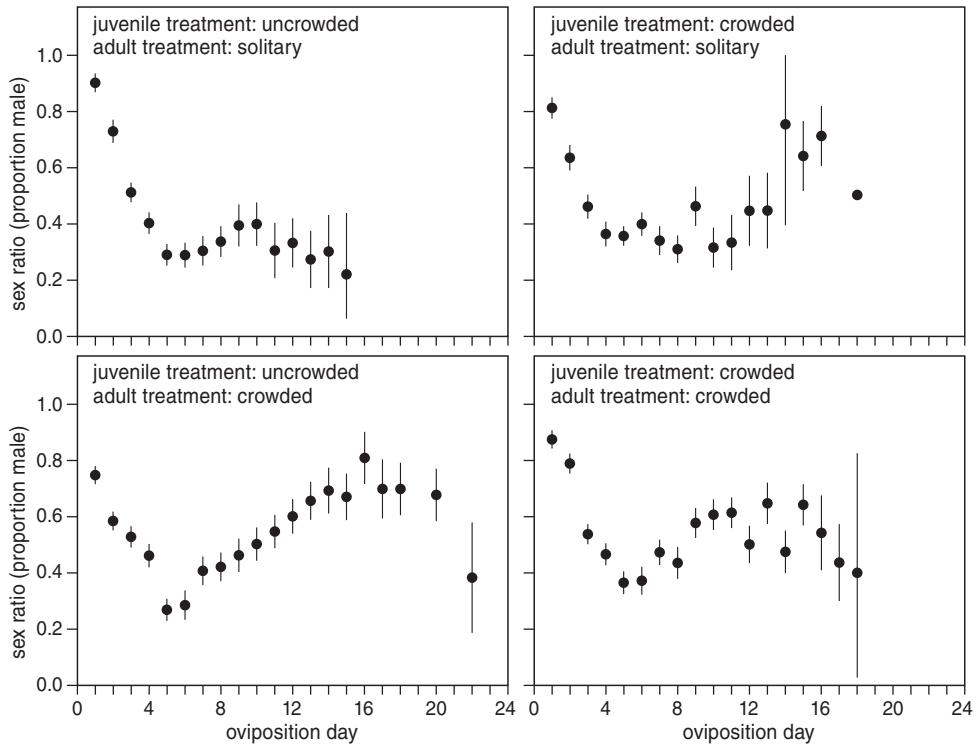

Figure 7.4 Sex ratio (proportion male) plotted against oviposition day for each of the four treatment combinations. Error bars show the 95% confidence intervals.

Table 7.1 The minimal adequate model for sex ratio with respect to both treatment and laying day. The model is a linear mixed effects model with arcsine-transformed sex ratios and female ID as a random factor. Non-significant interaction and main effects were deleted from the model ($P > 0.05$). JD and AD are juvenile density and adult density respectively.

	NumDF	DenDF	F-value	*P*-value
(Intercept)	1	483	1237.31	<.0001
Adult Density [or AD]	1	44	11.41	0.0015
Laying Day	1	483	56.38	<.0001
(Laying Day)^2	1	483	135.92	<.0001
Potato weight JD	1	44	10.40	0.0024
Potato weight AD	1	44	3.44	0.0704
Adult treatment: Day	1	483	4.29	0.0389
Adult treatment: Day^2	1	483	48.71	<.0001
Day: potato weight JD	1	483	20.15	<.0001
Day: potato weight AD	1	483	12.57	0.0004

effect of treatment on sex ratio per day using a linear mixed effects model. The results are shown in table 7.1 and figure 7.4. There was a strong effect of laying day on the sex ratio of the clutch and the effect was also nonlinear (both day and day^2 were significant, see table 7.1). Otherwise the results were largely consistent with the previous analysis. There was also a significant effect of adult density ($F_{1,44} = 11.41$, $P = 0.002$) but not of juvenile density ($F_{1,43} = 0.09$, $P = 0.77$; Figure 7.4) and there was a significant effect of potato weight during juvenile stage ($F_{1,44} = 10.40$, $P = 0.002$), but not during the adult stage ($F_{1,44} = 3.44$, $P = 0.07$), with females growing on larger potatoes producing a more female biased sex ratio. There were significant interaction effects between adult treatment and day and day^2 (Table 7.1) with females under crowded conditions having a stronger rise in the number of sons they produce late in life (Figure 7.4). There were also significant interactions between the potato weight at both life stages and day (see Table 7.1). Females fed on larger potatoes during the adult stage started to produce male biased sex ratios earlier and females raised on big potatoes during the juvenile stage showed no rise in number of sons produced, while females raised on smaller potatoes did.

DISCUSSION

We found that sex ratio varied with the crowding of the local population, with females producing more male-biased clutches under high adult crowding. This result supports the interpretation that female *P. citri* can facultatively adjust the sex ratio of their offspring and are not constrained by their genetic system. We also found a strong effect of age on the sex ratio produced by females, with females initially producing more male offspring before switching to a female biased sex ratio, with older females then producing more male-biased sex ratios again (Figure 7.4). Despite these dynamic changes in sex ratio, the overall sex ratio was 0.51 (incidentally highlighting the difficulties of interpreting lifetime sex ratios).

The shifts in sex ratios we observed contradict those expected under the classic local resource competition (LRC) model, which assumes that increasing colony density signifies a reduction in interactions among kin, which it did in our experiment design. When a greater proportion of unrelated females compete for resources (as in our high density treatments), sex ratios should be less biased, and yet we observed a greater male bias under low LRC conditions (Figure 7.3). However these results are consistent with density influencing sex ratio through a more general effect on competition among female offspring, such that when densities are high, male offspring will be of greater reproductive value to mothers as they need fewer resources and are able to migrate away from dense colonies.

Varndell and Godfray (1996) tested LRC using a slightly different experimental protocol, such that individuals in their high-density adult treatments were either closely related to each other (being derived from one egg sac in the low density juvenile treatment), or a mix of kin and non-kin (if derived from the 5 eggs sacs of the

high density juvenile treatment). This design risked confounding the effects of competition among relatives (the basis for LRC) with (increasingly "global") competition for resources among unrelated individuals, as increases in density represented by the two adult treatments should have different effects on the extent of LRC signalled by high mealybug density. In the treatment where adults were derived from one egg sac, LRC remained high and sex ratios should be relatively male-biased (and certainly more male-biased than the high-density treatment drawn from 5 unrelated egg sacs): the data from Varndell and Godfray (1996) are in the opposite direction. We tried to limit this by generating interactions between less related individuals in the treatments at both the juvenile and adult stages. For us therefore, increasing density meant a decrease in the degree of competition between related females and so a decrease in LRC; in the Varndell and Godfray (1996) experiment, the high adult density treatments included both low and high LRC environments. Whilst our current experiment has at least begun to address relatedness and density, we note that further experiments explicitly manipulating relatedness in more detail may be called for. For instance, high relatedness among ovipositing females could potentially be important for the predicted effect of LRC, since their offspring would be related and therefore sex ratios would be predicted to remain male-biased even with increasing population density. Although sex allocation responses to relatedness are not always present (e.g. in the parasitoid wasp Nasonia vitripennis: Reece *et al.*, 2004; Shuker *et al.*, 2004a; Shuker *et al.*, 2004b), Kasuya (2000) showed that another scale insect, the mango shield scale (*Milviscutulus mangiferae*) does discriminate kin and shows differential migration behaviour to avoid kin-competition.

Taken together however, both experiments question the importance of LRC theory for *P. citri* sex ratios, albeit in different ways. In our experiment, increased density led to a reduction in the production of females, suggesting that increased resource competition (but not increased *local* resource competition *sensu stricto*) favours a greater proportion of sons. Perhaps the most likely explanation is that the fitness gains through female offspring decline with increased competition, regardless of interactions with relatives.

We found an average overall sex ratio of 0.51. This again differs from the results of Varndell and Godfray who found an overall sex ratio of 0.32. Several other studies have looked at sex ratio in *P. citri* and there seems to be substantial variation between the results of different studies (Table 7.2). Nelson-Rees found a slightly female biased sex ratio of 0.43 (Nelson-Rees, 1960), while James (1937; 1938) and Schrader (1922) both observed equal sex ratios in their studies. It is of course hard to compare these earlier studies because they were testing different things, and were also all based on secondary sex ratios so that effects of differential mortality cannot be excluded. In addition, different authors also used different female densities in their experiments. Table 7.2 gives an overview of the different studies and the densities used (only adult density is considered because juvenile density is often not stated). However, taken together these results show that the sex ratios observed in this present study are more consistent with the earlier studies, with young solitary

females producing an equal or slightly female-biased sex ratio, as compared to the strongly female-biased sex ratios observed by Varndell and Godfray (1996). In order to better understand the observed differences in sex ratio between different studies and to establish how much of the variation in sex ratios can be explained by genetic differences between populations, we are currently exploring genetic variation for sex allocation among *P. citri* strains drawn from three independent populations.

Table 7.2 Sex ratios have varied across studies of sex allocation in *P. citri*. This table gives an overview of the available sex ratio data and also includes factors that have previously been shown to influence sex allocation. Sex ratios were calculated from the raw data presented in the papers or obtained from the figures.

Author	Sex ratio	Type sex ratio	Aim of study	Density manipulation	Culture temp.	Age of female when mated
This study	0.46	Primary sex ratio	Effect of density and female age	Solitary as adults; juvenile density varied	25°C	30 days
This study	0.54	Primary sex ratio	Effect of density and female age	Crowded as adults (50); juvenile density varied	25°C	30 days
Varndell & Godfray 1996	0.32–0.38	Primary sex ratio	Effect of density	Solitary as adults juvenile density varied	25°C	30 days;
Varndell & Godfray 1996	0.25–0.29	Primary sex ratio	Effect of density and female age	Crowded as adults (20); juvenile density varied	25°C	30 days
Schrader 1922	0.47	Secondary sex ratio	Determining sex ratio	Solitary as adult	Unknown	Directly after maturation
James 1937	0.5	Secondary sex ratio	Effects of female age at mating	Solitary as adult	20.5–26.8°C	Directly after maturation
James 1937	0.63–0.83	Secondary sex ratio	Effects of female age at mating	Solitary as adult	20.5–26.8°C	Mating delayed 42–70 days after maturation
Nelson-Rees 1960	0.43	Secondary sex ratio	Effects of female temperature and age at mating	Solitary as adult	20.0–26.0°C	Directly after maturation (41 days)
Nelson-Rees 1960	0.37–0.84	Secondary sex ratio	Effects of temperature and female age at mating	Solitary as adult	20.0–26.0°C	Varied (25-148 days)
Nelson-Rees 1960	0.61–0.68	Secondary sex ratio	Effects of temperature and female age at mating	Solitary as adult	29.1-30.2°C	Directly after maturation

Why might this general female bias be present? One possibility is that another form of local resource competition, namely local mate competition (LMC) is playing a role. Competition among related males is predicted to select for female-biased sex ratios when only one or a few females contribute offspring to a patch (Hamilton, 1967, 1979). However, the observed female bias is small, and given male dispersal in this species, LMC among sons is perhaps unlikely to be very important. As mentioned in the introduction, Varndell and Godfray (1996) offered an alternative explanation for their data. They suggested that perhaps females do not compete with each other, but instead cooperate, possibly because it is easier to obtain resources from their host plant when feeding in groups (i.e. local resource enhancement, LRE). However, this hypothesis could not be confirmed in later studies (Varndell, 1995) and it could also not explain the opposite effect of juvenile and adult crowding on the sex ratio. It is also not supported by the data we have presented here.

Local interactions among relatives are not the only potential factors influencing optimal sex allocation in mealybugs however. Since Varndell and Godfray (1996) published their study, the potential for intra- and inter-genomic conflicts over sex ratio has become clear in *P. citri*. For example, recent work by Buglia and Ferraro (2004b) suggests that males might be able to control the sex ratio of their offspring as well. This would be selectively advantageous to males because under PGE their genes are not transmitted through sons and so males are selected to favour female offspring (similar intra-genomic conflicts over sex ratio exist in true haplodiploids, e.g. Shuker *et al.*, 2009; Shuker *et al.*, 2006). Moreover, inter-genomic conflict over sex allocation could arise between the host and their endosymbiotic bacteria since endosymbionts are also only transmitted though females (Normark, 2004a). Currently, there is little direct evidence for the influence of either males or endosymbionts on sex allocation (but see below).

In addition to our density treatment effects on sex ratio, we also found that female condition affects sex ratio. First of all, larger females produced a more female-biased sex ratio, but not more eggs. Second, the size of the potato a female was reared on also influenced sex ratio, with females raised on bigger potatoes producing a more female-biased sex ratio. According to Trivers and Willard (1973), females should adjust the sex ratio according to their condition when one sex benefits more from a mother in a particular condition than the other. The results found in this study might suggest that female offspring benefit more from the good condition of their mother. Thus yet another strand of sex allocation theory might also influence mealybug sex allocation. However Varndell and Godfray (1996) found the opposite pattern, with larger, more fecund and long-lived mothers producing more male offspring. The reason for these differences is not clear.

We also found a strong age effect on the sex ratio that a female produces, with females first producing males, followed by a female-biased sex ratio for several days, and finally producing a more male-biased sex ratio again at the end of their life (although there is an increase in variation of the sex ratios produced by older females). Nelson-Rees (1960) found a similar pattern of early male production for

females mated as soon as they reached maturity, although an increase in male production towards the end of a female's oviposition period was only seen when females were prevented from ovipositing for a period. The pattern observed by Varndell and Godfray (1996) falls somewhere in between, with no clear peak of early male production, but a rise in males towards the end of the oviposition period (with some variation among treatments as seen here).

A possible explanation for these observations, and for those from earlier work on temperature (Table 7.2), is that the sex determination system undergoes changes or deteriorates with age or stress. Kono *et al.* (2008) used quantitative PCR to determine the number of endosymbionts in mealybugs in different developmental stages and found that in older females the number of endosymbionts strongly decreases. Similarly, high temperature leads to a more male biased sex ratio (Nelson-Rees, 1960), as well as deterioration of the endosymbionts (Buchner, 1965). The extent to which these associations are circumstantial or causal remains to be evaluated, but links to the hypothesis that endosymbiotic bacteria influence sex determination in some way are apparent (Normark 2004) and we are currently exploring the role of environmental stress on both female condition and sex allocation in more detail.

In summary, sex ratios in *P. citri* are clearly influenced by the environment, both in terms of more direct effects on females (including age and condition) and also in terms of local population density. We have perhaps uncovered more complexity than we have been able to explain, but nonetheless suggest that the role of the environment in altering the value of female versus male offspring via global competition among females is the most likely explanation for much of the sex ratio data presented here.

Acknowledgements

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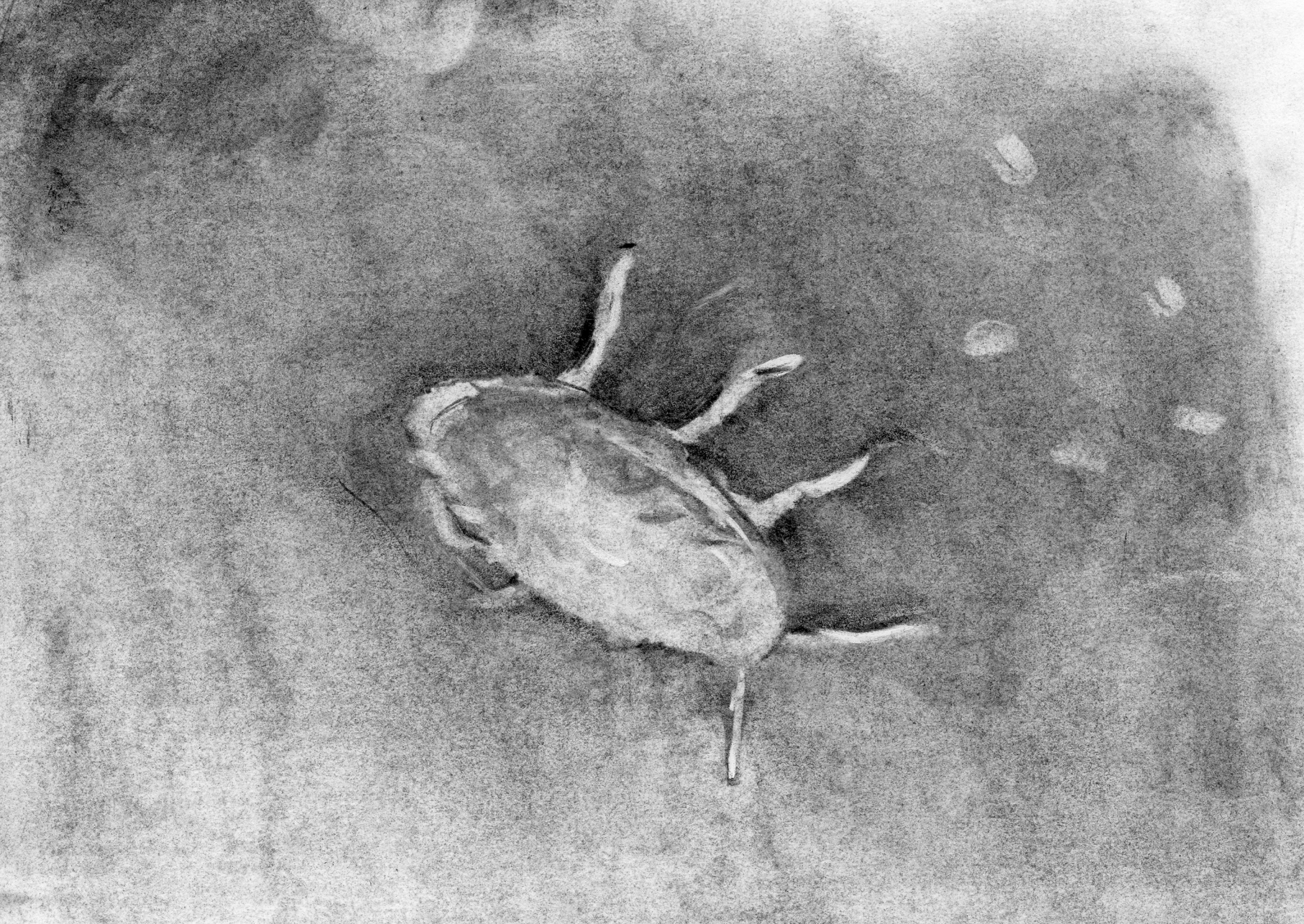

CHAPTER 8

Sex-specific dispersal behaviour of crawlers in the mealybug *Planococcus citri*.

Laura Ross, Ido Pen and David M. Shuker.

ABSTRACT

Sex-specific dispersal can have important evolutionary and ecological implications, influencing local population structure and sex ratio, as well as the speed at which new habitats can be colonized. In scale insects, first instar larvae (crawlers) are assumed to be the main dispersal stage. Although all scale insects are extremely sexually dimorphic, in most species the sexes are indistinguishable as crawlers. Here we consider the mealybug *Planococcus citri*, and dispersal by crawlers to or from resource patches. The aim of this study was to test if: (1) crawler dispersal behaviour differs between the sexes and how this is affected by local conditions (population density and sex ratio); (2) there is a difference between the sexes in crawler migration success to a new host plant. Using two experiments, which differed in how resources were spread between dispersal sources and sinks, we show that male and female larvae do not differ in their dispersal behaviour or in their dispersal success when dispersal is via crawler locomotion. These laboratory experiments are an important starting point for understanding the evolution of dispersal behaviour of *P. citri* in the wild, suggesting that more attention might need to be paid to different methods of dispersal as well as crawler locomotion.

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INTRODUCTION

Patterns of dispersal are important factors shaping both population structure and the opportunity for and speed of colonisation of new habitats (2002). There has been substantial work on the dispersal behaviour of scale insects. Many studies have identified that the juvenile stages are the main agents of dispersal (Beardsley & Gonzalez, 1975; Greathead, 1990, 1997; Gullan & Kosztarab, 1997). First instar larvae (crawlers) have been found to possess numerous characteristics that have been considered adaptations for dispersal behaviour, including long legs and antennae (relative to later instars: Beardsley & Gonzalez, 1975; Gullan & Kosztarab, 1997). Additionally, dispersal strategies may be more passive, and crawlers of several species have found to exhibit behaviours that increase wind dispersal and to use wind dispersal to migrate for several kilometres (Washburn & Washburn, 1984). Crawlers from several species have also been found to be able to survive without food for extended periods, which should again enhance their dispersal success (Gullan & Kosztarab, 1997).

Several studies have attempted to estimate crawler dispersal rate and distance under both laboratory and field conditions, as well as to establish the factors influencing crawler dispersal (reviewed by Greathead, 1990, 1997). However, experimental tests are rare (but see Washburn & Frankie, 1981; Washburn & Washburn, 1984). As such, one aspect of the crawler dispersal behaviour has yet to be seriously addressed: the potential difference in dispersal behaviour between the sexes during the crawler stage. If there is such a difference, this would be important as it will alter the predicted effects of dispersal rates on colonization of new habitats and on the population structure. For example, sex-biased dispersal might change the local operational sex ratio, with the source population being biased towards the least migratory sex, while newly established populations will be biased towards the migratory sex (Leturque & Rousset, 2003). However sex-biased migration can also affect sex ratio in a different way. Since related crawlers might compete locally for resources where they hatch, if one sex has a lower dispersal rate than the other, then this sex is expected to experience higher levels of kin competition (Hamilton, 1967). This in turn can lead to biased primary sex ratios as ovipositing females are expected to bias the sex ratio of their offspring they produce in order to minimize competition among their offspring (Clark, 1978; Hamilton, 1967; West, 2009). Indeed, primary sex ratio adjustment to local density has been observed in the mealybug *Planococcus citri* (Ross *et al.*, 2010a; Varndell & Godfray, 1996). Additionally recent theoretical work has shown that sex ratio and sex-biased dispersal can coevolve, and that biased sex ratios can select for sex-biased dispersal and *vice versa* (Leturque & Rousset, 2003, 2004).

But why would there be differences between dispersal behaviour of male and female crawlers? Male and female scale insects differ in several important ways. First of all, males stop feeding after the second instar. Because of this they need fewer resources, and need them for a smaller proportion of their life. Therefore they are potentially less sensitive to both resource competition and food shortage compared to females and might be less inclined to risk dispersion under those circumstances.

