Evolution and Ecology
of Sex Allocation

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Submitted for the Degree of Doctor of Philosophy
University of Edinburgh 2003
ABSTRACT

In sexually reproducing organisms, the allocation of resources to male and female reproduction can have direct and considerable effects on an organism’s fitness. Consequently, females are expected to allocate their resources to the production of sons and daughters (sex allocation) in such a way as to maximise their fitness. The field of sex allocation consists of a large body of theoretical and empirical research. This has resulted in sex ratio evolution becoming one of the most well understood areas of evolutionary biology, providing some of the clearest evidence to support evolution by natural selection. In addition, the success of sex allocation theory and the ease of collecting data (it is often fairly easy to count and sex offspring), has allowed evolutionary biologists to use it as a tool to answer more general questions. This approach can be extrapolated to answer a number of questions in any sexually reproducing organism, as the same general principles underlie sex allocation in dioecious and hermaphroditic organisms throughout animal, plant and protozoan taxa. I have investigated these principles in parasitoid wasps, sea turtles and malaria parasites. Experiments to test whether females of the gregarious parasitoid wasp, *Nasonia vitripennis*, adjust their offspring sex ratio in response to whether they mate with a sibling or a non-relative reveals that they are unable to discriminate kin. Field studies provide the first set of comprehensive data concerning the field sex ratios of 2 species of sea turtle (*Chelonia mydas* and *Caretta caretta*) nesting in the Mediterranean and an additional assessment of using indirect methods to measure sex ratios in the field. These observations are extended to test whether the differential fitness theory of environmental sex determination applies to *Caretta caretta*. Lab experiments using the rodent malaria (*Plasmodium chabuadi*) investigate: (1) facultative sex allocation in malaria parasites where parasites respond to host anaemia, (2) methods to estimate the sex ratio of malaria parasites, (3) whether the assumptions of sex allocation theory are appropriate to malaria parasites. Theory developed shows that malaria parasites should alter their sex allocation in response to intrinsic and host factors that could impair fertilisation in the mosquito.
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DECLARATION

It was only possible to undertake a lot of my research through collaborations, and details of these are provided below.

CHAPTER 2
Experiment 1 was carried out with an honours student, Clarissa Batchelor. Experiments 2 and 3 were carried out in collaboration with Dr David Shuker, and 2 honours students; Amir Choudhary and Alison Duncan. Dr Ido Pen provided the mathematical theory for figure 1 and the appendix of the subsequent manuscript.

CHAPTERS 3 & 4
I collected all data as part of the long term Marine Turtle Research Group, a project run by Drs Brendan Godley and Annette Broderick. I analysed all data and produced subsequent manuscripts.

CHAPTERS 5 & 7
I carried out all experiments, analysed the data and produced subsequent manuscripts.

CHAPTER 6
I began the project by writing simulations in collaboration with Andy Gardner. Andy was then able to use the simulation to develop an analytical solution to our question. The manuscript was written using my biological knowledge and Andy’s maths.

I have also been involved in producing several other papers during my thesis, these appear as the following appendices:

ACKNOWLEDGEMENTS

Firstly, huge thanks to my supervisor, Stu West, for his un-ending encouragement, patience and his ability to explain complex issues in a simple way. If it wasn't for Andrew Read taking me under his wing too, I wouldn't have become interested in malaria parasites (for which, I think I am grateful!). Thanks also to Andrew for sensibly dealing the various crises that arose with regard to the malaria work on my behalf. Both supervisors provided endless moral boosts and the financial support for carrying out the work and attending various conferences.

Many thanks to everyone I collaborated with, for data collection, extremely useful discussions and writing papers: Stu West, Andrew Read, David Shuker, Andy Gardner, Ido Pen, Brendan Godley, Annette Broderick.

I have a huge debt of gratitude to a large number of people without whom collecting data would have been impossible. These hardworking and generous folk include: Aleta Graham and Ali Duncan who helped collect malaria and wasp data. John Tweedie and Sheena Booth for caring for my animals. All of the honours students I worked with in the lab (Clarissa Batchelor, Ali Duncan and Amir Choudhary) on the kin recognition project. The volunteers on the year 2000 Marine Turtle Conservation Project in Northern Cyprus who worked ‘24 hours a day’ to collect the sea turtle data. Thanks to Ewan Dennis and Andy Gardner for teaching me my rudimentary simulation skills.

Thanks also to various friends for all their support, patience and advice, especially Ewan Dennis, Tim Vines and Dave Shuker. Brian Chan, Lucy Crooks, Heather Ferguson, Meghan Gannon, Andrea Graham and Jaap de Roode provided me with encouragement and entertainment that kept me going in the lab. Finally, thanks to the NERC and Edinburgh University for giving me the studentship.
CHAPTER 1

Introduction

1.1 Summary

In sexually reproducing organisms, the allocation of resources to male and female reproduction has direct and considerable effects on an organism’s fitness. Consequently, females are expected to allocate their resources to the production of sons and daughters (sex allocation) in such a way as to maximise their fitness (Charnov, 1982). Following this premise, we can explain why an equal sex ratio is commonly observed, and also when and why unequal sex ratios should occur, in taxa ranging from protozoa to mammals. The field of sex allocation consists of a large body of theoretical and empirical research and the same general principles underlie sex allocation in dioecious and hermaphroditic organisms throughout animal, plant and protozoan taxa. This has resulted in sex ratio evolution becoming one of the most well understood areas of evolutionary biology, providing some of the clearest evidence to support evolution by natural selection (Charnov, 1982; Werren, 1987; Godfray and Werren, 1996; West et al., 2000a). In addition, the success of sex allocation theory and the ease of collecting data (it is often easy to count and sex offspring) has allowed evolutionary biologists to use it as a tool to answer more general questions (West et al., 2000a).

I have investigated how natural selection has shaped sex allocation in a parasitoid wasp (chapter 2), sea turtles (chapters 3 and 4) and malaria parasites (chapters 5-7). In chapter 2 I test whether females of the gregarious parasitoid wasp, Nasonia vitripennis, adjust their offspring sex ratio in response to whether they mate with a sibling or a non-relative. This is an example of how sex allocation theory can be used to answer general questions – in this case, whether Nasonia vitripennis can recognise kin. In chapter 3, I present the first set of comprehensive data concerning field sex ratios of 2 species of sea turtle (Chelonia mydas and Caretta caretta) nesting in the Mediterranean, and an evaluation of indirect methods used to
measure sex ratios in the field. In chapter 4, I test whether Charnov and Bull’s (1977) theory for environmental sex determination applies to Caretta caretta. In chapters 5-7, I examine sex allocation in malaria parasites. It has been proposed that sex allocation in these species may be facultative, with parasites responding to host factors that could impair fertilisation in the mosquito. In chapter 5, I consider how to estimate sex ratio in Plasmodium chabaudi, a rodent malaria parasite, and address the assumption that there is no differential mortality in male and female gametocytes (the sexual stages of malaria parasites). In chapter 6 I develop theory to investigate how selection is expected to shape sex allocation in malaria parasites in response to host factors that could impair fertilisation in the mosquito. In chapter 7, I test whether Plasmodium chabaudi shows facultative sex allocation in response to the host hormone erythropoetin, which is produced when hosts become anaemic, and could correlate with the start of reduced fertilisation efficiency (see Reece and Read, 2000 – A5). In the remainder of this chapter I shall describe the relevant aspects of sex allocation theory.

1.2 Fisher’s theory of equal investment

Ronald Fisher (1930) provided a clear explanation of why an equal number of male and female offspring are produced in most species, although, Fisher’s (1930) formulation was predated by Darwin’s (1871) verbal argument proposed in The Descent of Man. This was in turn mathematically formulated by Dusing in 1883 (Edwards, 2000). Imagine a population where sons and daughters are equally costly to produce and the sex ratio is female biased: sons will (on average) obtain more than 1 mate, so females gain a greater fitness return from their sons compared to their daughters. Therefore, mothers who invest more resources in sons (the rarer sex) will have a greater fitness and be favoured by natural selection. Conversely, if females are the rarer sex, females who bias their offspring sex ratio toward daughters are favoured by selection. Therefore, the fitness return from sons and daughters is only identical when resources are allocated equally to each sex, which is termed the unbeatable (Hamilton, 1967), or evolutionarily stable strategy (ESS; Maynard Smith, 1982). As a result of this frequency dependant selection, the ES sex ratio is a weakly stable equilibrium and thus cannot be invaded by a mutant that produces a different offspring sex ratio.
Fisher’s model predicts equal allocation of resources to the sexes during the period of parental investment (the primary sex ratio), therefore it is not affected if there is differential mortality between males and females after parental investment has ended (secondary sex ratio; Leigh, 1970; Wildish, 1976). When a son and a daughter cost the same amount in terms of parental resources, the ESS is an equal sex ratio, but if a different amount of parental resources are required to produce a member of each sex, then the ESS is to invest an equal amount in each sex – which may result in a biased numerical sex ratio (Trivers and Willard, 1973). This situation is theoretically identical but empirically more complex as the ‘currency’ of investment must be identified and progeny cannot simply be counted and sexed. One such complex scenario occurs in parasitoid wasps of the genus *Achrysocharoides*, where females lay single sex clutches. In this case, females should adopt a sex allocation strategy appropriate to the type of resource limitation that they face. Egg limited females are predicted to produce an equal number of male and female eggs and host limited females should lay an equal number of male and female clutches (Godfray, 1994; West *et al.*, 1999).

### 1.3 Biased sex ratios

Fisher’s principle illustrates the frequency dependent nature of selection on the sex ratio, and it also provides a null model (equal investment in the sexes) which is the foundation upon which most areas of sex allocation research are built. There has been relatively little empirical work on Fisher's principle itself (Basolo, 1994; Carvalho *et al.*, 1998; Blows *et al.*, 1999). Instead, the most productive research has investigated scenarios where the explicit and implicit assumptions of Fisher's principle are violated (Bull and Charnov, 1988; Frank, 1990). For example, when the fitness of each sex is influenced by a property of the environment, parental condition or status, the fitness returns from each sex are unequal or non-linear (Trivers and Willard, 1973; Bull, 1980; Charnov, 1982). In these cases, Fisher’s principle does not apply and the ES sex ratio can be biased at the individual brood or population level (Frank, 1987 and 1990). In the rest of this chapter I shall describe why skewed sex ratios can be the unbeatable sex allocation strategy and illustrate them with examples from animal and protozoan taxa.
Throughout this thesis (unless otherwise stated) when sex ratios are given, they refer to the proportion of males.

1.3.1 Local resource enhancement

Local resource enhancement (LRE) occurs when cooperative interactions between relatives occur. For example, if a daughter helps her parents rear their offspring, then her help goes some way to repaying the cost her parents incurred in rearing her. Here, the unbeatable strategy is to invest more in the sex that increases the resources available to the parents (Trivers and Willard, 1973). Most reports of LRE concern birds that help at the nest after fledging (Trivers & Willard, 1976; Gowaty and Lennartz, 1985; Lessels and Avery, 1987; Komduer et al., 1997; West and Sheldon 2002), but LRE is not restricted to birds. Female African wild dogs (Lycaon pictus) disperse from the pack after weaning but males stay to help and the average sex ratio observed in the field is 0.6 (Malcom and Marten, 1982). LRE through defence against predators occurs in allodopine bees (Exoneura nigrescens) – the more sisters that nest together in a burrow makes for a bigger burrow that has better defence (Schwarz, 1988). This leads to higher productivity per female and the observed mean field sex ratio of these bees is 0.2 (Schwarz, 1988). In addition, females are produced first, which could be a form of brood insurance – if the mother dies, she will have daughters mature enough to take care of the rest of her brood (Bull and Schwarz, 2001). Biased sex ratios are predicted at the individual brood level, but because different females should adopt different strategies depending on how many helpers they have, it is very hard to predict what the population sex ratio should be (Pen and Weissing, 2000).

1.3.2 Local resource competition

Local resource competition (LRC) occurs when an unequal sex ratio is favoured due to the detrimental effects of relatives competing for a limiting resource. Specifically, the fitness return (per unit of investment) from sons and daughters is unequal as a result of competition within one sex for a local resource. In this situation, parents who invest more in the sex that faces the least (or suffers the least) from this competition are favoured by natural selection (Hamilton, 1967; Taylor and Bulmer, 1980; Werren, 1980; Taylor, 1981; Frank, 1990). Competition for a variety of local resources has been documented, for example: Male African
bushbabies (genus *Galago*), disperse, but daughters do not disperse compete for food, thus the observed mean sex ratio is 0.7 (Clark, 1978). Habitats with high food availability contain the largest population densities of brush–tailed possums (*Trichosurus vulpecula*), and local dens are likely to be saturated. In this situation, females preferentially produce the sex that disperses (males), rather than daughters who would compete with their mother for den sites (Johnson et al., 2001). In many species, sons must compete to mate with their sisters, this leads to female biased sex ratios (Hamilton, 1967). This type of LRC is given special consideration below.

### 1.3.3 When LRE and LRC interact

The sex ratio predicted under LRE through helping can depend on the number of helpers present and the extent to which they increase the fitness of their parents – in some cases the sex ratio can be biased towards the non-helping sex. Sex allocation in the Seychelles warbler (*Acrocephalus sechellensis*), is an example of both LRE and LRC. When local territories are saturated, daughters remain on their parent’s territory to help rear their siblings, whereas sons disperse, so we expect a female bias. However, the number of helpers that can be supported by a territory depends on its quality. Good quality territories contain enough food to support helpers and 90% of offspring are female, but on poor quality territories 80% offspring are male because helpers increase the competition for food which negates the benefits of helping (Komdeur, 1998; Pen and Weissing, 2000).

### 1.4 Local mate competition

A special case of LRC is Local Mate Competition (LMC), which occurs in subdivided populations when sons compete to mate with their sisters. In this case the ES sex ratio is given by equation one (Hamilton, 1967):

\[
r = \frac{n-1}{2n},
\]

where \( n \) represents the number of foundresses whose offspring mate in the patch. When there is only one foundress, \( r = 0 \); which is interpreted as ‘a foundress should produce the minimum
number of sons required to fertilise all of her daughters’ (Hamilton, 1967). As \( n \) increases, the unbeatable sex ratio becomes less female biased because there are more opportunities for sons to outbreed. Several different, but equally valid conceptual tools can explain why LMC leads to female biased sex ratios (Frank, 1996 and 1998). Explanations based on individual selection suggest that a female biased sex ratio reduces competition between sons for mates and provides sons with more females to mate with (Taylor, 1981). An alternative explanation emphasises selection within and between groups: Producing a female biased sex ratio decreases fitness relative to other members of the same group (patch), but increases the overall productivity of the group (Hamilton, 1979; Taylor and Bulmer, 1980; Colwell, 1981; Frank, 1986 and 1998). LMC has been applied to explain why female biased sex ratios occur in a wide range of organisms including: parasitic wasps, fig wasps, protozoan parasites, beetles, spiders, barnacles, snakes, aphids and mites (Werren, 1980 and 1983; Charnov, 1982; Herre, 1985; Madsen and Shine, 1992; Wrensch and Ebbert, 1993; Shuter and Read, 1998, Nee et al., 2001). It appears that LMC is the most common form of LRC, but this may be an artefact due to the ease of identifying competition for females in such subdivided mating groups.

\subsection*{1.5 Inbreeding and the sex ratio}

\subsubsection*{1.5.1 Inbreeding in haplodiploids}

In haplodiploid organisms (such as the Hymenoptera), inbreeding also leads to selection for female biased sex ratios. When sex is determined by haplodiploidy, daughters are produced from fertilised eggs and sons from unfertilised eggs. When sibmating occurs, females are relatively more closely related to their diploid daughters than to their haploid sons, who have no paternal genetic contribution (Werren, 1980; Werren, 1983). For a haplodiploid female her unbeatable sex ratio is linked to the level of LMC by equation two and shown in Figure 1.1:

\[
 r = \frac{(n - 1)(2 - s)}{n(4 - s)},
\]

where \( s \) represents the probability of sibmating and \( n \) represents the number of foundresses in the patch (Hamilton, 1979; Frank, 1985; Herre, 1985; Werren, 1987). When \( s = 0 \), the optimal
sex ratio reduces to the situation given in equation one for diploids. LMC can lead to inbreeding and both processes favour female biased offspring sex ratios (Herre 1985; Greeff, 1996). The clearest empirical work that has teased apart the effects of inbreeding and LMC on the sex ratio is on fig wasps (Herre, 1985 and 1987). In fig wasps, the sex ratio becomes less female biased as number of foundresses increases and for a given number of foundresses as the probability of inbreeding decreases. It is unclear if the increased bias is a result of selection on the average strategy, or a facultative adjustment made in response to sibmating. This question is addressed in chapter 2.

![Figure 1.1: The unbeatable sex ratio where the probability of inbreeding ranges from 0 (always outbreed) to 1 (always sibmate), for haplodiploid females under various levels of LMC (foundress number).](image)

1.5.2 LMC and the inbreeding co-efficient

In other taxa (such as haploid malaria parasites), it can be difficult to estimate the number of females whose offspring mate in a patch. In this scenario, Wright’s inbreeding co-efficient ($f$) can be a more useful parameter to measure. When all females contribute an equal number of offspring to the mating group, then $f = 1/n$, where $n$ is the number of foundresses. Therefore, $f$ can be used as a measure of LMC and the unbeatable sex ratio is given by:

$$ r = \frac{1-f}{2} $$

for haploid or diploid organisms, and:

$$ r = \frac{(1-f)(2-f)}{4-f} $$

for haplodiploids.
This formulation is especially useful for haploid malaria parasites as the population level inbreeding coefficient correlates with the level of LMC and is a more informative parameter to measure than the number of genotypes in an infected host (Nee *et al.*, 2002). It should be noted that in haploids and diploids, inbreeding does not lead to a relatedness asymmetry between parents and offspring, so we do not observe a female bias in addition to that predicted by the level of LMC.

1.6 **Sex allocation in response to the environment**

Trivers and Williard (1973) described why it would be beneficial for female mammals to facultatively control the sex of their offspring and this rational has been used to explain a variety of patterns of parental and offspring control over sex allocation. This principle applies in all cases where sex is conditionally adjusted in response to an environmental parameter, and is best explained with an example (see also figure 1.2). In the parasitoid wasp *Spalangia cameroni*, daughters achieve higher fitness than sons from developing in a large host. Therefore, females achieve the highest fitness returns from laying daughters in relatively large hosts and sons in relatively small hosts (Charnov, 1981; Godfray, 1994). In birds, mammals and many insects when facultative sex allocation occurs, sex is determined by chromosomes but the females have a mechanism to bias their offspring sex ratio. In mammals and birds this mechanism is unknown, but there is evidence to suggest that birds have control over what chromosome each egg carries (Komdeur, 2002), and female brush tailed possums are biasing their offspring sex ratios before birth (Johnson and Ritchie, 2002).
Figure 1.2: Sex bias in response to a characteristic of the environment. The fitness of one sex (in this case males) does not vary according to the environment (patch type), but the fitness of the other sex (females) does correlate with patch type. In this case, the unbeatable strategy is produce the sex that will do best in each patch. The correlation between fitness and patch type can be linear or non-linear, positive or negative.

1.6.1 Parental characteristics

The Trivers and Willard (1973), hypothesis also explains why facultative sex allocation occurs in response to parental phenotypic characters. Some of the clearest examples of this come from birds, in which females should bias their investment towards sons when they mate with a high quality male. The reason being, if her mate’s attractive traits are heritable, they are very beneficial to her sons and may even be detrimental to her daughters (Burley, 1981, 1986; Moller and Ninni, 1998; West and Sheldon, 2002). Maternal rank can also have implications for sex allocation. If daughters inherit their social rank from their mothers, only high ranking females should specialise in producing daughters (Trivers and Willard, 1973). Recently, it has become unclear if female primates are responding to maternal rank, as previously thought - due to the probability of type I errors arising from small sample sizes (Brown and Silk, 2002).

Maternal condition is another parental characteristic that can have implications for sex allocation. In species where male reproductive success has a high variance and the most successful males tend to be the largest and most vigorous, only when females are in good enough condition to provide the level of resources required to produce a high quality male, should they invest in a son (Clutton-Brock and Iason, 1986). There is evidence to suggest that this applies to animals such as wild horses (*Equus caballus*; Kohlman, 1999), and elk (*Cervus*...
An alternative hypothesis suggests that females in good condition should invest in daughters, even if a son’s reproductive success could be higher. This is because the fitness of a son’s offspring depends on the female he mates with, but the fitness of her daughter’s offspring depends on her condition (Leimar, 1996). Individual females will differ in condition, rank and mate quality, so it is not possible to make general predictions about when or what sex biased investment should be observed (West and Sheldon, 2002). This means that competing hypotheses can only be tested on a case by case basis.

However, there are many instances of condition related sex ratio bias in the literature. For example, female blue footed boobies (Sula nebouxii) suffer a greater fitness loss than males when their mother is in poor condition and subsequently females bias their sex allocation according to their condition (Velando, 2002). Also, in the freshwater turtle (Malaclemys terrapin) females in good condition choose warmer nest sites (which result in female offspring), than when in poor condition – as (presumably) females benefit more than males from being larger through increased fecundity (Roosenberg, 1996). Recent evidence suggests that female kakapos (Strigops habroptilus) that are in good condition bias their investment towards sons. Unfortunately for conservationists, an excess of males does not facilitate population growth but ironically their supplementary feeding has improved the condition of many females (Clout et al., 2002).

### 1.7 Environmental sex determination

When sex is determined by an environmental characteristic (environmental sex determination; ESD), offspring sex is a trait acquired after conception and not as a result of direct parental control. An ESD system is favoured when an environmental parameter has a differential effect on male and female fitness (Charnov and Bull, 1977), in the same way as maternal condition in the previous section (Trivers and Willard, 1973; figure 1.2). Patch differences in the ratio of male/female fitness maintains selection for the different sexes to be produced in different ranges of the environment regardless of the absolute values of male and female fitness (Bull, 1980, 1982). With ESD, sex chromosomes do not seem to play a role in sex
determination so offspring of any genotype can develop into a male or into a female. However, we assume that there is an interaction between the environment and genes in determining phenotype, so it might be more useful to view ESD and genetic sex determination (GSD) at either end of a sex determination continuum (Bull, 1980). There is some evidence that sex could be heritable when determined by ESD (Rhen, 1998), and that GSD can be disrupted by hormones in the maternal environment (Clark and Galef, 1995).

