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.

Contents

List of figures and tables

Declaration

It was only possible to undertake a lot of my research through collaborations, details 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:

A1 West S. A., Reece S. E. & Sheldon B. C. 2002. Sex ratios. *Heredity*, 89, 117-124.

A2 Broderick A.C., Godley B.J., Reece S. E. & Downie J. R. 2000. Incubation periods and sex ratios of green turtles: highly female biased hatchling production in the eastern Mediterranean. *Marine Ecological Proceedings*, 202, 273-281.

A3 Godley B. J., Broderick A. C., Downie J. R., Glen F., Hays G. C., Houghton J., Kirkwood I. & Reece S. E. 2001. Thermal conditions in nests of loggerhead turtles: further evidence suggesting skewed sex ratios of hatchling production in the Mediterranean. *Journal of Experimental Marine Biology and Ecology*, 263, 45-63.

A4 West S. A., Reece S. E. & Read A. 2001. Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends in Parasitology*, 17, 525-531.

A5 Reece S. E. & Read A. 2000. Malaria sex ratios. *Trends in Ecology & Evolution*, 15, 259-260.

A6 West S. A., Reece S. E. & Read A. 2003. *Toxoplasma gondii*, sex and premature rejection. *Trends in Parasitology*, In press.

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 then 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):

$$r = \frac{n-1}{2n}, \qquad (1)$$

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; Shutler 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.

1.5 Inbreeding and the sex ratio

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:

$$r = \frac{(n-1)(2-s)}{n(4-s)}, \qquad (2)$$

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.

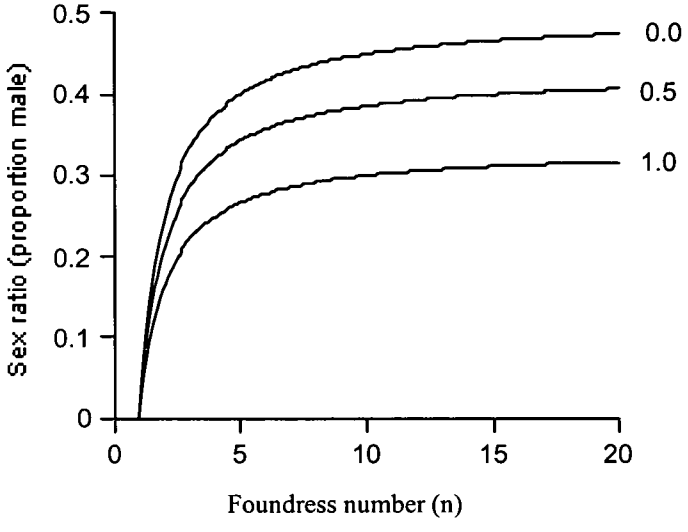


Figure 1.1: The unbeatable sex ratio where the probability of inbreeding ranges from 0 (always outbreed) to 1 (always sibmate), for haplodiploid females under various levels of LMC (foundress number).

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: (3)

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

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).

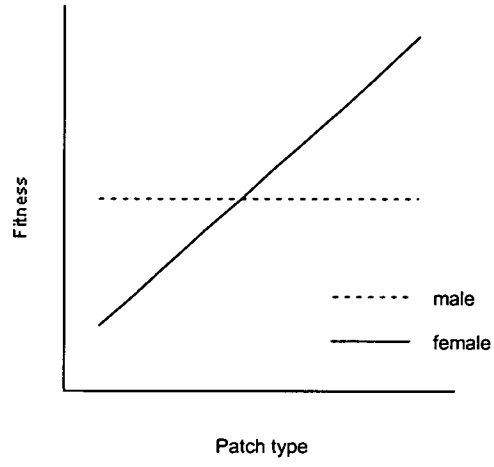


Figure 1.2: Sex bias in response to a characteristic of the environment. The fitness of one sex (in this case males) does not vary according to the environment (patch type), but the fitness of the other sex (females) does correlate with patch type. In this case, the unbeatable strategy is produce the 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 comes 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 (*Equs caballus;* Kohlman, 1999), and elk (*Cervus elaphus;* Cameron and Linklater, 2000). 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 (Charnier, 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 el., 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) is $r = (N-1)/2N$, 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 $r = (N-1)(2-p)/N(4-p)$, 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 $N < (5-2p)(1-p)$, then

$$s_0{}^* = \frac{(N-1)(2-p)}{N(4-p)^2}(Np-2p+4) \qquad \text{and}$$

$$s_1{}^* = \frac{(N-1)(2-p)}{N(4-p)^2}(N-Np-2p+5). \tag{2.1}$$

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

$$s_0{}^* = \frac{1}{2}\frac{N(1-p)-1}{N(1-p)^2}. \tag{2.2}$$

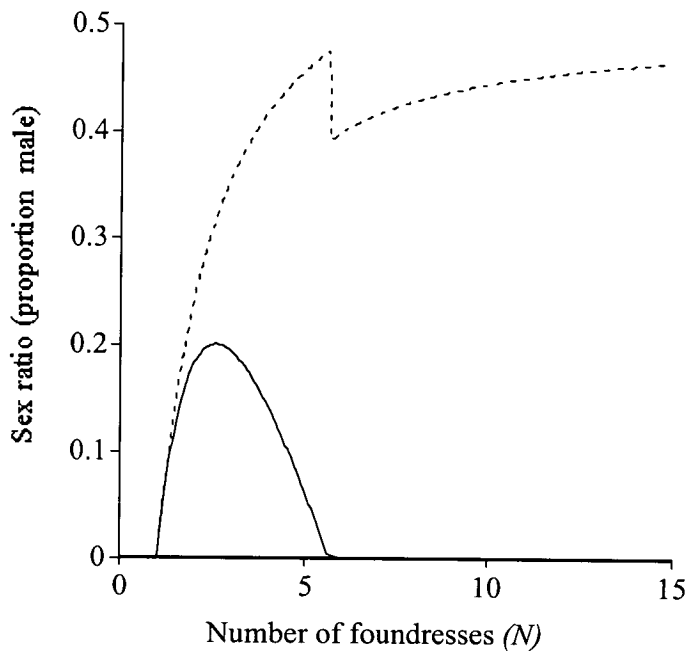


Figure 2.1: Inbreeding and facultative sex ratio adjustment. The predicted ES sex ratios for females who have mated with a sibling (unbroken line), or a non relative (dotted line), is plotted against the number of foundresses (N). The probability of sibmating (P) is assumed to be $1/N$.

2.3 Methods

2.3.1 Study organism

Nasonia vitripennis (Hymenoptera: Chalcidoidea) is a gregarious parasitoid wasp that parasitises a range of dipteran pupae including *Calliphora* and *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 *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 24hours. 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.

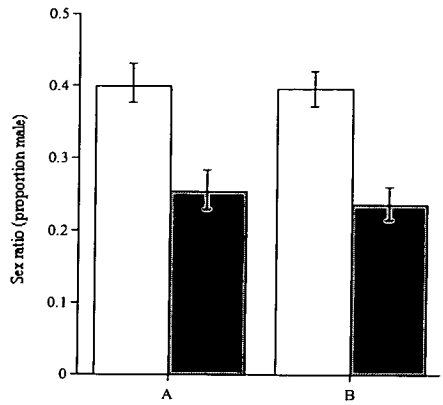


Figure 2.2: The mean sex ratio for lines R6 (unshaded), and LabII (shaded) for each treatment in experiment 1. Treatments are represented by A: sibmating and B: non sib mating. Bars are 95% confidence intervals.

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).

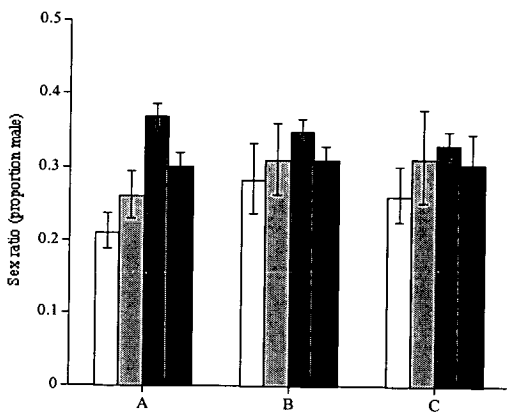


Figure 2.3: The mean sex ratio for each treatment in experiment 2, for all lines. For each treatment lines are B5, HV287, HV395 and HV55 from left to right. Treatments consist of A: siblings developing in different hosts, B: non siblings and C: siblings developing in the same host and removed prior to 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).

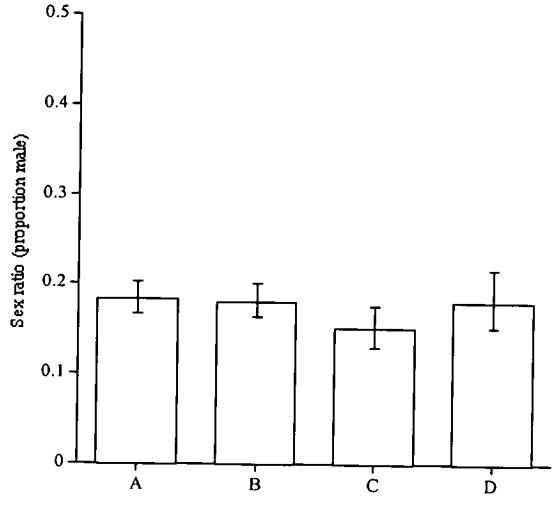


Figure 2.4: The mean sex ratio for each treatment in experiment 3, as there was no significant difference between the sex ratios produced by each line, their data has been amalgamated. A: sibmate and female bias, B: sibmate and equal sex ratio, C: non sibmate and female bias and D: non sibmate and equality. 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 α set at 0.05. The minimum significant difference I could detect between treatments in each experiment was 0.025 or less (with α = 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):

$$W_i(s_i,\bar{s}) = v_f W_{f_i}(s_i,\bar{s}) + v_m W_{mi}(s_i,\bar{s}). \qquad \text{(A1)}$$

The number of mated females obtained through daughters is simply proportional to the number of daughters produced:

$$W_{f_i} = 1 - s_i. \qquad \text{(A2)}$$

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

$$W_{mi} = s_i \frac{1-\bar{s}}{\bar{s}} = s_i \frac{1-(1-p)\bar{s}_0 - p\bar{s}_1}{(1-p)\bar{s}_0 + p\bar{s}_1} \tag{A3}$$

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

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

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

$$\begin{aligned} \left.\frac{dW_0}{ds_0}\right|_{s_0=\bar{s}_0=s_0^*} &= -v_f r_{f0} + v_m r_{m0} \frac{N(1-\bar{s}^*)-(1-p)}{N\bar{s}^*} \\ \left.\frac{dW_1}{ds_1}\right|_{s_1=\bar{s}_1=s_1^*} &= -v_f r_{f1} + v_m r_{m1} \frac{N(1-\bar{s}^*)-(1-p)}{N\bar{s}^*} \end{aligned} \tag{A5}$$

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, $r_{fo} = 1/2$ and for sibmated females I obtain

$$r_{f1} = \frac{\text{random allele daughter IBD to random allele mother}}{\text{2 random alleles mother are IBD}} = \frac{3+5\bar{F}}{4+4\bar{F}} \qquad \text{(A6)}$$

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

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

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

$$\bar{F}' = \frac{\sum q_j N_j F'_j}{\sum q_j N_j} = \frac{(1+3\bar{F})}{4\sum q_j N_j} \qquad \text{(A8)}$$

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

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

Substitution in (A6) gives

$$r_{f1} = \frac{1}{2}\left(\frac{3\bar{N}-1}{2\bar{N}-1}\right). \qquad \text{(A10)}$$

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

$$s^* = \frac{(N-1)(2-p)}{N(4-p)}. \qquad \text{(A11)}$$

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

$$\begin{aligned} s_0^* &= \frac{(N-1)(2-p)}{N(4-p)^2}(Np-2p+4) \\ s_1^* &= \frac{(N-1)(2-p)}{N(4-p)^2}(Np-N-2p+5) \end{aligned} \qquad \text{(A12)}$$

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

$$s_0^* = \frac{1}{2}\left(\frac{N(1-p)-1}{N(1-p)^2}\right). \qquad \text{(A13)}$$

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 χ^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 ($\chi^2_{(2)}$ = 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).

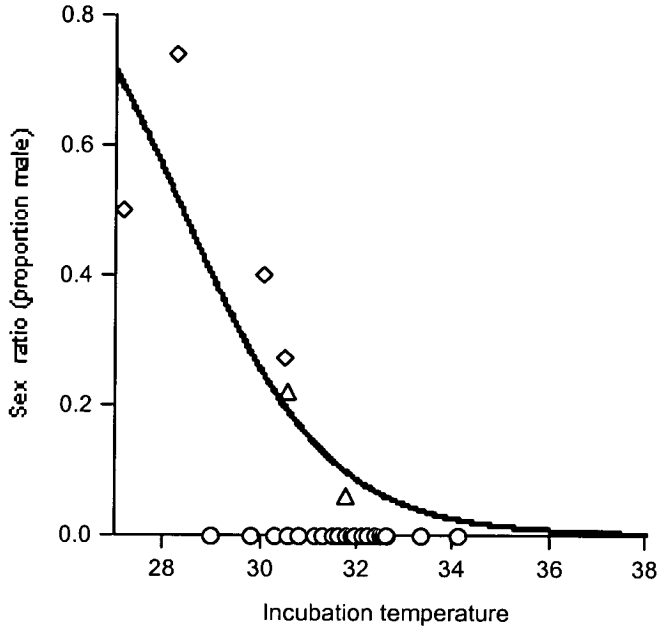


Figure 3.1a: Shows the relationship between nest temperature (°C) and sex ratio for green turtles. The line was fitted from the logistic regression model, Sex ratio = $e^{(a+bx)} / 1+e^{(a+bx)}$, where a = 32.45 b = -1.13 and x represents temperature; r^2 = 0.60 and plotted data are those collected from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

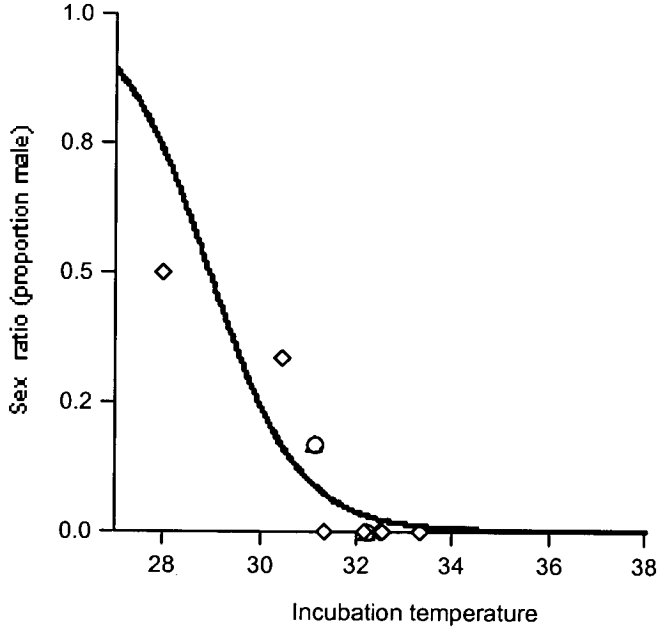


Figure 3.1b: Shows the relationship between nest temperature (°C) and sex ratio for loggerhead turtles. The line was fitted from the logistic regression model, Sex ratio = $e^{(a+bx)} / 1+e^{(a+bx)}$, where a = 27.66, b = -0.96 and x represents temperature; r^2 = 0.62 and plotted data are those collected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

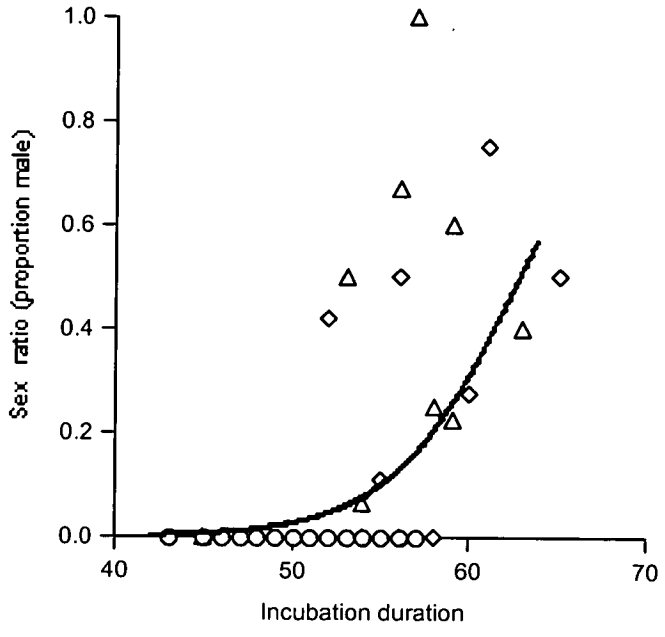


Figure 3.2a: Shows the relationship between incubation duration (days) and sex ratio for green turtles. The line was fitted from the logistic regression model, Sex ratio = $e^{(a+bx)} / 1+e^{(a+bx)}$, where a = -14.82, b = 0.23 and x represents incubation duration; r^2 = 0.53 and plotted data are those collected from 56 nests: o = site 1, Δ = site 2 and ◊ = site 3.

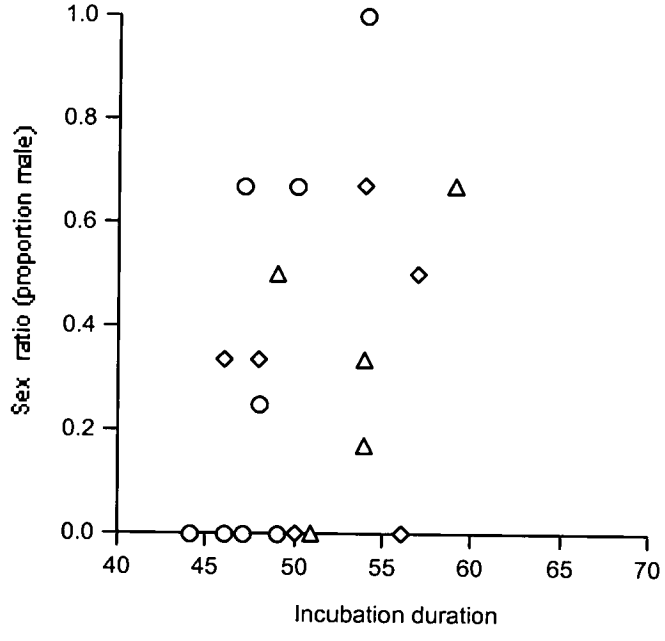


Figure 3.2b: Shows data for incubation duration (days) and sex ratio of loggerhead turtle nests. r^2 = 0.14 and plotted data are those collected from 20 nests: o = site 1, Δ = site 2 and ◊ = site 3.

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.

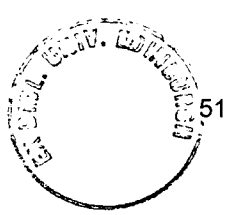
4.3.2 Experimental design

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 0.3°C) and water content was calculated from the difference in weight of a 75 g sand sample after dehydration for 4 h at 250°C. 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, $F_{1,27}$ = 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).

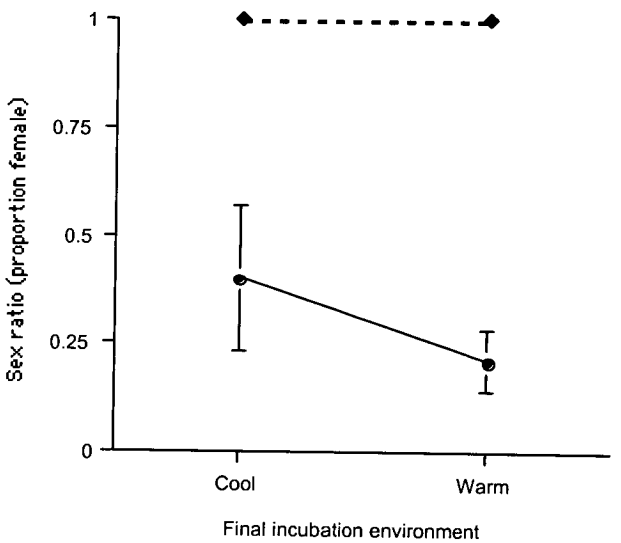


Figure 4.1: Mean sex ratio of a sample of clutches used in the experiment. Sex was determined in the warm environment for females and the cool environment for males. From the female-producing treatment, I sexed samples from four clutches in the warm environment and three clutches from the cool environment. 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.

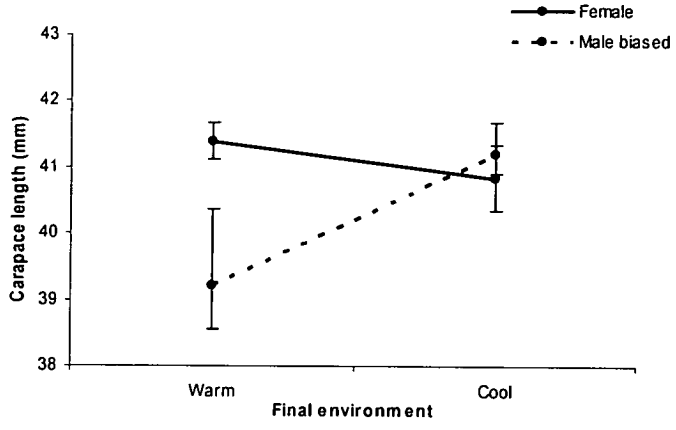
4.4.3 *Phenotype of experimental clutches*

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.