Secondly, adult males are winged while adult females are almost completely sedentary in most species, and males also have a very sensitive pheromone receptor system and are able to find females at large distances (Branco *et al.*, 2006). Suggesting that young males do not need to disperse to find mates. Based on these two factors we predict that female crawlers are more likely to disperse than male crawlers. Although there has yet to be an experimental study on sex-specific migration in scale insects, there are suggestive observations of a few species in which there is a morphological difference between the sexes at the early juvenile stage (Brown & Bennett, 1957; Greathead, 1990; Gullan & Kosztarab, 1997). However all these species are exceptional in that their crawlers are sexually dimorphic. The dimorphism has allowed easy observation of behavioral differences between the sexes, but this might not be representative for other scale insects, as the dimorphism might have evolved as a result of the differential behaviour specific to these species. It is therefore important to test for sex-specific crawler dispersal in species that do not show sexual dimorphism at the crawler stage (as is the case in the large majority of species).

Usually crawlers are expected to settle close to where they were born (Beardsley & Gonzalez, 1975; Gullan & Kosztarab, 1997; Nestel *et al.*, 1995), possibly because dispersal is risky. However, presumably local conditions will influence dispersal behaviour under some circumstances (Comins, Hamilton & May, 1980; Hamilton & May, 1977). There are two situations when dispersal might be selected for: (1) high local density, as this might result in high levels of competition (both between kin and between unrelated individuals); (2) local exhaustion of resources (e.g. due to death of the host plant) (Washburn & Washburn, 1984). Based on the predictions described above we expect that female crawlers are under stronger selection pressure than males to disperse in order to avoid the adverse effects of crowding and lack of resources.

In this paper we present the results of two experiments that test for sex-specific differences in dispersal rate, addressing both crowding and resource availability and the associated effects on mortality. The first experiment tested the effect of crawler density and sex ratio on the migration probability of both sexes in a fully factorial design with two density treatments and two sex ratio treatments (male biased versus female biased). The second experiment tested for a difference in dispersal success between males and females when there was no local food source, again considering two sex ratio treatments (male biased versus female biased). We also consider the role of differential mortality, by comparing both primary sex ratio (determined at the egg stage) and secondary sex ratio (determined at the third instar).

MATERIALS AND METHODS

Study system

The experiments where conducted using the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Coccoidea: Pseudococcidae). This cosmopolitan species can parasitize a wide variety of host species and is easily cultured in the lab. *P. citri* has strictly sexual

reproduction and an unusual genetic system in which the chromosomes inherited from the father are deactivated (condensed) during early development in males and not passed on in the germ line (Brown & Nelson-Rees, 1961; Schrader, 1921, 1922). This gives the opportunity to determine the primary sex ratio, by staining the chromosomes of the embryos and inspecting them for the condensed paternal chromosomes (indicating that the embryo is male).

Culture conditions

The culture used for this experiment was obtained from a laboratory culture at Wye College, U.K. All mealybugs were cultured on sprouting potatoes (cultivar Desiree) in rectangular plastic boxes (3.2 litres in volume) covered with fine mesh. Cultures were kept at 25°C and 70% relative humidity under a 12-h light/12-h dark regime.

Experimental setup

The setup for both experiments consisted of two small plastic rectangular boxes connected by a plastic tube with a diameter of 1 cm (see figure 8.1 for further measurements), to allow for dispersal between them. In both experiments one of the 2 boxes was randomly assigned the source box and this was where the egg masses were placed to initiate the experiments. In order to obtain individuals of known and equal age for each experiment, 20 ovipositing females were removed from the mass culture and placed on fresh potatoes where they were allowed to oviposit for 24 hours. Following oviposition, offspring were allowed to develop for 30 days, by which time they will have reached reproductive maturity and been mated. Females were then isolated from the main culture and placed on a single potato sprout in individual glass tubes and checked daily for signs of oviposition. As it is difficult to handle individual mealybug eggs, density in our experiments was manipulated by varying the number of resulting egg masses from these females to initiate each replicate. From previous experiments we know that female age strongly influences clutch sex ratio (Ross *et al.*, 2010a). This

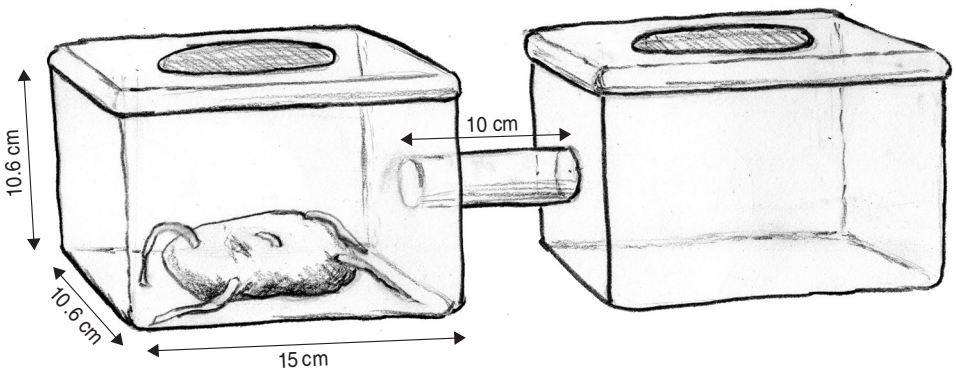

Figure 8.1 Schematic representation of the experimental setup used for both experiments. Drawing by Frans Ross.

knowledge can be used to manipulate the population sex ratio, by using egg masses of females from different ages. We used eggs from females on the first and sixth day of oviposition. A previous study showed that the average sex ratio (fraction males) produced by the females on the first day is 0.81 and the sex ratio of the sixth day is 0.34, while the average clutch sizes are similar (Ross *et al.*, 2010a). For the male biased treatment, we therefore isolated egg masses from females on their first day of oviposition, while for the female biased treatments we used egg masses produced one the sixth day (these females will be referred to as one day old and six days old).

Experiment 1: Density and sex ratio

For the first experiment a single potato was placed in each of the 2 chambers of the migration box. The potatoes were weighed at the start of the experiment and matched in size so that the difference in weight between the two potatoes in the chambers was less than 10 grams. Depending on the treatment either 10 or 20 egg masses from either one or six day old females were placed on the potatoes in the source box, resulting in four treatments: female biased low density, female biased high density, male biased low density and male biased high density. The eggs were then allowed to hatch and the larvae to develop and migrate between the boxes. Fifteen days after the boxes had been founded, the migration tube was blocked to avoid migration of later instars and after twenty days the number of males and females was determined based on their morphological differences. The total sample size was 27 across the four treatment combinations.

Experiment 2: Starvation and sex ratio

In the second experiment we tested for sex differences in migration success when no local food source is available, by comparing the sex ratio of crawlers that migrated successfully to a new food source with those in the control that did not have to migrate to find food. The experiment consists of one migration treatment and two control treatments: a secondary sex ratio control (determined at the third instar) and a primary sex ratio control (determined at the embryo stage). For each of these treatments there are two sex ratio conditions (male biased and female biased, as described previously). For the migration treatment the same setup was used as for the first experiment, although in this experiment a potato was placed only in the sink chamber, while the source chamber remained empty. Depending on the sex ratio treatment, a single egg mass of either a one day or six day old mother was placed in the empty source chamber. For the secondary sex ratio control a potato was placed in a single box, without a migration tube and the egg mass was placed directly on the potato; for the primary sex ratio control treatment the eggs were taken directly from the ovipositing female (either one or 6 days old) and transferred to fixative and subsequently stained, sexed and counted (for a detailed description of the methods see Ross *et al.*, 2010a). The sex ratio of the secondary sex ratio and the migration treatments were counted 20 days after the populations were founded, again based on morphological differences between the sexes. The total sample size of the experiment was 50.

Data analysis

All data were analyzed using R (R Development Core Team, 2008). All sex ratio data were analyzed using a generalized linear model approach with a quasibinomial error structure to correct for overdispersion. All other data were analyzed using a general linear model with a Gaussian error structure. For the first experiment we calculated the dispersal probabilities of males and females by dividing the number of successful dispersers of a certain sex by the total number of that sex (summed over the two boxes). For the second experiment we fitted a generalized linear model to test for the treatment effects on sex ratio. We assumed that if there was a general mortality difference between the sexes we would expect to see a difference between the primary SR control treatment and the other two, while if the mortality difference was mainly associated with different success rates of dispersal, then the two controls (primary and secondary sex ratio) should be similar, but the sex ratio in the migration treatment different.

RESULTS

Experiment 1: Density and sex ratio

TOTAL SEX RATIO: To manipulate the sex ratio of the source populations egg masses of females of two different ages were used to setup the source population. This manipulation was successful, as there was a significant effect of the age of the mothers used on the population sex ratio ($F_{1,20} = 126.4$, $P < 0.001$; Figure 8.2). The boxes founded by females on their first day of oviposition had an average sex ratio of 0.83, whilst the boxes founded by females on their sixth day of oviposition had an average sex ratio of 0.42. To manipulate the density of the populations, boxes were founded with either 10 or 20 egg masses. The total population size in the low-density box was on average 209 (+/– 36 s.e.) and in the high-density box 491 (+/– 61 s.e.). The density treatment did not affect the sex ratio of juveniles ($F_{1,21} = 0.88$, $P = 0.36$), however there was a significant interaction effect between density and the mother's age on sex ratio ($F_{1,19} = 6.77$, $P = 0.018$; see also figure 8.2). As such, it seems that under high density and a male biased sex ratio, females have a relatively higher mortality, while at a slightly female biased sex ratio, higher density leads to relatively higher mortality for males. However without knowing the primary sex ratio of the treatments it is hard to confirm that the observed results are the result of differential mortality.

DISPERSAL: The number of individuals that migrated to the second box was very low for all treatments with an overall average of 4.22 (+/– 1.07 s.e.) individuals moving boxes and an average migration probability of 0.013. There was no effect of either the density of the population, the age of the founding females or the weight of the potato in the source box on the total migration rates (age: $F_{1,20} = 0.07$, $P = 0.79$, density: $F_{1,21} = 0.37$, $P = 0.55$, potato: $F_{1,19} = 0.88$, $P = 0.36$) and nor were there any significant interactions between those factors.

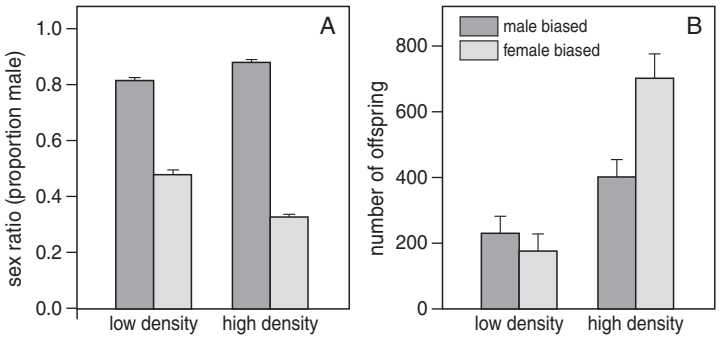

Figure 8.2 Results from the first experiment showing (A) The average sex ratio (error bars showing binomial standard errors) across both source and sink box and b) the total number of offspring summed over the source and sink boxes (error bars showing standard errors) for the two different density treatments and for mothers of two different ages (day 1 and 6). Sex ratios and number of offspring were counted at the age of 20 days, after individuals were allowed to disperse.

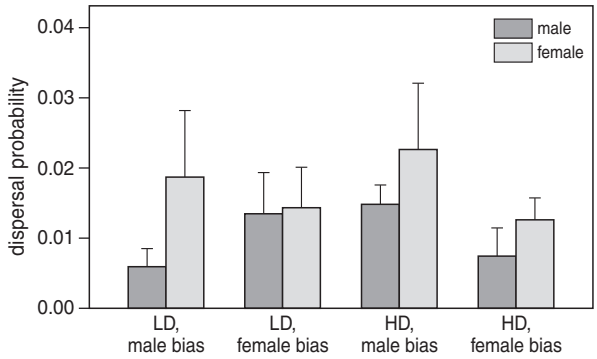

Figure 8.3 Dispersal probability of male and female crawlers for each of the four treatment combinations in experiment 1 (LD: low density, HD: high density). The error bars show the binomial standard errors.

In order to test for a difference between the dispersal rates of the sexes and the possible effect of density and sex ratio on this difference, we compared the sex ratio between the source and sink boxes. We fitted the sex ratio in the sink population as response variable, with density and the age of the mother as explanatory variables and the sex ratio in the source population as covariate. The only factor with a significant effect on the sink sex ratio was the sex ratio in the source box ($F_{1,14} = 18.94$, $P = 0.0009$) and the correlation between source and sink sex ratios did not differ from unity (1.12 +/– 0.51 s.e.). This shows that there is no significant difference between the dispersal rates of the sexes and neither density or the sex ratio treatment significantly affect the sex ratio of dispersers (density: $F_{1,13} = 0.13$, $P = 0.725$, sex ratio treatment: $F_{1,12} = 0.18$, $P = 0.679$).

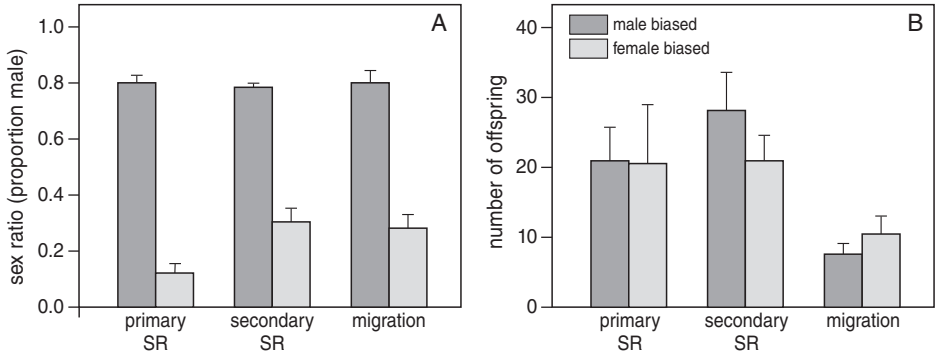

Figure 8.4 (A) The average sex ratio of surviving offspring (error bars showing binomial standard errors) and (B) the total number of offspring (error bars showing standard errors) for the two controls (primary and secondary sex ratio) and the migration treatment for mothers of two different ages (day 1 and 6) for experiment 2.

Experiment 2: Starvation and sex ratio

The number of surviving offspring in the dispersal treatment was lower than the number in the two controls ($F_{1,47} = 5.38$, $P = 0.008$, figure 8.4B), suggesting that only a proportion of the crawlers originally present were able to successfully migrate and find the food source. However the sex ratio treatment did not affect the number of successful dispersers ($F_{1,46} = 0.16$, $P = 0.69$).

In order to compare if there was a difference in mortality between the sexes and how much of the mortality is caused by a failure to disperse to the new food source, we compared the sex ratio between the two controls and the migration treatment. There was a significant difference in sex ratio between the female and male biased sex ratio treatments as expected ($F_{1,45} = 69.65$, $P << 0.001$). However, there were no significant differences among the three migration treatments (migration and the two controls: $F_{1,46} = 1.93$, $P = 0.157$) and additionally no interaction between the sex ratio treatments and the three migration treatments ($F_{1,43} = 1.31$, $P = 0.28$). Since within each of the two sex ratio manipulations (male and female biased clutches) there was no difference in the sex ratio between control clutches sexed as embryos (primary sex ratio control), control clutches sexed as adults (secondary sex ratio control) and the actual migration treatment, migration success does not differ between the sexes and we can also be confident that differential male and female mortality is not confounding these results.

DISCUSSION

Neither of the two experiments revealed a difference between the sexes in either the dispersal rates or dispersal success, although it must be noted that the number of dis-

persers in both experiments were relatively small. The first experiment showed some suggestion that there might be a mortality difference between the sexes, with males having a slightly increased mortality rate, however as our experiment was not specifically designed to test for difference in mortality, alternative explanations cannot be excluded. Our second experiment on the other hand suggests that differential mortality is unlikely to be a major confounding variable.

Based both on behavioural and life-history differences between the sexes, we expected that female crawlers would have a higher dispersal rate than males. This pattern has earlier been observed in a few scale insect species that have sexually dimorphic crawlers: First of all, a few species of armoured scale insects (Coccoidea: Diaspidae) have crawlers that are sexually dimorphic in colour. In these species it has been observed that female crawlers are more likely to disperse away from the place where they were born than brothers (Brown & Bennett, 1957; Gullan & Kosztarab, 1997). Secondly sex-specific dispersal has been observed in Dactylopius austrinus. In this species, female crawlers develop long wax threads, which aid dispersal, while this adaptation is absent in male crawlers (Greathead, 1997; Moran, Gunn & Walter, 1982). Sex-specific crawler dispersal has also been observed in two species of gall-forming Eriococcids. In these species males are found to induce a gall on the gall of their mother or develop within their mother's gall and therefore generally stay close to the place there were born, while female crawlers disperse and form galls independently (Cook, Gullan & Stewart, 2000). Additionally, a remarkable form of sex-specific dispersal strategy has also been observed in another Eriococcid genus (Cystococcus), whereby female crawlers are carried to new feeding sites by their adult (winged) brothers (Gullan & Cockburn, 1986). All these observations suggest that female crawlers are more likely to disperse than males.

However in our experiment we were unable to observe a difference between the sexes, although the sample size used was relatively small and the low dispersal rate observed reduced the power of the experiment. One possible explanation for the contrast between our results and earlier observations is that strong differences in crawler dispersal rates between the sexes are only present in species with sexual dimorphic crawlers. Another aspect that could have affected the results of our experiments is that we only considered dispersal distance while there might be sex differences in dispersal behaviour that do not necessarily affect the total distance crawler travel but does affect the distribution of male and female crawlers on the host plant. For example in many armoured scale insects it has been shown that male and female crawlers might differ in how they are attracted to light and in which areas of the host plant they prefer to settle (Beardsley & Gonzalez, 1975). It has also been shown that in the armoured scale insect Pseudaulacaspis pentagona, male crawlers congregate in clusters while female crawlers settle more scattered across the host plant (Beardsley & Gonzalez, 1975). This suggests that there could also be more subtle behavioural differences between male and female crawlers in P.citri, that our experiment is unable to reveal. Finally we would like to point out that in the experiments presented in this paper we only examine dispersal behaviour via walking, while other forms of

dispersal, for example wind dispersal might also be important and potentially more prone to sex differences.

The overall dispersal rate in the first experiment was very low with no dispersal events in 30% of the boxes. However, the relative higher numbers of dispersers observed in the second experiment using the same setup did show that crawlers are able to migrate between the boxes. The difference between the experiments, in which a food resource either was or was not present in the source box, suggests that even under relatively high densities, crawlers tend to settle nearby, rather than trying to find a less crowded spot. Moreover, the fact that density did not have any effect on the total migration rate might suggest that the crowding in the high density treatment was insufficient to induce enough competition. Judging what constitutes biologically meaningful levels of density however is difficult (Ross *et al.*, 2010a), as estimates of natural density levels are rare (but see Nestel *et al.*, 1995). Other studies considering dispersal behaviour of scale insects crawlers showed a range of factors influencing dispersal. In the armoured scale insect Aonidiella aurantii crawlers start wandering shortly after emergence but the wandering time is affected by host plant species and quality, with longer wandering times on lower quality host (Willard, 1973).

To our knowledge this paper presents the first experimental attempt to test for a potential difference in dispersal behaviour between male and female crawlers in scale insects. The results presented suggest that there is no substantial difference, neither in the dispersal probability nor its success. However, these experimental results need validation under more natural situations. In order to test sex specific migration under field conditions it might be possible to use the cytological techniques described in this paper to sex crawlers found at varying distances from a known source populations. In addition, population genetic techniques, at a sufficient degree of resolution, may also shed light on dispersal patterns. One could compare nuclear and mitochondrial sequences (or endosymbiont) sequences to compare the geographic structuring of female lineages across a population, assuming sufficient variation is present (and it may well not be). Unfortunately population genetic studies on scale insects are rare (but see Cook & Rowell, 2007) and to date no study has attempted to estimate sex specific dispersal rates in scale insects using these techniques, and it will no doubt be difficult to get sufficient material to provide a fine-scale of resolution. Additionally although these techniques can shed light on overall differences in dispersal rates between the sexes, they will not be able to reveal at which developmental stage the difference in dispersal rate is most pronounced. Progress will probably be most rapid by exploring the diversity of dispersal strategies observed in nature, in particular in exploring the extent to which crawlers actively or passively disperse, which might then allow more sophisticated experimental manipulations to be undertaken.

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CHAPTER 9

Temporal variation in sex allocation in the mealybug *Planococcus citri*: adaptation, constraint, or both?

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ABSTRACT

Sex ratio theory has been very successful in predicting under which circumstances parents should bias their investment towards a particular offspring sex. However, most examples of adaptive sex ratio bias come from species with well-defined mating systems and sex determining mechanisms, while in many other groups there is still an ongoing debate about the adaptive nature of sex allocation. Here we study the sex allocation in the mealybug *Planococcus citri*, a species in which it is currently unclear how females adjust their sex ratio, even though experiments have shown support for facultative sex ratio adjustment. Previous work has shown that the sex ratio females produce changes over the oviposition period, with males being overproduced early and late in the laying sequence. Here we investigate why females change their sex ratio is such complex manner. We first show that this sex allocation behaviour is consistent across lines from three geographical regions. Second, we test whether females produce sons first in order to synchronize reproductive maturation of her offspring, although our data provide little evidence for this adaptive explanation. Finally we test the age at which females are able to mate successfully and show that females are able to mate and store sperm before adult eclosion, and that mating success is increased when females mate with related males from the same population. Whilst early-male production may still function in promoting protandry in mealybugs, we discuss whether mechanistic constraints limit how female allocate sex across their lifetime.

Unpublished manuscript

INTRODUCTION

The allocation of resources between male and female offspring constitutes an important life history decision for sexually reproducing organisms (West, 2009). Under many circumstances equal investment in both offspring sexes is expected (Fisher, 1930), and this is reflected in the appearance of genetic sex determining systems that lead to equal sex ratios (Bull, 1983; Uller *et al.*, 2007). However, there are numerous circumstance under which individuals are predicted, and have been observed, to bias their sex ratio toward a particular offspring sex (West, 2009) (West *et al.*, 2000) (West & Sheldon, 2002). This is because parents are selected to bias the sex ratio of their offspring if one offspring sex is expected to have a higher reproductive value than the other sex (Hamilton, 1967; Trivers & Willard, 1973; West, 2009). The differences in reproductive value of offspring that drive the evolution of adaptive sex allocation can be caused by a variety of factors. For example when offspring compete among each other, the offspring sex that suffers most from this competition will have a lower reproductive value (Clark, 1978; Hamilton, 1967). Alternatively the condition of parents can affect the reproductive value of their offspring sexes differently if one sex suffers more from reduced investment by the parents (Trivers & Willard, 1973). These factors can drive selection on parents to adjust their sex ratio to local conditions.