Just as there are many scenarios in which parents will alter the sex ratio of their offspring there are several environmental cues that can determine sex. For example, sex is determined by photoperiod in *Gammarus duebeni* – sex ratio decreases with photoperiod length (Bullheim and Bull, 1967). The fitness of males is greater than for females at a given body size as large males can pair with more fecund females. Hatching early in the spring (cued by short photoperiod) allows a longer development to reach a larger size before mating (McCabe and Dunn, 1997). The sex ratio in the nematode *Paramermis contorta* decreases as their host environment becomes increasingly more nutritious or less crowded as females benefit from an increase in body size more than females (Caullery and Comas, 1928; Christie, 1929). In the marine worm, *Bonellia viridis*, larvae that settle on adult females differentiate into males whereas larvae that settle alone become females (Bacci, 1965). Females can exist as independent organisms, but males must live a parasitic existence on females.

1.7.1 Temperature sex determination

One of the most well known examples of ESD is temperature sex determination (TSD), which occurs throughout reptilian taxa (Charrier, 1966; Pieau, 1971; Bull, 1980) and in the Atlantic silverside fish, *Menidia menidia* (Conover and Kynard, 1981). Despite being well known, a clear understanding (and empirical support) of why temperature has different fitness consequences for male and female reptiles has remained elusive (Bull, 1980; Janzen, 1996; Shine, 1999). For example, in leopard geckos (*Eublepharis macularius*), males develop from the temperature range optimal for growth rate and females from temperatures above and below this range (Gutzke and Crews, 1988; Viets, 1993). Presumably, males gain greater fitness then females from large body size as they must compete for mates and defend territories – but this test has not been reported in the literature.
In sea turtle species, warm temperatures result in females and cool temperatures in males, but vice versa in alligators and some lizards. However, in crocodiles (*Alligator mississippiensis*) and snapping turtles (*Chelydra serpentina*) females are produced from both extremes of the thermal range and males from intermediate temperatures (Ferguson, 1982). A characteristic of all temperature–sex functions is a narrow transitional range, i.e. temperatures from which a mixed sex ratio is produced. For example, in sea turtles, an equal sex ratio is produced at 29°C, and single sex clutches from temperatures 2-3°C either side of this ‘pivotal’ temperature (Pieau and Dorizzi, 1981; Yntema and Mrosovsky, 1982). This often leads to extreme temporal skews in sex ratio and single sex clutches, and this reduces the rate at which the sex ratio can evolve (Bull, 1980). When embryos are incubated near the threshold temperature, the sex ratio is a product of embryonic differences in the temperature response, but in extreme temperatures all offspring become the same sex regardless of genotype. Attempts to quantify the amount of heritable variation in this response have produced mixed results. Janzen (1992), showed that the sex ratio in the common snapping turtle (*Chelydra serpentina*) has a low heritability, where as Rhen (1998), interpreted high among-family variation in sex ratio in the snapping turtle as potential for sex ratio evolution. However, Rhen’s (1998), study may have confounded the consequences of maternal effects with genetic (heritable) effects.

### 1.8 The precision of sex allocation

Until recently it was assumed that chromosomal sex determination was a constraint that would remove parental control over the sex ratio, and this assumption was supported by unbiased sex ratios observed in many vertebrates at the population level (Williams, 1979; Bull and Charnov, 1988). Evidence now suggests that facultative sex allocation is not just restricted to haplodiploid groups, such as the Hymenoptera, protozoa, mammals, birds, frogs, lizards, aphids, snakes and spiders are adjusting their offspring sex ratios too (Madsen and Shine, 1992; Johnson et al., 2001; West and Sheldon, 2002; Hardy, 2002; West *et al.*, 2002a – A1). In some taxa this adjustment is not apparent at the population level as some individuals are predicted to preferentially invest in daughters, and others in sons. Examining the precision
with which individuals adjust their brood sex ratios has increased the level at which we can explain sex ratio variation. For example, when sex ratio bias is observed in response to LRE in birds, it is consistent with theoretical prediction (West and Sheldon, 2002).

However, it is also clear that extreme sex ratio shifts occur in some species more than in others and understanding this is still a major challenge (see appendix 1A). There are several potential and non-exclusive explanations for this. First, if the environmental factor that causes selection to act on the sex ratio is for females hard to assess, then it may be too risky to undertake extreme biases in offspring sex ratio. However, this may depend on the type of environmental uncertainty – for example, whether the environmental cue varies between or within years (Pen et al., in prep). Second, in the Hymenoptera, the cost of fertilised and unfertilised eggs is assumed to be the same, but for species with chromosomal sex determination, we do not know the mechanism of sex bias. If there are costs involved, perhaps only certain conditions provide large enough benefits from sex bias to overcome the costs involved. Third, in fig wasp species that respond to LMC, females are better at producing the unbeatable sex ratio when ovipositing with the co-foundress number that their species encounters most frequently (Herre, 1987). Species in which females regularly encounter more variation in number of co-foundresses produce the unbeatable sex ratio in a wider range of LMC conditions, suggesting that more plasticity in their response to LMC has been selected for. Finally, species with complex life histories that involve many trade-offs or substantial dispersal obscure whether selection favours biased sex ratios and what the population sex ratio should be (Frank 1987, 1990; Pen and Weissing 2000; Cockburn et al., 2002; West and Sheldon 2002; West et al., 2002a – A1). Once more progress is made into understanding the mechanisms for sex ratio bias, it should be easier to test for taxonomic wide trends and address unexplained sex ratio variation. For example, how do haploid malaria parasites produce haploid gametes and why do observed sex ratios vary more than predicted by their inbreeding rate (this variation is addressed in chapters 5-7)?
1.9 Thesis aims

In this thesis, I have added to our understanding of the evolution and ecology of sex allocation in several taxa by addressing the following topics:

1. Using sex allocation theory to test for kin discrimination in a parasitoid wasp.
2. What are the field sex ratios of Mediterranean sex turtles and how should their sex ratios be estimated?
3. Does Charnov and Bull’s (1977) differential fitness hypothesis explain why the sex of sea turtles is determined by temperature?
4. How should sex ratios of malaria parasites be estimated, and how appropriate are these estimates for testing sex allocation theory?
5. How could selection have shaped sex ratios in malaria parasites when fertilisation efficiency is reduced?
6. Does host anaemia play a role in shaping sex ratios in malaria parasites?
CHAPTER 2

Kin discrimination and sex ratios in a parasitoid wasp

This chapter has been submitted to the Journal of Evolutionary Biology, as: S.E. Reece, D.M. Shuker, I. Pen, A.B. Duncan, A. Choudhary, C.M. Batchelor and S.A. West. Kin discrimination and sex ratios in a parasitoid wasp.

2.1 Summary

Sex ratio theory provides a clear and simple way to test if non-social haplodiploid wasps can discriminate between kin and non-kin. Specifically, if females can discriminate siblings from non-relatives, then they are expected to produce a higher proportion of daughters if they mate with a sibling. This prediction arises because in haplodiploids, inbreeding (sibmating) causes a mother to be relatively more related to her daughters than her sons. Here I formally model this prediction for when multiple females lay eggs in a patch, and test it with the parasitoid wasp Nasonia vitripennis. My results show that females do not adjust their sex ratio behaviour dependent upon whether they mate with a sibling or non-relative, in response to either direct genetic or a range of indirect environmental cues. This suggests that females of N. vitripennis cannot discriminate between kin and non-kin. The implications of my results for the understanding of sex ratio and social evolution are discussed.
2.2 Introduction

The evolution of biased sex ratios in spatially structured populations has proved to be one of the most productive areas of evolutionary ecology (Charnov, 1982; Godfray, 1994, West et al., 2000a). Hamilton (1967), was the first to show that when the offspring of one or a few mothers mate amongst themselves in their natal patch, before their daughters disperse, a female biased sex ratio is favoured by natural selection. A useful way of conceptualising this is that the female bias arises because it reduces competition among a female's sons for mates, and because it increases the number of mates for each of the sons (Frank, 1998; Taylor, 1981). Together these processes have been termed local mate competition (LMC; Hamilton, 1967), and can be formalised with the prediction that the unbeatable sex ratio (proportion of males) on a patch \( r = \frac{(N - 1)}{2N} \), where \( N \) is the number of foundress females that lay eggs on the patch. There is considerable evidence from a variety of organisms that this prediction can explain sex ratio variation across species/populations, and also that individuals facultatively adjust their offspring sex ratios in response to the number of females laying eggs per patch (e.g. wasps, ants, beetles, spiders, mites, malaria and related protozoan parasites, snakes and flowering plants; Charnov, 1982; Hardy, 2002).

In contrast, there is a lack of evidence for the importance of an additional factor that can explain sex ratio variation – inbreeding. In haplodiploids, the sex of an egg is determined by whether it is fertilised, with males and females developing from unfertilised (haploid) and fertilised (diploid) eggs respectively. A consequence of this is that inbreeding causes mothers to be relatively more related to their daughters than their sons, and so in haplodiploids, a more female biased sex ratio is favoured than in diploids (Frank, 1985; Herre, 1985). The combined effects of LMC and inbreeding can be formalised with the prediction \( r = \frac{(N - 1)(2 - p)}{N(4 - p)} \), where \( p \) is the proportion of individuals that are sibmated (Frank, 1985; Herre, 1985; Werren, 1987). The only evidence for the separate effects of LMC and inbreeding come from Herre’s work on fig wasps, where for a given number of foundresses \( N \), sex ratios produced by inbred species were more female biased (Herre, 1985, 1987; Herre et al., 2001). However, Greeff (1996), has shown theoretically that individuals can be selected to facultatively adjust their sex ratio in response to whether they mate with a sibling or a non
relative. Greeff’s (1996), model predicts split sex ratios, with sibmated (inbred) females producing a more female biased sex ratio then females who do not mate with sibs (outbred). The pattern found by Herre in fig wasps could therefore be explained either by females adjusting their sex ratio in response to the average level of inbreeding, or females facultatively adjusting their sex ratio in response to sibmating.

As well as explaining sex ratios, an understanding of whether individuals show facultative adjustment of the sex ratio in response to sibmating is important for three more general reasons. First, it provides a relatively easy way to examine if non-social wasps can discriminate between kin and non-kin (West and Herre, 2002; West et al., 2000a; Greeff, 1996). Our understanding of kin discrimination in non-social species (and hence its possible importance in the evolution of sociality), is extremely poor, especially when compared with work on social species (Fellowes, 1998). This is largely because the specialised behaviours associated with sociality, such as helping, offer relatively easy ways to test for kin discrimination (Clutton-Brock, 2002; Griffin and West, 2002; Bourke and Franks, 1995). Second, it can lead to split sex ratios, with some females producing more female biased offspring sex ratios than the population average, and some less female biased. This can facilitate the evolution of eusociality in haplodiploid species such as wasps, ants and bees (Grafen, 1986; Greeff, 1996; Seger, 1983). Third, it can help explain the controversial genetic variation that has been observed in the sex ratio behaviour of parasitic wasps (Hardy, 1992; Orzack et al., 1991), a point that I shall return to in the discussion.

Here, I present the first empirical test of whether individuals facultatively adjust their sex ratio as predicted by Greeff (1996), in response to whether they mate with a sibling. Greeff’s (1996), prediction was not developed for parasitoid and fig wasps where multiple females lay eggs per patch (see section 2.2.1). Consequently, my first aim is to develop theory that predicts how females should adjust their offspring sex ratios in response to sibmating, when $N$ females lay eggs per patch. The model is easily tested in a variety of haplodiploid organisms. For facultative sex ratio adjustment in response to sibmating to evolve, individuals would have to be able to discriminate between siblings and non-relatives. Such kin discrimination can occur via direct genetic cues, or via indirect environmental cues. For example, Ode et al.
(1995) have shown that the parasitic wasp *Bracon hebetor* uses an indirect cue to assess relatedness – females avoid inbreeding by preferring to mate with males that developed in a different host, with host odour rather then genetic relatedness providing the cue for kin discrimination. I carry out 3 experiments on the parasitoid wasp *Nasonia vitripennis* to determine if individuals adjust their sex ratio in response to whether they mate with a sibling. I examine behaviour in response to both direct genetic cues as well as 3 indirect environmental cues: (i) host developed in; (ii) time between emergence and mating; (iii) sex ratio upon emergence.

### 2.2.1 Sibmating and sex ratios

Greeff (1996) has modelled sex ratio behaviour for a situation in which a proportion of an individual’s offspring sibmate, and the rest mate with non relatives. This model shows that females are predicted to adjust their sex ratio depending on whether they mate with a sibling or non-relative. Here, I develop theory that allows the level of LMC (*N*) and sib-mating (*p*) to vary independently, and therefore, is more suited to organisms with which this theory can be tested, such as parasitoid or fig wasps. The predictions of my model will differ quantitatively from Greeff’s (1996) model because the Evolutionary Stable sex ratio (Maynard Smith, 1982) for a given female will depend not only upon her own mating status (mated with sibling or non-relative), but also upon the mating status of other females on the patch.

Following the basic life cycle of Hamilton’s (1967) original formulation of LMC, I assume that: (i) mated females form groups of variable size (*N*) in discrete patches where they lay their eggs; (ii) sons and daughters mate at random in their natal patch, after which the newly mated females disperse; (iii) the mating structure, distribution of *N*, leads to an average probability of sib-mating *p*. I wish to predict how the sex ratio behaviour of a female should depend upon whether she has mated with a sibling or non-relative, for given values of *N* and *p*. I label the ESS sex ratio for a sib-mated female as *s*₁*, and for a female who has mated with a non-relative as *s*₀*. In the appendix I derive the following results. If *N* < (5 − 2*𝑝*)(1 − *p*), then

\[ s_0^* = \frac{(N - 1)(2 - p)}{N(4 - p)^2}(Np - 2p + 4) \]

and
\[ s_1^* = \frac{(N-1)(2-p)}{N(4-p)} (N - Np - 2p + 5). \quad (2.1) \]

For \( N > (5 - 2p)(1-p) \) I get \( s_1^* = 0 \), and

\[ s_0^* = \frac{1}{2} \frac{N(1-p) - 1}{N(1-p)^2}. \quad (2.2) \]

Figure 2.1: Inbreeding and facultative sex ratio adjustment. The predicted ES sex ratios for females who have mated with a sibling (unbroken line), or a non relative (dotted line), is plotted against the number of foundresses (\( N \)). The probability of sibmating (\( P \)) is assumed to be \( 1/N \).

2.3 Materials and Methods

2.3.1 Study organism

\textit{Nasonia vitripennis} (Hymenoptera: Chalcidoidea) is a gregarious parasitoid wasp that parasitises a range of dipteran pupae including \textit{Calliphora} and \textit{Sarcophaga} species. Female wasps lay clutches of 20-40 eggs in each host and avoid ovipositing in previously parasitised hosts (superparasitism). Females mate once and then disperse to find oviposition sites. Sex allocation in \textit{Nasonia} is well understood, with females responding facultatively to LMC cues
(Werren, 1980; Werren, 1983; Werren, 1984; Orzack and Parker, 1990; Orzack et al., 1991; King and Skinner, 1991; King, 1993a; Orzack and Gladstone, 1994; Molbo and Parker, 1996; Flanagan et al., 1998). I cultured wasp lines in 16hr light/8hr dark cycles at 25°C, in which male offspring emerge after 14 days and mate with females as they emerge the following day. In my 3 experiments I used 6 recently isolated field lines; 1) R6 from Rochester, USA, 2000, 2) B5 from Elspeet, Netherlands, 2001, and 3) HV287, 4) HV395, 5) HV55 from Hoge Veluwe, Netherlands, 2001, and 6) LabII, an inbred line from Leiden, founded circa 1970. In addition, I used a red-eyed mutant strain (STDR) to allow us to examine the behaviour of individuals when ovipositing in groups. I screened each field line for the absence of sex ratio distorters prior to experiments. Experiment 1 was carried out in March 2000 and experiments 2 and 3 in March 2002. I used a relatively large host species for my experiments (Calliphora vicina and C. vomitoria), to minimise any effect of differential mortality (Werren, 1983).

### 2.3.2 Experiment 1: Sibmating, host cues and mating delay

In this experiment I simultaneously manipulated whether a female was mating with a sibling or non-relative and two indirect cues that may indicate sibmating: (i) host developed in – individuals from the same host are more likely to be siblings than individuals from different hosts, so mating with an individual from the same host may indicate sibmating (Ode et al., 1995); (ii) delay between emergence and mating – males wait for females to emerge on the host they developed in, so females mating immediately upon emergence are more likely to be sibmating than females mating after a delay. This experiment consisted of 2 treatments, each replicated with two different wasp lines, R6 and LabII. In A, the sib mating treatment, females were mated with brothers that had developed in the same host, and were allowed to mate immediately upon emergence. In B, the non-sib mating treatment, females mated with a male from the other line who had developed in a different host species, and mating was delayed until 48 hours after emergence.

For each line I set up 300 singly mated females in individual oviposition patches (tubes containing 3 hosts). Offspring from each female were used for one mating group replicate only, with one female from each replicate providing sex ratio data, to avoid pseudoreplication (Hurlbert, 1984). I prepared the mating group treatments by removing wasps at the late pupal
stage from hosts, approximately two days prior to emergence. To set up the sib mating treatment a single host was placed in a tube to allow the offspring to emerge and mate. To set up the non-sib mating treatment I placed 5 sisters in a tube and added 5 unrelated males (from the other line) 48 hours after the sisters emerged.

I allowed wasps to mate for 48 hours in their mating group treatments, by which time all females were mated. One female per mating group replicate was randomly chosen and “pre-treated” individually. This process allows females to host feed and mature eggs and had 2 stages: (a) placing females in individual tubes with a single host for 24 hours; (b) replacing the host with honey solution for a further 24 hours. After pre-treatment, each female, together with a red eye mutant marker female (also pre-treated), was put into a test tube with 8 hosts (hereafter termed the patch) that had a one-way escape tube to allow females to disperse after oviposition and prevent superparasitism (Werren, 1980; Werren, 1983; Werren, 1984; Godfray, 1994). I removed any females remaining in the patch after 48 hours and incubated all clutches at 25°C. I sexed the offspring of experimental females and also recorded the number of marker female offspring post emergence to control for any influence of relative fecundity on offspring sex ratios (Flanagan et al., 1998).

2.3.3 Experiment 2: Sibmating and host cues

In this experiment I separately manipulated whether a female was mating with a sibling or non-relative and an environmental cue, the host developed in. First, I set up mated females to produce full sib families as detailed for experiment 1, using lines B5, HV287, HV 395 and HV 55. This experiment consisted of 3 mating group treatments; A) 8 sisters and 2 brothers which developed in different hosts, B) 8 sisters and 2 males from the other line, C) 8 sisters and 2 brothers who developed in the same host. The sex ratio of 8:2 was chosen to resemble that found in the field (Molbo and Parker, 1996). As in experiment 1, each family provided wasps for 1 mating group replicate in 1 treatment. I allowed wasps to mate for 48 hours from emergence. Subsequent pre-treatment, and collection of sex ratio data were carried out as detailed for experiment 1.
2.3.4 Experiment 3: Sibmating and emergence sex ratio

In this experiment I separately manipulated whether a female was mating with a sibling or non-relative and an environmental cue that may indirectly suggest sibmating, the sex ratio upon emergence. If a female emerges into a highly female biased mating group it may indicate that her group was founded by 1 or a few females, thus sibmating is likely. Where as a mating group with an equal sex ratio suggests multiple foundresses and a higher probability of mating with a non-relative. This experiment was carried out using lines B5 and HV287, setting up mated females as previously described. I utilised two treatments (mating with a sib or non-relative from the other line), each with two levels (female biased or equal sex ratio), giving four groups: (A) Sibmate and female bias (8 sisters and 2 brothers); (B) Sibmate and equal sex ratio (5 sisters and 5 brothers); (C) Non-sib mate and female bias (8 sisters 2 unrelated males from the other line); (D) Non-sib mate and equal sex ratio (5 sisters and 5 unrelated males from the other line). I allowed wasps to mate for 48 hours from emergence. Subsequent pre-treatment, and collection of sex ratio data were carried out as detailed for experiment 1.

2.3.5 Analysis

I discarded clutches produced by unmated females (all male offspring) from the analysis. Sex ratio data usually have non-normally distributed error variance and unequal sample sizes. This can be accounted for by assuming binomial errors and a logit link function in a general linear model analysis of deviance (whilst retaining maximum statistical power; (Crawley, 1993). Using S-Plus 6 (Insightful Corporation), a full model was fitted, including interactions, and terms deleted in a stepwise fashion (Crawley, 2002). Significance was assessed by examining the change in deviance following removal of each term from the minimal model. After fitting the full model I compared the residual deviance and residual degrees of freedom. Relatively large values of residual deviance indicate overdispersion and potential overestimation of the significance level. To account for this the residual deviance is rescaled by the Heterogeneity Factor (HF; ratio of residual deviance to degrees of freedom), and consequently, an F test was used to test whether the removal of a term caused a significant increase in deviance.
2.4 Results

2.4.1 Experiment 1: Sibmating, host cues and mating delay
There was no significant effect of treatment ($F_{1,194} = 0.78, P = 0.38, HF = 3.86$) or the clutch sizes of both the marker females ($F_{1,195} = 1.33, P = 0.25$) and experimental females ($F_{1,196} = 1.69, P = 0.20$) on sex ratio. Line R6 had a significantly higher sex ratio than LabII ($F_{1,197} = 128.23, P < 0.0001$; see figure 2.2). In addition, the sex ratio of the ‘family’ each female came from did not influence offspring sex ratio ($F_{1,193} = 0.40, P = 0.53$), consequently this data was not collected in subsequent experiments.

![Figure 2.2: The mean sex ratio for lines R6 (unshaded), and LabII (shaded) for each treatment in experiment 1. Treatments are represented by A: sibmating and B: non sib mating. Bars are 95% confidence intervals.](image)

2.4.2 Experiment 2: Sibmating and host cues
Treatment did not have a significant effect on sex ratio ($F_{2,593} = 1.37, P = 0.25, HF = 4.52$; figure 2.3). There was a significant effect of line on sex ratio ($F_{3,595} = 8.76, P < 0.001$; means: HV395 = 0.35; HV55 = 0.31; HV287 = 0.30 and B5 = 0.25; figure 3), a weak positive effect of marker female clutch size ($F_{1,595} = 6.68, P = 0.01$) and no significant effect of experimental female clutch size ($F_{1,592} = 0.18, P = 0.67$).
Figure 2.3: The mean sex ratio for each treatment in experiment 2, for all lines. For each treatment lines are B5, HV287, HV395 and HV55 from left to right. Treatments consist of A: siblings developing in different hosts, B: non siblings and C: siblings developing in the same host and removed prior to emergence. Bars are 95% confidence intervals.

2.4.3 Experiment 3: Sibmating and emergence sex ratio

Marker female clutch size had a significant positive effect on offspring sex ratio ($F_{1,345} = 8.06$, $P = 0.005$, $HF = 2.83$). Line and experimental female clutch size did not have significant effects on sex ratio ($F_{1,341} = 0.04$, $P = 0.84$ and $F_{1,342} = 0.21$, $P = 0.65$ respectively). Neither mating group sex ratio or mate relatedness had a significant effect on sex ratio ($F_{1,343} = 0.26$, $P = 0.61$ and $F_{1,344} = 1.06$, $P = 0.30$; see figure 2.4).