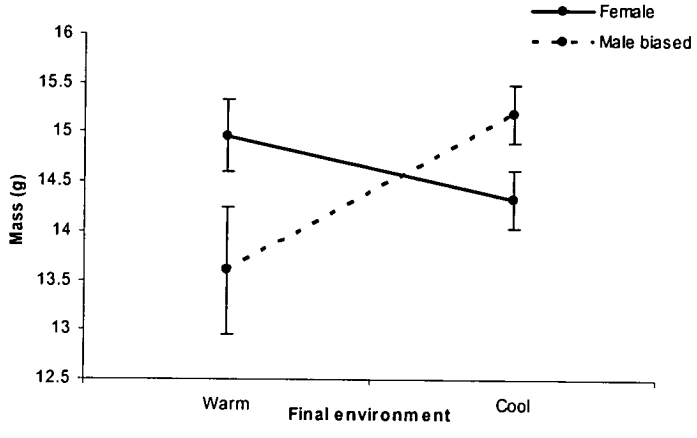
	Sex	Final environment	Sex~Final environment
Carapace length	$F_{(1,9)} = 2.57$	$F_{(1,14)} = 0.92$	$F_{(1,14)} = 2.83$
	$P = 0.143$	$P = 0.359$	$P = 0.116$
Mass	$F_{(1,10)} = 0.28$	$F_{(1,14)} = 2.72$	$F_{(1,14)} = 20.41$
	$P = 0.608$	$P = 0.121$	$P = 0.001$
Body fat	$F_{(1,10)} = 0.01$	$F_{(1,13)} = 0.005$	$F_{(1,13)} = 1.58$
	$P = 0.922$	$P = 0.915$	$P = 0.232$

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.

(a)



(b)



(c)

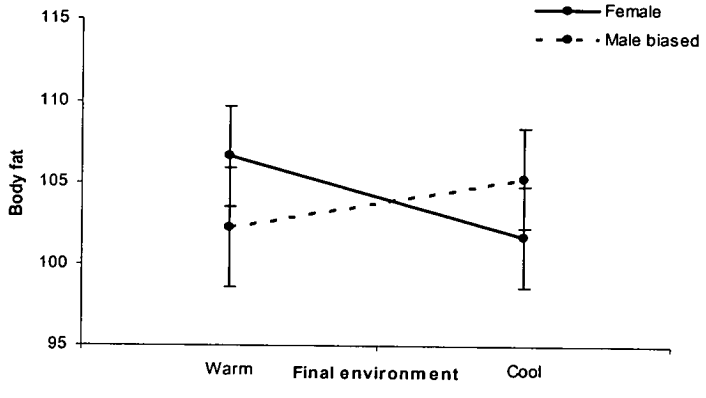


Figure 4.2: (a) Overall mean carapace length, (b) overall mean mass and (c) overall mean body fat for both sex ratios in each final incubation treatment. Bars represent the standard error of the mean.

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, $F_{1,102} = 0.06$).

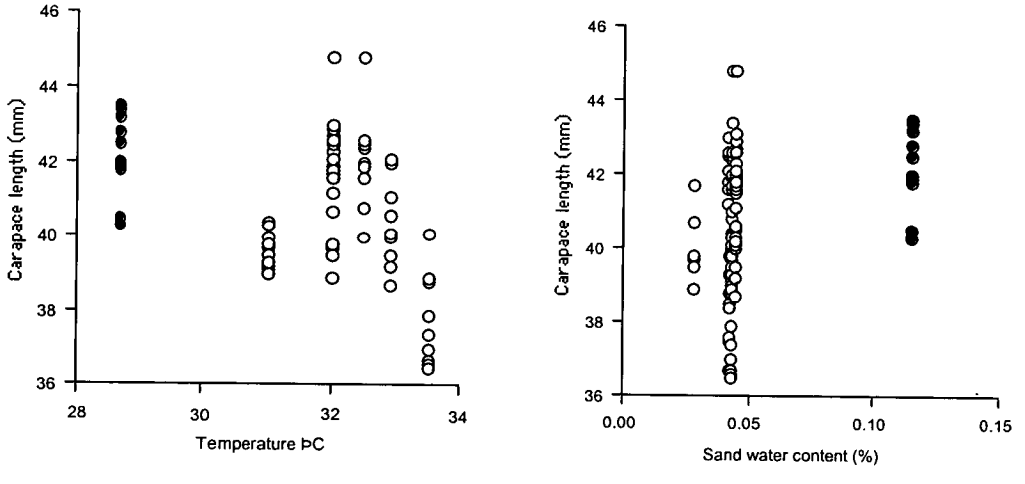


Figure 4.3: Left: Plot of temperature and right, plot of percent water content against carapace length of individual hatchlings from natural nests. There were significant correlations with this data set, but not when using mean carapace length for each nest. Open circles, data from warm beach; closed 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 vitro.* 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 Wrights 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 (Ranford-Cartwright *et al.*, 1993; Smith *et al.*, 2000). 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 Ca Cl_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.

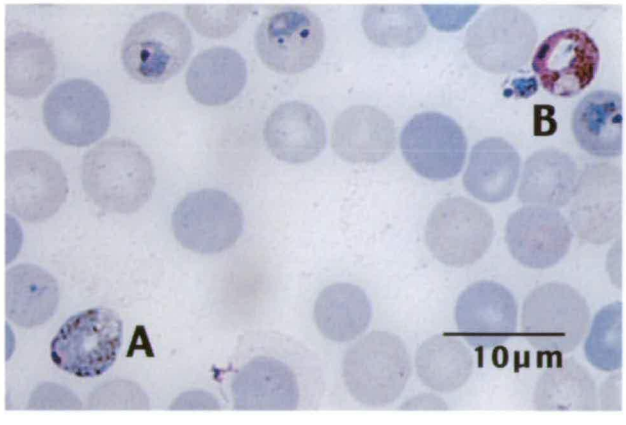


Figure 5.1: Gametocytes in a standard ('immediate' smear, see text). A) female gametocyte and B) male gametocyte. See text for sexing criteria.

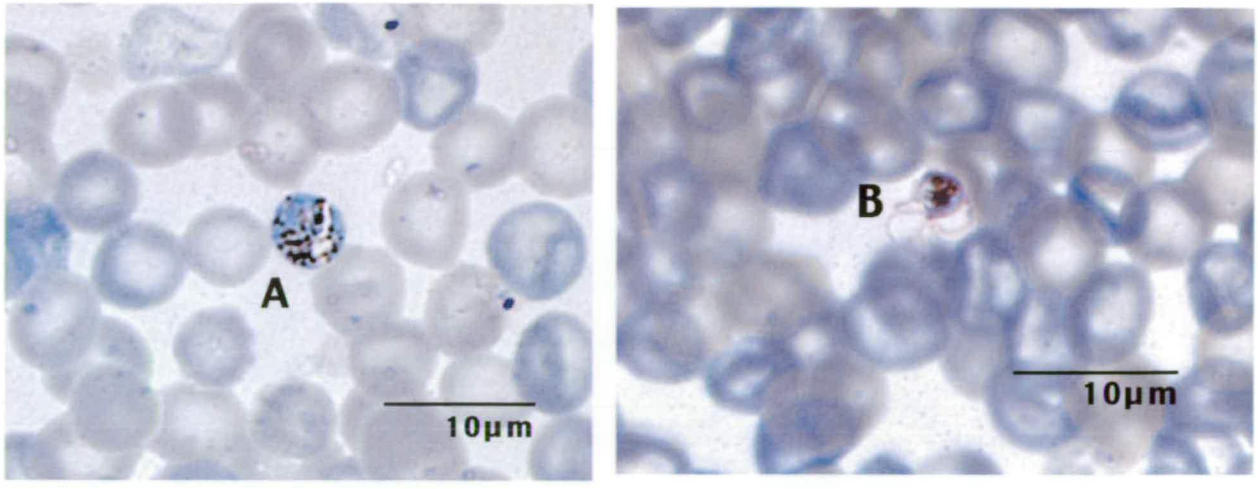


Figure 5.2: Gametocytes in a 'delayed' smear' (see text), in which gametocytes are undergoing gametogenesis. A) female gamete and B) male gametocyte exflagellating to produce male gametes from its enlarged nucleus.

5.3.3 Differential mortality experiment

Each mouse in the treatment (CQ) group was given a curative dose of chloroquine sulphate (Nivaquine[tm]) 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 $x10^6$ 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 ($T_{1/2}$; time required for the gametocyte density to decrease to half of the initial density) using the function: $T_{1/2} = \frac{-\ln(1/2)}{b}$.

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).

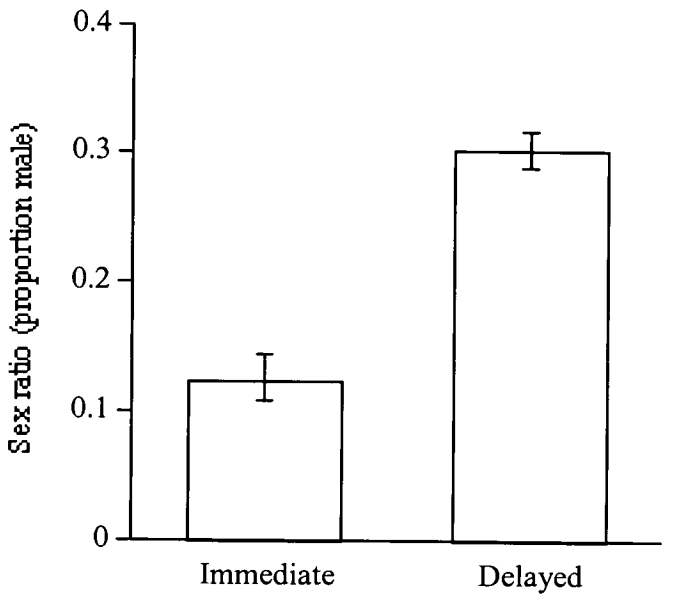


Figure 5.3: Mean sex ratio estimated from thin smears made with blood immediately after being taken from the tail vein (immediate), and smears made from blood in which gametogenesis had begun (delayed). Bars are the standard error of the mean, n = 28 pairs.

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 $x10^6ml^{-1}$; mean for control = -0.008 ± 0.0008; n = 16 mice, T = -4.16, P = 0.001). 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:

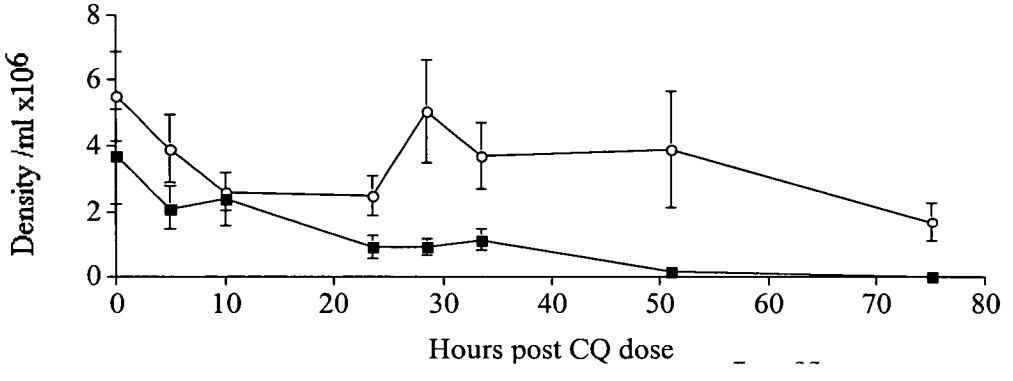


Figure 5.4b:

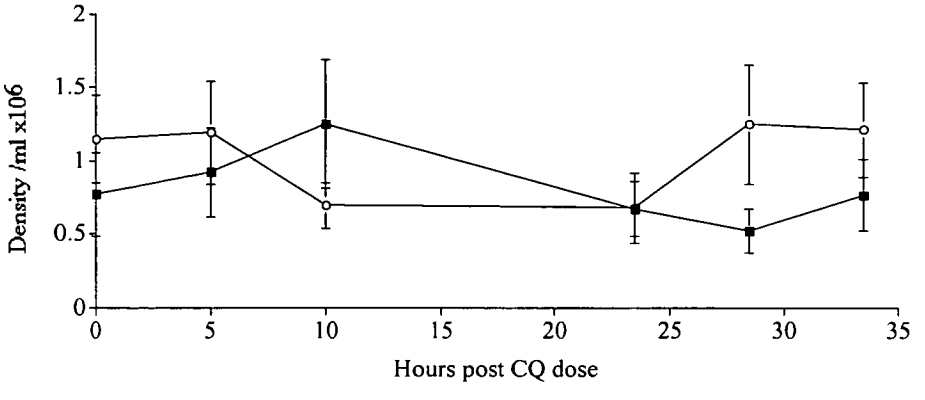


Figure 5.4c:

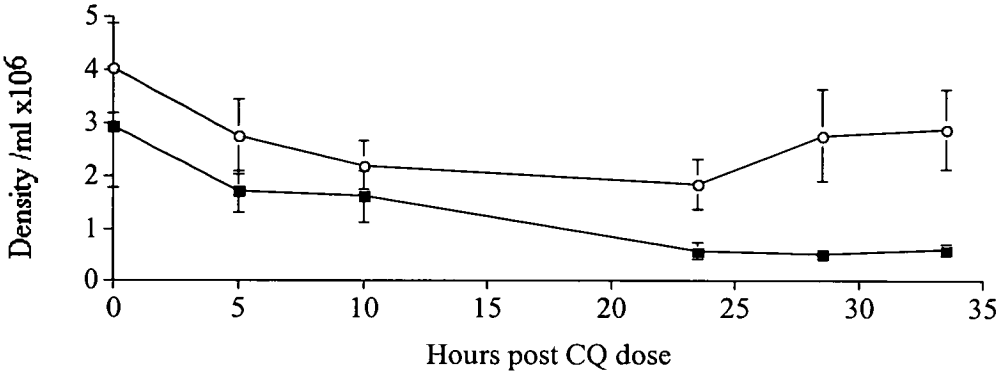


Figure 4: a) Total, b) male and c) female gametocyte density throughout the sampling period in infections treated with chloroquine (closed symbols) or distilled water (open symbols). The density in the chloroquine group decreased at a significantly faster rate than the control group. Error bars are standard 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, $b\pm$ S.E., for females, = 0.09 ± 0.009, n = 10; mean for males = 0.04 ± 0.01, n= 9; paired t-test, T = 3.94, n = 9 pairs, P = 0.004). The half life for female gametocytes was 8hrs (95% c.i. 5-13 hrs), where as 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.

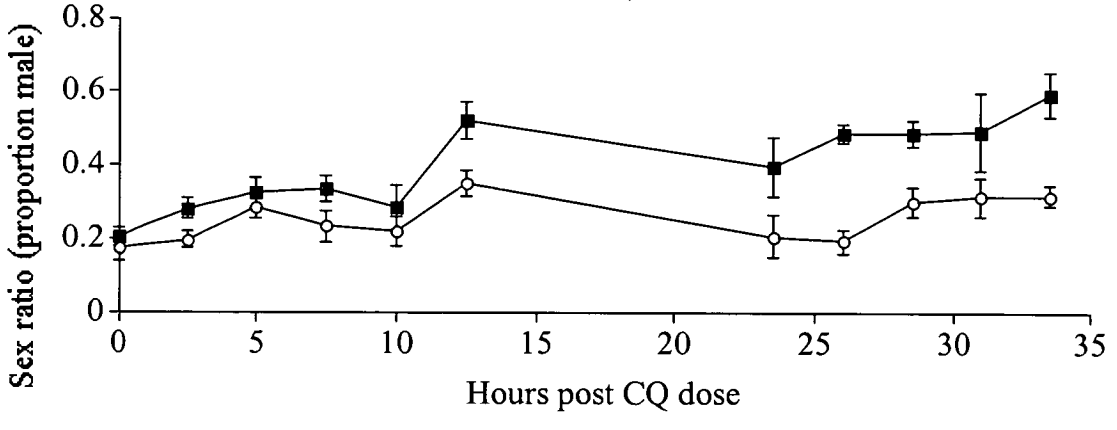


Figure 5.5: Sex ratio of the chloroquine group (closed symbols) become less female biased over the sampling period than the control group (open symbols). Error bars are standard error of the mean.

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: $Male = {}^{rc}/_{b_{male}}$ and $Female = \frac{c(1-r)}{b_{female}}$ 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).

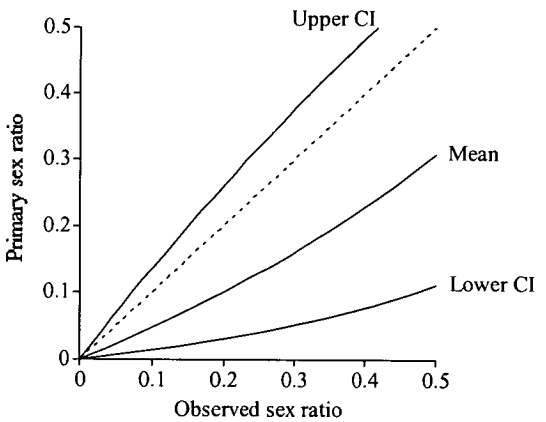


Figure 5.6: For asynchronous gametocyte production. The unbroken lines show the primary sex ratio (before mortality), for a range of observed (secondary) sex ratios and the 95% confidence intervals for this relationship. The dashed line is $y = x$, i.e. when the primary and secondary sex ratios are equal.

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 seconday 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).

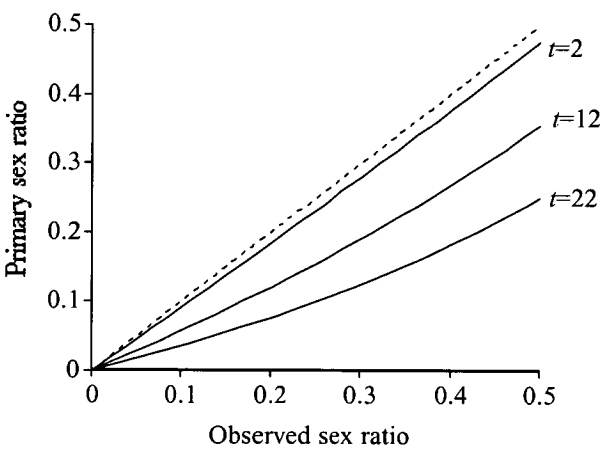


Figure 5.7: The relationship between the primary sex ratio (before mortality), for a range of observed (secondary) sex ratios for 3 times (t hours) since synchronous gametocyte production. The dashed line is $y = x$, i.e. when the primary and secondary sex ratios are equal or when t = 0hrs.

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 extracelluar 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; 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 a vector. 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).

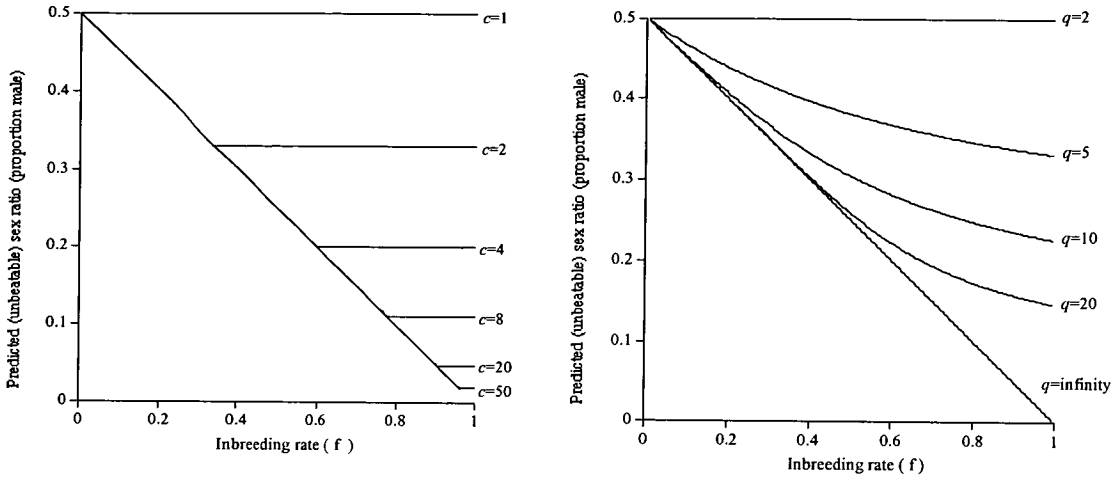


Figure 6.1: The relationship between the predicted unbeatable sex ratio (proportion of gametocytes that are male; z*) and the inbreeding rate (*f*). Left shows the unbeatable sex ratio when the number of gametes produced by each male gametocyte (*c*) varies and gametes from all gametocytes in a very large group can interact ($q \rightarrow \infty$; Read *et al.* 1992). Right shows the unbeatable sex ratio when the number of gametocytes whose gametes can interact (*q*) is limited and the number of gametes produced by each male gametocyte (*c*) is not limiting (West *et al.* 2002b).

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 $m \rightarrow 0$, the fitness of the Null is dominated by its success in vectors feeding upon hosts containing no Mutant lineages.

$$w_N \approx \frac{1}{n} S_{N,0} = f\, S_{N,0} \qquad (1)$$

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.

$$w_M \approx S_{M,1} \qquad (2)$$

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 ϕ_N female gametes able to interact in the midgut. The number of zygotes, ζ, is the smaller of these two values, and since zygotes are diploid the number of Null alleles formed in that vector is $2\ \zeta$.

$$S_{N,0} = \sum_{\mu_N=0}^{q} \binom{q}{\mu_N} z_N^{\mu_N} (1-z_N)^{q-\mu_N} 2\min\{c\,\mu_N, q-\mu_N\} \qquad (3)$$

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, ζ, is then the lower of the two values $c\ (\mu_M + \mu_N)$ and $\phi_M + \phi_N$, meaning that there are ζ successful male gametes and ζ successful female gametes. Of the former, a proportion $\varpi_{M,1} \sim HypGeo(\zeta, c\ \mu_M, c\ (\mu_M + \mu_N))/\zeta$ will be Mutant, and of the latter a proportion $\varpi_{M,0} \sim HypGeo(\zeta, \phi_M,$

$\phi_M + \phi_N)/\zeta$ will be Mutant. The success of the Mutant is simply $\zeta(\varpi_{M,1} + \varpi_{M,0})$ (Taylor, 1981; Charnov, 1982).

$$S_{M,1} = \sum_{\tau_M=0}^{q} \sum_{\mu_M=0}^{\tau_M} \sum_{\mu_N=0}^{q-\tau_M} \binom{q}{\tau_M} f^{\tau_M}(1-f)^{q-\tau_M} \binom{\tau_M}{\mu_M} z_M^{\mu_M}(1-z_M)^{\tau_M-\mu_M} \binom{q-\tau_M}{\mu_N} z_N^{\mu_N}(1-z_N)^{q-\tau_M-\mu_N} \min\{c(\mu_M+\mu_N), q-\mu_M-\mu_N\}(E[\varpi_{M,1}]+E[\varpi_{M,0}]) \quad (4a)$$

where

$$E[\varpi_{M,1}] = \begin{cases} \dfrac{\mu_M}{\mu_M+\mu_N} & if \quad \mu_M+\mu_N > 0 \\ 0 & \mu_M+\mu_N = 0 \end{cases} \quad (4b)$$

$$E[\varpi_{M,0}] = \begin{cases} \dfrac{\tau_M-\mu_M}{q-\mu_M-\mu_N} & if \quad q-\mu_M-\mu_N > 0 \\ 0 & q-\mu_M-\mu_N = 0 \end{cases} \quad (4c)$$

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 ω 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 6.1: Definition of each parameter / variable referred to in the methods and appendix.