Utilising this well-developed theory base, sex allocation studies have provided much evidence of adaptive behaviour, especially in organisms with well-defined mating systems and mechanisms of sex determination, in particular hymenoptera, including fig wasps, parasitoid wasps and the social Hymenoptera (Bourke & Franks, 1995; Godfray, 1994; King, 1993; West, 2009; West *et al.*, 2000). However, outside these groups, there is still an ongoing debate over the adaptive nature of the observed patterns of sex allocation (Ewen, Cassey & Moller, 2004; West & Sheldon, 2002). This is particularly true for systems where the sex determination and especially the mechanisms for adaptive sex allocation are unknown (including mammals and birds, as well as many insects (Cockburn, Legge & Double, 2002; Pike & Petrie, 2003; West, 2009). As such, when patterns fit with theoretical expectations, limitations of our understanding of mechanism may be partly put to one side; however, when sex ratio patterns are complex or non-intuitive, the need for a more integrated understanding of sex allocation, putting the phenotype in to the context of a species' biology and mechanisms of sex determination and sex allocation becomes more obvious (West, 2009). Only then can adaptive explanations be more fully tested, and the role of mechanistic constraints (for instance in terms of the role of the genetic system, or information-processing: West et al 2005; Shuker and West 2004) be more fully understood.

In this paper we are interested in trying to explain the pattern of sex allocation observed in a species with an unusual type of sex determination in which the mechanism of facultative sex allocation is unknown. The mealybug *Planococcus citri* exhibits paternal genome elimination (PGE), where although both sexes develop from fertilized eggs and are diploid, males do not transmit their father's chromosomes (Brown

& Nelson-Rees, 1961; Schrader, 1921). Therefore, the method by which female mealybugs adjust their sex ratio is different from that used by females of haplodiploid species (the genetic system found in many species with adaptive sex allocation, such as the Hymenoptera), who can simply control egg fertilisation (Bull 1983). Despite this, there is evidence for adaptive, facultative changes in sex allocation, most notably towards density (Ross *et al.*, 2010a) and mating age (Ross et al submitted). However, previous experiments have also shown a noticeable pattern of sex allocation changing across the oviposition period, with male biased sex ratios being produced early and late in the oviposition period, although the (historical) data are somewhat equivocal (Nelson-Rees, 1960; Ross *et al.*, 2010a; Varndell & Godfray, 1996). We also have recent evidence that suggests that this temporal pattern of sex allocation can influence sex ratio to give the impression of adaptive allocation (Ross et al submitted). Here therefore we would like to investigate why females change their sex ratio during the oviposition period and how this affects their ability to facultatively change their sex ratio. Given that we know rather little about how sex is determined or allocated, here we test whether temporal patterns are (i) consistent across a sample of geographic populations; (ii) associated with male and female development such that reproductive maturity of the sexes is synchronised.

We present the results from two experiments. The first experiment explores variation in sex allocation patterns between lines from three geographic regions to see if the unusual pattern of sex ratio change over the oviposition period is consistent across lines. This was done by determining the primary sex ratio in 15 genetically distinct inbred lines from three populations, focussing both on the total sex ratio produced and on the patterns across the oviposition period. In the second experiment, we consider the function of early male production by comparing the age at which male and female are able to mate successfully. Here we used the same lines to determine both male and female development times and how they are affected by genetic and environmental factors. Finally we also test for factors determining female fertilization success and how this is affected by mating age and the whether her mate is from the same or different line.

METHODS

Study organism

The citrus mealybug *Planococcus citri* (Hemiptera: Coccoidea) is a cosmopolitan species that feeds on wide range of host plants and causes economical damage to both crops and ornamental plants (Ben-Dov *et al.*, 2009). *P. citri* reproduces sexually and there is a strong dimorphism between the sexes both in life history and behaviour (Ross *et al.*, 2010a). While the sexes are indistinguishable as nymphs, males undergo a form of metamorphosis after the second instar and adult males are winged, while the females do not undergo metamorphosis and grow much larger than the males (Sutherland, 1932). Additionally males do not feed after their second

instar and adult males lack functional mouthparts, while females continue feeding until they die. This results in a large difference in lifespan between the sexes, with males only living up to 3 days after eclosion while females can live several weeks after becoming reproductively mature (Nelson-Rees, 1960). Adult female are almost completely sedentary and the highly mobile crawlers (first instar nymphs) are assumed to be the main agent of dispersal (Gullan & Kosztarab, 1997).

Lines used

All specimens used were cultured on sprouting potatoes (cultivar Desiree) in plastic food store boxes covered with fine mesh. For both experiments, lines from three different geographical areas were used to test genetic variation in both sex ratio and development time. They originated from Portugal, Israel, and the UK. The line from Portugal had been collected from a citrus orchard about three months before the start of the experiment, while the lines from Israel and the UK originated from long-term laboratory populations and were founded by specimens collected in glasshouses. From each area, six isofemale lines were created by allowing a single egg-laying female to oviposit for three days in a new box containing fresh potatoes, after which the female was removed and her offspring allowed to grow up. This resulted in 15 viable isofemale lines (Table 9.1). These lines were used to test for variation between the lines in sex allocation behaviour (experiment 1) and development time and survival (experiment 2).

Table 9.1 Origin and sample size (N) of the 15 isofemale lines in both experiments (E1 and E2). PT: Portugal; UK: United Kingdom.

Code	Origin	N female(E1)	N female (E2)	N male (E2)
IA1	Israel	5	83	115
IA2	Israel	6	62	67
IA3	Israel	9	48	40
IB1	Israel	2	61	61
IB2	Israel	4	165	36
IB3	Israel	1	-	-
M1	Mafra (PT)	5	18	20
C1	Camarate (PT)	7	33	24
C2	Camarate (PT)	1	57	12
S1A	Silves (PT)	5	-	-
S1B	Silves (PT)	6	70	62
W1	Wye (UK)	5	121	77
W2	Wye (UK)	4	30	8
W3	Wye (UK)	8	17	10
W5	Wye (UK)	6	-	-
Total		**74**	**765**	**532**

Experiment 1 – Primary Sex Ratios

The goal of this experiment is to partition variation in sex ratio within and between lines and to test if the pattern with which females adjust their sex ratio over time is consistent across lines. To this end, the primary sex ratio was measured for the entire ovipositing duration of approximately 10 females from 15 isofemale lines from three geographic locations. In order to obtain virgin females for mating, 3rd instar females were isolated from their isofemale line boxes 20–25 days after hatching, and males were also isolated at this time. After isolation, females were kept in large glass tubes and provided with a potato sprout, while males were kept in small tubes without food (as they do not feed after their second instar). Ideally, 10 females and 15 males per isofemale line were separated, but due to low male and female availability in some lines, 6–10 females per line and 3–17 males per line were separated.

When the males reached maturity, they were added to the tube of a female of the same isofemale line (their full-sib) in order to inseminate her. Successfully mated (egg-laying) females were used for the rest of the experiment (see Table 9.1). Unfortunately the methods used was not sufficient to ensure female virginity of all females as females were found able to mate very prematurely (see experiment 2) and therefore were already fertilized before they were isolated. However as these females were mated by a full-sib just like the intentionally mated females, we chose to include them in the analysis and corrected for the possible effect of early mating by including mating age as a covariance in the analysis.

After females were mated they were checked daily for signs of oviposition. Once females commenced oviposition, eggs were collected daily from the start of ovipositing until the female died. Collected eggs were fixed in Carnoys fluid (4:3:1, Chloroform:Ethanol:Glacial acetic acid) for four days and subsequently stored in 90% ethanol. Primary sex ratios of oviposition days 1, 3, 6, 9 and 12 of ovipositing were later counted under a fluorescent microscope by staining the egg's DNA with DAPI dissolved in phosphor buffered saline (PBS) (ratio: 1:1000). This way, male eggs can easily be identified by their heterochromatized chromosomes (see Ross *et al.* 2010 for more details on staining methods).

Experiment 2 – Development time

In experiment 2 we measured the development times and mortality rates of males and females from different isofemale lines to determine variation within and between lines. We also considered how development time and mortality of males and females was influenced by the density and sex ratio of the population in which they were raised. Twelve of the isofemale lines from experiment 1 were used in this experiment (see Table 9.1 for the lines used and sample sizes) and the experiment was performed in two blocks, which was controlled for in the analysis. For each line, three boxes were set up containing three randomly selected, egg-laying founding females without their clutch. Females were allowed to oviposit for exactly one day and where then removed, leaving their clutch behind. This way the exact age of all offspring is known and all males and females uses in the experiment are the same age. Boxes

were checked every day so males could be isolated into glass tubes on the very day they started spinning their cocoon. Females were isolated when the first male in their box pupated. All males and females from a box were counted in order to gain secondary sex ratio and density data.

For males, the following data were collected: age at start of pupation and age at reproductive maturity (defined as one day after emergence from the pupa). For females, we collected data on mating age (ability to mate successfully, obtained by mating random females of the same line over a range of ten days and determining a posterior if the mating was successful (eggs) or not (no eggs)) and age at the start of oviposition (for females that were mated successfully).

Data analysis

Data analysis was performed using R (R Development Core Team, 2010). All data was analysed using general and generalized linear mixed effects models. For the analysis of general mixed effect models the R package nlme (Pinheiro *et al.*, 2007) was used. For the analysis of generalized mixed effect models (glmm) we used the package MCMCglmm (Hadfield, 2010a), this package analyses mixed models in a Bayesian framework while allowing a range of possible error-structures.

The data on sex ratio, mortality and mating success were analyzed using a GLMM with quasi-binomial error structure, to avoid effects of over-dispersion. Data on development times for both sexes were analysed using a GLM with a normal error structure (and log transformation). In order to avoid pseudo-replication, the sex ratio data was analyzed with Female ID and line as random effects and country of origin as a fixed effect. All continuous covariates included in the analyses were centered. In all analyses of the second experiment line, box and tube (for female development) were fitted as random effects (with box and tube nested in line) and experimental block as a fixed effect. For the analysis of female mortality and mating success we used the number of surviving/successfully mated female per tube and used a binomial error structure, while for male mortality we also fitted a binomial model using the proportion of surviving males per box. For the Bayesian analysis using MCMCglmm each model was run for a million iterations with a burn-in time of 200,000. A weak informative parameter-expended prior was used for the random effects (Hadfield, 2010b). Additionally, for each model in MCMCglmm, convergence of the chain was tested by using an autocorrelation statistic. For the fixed effects estimated using MCMCglmm we report the probability that the estimate is larger than zero and refer to this as p_{MCMC} (this can be interpreted as a Bayesian equivalent of a p-value (Hadfield, 2010b)). For the random effects, we report the percentage of variance explain by each factor. For the random effects estimated with MCMCglmm we also give the 95% credibility interval for these estimates while for the analysis using LME we test the significance of the random effects by comparing models with and without the random effect with a likelihood test. A wide credibility interval (including a very small lower bound, less than 0.001 for instance) suggests limited or low power to estimate the random effect; to put in analogous frequentist terms, this can be interpreted as not significantly different to zero.

RESULTS

Experiment 1: Sex ratio variation

Oviposition day had a strong effect on sex ratio (figure 9.1B,C,D) and the relationship was polynomial rather than linear as both the linear and quadratic effects of day were significant (see table 9.2). When a female started ovipositing, she laid a highly male biased clutch (day 1: 0.85), which quickly decreased (day 3: 0.54) towards a very female bias (day 6: 0.23, day 9: 0.28). Around the tenth day after ovipositing, sex ratios started becoming more male biased again (day12: 0.32, day15: 0.47) (see figure 1b,c,d). In order to test if this pattern was consistent across the lines, we estimated how much of the variation in sex ratio can be explained by line effects (Figure 1a). We estimated this in two different ways (see methods). A simple mixed model with arcsin square root transformation estimates that line explains 4.88% of the variation in sex ratio and this effect was marginally significant ($LR_{6,7}$ = , P = 0.048). However the estimate of line effect with a Bayesian GLMM gives an estimation of 0.19% (CI 95%: 0.00096 – 22.15%) of the total variance, suggesting the variance explained was not significantly different to zero. So although there is some suggestion

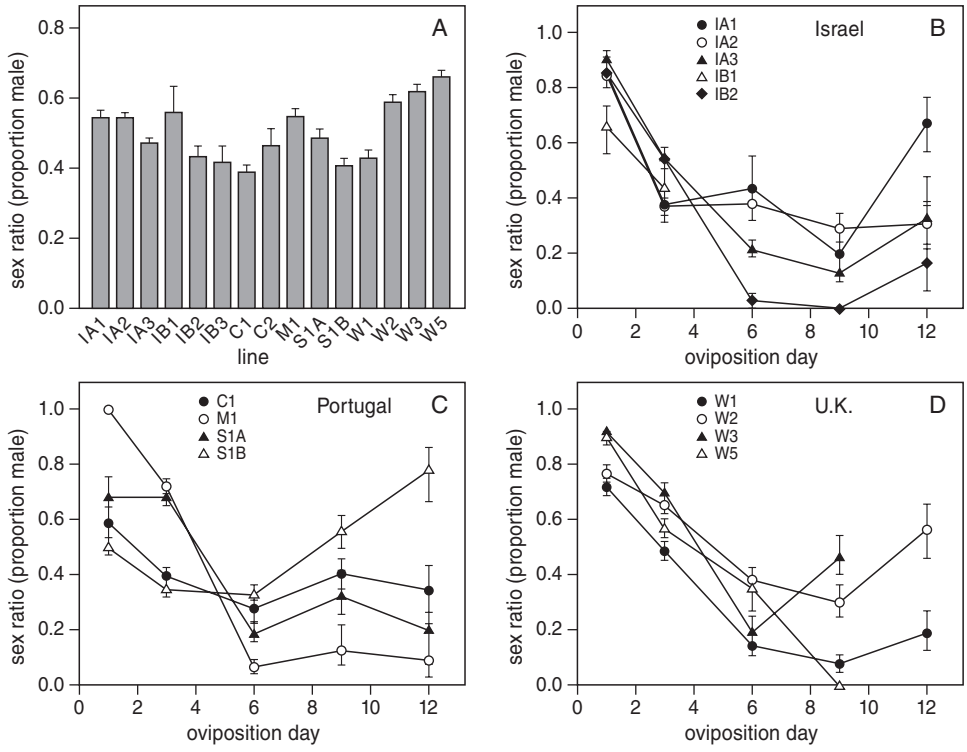

Figure 9.1 (A) Average total sex ratio per line the error bars show the binomial standard error. (B-D) Sex ratio per oviposition day for all lines divided per country of orgin: (B) Israel, (C) Portugal and (D) U.K.

that the total sex ratio produced differed slightly between lines (Figure 9.1), the pattern with which sex ratio changes over time is similar between the lines (see Figure 9.1B,C,D), with in all cases males being produced first. We also tested for differences in sex ratio between the countries of origin and found that there are no significantly differences in sex allocation (Portugal: 0.45 ±0.0098 s.e., Israel: 0.51 ±0.0094 s.e., U.K.: 0.57 ±0.0098, difference Portugal-Israel: $P_{MCMC} = 0.384$, difference Portugal-U.K.: $P_{MCMC} = 0.254$, see table 9.2).

One factor that did affect the pattern of sex allocation however was the day on which females were mated. There was a significant interaction between mating day and oviposition day ($P_{MCMC} = 0.012$, table 9.2), with female that were mated late changing sex ratios throughout their oviposition period at a steeper slope than females that were mated early. Mating day did not change the total sex ratio produced however ($P_{MCMC} = 0.106$, table 9.2) and neither did it change the overproduction of male offspring at the beginning and end of the oviposition period.

Experiment 2: Development time and mortality

MALE DEVELOPMENT

Total male development time – the amount of days from hatching until reproductive maturity – did not differ between lines ($LR_{8,7} = 0.35$, $P = 0.554$, Figure 9.2A) and explains only 4.55% of the variance. Male development was instead influenced by environmental factors. Density in the box the males were raised in influenced their development time with males developing faster at higher densities ($F_{1,20} = 18.79$, $P = 0.0003$). Additionally, rearing box explained a significant proportion of the variance in development time (box: 14.08% of the total variance, $LR_{10,9} = 16.42$, $P < 0.0001$) and males from the two experimental blocks differed in their development time ($F_{1,20} = 9.98$, $P = 0.005$). Average development time was 25.0 days (±2.0 SD), with a minimum time of 21 days and a maximum of 31 days.

Table 9.2 Summary table of the fixed effects of a bionomial mixed model with line and female id as random effects. The intercept shows the estimate for Portugal and the other two countries show the difference with this estimate. The table shows the posterior estimate of the effect the 95% credibility interval and the chance that the effect is different from the null hypothesis (effect= 0).

	posterior.mean	lower 95% CI	upper 95% CI	P_{MCMC}
(Intercept)	–1.382	–1.912	–0.934	0.001
Day	–0.245	–0.282	–0.208	0.001
Day2	0.019	0.016	0.022	0.001
Israel	0.271	–0.472	0.876	0.384
U.K.	0.410	–0.332	1.107	0.254
Mate day	0.086	–0.020	0.173	0.106
Mate day : Day	0.018	0.003	0.034	0.012

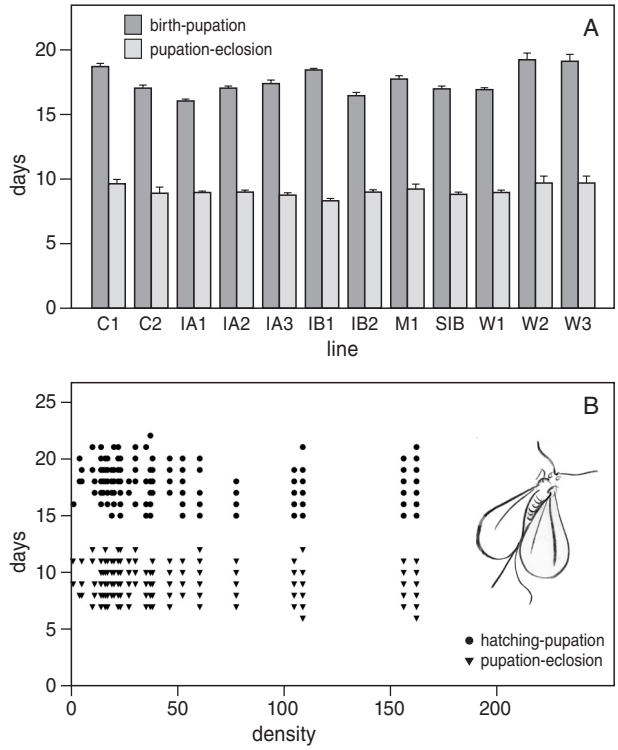

Figure 9.2 (A) Male development times per line. The blue bars shows the average time (in days) between hatching and pupation, while the purple bars shows the average time (in days) between pupation and eclosion. Error bars show the standard error. (B) The relationship between population density and male development time. Plot shows the development times of the males (circles show the time between hatching and pupation, while the triangles show the time between pupation and eclosion) plotted against the density of the box they were raised in.

Total development time in males can be broken up into two meaningful developmental stages: the time from hatching to mid-second instar, during which they disperse and feed much like females do, and the time from mid-second instar to maturity, where they pupate inside a cocoon of wax threads (Gullan & Kosztarab 1997). During the first stage, development time is influenced strongly by environmental factors (see table 9.3) with box explaining a significant percentage of the variance (20.36%, $LR_{1,10} = 38.16$, $P < 0.001$), while during the pupating stage, the box males were raised in had little effect on development time (0.48%, $LR_{1,10} = 0.034$, $P = 0.853$) but the experimental block did ($F_{1,20} = 23.30$, $P = 0.0001$, Table 9.3). Higher density in the rearing box reduced the development time of males during both developmental stages (Hatching-Pupation: $F_{1,20} = 13.63$, $P = 0.001$, Pupation-Eclosion: $F_{1,20} = 4.50$, $P = 0.047$, Figure 9.2B). Line did not explain a significant proportion of the variance in either (Hatching-Pupation: LR1,10 = 0.74, $P = 0.388$, Pupation-Eclosion: $LR_{1,10} = 0.024$, $P = 0.877$, Table 9.3, Figure 9.2A).

Mortality during pupation was very low, only around 6%, and line only explained 0.59% of the variance in male mortality, with a very large credibility interval ($CI_{95\%}$: 0.0004 – 86.81%). Male mortality was also not influenced by environmental factors experienced in the rearing box (for density, sex ratio and their interaction, all $p_{MCMC} > 0.2$) and the difference in male mortality between the two experimental blocks was not significant ($p_{MCMC} = 0.120$, 8% for the first block and 4% for the second).

Table 9.3 Male development time. Results from two log transformed mixed models. The top panel shows the results for the time between hatching and pupation and the second panel shows the results for the time between pupation and eclosion. For each model the random effects are shown as the percentage of the variance explained and their significance derived from a likelihood test. The fixed effects are shown in a ANOVA table..

Hatching-Pupation				
Random effects	% var explained		likelihood ratio	*P*-value
Line	7.24		0.74	0.388
Box	20.36		38.16	<0.001
Residual	72.39			
Fixed effects	numDF	denDF	F-value	*P*-value
(Intercept)	1	496	72241.83	<0.0001
SR	1	20	0.44	0.516
Density	1	20	13.63	0.0014
Country	1	9	1.02	0.399
Block	1	20	1.91	0.182
SR:Density	1	20	0.05	0.828
Pupation-Eclosion				
Random effects	% var explained		likelihood ratio	*P*-value
Line	0.41		0.024	0.8765
Box	0.48		0.034	0.8527
Residual	99.11			
Fixed effects	numDF	denDF	F-value	*P*-value
(Intercept)	1	464	93480.79	<.0001
SR	1	20	1.43	0.2461
Density	1	20	4.50	0.0466
Country	1	9	1.02	0.3974
Block	1	20	23.30	0.0001
SR:Density	1	20	2.59	0.1232

FEMALE DEVELOPMENT

We analysed female development time as the time between hatching and the start of oviposition. Total development time from hatching to reproductive maturity averages 33.69 days (±3.60 SD). Female development did not differ between lines ($LR_{10,9} < 0.0001$, $P > 0.99$), but the box a female was raised in explained a significant portion of the variance (Box: 19.48%, $LR_{12,11} = 6.96$, $P = 0.008$), additionally there was a marginally non-significant effect of the density in the rearing box, with females developing faster at higher densities ($F_{1,14} = 3.76$, $P = 0.073$). There was also a difference between the two experimental blocks (block 1: 32.97 ±2.66 SD, block 2: 34.61 ±4.35 SD, $F_{1,14} = 10.01$, $P = 0.007$).