Figure 2.4: The mean sex ratio for each treatment in experiment 3, as there was no significant difference between the sex ratios produced by each line, their data has been amalgamated. A: sibmate and female bias, B: sibmate and equal sex ratio, C: non sibmate and female bias and D: non sibmate and equality. Bars are 95% confidence intervals.
2.4.4 Power analyses

For each experiment I performed a power analysis to explore how big a difference in sex ratio I could detect between treatments (using S-Plus 6; Insightful Corporation). From the theory outlined in section 2, the predicted difference in sex ratio allocation between sibmated and outbreeding females, in a two foundress patch, is 0.061 (i.e. a 6.1% difference in % male). For all three experiments, the power to detect a significant difference in sex ratio between treatments of this magnitude was > 0.99, with \( \alpha \) set at 0.05. The minimum significant difference I could detect between treatments in each experiment was 0.025 or less (with \( \alpha = 0.05 \), and power = 0.8).

2.5 Discussion

I have shown that when multiple females lay eggs on a patch, females are expected to adjust their offspring sex ratio depending upon whether they mate with a sibling or non-relative, producing a more female biased sex ratio when mating with a sibling (Figure 2.1; section 2.2.1; extending Greeff, 1996). However, in contrast to this prediction, females of the parasitoid \( N. vitripennis \) did not adjust their sex ratio depending upon: (a) whether they mated with a sibling or non-relative, or (b) several environmental cues that may suggest a high or low likelihood of mating with a sibling (host developed in, time between emergence and mating, sex ratio upon emergence). This suggests that females cannot use direct genetic or indirect environmental cues to discriminate kin from non-kin.

My results have two implications for our understanding of sex ratio behaviour in haplodiploids. First, in fig wasps, more inbred species are observed to have more female biased sex ratios in more inbred species (Herre, 1985; Herre, 1987). If fig wasps also cannot discriminate between kin, then this pattern must be explained by selection on females to adjust their offspring sex ratios in response to the average level of inbreeding in their population. Second, much debate has focused on understanding the variation in offspring sex ratios produced by \( N. vitripennis \) females when ovipositing under the same conditions (Orzack and
Parker, 1990; Orzack et al., 1991); see also figure 3 for repeatable between line differences in sex ratio). This variation could arise if some females were sib-mated and produced different sex ratios in response to this cue – however, my experiments suggest that this explanation is unlikely. Nonetheless, inbreeding could still help maintain genetic variation if the amount of inbreeding varies spatially or temporally – i.e. through genotype by environment interactions (see West and Herre, 2002).

Clearly more experimental work will be required to test the generality of whether haplodiploid females adjust their offspring sex ratios in response to mating with siblings (Greeff, 1996; section 2.2.1). Molecular markers such as microsatellites would enable such studies on natural populations. One interesting study from this point is that of Roeder et al. (1996) on the mite Tetranychus urticae. They showed that females produced a more female biased sex ratio when they were related to the other females laying eggs on the patch, and argued that their data supported theory which predicts this pattern (Frank, 1985; Frank, 1986; Taylor and Frank, 1996; Courteau and Lessard, 2000). However, Roeder et al.’s (1996), experimental treatments confounded the relatedness between females with whether they mated with a sibling or non-relative. Consequently, their result could also be explained by the effect of sib-mating, as described in section 2.2.1.

I conclude with two general points that arise from my observation that N. vitripennis females cannot discriminate kin from non-kin mates. First, this result is not inconsistent with the observation in social insects that workers adjust the sex ratio of reproductives in response to their relative relatedness to males (brothers) and females (sisters; Chapuisat and Keller, 1999; Sundstrom and Boomsma, 2000). The reason for this is that workers appear to assess genetic variability within a colony and adjust their behaviour accordingly, rather than assessing genetic relatedness directly (Keller, 1997). Second, if kin discrimination is not common, then the evolution of kin selected social behaviour in the Hymenoptera is more likely to have arisen through limited dispersal making individuals interact with relatives (Hamilton, 1964; Hamilton, 1972). Although limited dispersal can also lead to increased competition between relatives, negating such selection for altruism (West et al., 2001b; West et al., 2002c), the life cycle of many Hymenoptera may avoid this problem by a dispersal phase that separates
altruism from competition (Queller, 1992; West et al., 2002c) and examining this problem in facultatively social species remains a major task.

2.6 Appendix

2.6.1 Life cycle
Mated females form groups of variable size in discrete patches where they lay their eggs. Sons and daughters mate at random in their natal patch whereupon the newly mated females disperse to a random location (island model of dispersal), and the cycle starts again. I want to know how females should adjust the sex ratio of their offspring according to whether they have mated with a sibling or a non-relative.

2.6.2 Evolutionary equilibrium conditions
I focus on a random patch and a random female in that patch. The subscript \(i\) will be used to denote the focal female’s mating state: sib-mated \((i=1)\) or not \((i=0)\). The patch contains \(N\) females, a proportion \(p\) of which is sib-mated. Let \(s_i\) denote the proportion of sons in the focal female’s clutch and \(\bar{s}_i\) the average sex ratio of all state-\(i\) females (including the focal female) in the focal patch. The average sex ratio of all females in the patch is then given by \(\bar{s} = (1-p)\bar{s}_0 + p\bar{s}_1\). The focal female’s fitness is her contribution to the pool of mated females in the next generation and I denote it by \(W_i(s_i,\bar{s})\) to remind us that it depends on her own sex ratio \(s_i\) and the average sex ratio \(\bar{s}\) in the patch. Total fitness can be decomposed into fitness obtained through daughters \((W_{fi})\) and through sons \((W_{mi})\), weighted according to sex-specific reproductive values \((v_f\) for daughters, \(v_m\) for sons):

\[
W_i(s_i,\bar{s}) = v_f W_{fi}(s_i,\bar{s}) + v_m W_{mi}(s_i,\bar{s}).
\]  

(A1)

The number of mated females obtained through daughters is simply proportional to the number of daughters produced:
\[ W_{fi} = 1 - s_i. \] (A2)

The number of females mated by sons equals the number of sons (proportional to \( s_i \)) times the average number of mates per son \((1 - \bar{s})/\bar{s} \):

\[ W_{mi} = s_i \frac{1 - \bar{s}}{\bar{s}} = s_i \frac{1 - (1 - p)\bar{s}_0 - p\bar{s}_i}{(1 - p)\bar{s}_0 + p\bar{s}_i} \] (A3)

I use the direct fitness approach (Taylor and Frank, 1996) to obtain the selection differentials:

\[
\frac{dW_i}{ds_i} = v_f \left[ r_{fi} \frac{\partial W_{fi}}{\partial s_i} + \bar{r}_{fi} \frac{\partial W_{fi}}{\partial \bar{s}_i} \right] + v_m \left[ r_{mi} \frac{\partial W_{mi}}{\partial s_i} + \bar{r}_{mi} \frac{\partial W_{mi}}{\partial \bar{s}_i} \right].
\] (A4)

Evaluated at \( s_i = \bar{s}_i = s_i^* \). The \( r_{ji} \) are the coefficients of relatedness of a state-\( i \) mother to her sex-\( j \) offspring, and \( \bar{r}_{ji} \) are the average coefficients of relatedness of a state-\( i \) mother to any sex-\( j \) offspring (including her own) born the focal patch. Since I assume that females in the same patch are a random sample of the population at large, I know that \( \bar{r}_{ji} = r_{ji} / N \). Working out (A4) then gives

\[
\frac{dW_0}{ds_0} \bigg|_{s_0 = \bar{s}_0 = s_0^*} = -v_f r_{f0} + v_m r_{m0} \frac{N(1 - \bar{s}^*) - (1 - p)}{N\bar{s}^*}
\]
\[
\frac{dW_1}{ds_1} \bigg|_{s_1 = \bar{s}_1 = s_1^*} = -v_f r_{f1} + v_m r_{m1} \frac{N(1 - \bar{s}^*) - (1 - p)}{N\bar{s}^*}
\] (A5)

### 2.6.3 Coefficients of Relatedness

For haplodiploid species, \( v_f = 2v_m \) and \( r_{mi} = 1 \) (e.g. Taylor 1988). I can arbitrarily set \( v_f = 1 \), therefore it remains to calculate the \( r_{fi} \). For non-sibmated females, \( r_{f0} = 1/2 \) and for sibmated females I obtain

\[ r_{f1} = \frac{\text{random allele daughter IBD to random allele mother}}{2 \text{ random alleles mother are IBD}} = \frac{3 + 5F}{4 + 4F} \] (A6)
where $\bar{F}$ is the average inbreeding coefficient (the probability that 2 alleles at the same locus are identical by descent; IBD). The inbreeding coefficient $F_j'$ among daughters born in a patch of size $N_j$ is given by

$$F_j' = \frac{1}{N_j} \left[ \frac{1}{2} \left( \frac{1}{2} + \frac{1}{2} \bar{F} \right) + \frac{1}{2} \bar{F} \right] = \frac{1}{4N_j} (1 + 3\bar{F})$$  \quad (A7)

Then the change in the average inbreeding coefficient from one generation to the next is given by

$$\bar{F}' = \frac{\sum q_j N_j F_j'}{\sum q_j N_j} = \frac{(1 + 3\bar{F})}{4 \sum q_j N_j}$$  \quad (A8)

Where $q_j$ is the relative contribution of patches of size $j$ to the next generation pool of mated females ($\sum q_j = 1$). In general, the $q_j$ will depend positively on the number of females produced in patches of size $j$. However, since larger patches are expected to produce less female-biased sex ratios, the $q_j$ are likely to depend only weakly on the sex ratio. Therefore, in the calculation below I assume that the $q_j$ are in fact independent of the sex ratio. If I write $\bar{N} = \sum q_j N_j$ then the equilibrium ($\bar{F}' = \bar{F}$) average inbreeding coefficient is given by

$$\bar{F} = \frac{1}{4\bar{N} - 3}.$$  \quad (A9)

Substitution in (A6) gives

$$r_{j1} = \frac{1}{2} \left( \frac{3\bar{N} - 1}{2\bar{N} - 1} \right)$$  \quad (A10)
2.6.4 Solutions

Under random mating, the frequency of sibmated in patches of size \( j \) is \( 1/N_j \). Thus, if I write \( p = 1/\bar{N} \), then \( p \) is the harmonic mean frequency of sibmating. To find the equilibrium sex ratios \( s_i^* \) as a function of \( p \) and patch size \( N \), I substitute (A10) and the other coefficients in the right-hand sizes of equations (A5), set the result equal to zero and solve for the \( s_i^* \).

If I assume that females do not adjust the sex ratio facultatively to their mating-state \( (s_0 = s_1 = s) \) then I get Herre’s (1985) result

\[
\frac{s^*}{s} = \frac{(N-1)(2-p)}{N(4-p)}. \tag{A11}
\]

If females do adjust the sex ratio facultatively, I get for \( N < (5-2p)/(1-p) \)

\[
\begin{align*}
\frac{s_0^*}{s} &= \frac{(N-1)(2-p)}{N(4-p)^2}(Np - 2p + 4) \\
\frac{s_1^*}{s} &= \frac{(N-1)(2-p)}{N(4-p)^2}(Np - N - 2p + 5) \\
\end{align*}
\tag{A12}
\]

For \( N > (5-2p)(1-p) \) I get \( s_1^* = 0 \) and

\[
\begin{align*}
\frac{s_0^*}{s} &= \frac{1}{2} \left( \frac{N(1-p) - 1}{N(1-p)^2} \right). \\
\end{align*}
\tag{A13}
\]
CHAPTER 3

Extreme sex ratios of green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) sea turtle nests in the Mediterranean and indirect methods for estimating sex ratios.

This chapter has been submitted to Biological Conservation, as: S.E. Reece, A.C. Broderick, B.J. Godley and S.A. West. Extreme sex ratios of green (*Chelonia Mydas*) and loggerhead (*Caretta caretta*) sea turtle nests in the Mediterranean and indirect methods for estimating sex ratios.

3.1 Summary

I used gonadal histology to directly estimate the sex ratios of green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) turtles hatching from nests in Northern Cyprus, eastern Mediterranean. My data showed sex ratios that were markedly skewed with relatively few males being produced. The mean sex ratio (proportion male) of hatchling green turtles was 0.10 and the mean sex ratio of hatchling loggerhead turtles was 0.21. These highly skewed sex ratio estimates are discussed in the context of conservation and current sex allocation theory applicable to sea turtle sex determination. Nest temperature showed significant correlation with the sex ratio of green and loggerhead turtle nests allowing the pivotal temperature to be estimated as 28.6°C for green turtles and 28.9°C for loggerhead turtles. Although incubation duration showed a significant correlation with the sex ratios of green turtles, this was not demonstrated for loggerhead turtle nests. I discuss the value of my data in making indirect sex ratio estimates.
3.2 Introduction

In order to successfully conserve endangered species, it is important to identify the factors that threaten population variability and viability. One demographic variable that is particularly vulnerable to environmental stochasticity is the sex ratio. The sex of many reptiles including sea turtles is determined by their incubation temperature (temperature dependant sex determination; TSD; Charnier, 1966; Bull, 1980; Pieau, 1971; Yntema and Mrosovsky, 1980), in which females are produced from relatively warm nests. Consequently, the sex ratio will vary with environmental fluctuations and concerns have been raised as to whether this could reduce population viability and at worst contribute to population extinction (Janzen, 1994; Berec, 2001; Freedberg and Wade, 2001). If this is a real threat, then manipulation of the sex ratio could become a potential tool for conservation (Mrosovsky et al., 1984b; Vogt, 1994; Wedekind, 2002; but see also Mrosovsky and Godfrey, 1995; Girondot et al., 1997). A fundamental first step in understanding whether the sex ratio can influence population viability is to accurately document field sex ratios (Charnov, 1982). To date, studies of sea turtles throughout the world have reported: (a) pivotal temperatures (that which produces an equal sex ratio) of around 29°C; (b) nest temperatures that are generally in excess of the pivotal temperature, and (c) a concurrent female biased sex ratio (Mrosovsky et al., 1984a; Mrosovsky and Provancha, 1988; Mrosovsky and Provancha, 1992; Godfrey et al., 1996 and 1999; Binkley et al., 1998; Kaska et al., 1998; Casale et al., 2000; Godley et al., 2002). Unfortunately, a multitude of different methods have been employed to estimate the sex ratio of hatchling populations (Mrosovsky and Godfrey, 1995). Furthermore, these studies are often restricted to a subset of nesting beaches that does not encompass all the variation in environmental characteristics encountered by the study population (Broderick et al., 2000 – A2; Godley et al., 2001a – A3).

Here, I investigate the hatchling sex ratios of green turtle, Chelonia mydas, and loggerhead turtle, Caretta caretta, populations nesting in Northern Cyprus, eastern Mediterranean. Previous work in Northern Cyprus has revealed a female bias in green turtles and both nest temperatures and incubation durations expected to produce a female bias in loggerhead turtles.
(Kaska et al., 1998; Broderick et al., 2000 – A2; Godley et al., 2001a,b). However, these studies were performed only on a small subset of nesting beaches in Northern Cyprus. My first aim was to extend these results with data for green and loggerhead turtles nesting at sites, which allowed incorporation of more variation in sand albedo (reflectance of solar radiation; Hays et al., 2001) than previous studies. In particular, I investigate previously neglected beaches with a higher sand albedo, as these sites are expected to be important for the production of males. Mean absorption of incident solar radiation values range from 69 – 91% throughout the nesting beaches in Northern Cyprus (Hays et al., 2001). Furthermore, these incident solar radiation absorption estimates correlate with sand temperature at green turtle nesting depth with an $r^2$ of 0.79 (Hays et al., 2001).

My second aim is to compare the correlations between sex ratios estimated by a direct method (gonadal histology) and environmental parameters that may be useful for indirectly estimating sex ratios. Specifically, nest temperature and incubation duration have been shown to correlate with sex ratio (Marcovaldi et al., 1997; Godfrey et al., 1999; Mrosovsky et al., 1999). Comparing the utility of such indirect methods is important for several reasons: (1) for financially constrained conservation projects it is only feasible to employ cheap and rapid techniques in the field; (2) it is often ethically unacceptable to sacrifice individuals from an endangered species for direct sexing, and (3) making large scale sex ratio estimates with direct methods is logistically impractical. If environmental characteristics of nests are highly correlated with sex ratios then they would provide useful indirect methods to; (1) estimate population sex ratios, and (2) monitor the potential effects of rapid global warming.

### 3.3 Methods

#### 3.3.1 Study sites

Data were collected from 3 study sites in Northern Cyprus, eastern Mediterranean; (1) a 2.5km beach with 2 coves, on the north coast at Alagadi; 35°33’N, 33°47’E, (2) 2 beaches, 2.1km in total, on the west coast near Akdeniz; 35°38’N, 32°92’E, and (3) 2 beaches, 0.3km in total, also on the west coast and near Korucam; 35°40’N, 32°92’E. Only site 1 experienced
recreational use and beach umbrellas were used outside the nesting zone to prevent shading and damage to nests. The 3 sites represent nesting zones with markedly different sand albedos (Hays et al., 2001): site 3 > site 2 > site 1, i.e. the sand at site 1 is expected to experience the highest temperatures.

3.3.2 Data collection
I monitored site 1 at Alagadi every day and night during the nesting season (May – early August) and the hatching season (late July - October). I patrolled sites 2 and 3 every 3 days during the nesting season and daily during the hatching season. After laying, all nests were measured to marker posts at 50m intervals at the back of the beach and given a numbered tag for identification. Details of the protocol for recording nesting and hatching events are described in detail by Broderick and Godley (1996). I used only natural nests left ‘in situ’ in this study, and I calculated incubation durations as the date the first hatchlings emerged minus date laid, and the date prior to midnight was always used. I recorded the hourly temperature of 29 nests (17 green and 12 loggerhead) using Tinytalk data loggers (Orion components, accuracy: 0.1, ± 0.3°C), inserted into the centre of the clutch within 3 days of laying and retrieved after all hatchlings had emerged.

The most accurate method for sexing hatchlings is gonadal histology (Yntema, 1976; Yntema, 1981; Yntema and Mrosovsky, 1980; Mrosovsky and Benabib, 1990; Mrosovsky et al., 1999). Given the endangered status of both species I only collected naturally deceased hatchlings for sexing. Although, this could introduce sampling bias, the physiology of TSD is highly conserved in all species of sea turtle (Bull et al., 1982b), and the results show that the relationships with sex, temperature and incubation period are consistent with those from studies where random hatchlings were sacrificed (Kaska et al., 1998). I collected dead hatchlings from nests subject to temperature monitoring and/or of known incubation period and undertook histology whilst in Northern Cyprus. I collected all histological data regarding loggerhead turtles in the 2000 nesting season, and in 1998 and 2000 for green turtles. In addition, I was able to augment sample sizes in several analyses with previously collected data (Kaska et al., 1998; Broderick et al., 2000 –A2).
3.3.3 Analysis
Sex ratio data usually have non-normally distributed error variance and unequal sample sizes. This can be accounted for by assuming binomial errors and a logit link function in a general linear model analysis of deviance (whilst retaining maximum statistical power; Crawley, 1993). Using GLMstat (http://www.ozemail.com.au/~kjbeath/glmstat), a full model was fitted, including interactions, and terms deleted in a stepwise fashion (Crawley, 1993). I used $\chi^2$ to test whether the removal of a term caused a significant increase in deviance. After fitting the full model, I compared the residual deviance and residual degrees of freedom. Relatively large values of residual deviance indicate overdispersion and a potential overestimation of the significance level. To compensate for this, the residual deviance is rescaled by the Heterogeneity Factor (HF; ratio of residual deviance to degrees of freedom) and significance testing carried out with an $F$ test (Crawley, 1993). I checked all other data for normality and applied linear regression techniques.

3.4 Results
I analysed data from a total of 204 green turtle nests; 4 from 1996 (Kaska et al., 1998), 52 from 1998 (Broderick, et al., 2000- A2), and 148 from 2000 (this study), and data from 116 loggerhead turtle nests; 2 from 1996 (Kaska et al., 1998), 23 from 1999 (Godley et al., 2001a –A3) and 91 from 2000 (this study).

3.4.1 Sex ratios from gonadal histology
The overall mean (-S.E.,+S.E.; confidence limits are asymmetric due to binomial data) sex ratio, as proportion male, for green turtle nests sexed was 0.10 (-0.09,+0.12, $n = 60$ nests, 385 hatchlings) and 0.21 (-0.16,+0.28, $n = 21$ nests, 84 hatchlings) for loggerhead turtle nests. For green turtle nests, the mean sex ratio produced was female biased at most sites (site 1: 0.00 ± 0.00, $n = 48$ nests; site 2: 0.22 -0.18,+0.26, $n = 8$ nests; site 3: 0.52 -0.43,+0.61, $n = 4$ nests). For loggerhead turtle nests, the mean sex ratio produced at all sites was also female biased (site 1: 0.22 -0.15,+0.31, $n = 11$ nests; site 2: 0.19 -0.14,+0.25, $n = 6$ nests; site 3: 0.33 -0.20,+0.50, $n = 4$ nests). For green turtles, there were significant differences in the sex ratios produced from the different sites when controlling for year ($\chi^2_{(2)} = 19.77$, $p < 0.0001$, $n = 60$, $HF = 0.25$), but this was not apparent for loggerhead turtle nests ($F_{2,18} = 0.22$, $P = 0.803$, $HF$
= 1.7). In addition, there was a correlation between year of study and sex ratio for green turtle nests ($\chi^2 = 7.60, P = 0.022, n = 60, HF = 0.25$), but not loggerhead turtle nests ($F_{2,17} = 3.17 P = 0.068, HF = 1.7$). A significant effect of year is expected for green turtles as not all years are represented by data from each site.