Symbol	Definition
$Bi(k,\pi)$	Binomial distribution: k trials and probability of success π
c	Number of viable male gametes per male gametocyte
f	Inbreeding coefficient; $f = n^{-1}$
g_X	Number of X-allele male gametes remaining viable
$HypGeo(\alpha,\beta,\gamma)$	Hypergeometric distribution: α trials, and β potential successes out of γ
M	The Mutant allele
m	Population frequency of the mutant
N	The Null allele
n	Number of independent lineages per vertebrate host
p	Probability of male gamete survival
q	Number of gametocytes whose gametes can interact in the vector
$S_{X,y}$	Success of the X-allele in a host containing y Mutant infections
w_X	Absolute fitness of the X-allele
z	Sex ratio (proportion male gametocytes per lineage)
z^*	Evolutionarily stable (ES) sex ratio
z_X	Sex ratio employed by the X-allele
χ	Species-specific number of gametes released per male gametocyte
ϕ_X	Number of X-allele females in a mating group
μ_X	Number of X-allele males in a mating group
τ_X	Total number of X-allele gametocytes in a mating group
$\varpi_{X,y}$	Frequency of X-alleles in successful male (y=1) or female (y=0) gametes
ω	Relative fitness of the Null, w_N / w_M; Mutant invades if $\omega < 1$
ζ	Number of zygotes produced by the mating group

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 μ 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 $q \to \infty$ 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.

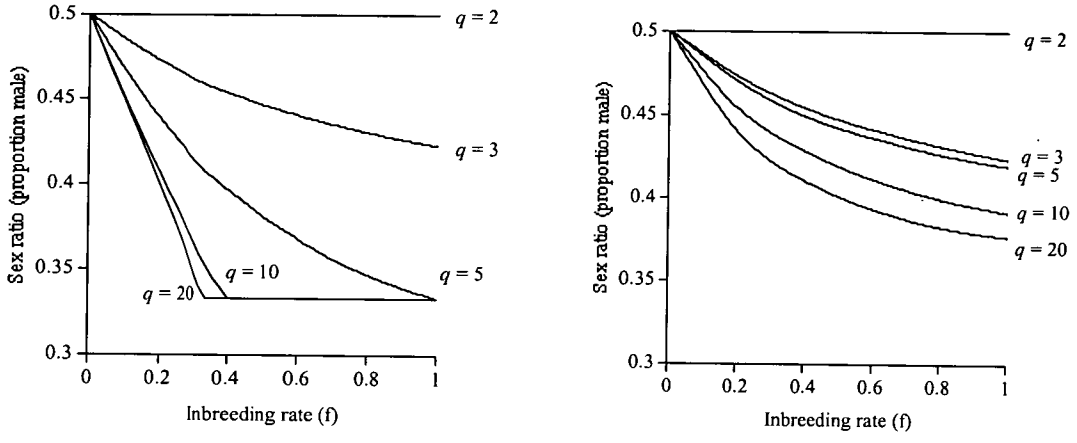


Figure 6.2: Left shows the relationship between predicted sex ratio and inbreeding rate, for given values of q when c = 2 assuming no interaction between the two types of fertility insurance and right shows the relationship between ES sex ratio and inbreeding rate arising from equations 1-4, for given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

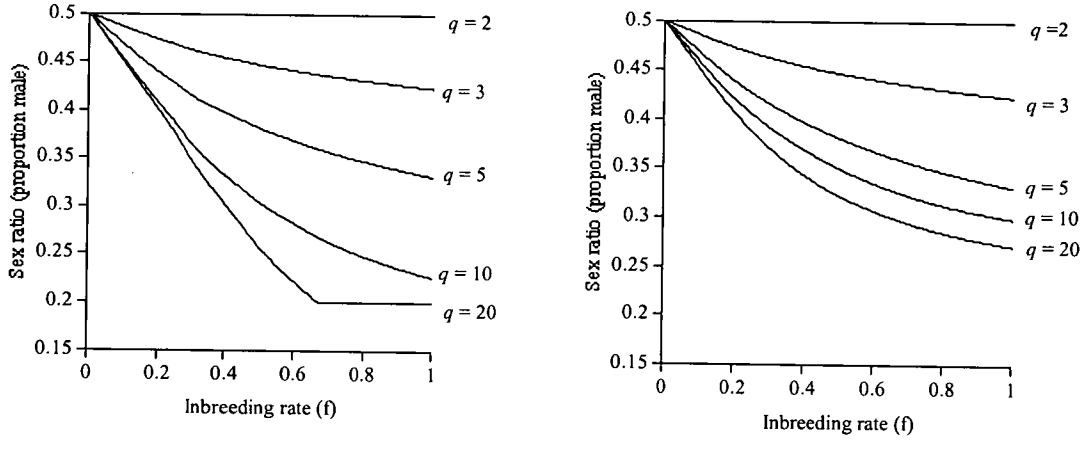


Figure 6.3: Left shows the relationship between predicted sex ratio and inbreeding rate, for given values of q when c = 4 assuming no interaction between the two types of fertility insurance and right shows the relationship between ES sex ratio and inbreeding rate arising from equations 1-4, for given values of q when c = 4.

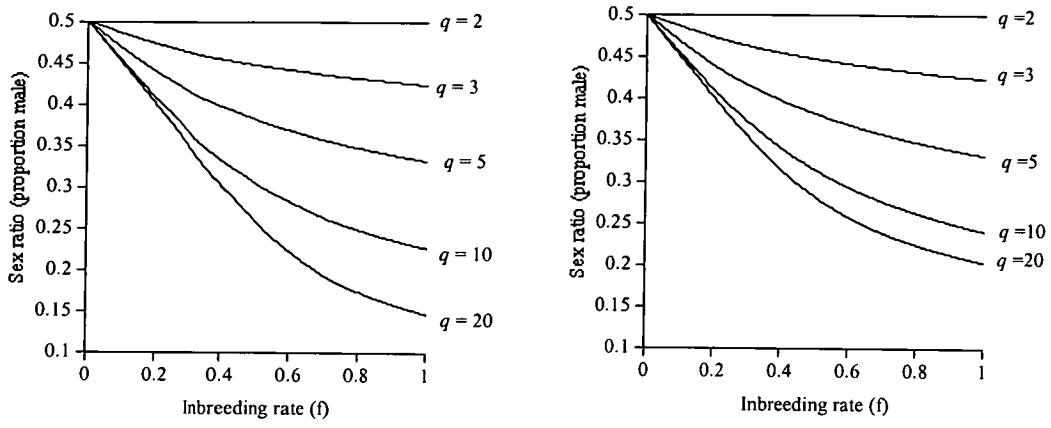


Figure 6.4: Left shows the relationship between predicted sex ratio and inbreeding rate, for given values of q when c = 8 assuming no interaction between the two types of fertility insurance and right shows the relationship between ES sex ratio and inbreeding rate arising from equations 1-4, for given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

$$S_{N,0} = \sum_{\mu_N=0}^{q} \binom{q}{\mu_N} z_N^{\mu_N} (1-z_N)^{q-\mu_N} 2\zeta \qquad \text{(A.1a)}$$

where

$$\zeta = \begin{cases} q-\mu_N & if \quad \mu_N > 0 \\ 0 & \mu_N = 0 \end{cases} \qquad \text{(A.1b)}$$

and

$$S_{M,0} = \sum_{\tau_M=0}^{q} \sum_{\mu_M=0}^{\tau_M} \sum_{\mu_N=0}^{q-\tau_M} \binom{q}{\tau_M} f^{\tau_M} (1-f)^{q-\tau_M} \binom{\tau_M}{\mu_M} z_M^{\mu_M} (1-z_M)^{\tau_M-\mu_M} \binom{q-\tau_M}{\mu_N} z_N^{\mu_N} (1-z_N)^{q-\tau_M-\mu_N}$$
$$\zeta(E[\varpi_{M,1}]+E[\varpi_{M,0}])$$

(A.2a)

where

$$\zeta = \begin{cases} q - \mu_M - \mu_N & if \quad \mu_M + \mu_N > 0 \\ 0 & \mu_M + \mu_N = 0 \end{cases} \tag{A.2b}$$

$$E[\varpi_{M,1}] = \begin{cases} \dfrac{\mu_M}{\mu_M + \mu_N} & if \quad \mu_M + \mu_N > 0 \\ 0 & \mu_M + \mu_N = 0 \end{cases} \tag{A.2c}$$

$$E[\varpi_{M,0}] = \begin{cases} \dfrac{\tau_M - \mu_M}{q - \mu_M - \mu_N} & if \quad q - \mu_M - \mu_N > 0 \\ 0 & q - \mu_M - \mu_N = 0 \end{cases} \tag{A.2d}$$

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

$$S_{N,0} = 2q \min\{c\, z_N, (1 - z_N)\} \tag{A.3}$$

and

$$S_{M,1} = q \min\{c(z_M f + z_N(1-f)), (1-z_M)f + (1-z_N)(1-f)\} \left(\frac{z_M f}{z_M f + z_N(1-f)} + \frac{(1-z_M)f}{(1-z_M)f + (1-z_N)(1-f)} \right) \tag{A.4}$$

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 ω 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

$$S_{N,0} = \sum_{\mu_N=0}^{q} \sum_{g_N=0}^{\chi\mu_N} \binom{q}{\mu_N} z_N^{\mu_N} (1 - z_N)^{q-\mu_N} \binom{\chi\mu_N}{g_N} p^{g_N} (1-p)^{\chi\mu_N - g_N} 2\min\{g_N, q - \mu_N\} \tag{A.5}$$

and

$$S_{M,1} = \sum_{\tau_M=0}^{q} \sum_{\mu_M=0}^{\tau_M} \sum_{\mu_N=0}^{q-\mu_M} \sum_{g_M=0}^{\chi\mu_M} \sum_{g_N=0}^{\chi\mu_N} \binom{q}{\tau_M} f^{\tau_M} (1-f)^{q-\tau_M} \binom{\tau_M}{\mu_M} z_M^{\mu_M} (1-z_M)^{\tau_M-\mu_M} \binom{q-\tau_M}{\mu_N} z_N^{\mu_N} (1-z_N)^{q-\tau_M-\mu_N}$$
$$\binom{\chi\mu_M}{g_M} \binom{\chi\mu_N}{g_N} p^{g_M+g_N} (1-p)^{\chi(\mu_M+\mu_N)-g_M-g_N} \min\{g_M+g_N, q-\mu_M-\mu_N\} (E[\varpi_{M,1}] + E[\varpi_{M,0}])$$

(A.6a)

where

$$E[\varpi_{M,1}] = \begin{cases} \dfrac{g_M}{g_M+g_N} & \text{if} \quad g_M+g_N > 0 \\ 0 & \quad g_M+g_N = 0 \end{cases} \tag{A.6b}$$

$$E[\varpi_{M,0}] = \begin{cases} \dfrac{\tau_M-\mu_M}{q-\mu_M-\mu_N} & \text{if} \quad q-\mu_M-\mu_N > 0 \\ 0 & \quad q-\mu_M-\mu_N = 0 \end{cases} \tag{A.6c}$$

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, erythropoesis 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 vinckei* 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 erythropoesis (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 chabuadi*, 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 Cl_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, 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 15mins 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 15mins, 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). I 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 $F_{1,13} = 3.03$, $P = 0.1.05$ respectively), or NO level (figure 7.3; $F_{1,13} =$

2.01, P = 0.1.80). 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.

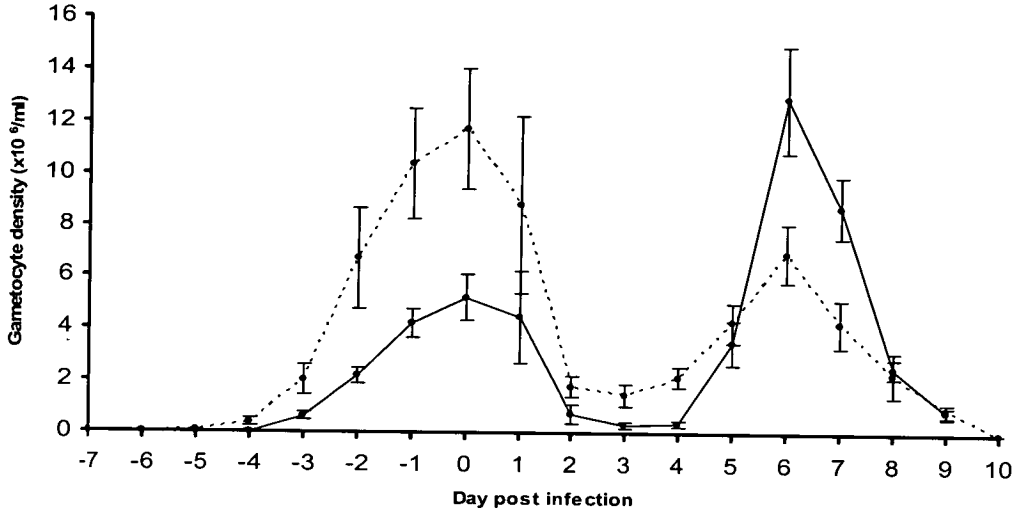


Figure 7.1a: The gametocyte density in the Epo treated group (dashed line), and control group (solid line), during experiment 1. The x-axis is day post infection relative to max anaemia, i.e. scaled so that all infections reached maximum anaemia on day 0, and bars are standard error of the mean.

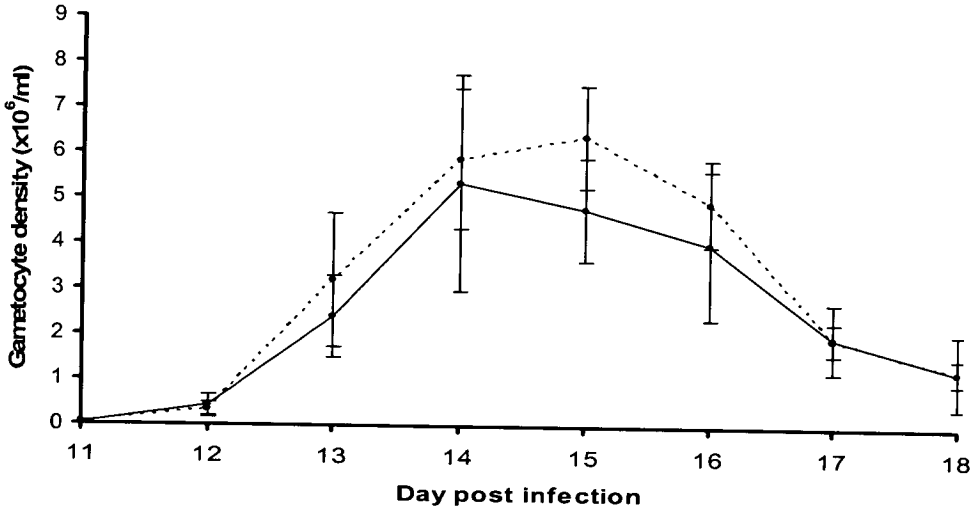


Figure 7.1b: The gametocyte density in the Epo treated group (dashed line), and control group (solid line), during experiment 2. The x-axis shows day post infection, and bars are standard error of the mean. The period on the x-axis corresponds to ~ 4-9 in figure 7.1a.

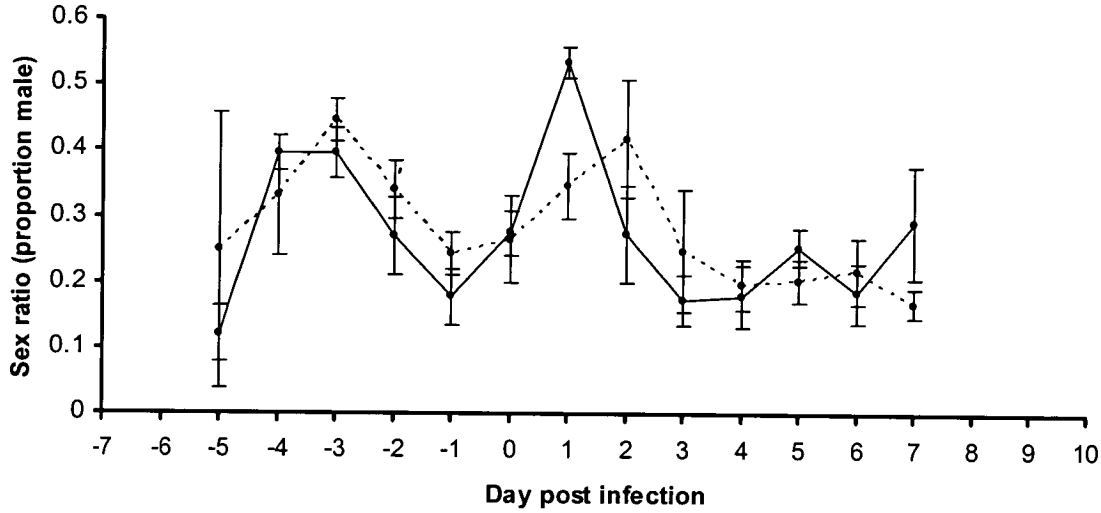


Figure 7.2a: The mean sex ratio during the course of experiment 1, for the Epo group (dashed line), and control group (unbroken line). The x-axis is scaled so that all infections reached maximum anaemia on day 0, and bars are standard error of the mean.

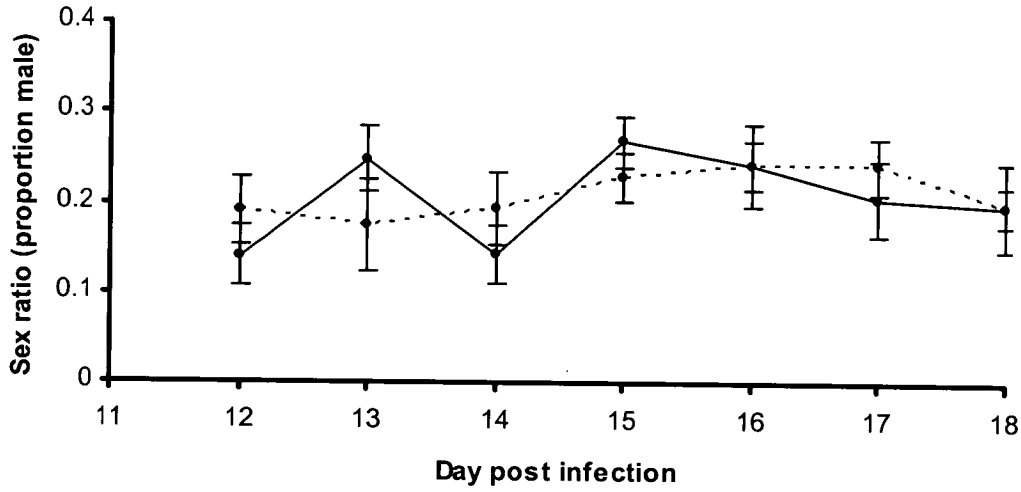


Figure 7.2b: The mean sex ratio during the course of experiment 2 for the Epo group (dashed line), and control group (unbroken line). The x-axis shows day post infection, and bars are standard error of the mean. The period on the x-axis corresponds to ~ 4-9 in figure 7.2a.

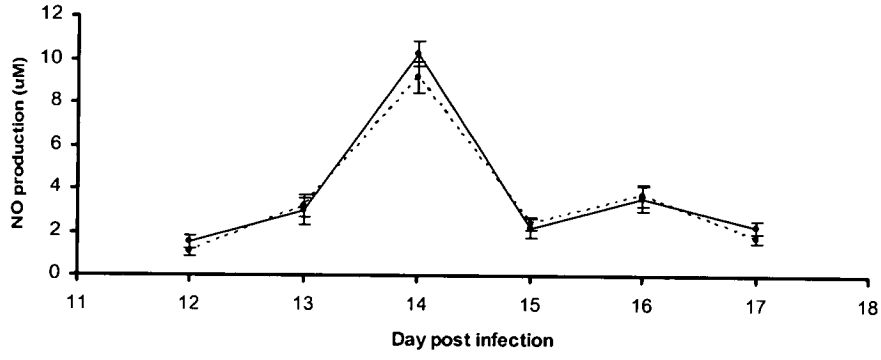


Figure 7.3: Mean nitric oxide production of the Epo group (dashed line), and control group (unbroken line), during experiment 2. The x-axis shows day post infection, and bars are standard error of the mean.

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).

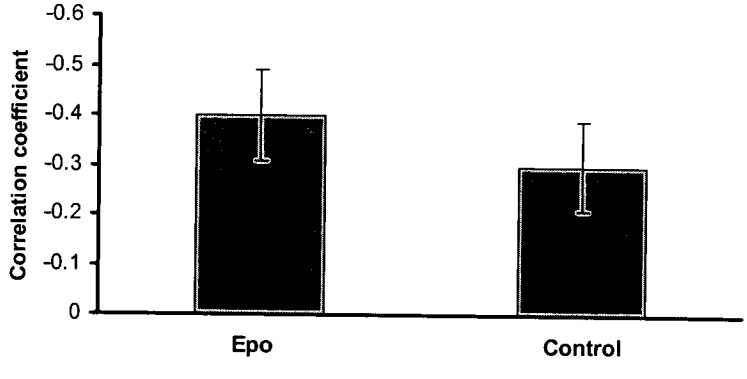


Figure 7.4: Shows the negative correlation between reticulocyte density and the sex ratio at t +2 for each mouse in the Epo and control group. Bars are standard error of the mean.

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.