In order to test the age at which females were able to mate we introduced males at different ages. On average, females mated successfully at the age of 25.3 days (± 2.2 SD), although some would mate after 21 days (see figure 9.4A). Mating age had a significant effect on the age at which females started ovipositing, because females could only start reproducing once mated ($F_{1,66} = 8.56$, $P = 0.005$). However, the ages at which females were mated did affect the time between mating and oviposition. Females take an average of 8.36 days (±3.91 SD) to start producing offspring, but females that were mated young take significantly longer ($F_{1,66} = 21.82$, $P < 0.001$, see Figure 9.5). This suggests that females are able to mate prematurely and store sperm until their oocytes have developed. There was also a marginal difference in the time between mating and the start of oviposition between the different countries (Portugal: 9.00 ±3.90 SD, Israel: 7.28 ±3.56 SD, U.K.: 10.17 ±4.09 SD, $F_{2,9} = 3.66$, $P = 0.069$). Finally, density in the rearing box reduced the time between mating and oviposition ($F_{1,14} = 14.92$, $P = 0.002$).

Females experienced considerable mortality from the time they were isolated with only 45% of females surviving until adulthood. We tested for factors affecting this mortality. Neither the density nor the sex ratio in the box in which the females developed affected their later survival (for both $p_{MCMC} > 0.1$), and box only explained 0.38% (CI95%: 0.00003 – 51.64%) of the variation in mortality rate. The mortality

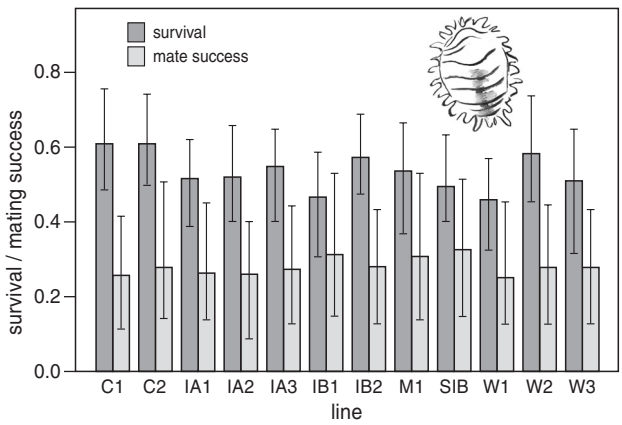

Figure 9.3 Average female survival and mating success per line. Error bars show the binomial standard error.

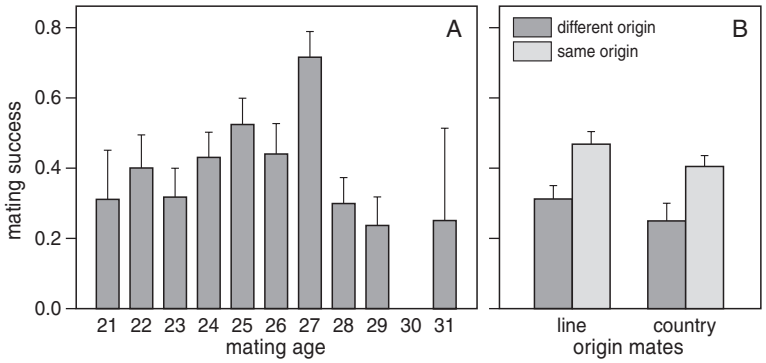

Figure 9.4 (A) Average female mating success (proportion of females that were successfully fertilized) for different mating ages and for female mated with males from the same line and from a different line. (B) Average mating success for matings between females and male from the same or different lines and for mates from the same or different country of origin. Error bars show the binomial standard errors.

rates for different lines are shown in Figure 9.3. Line did not explain any of the variance in mortality 0.40% (CI95%: 0.0002 – 71.10%, figure 9.3). There was also no difference in mortality rates of females between the two blocks (p_{MCMC} = 0.136).

Mating success

The above analyses all took into account only those females who had mated successfully and hence produced eggs, but did not explain why some females were fertilized and others were not. Therefore we tested the factors determining fertilization success. Females were mated at different ages and an important objective of our experiment was to test at what age females can be mated and if mating age affects fertilization success. We found that in our experiment age at mating of the female did not affect fertilization success (p_{MCMC}= 0.976, see figure 9.4A), suggesting that females are probably able to mate even earlier than the minimum age observed in this experiment. The fertilization success for the different lines is shown in figure 9.3. Line only explained about 2.92% of the variation in fertilization success (CI95%: <0.00001 – 37.00%). However, the success of a mating does depend on the line of the father, specifically whether the father was of the same line as the mother or not (p_{MCMC}= 0.032, figure 9.4B). If the father was from the same line, an average of 47% of the matings were successful; if the father was from a different line, this was reduced to 31%; it did however not matter if the male was from the same or from a different country than the female (p_{MCMC}= 0.138, figure 9.4B)

Synchronised reproductive maturity

An important goal of this study was to see if the sexual dichronism, with males being produced first could be an adaptation to the difference in development time between the sexes. In order to test this we first of all wanted to see if males do indeed take

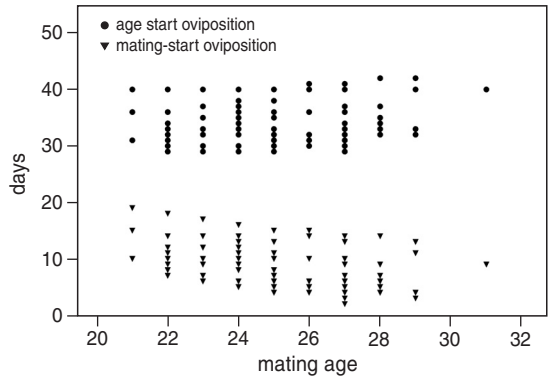

Figure 9.5 Female age at the start of oviposition and the time between mating and oviposition plotted against the age of female when they were mated.

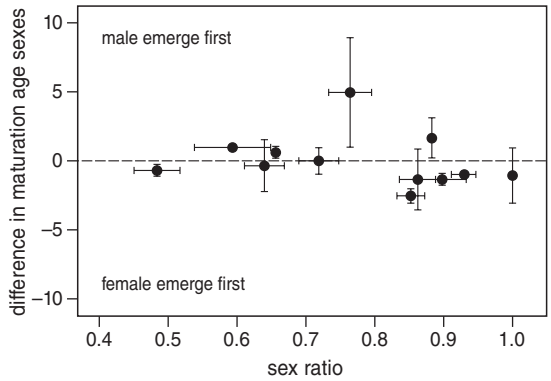

Figure 9.6 Mean day 1 sex ratio plotted against the mean difference between first male and female mating opportunity per line (average difference between the age of first male and first female per box successfully able to mate). Error bars show the (binomial) standard errors.

longer to develop. However just comparing development times of males and females might not be sufficient as earlier studies have suggested that females might be able to mate prematurely and store sperm. Therefore in this experiment we compare the minimum age at which females can be successfully fertilized with male eclosion times. We consider this on a box level, as within a box the same external conditions were experienced. In 16 out of 30 boxes that produced both viable males and females, some females could mate before some males could. This was revealed by taking the age of the first male to reach maturity and of the first female to mate successfully. In some boxes, males were mature 4 days before the first female could mate, while in another, some females were mature for 8 days already before the first male matured. Averaging over all boxes, however, there was no difference (0.0 days ± 2.6 SD) between first male and first female ability to mate: 23.5 days for both.

The second aim of the experiment was to try to link the difference between male and female development time to the pattern of sex allocation over the oviposition period and we predicted that if the early male production was an adaptation to relatively faster female development, lines that produced a more male biased sex ratio on the first day of oviposition would have a larger difference between male and female development time. Figure 9.6 shows the mean day1 sex ratio per line plotted against

the difference between the minimal male and female development time and there appears to be a relationship in the predicted direction, however this relationship is very weak. In order to test the significance of this relationship we wanted to test how early male production co-varies with the difference between male and female development time. Unfortunately we did not find any significant line effect in early male production: The line effect on sex ratio produced on day 1 of oviposition explains 45.04% of the variance but this estimate is not significantly different from zero (CI95%: 0.0002 – 77.89). Neither did we find any significant line effects on the development times of males or females (see above).

DISCUSSION

A systematic survey of replicate populations from three geographic regions revealed a rather consistent pattern of temporal variation in sex allocation. All females produced a roughly equal sex ratio and females from all lines produced male offspring first, before switching to a female biased sex ratio and then subsequently producing increasingly more males until they died. We hypothesized that this pattern of sex allocation behaviour with males being produced first might have been an adaptation to ensure that male and female offspring become reproductively mature at the same time to ensure mating opportunities. We found however that this is probably an unlikely explanation as males and females, when kept under similar environmental conditions, become reproductively mature at roughly the same age (i.e. males do not take longer to develop and do not need to be produced before females in order to synchronise reproductive maturity within a brood). Furthermore we also found that females are able to mate prematurely and store sperm, while previous experiments have shown that females are able mate successfully up to more than a month after they have become mature (Nelson-Rees, 1960)(Ross *et al. submitted*). Therefore as the window of female mating opportunity is so large, it seems unlikely that an adaptation to ensure synchronizing male and female maturation is necessary. However, even when males do not take longer to develop, it might still be beneficial for them to become reproductively mature slightly earlier than the females as this might avoid that their sisters are already mated with unrelated males by the time they emerge. Early-male emergence to enhance success in competitive mate-searching as a mating strategy, referred to as protandry, has been observed in many species across a wide taxonomic range (Morbey & Ydenberg, 2001; Thornhill & Alcock, 1983). This might explain why female produce sons first, although it does not explain the overproduction of sons late in life.

The temporal pattern of sex allocation does also affect how females can adjust their total sex ratio. Previous experiments have suggested that females can adjust their sex ratio with respect to a variety of environmental factors (Nelson-Rees, 1960; Ross *et al.*, 2010a)(Ross *et al. submitted*), however these factors (for example food restriction and high temperature, Ross *et al. submitted*), can often also affect the lifes-

pan. In these cases, it is hard to distinguish if females adaptively alter their sex ratio, or if these factors affect lifespan and thereby curtail the schedule of male and female production, skewing sex ratios (as suggested by Ross et al 2010 *submitted*). Therefore this may be a potentially non-adaptive side effect of the temporal order of sex allocation. Even if the sex ratio responses to environmental factors like food shortage and temperature are not an adaptive response, the resulting change in sex ratio will still have important demographic and ecological effects.

If the temporal pattern of sex allocation in *P. citri* is not adaptive, might it be associated with how sex is determined and controlled (or not) by females? Temporal patterns in sex allocation have been shown in other insects even in haplodiploid Hymenoptera, which have the ability to very precisely adjust their sex ratio. In parasitoid that adjust their sex allocation to avoid competition between male siblings, male production early in the laying sequence has been suggested to help increase the precision of adjustment (Chow & Mackauer, 1996; Waage & Lane, 1984). The more eggs a female is able to lay, the less likely she competes with other females for hosts and as a result of the laying sequence, the more female biased her sex ratio is. However this explanation might not hold for *P. citri*, as the sex-sequence observed here is more complex than that described for parasitoids and the temporal pattern mighty instead be a constraint rather than an adaptation to precise sex ratio adjustment. The sex determination mechanism in species with PGE is still almost completely unknown, but one possibility is that females add some type of maternal effect protein to those eggs determined to becoming male and that this causes the deactivation of the paternal genome in theses eggs (Buglia, Dionisi & Ferraro, 2009; Ross *et al.*, 2010b). If this is indeed the way that sex is determined, females might not be able to precisely alter the concentration of this substance in individual eggs but rather change the amount produced over time and this might explain why the sex ratio is biased across days and changes over time. This hypothesis is supported by the observation of gradual change in sex ratios over time, both under the standard laboratory conditions tested in the current study, but also under different environmental conditions (Ross *et al.*, 2010a)(Ross *et al.* submitted). So the way that females change their sex ratio over time might constrain the precision with which females can adjust the sex ratio of her offspring rather than an adaptive sex ratio response.

We found little evidence for population-level (and thereby genetic) variation in patterns of sex ratio or life-history. Sex ratio is well understood at a theoretical level, and to a large extent at an empirical level (West, 2009), but much less so at a genetic level (Pannebakker *et al.*, 2008). We know rather little about the underlying genetics of both sex ratio itself, as well as variation in the ability of individuals to adjust it. A lack of genetic variation in sex ratio is not unheard of although there is a clear genetic component in the sex allocation behaviour of some species, for example the haplodiploid parasitoid wasp *Nasonia vitripennis* (reviewed in Pannebakker *et al.*, 2008). The present experiment shows that even if there is a genetic basis for sex ratio variation in *P. citri*, this effect is small. The same is possibly true for the variation in several other life history traits that we considered, as we found that although there was con-

siderable variation between individuals, possible line (= genetic) effects explained little of this variation. We found instead that environmental effects were more important in explaining both development times and mortality rates. One factor that was found particularly influential was the density experienced during development on development time. We found that for both males and female at least some part of the development was sped up when they developed under higher densities, although the reason for this finding is currently unclear. Effects of density on development time are commonly found across insects, but the direction of these effects varies; in some taxa there is a increase in development time due to crowding, possibly as an effect of competition, however in other taxa density can reduce development time, which has been suggested an adaptive response to avoid competition (Peters & Barbosa, 1977)

Finally, our timing of mating experiments also showed an intriguing effect of line on the outcome of mating interactions, with higher fertilization rates observed when mates were from the same line. Mating success can first of all be influenced by both male and female mating choice. There is a wealth of literature on mate choice in relation to inbreeding avoidance, as individuals are believed to avoid incestuous matings to avoid inbreeding depression. However it has recently been suggested that inbred matings might, under some conditions be selected for, as matings with a related individuals lead to indirect fitness benefits from helping a related individual reproduce, as well as the direct benefit of reproduction (Kokko & Ots, 2006). Experimental results showing this pattern are almost completely absent though (Kokko & Ots, 2006). In our experiment if mates were from the same line they both originated from the same population and were full-sibs and therefore relatedness and population effects might be slightly confounded. We found that both line of origin and country of origin seem to affect mating success, although the country effect was not significant. As we did not directly observe matings, our results could either be due to a failure to mate, or to a failure of fertilization. A failure to mate could have come about by either female, male or mutual mate choice and would suggest that individuals prefer to mate with their siblings over unrelated individuals, possibly to increase their inclusive fitness . However, mating and insemination might be more common but our data explained by a failure of fertilization (post-copulatory choice (Eberhard, 1996) or incompatibility (Tregenza & Wedell, 2000)). As the lines originate from different geographical locations, this might indicate some low level of reproductive isolation between populations. *P. citri* is generally assumed to be a cosmopolitan species but several closely related species have been described (Cox, 1981) and some gene flow might occur between these species in some regions (Mendel personal communication). However, information on gene flow between *P. citri* populations from different regions is limited.

Acknowledgements

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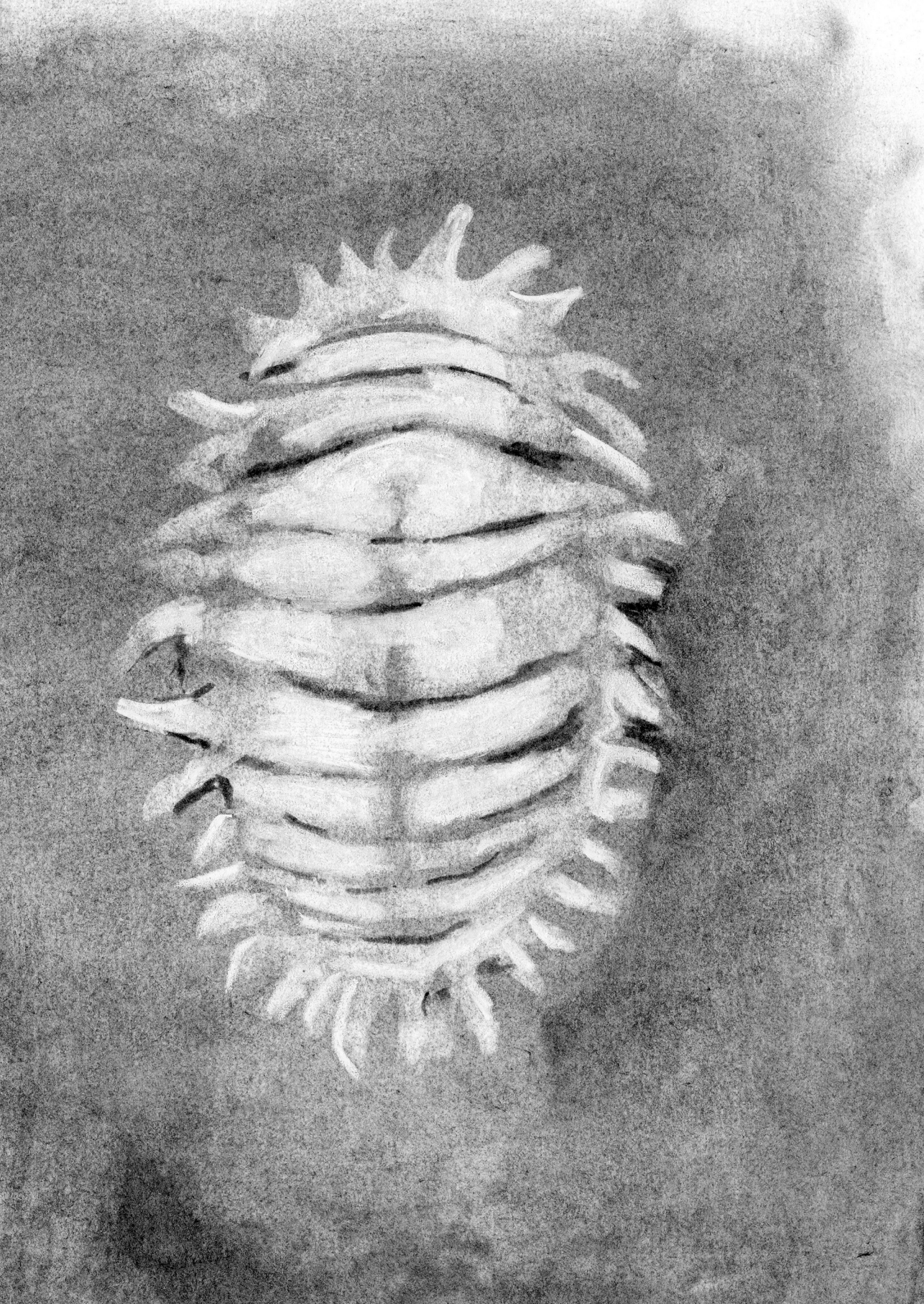

CHAPTER 10

Temperature, age of mating and starvation determine the role of maternal effects on sex allocation in the mealybug *Planococcus citri*

Laura Ross, Elizabeth J. Dealey, Leo W. Beukeboom and David M. Shuker

ABSTRACT

Environmental effects on sex allocation are common, yet the evolutionary significance of these effects remains poorly understood. Environmental effects might influence parents, such that their condition directly influences sex allocation by altering the relative benefits of producing sons versus daughters. Alternatively, the environment might influence the offspring themselves, such that the conditions they find themselves in influence their contribution to parental fitness. In both cases parents might be selected to bias their sex ratio according to the prevailing environmental conditions. Here, we consider sex allocation in the citrus mealybug *Planococcus citri*, a species with an unusual genetic system in which paternal genes are lost from the germline in males. We test environmental factors that may influence either female condition directly (rearing temperature and food restriction) or that may be used as cues of the future environment (age at mating). Using cytological techniques to obtain primary sex ratios we show that high temperature, older age at mating and starvation all affect sex allocation, resulting in female biased sex ratios. However, the effect of temperature is rather weak, and food restriction appears to be strongly associated with reduced longevity and a truncation of the usual schedule of male and offspring production across a female's reproductive lifetime. Instead, facultative sex allocation seems most convincingly affected by age at mating, supporting previous work that suggests that social interactions experienced by adult *P. citri* females are used when allocating sex. Our results highlight that even within one species different aspects of the environment may have conflicting effects on sex allocation.

Behavioral Ecology and Sociobiology, in press

INTRODUCTION

Sex allocation is an important reproductive decision that can have significant effects on an individual's fitness. There is a rich theory-base predicting optimal sex ratios under different conditions and observed sex allocation patterns often closely match the theoretical predictions, especially in insects (West, 2009). The different aspects of sex allocation theory all share conceptual space in terms of fitness returns through sons and daughters, with different "theories" basically dealing with the different ways in which male and female offspring can contribute to parental fitness and how parents and the environment influence this fitness return (Charnov, 1982; Hamilton, 1967; West, 2009).

Environmental factors acting on parents can affect sex allocation through parental condition: Trivers and Willard (1973) showed that if there is variation in parental condition and if the fitness of one offspring sex is more strongly affected by their parent's condition, then parents should bias their offspring sex ratio towards the sex that either benefits most or suffers least from their condition. In other words, if the environment affects the condition of a female in such a way that she must invest less in her offspring and one sex suffers less from this reduced investment, a female should bias her sex ratio towards that sex. Several (environmental) factors are known to affect both parental condition and offspring sex ratio. These factors include extreme temperature, drought, parental age and lack of resources (Cockburn *et al.*, 2002; Sabelis, Nagelkerke & Breeuwer, 2002; Sheldon & West, 2004; West, 2009; West & Sheldon, 2002).

Alternatively, environmental factors acting upon the offspring themselves can alter sex allocation, as such effects might again influence the fitness return parents get from their offspring (Trivers & Willard, 1973). If environmental conditions affect the fitness of male and female offspring differently, and parents can predict the environment their offspring will experience, parents should bias the sex ratio towards the offspring sex with the highest fitness under these conditions. A variety of factors could have differential fitness effects on offspring. One particular factor that has been the focus of many sex allocation studies is the level of competition between kin, as competition between siblings can reduce the fitness return of offspring to their parents, and parents are therefore expected to overproduce the sex that suffers least from kin competition (local resource competition,theory, LRC: Charnov, 1982; Clark, 1978; Hamilton, 1967; West, 2009). Data supporting the role of different forms of LRC are abundant, with many of the best examples coming from parasitoid wasps (West, 2009; West *et al.*, 2005). Other environmental factors can have similar sex-specific fitness effects, for instance if the environment influences offspring survival, reproductive success or dispersal ability. These differential effects on offspring fitness have been used to explain environmental effects on sex allocation in a variety of taxa. Examples include the effect of high temperature in the spider mite *Tetranychus mcdanieli* (Roy, Brodeur & Cloutier, 2003), host size and quality in several species of parasitoid wasps (Charnov *et al.*, 1981; Murdoch *et al.*, 1992; West, 2009) and female mating age in the mealybug *Planococcus citri* (Werren & Charnov, 1978).