### 3.4.2 Nest temperature and sex ratios

The mean (± S.E.) nest temperature from the middle third of incubation (during the period when sex is determined) was 30.97°C (± 0.23, n = 50) for green turtles, and 31.71°C (± 0.21, n = 36) for loggerhead turtles. For green turtle nests, the mean nest temperatures at each site was: site 1 = 31.46°C (± 0.19, n = 39 nests), site 2 = 30.90°C (± 0.32, n = 5 nests), site 3 = 27.85°C (± 0.35, n = 6 nests) and for loggerhead turtle nests; site 1 = 31.87°C (± 0.20, n = 32 nests), site 2 = 31.65°C (± 0.55, n = 2 nests), site 3 = 29.23°C (± 1.26, n = 2 nests). As expected, nest temperature showed significant variation between sites for both green and loggerhead turtles ($F_{1,46} = 26.40, P < 0.0001$, and $F_{2,32} = 7.18, P = 0.003$, respectively). Again, I found significant differences between years for green turtle nests but not loggerhead turtle nests ($F_{1,46} = 11.26, P = 0.002$ and $F_{1,32} = 3.9, P = 0.057$, respectively). Temperature had a significant negative correlation with the sex ratio (proportion males) of samples from green turtle nests ($\chi^2(1) = 43.52, P < 0.000, n = 31, HF = 0.8, r^2 = 0.60$; see figure 3.1a) and samples from loggerhead turtle nests ($\chi^2(1) = 11.52, P < 0.0001, n = 9, HF = 0.5, r^2 = 0.62$; see figure 3.1b). The logistic regression model estimate of pivotal temperature was 28.6°C for green turtles and 28.9°C for loggerhead turtles.

### 3.4.3 Incubation duration and sex ratios

For green turtle nests, the mean (± S.E.) incubation duration was 50.0 days (± 0.5, n = 107), and for loggerhead turtle nests, 48.1 days (± 0.5, n = 38). For green turtle nests, the mean incubation durations at each site were: site 1 = 48.2 (± 0.4, n = 58 nests), site 2 = 52.3 days (± 1.1, n = 20 nests), site 3 = 52.1 days (± 1.4, n = 29 nests), and for loggerhead turtle nests; site 1 = 46.4 days (± 0.5, n = 24 nests), site 2 = 49.6 days (± 2.3, n = 7 nests), site 3 = 52.6 days (± 2.5, n = 7 nests). This variation between sites was significantly different for green and loggerhead turtles ($F_{2,104} = 8.8, P < 0.0001$, and $F_{2,37} = 5.3, P = 0.009$, respectively) and all data were from 2000. Incubation duration had a positive correlation with the sex ratio
(proportion males) of green turtle nests ($\chi^2_{(1)} = 29.27$, $P < 0.0001$, $n = 56$, $HF = 1.0$, $r^2 = 0.53$; see figure 3.2a) but not the sex ratio of samples from loggerhead turtle nests ($F_{1,19} = 2.60$, $P = 0.123$, $HF = 1.6$, $r^2 = 0.14$; see figure 3.2b). The logistic regression model estimate of pivotal incubation duration is 64 days for green turtles. Temperature had a negative correlation with incubation duration for green turtle nests ($F_{1,50} = 21.44$, $P < 0.000$, $r^2 = 0.43$), and loggerhead turtle nests ($F_{1,35} = 13.65$, $P = 0.001$, $r^2 = 0.39$).

Figure 3.1a: Shows the relationship between nest temperature (°C) and sex ratio for green turtles. The line was fitted from the logistic regression model, Sex ratio = e^{(a+bx)} / 1+e^{(a+bx)}, where $a = 32.45$ $b = -1.13$ and $x$ represents temperature; $r^2 = 0.60$ and plotted data are those collected from 31 nests: o = site 1, Δ = site 2 and ◊ = site 3.
Figure 3.1b: Shows the relationship between nest temperature (°C) and sex ratio for loggerhead turtles. The line was fitted from the logistic regression model, 
\[
\text{Sex ratio} = \frac{e^{(a+bx)}}{1+e^{(a+bx)}},
\]
where \( a = 27.66 \), \( b = -0.96 \) and \( x \) represents temperature; \( r^2 = 0.62 \) and plotted data are those collected from 9 nests: \( o = \) site 1, \( \Delta = \) site 2 and \( \diamondsuit = \) site 3.

Figure 3.2a: Shows the relationship between incubation duration (days) and sex ratio for green turtles. The line was fitted from the logistic regression model, 
\[
\text{Sex ratio} = \frac{e^{(a+bx)}}{1+e^{(a+bx)}},
\]
where \( a = -14.82 \), \( b = 0.23 \) and \( x \) represents incubation duration; \( r^2 = 0.53 \) and plotted data are those collected from 56 nests: \( o = \) site 1, \( \Delta = \) site 2 and \( \diamondsuit = \) site 3.
Figure 3.2b: Shows data for incubation duration (days) and sex ratio of loggerhead turtle nests. $r^2 = 0.14$ and plotted data are those collected from 20 nests: $o =$ site 1, $\Delta =$ site 2 and $\diamond =$ site 3.

3.5 Discussion

3.5.1 Extreme sex ratios in the Mediterranean

My sex ratio estimates for both green and loggerhead turtles hatching in Northern Cyprus are strongly female biased. Such an extreme sex ratio is expected as mean nest temperatures exceed both my estimates of pivotal temperature and prior published pivotal temperatures, for both species (Marcovaldi et al., 1997; Mrosovsky and Pieau, 1991; Yntema and Mrosovsky, 1982; Godley et al., 2002). My sex ratio estimate for green turtles of 10% male supports the female bias estimated in Broderick et al., (2000 - A2), of 4-14% males. Sex ratios from the warmer sites (1 and 2) were more female biased than at the cooler site (site 3) for both species. Previously, extremely skewed sex ratios in loggerhead turtle nests species have only been inferred (Godley et al., 2001a, b), but this is the first time that a number of loggerhead nests have been subject to sexing by histology since the preliminary work by Kaska et al., (1998). Consequently, this has enabled the first elaboration of an estimate of a pivotal temperature for the Mediterranean population.
3.5.2 Using temperature and incubation duration

Directly measuring sex ratios by gonadal histology is the most accurate method, but is also inappropriate for an endangered species. I have shown that nest temperatures show a significant correlation with the sex ratios of green ($r^2 = 0.60$) and loggerhead ($r^2 = 0.62$) turtles. This relationship indicates that if the mean temperature of a nest is known, an estimate of the sex ratio can be made with some confidence. As I have shown, this relationship applies to turtles nesting in the Mediterranean and it would be useful to extend this relationship to other populations to obtain and compare wider spatial and temporal sex ratio estimates. My data, which encompasses nesting areas that produce different sex ratios, have enabled us to improve the accuracy of previous estimates for the pivotal temperatures of Mediterranean green and loggerhead turtles (28.6°C and 28.9°C respectively). It should now be possible to extend sex ratio estimates throughout the region to incorporate sites for which the nest temperature can be estimated reliably from beach sand temperatures. Such estimates must incorporate internest variability throughout the nesting beaches of a population (Godley et al., 2002) and incorporate the possible effects of metabolic heating (Godfrey et al., 1997; Booth and Astill, 2001; Broderick et al., 2001)

I found incubation duration showed a weaker correlation with sex ratio than nest temperature for green turtles ($r^2 = 0.53$) and no correlation for loggerhead turtles ($r^2 = 0.14$). This relationship allowed us to estimate the pivotal incubation duration for green (64 days) but not loggerhead turtles. There are several reasons why the correlation between incubation duration and sex ratio may be weaker (or absent) than that for nest temperature despite the prior demonstration of the correlation between incubation temperature and incubation duration in both species. Firstly, there is a pronounced seasonal component to sand temperature regimes experienced in Northern Cyprus (Godley et al., 2001a – A3) and development rate, as measured by incubation duration, will be affected by temperature throughout incubation as opposed to sex ratio which is determined by temperature in the middle third of incubation. Secondly, the monitoring regime may have introduced variance into the methodology for sites 2 and 3, where incubation durations had an error of ca. 4% as they were monitored every 3 days, rather than daily as for site 1. Thirdly, loggerhead turtle nests are affected by diel variation because they are shallower than green turtle nests (Godley et al., 2001a – A3). As
development rate has a positive correlation with temperature, excursions above the pivotal may have a stronger affect on sex ratio than excursions of an equal duration below the pivotal. Finally, studies have shown that loggerhead hatchlings spend several days in the sand before emerging (Godfrey and Mrosovsky, 1997), so field variation in this hatch-emergence period may also confound incubation duration data.

3.5.3 Why are sea turtle sex ratios so extremely female biased?

It is commonly assumed that Fisher’s (1930) theorem of equal investment applies to sea turtles, in which case a sex ratio of 50% males would be predicted. However, species with ESD violate a fundamental assumption of Fisher’s theory because selection responds to the fitness difference between males and females in each type of environment, rather than the frequency of males in females (Charnov and Bull, 1989a, 1989b; Frank and Swingland, 1988). The expected population sex ratio can depend upon the reason for ESD. One explanation for ESD is that the environment has differential effects on the fitness of males and females - if one sex gains more than the other from developing in a certain environment then it should be preferentially produced in those conditions (Charnov and Bull, 1977). This idea has been able to explain ESD in many groups of animals (e.g. shrimps, fish, nematodes, other reptiles; Caullery and Comas, 1928; Christie, 1929; Conover and Kynard, 1981; Viets et al., 1993; McCabe and Dunn, 1997). In this case theory predicts that the population sex ratio should be biased towards the sex that is produced in poorer conditions, and this prediction has been supported by data from fish and invertebrates (Charnov and Bull, 1989a; Charnov 1993). However, the explanation for ESD in reptiles is less clear (Bull, 1980; Janzen, 1995; Janzen, 1996; Shine, 1999). Even if the differential fitness idea applies, it is not clear that it necessarily predicts a female biased sex ratio in reptiles, let alone one so extreme (Freedberg and Wade 2001; Freedberg et al., 2002; Reece et al., 2002). In particular, more complex models suggest that the population sex ratio can depend upon the details of male and female life histories and how the sex ratio produced interacts with other aspects of the organism’s life history (Frank 1987, 1990; Pen and Weissing 2000; West and Sheldon 2002; West et al., 2002a - A1).
Another possible explanation for extremely female biased sex ratios in sea turtles is cultural inheritance (Freedberg and Wade, 2001). Sea turtles exhibit nest site philopatry, the phenomenon where daughters return to nest in their natal site. Consequently, the more females produced in a particular site, the more popular this site is in the next generation, and the female bias perpetuates (Reinhold, 1998; Freedberg and Wade, 2001). In this situation we would expect selection to favour rogue females that produce rare males, but such frequency dependant selection could be reduced by: (1) the cost of nesting in novel (potentially unsuitable) sites; (2) sex is not chromosomally inherited and so selection can only act on nuclear genes that indirectly affect sex allocation, and (3) selection on the response to temperature is constrained due to an extremely low heritability of the pivotal temperature (Bull et al., 1982; Janzen, 1992). Although this idea is intuitively appealing, testing its assumptions in long-lived field populations remains a major challenge. Nonetheless, the crucial point remains that natural selection does not necessarily favour sea turtle sex ratios of 50% males.

3.5.4 Should conservationists take action?

My results have implications for sea turtle conservation. Firstly I have further confirmed that primary sex ratios in both populations of turtles nesting in Northern Cyprus are likely to be highly female skewed. By the detailed elaboration of pivotal temperatures in both populations I have confirmed that this trait is well conserved in marine turtles allowing wider inference to be made by other Mediterranean studies.

In the past, many conservation protocols were concerned with bringing the hatchling sex ratio closer to equality, either by artificial incubation or translocating nests to cooler nest sites (see Vogt, 1997). I have reasoned that equality is not necessarily the sex ratio favoured by natural selection, so these conservation strategies are not necessarily appropriate. Also, given that there is no evidence that breeding populations are male limited, a female bias is likely to promote population growth – a primary aim of sea turtle conservation strategies. In situations where artificial incubation is favoured due to high levels of nest predation or unsuitable nest sites, the temperature regimes chosen have a direct effect on sex allocation. I have shown that using temperature data - and to some extent incubation durations, it will be easier to estimate natural sex ratios and replicate them in any management strategy. The occurrence of rapid
global warming may perpetuate and further skew these extreme sex ratios and this could proceed unnoticed without comprehensive field sex ratio data for making appropriately timed comparisons. My data suggest that nest temperature will provide an excellent tool for undertaking the necessary monitoring of hatchling sex ratios in the Mediterranean.
CHAPTER 4

The effects of incubation environment, sex and pedigree on the hatchling phenotype in a natural population of loggerhead turtles

This chapter appears as the following publication: S.E. Reece, A.C. Broderick, B.J. Godley and S.A. West. 2002. The effects of incubation environment, sex and pedigree on hatchling phenotype in a natural population of loggerhead sea turtles. *Evolutionary Ecology Research*, 4, 737-748.

4.1 Summary

Explaining environmental sex determination (when offspring sex is determined by a property of the embryonic environment) in reptiles remains one of the greatest problems in the field of sex allocation. I tested Charnov and Bull’s differential fitness hypothesis in a natural population of loggerhead sea turtles in the field. This hypothesis states that the embryonic environment affects a trait that has different fitness consequences for males and females. I experimentally manipulated the incubation environment experienced by each sex and measured the phenotypic variation observed in hatchlings from experimental clutches and additional natural nests. Sand temperature had a negative correlation and percent water content has a positive correlation on the size of hatchlings from natural nests, and there was a significant interaction between sex and sand temperature on mass. This suggests that females, who develop in warm temperatures, are larger than males at hatching. The Charnov and Bull (1977), hypothesis would explain this pattern of environmental sex determination if larger size at hatching leads to a greater increase in lifetime fitness for females than males.
4.2 Introduction

In some species, the sex of offspring is determined by the environment in which embryonic development occurs; this is known as environmental sex determination (Charnov and Bull, 1977). Charnov and Bull’s (1977) differential fitness hypothesis provides an extremely general explanation of why environmental sex determination can be favoured over other methods of sex determination, such as genetic sex determination. Their hypothesis states that environmental sex determination is advantageous when the relationship between fitness and the embryonic environment is different for males and females. A clear case of the differential fitness hypothesis is found in Gamarus dubeni, where photoperiod is the environmental variable influencing sex determination (Bullheim and Bull, 1967). Competition for mates results in males gaining a greater fitness benefit than females from being large (McCabe and Dunn, 1997). Offspring produced at the start of the breeding season have the longest growth period and can reach a large size at the peak of the breeding season. As a short photoperiod is associated with the start of the breeding season, males are produced under conditions with a short photoperiod. This differential fitness concept has also been used to explain several cases of environmental sex determination, including that in nematodes (Caullery and Comas, 1928; Christie, 1929) and Atlantic silverside fish (Conover and Kynard, 1981). However, attempts to explain environmental sex determination in reptiles have been less successful, and this remains one of the greatest problems for sex allocation theory (Janzen and Paukstis, 1988, 1991a; Shine, 1999; West et al., 2002a- A1; Janzen and Krenz, in press). In many reptiles, offspring sex is determined by incubation temperature, or temperature sex determination (Bull, 1980). The relationship between sex and incubation temperature is characterized by the ‘pivotal’ temperature, which produces an equal sex ratio, and the narrow, ‘transitional’ temperature range, which produces a mixed sex ratio (Bull, 1980). Across reptiles, three patterns of temperature sex determination have been observed: (1) male sea turtles are produced from nests with temperatures below the pivotal (29°C; Yntema and Mrosovsky, 1982; Mrosovsky and Pieau, 1991; Mrosovsky, 1994; Ackerman, 1997); (2) male lizards and alligators are produced from nests above the pivotal (Charnier, 1966; Raynaud and Pieau, 1972; Wagner, 1980); and (3) male leopard geckos and crocodiles are produced only from the middle range of nest temperatures (this pattern has two pivotal temperatures; Ferguson and
Joanen, 1982; Gutzke and Paukstis, 1984). Unfortunately, because of the difficulty of experimentally separating the different effects of incubation temperature and sex on fitness, minimal progress has been made in determining the fitness advantages of temperature sex determination (Janzen, 1995; Shine, 1999). Ironically, species with genetic sex determination have provided some of the clearest evidence that temperature differentially affects the sexes (Burger and Zappalorti, 1988; Shine et al., 1997; Elphick and Shine, 1999). Here, I test Charnov and Bull’s (1977) hypothesis in loggerhead turtles (Caretta caretta), a species with environmental sex determination. The temperature–sex function and the period when sex is determined are well documented in this species. (Yntema and Mrosovsky, 1980, 1982; Mrosovsky and Provancha, 1992). I was able to experimentally separate the effects of incubation environment and sex by manipulating incubation environment after sex had been determined. In long-lived species, such as sea turtles, it is very difficult to study lifetime reproductive success, especially with respect to juvenile traits. Several traits of hatchlings (e.g. size, mass, residual yolk content) may correlate with fitness, and incubation environment may affect these traits differently in males and females (see Shine et al., 1997; Elphick and Shine, 1999). Similar instances, as well as the implications for sex allocation, are well documented in other organisms; for example, the size of parasitoid wasps at emergence has a strong influence on female survival and fecundity (Godfray, 1994; Visser, 1994; West et al., 1996), but less of an effect on male mating success (Charnov et al., 1981; Godfray, 1994). To provide a context for my experiment, I also documented phenotypic variation in hatchlings from natural nests within the population.

4.3 Methods

4.3.1 Study site

I carried out both the experiment and natural nest study on the beaches of Northern Cyprus, eastern Mediterranean. I collected 18 whole clutches to use in the experiment, within 3 days of laying, from a 5 km stretch of beach (35°28’N, 32°E) that has a very low hatching success due to a high level of canine predation and regular inundation for the whole beach. I split each experimental clutch, containing 50–100 eggs, into two equal groups and
buried them in artificial nests at a depth of 55 cm at two sites: (1) a warm site, a beach with female-producing incubation temperatures, at Alagadi (35°33’N, 33°47’E); and (2) a cool site, a beach with male-producing incubation temperatures, near Korucam (35°40’N, 32°92’E). Incubation temperatures were based on previously collected temperature and sex ratio data (Kaska et al., 1998; Godley et al., 2001a,b; Hays et al., 2001). In addition, I examined phenotypes from natural nests laid at these two sites.

4.3.2 Experimental design

At both of these sites, I buried split clutches in hatcheries approximately 8 x 5 m in size (rather than randomly on the beaches), as a concentrated area can be protected against disturbance by humans, nesting turtles, predation by dogs, foxes and crabs as well as be efficiently monitored in the dark (when hatchlings emerge). This method means that replication was not carried out at the hatchery level, a potential problem that I return to in the Discussion. I assessed whether each hatchery was representative of the surrounding beach in terms of percent water content and temperature (the two main environmental variables known to affect phenotype). I calculated the mean of three temperature readings and three water content readings for 30 random samples of each beach and each hatchery. Temperature was measured with a Hanna temperature probe (accurate to 0.3ºC) and water content was calculated from the difference in weight of a 75 g sand sample after dehydration for 4 h at 250ºC. Both temperature and percent water content data were collected for 20 beach and 20 hatchery sites at the cool hatchery. I measured temperature at 16 beach and 16 hatchery sites, and percent water content at 14 beach and 15 hatchery sites, on the warm beach.

Sex is determined during the middle third of the incubation period (Bull, 1981; Pieau,1982; Yntema and Mrosovsky, 1982; Vogt and Bull, 1984), so I used the date each half clutch was laid in conjunction with temperature data from each hatchery to calculate when each half clutch had completed two-thirds of its incubation, based on previous temperature and incubation data from these sites (Kaska et al., 1998; Godley et al., 2001a,b). When each half clutch had reached this point, it was excavated and split into two new groups (making a total of four groups from each original clutch). One of these new groups was reburied in the same place and the other group moved to the hatchery on the other beach. This
created a factorial design with four treatments: (1) females incubated at female-producing temperatures, (2) females incubated at male-producing temperatures for the final third of their incubation, (3) males incubated at male-producing temperatures and (4) males incubated at female-producing temperatures for the final third of their incubation. At each split, the identity of the original clutch that each group came from was maintained.

When each experimental clutch began to hatch, I collected all emerging hatchlings and took measurements from a random sample of up to 10 individuals. For each hatchling, the mean was calculated from each of three measurements of maximum straight carapace (dorsal shell) length, total body mass and total body fat. I made carapace measurements with callipers accurate to 0.1 mm and mass was measured using an Ohaus balance (accurate to 0.1g). Body fat was measured using total body electrical conductivity (TOBEC), using an EM-SCAN model SA-3000 TOBEC meter for live animals. The TOBEC meter readings are relative values and require species-specific calibration for transformation to absolute values. Calibration involves calculating a regression equation relating TOBEC value to absolute fat content from a sample of dead hatchlings (which are hard to obtain outside the field). As TOBEC increases linearly with fat content, I was able to use relative TOBEC values in my analysis. I measured 238 hatchlings from 40 successful (produced live hatchlings) quarter clutch groups. Of the original 18 whole clutches, 3 were unsuccessful, 3 had one successful quarter clutch group, 3 had two successful quarter clutch groups, 5 had three successful quarter clutch groups and 4 had four successful quarter clutch groups.

I collected dead hatchlings for sexing by histology (Yntema, 1976, 1981; Yntema and Mrosovsky, 1980; Mrosovsky and Benabib, 1990; Mrosovsky et al., 1999), to verify that the warm hatchery produced females and the cool hatchery produced males. I sexed 44 hatchlings from seven warm hatchery clutch groups and 16 cool hatchery quarter clutch groups.

4.3.3 Natural nest study
I also measured the phenotypic traits of up to 10 hatchlings from 12 nests in the same way as for the experimental clutches. At laying, I inserted Tinytalk data loggers (Orion components, accuracy 0.1 ±0.3°C) into the centre of the clutch to give hourly nest temperature readings and
calculated percent water content from a 3 x 75g sample of sand taken from the egg chamber. In total, I measured 114 hatchlings from 11 nests at the warm beach and one nest at the cool beach. Percent water content was calculated for all 12 nests, but temperature data were only collected for eight nests, so I carried out analyses using temperature only on this subset.

4.3.4 Analysis
I analysed all data in GLMstat, a program that uses generalized linear modeling (GLM; Crawley, 1993) techniques. For the sex ratio (proportion) data, I used binomial errors and a logit link function in an analysis of deviance, as proportion data often have non-normally distributed error variance and unequal sample size and this method retains maximum power (Crawley, 1993). A $\chi^2$-test (proportion data) or $F$-test (parametric data) was used to assess whether the removal of a term caused a significant increase in deviance. The suitability of using binomial errors was assessed after fitting the full model by comparing the residual deviance and residual degrees of freedom. Relatively large values of residual deviance indicate overdispersion and a potential overestimation of significance. To account for this, the residual deviance is rescaled by the heterogeneity factor (ratio of residual deviance to degrees of freedom) and significance testing carried out with an $F$-test (Crawley, 1993). For each of the continuous variables (carapace length, mass and body fat), I assumed normal errors and carried out a split-plot analysis (for nested data) to retain maximum power and avoid pseudoreplication at the within-clutch (block) level. To avoid problems associated with multiple analysis of the same data set, I only report $P$-values that were significant after Bonferroni correction.