Effect of Epo on:	**Exp**	**Test details**	**Mean & s.e.m.**
Anaemia, max RBC loss (RBCx10^9/ml)	1	T=-0.49 P=0.636	E 5.81 ±0.54 C 6.23 ±0.69
Max mass loss (g)	1	T = -1.75, P = 0.110	E 3.33 ±0.34 C 4.63 ±0.54
Total parasite density (x10^9/ml)	1	T= 0.91, P = 0.380	E 7.37 ±0.84 C 6.48 ±0.55
Total reticulocyte density (x10^9/ml)	1	T= 2.56, P = 0.025	E 18.09 ±0.66 C 15.45 ±0.78
Total pre crisis gametocytes (x10^6/ml)	1	T= 3.55, P = 0.004	E 42.05 ±7.41 C 17.18 ±4.28
Total post crisis gametocytes (x10^6/ml)	1	T= -1.72, P = 0.110	E 20.68 ±2.05 C 28.45 ±4.20
Total gametocytes (x10^6/ml)	1	T= 1.63, P = 0.130	E 61.38 ±23.10 C 45.63 ±5.57
Sex Ratio: mean	1	T=1.22 P=0.245	E 0.25±0.02 C 0.25±0.02
Sex Ratio: upper 95% CIi for mean	1	T=0.914 P=0.379	E 0.37±0.02 C 0.34±0.03
Sex Ratio: lower 95% CI for mean	1	T=1.43 P=0.178	E 0.18±0.02 C 0.15±0.02
Sex Ratio: median	1	T=0.376 P=0.714	E 0.24±0.02 C 0.23±0.02
Sex Ratio: 90th percentile	1	T=1.07 P=0.304	E 0.48±0.02 C 0.44±0.03
Sex Ratio: 10th percentile	1	T=1.57 P=0.143	E 0.08±0.03 C 0.03±0.02
Sex Ratio: @ peak	1	T=1.88 P=0.084	E 0.29±0.05 C 0.17±0.05
Anaemia, max RBC gain (RBC x10^9/ml)	2	T=-1.14 P=0.274	E 5,86 ±0.72 C 4.8 ±0.79
Total parasite density (x10^9/ml)	2	T=-2.95 P=0.011	E 2.51±0.13 C 0.82±0.51
Total gametocyte density (x10^6/ml)	2	T=-5.13 P=0.0002	E 64.7±7.54 C 19.5±3.04
Total reticulocyte density (x10^9/ml)	2	T=-5.45 P=0.0001	E 36±3.98 C 11.8±0.79
Total NO production (μM)	2	T=1.425 P=0.178	E 17.58±1.20 C 20.8±0.77

Max NO production (μM)	2	T=1.195 P=0.2571	E 9.15±0.59 C 10.26±0.57
Sex Ratio: mean	2	T=-0.30 P=0.770	E 0.21±0.01 C 0.21±0.02
Sex Ratio: upper 95% CI for mean	2	T=0.05 P=0.96	E 0.28±0.02 C 0.28±0.02
Sex Ratio: lower 95% CI for mean	2	T=-0.67 P=0.515	E 0.14±0.02 C 0.13±0.02
Sex Ratio: median	2	T=-0.884 P=0.390	E 0.21±0.01 C 0.20±0.01
Sex Ratio: 90th percentile	2	T=0.118 P=0.908	E 0.30±0.02 C 0.30±0.02
Sex Ratio: 10th percentile	2	T=-0.301 P=0.768	E 0.12±0.02 C 0.11±0.02
Sex Ratio: @ peak	2	T=0.09 P=0.99	E 0.21±0.03 C 0.21±0.04

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.

	Exp	**Factor**	**Result**
Anaemia (RBCx10^9/ml)	1	Epo	$F_{(1,13)} = 0.67, P = 0.428$
		Day	$F_{(1,13)} = 15.15, P = 0.002$
		Mouse	$F_{(13,14)} = 1.22, P = 0.357$
Parasite density (x10^9/ml)	1	Epo	$F_{(1,13)} = 1.27, P = 0.280$
		Day	$F_{(1,13)} = 16.42, P = 0.001$
		Mouse	$F_{(13,14)} = 0.91, P = 0.565$
Reticulocyte density (x10^9/ml)	1	Epo	$F_{(1,13)} = 1.42, P = 0.255$
		Day	$F_{(1,13)} = 236.09, P = 0.0000$
		Mouse	$F_{(13,14)} = 1.75, P = 0.156$
Gametocyte density (x10^6/ml)	1	Epo	$F_{(1,13)} = 3.54, P = 0.082$
		Day	$F_{(1,13)} = 19.70, P = 0.001$
		Mouse	$F_{(13,13)} = 8.62, P = 0.012$
		Epo:day	$F_{(1,13)} = 3.02, P = 0.028$
Sex Ratio	1	Epo	$F_{(1,13)} = 1.17, P = 0.299$
		Day	$F_{(1,13)} = 67.49, P = 0.0000$
		Mouse	$F_{(13,14)} = 1.91, P = 0.121$
Anaemia (RBCx10^9/ml)	2	Epo	$F_{(1,13)} = 4.75, P = 0.048$
		Day	$F_{(1,13)} = 91.72, P = 0.0000$
		Mouse	$F_{(13,13)} = 2.80, P = 0.037$
		Epo:day	$F_{(1,13)} = 9.79, P = 0.008$
Parasite density (x10^9/ml)	2	Epo	$F_{(1,13)} = 0.05, P = 0.827$
		Day	$F_{(1,13)} = 11.11, P = 0.005$
		Mouse	$F_{(13,14)} = 0.63, P = 0.794$
Reticulocyte density (x10^9/ml)	2	Epo	$F_{(1,13)} = 3.03, P = 0.105$

		Day	$F_{(1,13)} = 1.22, P = 0.289$
		Mouse	$F_{(13,14)} = 4.03, P = 0.007$
Gametocyte density ($x10^6$/ml)	2	Epo	$F_{(1,13)} = 1.00, P = 0.336$
		Day	$F_{(1,13)} = 5.09, P = 0.042$
		Mouse	$F_{(13,14)} = 0.81, P = 0.645$
Sex Ratio	2	Epo	$F_{(1,13)} = 0.01, P = 0.922$
		Day	$F_{(1,13)} = 1.67, P = 0.218$
		Mouse	$F_{(13,14)} = 1.58, P = 0.203$
NO(μM)	2	Epo	$F_{(1,13)} = 2.01, P = 0.180$
		Day	$F_{(1,13)} = 3.78, P = 0.074$
		Mouse	$F_{(13,14)} = 5.75, P = 0.001$

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*.

Relationship	**Results**	**Mean & 95% CI**
Reticulocytes and NO	$T = 3.60, P = 0.003$	0.24 [0.10, 0.38]
Gametocytes @t+2 and red blood cell density	$T = -3.10, P = 0.008$	-0.29 [-0.48, -0.09]
Reticulocytes and day post infection	E: $T = 0.61, P = 0.001$ C: $T = 15.55, P = 0.000$	E: 0.51 [0.30, 0.70] C: 0.75 [0.63, 0.86]
Sex ratio @ t+2 and reticulocytes	$T = -5.13, P = 0.0002$	-0.33 [-0.47, -0.20]

7.5 Discussion

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.

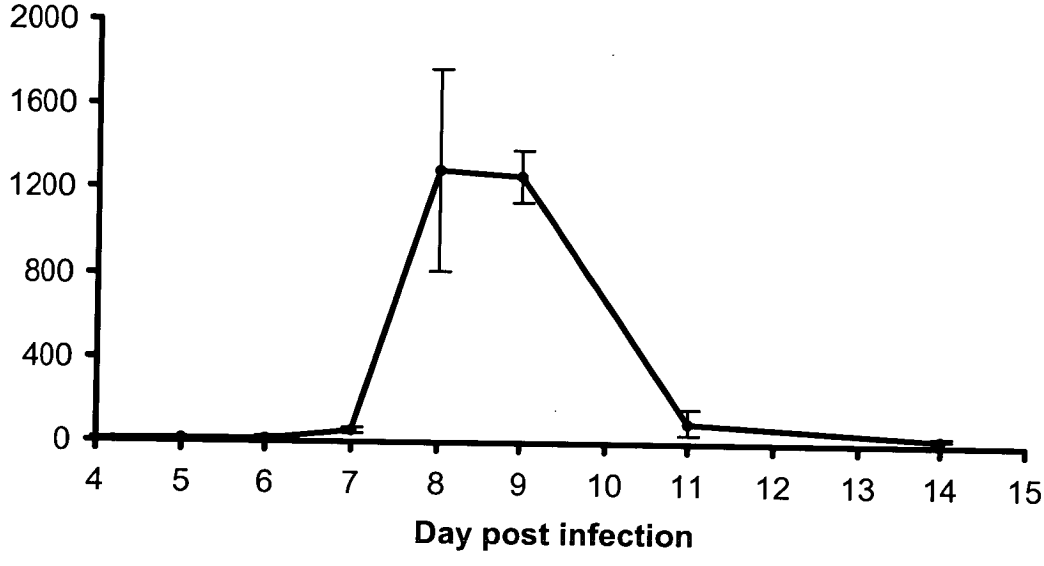


Figure 7.5: The mean Epo level during a *P. chabaudi* infection in C57 Black mice, initiated with $1x10^6$ parasites, bars are standard error of the mean. Courtesy of Chang and Stevenson (2002).

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 ~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 hatchling 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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Toxoplasma gondii, sex and premature rejection

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Adaptive sex ratio theory explains why gametocyte sex ratios are female-biased in many populations of apicomplexan parasites such as *Plasmodium* and *Toxoplasma*. Recently, Ferguson has criticized this framework and proposed two alternative explanations – one for vector-borne parasites (e.g. *Plasmodium*) and one for *Toxoplasma*. Ferguson raises some interesting issues that certainly deserve more empirical attention. However, it should be pointed out that: (1) there are theoretical and empirical problems for his alternative hypotheses; and (b) existing empirical data support the application of sex ratio theory to these parasites, not its rejection.

Ferguson suggests that natural selection has favoured female-biased sex ratios in *Plasmodium* and related Haemospororin parasites to promote inefficient fertilization as a form of reproductive constraint [1]. He argues that this is advantageous because it reduces the number of oocysts formed, which avoids compromising vector fitness. Even if there is selection to reduce parasite burdens within mosquitoes (a possibility that remains to be theoretically or empirically demonstrated), there are two problems with Ferguson's suggestion. First, it would be much easier and less wasteful to constrain oocyst numbers by decreasing the number of gametocytes produced. There is abundant evidence that *Plasmodium* parasites are able to adjust their rate of gametocytogenesis adaptively in response to environmental conditions [2–4]. Second, reproductive restraint achieved through sex ratio adjustment is not necessarily evolutionarily stable. A population minimizing fertilization success with a female-biased sex ratio could easily be invaded by a less-female-biased mutant. This is because the increased mating success of the mutant would more than compensate for the reduction in total transmission from the host.

Ferguson made an analogy with the problem of how many eggs an insect should lay per 'patch' (the clutch size), but this analogy illustrates the problems with his suggestion. First, insects do appear to adjust their clutch size so as not to overexploit local resources [5]. However, they do so by adjusting the number of eggs that they lay (analogous to the number of gametocytes produced) and not by adjusting embryo mortality (analogous to Ferguson's idea of reproductive constraint through female-biased sex ratios). Second, Ferguson's argument appears to rely on the assumption that if natural selection is acting on the clutch size, then it cannot simultaneously act upon the sex ratio. However, there is abundant evidence suggesting that insects adjust clutch size and sex ratio simultaneously, with the sex ratio conforming to sex allocation theory [5].

The idea that female-biased sex ratios are required to keep vectors alive cannot apply to apicomplexans that are not vector-borne. Instead, Ferguson argues that, in *Toxoplasma gondii*, the female bias also results in inefficient fertilization, but the unfertilized female gametes reproduce parthenogenetically. There is no empirical evidence for this novel prediction; indeed, as far as we are aware, sexual reproduction characterized by meiosis and syngamy is an obligate part of the life cycle of all apicomplexans. But, even if it is not, the existence of parthenogenetic reproduction provides no explanation of how selection has generated the observed sex ratios.

By contrast, the sex ratio hypothesis is well supported by theory and can, without invoking any undiscovered life cycles, explain a wide range of sex ratio patterns observed in Apicomplexa [6,7]. Ferguson rejects the conventional sex ratio theory because he states that it always assumes that there are enough male gametes to fertilize all the female gametes. This is not true. Elsewhere, we have incorporated this fertilization problem into sex ratio theory. We demonstrated that if there are not enough male gametes (as, for instance, there might not be enough male gametes in small bloodmeals or at low gametocyte densities), then less-female-biased sex ratios are favoured by natural selection [6,8,9] (Gardner, A. *et al.*, unpublished). However, to account for the female-biased sex ratios seen in many apicomplexan populations, we contend that usually there must be enough males to fertilize all the female gametes. This is because when mating takes place among clone mates (extreme inbreeding), natural selection will favour the sex ratio that maximizes zygote number. Ferguson contends instead that males are normally limiting, so that observed sex ratios are not maximizing oocyst number. In fact, experimental data from *Plasmodium* shows the opposite pattern, consistent with adaptive sex ratio theory: experimental manipulation giving a less-female-biased sex ratio led to a decrease in oocyst load [10]. More generally, data on fertilization efficiency, or zygote, gamete or gametocyte death merely show that, as with free-living organisms, not all apicomplexan gametes produce reproductive offspring. Such data most assuredly do not demonstrate that males are selectively limited.

Nonetheless, we are only too aware that data in this area are woefully inadequate [6,7]. For instance, Ferguson asserts that, in *T. gondii*, observed sex ratios would lead to female gametes outnumbering male gametes, and so large numbers of unfertilized female gametes remain, irrespective of the efficiency of fertilization. We are unconvinced. When surveying the literature, we found that most studies did not quantify the sex ratio or number of microgametes per microgametocyte (a tradition Ferguson maintains). However, our own counts suggested 1.9–2.6 male gametes per female gamete [11]. It is also worth noting that such data can overestimate the female bias in the sex ratio [12], and hence underestimate the ratio of male

to female gametes. Based on these data, the highly inbred nature of *T. gondii* population [13] and our theoretical arguments, we assert that the number of male gametes must be equal to or greater than the number of female gametes in *T. gondii*. Otherwise, clones producing a less-female-biased sex ratio would have invaded the population. Actually, the crucial point is not whether there are unfertilized females, but rather whether sex ratios less-female-biased than those found naturally would result in more zygotes. If they did, the sex allocation theory would have some problems [7]. But until that is demonstrated, rejection is premature. Again, comparison with insects illustrates this point, where it is well understood that adaptive female-biased sex ratios can lead to high levels (>30%) of females remaining unfertilized (virgins) [14].

Nonetheless, Ferguson is right that most free-living organisms make use of a vast excess of male gametes in the mating arena. If we are right in that there are sufficient males to fertilize all the females gametes in apicomplexan mating arenas, despite the female-biased gametocyte sex ratios we see in Nature, then apicomplexan gametes must have some very interesting ways of locating each other and for male gametes to move on from already mated (or mating) female gametes. The sex allocation view is well-founded in theory, does not require us to invoke novel life cycles, and has made several novel predictions that have subsequently proved to be quantitatively correct [6,7]. We make another prediction: the biology of gamete–gamete interactions in Apicomplexa will be more fascinating and possibly more complex than anything seen in species that apparently require vast numbers of male gametes to get the job done.

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Heredity (2002) 88, 117–124

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Sex ratios

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Sex ratio theory attempts to explain variation at all levels (species, population, individual, brood) in the proportion of offspring that are male (the sex ratio). In many cases this work has been extremely successful, providing qualitative and even quantitative explanations of sex ratio variation. However, this is not always the situation, and one of the greatest remaining problems is explaining broad taxonomic patterns. Specifically, why do different organisms show so much variation in the amount and precision with which they adjust their offspring sex ratios?
Heredity (2002) **88**, 117–124. DOI: 10.1038/sj/hdy/6800018

Keywords: adaptation; birds; constraints; natural selection; sex allocation; wasps

Introduction

Given that an organism reproduces sexually, how should it allocate resources to male and female reproduction? This decision has been termed sex allocation, and involves many related questions, ranging from what mating system is favoured (eg, separate sexes, or hermaphrodites with male and female reproductive organs), to how parental investment in individual offspring is expected to differ depending upon their sex (Charnov, 1982). Here we focus on the area of sex allocation research that has attracted the most attention: how individuals in species with separate sexes vary the proportion of their offspring that are male (termed the sex ratio).

Work on sex ratios has often been extremely successful (Charnov, 1982; Werren, 1987; Godfray and Werren, 1996; West *et al*, 2000a). Time and time again, sex ratio theory has been able to explain and predict variation in the sex ratio across species and populations, as well as facultative adjustment of offspring sex ratios by individuals in response to environmental conditions. This is best illustrated by considering some examples.

(1) Parasitic wasps (parasitoids) are insects whose larvae develop by feeding on the bodies of other arthropods, usually insects (Godfray, 1994). In many parasitic wasp species, where only one individual can develop per host, the size of the adult wasp depends upon the size of the host in which it was laid. It is thought that females gain a greater benefit from larger body size than males (although this has yet to be shown in the field; Godfray, 1994; West *et al*, 1996), in which case it would be advantageous to lay females in relatively large hosts and males on relatively small hosts. This pattern has been observed in a large number of species (Charnov *et al*, 1981; Godfray, 1994; West and Sheldon, submitted) (Figure 1a).

(2) The Seychelles warbler is a territorial bird endemic to a few islands in the Seychelles. In this species, daughters help their parents raise subsequent offspring, whereas sons disperse. In a high-quality territory (one with a high density of insect prey), having a helper is advantageous and so predominantly (90%) females are laid, whereas in a low-quality territory (relatively few insects) the increased competition for food means that a helper is disadvantageous and it is mainly (80%) males that are reared (Komdeur, 1996; Komdeur *et al*, 1997) (Figure 1b).

(3) Malaria (*Plasmodium*) and related protozoan parasites often have population structures that lead to considerable inbreeding (West *et al*, 2001a). When inbreeding occurs it leads to competition for mates between related males (ie brothers), which reduces the fitness return from producing males and favours the production of a female-biased sex ratio (Hamilton, 1967; Read *et al*, 1992). Higher levels of inbreeding favour more female biased sex ratios, and consistent with this prediction, more female biased sex ratios are observed in populations where the prevalence of transmission stages (gametocytes) is lower and inbreeding rates are likely to be higher (Read *et al*, 1995) (Figure 1c).

One notable aspect of sex ratio theory is that relatively simple models are often able to predict patterns in empirical data extremely well (Charnov, 1982; Godfray, 1994; Chapuisat and Keller, 1999; West *et al*, 2000a; Herre *et al*, 2001). The main reasons for this success are that, in many cases, the predictions of sex ratio theory depend upon: (i) a simple and unavoidable trade-off (an offspring can be either male or female), and (ii) a small number of crucial variables which are easy to measure (eg, the size of a host, or the number of females on a patch) (Charnov, 1993; Seger and Stubblefield, 1996; Frank, 1998; West *et al*, 2000a). Indeed, in some areas the fit of data to theoretical predictions can be expected to be so close that sex ratio theory has been argued to have a predictive power almost comparable to that of the 'hard' sciences of chemistry and physics (Hamilton, 1996). This has allowed work on the sex ratio to (a) provide some of the best quantitative evidence for the relative importance of natural

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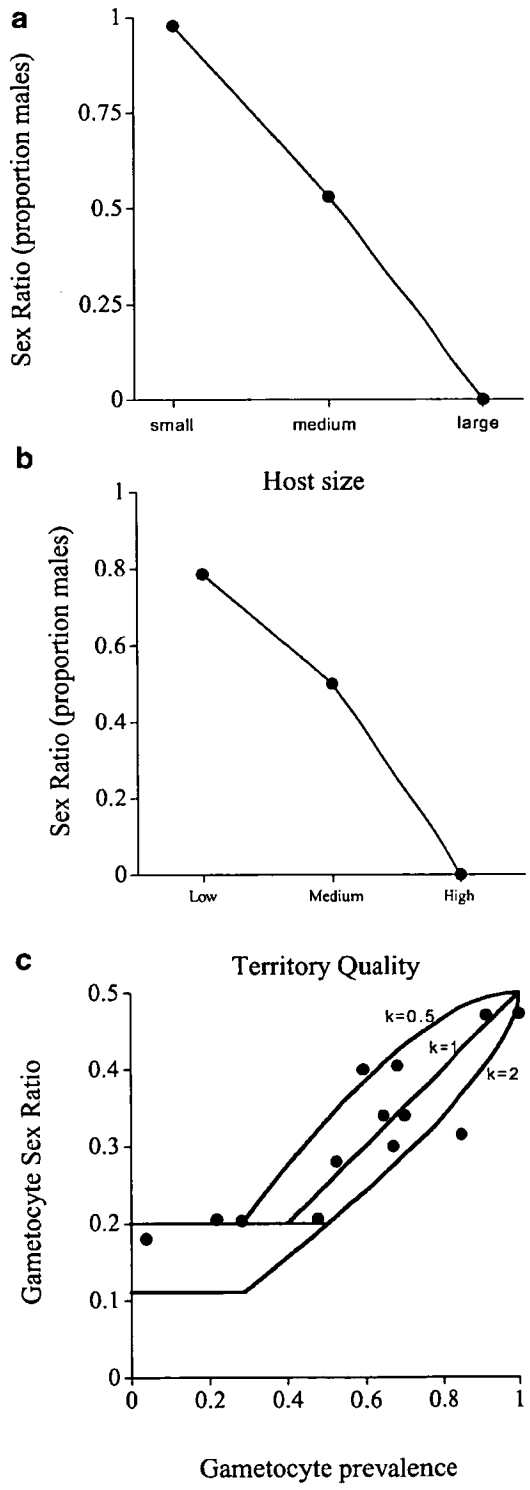
selection at the gene, individual, kin and population levels (Leigh *et al*, 1985; Beukeboom and Werren, 1992; Seger and Stubblefield, 1996; Chapuisat and Keller, 1999; West *et al*, 2000a), and (b) to address very general questions about the precision of adaptation and the limits on natural selection (Herre, 1987; West and Herre, 1998; Herre *et al*, 2001). In addition, reasoning has been reversed and observed sex ratios have been used to estimate parameters upon which the sex ratio is predicted to depend, but which can be difficult to measure directly (eg, the inbreeding rate in malaria parasites, which is a parameter of clinical importance (Read *et al*, 1992; West *et al*, 2000b, 2001a), the factor limiting reproduction in parasitic wasps (West and Rivero, 2000), or the relatedness between competing male fig wasps (West *et al*, 2001b)).