Environmental conditions experienced by parents can therefore influence their sex allocation decisions in two ways: either directly by influencing parental condition, or indirectly by being used as a cue of offspring fitness. In the latter case, parental condition may be completely unaffected, but the environment a parent experiences nevertheless predicts the fitness returns through sons and daughters. Of course, effects at these two levels might interact if parental condition also reflects the offspring environment, making it hard to disentangle the underlying mechanisms (Wild & West, 2007). While anecdotal evidence of effects of environmental conditions on sex ratio are numerous, many of these results come from studies focussing on other aspects of life history and experimental studies focussing directly on its effects on sex ratio are rare (Roy *et al.*, 2003). Finally, the underlying mechanisms are often obscured, as in many studies it is impossible to separate the effects of differential mortality from the effect of adaptive sex ratio adjustment. Here we present the results of an experimental study on the effect of a variety of environmental conditions on sex allocation in the mealybug *Planococcus citri*.

In *P. citri.* several factors experienced by females have been found to affect sex allocation. These include population density (Ross *et al.*, 2010a; Varndell & Godfray, 1996), temperature (James, 1937; Nelson-Rees, 1960), and age (James, 1938; Nelson-Rees, 1960; Ross *et al.*, 2010a). The observed effects are of particular interest for two reasons. First, mealybugs have an unusual genetic system, paternal genome elimination (PGE), whereby both sexes develop from fertilized eggs but in males the paternal genome is deactivated during development and lost from the germline during spermatogenesis (Schrader, 1921). As a consequence, males have haploid gene expression and only transmit maternal genes (i.e. PGE is akin to true haplodiploidy in terms of transmission genetics and gene expression patterns) (Brown & Nelson-Rees, 1961; Brown & Nur, 1964; Nur, 1980). An increased understanding of sex allocation patterns in mealybugs might therefore yield insight into the potential evolutionary advantage of this extraordinary mode of sex determination.

Second, there is strong sexual dimorphism in several morphological and life history traits in mealybugs suggestive of different patterns of selection on males and females (Gullan & Kosztarab, 1997). Adult males are winged while adult females typically lack wings, have reduced antennae and legs, and exhibit a mostly sedentary habit. In addition, males only feed for the first two instars, whilst females feed throughout their life (Gullan & Kosztarab, 1997). These adaptations indicate different resource requirements and dispersive abilities of males and females, such that maternal condition and the environment an offspring finds itself in may differentially influence the fitness returns of sons and daughters (see methods for more details on P.citri life history). Due to their dispersive abilities and lesser reliance on larval feeding, males may be less influenced by the environment but more by maternal condition (if that influences egg size and provisioning). Reduced maternal condition may therefore favour daughter production, whilst poorer quality offspring environments may favour male production. That said, earlier studies showed that both high temperature and ageing of mothers, which we might expect to be related with reduced maternal

condition, result in a male biased sex ratio (Nelson-Rees, 1960). More recently, two studies have found effects of density on sex allocation (Ross *et al.*, 2010a; Varndell & Godfray, 1996), although the results differed and the cause for this difference could not be identified. Density can influence both maternal quality (if resource competition in high density patches influences maternal condition) and can also be an indicator of the degree of competition that offspring will experience. Previous experiments did not make this distinction between possible effects explicit. However, both studies suggested that resource availability might have a profound effect on sex allocation and we felt that a renewed attempt to test environmental effects on sex allocation was necessary in order to understand sex allocation patterns in *P. citri.*.

We performed two experiments that manipulated three environmental factors that differed in whether they directly influenced maternal condition (rearing temperature and food deprivation) or whether they might signal aspects of the environment (availability of males, manipulated as age at first mating, and representing a measure of population density). In the first experiment, we manipulated temperature and age at first mating in a fully factorial experiment. In this experiment we used two temperature treatments: 25°C, which is the temperature at which the stock cultures are kept and 30°C, as previous experiments have shown that females kept at this temperature have a reduced life span and reproductive success. In our second experiment, we tested the role of food deprivation on sex allocation. If maternal condition influences sex allocation in *P. citri.*, we predict sex ratio changes associated with rearing temperature and food deprivation. As male offspring are thought to depend more on maternal investment than female offspring (which feed throughout their lives), we predict that maternal stress is associated with a greater production of female offspring. If sex allocation is more strongly influenced by cues about the offspring environment, delayed mating (due to low availability of males) might signal low density and thus we predict the greater production of female offspring (Ross *et al.*, 2010a).

METHODS

Study organism

The citrus mealybug, *Planococcus citri* (Pseudococcidae: Coccoidea: Hemiptera) is a cosmopolitan sap-feeding plant pest species (Gullan & Kostarab, 1997). It is sexually dimorphic, with the males being small and wingless whilst the females are larger, sedentary and covered in wax (see Figure 1 for an illustration of both sexes). The adult males become sexually mature at approximately 29 days (when reared at 25° C) and have a short life span of approximately 2 days, during which they do not feed. The females become sexually mature at approximately the same time, and have been reported to survive up to 120 days under laboratory conditions (Nelson-Rees, 1960). Females can lay several hundred eggs during their lifetime, and these eggs are laid in a fibrous ovisac located under and behind her body. The larvae ('crawlers') hatch about 2–3 days after egg laying, and the first instar larvae are highly mobile (Gullan

& Kostarab, 1997), although generally crawlers settle closely to where they where born. The two sexes become distinguishable in the late second instar at approximately 16–20 days post-oviposition when the males pupate (Ross *et al.* in prep). The laboratory stock culture of *P. citri.* used for the experiments was obtained from Wye College, University of London and originally collected from a nearby glasshouse, and has been maintained through mass cultures on potato (cultivar Desiree) at 25°C and 60–70% humidity, under a 12:12 light:dark cycle.

Experiment 1: Temperature and age at mating

The first experiment consisted of two rearing temperatures and two mating ages in a fully-factorial design. The two rearing temperatures were 25°C and 30°C, and the two mating ages were 29 days and 63 days (see Figure 10.1). The whole experiment was repeated twice, resulting in two blocks. The earlier mating age was chosen as females become sexually mature at approximately 29 days; the older age of 63 days was selected following the data from Nelson-Rees (1960), which shows that the sharpest rate of increase in proportion of males occurs after 50 days. Effects of mating age and temperature on males were controlled for by mating all experimental females with 29 day old males which had been raised at 25°C.

The females used in all treatments were raised at 25 degrees till they became detectably female (day 20), then females were isolated and transferred to either the 25 or 30 degree treatment group. Females were mated and remained at these temperatures until they finished ovipositing. All mealybugs used in the experiment were obtained by allowing 50 randomly selected females to oviposit overnight. The egg masses from these females were transferred to fresh potatoes in order to obtain the stock used for the experiments, ensuring constant age for all individuals used.

At day 20, when the sexes of the crawlers became distinguishable, females were randomly selected from the experimental stock and each individual female was assigned to a box in one of the four treatment groups, so that each box contained one solitary female. Treatment boxes (plastic boxes with a volume of 1.1 litres, covered with fine mesh) were set up as follows: a single freshly sprouted potato was randomly selected, the weight recorded, and then placed in the box on a single sheet of paper towel. The box was then randomly assigned to a treatment and 30 boxes were set up for each treatment group. The treatment boxes were then placed at either 25 or 30°C, both at 60–70% humidity and under a 12:12 light: dark cycle. In addition, males were isolated from the experimental stock and placed into individual glass tubes, which were then maintained at 25°C, 60–70% humidity and under a 12:12 light:dark cycle. Both sexes were then allowed to develop for a further 9 days. Additionally, at day 34 a further 50 ovipositing females were randomly selected from the stock cultures and given fresh sprouting potatoes, as described before, in order to generate males for the late mating treatments. The egg masses from these females were again removed after one day and placed on fresh potatoes.

For the early mating treatments at day 29, two males were randomly selected and released into each of the boxes in the early mating treatments (two males were used

to ensure that all females were inseminated). For the late mating treatments males were introduced into the female boxes at day 63 (figure 10.1). All females were checked daily, from the day after mating for signs of oviposition. As soon as the females began oviposition, the egg masses were collected daily until the female died (figure 10.1). Egg masses were fixed and stored prior to sexing (see below for fixation and staining methods).

In total 95 females successfully started oviposition and were included in the analysis. Mortality before the start of oviposition was high for the late mating treatments, especially those kept at 30°C. Additionally, in the late mating treatments several females failed to start ovipositing. In a few cases females in the late mating treatment started ovipositing before males were introduced, as P.citri reproduction is strictly sexual (Borges da Silva, Mendel & Franco), this was probably due to males managing to entered the treatment boxes. These females were excluded from the experiment.

Experiment 2: Food restriction

To test the effect of food restriction, we either removed mated females from their food source (food restricted treatment) or moved mated females to a new food source (control). For the control females we also noted daily if females were able to feed (present on the potato); since mealybug females are almost completely sedentary this was assumed to provide reasonable information on their opportunity to feed.

In order to obtain individuals of known age that could be used for the experiment, we randomly took 20 egg-laying females from the mass culture and provided them with fresh potatoes. The females were allowed to lay eggs for 24 hours. The eggs were allowed to hatch and the crawlers raised until 19 days after they were laid. By this time males had pupated and were easily recognizable and males and females were isolated in order to avoid early mating: females were removed from the mass culture and each individually placed on a new potato in a smaller box. Males were isolated in small glass tubes. After 29 days 2 adult males were introduced into each box again to make sure that all females would be inseminated. The next day (day 30) we removed the males, and the females were randomly assigned to one of the two treatments: food restriction or the control. In the food restriction treatment females were removed from the potato and placed back in their box on a piece of paper towel, while in the control treatment females are removed from the potato and placed back in their box on a new potato. Subsequently, females were checked each day for signs of egg laying and eggs were removed and fixed. Additionally, for the control females we also noted whether individuals were sitting on the potato or on the paper towel. In total, 50 out of the 56 females in the experiment started oviposition, with 11,189 eggs being counted and 98.5% of the eggs successfully sexed. Eggs were counted on the first and every alternating day until the females died.

Fixation and staining

Immediately following collection, we placed a few drops of fixative solution (Carnoy's fluid: 4 parts chloroform: 3 parts ethanol: 1 part glacial acetic acid) on the

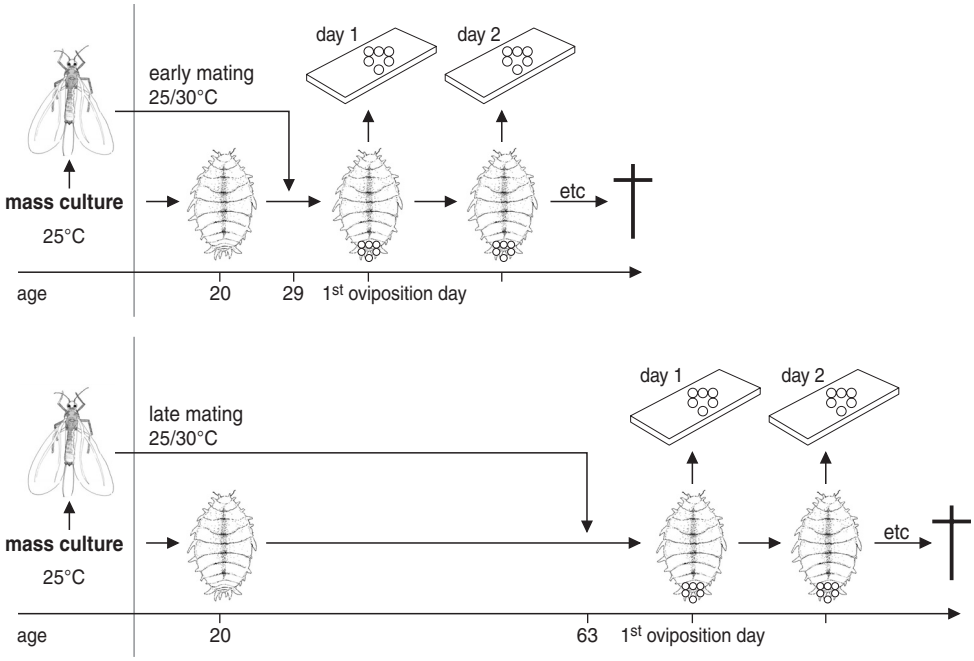

Figure 10.1 A schematic representation of experiment 1 manipulating temperature and mating age. The axis shows the age of the females. The top panel shows the timing of events -- when females are isolated from the mass culture, when males are introduced and when females started ovipositing and eggs were collected -- for the early mating treatments, while the bottom panel shows the late mating treatments. Temperatures during each of the experimental treatments are indicated in the figure: 25°C in the mass culture and during the rearing of males and either 25°C or 30°C during the experimental stage, depending on the temperature treatment in both the early and late mating treatments.

egg mass and removed the protecting fibres using a small rod under a dissecting microscope. We then transferred the eggs to an eppendorf tube containing the fixative solution, and stored them in a fridge at 4°C for at least 24 hours and up to a maximum of 3 days. At the end of this period, the eggs were transferred into 90% ethanol solution to prevent DNA degradation and stored at 4°C.

To determine the sex of each embryo, we transferred eggs from the ethanol solution to a glass slide, stained them with DAPI (Sigma D9564, diluted 1:1000 in PBS) and then sealed a cover slip over the stained embryos. We then examined the eggs under a fluorescence microscope at 200× magnification and determined the sex of each embryo. The males can be differentiated from the females as their condensed paternal genome stains heavily and forms a brightly coloured body in the nuclei of male cells (Ross *et al.*, 2010a). The sex of each embryo (female, male, unknown) was recorded for every clutch (experiment 1) or every other clutch (experiment 2) from all females in the treatments. For experiment 1 we sexed 28086, with a 98% success rate, while for experiment 2 we sexed 11189 embryos with a 98.5% success rate.

Data analysis

All data analyses were performed using the statistical program R (R Development Core Team, 2010). For generalized linear mixed models the R package nlme was used (Pinheiro *et al.*, 2007). The relationships between the life history traits – fecundity, longevity, oviposition – were explored using generalized linear models with Gaussian error structures with a log transformation, or with quasi-poisson error structures in the case of clutch size and we consider these factors as measures of maternal condition. The lifetime sex ratios were analysed using generalized linear models with quasi-binomial error structure to correct for overdispersion. The sex ratio data per day were analysed by using a generalized linear mixed model approach with female identity fitted as a random effect and with arcsin square root transformed sex ratios, assuming a Gaussian error structure. We also used a correction for autocorrelation (corCAR1 function in nlme: Pinheiro *et al.*, 2007). For the analysis of the effects of food restriction on sex ratio, in addition to the treatment, for the control females we also considered female feeding location (on or off the potato). For the analysis per female we used fitted fraction days feeding (number of days on potato/ length oviposition), while for the analysis per day we fitted the feeding position of the female for each day. These factors were both fitted for control females only, using the "at.level" function in the R package MCMCglmm (Hadfield, 2010a). Throughout we consider sex ratio as the fraction of offspring that are male.

RESULTS

Experiment 1: Temperature and mating age

Effects on maternal condition

The rearing temperature of female mealybugs significantly affected aspects of development, but it was not clear that maternal condition was adversely affected by high temperature. Females reared at the higher temperature lived less long and had a shorter oviposition period ($F_{1,90} = 16.86$, $P < 0.001$; Figure 10.2), but they did not produce fewer offspring (Mean number of offspring: 25°C: 357.6, s.e= 34.0, 30°C: 348.5, s.e= 32.2, $F_{1,90} = 0.93$, $P = 0.34$), and they did not take significantly longer to commence oviposition ($F_{1,90} = 3.45$, $P = 0.07$). There was a significant interaction with age at mating however (see below).

Age of mating was also associated with aspects of maternal reproductive behaviour and life history. Mating age was strongly associated with female lifespan, consistent with there being a cost of reproduction. 79% of females in the late mating treatment survived until day 63, when the males were introduced. By this time only three out of 58 of the early mating females were still alive. However, females from the early and late mating treatments did not differ in the length of their oviposition period post-insemination ($F_{1,89} = 0.008$, $P = 0.93$). Age at mating was also associated with female egg production, with females producing fewer offspring when mated later in life ($F_{1,89} = 15.86$, $P < 0.01$), with the number of offspring even more

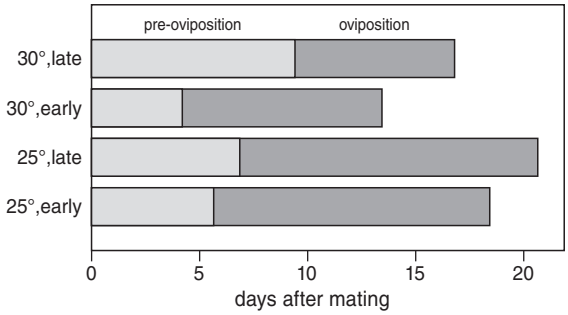

Figure 10.2 Mean time between mating and oviposition (light grey bars, "pre-oviposition") and the length of oviposition (dark grey bars, "oviposition") for each treatment in experiment 1. Times are presented as time after mating (day 29 for the early mating treatments and day 63 for the late mating treatments). Treatments shown from top to bottom: high temperature and delayed mating ("30, late"), high temperature and early mating ("30, early), low temperature and delayed mating ("25, late") and low temperature and early mating ("25, early").

reduced for the late mating females kept at 30 degrees (Temperature × Mating time: $F_{1,87} = 13.59$, $P < 0.01$). Finally, females in the early mating treatment reared at 30°C started ovipositing following insemination earlier than those raised at 25°C, although females in the late mating treatment took longer to start ovipositing at higher temperature (Temperature × Age at mating: $F_{1,87} = 17.1$, $P < 0.001$, time post-insemination; Figure 10.2). Potato weight did not affect any of these maternal condition factors (all $P < 0.2$). In summary, temperature appeared to have at most small effects on maternal condition (e.g. no loss of fecundity), whilst females that mated later in life produced fewer offspring, presumably due to that delay.

Sex ratio

Considering the overall sex allocation across a female's life (see Figure 10.3), there was no effect of rearing temperature on sex ratio ($F_{1,93} = 1.12$, $P = 0.293$), despite a marginally non-significant interaction between temperature and age at mating ($F_{1,91} = 3.51$, $P = 0.064$). There was a significant effect of time of mating on life-time sex ratio, with females that mated later in life producing a more female biased sex ratio ($F_{1,92} = 6.35$, $P = 0.014$).

We also analysed the sex ratio per day and the results are shown in table 10.1 and figure 10.4. The number of days since the commencement of oviposition ("oviposition day") was strongly associated with sex ratio (Table 10.1) and the effect was linear (the quadratic term was non-significant, although there was a significant difference between the quadratic terms of the two temperature treatments: Table 10.1). In this analysis, temperature was again not significantly associated with sex ratio as a main effect, although both mating time and the interaction between mating time and temperature were significant and all of these factors resulted in a more female biased sex ratio. The results also show that the observed effects on the total sex ratio cannot

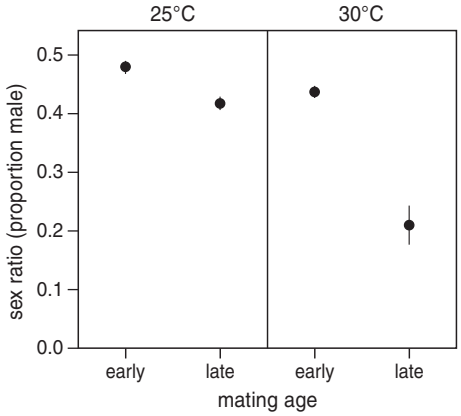

Figure 10.3 Mean life-time sex ratio for each of the four treatment combinations of experiment 1. Results of the low temperature treatments are shown in the left panel of the graph while the results of the high temperature treatments are shown in the right panel. The age at which females were mated (early and late) is shown on the x-axis. Sex ratios are the proportion of offspring that are male. Error bars show the binomial standard errors.

be fully explained by the different length of the oviposition period between the treatments. Figure 10.4, panel (A) shows the pattern of sex ratio production at 25°C with females mating at reproductive maturity, with males predominating early on, followed by a female bias, and then a tendency to produce more males again (see also Ross *et al.*, 2010a for discussion of this "standard" pattern). Panels (B-D) show the sex ratio patterns for the other three treatments. The most notable difference to panel

Table 10.1 Anova table showing the results of experiment 1 of temperature and mating age. GLMM with arcsin square root transformed sex ratio, female id fitted as a random effect and Gaussian errors corrected for autocorrelation.

	NumDF	DenDF	F-value	*P*-value
(Intercept)	1	823	582.33	<.0001
Temperature	1	87	2.43	0.123
Mating age	1	87	23.85	<.0001
Laying day	1	823	87.42	<.0001
Laying day^2	1	823	0.74	0.390
Potato weight	1	823	1.46	0.228
Block	1	87	2.53	0.116
Temperature: Mating age	1	87	5.22	0.025
Temperature: Laying day	1	823	0.00	0.955
Temperature: Laying day^2	1	823	4.48	0.035
Mating age: Laying day	1	823	2.26	0.133
Mating age: Laying day^2	1	823	0.94	0.334

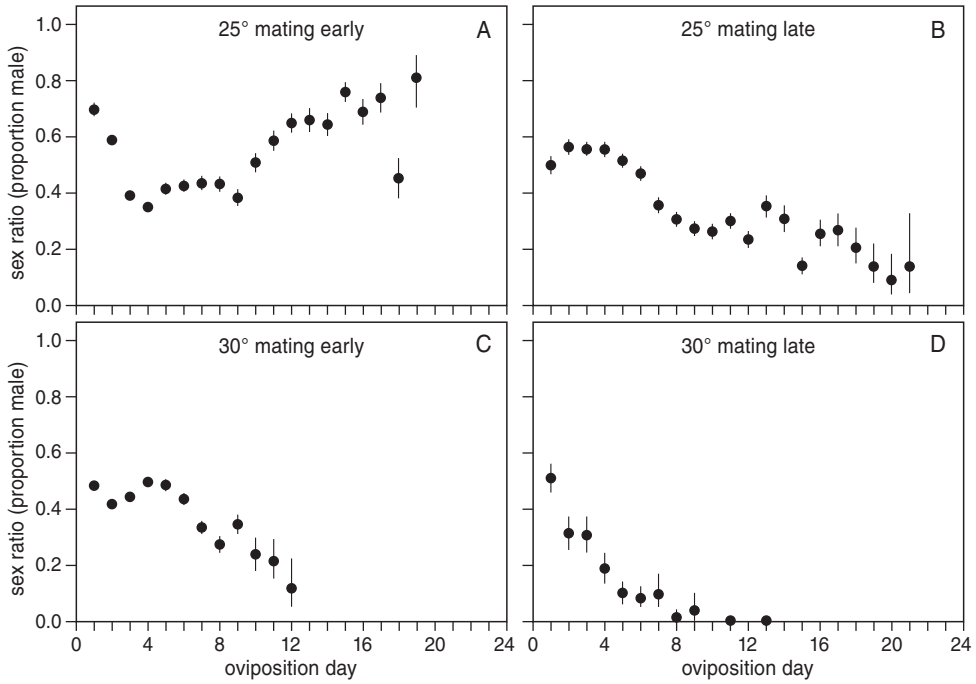

Figure 10.4 Mean sex ratio per day with respect to oviposition day (days since the start of oviposition) for each of the four treatment combinations of experiment 1: (A) Low temperature and early mating; (B) Low temperature and delayed mating; (C) High temperature and early mating; (D) High temperature and delayed mating. Error bars show the binomial standard errors.