4.4 Results

4.4.1 Environmental characteristics of study sites
The warmer beach had a significantly higher mean (± standard error) sand temperature (warm beach: 31.71 ± 0.11°C, $n = 32$; cool beach: 30.97 ± 0.84°C, $n = 40$: $F_{1,70} = 16.79$, $P < 0.01$) and a significantly lower mean percent water content (warm beach: 1.7 ± 0.10g, $n = 29$; cool beach: 2.2 ± 0.10g, $n = 40$: $F_{1,67} = 12.23$, $P < 0.01$) than the cooler beach. Within the
beaches, each hatchery and its surrounding beach did not differ in mean sand temperature or mean percent water content (warm: temperature, $F_{1,30} = 3.18, P > 0.05$; percent water, $F_{1,37} = 0.08, P > 0.05$; cool: temperature, $F_{1,38} = 1.95, P > 0.05$; percent water, $F_{1,38} = 0.31, P > 0.05$).

**4.4.2 Sex ratios of experimental clutches**

I successfully produced female- and male-biased clutches from my two hatchery sites. First, the sex ratio of each experimental clutch was significantly influenced by the temperature of the hatchery in which the sex-determining period was spent (warm hatchery mean = 0.00 ± 0.0, n = 7 nests, 10 hatchlings; cool hatchery mean = 0.71 ± 0.08, n = 16 nests, 34 hatchlings: $F_{1,21} = 29.91, P < 0.01, HF < 1$). The warm hatchery produced entirely female clutches; a high percentage of males was produced from clutches in the cooler hatchery (Figure 4.1). Second, the hatchery where the final third of incubation was spent had no effect on sex ratio ($F_{1,20} = 0.07, P > 0.05, HF < 1$).

![Figure 4.1: Mean sex ratio of a sample of clutches used in the experiment. Sex was determined in the warm environment for females and the cool environment for males. From the female-producing treatment, I sexed samples from four clutches in the warm environment and three clutches from the cool environment. From the male-producing treatment, I sexed samples from seven clutches in the cool environment and nine clutches in the warm environment. Asymmetric standard error bars are shown for treatments where the standard error is greater than 0.0. Unbroken line, cool environment; broken line, warm environment.](image)
4.4.3 Phenotype of experimental clutches

I carried out a nested analysis with clutch identity as the highest block, sex nested within clutch and final incubation environment nested within sex. Table 4.1 shows the relationships between each phenotypic trait and experimental treatment (see also figures 4.2a–c). The four experimental treatments are represented as the two factors; sex, final environment and their interaction, each with two levels (female/male and warm/cool, respectively). For all phenotypic traits, neither the final environment nor sex had a significant effect. The interaction between sex and final incubation environment had a significant effect on hatchling mass (not length or body fat; figures 4.2a-c). The mass of both male and female hatchlings that spent their final third of incubation in the warm environment was greater than that of same-sex hatchlings in the cool environment; this effect was more pronounced in male than female hatchlings. There was no significant effect of original clutch (i.e. block effect) on phenotypic traits. I also calculated narrow sense heritabilities for phenotypic traits (Roff, 1997). Heritabilities of male hatchlings were: carapace length = 0.02, mass = 0.03, body fat = 0.01. Those for female hatchlings were: carapace length = 0.01, mass = 0.03, body fat = 0.01.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Sex</th>
<th>Final environment</th>
<th>Sex–Final environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carapace</td>
<td>$F(1,9) = 2.57$</td>
<td>$F(1,14) = 0.92$</td>
<td>$F(1,14) = 2.83$</td>
</tr>
<tr>
<td>length</td>
<td>$P = 0.143$</td>
<td>$P = 0.359$</td>
<td>$P = 0.116$</td>
</tr>
<tr>
<td>Mass</td>
<td>$F(1,10) = 0.28$</td>
<td>$F(1,14) = 2.72$</td>
<td>$F(1,14) = 20.41$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.608$</td>
<td>$P = 0.121$</td>
<td>$P = 0.001$</td>
</tr>
<tr>
<td>Body fat</td>
<td>$F(1,10) = 0.01$</td>
<td>$F(1,13) = 0.005$</td>
<td>$F(1,13) = 1.58$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.922$</td>
<td>$P = 0.915$</td>
<td>$P = 0.232$</td>
</tr>
</tbody>
</table>

Table 4.1: $F$-ratios and $P$-values (significant after Bonferroni correction) for each measure of phenotype and potential explanatory variables. Sex–final environment refers to the interaction between hatchling sex (determined by incubation temperature for two-thirds of incubation duration) and the incubation environment for the final third of incubation.
Figure 4.2: (a) Overall mean carapace length, (b) overall mean mass and (c) overall mean body fat for both sex ratios in each final incubation treatment. Bars represent the standard error of the mean.
4.4.3 Phenotype of natural nests

Across nest means, temperature did not correlate significantly with any phenotypic traits (length, $F_{91,6} = 0.90$; mass, $F_{1,6} = 0.23$; body fat, $F_{91,6} = 1.19$). Water content had a significant positive correlation with body fat ($F_{1,10} = 7.72, P < 0.05$), but not with length ($F_{1,10} = 0.71$) or mass ($F_{1,10} = 1.27$). I also analysed these data using individuals as data points and clutch identity as a factor. Although these analyses were not independent from those above and involved pseudoreplication at the within-clutch level, they show the relative importance of environmental and clutch effects on phenotype. The analysis of individuals showed a strong effect of clutch identity with all three phenotypic traits (carapace length: $F_{11,102} = 22.39, P < 0.01$; mass: $F_{11,102} = 37.35, P < 0.01$; body fat: $F_{11,102} = 5.27, P < 0.05$). In this analysis, temperature had a significant negative correlation and percent water content a significant positive correlation with carapace length (temperature: $F_{1,69} = 6.75, P < 0.05$, figure 4.3a; percent water: $F_{1,102} = 6.80, P < 0.05$, figure 4.3b), but not body mass (temperature, $F_{1,69} = 1.15$; percent water, $F_{1,101} = 1.00$) or body fat (temperature, $F_{1,69} = 0.04$; percent water, $F_{1,102} = 0.06$).

Figure 4.3: Left: Plot of temperature and right, plot of percent water content against carapace length of individual hatchlings from natural nests. There were significant correlations with this data set, but not when using mean carapace length for each nest. Open circles, data from warm beach; closed circles, data from cool beach.
4.5 Discussion

I successfully produced markedly different sex ratios in the different experimental hatcheries (warm = 100% female, cool = 70% male; figure 4.1). Manipulating incubation environment showed an interaction between environmental variables and the phenotype (mass) of male and female hatchlings ($P < 0.001$ after Bonferroni correction). The narrow sense heritabilities were low for males and females (<0.04), encompassing inheritance from both genes and maternal effects. The greater increase in mass (from the warm environment) gained by male than female hatchlings suggests that: (1) environmental temperature is less important in terms of body mass for female than male hatchlings and (2) to fit the Charnov and Bull (1977) hypothesis, it is less beneficial for males to be very heavy at hatching. For example, male turtles may metabolize too much of their yolk reserves if they develop in warm temperatures, whereas female fitness is enhanced or not adversely affected by warm temperatures. Conversely, in the analysis of individual hatchlings from natural nests, temperature had a negative correlation and percent water content a positive correlation with carapace length (Figures 4.3a,b). In the analysis of nest means, the only significant result was a positive effect of percent water content on body fat, but this effect was not observed in individuals. I also found that it is possible to manipulate the incubation environment in the field after sex has been determined, and I hope this will provide a useful base for further field studies.

To my knowledge, this is one of only a few entirely field-based studies to address why the sex of reptiles is determined by an environmental variable. However, working on a field population of an endangered species constrained my experimental design; I could not replicate my two hatchery treatments and adequately protect hatchlings while minimizing the number of clutches used. This problem (Hurlbert, 1984) is common in studies of environmental sex determination; for example, laboratory studies commonly use data from multiple animals in a single incubator, as finances constrain the number of incubators per treatment. Although greater replication at the hatchery/block level is the best solution (e.g. by blocking the experiment and carrying out different treatments in the same incubator sequentially, or by
repeating the experiment over multiple years), another approach has been to use indirect evidence to verify that treatment variables are the most important causal factors (e.g. Shine and Elphick, 2001). I found no significant difference between each hatchery and its surrounding beach in terms of water content and temperature, which are known to be important causal variables.

I have shown that the incubation environment can differentially influence the phenotype of male and female hatchling loggerhead turtles; if warm nests consistently produce large and heavy hatchlings, then these hatchlings are likely to be female and males (from cool nests) are likely to be smaller. The Charnov and Bull (1977) hypothesis could explain why environmental sex determination exists in this population if a larger size at hatching confers a greater lifetime fitness gain to females than males. However, there are several reasons to suspect that their hypothesis might not be important for this population (but see Rhen and Lang, 1995; Shine et al., 1995; Janzen, 1996): (1) the difference in magnitude of environmental and clutch effects on phenotype; (2) it is possible that carapace length, mass and body fat at hatching are traits that do not persist into adulthood – especially as females are the larger sex at maturity (Godley et al., 2002); (3) also, it is possible that the small effects of temperature and humidity I detected indicate that the environment is important, but that Northern Cyprus may have a ‘homogeneous incubation environment’, and the environmental variation between nest sites is not great enough to generate large differences in phenotype. Stronger effects on phenotype may have been induced if I had manipulated the environment earlier in development (Shine and Elphick, 2001). Unfortunately, measuring lifetime reproductive success is currently impossible in long-lived species such as sea turtles, let alone associating variation in lifetime reproductive success with hatchling traits (Shine, 1999).

Current literature on environmentally induced variation in phenotype generally reports that large hatchling size correlates with: (1) water available to facilitate yolk metabolism (Morris et al., 1983; Miller and Packard, 1992; Packard et al., 1993; Packard, 1999); (2) cool temperatures, which are associated with humidity and longer incubation (Packard, 1999); (3) egg size and yolk provisioning (Packard et al., 1993; Roosenburg, 1996; Steyermarker and Spotila, 2001). The relative contributions of the environment and maternal effects to
phenotype are unclear and all possibilities, from equality to strong skews, have been documented in reptiles (Morris et al., 1983; Janzen, 1993; Shine et al., 1997; Packard, 1999). How these variables interact with fitness is unclear; for example, large hatchlings can run faster and escape predators (Miller et al., 1987; Janzen, 1993; Packard, 1999), but have consumed more yolk and may need to feed sooner after emergence than small hatchlings that have larger yolk reserves.

My experimental results contradict the sparse existing literature, because hatchlings spending the final third of their incubation period in the warm environment were heavier than their counterparts in the cool environment regardless of sex. I suggest two possible explanations for this observation. First, clutches in the cool hatchery may have experienced a detrimental level of sand water content. McGhee (1990) demonstrated that in loggerhead turtle nests the field mean for water content was 18% and levels exceeding 25% impaired growth. Second, there was an interaction between the environment and stage of development, so warm temperatures towards the end of incubation boosted hatchling size. My paradoxical experimental results emphasize the need for interpreting the fitness consequences of phenotypic variation with caution and that further experimental investigation is merited.
CHAPTER 5

Sex ratios in the rodent malaria parasite, *Plasmodium chabaudi*

This chapter has been submitted to Parasitology as: S.E. Reece, A.B. Duncan, S.A. West and A.F. Read. Sex ratios in the rodent malaria parasite, *Plasmodium chabaudi*

5.1 Summary

The sex ratios of malaria and related Apicomplexan parasites play a major role in transmission success. Here, I address two fundamental issues in the sex ratios of the rodent malaria parasite, *Plasmodium chabaudi*. First I test the accuracy of empirical methods for estimating sex ratios in malaria parasites, and show that sex ratios made with standard thin smears may overestimate the proportion of female gametocytes. Second, I test whether the mortality rate differs between male and female gametocytes, as assumed by sex ratio theory. Conventional application of sex ratio theory to malaria parasites assumes that the primary sex ratio can be accurately determined from mature gametocytes circulating the peripheral circulation. I stopped gametocyte production with chloroquine in order to study a cohort of gametocytes *in vivo*. The mortality rate was significantly higher for female gametocytes, with an average half life of 8 hours for female gametocytes and 16 hours for male gametocytes. While my results do not invalidate recent evidence that sex ratio data provide a more accurate method of estimating the inbreeding rate than molecular genetic data, they do indicate possible biases associated with standard methods for estimating sex ratios.
5.2 Introduction

In order for malaria parasites to transmit to new vertebrate hosts, a round of sexual reproduction must be undertaken in the mosquito vector. Sexual stages, termed gametocytes, are produced from the parasite’s asexual cycle and are the functional equivalents of males and females. Within 20 minutes of being taken up in the bloodmeal of a mosquito, the gametocytes have differentiated into gametes (Micks, et al., 1948; Billker et al., 1997). Each female gametocyte produces 1 female gamete and each male gametocyte can produce up to 8 male gametes (Janse et al., 1989; Schall, 2000; Sinden, 1975; Sinden et al., 1978). Male gametes are motile (each has a flagellum), and can fertilise female gametes either from the same clonal lineage (genotype), or outcross with other genotypes. The fertilised zygotes undergo several stages of asexual replication and meiosis before migrating to their vector’s mouthparts, ready to infect a new host. The gametocyte sex ratio (defined as proportion male gametocytes) is an important factor in determining how well a parasite genotype maximises its genetic representation in the population of new infections (Robert et al., 1996; Schall, 2000; Paul et al., 2000; Paul, et al., 2002). Recently there has been an increased interest in using evolutionary theory to explain the sex ratios observed in malaria parasites (reviewed by Read et al., 2002; West, et al 2001). Theory predicts that the sex ratio ($r^*$), should be related to the inbreeding rate by the equation $r^* = (1 − F)/2$, where $F$ is Wright’s coefficient of inbreeding (the probability that two homologous genes in two mating gametes are identical by descent; Dye & Godfray, 1993; Nee, et al., 2002). Whilst this relationship has enabled a broad scale understanding of the sex ratios observed in malaria and related apicomplexan parasites, there are discrepancies that demand understanding (Paul et al., 2002a; West et al., 2001 - A4). To date, there has been little work on how appropriate standard empirical measurements of apicomplexan sex ratios are for sex allocation theory (Read at al., 2002). Here, I begin to address these issues.

First, I test the accuracy of standard methods for estimating gametocyte sex ratios. The sex of gametocytes is usually assigned by examination of thin blood smears stained with Giemsa solution. However, this method may be inaccurate as sex ratio estimates made with specific molecular markers are significantly less female biased than when made with thin blood smears
In addition, sex ratio estimates can be much more female biased than expected – in extreme cases no males are observed amidst hundreds of gametocytes (Pickering et al., 2000); these observations could arise if young male gametocytes resemble females and are sexed wrongly from thin smears (Dearsly et al., 1990; Schall, 1989). I tested this possibility using the rodent malaria parasite *Plasmodium chabaudi*, by comparing the sex ratios observed in thin smears with sex ratios observed using a method in which gametocytes are allowed to partially differentiate into gametes to reveal their sex more clearly.

Second, I test the assumption that the mortality rate of male and female gametocytes is equal in *P. chabaudi*. Sex ratio theory is concerned with predicting the sex ratio at the point when sexual differentiation occurs (defined as the primary sex ratio), and differential mortality results in the observed sex ratio (the secondary sex ratio), differing from the primary sex ratio. Primary and secondary sex ratios may differ for a number of non-exclusive reasons. In most organisms, females live longer than males (Owens, 2002). For malaria parasites, sex biased mortality could occur in a number of ways. Sex specific antigens exist (Severini et al., 1999), and male and female gametocytes could be killed by host immunity at different rates (Paul et al., 2002; Paul et al., 2000; Reece & Read, 2000 - A5). There may also be sex specific rates for sequestration in the capillaries. If the mortality rate is greater for male gametocytes, natural selection could favour a less female biased sex ratio for a given level of inbreeding to insure there are enough male gametes in the bloodmeal to fertilise all the females (West et al., 2001 - A4; West, et al., 2002; Paul et al., 2002; Gardner et al., submitted;).

### 5.3 Methods

#### 5.3.1 Parasites and hosts

I gave 11-week-old female C57 black mice (Harlan-Olac, UK) an intra-peritoneal inoculation of $10^6$ red blood cells parasitised with *Plasmodium chabaudi*, clone ER (WHO Registry of Standard Malaria Parasites, University of Edinburgh, UK). I administered parasites in 0.1ml doses consisting of 47.5% Ringers (27mM KCl, 27mM CaCl$_2$, 0.15M NaCl), 50% heat
inactivated calf serum and 2.5% heparin (200 units ml\(^{-1}\)). I housed all mice in groups of 5 at 20°C with a 12hr light /12hr dark cycle. I provided food (41B, Harlan-Teklad, UK) and water with 0.05% pABA (to enhance parasite growth) ad lib.

### 5.3.2 Comparison of methods for sexing gametocytes

I took blood samples from the tail veins of five mice on six occasions during days 15-17 post infection (P.I.). For each mouse at each sampling point, I made three blood smears immediately (‘immediate’ smears), and three smears after 15mins when gametocytes began differentiation (‘delayed’ smears). I recorded the total numbers of male and female gametocytes in the three smears from each sampling point for each method. I made the delayed smears according to the following protocol. ~1μl blood was taken from the tail vein and kept in the cap of a 0.5ml eppendorf tube containing warm water (humidity prevents blood clotting), and the cooling blood was dropped onto 3 slides after 15 minutes, using a 20μl pipette and then smeared. I fixed all smears in methanol and stained them with 10% Giemsa solution.

For each ‘immediate’ smear, I sexed gametocytes according to published criteria: (1) the cytoplasm of males is pink and females a pale blue; (2) males tend to be crescent shaped and females ring shaped and (3) females have a compact nucleus with a vacuole beside it and the nucleus in males is dispersed with pale rim around it (figure 5.1; Dearsly et al., 1990; Landau and Boulard, 1978; Schall, 1989; Sinden et al., 1978). In contrast, gametocytes in ‘delayed’ smears had begun to differentiate into gametes and take on a different morphology (figure 5.2). Gametocytes require a 5°C drop in temperature and an increase in pH to become activated (Billker et al., 1997; Ogwan'g et al., 1993). The temperature drop was achieved as soon as blood was removed from the tail vein and the pH increased as CO\(_2\) levels dropped. Females either remained in their red blood cell and appeared as described above, or had burst out and “rounded up,” where they condense into a circular shape and stain a more intense blue (Sinden, 1975; Sinden, 1983; Sinden et al., 1978). Males undergo rapid DNA proliferation and, as their nucleus expands, the cytoplasm becomes pushed away and forms a ring around the large circular nucleus (Kawamoto et al., 1992). After 15-20mins the male gametocyte begins to package up DNA into separate flagella which extend from the nucleus and stain red-
purple (Janse et al., 1986; Kawamoto et al., 1992). Male gametocytes eventually detach and swim off to locate female gametes but, at least in vitro, this takes longer than the 15mins allowed by my protocol.

![Gametocytes in a standard ('immediate' smear, see text). A) female gametocyte and B) male gametocyte. See text for sexing criteria.](image1)

![Gametocytes in a 'delayed' smear' (see text), in which gametocytes are undergoing gametogenesis. A) female gamete and B) male gametocyte exflagellating to produce male gametes from its enlarged nucleus.](image2)

5.3.3 Differential mortality experiment

Each mouse in the treatment (CQ) group was given a curative dose of chloroquine sulphate (Nivaquine™) dissolved in distilled water, and mice in the control group mice received distilled water. Curative chloroquine treatment kills all asexuals and very young gametocytes, leaving an infection composed of mature and maturing gametocytes. Sex specific clearance of gametocytes can then be measured directly (Smalley and Sinden, 1997). A trial with 6 mice showed that a dose of 30mg/kg clears asexual parasites to below detectable, for at least 2 days
post administration (asexual densities prior to treatment, 0.86 ±0.35; after 24hrs, 0.03 ±0.01; after 48 and 72hrs, no parasites). I administered drugs and placebos at 0800hrs on day 14 P.I., when peak gametocytaemia is expected, and then sampled each mouse throughout day 14 and 15 P.I. *P. chabaudi* gametocytes are thought to be produced synchronously at midnight, therefore, in this experiment I monitored the two cohorts of gametocytes produced over the two days prior to drug treatment.

To collect sex ratio data, I made smears following the method detailed above for ‘delayed smears’ for each mouse every 2.5hrs on both days, from 08:30hrs to 21:00hrs. I calculated gametocyte density from the product of red blood cell densities and gametocytaemia (proportion of red blood cells infected with gametocytes). I made thin smears every 5 hours (i.e. every other sampling point) for the first 36hrs and at 51 and 75hrs post treatment. I stained all smears using a 10% Giemsa buffered in 90% phosphate solution for 10mins.

### 5.3.4 Analysis

For both experiments I carried out the data analysis using S-Plus (Insightful Corporation). I compared the methods for sexing gametocytes using a paired *t* test, by arcsin square root transforming the sex ratios to allow the assumption of normal errors. For the differential mortality data, gametocyte densities decreased exponentially so I logarithmically transformed the data as ln(gametocyte density + 0.001). To calculate the lifespan of gametocytes I considered the gametocyte density data from the chloroquine group only. If I assume a constant mortality rate *b* (number of gametocytes x10⁶ hr⁻¹), and initial gametocyte density *a*, the density at time *x* equals *y*; given by: *y* = *ae⁻bx*. Taking the natural log of both sides gives ln (*y*) = ln (*a*) – *bx*, therefore the regression of time against ln(gametocyte density) provides the mortality rate (*b*: the slope) and initial gametocyte density (*a*: the intercept) for each mouse. Further analyses were then carried out using the regression data for each mouse to avoid pseudoreplication. From these mortality rates I calculated the half life of gametocytes in each mouse (T₁/₂; time required for the gametocyte density to decrease to half of the initial density) using the function: 

\[ T_{\text{1/2}} = \frac{-\ln(y)}{b}. \]
5.4 Results

5.4.1 Comparison of methods for sexing gametocytes
The mean (±S.E.) number of gametocytes sexed in each smear was 52.26 (±2.88) and I analysed 28 pairs of samples (2 pairs were lost). Sex ratios determined from smears made immediately were significantly more female biased than those determined from delayed smears (figure 5.3; mean difference ± s.e. = 0.12 ± 0.03, n = 28, paired T-test, $T = -4.92$, $P < 0.001$).

![Figure 5.3: Mean sex ratio estimated from thin smears made with blood immediately after being taken from the tail vein (immediate), and smears made from blood in which gametogenesis had begun (delayed). Bars are the standard error of the mean, n = 28 pairs.](image)

5.4.2 Differential mortality experiment
The gametocyte density of the chloroquine group decreased throughout the sampling period at a significantly faster rate than the control group (figure 5.4; mean ± S.E. slope for CQ = -0.02 ± 0.001 gametocytes x10$^6$ml$^{-1}$; mean for control = -0.008 ± 0.0008; n = 16 mice, $T = -4.16$, $P$
In the chloroquine group, asexuals were not observed post treatment. Thus, the drug treatment worked: asexuals were killed and surviving gametocytes died without replacement over the subsequent days. There was no significant difference in the change of red blood cell densities of the 2 groups throughout the sampling period (mean slope for CQ = -0.01 ± 0.003; mean slope for control = -0.01 ± 0.006; n = 20 mice, T = -0.98, P = 0.34).