Despite these successes, it must be emphasized that the success of sex ratio theory is limited to a number of theoretical areas and taxonomic groups. For example, striking sex ratio patterns are rarely observed in vertebrates (Williams, 1979; Charnov, 1982). In our view, one of the biggest problems remaining for research into sex ratios and sex allocation is explaining broad taxonomic patterns. Specifically, why do different organisms show so much variation in the amount and precision with which they adjust their offspring sex ratios? In the rest of this paper we will focus on a number of issues relating to this question.



Sex determination, adaptation and constraint in sex allocation

Do organisms with chromosomal sex determination show facultative sex ratio variation? The conventional wisdom is that the mechanism of sex determination is a powerful constraint that determines the degree of sex ratio adjustment shown by an organism. Specifically: (a) the most striking sex ratio patterns have been found in the Hymenoptera (ants, bees, wasps), where the haplodiploid genetic system would apparently allow a female precise control of the sex ratio of offspring by deciding whether or not eggs are fertilized (males are haploid and develop from unfertilized eggs, whereas females are diploid and develop from fertilized eggs); (b) interesting sex ratio patterns are rarely found in vertebrates such as mammals or birds because chromosomal (genetic) sex

Figure 1 Some striking sex ratio examples. (**a**) In many parasitic wasps where only a single wasp develops per host, males are laid on small hosts and females on large hosts. Shown here are data from the pine bark weevil, *Dolichomitus* sp., parasitising *Niphades variegates* (Kishi, 1970). (**b**) In the Seychelles warbler, daughters help their parents to rear subsequent offspring, and thereby increase their parents' fitness. However, helpers are only useful on high quality territories where there are plenty of insects to eat; on low quality territories the presence of helpers increases competition for food, leaving less for nestlings. Parents show strong, and reproducible, biases in the primary sex ratio of their offspring depending on the quality of territory that they breed on, producing daughters on high quality territories, and sons on low quality territories (Komdeur, 1996; Komdeur *et al*, 1997). (**c**) The observed relationship between the sex ratio and gametocyte (sexual transmission stage) prevalence across populations of *Leucocytozoon* and *Plasmodium* parasites (Read *et al*, 1995). This positive relationship is predicted because when fewer hosts are infectious, transmission rates will be lower, mixed infections rarer, and the rate of inbreeding higher (Read *et al*, 1995). The solid lines show the predicted relationship for various degrees of parasite genotype (clone) aggregation (*k* represents the aggregation parameter from the negative binomial equation).

determination (CSD) acts as a constraint to prevent facultative adjustment of offspring sex ratios (Maynard Smith, 1978; Williams, 1979; Charnov, 1982). It has been argued that this idea is supported by the fact that data on population sex ratios in birds and mammals show no consistent pattern, and represent just sample-size dependent noise around the sex ratio of 0.5 expected from a fair meiosis (Williams, 1979; Clutton-Brock, 1986; Bull and Charnov, 1988; Krackow, 1999; Palmer, 2000).

However, recent studies of taxa with CSD (mammals, birds, frogs, lizards, snakes, spiders) have reported shifts in offspring sex ratios consistent with adaptation (Madsen and Shine, 1992; Komdeur *et al*, 1997; Creel *et al*, 1998; Sheldon, 1998; Kruuk *et al*, 1999; Nager *et al*, 1999; Sheldon *et al*, 1999; Aviles *et al*, 2000; Sakisaka *et al*, 2000; Olsson and Shine, 2001). Furthermore, in some of these cases individuals appear to show extremely precise control of their offspring sex ratio. For example: (a) in the Seychelles warbler (described above), females vary the sex ratio of their offspring from 90% female to 80% male depending upon environmental conditions (Komdeur, 1996; Komdeur *et al*, 1997); (b) in the neotropical social spider *Anelosimus domingo*, mating occurs between the members of the same colony (between close relatives), so that females should only lay enough sons to mate their daughters – consistent with this prediction females produce, with very little variation, approximately one male for every nine females (Aviles *et al*, 2000); (c) for reasons that are unknown, *Eclectus* parrots often produce long unbroken runs of one sex that can only be explained by extremely precise control of the sex ratio at fertilization (eg, 20–30 males in a row) (Heinsohn *et al*, 1997). Although such extreme and precise sex ratio patterns are often observed in haplodiploid species, that they are seen in species with CSD suggests that constraints due to mechanisms of sex determination are not sufficient explanation for variation in sex ratio adaptation.

Resolution of whether species with CSD facultatively adjust their sex ratio in response to environmental conditions in a consistent manner requires comparison across studies. Unfortunately, previous comparisons across species have focused on population sex ratios (Williams, 1979; Clutton-Brock, 1986; Clutton-Brock and Iason, 1986; Palmer, 2000). This is problematic because in situations where we expect individuals to vary their sex ratio depending upon environmental conditions (as is the case with most vertebrate examples), with some individuals being expected to produce sons and others daughters (Trivers and Willard, 1973), it is extremely difficult to predict population sex ratios (Frank, 1990). Even in cases where it can be relatively easy to predict patterns of sex ratio variation across individuals, the overall population sex ratio is predicted to depend upon a variety of factors such as the details of male and female life histories, distribution of variation in maternal quality, the extent to which other behaviours (eg, clutch size) are facultatively adjusted, and the form of trade-off between current and future reproduction. (Frank, 1987, 1990; Frank and Swingland, 1988; Pen, 2000; Pen and Weissing, 2000a,b). Consequently, no *a priori* predictions can be made about population sex ratios, and inconsistent observations across species are not surprising (West and Sheldon, submitted).

West and Sheldon (submitted) have recently suggested that one way to solve this problem is to examine the precision with which individuals facultatively adjust their offspring sex ratios in response to environmental conditions (rather than population sex ratios). Using this methodology West and Sheldon (submitted) showed that birds consistently adjust their offspring sex ratios in the direction predicted by theory. This provides strong evidence that even in vertebrates, CSD is not an all-powerful constraint that prevents adaptive sex ratio manipulation.

How can we explain differences across taxa in the amount of sex ratio adjustment?

West and Sheldon (submitted) also suggested that a simple cost-benefit approach, which considers many possible factors, provides a unifying framework for understanding variation across taxa in how much individuals adjust their offspring sex ratios. Specifically, that facultative sex ratio variation will only be favoured when the fitness benefits of this behaviour are greater than its costs. In cases where facultative sex ratio variation is favoured, it will evolve to a level where the benefits of any further (marginal) increase in the precision of sex ratio adjustment would be exactly outweighed by its cost. Consequently, the most extreme and precise sex ratio variation will be seen in species where the fitness benefits of facultative sex ratio adjustment are high, and the costs low.

What kind of factors will influence the cost and benefits of facultative sex ratio adjustment? (1) The mechanism of sex detrmination will influence the cost of adjusting sex ratios (Maynard Smith, 1980; Charnov, 1982; Leimar, 1996; Pen *et al*, 1999; Pen, 2000; Pen and Weissing, in press). For example, CSD can impose a cost that is heavily dependent upon the mechanism (eg, the cost of aborting a fertilized egg or embryo would depend heavily upon at what stage this is done). (2) The benefit of facultative sex ratio variation will depend upon how much fitness gain is to be made from shifting offspring sex ratios, which will be influenced by the strength and form of selection involved. For example, if an environment is only encountered very rarely, there will be weak selection to produce the 'correct' sex ratio in that situation (Herre, 1987). (3) The benefit of facultative sex ratio variation will also depend upon environmental predictability – the more accurately individuals can assess the relevant environmental factors that influence the optimal sex ratio, more extreme sex ratio shifts would be predicted (Charnov, 1982; West *et al*, 2000a; West and Sheldon, submitted). This view thus suggests that the degree of precision of sex allocation should be viewed as a trait subject to natural selection.

Is there any evidence supporting the potential importance of these factors? First, more extreme sex ratio patterns are observed in situations where selection can be inferred to be stronger. For example, fig wasp species show: (a) more extreme sex ratio shifts in more variable environments, when there is stronger selection to adjust sex ratios in response to environmental conditions (Herre, 1987; Herre *et al*, 2001), and (2) more precise (lower variance) sex ratios in situations where selection for precise sex ratios (stabilizing selection) is greater (West and Herre, 1998). Furthermore, many cases of extreme (and precise) sex ratio variation in species where

the method of sex determination is likely to impose a heavy constraint (eg, CSD and pseudo-arrhenotoky, in spiders, aphids, snakes, mites), occur when there is intense competition between brothers for mates (local mate competition, LMC) and strong selection for extremely female biased sex ratios (eg, as low as 5% males) (Yamaguchi, 1985; Foster and Benton, 1992; Madsen and Shine, 1992; Nagelkerke and Sabelis, 1998; Aviles *et al*, 2000).

Second, data on the extent to which parasitic wasps adjust their sex ratio in response to host size, as described above (see Figure 1a), support the idea that more extreme sex ratio shifting is seen in more predictable environments. In some species (idiobionts) females kill the host at oviposition (egg laying), and in such cases host size will be a reliable cue as to the resources that offspring will have available for development. However, in other species (koinobionts) the host is not killed by the female, and so can continue to grow, in which case host size at oviposition is a less reliable predictor of the resources that their offspring will have available for development (King, 1989). Consistent with the possible importance of environmental predictability, species in which the host was killed at oviposition (idiobionts) were more likely to show facultative sex ratio variation than species in which the host was not killed (koinobionts) (King, 1989). More generally, it might be argued that in many cases, environmental predictability in factors influencing sex ratio behaviour is likely to be greater for invertebrates (eg, assessing the number of females on a patch or host size) than for vertebrates (eg, assessing the amount of lactation that a female will be able to provide, or the heritable genetic quality of her mate).

Clearly this is an area in which further theoretical and empirical work is required. For example:

(1) Much of the discussion above is based upon verbal arguments. Theoretical models are required that can be used to predict variation in the amount (and precision) of sex ratio adjustment. These models should allow for the cost of sex ratio adjustment (Maynard Smith, 1980; Charnov, 1982; Leimar, 1996; Pen *et al*, 1999; Pen, 2000; Pen and Weissing, in press), and variation in the benefit due to factors such as environmental predictability (West *et al*, 2000a; West and Sheldon, submitted). Also useful would be formal genetic models that incorporated the effect of genetic constraints such as mutation and pleiotropy (Barton and Turelli, 1989).

(2) The cost of sex ratio adjustment will depend heavily upon the mechanism with which it is carried out (Pen and Weissing, in press). However, very little is known about this mechanism in organisms with CSD (Krackow, 1995). Even in organisms with haplodiploid sex determination, there is very little knowledge of the underlying genetics (Orzack and Parker, 1990; Orzack and Gladstone, 1994; West and Herre, in press; for data in plants see Campbell, 2000). Estimating fundamental genetic parameters be particularly important if genetic constraints play an important role in explaining variation in the precision of sex ratio adjustment.

(3) A major problem is that in cases where the offspring sex ratio is expected to be adjusted in response to environmental conditions, the observed pattern is often a gradual shift in response to environmental quality (eg, host size, Figure 1a), whereas theory predicts a threshold shift from all male to all female offspring at a critical value of the environmental variable (eg, at a certain host size, Figure 2; Charnov *et al*, 1981). This remains the case even when there are costs to sex ratio adjustment (Pen, 2000; Pen and Weissing, in press). It is clear that theory needs to be developed that can adequately predict gradual shifts in offspring sex ratios in response to variation in environmental quality. One possibility is uncertainty, with individuals making mistakes (Charnov *et al*, 1981; Leimar, 1996; Pen, 2000; Pen and Weissing, in press). However, even with uncertainty the slopes frequently remain very steep (similar to a threshold) unless extreme assumptions are made (eg, if the cost of deviating from a sex ratio increases exponentially the further you are from 50% males). An alternative explanation might be genetic constraints, such as mutation or pleiotropy. Clearly formal genetic models of sex ratio adjustment are required, as well as empirical data to test them (eg, what is the mutability or pleiotropy of sex ratio behaviour?).

(4) In some vertebrates, the sex determination mechanism has evolved back and forth between CSD and environmental sex determination (ESD) on many occasions, especially in turtles (Janzen and Krenz, in press; Janzen, submitted). This suggests that the costs and benefits of different sex determination systems may vary dependent upon environmental conditions. This may depend upon selection for sex ratio adjustment, or in response to genetic (intragenomic) conflict over sex ratio adjustment (eg, mothers and offspring favouring a different sex ratio; Hamilton, 1967; Werren and Beukeboom, 1998; Werren and Hatcher, 2000; Beukeboom *et al*, 2001;

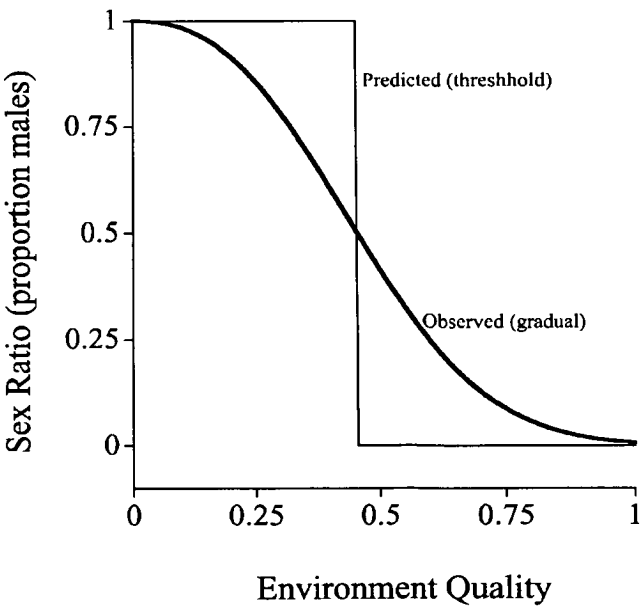


Figure 2 The pattern of conditional sex ratio adjustment – theory and reality. Plotted is the typical situation for the predicted and observed (thicker line) pattern of sex ratio adjustment in response to variation in environmental quality (eg, host size in parasitic wasps or maternal quality (dominance rank) in mammals). The theoretical prediction is usually to produce only one sex (in this case only males) below a threshold quality (eg, host size), and only the other sex (in this case only females) above that threshold. In contrast, empirical data usually shows a more gradual shift.

Werren *et al*, submitted), and even cultural inheritance (Freedberg and Wade, 2001) may play a role. Given this, it is clear that there may be complex interactions between selection on the sex determination system and the sex ratio, and that in some cases the evolution of these two traits will need to be studied simultaneously. This approach might even help solve the enormous problem of explaining the occurrence in reptiles of ESD, and in some cases extremely female biased sex ratios (Shine, 1999; Freedberg and Wade, 2001; Janzen, submitted).

(5) Greater emphasis is required on comparative studies that examine variation in the extent (or precision) of sex ratio adjustment (eg, Herre, 1987; West and Herre, 1998; West and Sheldon, submitted), rather than population sex ratios (eg, Williams, 1979; Clutton-Brock, 1986; Clutton-Brock and Iason, 1986; Palmer, 2000). Especially useful would be studies in areas where theory is well-developed, and unambiguous *a priori* predictions can be made (West and Sheldon, submitted). Meta-analyses provide a useful methodology for such work.

(6) Direct tests of theoretical predictions could be made by varying the benefit of sex ratio adjustment (eg, through varying environmental predictability) in controlled laboratory selection experiments, and observing the consequences for sex ratio behaviour.

(7) There are very little data on how selection acts on the sex ratio in natural populations, and a particular shortage of experimental work (Komdeur (1998) provides an exception; for laboratory studies see: Conover and van Voorhees, 1990; Basolo, 1994; Varandas *et al*, 1997; Carvalho *et al*, 1998; Komdeur, 1998; Blows *et al*, 1999). However, such work is of considerable importance because without evidence that selection favours particular variants in the sex ratio it is difficult to assess whether observed patterns may represent adaptation. Explaining sex ratio patterns (or their absence) then becomes largely an exercise in ingenuity.

Limits on our ability to explain sex ratios

As the last section has suggested, very little is known about the way in which selection acts on sex ratio variation, or on any putative decision rules underlying this variation, in natural populations. Even in the absence of this information, it is worth stressing that we should not expect to find adaptation in the sex ratio in all cases. In organisms with complex life-histories (overlapping generations, extensive dispersal, subject to multiple life-history trade-offs) a large number of factors could influence sex allocation and there is no reason to assume that selection would act to favour one form of sex allocation behaviour over another. In particular, if selection varies over time, or between genetically connected populations, then there may be little likelihood of populations reaching local optima. Hence, we reiterate the need for studies measuring selection on sex ratios and sex allocation, preferably replicated over time and space (especially in vertebrates).

A second limit to our ability to explain sex ratios may lie in the fact that models of optimal sex allocation generally assume that the sex ratio is a trait exclusively under parental control (Bull and Charnov, 1988). This assumption may be violated in several ways. As noted above, genetic conflict may influence the evolution of sex determination mechanisms, but this may equally apply to the evolution of sex allocation. There is considerable clear evidence for intragenomic conflict over sex allocation (Hamilton, 1967; Werren and Beukeboom, 1998) – indeed, sex ratio distorting elements provide some of the clearest examples of 'selfish genes' that distort the sex ratio in order to maximize their own transmission, but to the determent of the fitness of the individual carrying them (eg, Werren *et al*, 1981). As Leigh (Leigh *et al*, 1985; Leigh, 1986) suggested, a chromosomal sex ratio distorting mechanism might be vulnerable to invasion by selfish genetic elements, in which case suppression of the mechanism by autosomal genes would be favoured. Part of the variation between species in the extent to which they adjust the sex ratio might simply represent stochastic variation in whether they had been subject to invasion by selfish genetic elements followed by autosomal suppression.

Another level of conflict over the sex ratio may be between parents and offspring (Trivers and Hare, 1976; Werren and Beukeboom, 1998; Werren and Hatcher, 2000). It is well appreciated that in haplodiploid species, relatedness asymmetries may lead to parents and offspring having different optimum sex ratios (eg, outbred mothers are equally related to sons and daughters favouring a sex ratio of 0.5, whereas daughters are three times more related to sisters than brothers, favouring a sex ratio of 0.25; assuming the mother mated once). This has gained particular attention in the social insects, where theory is well developed, there is a large body of literature testing theory, and it seems that offspring can often win the conflict (Chapuisat and Keller, 1999; Sundstrom and Boomsma, 2000). Another reasonably clear example from the Hymenoptera arises in polyembryonic species (see Godfray, 1992; Grbic *et al*, 1992; Harvey *et al*, 2000). However, more generally, little is known about the resolution of parent–offspring conflict (ie who wins?). Furthermore, it is possibly less appreciated that if offspring are reared in family groups, interactions between different sexes of offspring may change the sex ratio, or the relative reproductive values of the two sexes of offspring (Pickering, 1980; Godfray, 1986; Ude *et al*, 1996; Nager *et al*, 2000). While the expected consequences of such intrabrood competition are worked out in some cases for the sex ratio at the level of the population (Godfray, 1986; West *et al*, 1999; Werren and Hatcher, 2000), it is not known how intersexual intrabrood competition would affect sex ratio adaptation at the level of individuals in response to environmental variation, particularly since the effects of competition between the sexes might be strongly context-dependent.

Conclusions

We have suggested that a major problem remaining for research into sex ratios and sex allocation is explaining why different organisms show so much variation in the amount and precision with which they adjust their offspring sex ratios. In order to address this problem we need to improve our understanding of constraints that may prevent 'perfect' behaviour, and to incorporate them into theory. This will require work from diverse areas –

for example, behavioural ecology, physiology, mechanisms of sex determination, genetics (eg, what is the importance of pleiotropy or mutation). Clearly this will not be a trivial undertaking.

However, this work is also important more generally because sex allocation theory offers some of the best opportunities for studying the nature of constraints on adaptation and evolution by natural selection (Herre, 1987; Bull and Charnov, 1988; Seger and Stubblefield, 1996; West *et al*, 2000a; Herre *et al*, 2001; West and Herre, in press). Although it is widely acknowledged that constraints are important in evolution (Gould and Lewontin, 1979; Maynard Smith *et al*, 1985; Partridge and Sibley, 1991; Orzack and Sober, 2001), little progress has been made outside of sex allocation theory in quantifying exactly why, when and to what extent constraints are important (Bull and Charnov, 1988). Work on sex allocation, and especially sex ratios, is useful in this respect because it is one of the few areas in life history theory (Stearns, 1992) where we can hope for a reasonably quantitative fit between empirical data and the predictions of simple theoretical models. Put simply, if we cannot understand sex ratios, we cannot hope to understand most other life history traits, whose evolution usually depend upon far more complex trade-offs.

Acknowledgements

This paper is based on a talk given at the Genetics Society meeting on Sex. We thank: Nick Barton, Jonathan Hodgkin, Anne McLaren and Linda Partridge for organising the meeting, and inviting the talk; John Brookfield for organising the special edition of *Heredity* that has arisen from this meeting, and for inviting our contribution; Dave Allsop, John Brookfield and Jack Werren for comments on the manuscript. We are funded by the BBSRC, NERC and Royal Society.

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Vol. 202: 273–281, 2000 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published August 28

Incubation periods and sex ratios of green turtles: highly female biased hatchling production in the eastern Mediterranean

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ABSTRACT: Marine turtles are globally endangered and subject to numerous conservation and management initiatives, yet many aspects of their life histories remain undescribed. All populations subject to investigation have been shown to have temperature dependent sex determination, and data in a number of cases have suggested that the sex ratio of hatchling production may be highly female biased. To date, the green turtle *Chelonia mydas* has been little studied in this respect. We recorded the temperature in 18 green turtle clutches laid at Alagadi Beach, Northern Cyprus using automated intra-nest recording devices. The temperatures experienced within these clutches ranged from 26.7 to 34.1°C with mean temperature ranging from 29.8 to 32.5°C. No regular diel thermal cycle was observed. Incubation periods at this site decreased as the season progressed and ranged from 43 to 60 d (n = 231; 1993 to 1998). In comparison to other published studies regarding temperature dependent sex determination in this species, these data are strongly suggestive of a highly female biased hatchling sex ratio. This hypothesis was partly confirmed utilising histological sexing of hatchlings found dead in nests (>99% female in 1998, n = 231). From these data we estimate that the pivotal incubation period for this population is ≥56 d and the pivotal temperature as below 29.2°C. Using a conservative assumption that 100% males are produced by nests with incubation periods ≥56 d and 100% females are produced by nests with shorter incubations, for the years 1993 to 1998, we estimate that at least 86 to 96% of hatchlings produced at this site were female.