(A) is that the initial peak of male bias is less obvious or absent, and that there is also no second peak of male production. As such, age of mating and to some extent temperature influences the ontogeny of sex allocation across much of the oviposition period, such that we see fewer males produced earlier and fewer males produced later as compared to "standard" lab conditions, generating a more female biased sex ratio overall.

Experiment 2: Food restriction

Effects on maternal condition

Food restriction significantly affected maternal condition in several ways, suggesting that food restriction did harm female condition. First of all, females that were starved lived significantly shorter than the control females ($F_{1,48} = 12.97$, $P < 0.001$). Food restriction also affected the number of eggs produced (control females: 276.63, s.e.= 26.03, starved females: 175.00, s.e.= 12.92; $F_{1,48} = 12.17$, $P = 0.001$). For the control females, the fraction of days spent on the potato was significantly positively associated with the number of offspring produced ($F_{1,47} = 8.60$, $P = 0.005$). Food restricted females started laying eggs significantly earlier than the control females

(mean number of days after mating for control females : 4.42, s.e. = 0.36; for starved females: 3.00, s.e. = 0.10; $F_{1,48} = 7.46$, $P = 0.009$). Among the food restricted females, those that started laying earlier laid more eggs over their lifetime ($F_{1,24} = 6.51$, $P = 0.018$).

Sex ratio

We again analysed sex ratio in two different ways. First of all we tested the effect of starvation on the overall sex ratio produced by each female, also fitting the proportion of days control females were observed on the potato (number of days feeding divided by the length of the oviposition period) to control for their feeding behaviour. Food restriction significantly influenced sex ratio, with food restricted (i.e. poor condition) females producing more female biased sex ratios (starvation: 0.47, s.e. = 0.017, control: 0.53, s.e. = 0.018, $F_{1,48} = 7.04$, $P = 0.011$). However, the fraction of days control females spent on the potato did not have a significant effect on the sex ratio those females produced ($F_{1,47} = 0.78$, $P = 0.382$).

We also analyzed the sex ratio per day using a GLMM with female identity as a random effect. For the control females we again fitted whether they were on the potato or not on each day. The results are shown in table 10.2. First of all, oviposition day had a significant non-linear effect on the sex ratio (Table 10.2, Figure 10.5). Food restricted females produced a more female biased sex ratio than the control, with food restricted females clearly missing the peak in male production late in life (Figure 5). The feeding opportunities of control females were not associated with sex ratio ($t_{217} = 0.89$, $P = 0.375$). As both the analysis considering the total sex ratio as the one looking at daily sex ratio patterns show a significant effect of food restriction it is

Table 10.2 Contrast table showing the results of experiment 2 of food restriction (starved vs. fed). GLMM with arcsin square root transformed sex ratio and Gaussian errors, corrected for autocorrelation. Table shows the (arcsin square root) transformed estimates (in bold) for the first factor level and the difference between the first and remaining factor levels (non-bold), e.g. "Fed: off potato", gives the difference between estimate for females that where starved ("Starved") and those that where not starved, but not feeding. The *P* value shows the significance of this difference. Likewise "Day: Starved" show the difference in slope between starved females and control females ("Day: Fed").

	Value	Std.Error	DF	t-value	*P*-value
Starved	**0.35**	**0.052**	**215**	**6.77**	**0.0000**
Day: Fed	**–0.06**	**0.008**	**215**	**–8.09**	**0.0000**
Day^2: Fed	**0.01**	**0.001**	**215**	**8.81**	**0.0000**
Day: Starved	0.02	0.015	215	1.4	0.1627
Day^2: Starved	0.02	0.003	215	5.25	0.0000
Fed: off potato	0.21	0.08	215	2.62	0.0095
Fed: on potato	0.30	0.075	215	4.05	0.0001

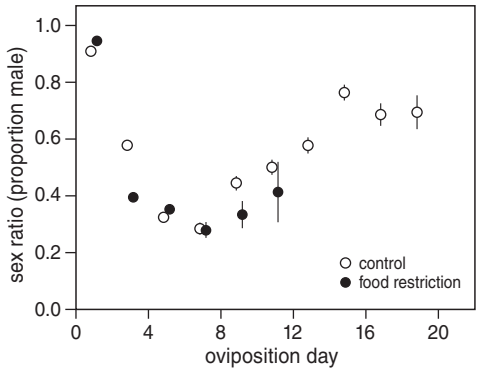

Figure 10.5 Mean sex ratio for each day plotted against oviposition day from experiment 2. The data presented are the number of days since the start of oviposition for the control females, and for those that were food restricted.

clear that although some of the effect of the total sex ratio might be influenced by differences in lifespan between the treatments (see Figure 10.5) this does not explain the whole effect.

DISCUSSION

In this paper we have considered how aspects of the maternal environment influence sex allocation in the mealybug *Planococcus citri*. Our clearest result is that food deprivation reduces maternal condition and leads to a greater production of female offspring. This result suggests that environmental impacts on adult female mealybugs can influence sex allocation, and in the predicted direction if males are more reliant on maternal resources than females. However, the extent to which this sex ratio shift is facultative is perhaps questionable, in that the reduced lifespan of these starved females appears to have merely truncated the schedule of production of male and female offspring across the oviposition period, rather than fundamentally changed the rate at which males and females are produced (Figure 10.5). The shift in sex ratio across a female's lifespan may then just be an artefact of curtailing the usual pattern of allocation, rather than a deliberate shift in sex ratio strategy.

The other factor that we manipulated and expected to influence maternal condition – rearing temperature – seemed to have a rather negligible effect on maternal condition, at least in terms of egg production (perhaps the closest measure we have to overall fitness). Likewise, temperature was only weakly associated with changes in sex allocation, only significant when interacting with the other factor we manipulated, namely age at mating. This questions the relevance of rearing temperature for sex allocation in *P. citri.* (see below). On the other hand, our environmental factor associated with mate availability – age at mating – although inevitably associated to some

extent with female condition in terms of age (but again see below), was indeed associated with changes in sex allocation. Females that experienced delayed mating were more likely to produce females and this result suggests that the frequency of interactions with males influences sex allocation, which can be interpreted in two ways. First, low encounter rate between adults can signal low population density, which has been shown to be associated with more female biased sex ratios before in *P. citri.*, as observed here (Ross *et al.*, 2010a). Secondly however, Werren and Charnov (1978) have previously interpreted data from *P. citri.* that showed *increased* male production by females that had experienced delayed mating (Nelson-Rees, 1960) as proof for an adaptive response by mothers to a shortage of males (invoking classic Düsing-Fisher frequency dependence), but clearly our data question the generality of that finding, at least in terms of the overall link between mate encounter rate and sex ratio.

The two experiments presented here, combined with our previous work (Ross *et al.*, 2010a) show that female age is associated with sex allocation, in as much as sex ratio varies across a female's oviposition period. If we assume that older females are in poorer condition due to the costs of reproduction, then we see that older females under "good" environmental conditions (mated promptly, reared at 25°C and not food restricted) tend to produce more male offspring at the end of their oviposition periods (Figures 10.3 and 10.4, and see Ross *et al.*, 2010a). The key question is whether this later phase of male production signifies, or is a result of, genuine "poorer" female condition? Relevant here are our age at mating treatments in experiment 1. By delaying mating, we delayed oviposition by in excess of 30 days, and yet the initial patterns of sex allocation were generally similar to (or *more* female-biased than) the beginning of the oviposition period for "standard" females. Thus, age *per se* does not appear to be associated with sex allocation pattern, rather it is when during the oviposition period that eggs are laid that is more crucial. Whilst one is not strictly comparing like with like (older, but unmated females will presumably have less resources available for reproduction when they finally commence oviposition), it is striking that panels (B) and (D) of Figure 10.4 are more similar to the first half of the oviposition period of panel (A), than the latter half. As such, female age in itself is perhaps less of an indicator of female quality; rather female age appears to interact with other factors such as age at mating and stage of the oviposition period.

As, alluded to above, the results concerning rearing temperature and age at mating found in experiment 1 differ from those observed in earlier studies of sex allocation in *P. citri* (James, 1937, 1938; Nelson-Rees, 1960). This could in part be due to the fact that our experiment used a different strain than the one that was used in previous studies. However another explanation is that in previous experiments brood sex ratios were determined at the beginning of the third instar when the sexes become distinguishable, while we determined the sex of embryos. This means that the difference might be explained by strong differential mortality between the sexes, caused by temperature (experienced by the mother) or mating age. If this is true we would expect the previous experiments to find a strong reduction in the number of surviving offspring between the treatments: this was indeed the case. For instance,

when comparing our results with those obtained by Nelson-Rees (1960), our study shows less of a reduction in clutch size as a result of high temperature and age at mating (see Table 10.3). This suggests that at least some of the differences between our studies might be explained by differential mortality between the sexes and that both the temperature a female has been raised at and her age at mating affect the mortality of male and female offspring differently. It is important to note that the suggested differential mortality effect of temperature is not the effect of temperature *per se* but of the temperature experienced by the mother, as in Nelson-Rees' experiments all clutches were raised at the same temperature. Thus daughters from mothers reared at higher temperatures may suffer from higher mortality than their male siblings. The reason for this is unclear. However it has recently been observed that the titre of the obligate endosymbionts that mealybugs need to survive are affected by host age and temperature, with a strong reduction in the number of endosymbionts in older females (Kono *et al.*, 2008) and in females raised at high temperatures (Buchner, 1965). Since it has been suggested that males might be less dependent on the endosymbionts than females (Ross *et al.*, 2010b), the differential survival effects might be explained by the number of endosymbionts a mother is able to transmit to her offspring.

The results from our experiments and earlier studies (Nelson-Rees, 1960; Ross *et al.*, 2010a; Varndell & Godfray, 1996) show that there is a strong effect of oviposition day on sex ratio and while the evolutionary significance of this pattern is currently unclear (and will be discussed elsewhere, Ross *et al.* in prep.) it is important to consider as it might affect the interpretation of sex allocation data. For example, in our experiment food restriction seems to give an adaptive pattern if overall sex ratio across a female's whole oviposition period is considered (assuming daughters favoured if mothers are in poor condition), whereas in fact it may be an artefact of a reduced lifespan interfering with the "usual" schedule of sex allocation. If this is right (and we can accept that it may not be), then adaptive facultative sex allocation with respect to food restriction is questionable. Order effects in sex allocation, where sex

Table 10.3 Comparison between clutch size effects of different environmental factors between our study and those obtained by Nelson-Rees (1960).

Nelson-Rees 1960		This study	
Treatment	Results	Treatment	Results
Temperature:	Clutch size:	Temperature:	Clutch size:
20–26 C:	527	25 C:	357.6 (+/– 34.0 s.e.)
29.1–30.1 C:	164.91	30 C:	348.5 (+/– 32.2 s.e.)
Mating age:	Clutch size:	Mating age:	Clutch size:
At maturation	420	At maturation	357.6 (+/– 34.0 s.e.)
70 days	220	63 days	268.0 (+/–43.5 s.e.)

ratios change during the oviposition period, are common in other taxa (Hardy, 1992) and our results highlight that care needs to be taken when interpreting sex ratios, especially if longitudinal patterns of sex allocation (but within individuals) are not adequately considered.

Our results do support the findings of earlier experiments (Ross *et al.*, 2010a; Varndell & Godfray, 1996) showing that female *P. citri* facultatively adjust their sex allocation depending on local conditions (aspects of population density). The mechanism by which they might do this is currently unclear though. Sex chromosomes in mealybugs are absent and it seems unlikely that autosomal genetic factors determine sex. Recently several histone proteins involved in the heterochromatization of paternal chromosomes in males have been shown to be present at higher concentrations in male embryos in *P. citri.* (Bongiorni *et al.*, 2004; Bongiorni *et al.*, 2001; Bongiorni *et al.*, 2007; Bongiorni & Prantera, 2003). It has therefore been suggested that these proteins are also involved in sex determination (Buglia & Ferraro, 2004a; Ross *et al.*, 2010b) and females might alter the concentration of these proteins in their eggs to change the sex ratio of their broods. Along these lines, Buglia *et al.*(2009) showed higher concentrations of the histone protein HP1 in eggs of females that were aged prior to mating. However in that study they assumed that these females produce male-biased clutches (although the sex ratio data were not provided), while in our study we observe the opposite effect of maternal aging prior to mating. It will be of great interest to test the effect of temperature and starvation on the concentration of HP1 in embryos and to directly link this to sex ratio data, as this will not only help to unravel the sex determination mechanism, but also how females are able to adjust their sex ratio according to environmental cues.

To conclude, our results show that even within a single species different components of the environment can influence sex allocation in complicated and apparently conflicting ways (for instance if one assumes that certain factors will always influence maternal condition in a negative way). As such, our work reinforces the emerging consensus that multiple forces of sex ratio selection (LRC, Trivers-Willard and so forth) may act simultaneously in populations (e.g. Pen & Weissing, 2002; Wild & West, 2007). Whilst this consensus may be intellectually refreshing, it will make determining patterns of causation difficult. Experimental approaches such as those undertaken here are a necessity, as will be a greater understanding of the mechanistic basis of how and when females allocate sex to their offspring (West, 2009). The more organisms we determine these mechanisms for, the better.

Acknowledgements

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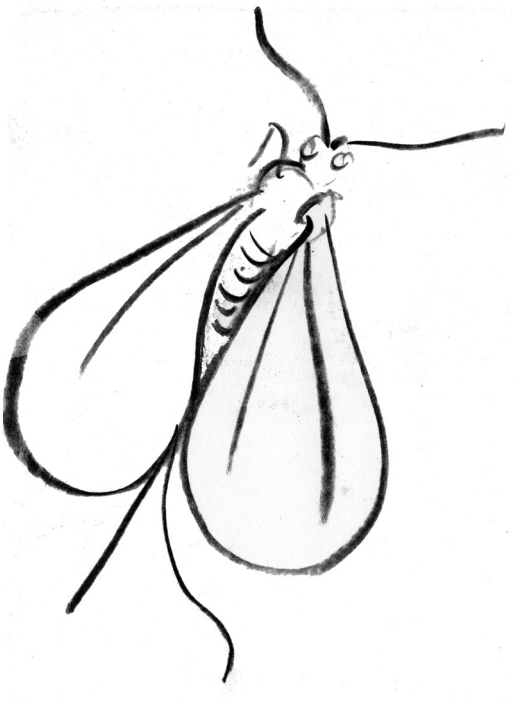

CHAPTER 11

Epilogue

Laura Ross

In toto intragenomic conflict can be seen as a perpetual driving force to evolutionary change. Hence, in opposition to more conventional views in which evolution is seen as adaptation to local environment, the view advocated here is that much of the evolution of genetical systems is internally driven and would continue without ecological and environmental variation. –

Hurst (1992)

INTRODUCTION

Genetic conflict is increasingly considered an important force in evolution (Burt & Trivers, 2006; Hurst & Werren, 2001; Hurst, 1992, 1995). Its effects have been suggested in the evolution of a wide range of biological phenomena; from the way genetic material is organized (Haig & Grafen, 1991) to the way organisms interact and reproduce (Bourke, 2009; Haig, 2000; Haig & Wilkins, 2000; Normark, 2006; Partridge & Hurst, 1998). In this thesis I have focussed specifically on the role of genetic conflict in the evolution of genetic systems. A species' genetic system is comprised of various aspects of genome organization and transmission, including ploidy level, asymmetry in ploidy and transmission between the sexes and the type of reproduction (sexual/asexual or a mixture). There is a remarkable variety in these systems across species (White, 1973) and the evolutionary significance of this variation remains poorly understood. In this thesis I have explored how genetic conflict could have caused several transitions between genetic systems by using a variety of different techniques. In this epilogue I will first of all briefly summarize the most important insights from my thesis work. I will then discuss some of the difficulties of studying genetic conflict and some of the approaches needed to study its role. Finally I will discuss how the role of genetic conflict in the evolution of novel genetic systems compares with alternative hypotheses and discuss the validity of the statement of Hurst that intragenomic conflict can be seen as a perpetual driving force to evolutionary change and that *much* of the evolution of genetical systems is internally driven and would continue without ecological and environmental variation.

MOST IMPORTANT INSIGHTS

I my thesis work I have investigated the role of genetic conflict on the evolution of alternative genetic systems. In order to do this I have used theoretical models where I explore the role of conflict on both the evolution and stability of two genetic systems. First of all I considered the role of conflict between maternal and paternal genes in males of species with PGE. I showed that apart from conflict over transmissions of the paternal genes, conflict can also exist over male survival, because paternal genes can gain indirect fitness benefits from the death of the male they are in, if this benefits their sisters. I showed how this conflict might have been responsible for the complete suppression of the paternal genome in males of many scale insects and mites (Norton *et al.*, 1993; Nur, 1980), and suggested it might also help to explain the evolution of split sex ratios in *Sciara* flies. These results also confirm the importance of sibling competition in determining the scope of genetic conflict in a given species.

In chapter 5 I considered the scenario where "infectious" sperm-producing tissue transferred during copulation can establish in a female and lead to the evolution of (a specific type of) hermaphroditism. This is the first suggestion that hermaphro-

ditism could have evolved as the result of conflict (Normark, 2009). I also showed that although the transition is originally a result of conflict, once it evolves the build-up of relatedness between the tissue and the individual it is in aligns their evolutionary interests, obviating conflict.

Apart from the theoretical work presented in this thesis which considers the role of genetic conflict more generally, I have also focused on the role of genetic conflict in shaping the variety of genetic systems in one particular group of insects: the scale insects. I chose to focus on this group as it has the largest variety of genetic systems of any group of comparable size. Furthermore, due to their biology and life history, a large scope for genetic conflict was expected (as detailed in chapter 2). A wealth of data on scale insects is available mainly from the first half of the twentieth century, but few of these data have been considered in light of genetic conflict. In chapter 2 of this thesis I review this available data and conclude that the data are often consistent with the hypothesis that genetic conflict has played an important role in the evolution of new genetic systems in scale insects. I also discussed cytological data, which suggested ongoing conflict and the presence of a continuing evolutionary arms race between different genetic elements (in particular between maternal and paternal genes in species with paternal genome elimination, or PGE). The main conclusion of the review was, therefore, that many of the genetic systems in scale insects may well have evolved as a result of conflict but that, in turn, these genetic systems may themselves have given rise to new conflicts. Because of this, the distribution of genetic and sex determination systems across scale insects is probably not stable and continuing co-evolutionary arms races may be taking place, further contributing to this diversification.

I have also attempted to use a formal comparative approach to test one particular hypothesis about the role of genetic conflict in scale insects, namely that between hosts and their endosymbionts which might have lead to the evolution of haplodiploidy and PGE (systems that are both characterized by asymmetric transmission). I showed that within scale insects, taxa that contained endosymbionts where indeed more likely to have a genetic system with asymmetric transmission than those without endosymbionts, consistent with their role in the evolution of these systems. However, unfortunately our analysis was limited to some extent by the lack of a well-resolved phylogeny. Hopefully though, with more biological and phylogenetic data becoming available, it will soon be possible to extend this analysis, both within scale insects and also across other taxa.

HOW CONFLICT CAN CAUSE TRANSITIONS

Although I have suggested that several of the transitions between genetic systems have resulted from conflict, exactly how conflict has led to these transitions might differ. In some cases, like endosymbiont-induced parthenogenesis as recently suggested for some species of armoured scale insects (Gruwell *et al.*, 2009), selection associated with the conflict may have directly caused a change in reproduction and

the outcome benefits the bacteria that has caused the transition. The evolution of hermaphroditism in *Icerya* may also have been a direct result of conflict between males and females over the fertilization rate (see chapter 5). However, although some transitions might be a direct result of the conflicting selection pressures on the interacting entities, and be associated with the outright win of one of the genetic entities involved, other transitions might have evolved not as a direct result of the manipulation of one of the entities but rather as an evolutionary response to avoid conflict: for instance, Normark (2006) suggested that asymmetric genetic systems like parthenogenesis could have evolved to avoid the spiteful interactions between half-siblings induced by the paternal genome for which they are unrelated (e.g. to increase the relatedness between competing relatives). Other examples of this conflict avoidance could be the evolution of split sex ratios in some Sciarid flies with PGE, as this might avoid the evolution of paternally-expressed male-suicide genes (see Chapter 4). Conflict avoidance might also help explain the remarkable biology of the scale insect family Stictococcidae. In these species, early instar offspring are fed directly by their mother. This could have caused strong selection for male-killing, both by endosymbionts and by paternally derived genes. Interestingly, Stictococcidae have lost endosymbionts from males, as well as reverted back from PGE to diplo-diploidy, and by doing so they have avoided selection for male-killing. Unfortunately, the order in which these three phenotypes – maternal provisioning, endosymbiont absence in males, and diplodiploidy – evolved is currently unclear and therefore other scenarios could explain their evolution as well. Finally, another scenario that could lead to the evolution of new genetic systems arises when different genetic entities are caught up in an evolutionary arms-race and the new genetic system evolves as a result of this arms-race, while not resolving, but merely changing the conflict (Conflict transformation: Hurst *et al.*, 1996). An example of this discussed in this thesis is the hypothesis by Normark for the role of endosymbionts on the evolution of haplodiploidy and PGE.