Figure 5.4a:

Figure 5.4b:
Figure 5.4c:

Figure 5.4: a) Total, b) male and c) female gametocyte density throughout the sampling period in infections treated with chloroquine (closed symbols) or distilled water (open symbols). The density in the chloroquine group decreased at a significantly faster rate than the control group. Error bars are standard error of the mean.

The mean gametocyte half life was 14hrs (95% c.i. 11-21hrs). The density of female gametocytes decreased significantly faster than male gametocytes (figure 5.5; mean mortality rate, \( b \pm \text{S.E.}, \) for females, \( = 0.09 \pm 0.009, \ n = 10; \) mean for males \( = 0.04 \pm 0.01, \ n = 9; \) paired t-test, \( T = 3.94, \ n = 9 \text{ pairs}, \ P = 0.004 \)). The half life for female gametocytes was 8hrs (95% c.i. 5-13 hrs), whereas the half life for males was 16hrs (95% c.i. 10-41 hrs). i.e. female gametocytes were lost from the circulation twice as fast as males.

Figure 5.5: Sex ratio of the chloroquine group (closed symbols) become less female biased over the sampling period than the control group (open symbols). Error bars are standard error of the mean.
5.4.3 Comparing primary and secondary sex ratios

I now estimate the consequences of this differential mortality for how much observed (secondary) sex ratios will differ from the sex ratio at gametocyte maturity (primary sex ratio). Although a cohort of parasites destined to become gametocytes will be initiated synchronously at shizogony, there are 2 scenario extremes for their recruitment as mature gametocytes: they mature at the same time (complete synchrony) or they have variable maturation times leading to continuous recruitment (asynchrony). I consider these 2 scenarios separately but reality is likely to lie between these extremes. Assuming recruitment is continuous, the initial density of each sex can be calculated for time $x$ from: $Initial = \int_0^\infty ce^{-bx} dx$, where $c$ is the sex specific recruitment value and $b$, the sex specific mortality rate. Integrating this function and incorporating the observed sex ratio $r$, gives an equation for the initial density of each sex:

$Male = \frac{rc}{b_{male}}$ and $Female = \frac{c(r-1)}{b_{female}}$ and the initial sex ratio is given by:

$R = \frac{r/b_{male}}{r/b_{male} + (1-r)/b_{female}}$, i.e. the lifespan of each sex weighted by their mortality weights.

Figure 5.6 shows the difference between the observed and primary sex ratio. The average sex ratio observed in the control group was 0.2, which corresponds to a primary sex ratio of 0.1 (95% c.i. 0.03 - 0.26).

![Figure 5.6](image-url)

Figure 5.6: For asynchronous gametocyte production. The unbroken lines show the primary sex ratio (before mortality), for a range of observed (secondary) sex ratios and the 95% confidence intervals for this relationship. The dashed line is $y = x$, i.e. when the primary and secondary sex ratios are equal.
If I assume all gametocytes mature synchronously, e.g. at shizogony (2400hrs), using the same logic as above, the primary sex ratio $R$, can be established from the function:

$$R = \frac{re^{tb_{male}}}{re^{tb_{male}} + (1-r)e^{tb_{female}}}$$

where $r$ is the observed sex ratio, $t$ the time since maturation and $b$ the sex specific mortality rates, i.e the same function for asynchronous recruitment but for a discrete time interval. Figure 5.7 shows the discrepancy between observed and primary sex ratio for synchronous recruitment at several different sampling points. The discrepancy between primary and secondary sex ratios increases with time since recruitment, for example, for a sex ratio of 0.2, observed 12 hours after recruitment, the primary sex ratio is 0.12 (95% c.i. 0.07 - 0.24).

![Figure 5.7](image-url)

Figure 5.7: The relationship between the primary sex ratio (before mortality), for a range of observed (secondary) sex ratios for 3 times ($t$ hours) since synchronous gametocyte production. The dashed line is $y = x$, i.e. when the primary and secondary sex ratios are equal or when $t = 0$ hrs.
5.5 Discussion

My first experiment demonstrates that sex ratios estimated immediately from tail blood may not be accurate. My second experiment shows that, at least for *P. chabaudi*, female gametocytes were lost from the circulation approximately twice as fast as males.

5.5.1 Comparison of methods for sexing gametocytes

My results suggest that standard methods for obtaining sex ratios lead to an over estimation of the extent of female bias in *P. chabaudi* gametocytes. This supports the observation made by Smith *et al.* (2000), who found that sex ratios of *P. falciparum* estimated from sex-specific molecular markers were less female biased than those from thin smears. One obvious case is the observation that sex ratios are so female biased that there can not be enough male gametes to fertilise all of the female gametes. (Burkot *et al.*, 1984). My results have implications for the theory showing that inbreeding rates of malaria and related Apicomplexans can be estimated more accurately and cheaply from sex ratio data than from molecular genetic methods (Read and Day, 1992; West *et al.*, 2000a; Nee *et al.*, 2002; Paul *et al.*, 2002). Clearly, this depends on the extent to which primary sex ratios are estimated without bias. Alternatively, the effects reported here could be incorporated in order to predict the secondary sex ratio that should be observed.

It is probable that male gametocytes were misidentified as females in smears made immediately from fresh blood (Schall, 1989; Dearsly *et al.*, 1990; Smith *et al.*, 2000). Giemsa stains female gametocytes blue because they are rich in basophilic structures (e.g. ribosomes and endoplasmic reticulum). If young male gametocytes are rich in ribosomes there may be a period when males resemble females and can begin gametogenesis (Sinden, 1975; Sinden, Canning & Spain, 1976; Aikawa *et al.*, 1981). Males may lose their ribosomes on maturation if they are not required for gamete production, but female gametes may need to retain them for zygote development. If gametocytes of other *Plasmodium* species share similar developmental pathways to *P. chabaudi*, then males may resemble females for a period of their time in circulation and lead to biased sex ratio estimates (Ranford-Cartwright *et al.*, 1993). A less plausible explanation for my results is that factors such as macrophages are
selectively attacking extracellular female gametocytes in delayed smears. I believe that this unlikely because there is no evidence that males or macrophages are preferentially attracted to females. Moreover, the activity of macrophages is likely to be severely restricted in rapidly cooling and clotting blood.

5.5.2 Differential mortality and lifespan of gametocytes

The discovery that female gametocytes are lost from the circulation at a faster rate than male gametocytes was unexpected. Female gametocytes could be lost from the circulation at a faster rate because they are larger at maturity and perhaps have a greater tendency than males to get “stuck” in peripheral blood vessels (Fallis & Desser, 1974; Schall, 1989; Bennett & Peirce, 1992; but see Read et al., 1992; Smith et al., 2000). Alternatively, they could be more antigenic or vulnerable to immune killing if they are more metabolically active (Severini et al., 1999). My mortality rate estimates show that the observed sex ratio may differ from the primary sex ratio whether gametocyte recruitment is synchronous or asynchronous. If gametocyte recruitment is asynchronous the primary sex ratio is approximately half the observed sex ratio where as the discrepancy increases with time since gametocyte production for synchronous recruitment. As recruitment of gametocytes is thought to be largely synchronous, sampling close to shizogony should minimise this discrepancy.

These data raise several issues worthy of investigation. Does differential mortality also occurs at the start of an infection, before host immunity appears? Determining this would go some way to establishing whether faster clearance of female gametocytes is due to host or intrinsic factors. Does greater female mortality significantly reduce the numbers of viable female gametes in the vector? Recent thinking has shown that parasites may need to invest more in males than predicted by their population structure in order to fertilise all female gametes (Paul et al., 2000; Paul et al., 2002; West et al., 2002b; Gardner et al., submitted) perhaps higher female mortality renders this unimportant. Finally, sex ratios in several Plasmodium species become less female biased as infections progress, and a variety of ideas have been suggested to account for this (Paul et al., 2000, 2002). Instead, could an accumulation of longer-lived male gametocytes account for these increases in sex ratio?
CHAPTER 6

Even more extreme fertility insurance and the sex ratios of protozoan blood parasites.

This chapter has been submitted to the Journal of Theoretical Biology as: A. Gardner, S.E. Reece and S.A. West. Even more extreme fertility insurance and the sex ratios of protozoan blood parasites.

6.1 Summary

Theory developed for malaria and other protozoan parasites predicts that the evolutionarily stable gametocyte sex ratio \( z^*; \) proportion of gametocytes that are male \( ) \) should be related to the inbreeding rate \( f \) by the equation \( z^* = (1-f)/2. \) Although this equation has been applied with some success, it has been suggested that in some cases a less female biased sex ratio can be favoured to ensure female gametes are fertilised. Such fertility insurance can arise in response to two factors: (i) low numbers of gametes produced per gametocyte and (ii) the gametes of only a limited number of gametocytes being able to interact. However, previous theoretical studies have considered the influence of these two forms of fertility insurance separately. I use a stochastic analytical model to address this problem, and examine the consequences of when these two types of fertility insurance are allowed to occur simultaneously. The results show that interactions between the two types of fertility insurance reduce the extent of female bias predicted in the sex ratio, suggesting that fertility insurance may be more important than has previously been assumed.
6.2 Introduction

One of the many successful applications of sex allocation theory has been the study of how competition for mates between related males can favour the evolution of female biased sex ratios (Charnov, 1982; Godfray, 1994; Hamilton, 1967; West et al., 2000a). Recent years has seen an increasing interest in applying this theory (local mate competition; LMC) to malaria and related protozoan parasites (Read et al., 2002; West et al., 2001 - A4). Here, the appropriate prediction is that the evolutionarily stable (ES; Maynard Smith, 1982) gametocyte sex ratio \( z^* \); proportion of gametocytes that are male) should be related to the inbreeding rate \( f \) by the equation \( z^* = (1-f)/2 \) (Hamilton, 1967; Nee et al., 2002; Read et al., 1992). When there is complete inbreeding \( f=1 \; \text{i.e. a single lineage or clone is selfing} \), the ES strategy is to produce the minimum number of males required to fertilise the available female gametes and thus, maximise the number of zygotes. Conversely, when gametes in the mating pool are of a mixture of lineages, \( f \) decreases and the sex ratio increases in order for each lineage to maximise its genetic representation in the zygote population. The relationship between the inbreeding rate and sex ratio has been able to explain a number of sex ratio patterns in Apicomplexan parasite populations (reviewed by West et al., 2001 – A4; Read et al., 2002). However, there are a number of observations that cannot be explained by this equation. In particular: (1) across Haemoproteus populations in birds the sex ratio does not correlate with an expected correlate of the inbreeding rate (prevelance; Shutler et al., 1995; Shutler and Read, 1998); (2) in malaria parasites, sex ratios within and between infections can be extremely variable (Osgood et al., 2002; Paul et al., 2002; Paul et al., 2000; Paul et al., 1999; Pickering et al. 2000; Schall, 1989; Taylor, 1997), and less female biased sex ratios can lead to greater transmission success (Robert et al., 1996).

A potential explanation for these contradictory observations is “fertility insurance” – the production of a less female biased sex ratio to ensure that all female gametes are fertilised (West et al., 2002b). Before describing how fertility insurance can influence the ES sex ratio it is necessary to describe the background biology. In malaria and related Haemospororin parasites, haploid sexual stages (gametocytes) are taken up from the host in the blood meal of
Once inside the midgut, the haploid gametocytes differentiate into haploid gametes and fuse to form zygotes. These resulting diploid zygotes undergo meiosis and asexual proliferation before migrating to the vector’s salivary glands where they wait to enter a new vertebrate host. Each female gametocyte (macro-gametocyte) will differentiate into 1 female gamete, whereas each male gametocyte (micro-gametocyte) will produce several motile male gametes. The number of viable gametes produced per male gametocyte varies enormously across species - 4-8 in mammalian malaria parasites (Read et al., 1992); ~2 in some lizard malarias (Schall, 2000); 5->1000 in Eimeriorin intestinal parasites (West et al., 2000b).

Fertility insurance can occur for two broad reasons – which are summarised here but discussed more fully in West et al. (2002b). First, the number of male gametes produced per gametocyte (c) may be a limiting factor (Read et al., 1992). If the mean number of viable gametes produced per male gametocyte is c, then the ES sex ratio must be $z^* \geq 1/(c+1)$, otherwise there will not be enough male gametes to fertilise the female gametes (fig 6.1; Read et al., 1992). Second, the ability of gametes to interact may be a limiting factor. West et al. (2002b) investigated this possibility by assuming that the number of gametocytes whose gametes can interact (q) is restricted. In this case a less female biased sex ratio is favoured to avoid the stochastic absence of males in a mating group of q gametocytes (figure 6.1; West et al., 2002b). A low q could occur for a number of reasons including low male gamete motility, high gametocyte or gamete mortality, low gametocyte density, or small blood meals (Shutler and Read, 1998; Paul et al., 1999, 2000, 2002; Reece and Read, 2000 – A5; West et al., 2001 – A4, 2002b). Recent attention has focused on how the host immune response may influence and vary the importance of these factors (Paul et al., 1999, 2000, 2002; Reece and Read, 2000 – A5).
In order to make their analyses mathematically tractable, previous studies have considered the influence of these two forms of fertility insurance separately. When examining the influence of male gametocyte fecundity \(c\), Read et al. (1992) assumed that the gametes from an infinite pool of gametocytes can interact \(q = \infty\), and when examining the influence of the number of gametocytes whose gametes can interact \(q\), West et al. (2002b) assumed that male gamete fecundity was not a limiting factor \(c = \infty\); i.e. one male gametocyte is able to provide enough gametes to fertilise all of the female gametes in its mating group arising from \(q\) gametocytes.

It has subsequently been assumed that the overall effect of these two factors can examined by seeing which is more constraining, and favours the least female biased sex ratio (West et al., 2002b). However, there is the possibility that these factors may interact – when both \(c\) and \(q\) are low, even if there are males in a mating group, these males may not be able to provide enough gametes to fertilise all the female gametes. Although this scenario could logically occur, it is not clear whether this interaction will significantly influence the ES sex ratio. I use a stochastic analytical model to address this problem and consider how the unbeatable sex ratio is influenced by the interaction of finite values for both \(c\) and \(q\). I use life history terminology associated with malaria parasites, but our results are applicable to any Apicomplexan parasite with dimorphic sexual stages.
6.3 Methods

I consider an infinite population of vertebrates harbouring malaria parasites and supporting an infinite number of blood-feeding dipteran vectors (effects due to finite numbers of vertebrate hosts is negligible unless the number of hosts is extremely small; Taylor and Bulmer, 1980). Every host contains an infinite pool of haploid gametocytes circulating in the peripheral blood, comprising \( n \) independent lineages (all notation is given in table 1). Within a lineage, all gametocytes are clonally derived from a single sporozoite founder individual. Each lineage produces a proportion \( z \) of male gametocytes and \( 1-z \) of female gametocytes, where \( z \) is determined by a single biallelic nuclear gene. A common 'Null' allele exists at frequency \( 1-m \) and has \( z = z_N \), and an infinitely rare 'Mutant' allele exists at frequency \( m \) and has \( z = z_M \). I may assign each host individual to one of \( n+1 \) classes on the basis of the number of Mutant lineages carried. Each host is fed upon by a large number of vectors, transmitting \( q \) gametocytes to each vector in the process. Once in the midgut of the vector, each male gametocyte gives rise to \( c \) male gametes and female gametocytes each give rise to a single female gamete. Random syngamy ensues, and the resulting next generation of zygotes are, following Read et al. (1992), assumed to reflect the genetic composition of the next generation of infections. It is worth noting that although each vector contains a single mating group of size \( q \) the predictions of this analysis will hold for any number of such groups, provided that there is no exchange of gametes between mating groups.

The fitness of the Null is the mean success of a Null lineage from each host-class weighted by the number of Null lineages in the host-class and the frequency of that host-class. As the mutant is infinitely rare, so that \( m \to 0 \), the fitness of the Null is dominated by its success in vectors feeding upon hosts containing no Mutant lineages.

\[
w_N = \frac{1}{n} S_{N,0} = f S_{N,0} \tag{1}
\]

where \( S_{N,0} \) is the mean number of zygotic Null alleles produced per vector feeding on a host harbouring zero Mutant lineages, and \( f \) is the degree of inbreeding. The Mutant never occurs in such hosts, and almost never occurs in hosts with other Mutant lineages, so its fitness is
dominated by its success in vectors feeding upon hosts with 1 Mutant lineage and n-1 Null lineages.

\[ w_M = S_{M,1} \]  

(2)

where \( S_{M,1} \) is the mean number of zygotic Mutant alleles derived from a vector feeding on a host containing one Mutant infection only. The Mutant invades if \( w_M > w_N \) and so the ES sex ratio \( z^* \) is the value of \( z_N \), such that \( \omega = w_N / w_M \) is not less than unity for all \( 0 \leq z_M \leq 1 \). Exact solutions for \( S_{N,0} \) and \( S_{M,1} \) will be determined, so that for known \( q, c \) and \( f \) pairs of sex ratio strategies may be compared.

A vector feeding on a Null-only host is assured of obtaining \( q \) Null gametocytes in its bloodmeal. \( \mu_N \sim Bi(q, z_N) \) are male, and the remaining \( \phi_N = q - \mu_N \) are female, so that there are \( c \mu_N \) male gametes and \( \phi_N \) female gametes able to interact in the midgut. The number of zygotes, \( \xi \), is the smaller of these two values, and since zygotes are diploid the number of Null alleles formed in that vector is 2 \( \xi \).

\[ S_{N,0} = \sum_{\mu_N = 0}^{q} \left( \frac{q}{\mu_N} \right) z_N^{\mu_N} (1 - z_N)^{q - \mu_N} 2 \min\{c \mu_N, q - \mu_N\} \]  

(3)

A vector feeding on a host containing 1 Mutant and \( n-1 \) Null lineage will obtain \( q \) gametocytes of which \( \tau_M \sim Bi(q, f) \) are Mutant and \( \tau_N = q - \tau_M \) are Null. These will comprise \( \mu_M \sim Bi(\tau_M, z_M) \) Mutant males and \( \phi_M = \tau_M - \mu_M \) Mutant females, and \( \mu_N \sim Bi(\tau_N, z_N) \) Null males and \( \phi_N = \tau_N - \mu_N \) Null females. The number of zygotes, \( \xi \), is then the lower of the two values \( c (\mu_M + \mu_N) \) and \( \phi_M + \phi_N \), meaning that there are \( \xi \) successful male gametes and \( \xi \) successful female gametes. Of the former, a proportion \( \sigma_{M,1} \sim HypGeo(\xi, c \mu_M, c (\mu_M + \mu_N)) / \xi \) will be Mutant, and of the latter a proportion \( \sigma_{M,0} \sim HypGeo(\xi, \phi_M, \phi_M + \phi_N) / \xi \) will be Mutant. The success of the Mutant is simply \( \xi (\sigma_{M,1} + \sigma_{M,0}) \) (Taylor, 1981; Charnov, 1982).

\[ S_{M,1} = \sum_{\tau_M = 0}^{q} \sum_{\mu_M = 0}^{\tau_M} \sum_{\mu_N = 0}^{\tau_N} \left( \frac{q}{\tau_M} \right) f^{\tau_M} (1 - f)^{q - \tau_M} \left( \frac{\tau_M}{\mu_M} \right) z_M^{\mu_M} (1 - z_M)^{\tau_M - \mu_M} \left( \frac{q - \tau_M}{\mu_N} \right) z_N^{\mu_N} (1 - z_N)^{q - \tau_M - \mu_N} \]  

\[ \min\{c(\mu_M + \mu_N)q - \mu_M - \mu_N\} (E[\sigma_{M,1}] + E[\sigma_{M,0}]) \]  

(4a)

where

\[ E[\sigma_{M,1}] = \begin{cases} \frac{\mu_M}{\mu_M + \mu_N} & \text{if } \mu_M + \mu_N > 0 \\ 0 & \mu_M + \mu_N = 0 \end{cases} \]  

(4b)
\[ E[\varpi_{M,N}] = \begin{cases} \frac{\tau_M - \mu_M}{q - \mu_M - \mu_N} & \text{if } q - \mu_M - \mu_N > 0 \\ 0 & \text{if } q - \mu_M - \mu_N = 0 \end{cases} \] (4c)

These expressions reveal whether the Mutant allele can invade a population fixed for the Null.

I determined the ES sex ratio iteratively, such that the value of \( z_N \) in each round is the sex ratio of the successfully invading Mutant or successfully defending Null of the previous round, and \( z_M \) is a randomly assigned value. After an indefinite number of rounds the Null will assume and subsequently retain the value of \( z^* \), so that at any time the currently unbeaten \( z \) can be tested for evolutionary stability by plotting \( \omega \) for \( z_N \) equal to the putative \( z^* \) against all \( 0 \leq z_M \leq 1 \) and rejecting if \( \omega < 1 \) for any \( z_M \).

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>( Bi(k,\pi) )</td>
<td>Binomial distribution: ( k ) trials and probability of success ( \pi )</td>
</tr>
<tr>
<td>( c )</td>
<td>Number of viable male gametes per male gametocyte</td>
</tr>
<tr>
<td>( f )</td>
<td>Inbreeding coefficient; ( f = n^{-1} )</td>
</tr>
<tr>
<td>( g_X )</td>
<td>Number of X-allele male gametes remaining viable</td>
</tr>
<tr>
<td>( HypGeo(\alpha,\beta,\gamma) )</td>
<td>Hypergeometric distribution: ( \alpha ) trials, and ( \beta ) potential successes out of ( \gamma )</td>
</tr>
<tr>
<td>( M )</td>
<td>The Mutant allele</td>
</tr>
<tr>
<td>( m )</td>
<td>Population frequency of the mutant</td>
</tr>
<tr>
<td>( N )</td>
<td>The Null allele</td>
</tr>
<tr>
<td>( n )</td>
<td>Number of independent lineages per vertebrate host</td>
</tr>
<tr>
<td>( p )</td>
<td>Probability of male gamete survival</td>
</tr>
<tr>
<td>( q )</td>
<td>Number of gametocytes whose gametes can interact in the vector</td>
</tr>
<tr>
<td>( S_{X,y} )</td>
<td>Success of the X-allele in a host containing ( y ) Mutant infections</td>
</tr>
<tr>
<td>( w_X )</td>
<td>Absolute fitness of the ( X )-allele</td>
</tr>
<tr>
<td>( z )</td>
<td>Sex ratio (proportion male gametocytes per lineage)</td>
</tr>
<tr>
<td>( z^* )</td>
<td>Evolutionarily stable (ES) sex ratio</td>
</tr>
<tr>
<td>( z_X )</td>
<td>Sex ratio employed by the ( X )-allele</td>
</tr>
<tr>
<td>( \chi )</td>
<td>Species-specific number of gametes released per male gametocyte</td>
</tr>
<tr>
<td>( \phi_X )</td>
<td>Number of ( X )-allele females in a mating group</td>
</tr>
<tr>
<td>( \mu_X )</td>
<td>Number of ( X )-allele males in a mating group</td>
</tr>
<tr>
<td>( \tau_X )</td>
<td>Total number of ( X )-allele gametocytes in a mating group</td>
</tr>
<tr>
<td>( \varpi_{X,y} )</td>
<td>Frequency of ( X )-alleles in successful male (( y=1 )) or female (( y=0 )) gametes</td>
</tr>
<tr>
<td>( \omega )</td>
<td>Relative fitness of the Null, ( w_N / w_M ); Mutant invades if ( \omega &lt; 1 )</td>
</tr>
<tr>
<td>( \zeta )</td>
<td>Number of zygotes produced by the mating group</td>
</tr>
</tbody>
</table>
To check our expressions, I derived (3) and (4) for the special cases where q or c are infinite, i.e. corresponding to the analyses of Read et al. (1992) and West et al. (2002b). These equations are presented in the appendix, and in all cases gave the same results as previous analyses.