KEY WORDS: Marine turtle · *Chelonia mydas* · Temperature · Hatching · Environmental sex determination

INTRODUCTION

In many reptilian species, including sea turtles, the sex of the offspring is dependent on incubation temperatures and is not genetically determined at the time of egg deposition. This temperature dependent sex determination (TSD) is exhibited in different patterns among different reptile groups (Janzen & Paukstis 1991). In the marine turtle species studied to date, higher temperatures have been shown to produce a greater proportion of females, with cooler temperatures producing more males (see Mrosovsky 1994 for review). The temperature experienced during the thermosensitive period, thought to occur during the middle third of incubation, is critical for sexual development. The temperature at which a 1:1 sex ratio is produced is termed the pivotal temperature (Yntema & Mrosovsky 1980, Miller & Limpus 1981, Spotila et al. 1987, Mrosovsky & Pieau 1991). Although the possibility for some degree of interpopulation variation in patterns of TSD in at least some sea turtle species exists (Chevalier et al. 1999), pivotal temperatures have been shown to be conservative (Mrosovsky 1994).

A number of population specific factors have been described as affecting nest temperatures and hence sex ratios of marine turtles. These include: latitudinal variation, seasonal temperature changes, shading by

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vegetation, sand colour, episodic events such as rain and depth of the eggs (Morreale et al. 1982, Mrosovsky et al. 1984, Mrosovsky 1988, Hays et al. 1999).

Studies determining the pivotal temperature and sex ratio of hatchling production in marine turtle populations have employed several different techniques including: laboratory incubation of eggs under controlled thermal regimes (Mrosovsky & Yntema 1980, Miller & Limpus 1981, Billett et al. 1992, Georges et al. 1994); inferences from long term control site temperature monitoring at nest depth (Limpus et al. 1983, Mrosovsky et al. 1984, Godfrey et al. 1996); intra-nest temperature logging (Spotila et al. 1987, Kaska et al. 1998); and inferences from incubation periods (Marcovaldi et al. 1997). This latter technique utilises the fact that the duration of incubation period is related to intra-nest temperature, so that a pivotal incubation period, i.e. that which produces a 1:1 sex ratio, can be calculated. This technique has recently been validated (Mrosovsky et al. 1999).

The major challenge to this area of study is the difficulty in accurately determining the sex of hatchlings. The most commonly used method is that of sexing hatchlings from gonadal morphology upon histological examination (Yntema & Mrosovsky 1980, Miller & Limpus 1981, Dutton et al. 1984, Billett et al. 1992). This usually necessitates the sacrifice of study animals and, given the endangered and protected status of marine turtles, has led to many studies based on small sample sizes. Although an alternative exists in the use of hormone assay (Wibbels et al. 1991), this is a relatively costly and logistically challenging technique. An additional opportunity arises to sample hatchlings which die in the sand column during emergence (Wibbels et al. 1999). Although it must be considered that these samples may be the result of a sex specific neonatal mortality factor, data derived from such studies can be from a large selection of nests and, in combination with other techniques, can give an insight into the likely sex ratio of hatchling production of the population.

Of the marine turtles, the most scrutinised has been the loggerhead turtle *Caretta caretta*, in which some populations have been demonstrated as showing extremely female biased hatchling production (Mrosovsky & Provancha 1989, 1992, Marcovaldi et al. 1997). Data regarding TSD in the green turtle *Chelonia mydas* are relatively scant. In a laboratory based study, Miller & Limpus (1981) suggested a pivotal temperature exists between 26 and 29°C for green turtles nesting on the Great Barrier Reef. Whilst Limpus et al. (1983) demonstrated spatial variation in the hatchling sex ratio of this population, no quantitative estimates of overall sex ratio were provided. Evidence from more recent studies on green turtles suggests that the pivotal temperature for this species is more likely to fall at the higher end of the range suggested by Miller & Limpus (1981).

Work over a number of seasons on the green turtles nesting in Suriname (Mrosovsky et al. 1984, Godfrey et al. 1996) has used progressive statistical refinements resulting in 2 separate estimates of a pivotal temperature of 29.1 or 29.4°C (Godfrey 1997). Godfrey et al. (1996) estimated that, dependent on variation in weather patterns, the ratio of female hatchlings produced in Suriname ranged between 20 and 90% in different years. In Tortuguero, Costa Rica, by measurement of intra-nest temperatures, an estimated pivotal range of 28 to 30°C was generated, and hatchling production was estimated as 67% female (Standora & Spotila 1985, Spotila et al. 1987). Higher male ratios (94%) were however recorded in nests shaded by vegetation. In a preliminary study of 5 green turtle nests in Northern Cyprus using intra-nest data loggers, placed at the top, middle and bottom of the clutch, Kaska et al. (1998) estimated the pivotal temperature in this population to be just below 29°C. No attempt was made in this study to generate overall sex ratios of hatchling production in the population. Although temperature differences were found within clutches, these were small (range ≤0.8°C; with cooler temperatures recorded at the bottom of clutches).

In the present study we examine the role played by temperature as an influence on the incubation periods and sex ratio of green turtle hatchlings produced at Alagadi Beach, Northern Cyprus in the eastern Mediterranean: (1) We investigate temperature regimes experienced in 18 green turtle nests utilising intra-nest temperature recording devices. (2) We describe incubation periods over 6 seasons (1993 to 1998) and investigate the role of temperature as an influence on the duration of incubation periods. (3) We describe the sex ratio in a large sample of non-invasively collected hatchlings from nests hatched in 1998. (4) From these data and known information regarding the role of temperature in the determination of sex in green turtle populations elsewhere, we draw wider inferences as to the likely sex ratio of hatchlings produced from this rookery and others in the region.

METHODS

Study site. This study was largely conducted on Alagadi Beach (35°33′N, 33°47′E), situated in Northern Cyprus in the eastern Mediterranean. There is a high level of human traffic on this 2 km beach; however, beach umbrellas and shades are only used at the water's edge, in the region where no green turtle clutches are laid. Additional limited data were collected from other more remote green turtle nesting

beaches on the island, which were not subject to any recreational use. No nests experienced any shading on these beaches.

Data collection. At Alagadi, both laying and hatching were monitored between May and October between 1993 and 1998 through nightly patrols and daily dawn beach surveys according to a previously described protocol (Broderick & Godley 1996). Other beaches were surveyed every 2 d throughout the season of 1998. Nest temperatures were recorded using 'Tinytalk' data loggers (Orion Components Ltd, Chichester, UK; accuracy ±0.4°C), which had been calibrated with a mercury thermometer of known accuracy (NAMAS certified to read within 0.1°C of the absolute temperature; Hays et al. 1999). The logger was placed into the middle of the clutch of eggs during oviposition, and the female was allowed to cover the nest. Temperature was recorded at sampling intervals of 48 min in a total of 18 clutches between 1995 and 1998. Data recorded from laying until midnight on the first night of hatchling emergence were included in our analysis. The positions of all nests were recorded in relation to marker posts placed at 50 m intervals at the back of the beach. In addition, a numbered plastic marker was placed in the sand above the egg chamber as the female began to cover her eggs. Upon hatching, nest contents, including dataloggers where present, were excavated and the nest tag located. Nest tags allowed 100% correlation with the laying event, a process difficult to ensure simply by using nest positions when high density nesting occurs. Incubation periods were calculated as the number of days between the night on which the clutch was laid until the night on which the first group of hatchlings emerged. The date prior to midnight of the night in question was taken in all cases.

Collection of hatchlings for sexing. Dead hatchlings were collected at post-hatching excavation (after a period of 48 h with no hatchling emergence) from nests of known incubation periods. The sex of hatchlings was determined using standard histological techniques (Yntema & Mrosovsky 1980, Miller & Limpus 1981, Dutton et al. 1984, Billett et al. 1992).

RESULTS

Temperatures

Lay dates, incubation periods and summary incubation temperature data recorded are presented in Table 1. Temperatures ranged between 26.7 and 34.1°C with mean incubation temperatures ranging from 29.8 to 32.5°C. During the middle third of incubation, temperatures ranged from 28.8 to 34.1°C with means ranging from 29.7 to 32.8°C. Fig. 1 illustrates the temperature profiles in 2 clutches (Clutches 9 and 18, the first and last laid in 1998) throughout the incubation period. No diel temperature cycle was recorded in any of the 18 study nests. Mean daily range for the 18 nests varied between 0.07 and 0.18°C (Table 1).

For the 10 clutches that we monitored in 1998, we averaged all the readings for each 48 min sampling interval to produce a composite seasonal profile of the

Table 1. Laying date, incubation periods and temperature data (°C) for the 18 clutches in which temperature was monitored. IP: incubation period

Nest	Lay date	IP (d)	Total IP				Middle third IP				Daily range			
			Mean	±SD	Min	Max	Mean	±SD	Min	Max	Mean	±SD	Min	Max
1	22 Jun 95	53	30.6	1.57	28.1	33.0	30.2	0.63	29.4	31.6	0.14	0.22	0	0.5
2	25 Jun 95	50	30.8	1.05	28.9	32.1	30.6	0.51	29.8	31.6	0.07	0.22	0	0.5
3	28 Jun 96	51	30.7	1.12	26.8	32.5	30.8	0.40	29.8	31.6	0.09	0.24	0	0.5
4	16 Jun 97	50	31.0	1.80	26.7	33.3	31.2	0.54	30.3	32.1	0.18	0.20	0	0.7
5	26 Jun 97	51	30.0	1.20	28.1	31.8	29.7	0.59	28.8	31.1	0.14	0.19	0	0.5
6	27 Jun 97	50	31.1	0.94	28.8	32.5	31.2	0.54	30.3	32.1	0.13	0.18	0	0.5
7	29 Jun 97	48	31.4	0.98	29.2	32.9	31.3	0.55	30.6	32.1	0.12	0.19	0	0.5
8	10 Jul 97	50	31.2	1.17	29.4	33.0	31.0	0.58	30.3	32.5	0.16	0.22	0	0.5
9	09 Jun 98	53	30.3	1.58	27.0	32.6	30.6	0.57	29.6	31.1	0.13	0.18	0	0.4
10	15 Jun 98	52	30.2	1.70	27.4	33.0	30.1	0.46	29.2	31.1	0.16	0.19	0	0.4
11	21 Jun 98	51	30.6	1.26	28.1	32.5	30.4	0.44	29.8	31.6	0.12	0.20	0	0.5
12	02 Jul 98	50	31.4	1.32	29.6	33.3	31.1	0.62	30.3	32.2	0.13	0.18	0	0.4
13	09 Jul 98	54	29.9	1.16	27.4	31.8	30.0	0.53	29.2	30.7	0.11	0.21	0	1.1
14	09 Jul 98	48	31.8	1.12	29.9	34.1	32.4	0.90	31.4	34.1	0.14	0.19	0	0.4
15	17 Jul 98	48	31.5	0.66	28.8	32.1	31.8	0.36	31.1	32.1	0.11	0.18	0	0.5
16	18 Jul 98	54	29.8	0.66	28.4	30.7	29.9	0.24	29.6	30.3	0.07	0.15	0	0.4
17	21 Jul 98	46	32.5	0.62	30.3	33.3	32.8	0.22	32.1	32.9	0.13	0.19	0	0.4
18	01 Aug 98	43	32.0	0.59	29.2	32.9	31.7	0.34	31.4	32.5	0.15	0.19	0	0.5

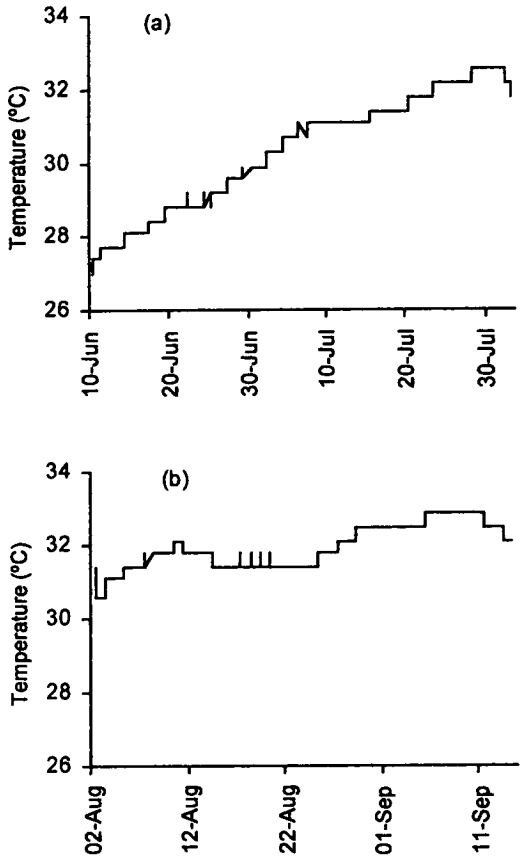

Fig. 1. Temperature profiles of (a) Clutch 9, laid on 9 June 1998 and hatched on 1 August 1998 after an incubation period of 53 d. (b) Clutch 18, laid on 1 August 1998 and hatched on 13 September after an incubation period of 43 d. Temperature shown is that recorded every 48 min throughout incubation

mean incubation temperatures experienced in this year (Fig. 2a). Apart from a period of 1 wk at the start and end of the season ≥2 nests were monitored concurrently. In general, the among nest variance was not high with mean range of temperature for all 48 min sampling intervals being 2.5°C (range 0.4 to 4.2, SD = 1.05, n = 2617). There was a pronounced seasonal pattern, with temperatures building through June and July, staying relatively high and constant in August, and gradually decreasing at the start of September. The increase in the variance of temperature on 5 September was due to the hatching of a nest, leaving only 2 study nests. The marked increase in temperature on 11 September was a result of the hatching of the penultimate nest.

To account for the temporal distribution of nesting, for each week of the 1998 season the proportion (%) of nests in the middle third of incubation was calculated (Fig. 2b). During this period few nests experienced thermal conditions likely to result in the production of a male biased sex ratio (>90% nests reach the middle third of incubation when mean temperatures are already well in excess of 29°C, the thermal region where most pivotal temperatures have been demonstrated; Mrosovsky 1994). This suggests that the sex ratio of hatchlings produced at this site will be heavily female biased.

Incubation periods

The incubation periods recorded at Alagadi Beach over the 6 yr of this study ranged from 43 to 60 d (Table 2). The mean incubation period recorded for each of the study years ranged from 48.0 to 51.4 d. Mean incubation period recorded in 1998 was significantly shorter than that of 1994 (ANOVA: $F = 3.25$, $p = 0.008$, df = 5, n = 231; followed by a Tukey test for unequal samples; Zar 1999).

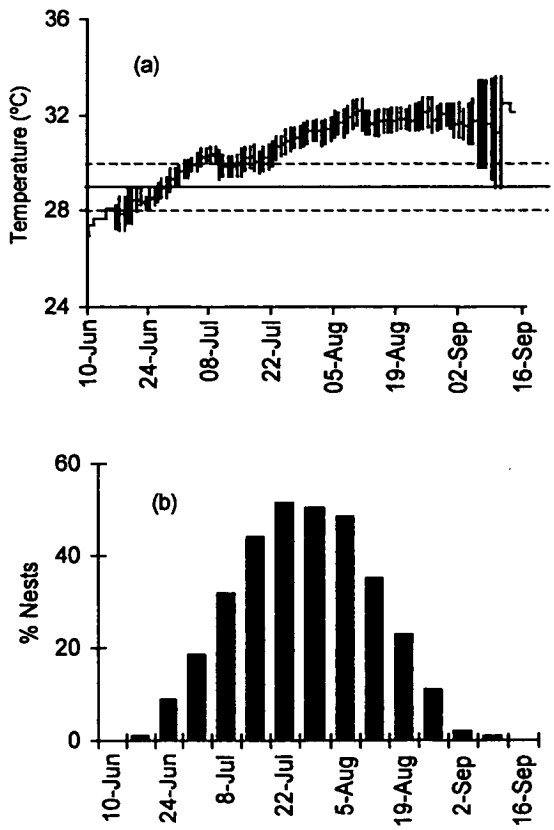

Fig. 2. (a) Composite seasonal profile of mean temperatures (with SE bars every 25th value) recorded within 10 clutches in 1998. Solid line indicates 29°C; the approximate pivotal temperature of green turtles previously reported (Godfrey 1997, Kaska et al. 1998). Broken lines signify 28 and 30°C; the range in which pivotal temperatures are likely to fall (Spotila et al. 1987). (b) Weekly distribution (%; n = 91) of clutches in their middle third of incubation, Alagadi 1998. Note: All clutches are represented in 2 or 3 weekly bins

Table 2. Mean incubation periods recorded at Alagadi Beach in each of the seasons 1993 to 1998. Parentheses give percentage of overall clutches laid that are included. IP: incubation period

Year	Mean IP	±SD	Min	Max	N (%)
1993	50.6	2.72	45	58	24 (48.0)
1994	51.4	3.57	45	59	45 (66.2)
1995	50.8	3.63	44	60	51 (79.7)
1996	48.0	5.07	44	58	7 (87.5)
1997	50.2	2.89	46	57	13 (100.0)
1998	49.3	3.48	43	58	91 (82.0)
Total	50.2	3.57	43	60	231 (73.6)

Seasonal variation in incubation periods

Fig. 3 illustrates the relationship recorded in 1998 between the date on which a nest was laid and incubation period. Regression analysis, conducted using day of the season (with Day 1 being that on which the first green turtle clutch was laid), showed this relationship to be statistically significant ($F = 54.57$, $p < 0.001$, df = 1, n = 91).

Relationship between incubation period and temperature

The regression line describing the relationship between mean incubation temperature and incubation period was calculated for the 18 nests containing temperature loggers ($F = 58.25$, $p < 0.001$, df = 1; Fig. 4a). Residuals were found to be normally distributed (Anderson-Darling $p > 0.05$). Furthermore, if incubation period is to allow reliable prediction of sex ratio, it must be strongly correlated with the mean temperature during the middle third of incubation. This was also found to be the case ($F = 29.19$, $p < 0.001$, df = 1; Fig. 4b).

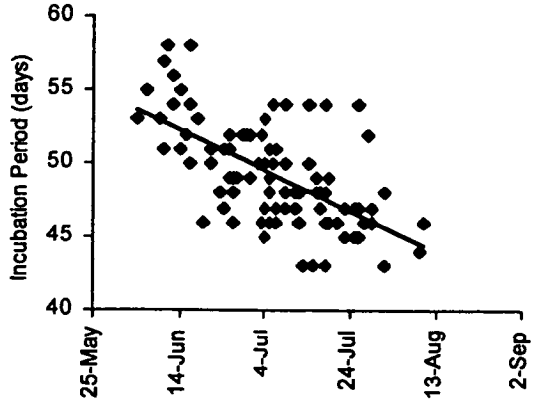

Fig. 3. Relationship between date on which a nest was laid and incubation period (IP) for 91 clutches laid in 1998, Alagadi Beach. (Eq. 1: IP = 53.8 – 0.138 date, $r^2 = 0.38$). For date, Day 1 = first day a green turtle clutch was deposited (4 June 1998)

Actual sex ratios

In 1998, 231 freshly dead hatchlings were collected from 44 nests of known incubation period at Alagadi and 13 hatchlings from 4 additional nests from the west coast of the island. The sex ratios of all nests, in comparison to incubation periods, are shown in Table 3. Males were only detected in nests with incubation periods of ≥56 d.

Estimating pivotal incubation period and pivotal temperature

Whilst it is not possible to derive an absolute pivotal incubation period using these data (as per Marcovaldi et al. 1997, Mrosovsky et al. 1999), as no males were

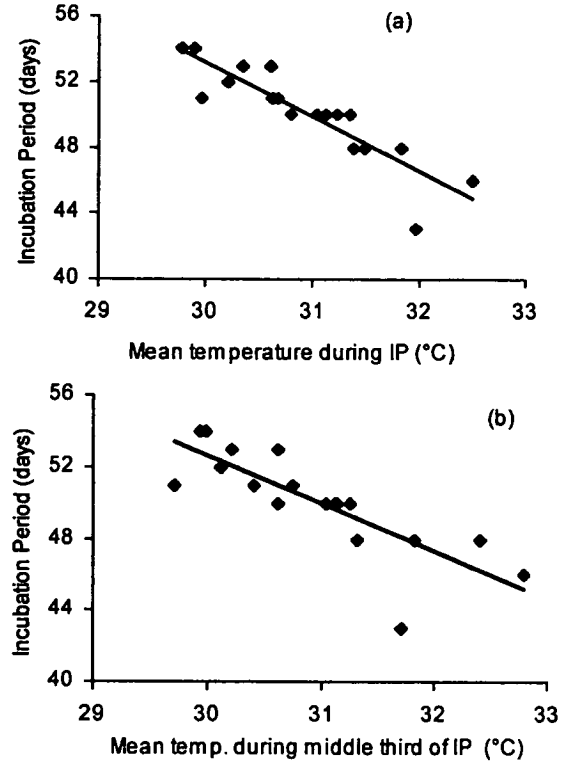

Fig. 4. (a) Relationship between incubation period and mean temperature throughout incubation period (IP). (Eq. 2: IP = 152 – 3.29 mean temperature throughout incubation, $r^2 = 0.79$). (b) Relationship between IP and mean temperature during the middle third of incubation (Eq. 3: IP = 132 – 2.65 mean temperature during the middle third of incubation, $r^2 = 0.65$)

Table 3. Results of histological sampling and sexing from nests of known incubation period (IP). Values in parentheses are number of hatchlings sampled from individual nests

IP (d)	Nests (n)	Hatchlings (n)	% female
43	2	7 (5, 2)	100
45	1	1	100
46	8	54 (7, 10, 10, 6, 8, 3, 5, 5)	100
47	6	32 (6, 4, 1, 7, 6, 8)	100
48	7	36 (8, 6, 6, 5, 2, 4, 5)	100
49	2	14 (8, 6)	100
50	2	9 (6, 3)	100
51	3	16 (4, 7, 5)	100
52	4	18 (7, 8, 2, 1)	100
53	1	5	100
54	4	12 (4, 4, 1, 3)	100
55	1	9	100
56	4	13 (1, 5, 5, 2)	100[a], 100, 60, 50[a]
57	1	8	100
58	2	10 (6, 4)	100[a], 75[a]

[a]Denotes samples from a nesting beach on the west coast of Cyprus

found in samples from nests with incubation periods less than 56 d, it is reasonable to assume that in this population the pivotal incubation duration is ≥56 d. However, this must be treated as a minimum value. Substituting this value in Eq. (2) (incubation period and mean temperature) we can derive a pivotal temperature of 29.2°C. However, this would be a maximum value. If we substitute 56 d incubation into Eq. (3) (incubation period and temperature during the middle third of incubation), a pivotal temperature of 28.7°C results. This too should be considered a maximum value.