KIN SELECTION AND INBREEDING

In the different chapters of this thesis it has become clear that interactions between individuals are crucial for understanding the scope for genetic conflict. I have shown that inbreeding can reduce the benefit of being selfish, such that inbreeding can limit the spread of paternally expressed suicide genes (Chapter 4). And in our model for the evolution of hermaphroditism, continual inbreeding between female and her infectious tissue promoted collaboration between the two (chapter 5). In both cases, inbreeding reduces conflict by inducing relatedness between genes that would have been in conflict otherwise, by coordinating the interests of the genes involved (Wenseleers & Ratnieks, 2001). This relatedness between genes, although it reduces conflicts that might lead to the evolution of novel genetic systems (Smith, 2000), might actually increase the selective benefit of some of these systems. Genetic

systems with asymmetric transmission (like haplodiploidy, PGE and parthenogenesis) might evolve more easily under high level of inbreeding, as relatedness between the maternal and paternal genome could allow the genome of one sex to sacrifice its reproduction altruistically on behalf of genome of the other (Bourke, 2009).

High levels of inbreeding are indeed often observed in species with asymmetric genetic systems (Hamilton, 1967, 1979, 1993), although there might be alternative explanations for this pattern. For example, haplodiploid species are generally assumed to be more resilient to inbreeding depression as recessive deleterious alleles are purged from males (Henter, 2003), so haplodiploid species might just be able to get away with higher levels of inbreeding. Alternatively, other factors that correlate with inbreeding might be responsible, for example a life history leading to high levels of inbreeding, might lead to high levels of sibling competition as well. In this thesis I have explored how under high levels of sibling competition conflict between host and endosymbiont might lead to the evolution of PGE and this instead of inbreeding itself might be the reason that these systems seem to correlate with inbreeding.

CONFLICT OVER SEX ALLOCATION

The comparative and theoretical work presented in this thesis focuses on the role of genetic conflict shaping genetic systems across species. However, as I discussed earlier, establishing the potential role of conflict is easier than establishing its actual role. In order to do so it is crucial to determine two things. First, that the natural or sexual selection optima for the traits expressed by the different interacting entities actually are different (i.e. experimentally manipulate traits to prove there actually is a manifest conflict occurring, rather than just predict one from first principles). Second, that each actor in the conflict has the power to manipulate or vary the trait or traits of interest. Determining this power as well as measuring the fitness optima for different genetic entities is far easier in an intra-specific context than across species as an experimental approach is often crucial. PGE is the most common genetic system in scale insects and although I have suggested that this system might have evolved as a result of conflict, its evolution has in turn given rise to new conflicts (see chapter 2 and 4). One trait over which we expect conflict in species with PGE is sex allocation (see chapter 3). Therefore I decided to focus my experimental work on this aspect and I chose to work with the citrus mealybug *Planococcus citri* as this species has PGE and therefore conflict between males and females over offspring sex ratio can be expected. Additionally, mealybugs also have maternally transmitted bacteria (von Dohlen *et al.*, 2001) that might have a stake in the sex allocation of their host (see Chapter 2). However, although some earlier experiments had identified environmental factors that affect sex allocation (James, 1937; Nelson-Rees, 1960) (Varndell & Godfray, 1996), it was still not understood why these factors had an effect, how sex was determined, and which parties (male, female and endosymbiont) could affect sex allocation.

In my experiments I showed that females are indeed able to adjust their sex allocation and identified several environmental conditions that influence sex allocation. I found that mothers produce more male-biased clutches when they experience high densities and interpreted this finding as an adaptive response to avoid the more severe negative effects of competition on female fitness (chapter 7). I also showed that female age strongly affects sex allocation, such that females change the sex ratio they produce during oviposition, with very male biased clutches being predominately produced at the beginning and the end of the oviposition period (chapter 7,8, 9 and 10). Finally, I also showed that the age at which females are mated affected sex allocation, with females that are older when mated producing more female-biased sex ratios (chapter 10). Although these sex allocation experiments showed clear and repeatable results, some of them contradicted earlier studies. This was why I attempted to see if this could be explained by genetic differences between populations. However, I did not find differences between populations from the U.K., Portugal and Israel (chapter 9). Taken together, these results highlight that experimental studies on sex allocation in a species for which a limited amount of life history data is available can be challenging, as in order to really understand observed patterns the biology of the organism is crucial. In the end, the full effects of (kin) competition and resource availability on the sex allocation decisions of females are still elusive, and without progress on them understanding sex allocation and the nature and extent of conflicts over sex allocation will remain partial at best. For instance, in order to determine the importance of sex ratio conflict it will be crucial to confirm the ability of the male and endosymbiont to influence the sex allocation. For this more experimental work will be needed; for example it might be possible to use antibiotic treatments to alter the number of endosymbionts transmitted to embryos and consider the affect on sex allocation (and pilot work at the end of my project has begun to consider this possibility). Male effects on sex allocation could be determined using quantitative genetics experiments to estimate the relative effect of male genotype on the sex ratio of their offspring. Only when we have determined the power of all the different entities will it be possible to consider the role conflict over sex allocation can have on of the mechanisms and stability of sex determination in mealybugs.

DIFFICULTIES CONSIDERING THE ROLE OF CONFLICT

Studying the effects of genetic conflict is challenging and this is why the importance of genetic conflict in evolution until now has mainly been considered by theoretical studies (e.g. Hurst, 1992, 1995; Hurst *et al.*, 1996). A theoretical approach for studying the role of genetic conflict has lead to important insights both on the possible role genetic conflict can have, but also in generating predictions for experimental studies. The main value of theoretical models is that they allow you to determine fitness optima for different entities and to determine how these optima will be affected by other biological factors, for example in chapter 4 we measured the strength of con-

flict under varying levels on inbreeding and recycling efficiency of sibling resources. Also by using inclusive fitness models (Taylor & Frank, 1996), as was done in this thesis it is possible to model both conflict with an individual but also the role interactions between relatives can play on the strength and effect of conflict. Models can also explore possible scenarios of conflict resolution and how this can lead to the changes in genetic system or other aspects of a species' biology. Finally another issue which might be possible to explore with a specific type of model is the effect that varying levels of power of different entities can have on the outcome of conflict. In order to do so it is important to incorporate detailed knowledge of the mechanism by which different entities can manipulate the conflict trait. These so called mechanistic models have recently been successfully deployed to understand the evolution of sex determining mechanisms (Kozielska *et al.*, 2009; Pen *et al.*, 2010), but will be suitable as well for other traits such as transmission. However although theoretical approaches can be very powerful in predicting the scope for conflict and suggesting possible scenarios for its effects, it does nothing to test the actual existence and effect of conflict. Therefore it will be crucial to use experimental approaches to test the predictions generated by the theoretical models, even if this is a much more challenging approach. In the remainder of the epilogue I will therefore explain what these challenges are and what approaches will be needed to overcome them and improve our understanding of the role of genetic conflict.

An important problem when studying the effect of conflict is that, the conflicts responsible for the evolutionary change of a trait might have been played out in the evolutionary past and evidence for conflict might have disappeared completely (conflict extinction Hurst *et al.*, 1996). A good example of this comes from a theoretical study by Kozielska and coworkers et (Kozielska *et al.*, 2009) which shows that the invasion of a sex ratio distorter might cause a change in the sex determination mechanism to suppress the drive induced by the distorter, but once this new system is in place the distorter itself is often lost from the population. Another problem is that at any given time most of the conflicts occurring may be relatively weak (i.e. the different actors have rather similar optima for the trait(s) associated with the conflict). For instance, selfish elements with a strong (fitness) effect, such as a male-killing endosymbiont, will cause very strong selection on the other genes in the genome to suppress the element or alternatively lead to population extinction (Hurst *et al.*, 1996). So even if such elements frequently arise and have profound effects on their hosts, there existence and importance might easily be overlooked. One of the rare cases where both the spread and the suppression of a selfish element could be studied in real time was that of a male killing endosymbiont in the butterfly *Hypolimnas bolina* (Hornett *et al.*, 2006). This male killer is found in populations of butterflies from several pacific islands, but the frequencies differ per population (Dyson & Hurst, 2004) and recently a suppressor genotype has been identified (Hornett *et al.*, 2006; Hornett *et al.*, 2008). This system has offered the unique opportunity to study the population effects and the suppression of a selfish element in a natural population.

As mentioned earlier, another problem commonly encountered when studying conflict but perhaps realized most clearly by the sexual conflict community (e.g. Arnqvist & Rowe, 2005; Chapman, 2006; Chapman *et al.*, 2003) is that in order for conflict to be important the competing entities both need to have the power to affect the trait under conflict. Determining the power of the different entities is therefore crucial to understand the effect conflict can have in any given system. However this can be challenging (Chapman, 2006; Hurst & Werren, 2001), as the use of an experimental approach is needed to determine the power of (one of the) genetic entities. The power of different entities is often hard to detect because even when both entities do have the ability to influence the conflict phenotype, due to continuing co-evolution they might have reached a stalemate (Hurst *et al.*, 1996), where genetic conflicts do not become resolved, nor do they lead to compromises. In cases like this conflicts and the power of different entities often only appear when the power of one of the actors is manipulated (Hurst & Werren, 2001).

Yet another aspect that can further hinder both our understanding of genetic conflict, as well as experimental attempts to study it, is the potential disconnect between the "conflict trait" (i.e. the trait over which entities come into conflict) and the traits that are then selected/deployed in the context of the conflict by the different entities. This means that, for the unwary, there can be confusion over what are the "conflict traits". For example, in chapter 4 where we discussed the evolution of male suicide genes, the "conflict trait" is male survival (the natural selection optimum for maternal versus paternal inherited genes for male survival is not the same), but the response of the maternal genes to suppress male suicide could be by evolving a different trait, like the suppression of paternal gene expression or the evolution of split offspring sex ratios. Thus, a conflict over a given trait arises if the selection surfaces for the transmission phenotypes differ between the two interacting entities (i.e. different optima). However, the traits that are involved in forming the architecture of the selection surface for the two entities may differ. The key thing is to separate out selection arising from an interaction or shared phenotype, and the different components of the phenotypes that different entities use or can employ during that interaction (or that make up the overall phenotype).

A final problem when studying the importance of genetic conflict in evolution is that it is difficult to quantify its importance as even when empirical evidence exists for the role of conflict in the evolution of a trait in one taxa, this provides no evidence for its role in the evolution of that same trait in other species. It will therefore be important to deploy methods that are able to measure the effect of conflict over a wide taxonomic scale.

HOW TO ESTABLISH THE ROLE OF GENETIC CONFLICT?

It is clear that studying the role of genetic conflict can often be challenging. However there are different approaches that can overcome at least some of the limitations and

can therefore help to improve our understanding of the effects of conflict. The use of these approaches has been expanding over the last few years, especially for studying sexual conflict, and in the next section I will discuss some of these approaches and how they could be deployed to improve our understanding of genetic conflict as a driving force in the evolution of genetic systems.

EXPERIMENTAL APPROACHES

As I have mentioned earlier, an experimental approach is often crucial to determine the power of different genetic entities and thereby to show the importance of conflict. In order to do so it is important to manipulate the power of the different entities. One way of doing this, which has proved particularly successful, is by studying hybrids (between distinct populations of the same species or between closely related species). This is often how, for example, segregation distorters are discovered (reviewed by Hurst & Werren, 2001). This technique might also be very useful to study conflict between genes of maternal and paternal origin in species with PGE. Recent experiments of hybrid crosses between mealybug species show that hybrid males prefer the pheromones from the paternal species (Mendel *et al. in prep*). This suggests that at least some paternal genes are expressed, possibly because maternal genes are less well able to suppress heterospecific paternal genes (i.e. the paternal genes now can influence the conflict trait). Apart from using hybrid crosses to study the power of different genetic entities, an experimental approach to directly manipulate the power of one entity directly could be an effective way to show the power of the other entity. Using an example discussed in Chapter 2, it might be possible to use recently developed RNAi techniques (Bongiorni *et al.*, 2007) to manipulate the deactivation of the paternal genome in males to see if the paternal genome might be able to make it into the sperm when it isn't heterochromatized.

Another experimental approach that is not just able to explore the presence of conflict but also the role it can have in trait evolution is experimental evolution. There are only few examples of studies using this approach and again most of these focus on sexual conflict. The classic examples of the use of this approach are the experiments on sexual conflict in *Drosophila* where the mating system was manipulated for several generations and the evolutionary response on the severity of conflict was measured by crossing mates from the artificially created monogamous and polygamous populations (Holland & Rice, 1999; Rice, 1996). One of the best examples of this approach studying the effect on selfish elements is the work of Price and colleagues (Price *et al.*, 2008) who used several replicated populations to show that polyandry repeatedly evolves in a population where segregation distorters are present at high frequencies, as by mating multiple times females reduced the chance their offspring would contain the distorter, as sperm that carried the distorter had a reduced competitive ability. To my knowledge, an experimental evolution approach has not yet been deployed to study the role genetic conflict can have in the evolution of

genetic systems. It might be that the approach is not a suitable for doing so as the evolutionary timescale on which genetic systems change might be too large to track in an experimental setting. However in a species where genetic systems are of relatively recent origin and which has a short generation time and can be kept at high densities in the lab such approach might still be valuable and both scale insects but also possibly mites might be good candidates for such approach.

COMPARATIVE APPROACHES: HOW TO COMPARE CONFLICT ACROSS TAXA?

Genetic conflict is increasingly considered as an important force driving the evolution of a wide variety of traits. However, although theoretical models have supported the plausibility of this claim for many traits, and empirical evidence has been shown for some, the real importance of genetic conflict remains unclear. Showing that for example the evolution of haplodiploidy in one particular taxonomic group is the result of genetic conflict does not mean the same mechanism is responsible for its evolution in other taxa. Therefore a comparative approach across a wide taxonomic range is crucial in order to test for the importance of genetic conflict for any particular trait. However, in order to do so it is crucial to be able to measure conflict and for this measure to be applicable across a range of taxa (i.e. provide some form of standardised measure or "effect size" of genetic conflict). In an ideal world the thing that we would like to quantify is the strength of conflict. However, it might often be easier to measure other factors that correlate with conflict and combine these to predict the strength of conflict rather than measure it directly. These factors include the number of genetic elements that could come intro conflict and the probability of asymmetries in transmission between them. Other important factors include the level of inbreeding and strength of competition between relatives and between mates (as determined by mating system and the gregariousness of broods). The problem is that, first of all, a considerable amount of knowledge on a species' biology is needed to determine these factors and, secondly, that it will be very challenging to use the same measurement across a wide taxonomic range. However as more phylogenetic data become available this type of analysis might be very powerful in determining the importance of conflict shaping a variety of biological phenomena.

CAN GENETIC CONFLICT EXPLAIN THE TAXONOMIC RANGE OF GENETIC SYSTEMS?

One of the main aims of my research has been to try to explain the patterns of variability in genetic systems across multi-cellular organisms. In order to do this I have focussed on one specific group, which is a particular hotspot of variation, and I have investigated if the scope for genetic conflict, based on various aspects of this biology,

could be responsible for this variation. However, even while I think it is plausible that genetic conflict has indeed played an important role, it is not yet possible to say definitively if the large variety of genetic systems has evolved because of conflict, or for another reason, and that the large scope for conflict as identified in this thesis is merely the result and not the cause of the variety in genetic systems. Currently the evidence is at best circumstantial. That is why I will finish this epilogue by discussing some the alternative explanations for the evolution of genetic systems and how these alternatives compare with the hypothesis that was focus of my thesis work.

In chapter 2 I have discussed some of the alternative explanations that have previously been suggested to explain the variety of genetic systems observed in scale insects, which include the fragility of males and the flexibility of sex ratio adjustment (Bull, 1983). I have argued that although some of these factors might certainly have been important in the determining the fitness effects of transitions between genetic systems, the explanatory power of these factors in the evolution of these systems is likely to be quite limited. Another possible explanation is that the observed distribution and variety of genetic system has evolved as a result of drift. The idea that a large amount of the observed variation in genome structure has evolved not as a result of natural selection but as the result of drift has recently received considerable attention, most notably by the publication of Michael Lynch's book(Lynch, 2008). If it is indeed true that genetic systems would merely have evolved as a result of drift, one of the most important predictions would be that taxa with smaller effective population sizes would show larger variability in genetic system, as due to increased drift different genetic systems could be fixed in different taxa. Although this prediction has not been formally assessed it does not seem to hold for the variety of genetic systems as insect which are probably the most variable in terms of genetic systems generally have large population sizes, as is also true more specifically for many scale insects (Gullan & Kosztarab, 1997; Normark & Johnson, 2010). Finally, another explanation for which at least some support exists is the importance of the molecular mechanism of chromosome transmission. Scale insects have both unusual holocentric chromosomes (chromosomes that lack centromeres) and inverse meiosis (where the mitotic phase of meiosis precedes the meiotic phase, instead of the other way around). Interestingly these two characteristics are also found in mites, another group with a large variety of genetic systems. In chapter 2 I have discussed that although chromosome structure might be able to explain variation in chromosome number, by itself it is an unlikely explanation for the variability of genetic systems. However, the chromosome structure and the type of meiosis might be important in determining the effects of genetic conflict. The way genes are organized on chromosomes, and the molecular mechanism by which these chromosomes are transmitted, might influence the power different entities have to affect the transmission leading to a stronger manifestations of conflict. Another factor that supports these ideas is the observation that scale insects have highly unusual number and arrangement of microtubules in the sperm and the paternal centriole and that a similar structure is found in other (unre-

lated) taxa with unusual genetic systems including PGE and haplodiploidy (Sciaridae (fungus gnats), Cecidomyiidae (gall gnats), whiteflies, oxyurid nematodes and thrips: Normark, 2009). Understanding the relationship between the abnormalities in the mechanism of transmission, genetic conflict and the diversity of genetic systems, might prove a crucial next step in order to understand the distribution of genetic systems.

FINAL REMARKS

I started this epilogue with a quote by Laurence Hurst, which stated that much of the evolution of genetical systems is driven by genetic conflict. From my thesis work I conclude that whilst some of the variation in genetic systems might indeed be driven by conflict, key experimental and comparative studies remain to be done to confirm that it explains the evolution of much of the variation in genetic systems. The work presented here hopefully provides a foundation for some of these future studies.

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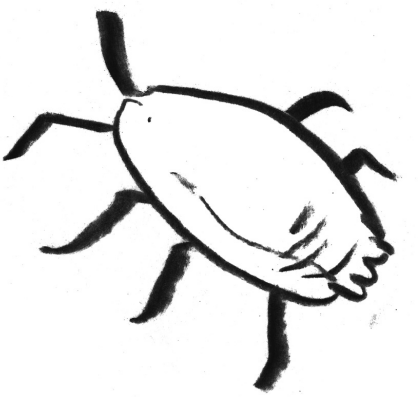

Samenvatting

INLEIDING

Het ontstaan van meercellige planten en dieren vanuit ééncellige voorouders was een belangrijke ontwikkeling in de evolutie van het leven op aarde. Om die stap te zetten moesten verschillende cellen samenwerken om te kunnen overleven en zich te kunnen voortplanten. Maar zelfs als samenwerking tussen alle cellen noodzakelijk is om te overleven betekent dat nog niet dat er geen concurrentie kan zijn tussen verschillende cellen onderling. Als een bepaalde cel een mutatie heeft waardoor zij sneller kan groeien dan de andere cellen in het lichaam zal deze cel in aantal toenemen en daarbij schade kunnen toebrengen aan het individu, tenzij dit proces wordt gestopt door de andere cellen. Dit soort 'op hol geslagen' cellen komen inderdaad regelmatig voor en veroorzaken vormen van kanker, in veel organismen een belangrijke doodsoorzaak. Gelukkig is over het algemeen de concurrentiestrijd tussen cellen in meercellige organismen vrij gering. Dat komt omdat in de meeste meercellige organismen alle cellen klonen van elkaar zijn en dus dezelfde genen hebben. Daardoor wordt competitie tussen cellen voorkomen omdat een gen dat bepaalde cellen sneller laat groeien niet in frequentie zal toenemen als dit andere cellen die hetzelfde gen bevatten benadeelt. Dus het feit dat alle cellen dezelfde genen bevatten bevordert de samenwerking tussen deze cellen. Desondanks kan er in een bepaalde situatie toch wel concurrentie tussen genen optreden, namelijk tijdens de voortplanting. Dit wordt vaak "genetisch conflict" genoemd, een term die hier verder gebruikt zal worden.

GENEN DIE VALS SPELEN

Verreweg de meeste meercellige organismen planten zich seksueel voort. Zo wordt in iedere generatie een nieuw individu gevormd door het samenvoegen van de helft van de genen van de vader met de helft van de genen van de moeder. Het gevolg is dat iedere ouder per nakomeling maar de helft van zijn genen kan doorgeven aan de volgende generatie. Maar hoe wordt bepaald welke genen dit zijn? Over het algemeen is dit een loterij, waarbij ieder gen een gelijke kans heeft om te worden doorgegeven. De afgelopen decennia zijn er echter verschillende genen gevonden die een "trucje" hebben waarmee ze hun kansen vergroten, met als resultaat dat deze genen zich snel door hun populatie verspreiden. Dit gebeurt zelfs als ze nadelig zijn voor het individu waarin ze zich bevinden.

EÉNCELLIGE MEELIFTERS

Afgezien van het ontstaan van meercelligen vond er nog een belangrijke ontwikkeling plaats waarbij twee verschillende cellen, ieder met hun eigen genen, begonnen samen te werken. Eén van deze twee (toen nog) bacteriën evolueerde om in de andere bacterie te leven. Hoe dit precies is gegaan is niet bekend maar het is mogelijk

dat de ene bacterie de ander inslikte om hem op te eten maar dat deze in leven bleef. In ruil voor een veilig onderkomen verzorgde de geïntegreerde bacterie nieuwe functies, zoals het produceren van energie uit suikers en zuurstof en uit zonlicht (in planten). Verschillende organellen (de "organen" van de cel), bijvoorbeeld de mitochondrieën en de chloroplasten in planten waren oorspronkelijk bacteriën en hebben nog steeds hun eigen genen. De cellen van meercelligen bevatten behalve de organellen met bacteriële oorsprong soms ook echte bacteriën. Veel insecten bijvoorbeeld herbergen zulke ééncellige gasten, endosymbionten genoemd, die hun gastheren helpen met bijvoorbeeld het produceren van essentiële voedingsstoffen en ook bij de verdediging tegen ziekteverwekkers. Vaak zijn deze bacteriën net als organellen met bacteriële oorsprong essentieel voor hun gastheer. Hoewel de gastheer dus baat heeft bij deze ééncellige symbionten kunnen ze ook voor problemen zorgen: dit komt omdat zowel organellen als endosymbionten alleen via vrouwelijke geslachtscellen (eicellen) worden doorgegeven aan het nageslacht. Dit betekent dat ieder individu alleen de organellen en symbionten van de moeder erft en niet die van de vader. Het betekent ook dat de endosymbionten die zich in een mannelijke gastheer bevinden nooit aan nageslacht zullen worden doorgegeven en dus op een evolutionair "dood spoor" zitten. Daardoor zal een symbiont die er voor kan zorgen dat hij in een vrouwtje in plaats van een mannetje terecht komt in frequentie toenemen. Er zijn inmiddels verschillende gevallen bekend waarbij endosymbionten de voortplanting van hun gastheer zo beïnvloeden dat ze meer dochters produceren. Sommige bacteriën zorgen er zelfs voor dat hun gastheer zich aseksueel voortplant, zodat mannetjes helemaal overbodig zijn geworden!