6.4 Results and Discussion

I have discriminated between two types of fertility insurance, in response to (i) low male gamete fertility (low c), and (ii) the ability of gametes to interact (low q). Previous theoretical work has examined the effect of these two types of fertility insurance separately. Specifically, West et al. (2002b) assumed that when both of these factors are operating, the effect for sex ratio evolution can be determined by seeing which leads to a greater reduction in the predicted female bias (i.e. which of figure 6.1 predicts the least female biased sex ratio). In contrast, our model explicitly allows for both types of fertility insurance to act simultaneously, and hence allows for any interactions. In figures 6.2-6.4 I give example predictions when the two types of fertility insurance are allowed to act separately as previously assumed by West et al. (2002b) (left part of figures 6.2-6.4) or simultaneously in our model (right part of figures 6.2-6.4). Our results show that when both c and q are low, the ES sex ratio may be higher than predicted when considering these two effects separately.

Why does the model predict a less female biased sex ratio? It has been assumed that one male gametocyte will be able to provide enough gametes to fertilise all the female gametes in the mating group that arises from q gametocytes. This is not the case if (q-1)\>c. More generally, the male gametocytes will not be able to fertilise all the female gametes when (q-\mu)\>c\mu, where \mu is the number of male gametocytes in a mating group. This risk of not having enough males to fertilise the females in the group leads to less female biased sex ratios being favoured. Another way of conceptualising this is that a finite q increases the potential for low c to be a
problem – when gametes can not interact as successfully (finite $q$), a mating group may contain only a single or small number of male gametocytes, and so the gamete fecundity ($c$) of these males is more likely to be a limiting factor.

The model shows that the interaction between the two types of fertility insurance can have a surprisingly large influence on the ES sex ratio. In the examples that I give, the predicted sex ratio can be up to 0.1 higher (figure 6.2, when $c=2, q=10$ and $f=0.3$). In this instance the sex ratio deviates from equality (0.5) by approximately half the amount inferred by West et al. (2002b). Although increasing $c$ proportionally reduces the degree of female bias, the complex interplay between male fecundity and size of mating groups makes it difficult to relate the magnitude of this effect to $q$. In the limit, as $q$ increases towards infinity, the effect dissipates as the predictions converge with those of Read et al. (1992). However, as $q$ rises it increases the propensity for $c$ to become limiting. The effect is therefore a dome-shaped function of $q$, although the exact relationship crucially depends upon the particular parameter values.

I also extended the model to allow stochastic variability in the number of viable gametes per gametocyte ($c$); see appendix, equations (A.5 and A.6). This could occur through variation in the number of gametes produced per gametocyte, or through mortality. Adding in this stochasticity (for invariant $E[c]$) gives further reduction in the female bias predicted, although this effect is negligible in all but the smallest of mating groups. However, a novel prediction arises from this form of stochasticity, as it allows the investigation of the mean value of $c<1$, so that male fecundity is lower than that of females. In this case, a male biased sex ratio is favoured. For the case of $q \rightarrow \infty$ equations A.3 and A.4 remain valid even for $c<1$, and male biased ES sex ratios are easily demonstrated. Switching the roles of males and females in the classic LMC relation, the result of Read et al. (1992) can be extended so that, as before, for $c \geq 1$ $z^* = \max\{(1-f)/2,1/(c+1)\}$, yet now for $c \leq 1$ $z^* = \min\{(1+f)/2,1/(c+1)\}$. This prediction contrasts with standard LMC models constructed for insects (e.g. Nagelkerke and Hardy, 1998; West and Herre, 1998), where male biased sex ratios are never predicted, due to the assumption that one male can mate any number of females (analogous to assuming $c=\infty$). Male biased sex ratios have been observed in some samples of lizard malaria (Paperna and
Landau, 1991), although the necessarily small sample sizes mean that these observations should be treated with caution.

To conclude, the analysis has revealed that fertility insurance can be a more potent evolutionary buffer to female biased sex ratios in malaria and related parasites than previously suggested. Clearly, the outstanding problem is to obtain empirical estimates of $c$ and $q$, and how their values are influenced by factors such as host immune responses. The literature has recently been reviewed on this (West et al., 2002b), and sadly very little is known.

![Figure 6.2](image1.png)  
**Figure 6.2:** Left shows the relationship between predicted sex ratio and inbreeding rate, for given values of $q$ when $c = 2$ assuming no interaction between the two types of fertility insurance and right shows the relationship between ES sex ratio and inbreeding rate arising from equations 1-4, for given values of $q$ when $c = 2$.

![Figure 6.3](image2.png)  
**Figure 6.3:** Left shows the relationship between predicted sex ratio and inbreeding rate, for given values of $q$ when $c = 4$ assuming no interaction between the two types of fertility insurance and right
shows the relationship between ES sex ratio and inbreeding rate arising from equations 1-4, for given values of \( q \) when \( c = 4 \).

Figure 6.4: Left shows the relationship between predicted sex ratio and inbreeding rate, for given values of \( q \) when \( c = 8 \) assuming no interaction between the two types of fertility insurance and right shows the relationship between ES sex ratio and inbreeding rate arising from equations 1-4, for given values of \( q \) when \( c = 8 \).

### 6.5 Appendix

In West et al. (2002b) the implications of finite mating group size for fertility insurance were made amenable for mathematical treatment by assuming infinite male fecundity. This represents a special case of our model, such that \( c = \infty \) and equations (3) and (4) reduce to

\[
S'_{N,0} = \sum_{\mu_N = 0}^{q} \binom{q}{\mu_N} \mu_N^{\mu_N} (1 - \zeta_N)^{q - \mu_N} 2 \zeta
\]  

(A.1a)

where

\[
\zeta = \begin{cases} 
q - \mu_N & \text{if } \mu_N > 0 \\
0 & \text{if } \mu_N = 0
\end{cases}
\]  

(A.1b)

and
\[ S_{M,0} = \sum_{\tau_{M}=0}^{\infty} \sum_{\mu_{M}=0}^{\infty} \sum_{\nu_{M}=0}^{\infty} \left( \frac{q}{\tau_{M}} \right)^{\nu_{M}} (1-f)^{\nu_{M}} \left( \frac{\tau_{M}}{\mu_{M}} \right)^{\mu_{M}} (1-z_{M})^{\tau_{M}-\nu_{M}} \left( \frac{q-\tau_{M}}{\mu_{N}} \right)^{\nu_{N}+\tau_{M}-\nu_{M}} \left( \frac{\tau_{N}}{\mu_{N}} \right)^{\nu_{N}} (1-z_{N})^{\nu_{M}+\tau_{M}-\nu_{N}} \right) \]  

where

\[ \zeta = \begin{cases} 
q - \mu_{M} - \mu_{N} & \text{if } \mu_{M} + \mu_{N} > 0 \\
0 & \text{if } \mu_{M} + \mu_{N} = 0 
\end{cases} \]  

(A.2b)

\[ E[\sigma_{M,1}] = \begin{cases} 
\frac{\mu_{M}}{\mu_{M} + \mu_{N}} & \text{if } \mu_{M} + \mu_{N} > 0 \\
0 & \text{if } \mu_{M} + \mu_{N} = 0 
\end{cases} \]  

(A.2c)

\[ E[\sigma_{M,0}] = \begin{cases} 
\frac{\tau_{M} - \mu_{M}}{q - \mu_{M} - \mu_{N}} & \text{if } q - \mu_{M} - \mu_{N} > 0 \\
0 & \text{if } q - \mu_{M} - \mu_{N} = 0 
\end{cases} \]  

(A.2d)

Conversely, in the deterministic analysis of Read et al. (1992), the fertility insurance consequences of limited male fecundity were investigated under the assumption of infinite mating group size. This special case, \( q = \infty \), reduces equations (3) and (4) to give

\[ S_{N,0} = 2q \min\{c, z_{N}(1-z_{N})\} \]  

(A.3)

and

\[ S_{M,1} = q \min\{c(z_{M}f + z_{N}(1-f)),(1-z_{M})f + (1-z_{N})(1-f)\} \]  

\[ \frac{z_{M}f}{(z_{M}f + z_{N}(1-f))} + \frac{(1-z_{M})f}{(1-z_{M})f + (1-z_{N})(1-f)} \]  

(A.4)

Although both \( S_{N,0} \) and \( S_{M,1} \) are linear functions of \( q \), and therefore have infinite solutions, the relative fitness of the Null allele may still be evaluated as \( \omega \) is the ratio of the two and hence is finite. The predictions converge with those of Read et al. (1992) for \( c \geq 1 \), but being more general, are able to predict the male biased ES sex ratio when males fecundity is more limiting than that of females, so that \( c < 1 \).

I considered the possibility of stochastic male fecundity, specifically, how accurately do expressions (3) and (4) predict the ES sex ratio when the value of \( c \) represents the expectation
of a random variable? Assuming that males all produce the same species-specific number (χ) of gametes of which a proportion p will be viable for fertilization, (3) and (4) become

\[ S_{N,0} = \sum_{\mu_N = 0}^{a} \sum_{g_N = 0}^{\mu_N} \left( q \right)^{\mu_N} \left( 1 - z_N \right)^{\mu_N} \left( \frac{\chi}{g_N} \right)^{\mu_N} \left( 1 - p \right)^{\mu_N - g_N} 2 \min \{g_N, q - \mu_N\} \]  

(A.5)

and

\[ S_{M,0} = \sum_{\tau_M = 0}^{b} \sum_{\mu_M = 0}^{\tau_M} \sum_{g_M = 0}^{\mu_M} \sum_{\tau_M = 0}^{\mu_M} \left( q \right)^{\mu_M} \left( 1 - f \right)^{\mu_M} \left( \frac{\tau_M}{\mu_M} \right)^{\mu_M} \left( 1 - z_M \right)^{\mu_M} \left( \frac{q - \tau_M}{\mu_M} \right)^{\tau_M} \left( 1 - z_M \right)^{\tau_M - \mu_M} \]

\[ \left( \frac{\chi}{g_M} \right) \left( \frac{\chi}{g_M} \right) \left( 1 - p \right)^{\mu_M} \min \{g_M, g_N, q - \mu_N\} \]  

(A.6a)

where

\[ E[\sigma_{M,1}] = \begin{cases} \frac{g_M}{g_M + g_N} & \text{if } g_M + g_N > 0, \\ 0 & \text{if } g_M + g_N = 0 \end{cases} \]  

(A.6b)

\[ E[\sigma_{M,0}] = \begin{cases} \frac{\tau_M - \mu_M}{q - \mu_M - \mu_N} & \text{if } q - \mu_M - \mu_N > 0, \\ 0 & \text{if } q - \mu_M - \mu_N = 0 \end{cases} \]  

(A.6c)
CHAPTER 7

Host anaemia and sex in malaria parasites.

7.1 Summary

Malaria and other haemosporin parasites must undergo a round of sexual reproduction in the vector in order to transmit to new hosts. Consequently, it is crucial that parasites produce the sex ratio (proportion male), that will maximise transmission to new hosts. There is some evidence to show that, consistent with evolutionary theory, the sex ratios of malaria parasites are negatively correlated to their inbreeding rate and this relationship has provided a broad scale understanding of protozoan sex ratios. However, observed sex ratios and those most infective to mosquitoes can be less female biased than theory predicts. It has recently been suggested that malaria sex ratios increase with the host’s anaemic response, and this is a facultative response to maintain transmission success. We tested the roles that host anaemia, erythropoiesis and nitric oxide play in shaping both the investment in sexual stages and sex allocation in the rodent malaria parasite *Plasmodium chabaudi*. 
7.2 Introduction

Sex allocation theory has been successfully applied in a broad range of taxa and provides some of the clearest examples of adaptation (Godfray and Werren, 1996; West, et al., 2000). The predictions of sex allocation theory often agree both qualitatively and in some cases quantitatively with observed sex ratios. In malaria and related Apicomplexan parasites, theory predicts that the gametocyte (sexual stage) sex ratio has been shaped by the inbreeding rate (Read and Day, 1992; Read et al., 1995; West et al., 2000b; Nee et al., 2002). When mating groups consist of gametocytes from one genotype, the unbeatable strategy is to produce just enough males to fertilise the females – which results in extremely female biased sex ratios (Hamilton, 1967). When the inbreeding rate is lower and males are fertilising unrelated females, a sex ratio closer to equality is favoured. This theory has provided a general understanding of protozoan sex ratios, but there are many discrepancies that are yet to be explained. For instance, sex ratios vary throughout an infection and the sex ratios most infective to mosquitoes are often less female biased than predicted by their inbreeding rate (Shutler et al., 1995; Robert et al., 1996; Shutler and Read, 1998).

Recently, Paul et al., (2000; reviewed in A7), have shown that sex allocation in both a rodent and an avian malaria parasite (Plasmodium vinckeii and P. gallinaceum), may be facultatively altered in response to host anaemia. When hosts are anaemic, the kidney and liver cells are stimulated to secrete erythropoetin (Epo) which initiates the differentiation of young red blood cells (reticulocytes) from precursor cells in a process termed erythropoiesis (Jelkmann, 1994); 3- 4 days after stimulation by Epo, reticulocytes appear in the circulation where they mature into erythrocytes. As the sex ratios observed in Paul et al.’s (2001) Epo group began to increase faster then the control group 1 day after Epo administration it appears that the parasites were responding to changes in Epo levels rather than changes in reticulocyte or erythrocyte density. Why should parasites increase their sex ratio in response to a rise their host’s Epo level? Paul et al., suggested that Epo levels in natural infections may correlate with the appearance of host factors that are detrimental to fertilisation success (Paul et al., 2000; Paul et al., 2002). Thus, in this environment, increasing investment in male gametocytes is a sensible strategy to ‘insure fertilisation’ (West et al., 2001b, 2002b). A number of studies have demonstrated a variety of host immune factors that impair fertilisation in the mosquito,
but it is not known if any of these factors are more detrimental to male gametes than to females.

There are a number of ways in which the host environment can impair fertilisation in the mosquito. A wealth of transmission-blocking studies has shown that host antibodies, especially immunoglobulins (IgG) can immobilise and agglutinate gametes in the blood meal (Gwadz, 1976; Aikawa et al., 1981; (Mendis and Targett, 1981). As male gametes swim around the blood meal in order to locate immotile female gametes, IgG could be more detrimental to males than females. However, whether host IgG levels correlate with Epo levels is not clear. The level of host nitric oxide (NO), increases during an infection, and NO has been shown to reduce transmission (Carter and Mendis, 1991). NO can impair DNA replication and could prevent male gametocytes from replicating their DNA during gametogenesis, but female gametogenesis does not involve DNA replication (Taylor-Robinson and Smith, 1999; Heyde et al., 2000). Slight anaemia facilitates vector feeding, but severe anaemia reduces blood meal size (Shieh and Rossignol, 1992) which could result in too few males being present in the blood meal (low q; see chapter 6). As hosts become more anaemic they produce more Epo and the reticulocyte density increases. Reticulocytes are a known cue for gametocyte production in many Plasmodium species and this may occur via an interaction with NO in the bone marrow (Millon and David, 1999). I conducted 2 experiments to test the roles that host anaemia, Epo, reticulocyte density and nitric oxide play in shaping both the investment in gametocytes and their sex ratios in the rodent malaria parasite Plasmodium chabaudi.

### 7.3 Methods

#### 7.3.1 Parasites and hosts

I gave C57 black mice at 10-12 weeks (Harlan-Olac, UK) an intra peritoneal inoculation of $10^6$ red blood cells parasitised with *Plasmodium chabaudi*, clone ER (WHO Registry of Standard Malaria Parasites, University of Edinburgh, UK), in 0.1ml doses consisting of 47.5% ringers (27mM KCl, 27mM Ca Cl₂, 0.15M NaCl), 50% heat inactivated calf serum
and 2.5% heparin (200 units ml\(^{-1}\)). I housed all mice in groups of 5 at 20°C with a 12hr light /12hr dark cycle, and provided food (41B, Harlan-Teklad, UK) and water with 0.05% pABA (to enhance parasite growth) \(ad\ lib\).

### 7.3.2 Experimental design

In experiment 1, I followed the methodology of Paul \(et\ al\). (2000), and administered Epo when parasites became detectable. By administering Epo before hosts became anaemic, I avoided confounding artificial and varying levels of naturally produced Epo. I used a total of 20 male mice, 10 in the Epo treatment and 10 in the control group. Treatment mice received 5x0.1ml intraperitoneal injections of 100units/ml of mouse recombinant Epo (Roche biochemicals, UK) from day 4-9 post infection (P.I.), and control mice received sham injections of distilled water, on days 4-9P.I. I sampled all mice daily from day 3P.I. to day 17P.I.

In experiment 2, I administered Epo prior to, and during, the gametocyte peak, when hosts had recovered from their anaemia crisis. I used a total of 15 female mice, 8 in the Epo treatment and 7 in the control group. Treatment mice received 5x0.1ml intraperitoneal injections of 100units/ml of mouse recombinant Epo (Roche biochemicals, UK) from day 11-15P.I., and control mice received sham injections of distilled water. I assayed the nitric oxide concentration in the blood of all mice from day 12-17P.I. This assay, based on the Greiss reagent, is suitable for samples with high protein content and low concentrations of nitric oxide (therefore appropriate for blood samples of 10μl; Oxford Biochemicals; USA). I sampled all mice daily from day 11P.I. to day 18P.I.

### 7.3.4 Data collection

On each daily sampling point I took thin blood smears from the tail vein of each mouse to determine the proportion of red blood cells infected with: gametocytes (gametocytaemia); asexual parasites (parasitaemia) and the proportion of red blood cells that were immature (reticulocytes). I stained smears using 10% Giemsa buffered in 90% phosphate solution for 15mins. I measured mass and calculated parasite, reticulocyte and gametocyte densities using red blood cell densities measured daily (Coulter Electronics).
7.3.5 Sexing parasites

I measured the sex ratio each day by taking ~1µl blood from the tail vein and let it stand for 15 mins in the cap of a 0.5ml eppendorf tube containing warm water. This procedure kept the blood humid whilst it cooled and the gametocytes inside began to differentiate into gametes, which facilitates accurate sexing of gametocytes. After 15 mins, the cooled blood was dropped onto 3 slides, smeared and stained as above. Gametocytes were not sexed in the standard way (from smears taken directly from the tail vein), as this method may lead to an over estimation of the proportion female in P. chabaudi (see chapter 5).

7.3.6 Analysis

In experiment 1, in order to present and analyse these data, each x axis for each infection was adjusted from ‘day post infection’ to day with respect to ‘maximum anaemia’. I did this for several reasons; (1) to follow similar methodology to Paul et al. (2001), who used day with respect to ‘peak parasitaemia’, (2) as Epo affects host anaemia, we expect anaemia to be driving any patterns in the data, and (3) it removed excess noise in the data set arising from hosts reaching the same point in their infection on different days. In experiment 2, data remained as ‘day post infection’ as I did not monitor infections prior to day 11P.I.

To investigate the effects of the Epo treatment in both experiments, I used generalised linear modelling techniques in S-Plus (Insightful corporation). I used F tests to assess the effect of each term in a nested model, to avoid pseudoreplication at the mouse level (Crawley, 1993; 2002). To retain maximum power when analysing sex ratio (proportion) data it is appropriate to assume binomial errors and a logit link function in an analysis of deviance, as proportion data often have non-normally distributed error variance and unequal sample size (Crawley, 1993).

In addition, I also used these data to calculate a summary statistic for each mouse, for each infection parameter, to assess the effects of Epo treatment. For asexual parasite, reticulocyte and total gametocyte densities, I calculated total production using the area under the curve for each mouse. In experiment 1, I investigated gametocyte production in more detail by splitting the total gametocyte density per infection into gametocytes produced during and after peak asexual density (day –7 to +2 and day +2 to +9 with respect to max anaemia, respectively).
used maximum weight loss and minimum RBC density for mice in experiment 1, and maximum RCB density for mice in experiment 2. I compared the summary statistics for each parameter using a 2 sample $T$ test.

To investigate hypothesised correlations between sex ratio, NO, anaemia, reticulocyte density and time, I calculated the correlation coefficient for each mouse, for each relationship investigated. This avoided pseudoreplication due to repeated measures on the same mouse. I arcsine-square root transformed the sex ratio to assume normal errors and tested for correlations using 2 methods. I used a 2 sample $T$ test to assess whether, for each correlation, the coefficients in the Epo and control group were significantly different. I then tested whether the coefficients for each correlation were significantly different from 0 with a 1- sample $T$ test, I did this for each treatment group where necessary. The co-ordinates in each correlation consisted of measurements taken on the same day. *P. chabaudi* gametocytes are thought to take 24-36hrs to mature and then spend a further 24-36hrs in the circulation. Therefore, depending on when sex is determined (between shizogony and maturity), both sex ratio and gametocyte production responses, would presumably, be more obvious in the 2 days following the stimulus than on the same day. To allow for this, I also correlated the sex ratios and gametocyte densities observed on both $t + 1$ and $t + 2$ days following the potential stimuli measured on day $t=0$.