Estimation of sex ratio

From these calculations it is possible to generate plausible estimates of sex ratio of hatchling production using incubation periods (at Alagadi in 1998: Fig. 5a; Alagadi between 1993 and 1998: Fig. 5b; and the rest of Northern Cyprus in 1998: Fig. 5c). If we assume that all nests with incubation periods of less than 56 d produce female biased offspring (considered as 100% females) and all nests of ≥56 d incubation period produced male biased offspring (considered 100% males), any estimate generated will give a conservative minimum proportion of females produced.

Based on these assumptions, it was estimated that >96% of hatchlings produced in the 91 nests laid at Alagadi in 1998 were female (Fig. 5a). Data from the years 1993 to 1998 produced estimates ranging from 86 to 96% female, with a combined data set of 231 nests likely to have produced a sex ratio >91% female (Fig. 5b). When nests from all other sites in Northern Cyprus (1998), excluding Alagadi were combined, they were estimated to have produced a sex ratio of >71% female (Fig. 5c; 120 nests). Overall for 1998 in Northern Cyprus this gives an estimated >82% females produced.

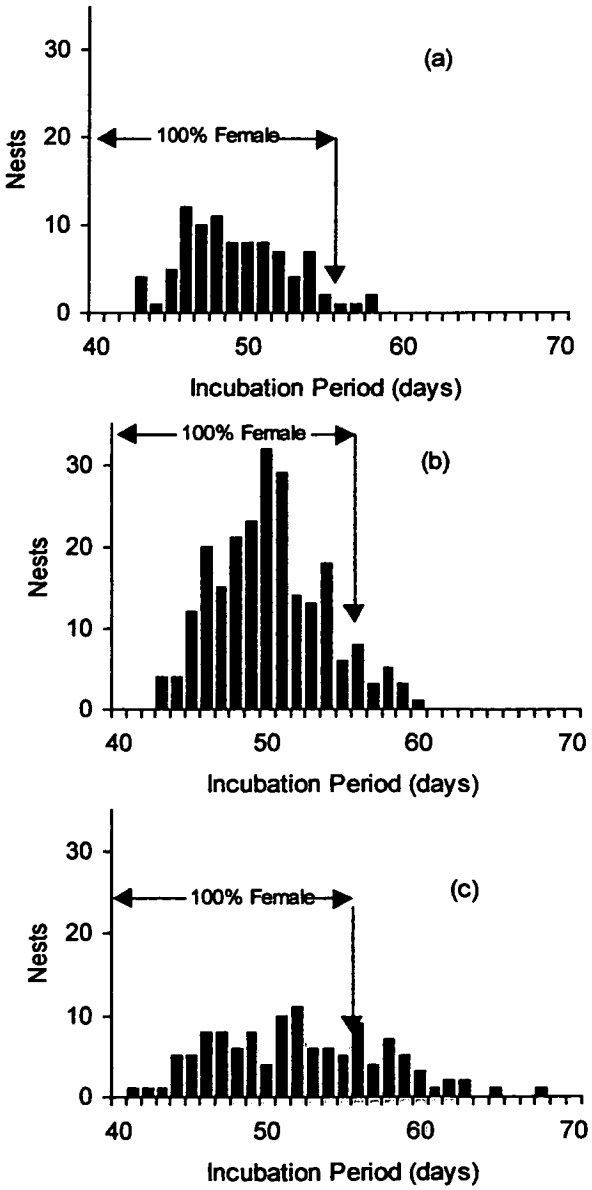

Fig. 5. Frequency histograms illustrating (a) incubation periods of 91 green turtle clutches in 1998 at Alagadi Beach, (b) incubation periods of 231 green turtle clutches over the study period 1993 to 1998 at Alagadi Beach, and (c) incubation periods of 120 green turtle clutches laid at other beaches around the island in 1998. Arrows signify the nests which, in the generation of sex ratios, were assumed to have produced 100% female hatchlings

DISCUSSION

Very few studies have examined natural nest temperatures where nest or egg manipulation has not taken place. The majority of the work conducted to date on TSD in marine turtles has been either laboratory based, with little attempt to replicate any seasonal or diel variation in temperature conditions, or based upon inferences made from sand temperatures. Conditions in the neighbouring sand are likely to vary from that of the nest owing to factors such as metabolic heat, decomposing bacteria and changes in moisture and porosity (McGehee 1990, Godfrey et al. 1997). In particular, the paucity of data based upon actual thermal conditions in nests of green turtles is apparent.

In this study we present the first substantial investigation of natural temperature regimes in nests of the green turtle in the Mediterranean. In this region, the only published work on this subject is that conducted by Kaska et al. (1998), who described the thermal conditions in 5 green turtle nests on a beach on the west coast of Northern Cyprus. However, the methodology of Kaska et al. (1998) involved both post-nesting and pre-hatching excavation to deploy and retrieve temperature loggers, and it is not known if this method of recording is free from artefact. Mean temperatures in our study (ranging between 29.8 and 32.5°C; n = 18) were comparable with those of Kaska et al. (1998) (29.5 to 31.3°C; n = 5). No regular diel variation was recorded in either study, similar to the findings of Hays et al. (1995) on Ascension Island. This is almost certainly due to the great depths at which green turtle clutches are laid, as diel variation has been recorded in the shallower nests of the loggerhead turtle in Cyprus (Broderick 1997, Kaska et al. 1998).

The composite thermal profile for 1998, produced from the temperature data recorded in the 10 study nests of this year, provides a clear illustration of the conditions experienced within clutches on this beach (Fig. 2a). Whilst the sample size may be small, it is apparent that nearly all clutches laid were likely to have been incubating at temperatures above 29°C during the middle third of incubation (Fig. 2b). The temperatures described are high and suggestive of female producing temperatures, when compared to previously published information regarding pivotal temperatures in this species (Miller & Limpus 1981, Mrosovsky et al. 1984, Standora & Spotila 1985, Spotila et al. 1987, Godfrey et al. 1996, Godfrey 1997, Kaska et al. 1998).

The clear relationship between temperature and incubation period recorded at this site and the high temperatures and short durations are indicative of a highly female skewed sex ratio. Indeed from this information alone we would expect this population to produce a higher proportion of females than any others of this species studied to date. The sexing of hatchlings supported these expectations, with >99% of those sampled at Alagadi Beach being female. It may be argued that through sampling only dead hatchlings, sex biased mortality is influencing our results. This is unlikely to be the case since the results obtained are similar to those of Kaska et al. (1998), who sacrificed live hatchlings in their study.

Our data give us a minimum pivotal incubation period of 56 d, which leads to a maximum pivotal temperature of 29.2°C. By comparison, Godfrey et al. (1996) estimated the pivotal incubation period in Suriname to be 58.5 d, which would translate to (using Eq. 2 calculated from this present study) a pivotal temperature of 28.4°C, similar to that estimated for that population (28.8°C, Mrosovsky et al. 1984; 29.1 to 29.4°C, Godfrey 1997). All previous studies on green turtles, barring that by Miller & Limpus (1981), suggest that the pivotal temperature for this species is somewhere between 28 and 30°C, which translates (using our temperature versus incubation period Eq. 2) to a pivotal incubation period of 53.3 to 60.2 d, encompassing the 56+ d estimate from this study.

Within the Mediterranean, the estimated annual female nesting population of green turtles is in the region of 300 to 500 individuals with the majority nesting in Turkey (Groombridge 1990) and Cyprus (approximately one-third; Broderick & Godley 1996), and a few nests recorded in Egypt and Israel (Kuller 1999, Clarke et al. 2000). Data regarding incubation periods in Turkey are sparse. However, those which have been published are from the most important single nesting beach in the region, Akyatan. There, mean incubation periods have been found to be 53.8 d (SE = 0.94, n = 23; Gerosa et al. 1995) and 53 d (SE = 0.56, n = 54; Gerosa et al. 1997), although in the second study some nests had been subject to prior disturbance by predators. In addition, clutches incubated at an Israeli hatchery also had relatively short incubation periods (mean 52.2 d, SD = 4.36, n = 21; Z. Kuller pers. comm.). Although conditions were sought which carefully mimicked those of the natural nests, caution should be used in comparing such an artificial environment. While these other Mediterranean sites have fairly short incubation periods, they are longer than those recorded in Cyprus, but still suggestive of female skewed hatchling production. Similarly the incubation periods of this population are below or at the lower end of the range of those found world-wide (50.6 to 59.5 d; see Hirth 1980 for a review).

The significant decrease in incubation periods within the 1998 season (Fig. 3) shows that clutches laid at similar times experience similar thermal conditions, and hence are likely to produce hatchlings of similar sex ratios. The decrease in incubation periods is likely

to be related to the seasonal profile of increasing temperatures (Fig. 2a). In 1994 and 1995, similar linear relationships were recorded; however in the later starting, longer season of 1993, the relationship between incubation period and laying date was quadratic rather than linear (Broderick 1997). Low levels of nesting in 1996 and 1997 precluded statistical analyses for these years. It is interesting to note that there was a closer relationship between mean temperature for the whole incubation period and incubation duration (Fig. 4a; $r^2 = 0.79$) than between mean temperature during the middle third of incubation and incubation duration (Fig. 4b; $r^2 = 0.65$). The effect of temperature on incubation duration is likely to be one integrated across the whole period, which will explain the increased proportion of variance explained by the former relationship. However, it is important to note that there is still a close relationship between the temperature in the middle third of incubation (the period when sex is determined) and the resultant incubation period.

Although the data collected were from a relatively large sample of nests and hatchlings (n = 48 nests; n = 244 hatchlings), because of the extreme skew in sex ratio, there was a paucity of male producing nests. Whilst it would be possible to calculate a pivotal incubation period mathematically as per Marcovaldi et al. (1997), this would have such a large confidence range as to have no utility. The fact that no males were produced in nests with incubation periods of below 56 d is convincing alone and is comparable with that found in the study of loggerhead turtles by Marcovaldi et al. (1997).

Although relatively crude, the minimum estimates of the ratio of female hatchlings produced across the different temporal scales are reliable and suggest that in the 6 yrs of this study Alagadi Beach has been producing a very high percentage of females (86 to 96%). Other beaches in the region may be predominantly male producing, possibly due to nests experiencing lower temperatures due to the sand being of a higher reflectance. This pattern has been found in studies on Ascension Island (Hays et al. 1995, 1999). Although other beaches in Northern Cyprus (Fig. 5c) have nests with incubation periods longer than those at Alagadi in the same season (Fig. 5a), they are still likely to have profoundly female biased hatchling production.

If the skewed sex ratio continues through to adulthood and natal philopatry for both sexes is in operation, it might be a concern that there would be too few males to allow successful fertilisation of available females. There is no evidence to suggest an infertility problem leading to low hatching success. Excluding nests which fail due to inundation or predation, nests in Cyprus hatch with an annual mean success of 83.8 to 85.3% (1992 to 1995; Broderick & Godley 1996).

Because of the discrete nature of the Mediterranean green turtle population (Bowen et al. 1992, Encalada et al. 1996), it is vital to discover if such highly biased hatchling production occurs elsewhere in the region. Given that the ratio of hatchlings produced in the Eastern Mediterranean would appear biased towards females, there is a need to investigate whether this bias persists in the wild population to the juvenile and adult stages. This would be possible using the techniques that demonstrated that, at least in some regions, the female bias of North American loggerhead turtles is present in the live juvenile population (Wibbels et al. 1991). Alternatively, sex ratios of stranded animals could be investigated.

Assuming these female biases are widely present in this relatively isolated population, plans for conservation management must take these results into account. Transplanting of nests may further bias sex ratios. Nests should be moved to an area where similar thermal conditions exist as at the primary site. Managers should be vigilant against transplanting from a possible male producing area to a safe female producing area, and only move nests if they are guaranteed to be lost without action. Clutches deposited early in the season may have a great level of importance with regard to producing enough males. This should be considered for example when prioritising limited management resources to prevent nest predation. The difficulty in determining a pivotal temperature for incubation period from a population with such a highly skewed sex ratio of hatchling production highlights the need for a laboratory based pivotal temperature to be demonstrated for this regional population.

Acknowledgements. The authors would like to thank all members of Glasgow University Turtle Conservation Expedition 1992 to 1998; special thanks to Damla Beton and Dr Tunay Beton and staff at Lefkosa Hospital for histological assistance. This work was supported by the British Association of Tortoise Keepers, British Chelonia Group, British Ecological Society, Carnegie Trust, Cross Trust, European Commission DG1B/1A, Institute of Biology, Glasgow Natural History Society, Glasgow University Court, MEDASSET UK, People's Trust for Endangered Species, North of England Zoological Society, and Zebra Foundation. Logistical support was given by the Department of Environmental Protection and the Society for the Protection of Turtles in Northern Cyprus; unpublished data were provided by Zeev Kuller, Israel Nature and National Parks Protection Authority.

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Editorial responsibility: John Austin (Assistant Editor), Oldendorf/Luhe, Germany

Submitted: October 15, 1999; Accepted: February 17, 2000
Proofs received from author(s): August 1, 2000

ELSEVIER

Journal of Experimental Marine Biology and Ecology
263 (2001) 45–63

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

Thermal conditions in nests of loggerhead turtles: further evidence suggesting female skewed sex ratios of hatchling production in the Mediterranean

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Received 21 September 2000; received in revised form 15 April 2001; accepted 28 April 2001

Abstract

Temperature was recorded in 23 nests of the loggerhead turtle (*Caretta caretta*) and control sites of nest depth at Alagadi (35°33′N, 33°47′E), Northern Cyprus, eastern Mediterranean. Control site sand temperature was found to be highly correlated with mean daily air temperature and mean nest temperature. Mean temperature in nests ranged from 29.5°C to 33.2°C, with mean temperature in the middle third of incubation ranging from 29.3°C to 33.7°C. Hatching success was significantly correlated with incubation temperature, with nests experiencing very high temperatures exhibiting low hatching success. All nests demonstrated regular diel variation in temperature with mean daily fluctuations ranging from 0.3°C to 1.4°C. Increase in temperature above that of the prevailing sand temperature attributed to metabolic heating was clearly demonstrated in 14 of 15 clutches, with the mean level of metabolic heating of all nests being 0.4°C. However, the level of metabolic heating varied markedly throughout the incubation period with levels being significantly higher in the final third of incubation. Incubation duration was found to be significantly correlated to both the mean temperature of nests throughout the incubation period and during the middle third of incubation. The relationship between incubation duration and mean incubation temperature was used to estimate mean incubation temperatures at

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0022-0981/01/$ - see front matter
PII: S0022-0981(01)00269-6

most major nesting sites throughout the Mediterranean from available data on incubation durations, showing that mean incubation temperature is likely to be above 29.0°C at most sites in most seasons.

Keywords: *Caretta caretta*; Incubation duration; Sea turtle; Sex ratios; Temperature-dependent sex determination (TSD)

1. Introduction

In marine turtles, as in many oviparous reptiles, the direction of sexual differentiation depends on the temperature prevailing during incubation; a phenomenon termed temperature-dependent sex determination (TSD; recently reviewed by Shine, 1999). In addition, the temperature of turtle nests also affects the rate of embryonic development (Ackerman, 1994, 1997), and phenotype of the offspring (Packard and Packard, 1988; Packard et al., 1999; McGehee, 1990). Recent studies to estimate sex ratio of hatchling production in marine turtles have employed several different techniques including: laboratory incubation of eggs (Mrosovsky and Yntema, 1980), inferences from long-term control site temperature monitoring at nest depth (Godfrey et al., 1996), intra-nest temperature logging (Spotila et al., 1987), and inferences from incubation durations (Marcovaldi et al., 1997).

Studies have indicated that nests which incubate at high temperatures (> 29.0°C) produce a larger proportion of females, with cooler nests (< 29.0°C) producing a greater proportion of males (Mrosovsky, 1994; Ackerman, 1997). The thermosensitive period when the sex is determined has been shown to occur during the middle third of incubation (Yntema and Mrosovsky, 1980; Mrosovsky and Pieau, 1991). The temperature at which a 1:1 sex ratio is produced is termed the pivotal temperature (Yntema and Mrosovsky, 1980; Miller and Limpus, 1981; Mrosovsky and Pieau, 1991). Although the possibility for some degree of inter-population variation in patterns of TSD in at least some sea turtle species exist (Chevalier et al., 1999), pivotal temperatures have been shown to be relatively conservative (Mrosovsky, 1994).

Of the marine turtles, the first to be intensively scrutinised concerning TSD was the loggerhead turtle (*Caretta caretta*) in which some populations have extremely female-biased hatchling production (Mrosovsky and Provancha, 1989, 1992; Marcovaldi et al., 1997; Hanson et al., 1998; Provancha and Corsello, 1998). The pivotal temperatures so far described in this species in the Atlantic are close to 29.0°C (Mrosovsky, 1994; Ackerman, 1997), with values of 29.2°C for Brazil (Marcovaldi et al., 1997), 29.0°C for the USA (Mrosovsky, 1994) and just below 29.0°C in the Mediterranean (Kaska et al., 1998). Although some estimates are present from the Pacific, they are more variable (28.9°C and 28.7°C in Australia: Georges et al. (1994); Limpus et al., 1983; and 29.7°C in Japan: Matsuzawa et al., 1998). Studies have involved laboratory incubations (Georges et al., 1994; Billett et al., 1992; Limpus et al., 1983; Mrosovsky and Yntema, 1980; Mrosovsky, 1988), extrapolation from control site temperatures (Baptistotte et al., 1999; Limpus et al., 1983; Mrosovsky and Provancha, 1989, 1992) or monitoring of a

relatively small number of nests in situ (Kaska et al., 1998). Few studies have actually looked at the thermal conditions in a large sample of nests (Maxwell et al., 1988; Hanson et al., 1998).

Field studies involving extrapolation of nest conditions through the monitoring of sand temperatures, have shown that several factors influence sand temperatures on nesting beaches including: latitudinal variation, seasonal temperature changes, shading by vegetation, sand colour, depth of the eggs and episodic events such as rainfall (Morreale et al., 1982; Mrosovsky et al., 1984a,b; Mrosovsky, 1988; Godfrey et al., 1996; Hays et al., 1999, 2001). In addition, an increase in temperature attributed to metabolic heat produced by developing embryos has been recorded in green (*Chelonia mydas*) (Hendrickson, 1958; Carr and Hirth, 1961; Bustard, 1972; Morreale et al., 1982; Kaska et al., 1998; Broderick et al., 2001), hawksbill (*Eretmochelys imbricata*) (Raj, 1976), loggerhead (Maxwell et al., 1988; Neville et al., 1988; Maloney et al., 1990, Hanson et al., 1998), and leatherback (*Dermochelys coriacea*) (Godfrey et al., 1997) turtles.

The loggerhead turtle is widely distributed and extends into the Mediterranean with major nesting sites in Cyprus, Greece, Libya and Turkey (Groombridge, 1990; Laurent et al., 1998; Margaritoulis et al., in press). The regional annual nesting population, excluding a large, and as yet unquantified population in Libya (Laurent et al., 1997), is estimated as 2000 females and is considered regionally endangered (Groombridge, 1990). Analysis of mitochondrial (mt) DNA suggests that loggerhead turtles nesting in different parts of the world are genetically distinct (Encalada et al., 1998; Laurent et al., 1998). Given that the population should be considered as a management unit at the regional or individual rookery scale (Laurent et al., 1998), it is of fundamental interest to ascertain the sex ratio of hatchling production.

Sex ratios of loggerhead turtles in the Mediterranean have, as yet, been little studied. Kaska et al. (1998) found a mean sex ratio of 81.6% female in a small sample ($n = 8$) of loggerhead clutches at four sites in Cyprus and Turkey. These preliminary data suggested that the pivotal temperature was in the region of 29.0°C. Using another approach, Godley et al. (2001) analysed data regarding incubation durations of nests at Alagadi, Northern Cyprus, over six seasons (1993–1998). Because incubation duration depends on temperature, using inferences from other studies (Godfrey and Mrosovsky, 1997; Godfrey et al., 1999; Marcovaldi et al., 1997, 1999), it was possible to convert data on durations to hatchling sex ratios, which resulted in an estimation that 89–99% of the hatchlings produced at this site were females. In addition, on beaches other than Alagadi throughout Northern Cyprus, mean incubation duration of 70 nests in 1998 was 48.8 days, suggesting a similar female-biased sex ratio was typical.

In this study, our aim was to examine the thermal environment in the nests of loggerhead turtles in the Mediterranean. (1) We investigate the seasonal pattern of temperatures in both control sites and in nests and investigate the link with air temperature. (2) We describe temperature regimes experienced in 23 loggerhead turtle nests utilising intra-nest temperature recording devices, detailing incubation temperatures, diel temperature fluctuations and metabolic heating. (3) We describe the relationship between temperature and incubation duration. (4) Using this empirical relationship, we estimate the incubation temperature in nests of loggerhead turtle rookeries through-

out the Mediterranean and draw wider inferences as to the likely sex ratio of hatchlings produced in the region.

2. Materials and methods

2.1. Study site

Fieldwork took place at Alagadi (35°33′N, 33°47′E) situated in Northern Cyprus in the eastern Mediterranean. This is one of the main nesting sites in Cyprus for loggerhead turtles (range in seasonal total of nests: 38–95 per season, 1993–1999). The beach consists of two short coves, 0.8 and 1.2 km long, separated by a rocky headland and backed by extensive dunes and low scrub. There is no natural shading of the beach and although there is a high level of human usage, beach umbrellas and shades are only used at the water's edge, a region in which no clutches are laid. The climate is typical of the eastern Mediterranean, with virtually no rain from May to October and mean temperature for each 24 h during this period is generally in the region of 20–30°C.