VOORPLANTING: CONFLICT OVER TOEGANG TOT DE VOLGENDE GENERATIE

Tijdens de voorplanting kunnen de verschillende genen, zowel die in de celkern als ook die in de organellen en endosymbionten, hun fitness (overleving en voortplanting) verhogen door de kans te vergroten om te worden doorgegeven naar de volgende generatie. Daarom is er onder evolutiebiologen recentelijk geopperd dat deze conflicten wel eens zouden kunnen verklaren waarom er zoveel variatie is in de manier waarop verschillende organismen zich voortplanten en hun genen doorgeven. Helaas is er tot nu toe nog maar weinig bewijs voor deze hypothese. Het probleem is dat verreweg de meeste biologen onderzoek doen aan een heel beperkt aantal organismen en dat de soorten die ze voor hun onderzoek gekozen hebben, in tegenstelling tot veel andere organismen, onderling nauwelijks verschillen in hun reproductie. Ze zijn vaak diploïd, sexueel en vrij van endosymbionten en zijn daarom minder geschikt om de variatie in het voortplantingsproces te bestuderen. Om daadwerkelijk aan te tonen dat genetisch conflict een rol speelt in de evolutie van nieuwe voortplantingssystemen, is het belangrijk om te kijken naar organismen die wel veel variatie hebben in de manier waarop ze reproduceren. Hiervan is sprake bij een groep

insecten, de schildluizen. Hoewel deze groep de laatste jaren door evolutiebiologen nauwelijks is opgemerkt, kenmerken schildluizen zich door enorme variatie in merkwaardige voortplantingsmechanismen (zie tabel 1). Daarom heb ik hen gekozen als object voor mijn onderzoek.

LUIZEN MET RARE SEKS

Er zijn ongeveer 8000 soorten schildluizen beschreven, en al deze soorten leven van sap dat ze uit planten zuigen. Schildluizen zijn klein en meestal onopvallend, maar ze kunnen grote schade toebrengen aan de planten waarop ze leven. Dit verklaart waarom het meeste onderzoek naar schildluizen de laatste jaren vooral gericht was op hun bestrijding. Zoals eerder gezegd variëren schildluizen enorm in de manier waarop zij zich voortplanten. Veel schildluizen planten zich seksueel voort, maar er zijn er ook die zichzelf kunnen kloneren. Weer anderen planten zich wel seksueel voort, maar alleen hun dochters ontwikkelen zich vanuit bevruchte eieren terwijl hun zonen zich uit onbevruchte eieren ontwikkelen en dus aseksueel (klonaal) worden gevormd (haplodiploidie: tabel 1). In weer andere schildluizensoorten lijkt de voortplanting op het eerste gezicht heel normaal. Vrouwtjes kunnen alleen eieren leggen als ze bevrucht zijn en beide seksen ontwikkelen zich uit bevruchte eieren. Pas als je beter kijkt zie je dat mannetjes de genen die ze van hun vader krijgen niet doorgeven aan hun nageslacht. Dit voortplantingsproces wordt paternale genoom eliminatie (PGE) genoemd, omdat de genen afkomstig van de vader – alhoewel ze wel aanwezig zijn in hun zonen – worden geëlimineerd voordat ze in hun zaadcellen terecht kunnen komen (tabel 1).

Table 1 De belangrijkste voortplantingssystemen in schildluizen

Voortplantingssystemen	Beschrijving
Seksuele voortplanting	Nakomelingen ontwikkelen zich vanuit bevruchte eieren en hebben zowel de genen van hun vader als hun moeder
Aseksuele voortplanting	Klonale voortplanting: nakomelingen ontwikkelen zich vanuit onbevruchte eieren
Haplodiploidie	Vrouwelijke nakomelingen ontwikkelen zich vanuit bevruchte eieren terwijl mannelijke nakomelingen zich uit onbevruchte eieren ontwikkelen
Paternale genoom eliminatie	Zowel vrouwelijke als mannelijke nakomelingen ontwikkelen zich vanuit bevruchte eieren, maar in mannetjes wordt de helft van hun genen (degene die ze van hun vader erfden) niet gebruikt en ook niet doorgegeven aan de volgende generatie
Hermafroditisme (tweeslachtigheid)	Individuen kunnen zowel zaadcellen als eicellen produceren en daardoor zichzelf bevruchten en nakomelingen produceren zonder met een ander individu te paren.

RESULTATEN

Tijdens mijn promotieonderzoek heb ik geprobeerd om de evolutie van een aantal van deze voortplantingssystemen te verklaren en om te begrijpen waarom juist schildluizen zoveel “rare” seks vertonen. Hiervoor heb ik verschillende onderzoeksmethodes toegepast. Ten eerste heb ik veel oudere bevindingen uit de literatuur ge(her)ïnterpreteerd. Veel interessante aspecten van de biologie van schildluizen zijn in het begin van de vorige eeuw beschreven, maar de evolutietheorie die nodig was om deze bevindingen te begrijpen was toen nog niet ontwikkeld (zie vooral hoofdstuk 2 en 3). Daarnaast heb ik wiskundige modellen gebruikt om te onderzoeken of nieuwe voortplantingssystemen inderdaad door genetisch conflict kunnen ontstaan (hoofdstuk 4 en 5). Tenslotte heb ik experimenten met schildluizen in het laboratorium uitgevoerd (hoofdstuk 7-10). Hieronder beschrijf ik mijn belangrijkste resultaten.

ENDOSYMBIONTEN EN DE VOORTPLANTING VAN HUN GASTHEREN EN -VROUWEN

Eén van de redenen waarom juist schildluizen zo’n grote variatie in voortplantingssystemen vertonen is wellicht het feit dat de meeste schildluizen bacteriën bevatten die binnenin hun cellen leven en dat die wel eens zouden kunnen proberen om de reproductie van hun gastheer te beïnvloeden. Om dit idee te testen hebben we een analyse uitgevoerd gebaseerd op reeds gepubliceerde gegevens over de aanwezigheid en het type bacteriën en het voortplanting systeem van verschillende soorten schildluizen (hoofdstuk 6). In deze analyse is ook rekening gehouden met de verwantschap tussen de soorten die zijn gebruikt, aangezien nauw verwante soorten een grotere kans hebben om dezelfde eigenschappen te delen. Daardoor zijn die gegevens niet als onafhankelijke data te beschouwen. In onze analyse vonden wij dat inderdaad schildluizen met endosymbionten vaker rare voortplantingsmechanismen (vooral haplodiploidie en PGE, zie tabel 1) hadden dan de schildluizen zonder deze microörganismen. Dit is een belangrijk resultaat aangezien het niet alleen helpt om de variatie van reproductieve systemen te verklaren maar ook laat zien hoeveel invloed endosymbionten kunnen hebben op de biologie van hun gastheer.

ZELFMOORDGENEN

Naast het bestaan van een conflict tussen symbiont en gastheer over de reproductie, kunnen ook de vader en moeder het soms oneens zijn over hun nakomelingschap. In sommige soorten kan zelfs binnen individuen een conflict ontstaan tussen de genen die van de vader en die van de moeder afkomstig zijn: mannetjes met PGE hebben hun vaders genen, maar geven ze niet door aan hun nageslacht omdat de zaadcellen die die genen bevatten niet tot ontwikkeling komen. Je zou daarom verwachten dat

er selectie is op paternale genen (de genen die afkomstig zijn van de vader) om dit proberen tegen te gaan met het doel om toch te worden doorgegeven. Maar zelfs als dat niet lukt hebben de paternale genen in mannetjes nog een andere optie om hun aandeel in de populatie te vergroten: exacte kopieën van de paternale genen komen namelijk ook voor in hun zusters en die kunnen de genen wel doorgeven. Dus, paternale genen in een mannetje die de fitness van hun zusters kunnen verhogen, verhogen daarmee ook hun fitness en zullen in frequentie toenemen. Maar hoe kunnen paternale genen in mannetjes hun zusters helpen? Dat lijkt lastig maar er zijn wel oplossingen denkbaar. Stel dat broers en zussen onderling concurreren om bijvoorbeeld voedsel, dan kan de dood van een broer positief uitpakken voor de zusters. Met behulp van een theoretisch model laten we zien dat paternale zelfmoordgenen die dat bewerkstelligen zich inderdaad op deze manier door een populatie kunnen verspreiden (hoofdstuk 4). We kijken ook hoe de genen afkomstig van de moeder dit proces zouden kunnen tegengaan door de expressie van paternale genen te onderdrukken. Dit zou een verklaring kunnen zijn waarom paternale genen in mannetjes met PGE aanwezig zijn in iedere cel, maar niet tot expressie komen (of te wel, de genen zijn aanwezig maar worden niet gebruikt).

BESMETTELIJK SPERMA

In bepaalde soorten kan ook conflict ontstaan tussen beide ouders over het relatieve aantal zonen en dochters in hun nageslacht. In soorten met PGE en in soorten waarbij mannetjes zich uit onbevruchte eieren ontwikkelen (haplodiplodie), dragen vaders niets bij aan het erfelijk materiaal van hun zonen en hebben daarom alleen evolutionair voordeel bij de productie van dochters, terwijl moeders juist evolutionair voordeel hebben bij de productie van zowel dochters als zonen. In soorten waarbij zonen zich ontwikkelen vanuit onbevruchte eieren kan een vader het geslacht van zijn kinderen beïnvloeden door te zorgen dat zijn partner vaker haar eieren bevrucht. Hij kan het echter ook anders aanpakken: sommige schildluizen zijn hermafrodiet, oftewel ze produceren zowel eicellen als zaadcellen. Nu blijkt dat een mannetje niet alleen zijn partner's eieren bevrucht maar ook extra zaadcellen aan haar overdraagt die uitgroeien tot weefsel dat weer nieuwe zaadcellen kan produceren in het nageslacht van de partner. Dus een individu kan testis weefsel overdragen aan zijn nakomelingen en op die manier kan deze via zijn mannelijke functie niet alleen zijn partner bevruchten, maar tegelijkertijd ook de eicellen van de opvolgende generaties. In een wiskundige model toon ik aan dat dit rare voortplantingssysteem inderdaad kan zijn geëvolueerd als gevolg van een conflict tussen de ouders over de bevruchting van de eieren (hoofdstuk 5). Ik laat tevens zien dat in dit systeem ook de symbiotische bacteriën weer een rol zouden kunnen spelen. Immers, het ontstaan van hermafrodieten levert ook evolutionair voordeel voor hen op doordat net als bij aseksuele voortplanting, mannetjes, die de bacteriën toch niet kunnen doorgeven, niet meer nodig zijn.

HOEVEEL ZONEN, HOEVEEL DOCHTERS?

Behalve theoretisch onderzoek en literatuurstudie heb ik tijdens mijn promotie project ook experimenteel onderzoek verricht. Voor deze experimenten heb ik gebruik gemaakt van de citruswolluis *Planococcus citri*. De citruswolluis leeft normaal gesproken op citrusvruchten/planten maar kan een groot aantal andere planten besmetten. Er is zelfs een aanzienlijke kans dat je hem kunt vinden op één van je kamerplanten. Wolluizen zijn klein en onooglijk. Volwassen vrouwtjes zijn zelfs nauwelijks herkenbaar als insecten, omdat ze haast geen poten of voelsprieten hebben (figuur 1). Maar hoewel ze er op het eerste gezicht misschien saai uitzien hebben ze een heel bijzondere biologie: net als andere wolluizen hebben ze PGE en als gevolg daarvan dragen mannetjes wel hun vader's genen maar geven ze deze niet door aan hun nageslacht. Net als de meeste schildluizen hebben citruswolluizen in hun cellen bacteriën die in hun dieet ontbrekende voedingstoffen voor ze produceren. De citruswolluis heeft zelfs twee verschillende soorten bacteriën, waarvan de ene bacterie binnenin de andere bacterie leeft! Dit is tot nog toe het enige bekende geval waarbij twee bacteriën op die manier samenleven. Net als andere endosymbionten worden ze alleen via de moeder doorgegeven. Aangezien ook paternale genen alleen via vrouwtjes worden doorgegeven betekent dit dat zowel vaders als endosymbionten er baat bij hebben dat hun partner en gastvrouw zo veel mogelijk dochters produceert. Maar wat is nu eigenlijk de ideale geslachtsverhouding voor de gastvrouw zelf en hoe wordt het geslacht van haar kinderen bepaald? Om deze vragen te beantwoorden, heb ik een aantal verschillende experimenten uitgevoerd. Om te begrijpen hoeveel zonen en dochters een moeder in het optimale geval zou moeten produceren is het belangrijk om eerst meer te weten te komen over de levenswijze van mannetjes en vrouwtjes. Onder de meeste omstandigheden is een gelijk aantal zonen en dochters de optimale "strategie" voor de moeder. Als er op een bepaald moment meer mannetjes dan vrouwtjes zijn, blijven sommige mannetjes ongepaard en hebben ouders er voordeel bij om meer dochters te produceren. Als er daarentegen meer vrouwtjes dan mannetjes zijn kan iedere zoon met meerdere vrouwtjes paren en daardoor meer kleinkinderen opleveren dan een dochter. Dit gaat echter niet op als onder bepaalde omstandigheden de fitness van mannetjes en vrouwentjes verschilt om een andere reden dan hun relatieve frequenties. Wolluis-mannetjes en -vrouwtjes verschillen enorm in lichaamsgrootte en uiterlijk, het is zelfs moeilijk te geloven dat ze tot dezelfde soort behoren (figuur 1). Het is daarom heel voorstelbaar dat omgevingsfactoren een verschillende invloed hebben op de fitness van mannetjes en vrouwtjes. Een factor waarvan we verwachten dat deze een dergelijk effect heeft is de populatiedichtheid. Een andere eigenschap waarin mannetjes en vrouwtjes namelijk verschillen is het feit dat mannetjes maar een beperkte tijd voeding nodig hebben om te overleven terwijl vrouwtjes gedurende hun hele leven moeten eten. We verwachten daarom dat mannetjes minder last hebben van competitie met soortgenoten, enerzijds omdat ze minder voeding nodig hebben, anderzijds omdat volwassen mannetjes – in tegenstelling tot vrouwtjes – kunnen vliegen en zo een rustiger plekje kunnen

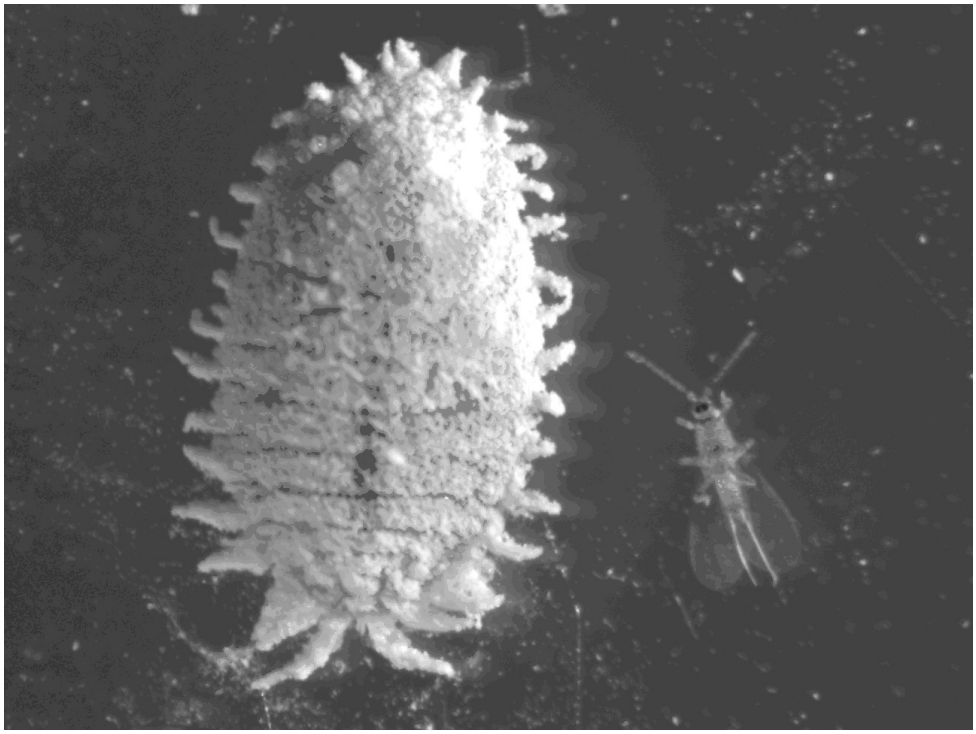

Figure 41 Citruswolluis, links een volwassen vrouwtje en rechts een volwassen mannetje

uitzoeken. We verwachtten daarom dat moeders onder hoge dichtheid meer zonen zullen produceren. Dit is inderdaad wat ik vind (hoofdstuk 7) en dit betekent ook dat vrouwtjes de mogelijkheid bezitten om het aantal zonen en dochters aan te passen al naar gelang de omstandigheden. Ik vind ook dat de leeftijd van moeders de geslachtsverhouding van haar nakomelingen beïnvloedt. Vrouwtjes die pas op late leeftijd door een mannetje bevrucht werden produceerden vooral dochters (hoofdstuk 10). Dit resultaat is waarschijnlijk op dezelfde wijze te verklaren als het effect van de populatiedichtheid. Wanneer een vrouwtje pas laat in haar leven paart komt dit waarschijnlijk omdat er maar weinig soortgenoten aanwezig zijn en het onder die omstandigheden beter is om meer dochters te produceren. Een andere factor die de geslachtverhouding beïnvloedt is gebrek aan voedsel. Vrouwtjes die weinig te eten hebben produceren vooral zonen (hoofdstuk 10). Dit resultaat zou kunnen worden verklaard doordat vrouwelijke nakomelingen meer voedingstoffen nodig hebben en het produceren van dochters dus risicovoller is. Tenslotte heb ik bestudeerd hoe vrouwtjes de geslachtsverhouding van hun nakomelingen variëren tijdens de periode waarin zij eieren leggen. Ik laat zien dat vrouwtjes de eerste paar dagen vooral zonen produceren, daarna vooral dochters en tegen het einde van hun leven weer vooral zonen. Ik vind ditzelfde patroon in vrouwtjes afkomstig uit ver-

schillende landen. Alhoewel wel we dit mechanisme nog niet helemaal begrijpen zou het mogelijk zijn dat een moeder eerst zonen produceert zodat deze met hun zusters kunnen paren voordat onverwante mannetjes hen voor zijn.

CONCLUSIES

Ik heb tijdens mijn promotieonderzoek verschillende technieken gebruikt om te onderzoeken hoe verschillende soorten conflicten over de voortplanting – tussen symbionten en gastheer, tussen mannetjes en vrouwtjes en tussen verschillende genen in eenzelfde individu – kunnen leiden tot de evolutie van nieuwe voortplantingssystemen. In de verschillende hoofdstukken van mijn proefschrift toon ik aan dat het ontstaan van nieuwe voortplantingssystemen in schildluizen inderdaad het gevolg kan zijn van dit soort conflicten. Mijn werk heeft nieuwe theorieën opgeleverd en met de enorme ontwikkelingen in genetische technieken kunnen veel daarvan hopelijk de komende jaren worden onderzocht. Verder heb ik aangetoond dat schildluizen de unieke mogelijkheid geven om theorieën over de rol van genetisch conflict te bestuderen. Ik hoop dat ik daarmee andere evolutiebiologen heb overtuigd om ook aan deze –onooglijke, vieze, maar unieke-- dieren te werken.

Hoewel ik me in dit proefschrift vooral gericht heb op de evolutie van voortplantingssystemen in schildluizen zijn mijn resultaten van bredere toepassing. Veel van de voortplantingssystemen die aanwezig zijn in schildluizen komen ook in andere organismen voor en dus is de kennis over de mechanismen en evolutionaire processen die ten grondslag liggen aan deze systemen ook voor die andere organismen van belang. Kortom, opheldering van de rol van genetisch conflict in schildluizen kan ons veel leren over het belang van genetisch conflict in de evolutie in het algemeen.


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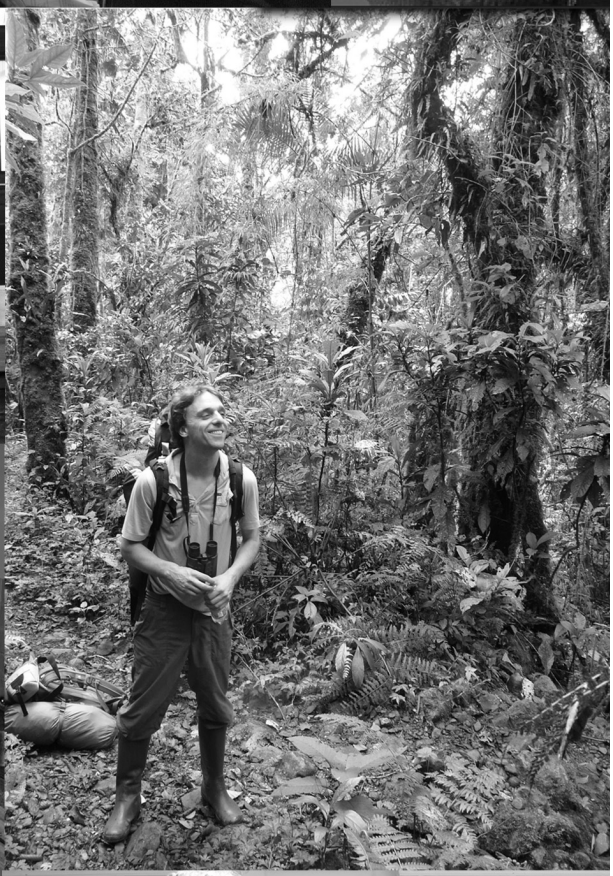

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