7.4 Results

7.4.1 Effects of Epo on summary statistics
The results for the effects of Epo on the summary statistics for each infection parameter, in both experiments are given in table 7.1. Briefly, Epo treatment had a significant effect on gametocyte, reticulocyte and asexual production. In experiment 1, total gametocyte production before crisis was higher in the Epo group ($T = 3.55$, $N = 14$, $P = 0.004$), but there was no significant difference in either post crisis or total gametocyte production. Total
gametocyte production was also higher in the Epo group in experiment 2 ($T = -5.13$, $N = 15$, $P = 0.0002$). Total reticulocyte production was significantly higher in the Epo group in both experiments ($T = 2.56$, $N = 14$, $P = 0.025$ and $T = -5.45$, $N = 15$, $P = 0.0001$ respectively). Total asexual parasite production was significantly higher in the Epo group in experiment 2 ($T = -2.95$, $N = 15$, $P = 0.011$).

7.4.2 Effects of Epo on parameters during the infection

The results for the effect of Epo treatment on the daily measurement data, analysed using nested GLMs, are given in table 7.2. In summary, Epo treatment did not have a significant effect in either experiment on gametocyte density (figure 7.1a&b; $F_{1,13} = 3.54$, $P = 0.082$ and $F_{1,13} = 1.00$, $P = 0.336$ respectively), sex ratio (figure 7.2a&b; $F_{1,13} = 1.17$, $P = 0.299$ and $F_{1,1} = 0.01$, $P = 0.922$ respectively), reticulocyte density ($F_{1,13} = 1.42$, $P = 0.255$ and $F_{1,13} = 3.03$, $P = 0.105$ respectively), or NO level (figure 7.3; $F_{1,13} = 2.01$, $P = 0.180$). Day post infection had a significant effect on all parameters in experiment 1, but not on sex ratio, reticulocyte density and NO level in experiment 2.

![Figure 7.1a](image)

Figure 7.1a: The gametocyte density in the Epo treated group (dashed line), and control group (solid line), during experiment 1. The x-axis is day post infection relative to max anaemia, i.e. scaled so that all infections reached maximum anaemia on day 0, and bars are standard error of the mean.
Figure 7.1b: The gametocyte density in the Epo treated group (dashed line), and control group (solid line), during experiment 2. The x-axis shows day post infection, and bars are standard error of the mean. The period on the x-axis corresponds to ~ 4-9 in figure 7.1a.

Figure 7.2a: The mean sex ratio during the course of experiment 1, for the Epo group (dashed line), and control group (unbroken line). The x-axis is scaled so that all infections reached maximum anaemia on day 0, and bars are standard error of the mean.
Figure 7.2b: The mean sex ratio during the course of experiment 2 for the Epo group (dashed line), and control group (unbroken line). The x-axis shows day post infection, and bars are standard error of the mean. The period on the x-axis corresponds to ~ 4-9 in figure 7.2a.

Figure 7.3: Mean nitric oxide production of the Epo group (dashed line), and control group (unbroken line), during experiment 2. The x-axis shows day post infection, and bars are standard error of the mean.

7.4.3 Correlation data
Consistent correlations with a $P$ value $< 0.01$ are reported in table 7.3. The sex ratio has a negative correlation with reticulocyte density (figure 7.4; $r = -0.33$, $T = -5.13$, $N = 14$, $P = 0.0002$). This relationship was significant when sex ratios were measured at $t=0$, $t+1$ and $t+2$ days after reticulocyte density. Reticulocytes also had a significant positive correlation with NO levels ($r = 0.24$, $T = 3.60$, $N = 14$, $P = 0.003$), and a positive correlation with day post
infection (Epo group: \( r = 0.51, T = 6.01, N = 7, P = 0.001 \); control group: \( r = 0.75, T = 15.55, N = 7, P = 0.0001 \)). Red blood cell density showed a significant negative correlation with the gametocyte density at \( t=0 \), \( t+1 \) and \( t+2 \) days later (\( t + 2: r = -0.29, T = -3.10, N = 14, P = 0.008 \)).

![Correlation Coefficient Graph](image)

Figure 7.4: Shows the negative correlation between reticulocyte density and the sex ratio at \( t +2 \) for each mouse in the Epo and control group. Bars are standard error of the mean.

Table 7.1: Shows the test details for the effect of Epo on all infection parameters measured in experiments 1 and 2. The mean and standards error for each parameter in the Epo group (E), and control group (C), are also given.

<table>
<thead>
<tr>
<th>Effect of Epo on:</th>
<th>Exp</th>
<th>Test details</th>
<th>Mean &amp; s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaemia, max RBC loss (RBCx10^9/ml)</td>
<td>1</td>
<td>( T = -0.49 ) ( P = 0.636 )</td>
<td>E 5.81 ±0.54 C 6.23 ±0.69</td>
</tr>
<tr>
<td>Max mass loss (g)</td>
<td>1</td>
<td>( T = -1.75 ) ( P = 0.110 )</td>
<td>E 3.33 ±0.34 C 4.63 ±0.54</td>
</tr>
<tr>
<td>Total parasite density (x10^9/ml)</td>
<td>1</td>
<td>( T = 0.91 ) ( P = 0.380 )</td>
<td>E 7.37 ±0.84 C 6.48 ±0.55</td>
</tr>
<tr>
<td>Total reticulocyte density (x10^9/ml)</td>
<td>1</td>
<td>( T = 2.56 ) ( P = 0.025 )</td>
<td>E 18.09 ±0.66 C 15.45 ±0.78</td>
</tr>
<tr>
<td>Total pre crisis gametocytes (x10^6/ml)</td>
<td>1</td>
<td>( T = 3.55 ) ( P = 0.004 )</td>
<td>E 42.05 ±7.41 C 17.18 ±4.28</td>
</tr>
<tr>
<td>Total post crisis gametocytes (x10^6/ml)</td>
<td>1</td>
<td>( T = -1.72 ) ( P = 0.110 )</td>
<td>E 20.68 ±2.05 C 28.45 ±4.20</td>
</tr>
<tr>
<td>Total gametocytes (x10^6/ml)</td>
<td>1</td>
<td>( T = 1.63 ) ( P = 0.130 )</td>
<td>E 61.38 ±23.10 C 45.63 ±5.57</td>
</tr>
<tr>
<td>Sex Ratio: mean</td>
<td>1</td>
<td>( T = 1.22 ) ( P = 0.245 )</td>
<td>E 0.25±0.02 C 0.25±0.02</td>
</tr>
<tr>
<td>Sex Ratio: upper 95% CI for mean</td>
<td>1</td>
<td>( T = 0.914 ) ( P = 0.379 )</td>
<td>E 0.37±0.02 C 0.34±0.03</td>
</tr>
<tr>
<td>Sex Ratio: lower 95% CI for mean</td>
<td>1</td>
<td>( T = 1.43 ) ( P = 0.178 )</td>
<td>E 0.18±0.02 C 0.15±0.02</td>
</tr>
<tr>
<td>Exp</td>
<td>Factor</td>
<td>Result</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>--------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Epo</td>
<td>$F_{(1,13)} = 0.67, P = 0.428$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Day</td>
<td>$F_{(1,13)} = 15.15, P = 0.002$</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Mouse</td>
<td>$F_{(13,14)} = 1.22, P = 0.357$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Epo</td>
<td>$F_{(1,13)} = 2.27, P = 0.155$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Day</td>
<td>$F_{(1,13)} = 12.22, P = 0.001$</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Mouse</td>
<td>$F_{(13,14)} = 0.91, P = 0.565$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Epo</td>
<td>$F_{(1,13)} = 1.42, P = 0.255$</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.2**: Shows the GLM results for the effect of Epo, day post infection and mouse on all infection parameters measured in experiments 1 and 2. The interaction between Epo and day post infection is included when significant.
<table>
<thead>
<tr>
<th>Relationship</th>
<th>Results</th>
<th>Mean &amp; 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocytes and NO</td>
<td>$T = 3.60, P = 0.003$</td>
<td>0.24 [0.10, 0.38]</td>
</tr>
<tr>
<td>Gametocytes @t+2 and red blood cell density</td>
<td>$T = -3.10, P = 0.008$</td>
<td>-0.29 [-0.48, -0.09]</td>
</tr>
<tr>
<td>Reticulocytes and day post infection</td>
<td>$E: T = 0.61, P = 0.001$</td>
<td>$E: 0.51 [0.30, 0.70]$</td>
</tr>
<tr>
<td></td>
<td>$C: T = 15.55, P = 0.000$</td>
<td>$C: 0.75 [0.63, 0.86]$</td>
</tr>
<tr>
<td>Sex ratio @ t+2 and reticulocytes</td>
<td>$T = -5.13, P = 0.0002$</td>
<td>-0.33 [-0.47, -0.20]</td>
</tr>
</tbody>
</table>

Table 7.3: Shows the significant correlations for relationships tested in experiments 1 and 2. Correlations are reported as significant when $P < 0.01$ and when correlations involved sex ratio or gametocyte density, the relationship must be consistent at day $t=0$, $t + 1$ and $t + 2$. 
Epo significantly increased total reticulocyte production in both experiments - therefore, the experimental manipulation was successful. In contrast to Paul et al.'s, results, these experiments show that Epo has no effect on the sex ratio of *P. chabaudi* gametocytes, but it does increase the timing and level of investment in gametocytes. In experiment 1, Epo treated infections produced more gametocytes early in the infection, but the asexual parasite density was not significantly different to that in the control infections. The total number of gametocytes produced post crisis and over the entire infection did not differ between the 2 groups, but there appears to be trend showing that the Epo infections produced their peak gametocytes much earlier than the control infections. This is unexpected as the control infections did not have more asexuals, which indicates that either the Epo group had a lower conversion rate at this point in the infection, or the conversion rate was the same but more gametocytes in the Epo group were cleared from the circulation. The latter could occur if the early peak in Epo gametocytes exceeded an ‘antigen threshold’ required for host recognition, and a stronger immune response was subsequently mounted. In addition, there was no difference in NO production between the Epo treated and control hosts.

### 7.5.1 Relationships between anaemia, Epo, reticulocytes, NO and gametocytes

Several patterns emerged from the correlational data: Firstly, sex ratio has a strong negative correlation with reticulocyte density. Reticulocyte density increased as the infection progressed, but the sex ratio showed a very weak negative correlation with day post infection, therefore day is unlikely to be causing the relationship between sex ratio and reticulocytes. This is not consistent with previous reported observations of the sex ratio becoming less female biased as infections progress, and that Epo elevates reticulocyte density whilst increasing the sex ratio (Paul *et al.*, 2001; 2002). Secondly, gametocyte density at $t+2$ showed a negative correlation with red blood cell density: i.e. more gametocytes were observed in anaemic hosts. This is consistent with my results showing that gametocyte production is enhanced by Epo, as anaemic hosts have high Epo levels. Chang and Stevenson (2002), have shown that Epo levels are elevated on days 8-10 post infection (figure 7.5),
therefore, unless mature gametocytes are first observed 6 days after Epo stimulus, we would expect peak gametocyte production to occur well before day 14 (see figure 7.1). Given observed timings, it seems more likely that reticulocytes act as a cue for gametocyte production: For example, Epo stimulus on day 8 will result in circulating reticulocytes on ~day 12, and resulting mature gametocytes will be observed 2 days later, on ~day 14, which fits the observed patterns. However, it is not clear if this is the case because I did not find a positive relationship between reticulocyte and gametocyte densities, unlike previous studies (Yap, 2000, Gautret et al., 1996). Finally, there was a significant positive correlation between NO and reticulocyte density. Both reticulocytes and gametocytes are thought to be produced in the bone marrow, and low levels on NO in the bone marrow have been suggested as a cue for gametocyte differentiation (Millon and David, 1999). There was no effect of Epo treatment on NO levels, so if this relationship is causal it suggests that both Epo and NO may act independently increase reticulocyte production, but not gametocyte production.

Figure 7.5: The mean Epo level during a *P. chabaudi* infection in C57 Black mice, initiated with 1x10⁶ parasites, bars are standard error of the mean. Courtesy of Chang and Stevenson (2002).
7.5.2 Contradictory results in different species?

This study shows that Epo increases gametocyte production, but has no effect on the sex ratio in *P. chabaudi*. Paul *et al.*’s experiments show that Epo increases the sex ratio but not gametocyte density in *P. vinkei* and *P. gallinaceum*. Other experiments in *P. berghei* showed the same pattern as I observed here, with gametocyte density increasing, and the sex ratio remaining unchanged (Janse, pers. comm.). Taken together, these diverse results indicate that these species may have different responses to stressful host conditions. Severe anaemia is one of the major causes of host death, if hosts survive this crisis period they go on to mount an effective immune response against their parasites (Cohen and Deans, 1988). During this period, the risk of host death or the subsequent immune response may select for parasites that increase investment in transmission at a cost to host survival or asexual growth. As hosts become more anaemic, the ratio of reticulocytes to mature red blood cells increases and parasite species that cannot invade reticulocytes may become resource limited. *P. chabaudi* and *P. berghei* do invade reticulocytes (*P. berghei* only invades reticulocytes), so these parasites may maintain or increase their investment in gametocytes through using reticulocytes. Where as *P. vinkei* and *P. gallinaceum* will not invade reticulocytes, so red blood cells may become too limited for increased investment in gametocytes, and increasing their sex ratio allows transmission success to be maintained. This hypothesis remains to be tested. However, due to the timing of the observed Epo responses in the *P. chabaudi*, *P. vinkei* and *P. gallinaceum*, it is unclear if they are responding to Epo itself or subsequent reticulocytes.

7.5.3 Sex ratio variation during the infection

Experiment 1 (figure 7.2a), shows that the sex ratio varies extensively, from 10 – 50% male, and stabilising at 20% during peak gametocyte production. This contrasts with results for *P. vinkei*, *P. berghei*, *P. gallinaceum* and *P. falciparum* in which the sex ratio increases to 0.5 as the infection progresses. When peak gametocyte density is observed in *P. chabaudi* (i.e. high *q*: chapter 6, the number of gametocytes whose gametes can interact), hosts have just recovered from their anaemia crisis, where as in the other species, gametocyte density and anaemia increase until host death. Thus, in *P. chabaudi*, there may be little reduction in fertilisation success due to anaemic blood meals containing too few gametocytes (a low *q*).
This would account for the high sex ratio observed during the anaemia crisis, and the low sex ratio observed when pre-crisis gametocyte density is highest. However, there was no correlation between sex ratio and red blood cell density or gametocyte density, suggesting that these factors may not be cues for sex allocation. In addition, according to theory, the unbeatable sex ratio for a single genotype at a high gametocyte density is \( \sim 0.1 \), yet observed sex ratios are \( \geq 0.2 \). This could indicate that *P. chabaudi* parasites have increased their sex ratio, even at peak density, when \( q \) is not expected to be limiting. However, it is also unclear why the sex ratio should vary so much at the start of the infection, before specific immune responses appear.

It is clear that much further work is required to tease apart the inter-correlated influences of reticulocytes, anaemia, Epo and NO on gametocytes and their sex ratio. In particular, experimental manipulations to address whether parasites use Epo or reticulocytes as a cue to shape their transmission strategies (gametocyte investment and sex ratio), are challenging but crucial to understanding transmission in malaria parasites. Given the differential mortality results obtained in chapter 5, it is possible that observed sex ratios are underestimating the female bias present in the primary sex ratio. Much further work needs to be done in order to identify why such differential mortality exists and whether is present throughout the infection, before beginning to address whether factors such as Epo interact with mortality rates. In *Plasmodium chabaudi* at least, it is unlikely that an accumulation of males is responsible for the sex ratio increases seen in figure 7.2a due to the half life of male gametocytes being relatively short compared to the time taken to reach maturity and their daily production cycle.
CHAPTER 8

Discussion

Discussions relating to the specific experiments in this thesis are given at the end of the relevant chapter. In this chapter I summarise the findings from each chapter and focus on some of the broader issues and questions arising. I also outline possible directions for addressing these questions.

8.1 Sex ratios in *Nasonia vitripennis*

The experiments presented in chapter 2 show that female *Nasonia vitripennis* do not alter their offspring sex ratio in response to the relatedness of their mate, which implies that they do not recognise kin. However, the model developed to predict the unbeatable sex ratios for sib and non-sib mated females shows that the expected difference when there are 2 foundresses is quite small (~5%). Even though a power analysis revealed that our sample sizes were high enough to detect an effect of this size, it would be ideal to replicate the experiments using more foundresses in the experimental patch. For example, if females can discriminate kin, we would expect to see a much greater difference in the sex ratios produced by sib and non-sib mated females from 4 or 5 foundress patches. An interesting extension to these experiments would be to investigate whether females can recognise their sisters. A female ovipositing on a patch with her sisters is predicted to produce a more female biased sex ratio than females on a patch with non-relatives (Taylor & Crespi, 1994; Frank 1995). This occurs because females should reduce competition for their sister’s sons as well as their own sons. If females continue to show no ability to discriminate kin there may be a constraint preventing kin recognition, or that females are producing the unbeatable sex ratio for an unknown trait. Understanding how females ‘optimise’ their sex allocation, clutch size and host choices simultaneously is crucial
to assessing whether females really are unable to respond to kin cues in the manner predicted by theory.

8.2 Sex allocation in sea turtles

Sea turtles exhibit temperature dependant sex determination, in which females are produced from warm nests and males from cooler nests. Sex ratios in the Mediterranean are extremely female biased as nest temperatures exceed the pivotal temperature (that which produces an equal sex ratio) for the majority of the nesting season at the majority of nesting sites in Northern Cyprus. Why sea turtles and other reptiles have temperature dependant sex determination remains unsolved. One of the most well supported hypotheses to explain the occurrence of environmental sex determination is Charnov and Bull’s (1977) differential fitness hypothesis. An experiment to investigate whether this hypothesis can explain sex determination in the loggerhead turtle revealed an interaction between sex and environment on mass at hatching, but not on other traits measured. These results are not consistent with previous observations in the literature and should be interpreted with caution. It is not possible to measure lifetime reproductive success (or many other traits) in adult turtles, so linking fitness with hatching traits is at best, approximate. Recent theory has shown that nest site fidelity could account for extreme female biases through a form of cultural inheritance: warm nests produce daughters, and daughters return to their nest site to lay their eggs, thus perpetuating the female bias (Freedberg and Wade, 2001). Unfortunately, the life history of sea turtles makes it unlikely that they can be used to test this hypothesis: but lizards with short generation times and nest site fidelity might be a useful field model organism. Explaining observed sex ratios is essential for conservation work - an important challenge is assessing whether population viability is threatened by a shortage of males in addition to the other factors reducing population size.

8.3 Sex allocation in malaria parasites

I have compared a new method for estimating sex ratios of Plasmodium chabaudi parasites with a traditional method and showed that the traditional method may lead to overestimations of the proportion of females. In light of these results I used the new method to test for
differential mortality in male and female gametocytes and investigate how host anaemia may influence the sex ratio. Unexpectedly, I found that the clearance rate of male gametocytes was significantly shorter that that of female gametocytes. This indicates that the secondary sex ratio may not be an accurate measure of the primary sex ratio - which may have implications for using secondary sex ratios to test sex allocation theory and estimate the inbreeding rate in malaria parasites. Data on whether gametocyte maturation is synchronous or asynchronous is urgently required. However, my results may only be applicable to Plasmodium chabaudi during days 14 – 16 post infection. Further work is required to test whether this pattern is present throughout the infection. If the differential mortality observed is a result of sex specific immune killing, it may not be present early in the infection, before hosts can mount antibody responses. Alternatively, the observed differential mortality may be a product of females sequestering in the capillaries at a higher rate than males.

The theoretical work revealed that there is an interaction between the number of gametocytes whose gametes can interact in the vector \((q)\) and the number of gametes produced by each male gametocyte \((c)\) on the unbeatable sex ratio. When \(q\) is small, a mating group may only contain 1 male gametocyte, which could result in \(c\) being limiting. These results show that when both \(c\) and \(q\) are low, the unbeatable sex ratio is much less female biased than previously expected. Empirical estimates of \(c\) and \(q\) are required to test this theory and further experiments can investigate how host factors may affect \(c\) and \(q\). It may be possible to estimate \(c\) by direct observation of exflagellating male gametocytes. It would be especially interesting to estimate \(c\) in the Haemoproteus species where sex ratios are often less female biased then expected (Shutler et al., 1995). Estimating \(q\) will be more challenging, but may be possible by using Epo to manipulate the gametocyte density during infections and comparing transmission success in control groups which have a lower \(q\) than Epo treated infections. The results presented in chapter 7 indicate that it may be possible to do this without altering anaemia.

Moreover, it is important to understand sex ratio variation within an infection, which may be achieved by focusing on the following questions: (1) How does transmission success vary throughout infections? This can be addressed by monitoring control infections. (2) How is
transmission success influenced by gametocyte density and the sex ratio throughout infections? Epo can be used to manipulate gametocyte density in P. chabaudi and sex ratio in P. vinkei. (3) Are parasites responding to reticulocytes, Epo or both as cues for sex ratio and gametocyte density? Host Epo levels can be manipulated easily and reticulocytes can be increased by transfusing blood from phenylhydrazine treated mice to experimental hosts. (4) How do transmission impairing factors such as NO, IgG and heparin, vary and do they correlate with the sex ratio and gametocyte density variation throughout infections? There are specific assays for these factors which are appropriate for small blood samples, thus allowing daily sampling of each host throughout infections. (5) Do different species of malaria parasite adopt different transmission strategies (sex ratio and gametocyte density) depending on their host cell preference and life history? This is much more challenging to address and may be possible through correlational studies or by administering chemicals that indicate ‘the quality of the host environment’ such as Epo, reticulocytes and chloroquine that are known to affect gametocyte density or sex ratio in different species. Many of these experiments may be easier to address using a system other than the ‘mouse – P. chabaudi model,’ as P. chabaudi has low gametocyte densities throughout most of the infection which lead to highly variable sex ratio estimates. ‘Lizard – malaria’ models provide longer lasting infections with higher gametocyte densities (Schall, 1989), which may reduce the risk of type 2 errors due to small host sample sizes and low gametocyte counts.

8.4 Concluding remarks

Given the wealth of empirical support already in the literature for sex allocation theory, it might be argued that future research in this field will only be useful for ‘dotting i’s and crossing t’s’. In this thesis I have used field, theoretical and lab approaches to examine sex ratios and test sex allocation theory in a broad range of taxa: parasitoid wasps, sea turtles and malaria parasites. The data reported here illustrate how much more work is required to address fundamental questions. For instance: Why is the sex of sea turtles and many reptiles, determined by nest temperature? Is sex environmentally determined in malaria parasites? What shapes sex ratios observed in infections of malaria parasites? What are the constraints on the unbeatable sex allocation strategies that are predicted by theory? These unanswered questions may be of importance for long term strategies to conserve threatened species and
intervention strategies to reduce the virulence or prevalence of protozoan parasites. In addition, sex ratio traits have a clear trade-off, and data are relatively easy to collect. If evolutionary biologists are still struggling to understand sex allocation it does not bode well for increasing our understanding of more complex traits such as parasite virulence.
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