2.2. Data collection

Nesting was recorded during nightly beach monitoring according to a previously described protocol (Broderick and Godley, 1996). Information on sand and nest temperature was gathered using 'Tinytalk' dataloggers (Orion Components, Chichester, UK) that recorded synchronously at 1-h intervals in 1999 and at 48-min intervals in 1996–1998. These dataloggers were calibrated with a mercury thermometer of known accuracy (NAMAS certified to read ± 0.1°C of absolute temperature; Hays et al., 1999). Dataloggers were placed in two control sites in the nesting zone from 30 May 1999 until 30 September 1999 (encompassing the incubation of loggerhead turtle clutches). Dataloggers were placed at a depth of 45 cm, the mean depth of the middle of loggerhead clutches at this site (Broderick, 1997). In each of the seasons 1996–1999, temperature dataloggers were placed in the centre of clutches of eggs as they were being laid (n: 2 in 1996; 4 in 1997; 2 in 1998; 15 in 1999). The female covered and camouflaged the nest herself. Deployment date of dataloggers was spread throughout the season to give as wide a temporal coverage as possible. On chosen deployment dates, study nests were chosen randomly, with the first clutch on a deployment date being used. Dataloggers measured $5 \times 5 \times 3$ cm (volume: 75 cm^3), representing some 3.3% of an average clutch as calculated conservatively by assessing the volume of eggs (mean clutch volume = 2306 cm^3; mean number of eggs per clutch = 84.2 eggs; mean egg diameter = 3.74 cm; egg volume = $4/3\Pi\ r^3$; Broderick and Godley, 1996; Margaritoulis et al., in press). Dataloggers started recording data at 6 am on the morning following deployment.

After 40 days, each nest was checked periodically throughout the night and again at dawn for signs of hatchling emergence. Incubation durations were calculated as the number of days between the night of laying and the night the first group of hatchlings emerged. Nest contents were excavated, nest chamber depth measured and dataloggers retrieved. Through a count of unhatched eggs and hatched shell fragments, clutch size (total number of eggs) and hatch success (number of eggs hatched/clutch size) were

calculated. In addition, the number of eggs with macroscopic dead embryos was enumerated.

Dataloggers were downloaded to a personal computer. For data manipulation purposes, each day was delineated at midnight, with the end of incubation being considered midnight on the night of the initial wave of hatchling emergence. Additional temperature data in the form of air temperature readings taken every 3 h at the Meteorological Centre at Kyrenia Harbour (16 km west of the study site) were made available by the North Cyprus Meteorological Service for May–August, 1999 from which a daily mean temperature was calculated.

3. Results

3.1. Basic nest parameters

Study nests had a mean incubation duration of 47.5 days (S.D.: 2.5, range: 42–52; Table 1) and a mean depth of 31 cm (S.D.: 7.8, range: 14–45 cm) and 48.5 cm (S.D.: 5.1, range: 38–57 cm) to top and bottom of the egg chamber, respectively. The mean

Table 1
Basic study nest information and descriptive statistics of temperature recorded throughout incubation, during the middle third of incubation and regarding mean daily range recorded

Nest	Lay date	ID (days)	Whole IP				Middle third IP				Daily range			
			Mean	±S.D.	Min	Max	Mean	±S.D.	Min	Max	Mean	±S.D.	Min	Max
1	13 June 1996	49	30.8	1.3	28.1	32.5	30.8	0.7	29.8	32.1	0.5	0.2	0.0	1.2
2	17 June 1996	48	30.8	1.5	27.7	33.0	30.8	0.5	29.8	32.1	0.4	0.2	0.0	0.9
3	10 June 1997	48	31.8	1.8	27.0	34.1	32.2	0.6	31.1	33.3	0.8	0.3	0.3	1.5
4	14 June 1997	42	31.8	1.3	27.0	34.1	32.1	0.5	30.6	32.9	0.9	0.7	0.3	4.9
5	27 June 1997	43	32.9	0.8	30.6	34.1	32.8	0.6	31.8	34.1	0.7	0.3	0.0	1.2
6	04 July 1997	44	32.9	0.5	31.6	34.0	32.9	0.2	32.5	33.5	0.3	0.3	0.0	1.0
7	09 June 1998	48	31.8	1.3	28.1	34.0	32.3	0.6	30.7	33.5	1.0	0.4	0.4	1.8
8	20 June 1998	50	32.2	1.6	28.8	34.9	31.8	0.9	30.3	33.7	0.7	0.2	0.3	1.2
9	30 May 1999	46	30.7	2.2	27.4	34.9	30.4	0.6	28.8	31.1	0.4	0.2	0.0	0.8
10	01 June 1999	50	29.5	1.6	26.3	32.2	29.3	0.5	28.1	30.3	0.4	0.3	0.0	1.4
11	02 June 1999	51	29.7	1.9	26.3	33.0	29.4	0.5	28.4	30.7	0.3	0.3	0.0	1.4
12	03 June 1999	46	32.0	1.9	28.1	35.7	31.5	0.9	29.2	33.0	1.4	0.5	0.4	3.8
13	05 June 1999	52	30.1	2.0	26.6	33.3	29.7	0.8	28.1	31.4	0.4	0.3	0.0	1.5
14	06 June 1999	52	30.8	1.8	26.6	33.3	30.4	1.0	28.4	32.2	0.6	0.3	0.3	1.5
15	13 June 1999	47	32.0	1.7	27.7	34.5	31.9	0.9	29.9	34.1	0.5	0.2	0.0	1.1
16	15 June 1999	48	31.3	1.4	28.1	33.7	31.4	0.9	29.9	33.3	0.9	0.3	0.4	2.6
17	15 June 1999	48	31.9	1.4	29.2	34.1	32.0	0.8	30.7	33.3	0.3	0.2	0.0	0.7
18	20 June 1999	46	31.8	1.2	29.0	33.5	32.2	0.5	31.0	33.0	0.4	0.2	0.0	1.1
19	23 June 1999	45	32.2	1.5	28.7	34.2	32.4	0.7	31.2	33.8	0.4	0.2	0.2	1.1
20	23 June 1999	47	32.8	1.2	29.0	34.5	33.7	0.5	32.6	34.5	1.0	0.2	0.5	1.6
21	27 June 1999	48	32.0	1.1	27.7	33.8	32.3	0.4	31.4	33.0	1.0	0.3	0.7	2.0
22	01 July 1999	46	33.2	0.8	29.9	34.5	33.5	0.4	32.6	34.1	0.9	0.3	0.4	1.9
23	10 July 1999	48	32.1	1.0	30.3	34.2	31.6	0.5	31.0	32.7	0.3	0.1	0.1	0.8

ID = incubation duration, IP = incubation period.

depth of study nests was, therefore, 39.8 cm. The mean clutch size was 84.7 eggs (S.D.: 16.0, range: 54–108 eggs) and clutches hatched with a mean hatching success of 0.77 (S.D.: 0.2, range: 0.27–0.98).

3.2. Seasonal profile in temperature at nest depth

The temperature recorded at the two control sites differed throughout the 1999 season (mean difference: 2.1°C, S.D.: 0.90; Fig. 1a). However, the hourly temperatures at these two sites followed similar seasonal patterns and were highly correlated during the period of study nest incubation (31st May–27th August: $r^2 = 0.82$, $F_{1,2128} = 9740$, $p < 0.001$) and the mean temperature of these two sites was used to illustrate the seasonal changes in sand temperature. When mean hourly temperature in nests throughout the 1999 season (range of n: 1–15; nest numbers: 9–23; Table 1) was compared with mean hourly control temperature (Fig. 1b), it was clear that although the two parameters were highly correlated ($R^2 = 0.99$, $F_{1,2128} = 187\,544$, $p < 0.001$; mean hourly nest temperature = 1.19 mean hourly control temperature -5.27), that after the first few weeks of monitoring, mean nest temperature was consistently higher than that of mean control temperature. In addition, the line of 29.0°C helps to illustrate that most nests will have been incubating above this threshold.

The mean daily sand temperature at the control sites was compared with mean daily air temperature during the same period (see Fig. 1c). Although air temperature is both lower than sand temperature and prone to greater short-term fluctuations, these parameters were highly correlated ($r^2 = 0.78$, $F_{1,87} = 311$, $p < 0.001$: mean daily sand temperature = 0.811 mean daily air temperature $+8.16$). Additionally, composite mean daily nest temperature correlated significantly with mean daily air temperature ($r^2 = 0.77$, $F_{1,87} = 294$, $p < 0.001$: mean daily nest temperature = 0.962 mean daily air temperature $+4.44$).

3.3. Intra-nest temperatures

Lay dates, summary incubation data and incubation temperatures for all 23 nests from four seasons are presented in Table 1. Temperatures ranged from 26.3°C to 35.7°C with mean temperatures of individual nests ranging from 29.5°C to 33.2°C (annual means: 1996, 30.8°C ($n = 2$); 1997, 32.4°C ($n = 4$); 1998, 32.0°C ($n = 2$); 1999, 31.5°C ($n = 15$); Table 1). During the middle third of incubation, temperatures ranged from 28.1°C to 34.5°C, with mean temperatures of individual nests during this period ranging from 29.3°C to 33.7°C (annual means: 1996, 30.8°C ($n = 2$); 1997, 32.5°C ($n = 4$); 1998, 32.1°C ($n = 2$); 1999, 31.4°C ($n = 15$); Table 1). Hatching success was not normally distributed (Anderson Darling, $p < 0.05$) so was subject to arcsine transformation. Arcsine hatching success of nests declined significantly with increasing temperature ($r^2 = 0.63$, $F_{2,20} = 17.4$, $p = 0.001$; Fig. 2). All nests experienced regular diel variation in temperature (see Fig. 3a and b) with mean values ranging from 0.3°C to 1.4°C in individual nests (annual means: 1996, 0.5°C ($n = 2$); 1997, 0.7°C ($n = 4$); 1998, 0.9°C ($n = 2$); 1999, 0.6°C ($n = 15$); Table 1). There was no correlation between the mean level of diel temperature change and either distance to the top ($r^2 = 0.01$,

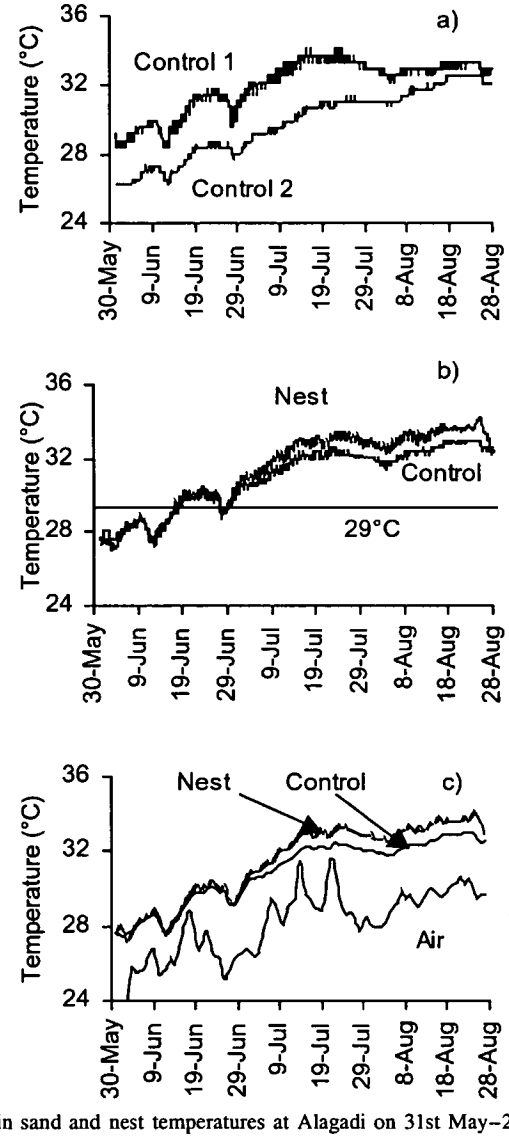

Fig. 1. Seasonal pattern in sand and nest temperatures at Alagadi on 31st May–27th August 1999 when the final study nest hatched. (a) Hourly temperature recorded at two control sites. (b) Mean hourly temperature at control sites and mean hourly nest temperature. (c) Mean daily nest temperature, mean daily control temperature and mean daily air temperature recorded at the Meteorological Centre at Kyrenia.

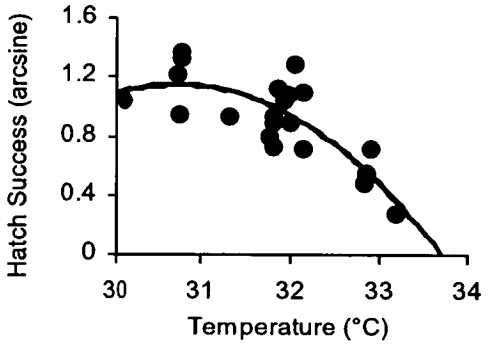

Fig. 2. Arcsine hatch success compared with mean nest temperature recorded throughout the incubation period. (Arcsine hatching success = 7.87 mean temperature − 0.13 mean temperature^2 − 120.)

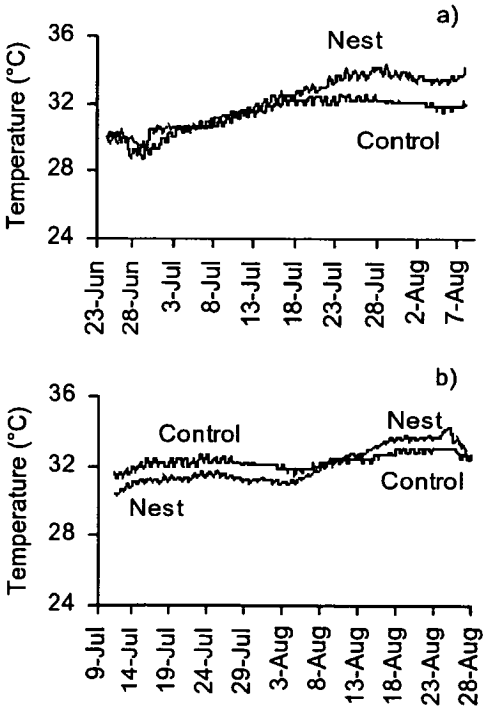

Fig. 3. Nest temperature profiles in comparison with mean control temperature. (a) Temperature recorded in nest 19. Clutch was laid on 23rd June and hatchlings emerged on 7th August 1999. (b) Temperature recorded in nest 23. Clutch was laid on 10th July and hatchlings emerged on 27th August 1999.

Table 2
Descriptive statistics of the level of metabolic heating recorded throughout incubation and during each third of incubation in study nests in 1999

Nest	Metabolic heating throughout IP				First third IP				Middle third IP				Final third IP			
	Mean	±S.D.	Min	Max	Mean	±S.D.	Min	Max	Mean	±S.D.	Min	Max	Mean	±S.D.	Min	Max
9	0.7	1.0	−0.6	2.3	−0.1	0.4	−0.6	0.8	0.2	0.6	−0.6	1.3	1.8	0.5	0.5	2.3
10	0.3	0.3	−0.6	0.7	0.3	0.2	−0.2	0.5	0.1	0.3	−0.6	0.6	0.5	0.2	0.2	0.7
11	0.7	0.5	−0.1	1.6	0.4	0.2	−0.1	0.7	0.5	0.2	0.0	0.7	1.3	0.2	0.9	1.6
12	0.2	0.6	−2.0	1.2	0.0	0.5	−1.4	−0.1	−0.2	0.6	−2.0	0.4	0.7	0.3	0.2	1.2
13	0.4	0.6	−0.1	1.5	0.0	0.3	−0.6	0.3	0.0	0.3	−0.8	0.4	1.1	0.3	0.5	1.5
14	0.1	0.5	−1.4	0.8	−0.1	0.4	−1.1	0.3	−0.2	0.4	−1.4	0.3	0.6	0.2	0.2	0.8
15	0.6	0.5	−0.9	1.5	0.2	0.4	−0.9	0.7	0.5	0.3	0.1	1.1	1.1	0.3	0.5	1.5
16	−0.4	0.4	−1.8	0.3	−0.6	0.5	−1.8	0.0	−0.5	0.3	−0.9	0.0	0.0	0.2	−0.3	0.3
17	0.0	0.3	−0.7	0.6	−0.3	0.3	−0.7	0.0	−0.1	0.2	−0.4	0.3	0.4	0.2	0.0	0.6
18	0.0	0.3	−0.9	0.4	−0.2	0.4	−0.9	0.2	0.0	0.1	−0.3	0.2	0.2	0.1	0.0	0.4
19	0.9	0.7	−0.5	2.0	0.3	0.4	−0.5	1.0	0.6	0.3	0.2	1.4	1.7	0.2	1.4	2.0
20	1.0	0.4	−0.7	1.6	0.8	0.6	−0.7	1.5	1.3	0.2	0.9	1.6	0.9	0.2	0.5	1.2
21	0.5	0.4	−0.5	1.1	0.0	0.3	−0.3	0.5	−0.2	0.2	−0.5	0.1	0.6	0.3	0.1	1.1
22	0.7	0.3	−0.1	1.1	0.7	0.4	−0.1	1.1	0.6	0.2	0.4	0.9	0.7	0.2	0.4	1.0
23	0.6	0.7	−0.2	2.0	0.0	0.1	−0.2	0.2	0.3	0.4	0.0	1.1	1.5	0.3	0.9	2.0

IP = incubation period.

$F_{1,21} = 0.17$, $p = 0.686$) or the bottom ($R^2 = 0.08$, $F_{1,21} = 1.75$, $p = 0.20$) of the egg chamber.

3.4. Metabolic heating

Illustrations of the typical relationship between the mean control site temperatures and within nest temperatures are given in Fig. 3a and b for two nests throughout the incubation period. Note the regular diel fluctuation in both the nest and control site temperatures and that although nest temperatures generally track the pattern of control site temperatures, there is a marked progressive increase in temperature in mid incubation. This increase was attributed to metabolic heating.

There are two main processes that can drive changes in nest temperature over extended time scales: (1) seasonal changes and (2) metabolic heating. Control loggers described the seasonal change in sand temperature. After 1 day of incubation, nest temperatures had equilibrated with the surrounding sand. To calculate the extent of metabolic heating, we simply determined the change in nest temperature from day 2 to day x of incubation and subtracted the seasonal change in sand temperature over the corresponding period.

Table 2 details descriptive statistics of estimated levels of metabolic heating for all 15 nests that were studied in 1999 for which 14 of the nests show demonstrable levels. Over the whole incubation period, mean levels of our index of metabolic heating ranged from −0.4°C to +1.0°C (mean: 0.4°C, S.D.: 0.4). However, the level of metabolic heating varied markedly throughout the incubation period with levels being significantly higher in the final third of incubation period (ANOVA: $F_{2,42} = 13$, $p < 0.001$; mean first third: 0.1°C, S.D.: 0.4; mean middle third: 0.2°C, S.D.: 0.5; mean final third: 0.9°C, S.D.: 0.5).

A multiple stepwise regression approach was used to investigate if there were any effects of arcsine hatch success, clutch size, number of hatchlings, or total number of embryos, i.e. hatchlings and partially developed embryos on overall mean metabolic

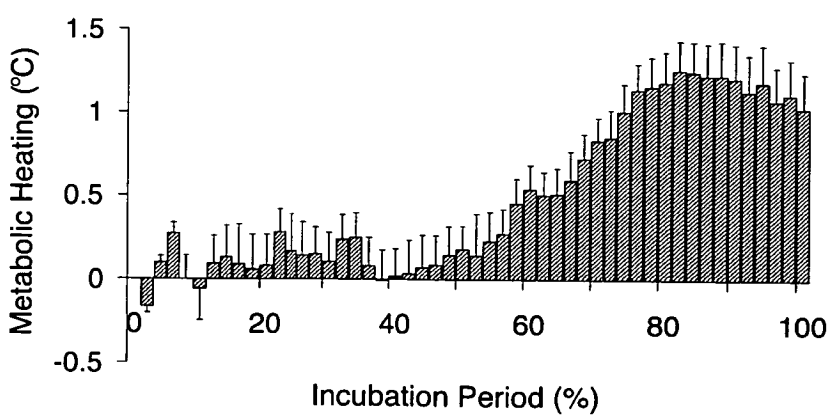

Fig. 4. Mean metabolic heating in each two percentiles of incubation in clutches ($n = 15$) studied in 1999. Error bars denote 1 S.D.

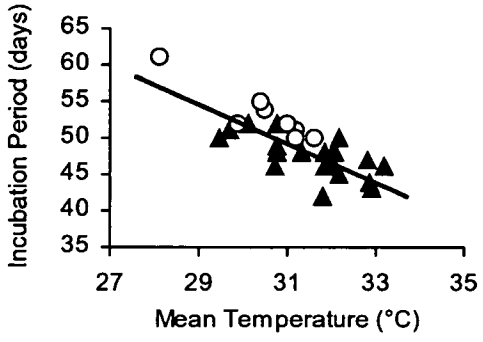

Fig. 5. Relationship between mean incubation temperature and incubation period in 23 clutches from this study (triangles) and 8 clutches (circles) from the study by Kaska et al. (1998). (Incubation period = −2.66 mean temperature+132.11.)

heating or the mean measure in either the first, middle or final third of incubation. None of the variables were found to exert a significant effect (regression analysis; $p > 0.05$).

To more clearly visualise the pattern of metabolic heating, we converted the incubation period in days into the proportion of incubation period. Fig. 4 shows the mean amount of metabolic heating in each two percentiles of incubation. Although there appears to be some level of metabolic heating from very early in incubation, it is variable and generally below 0.1°C until about 40% of the way through incubation. At this point, a steady rise in the level of heating is demonstrated to a peak of 1.0 °C, 84% of the way through incubation, at which point there is a slow decline, with levels of heating staying above 0.8°C until hatching.

3.5. Relationship between nest temperature and incubation period

Incubation durations (Table 1) were found to be significantly correlated to both mean temperature throughout the incubation period ($R^2 = 0.43$, $F_{1,21} = 15.87$, $p = 0.001$) and during the middle third of incubation ($R^2 = 0.44$, $F_{1,21} = 16.39$, $p = 0.001$). When we augment this data set with the eight nests studied by Kaska et al. (1998), we obtain similar relationships of higher significance, with incubation durations being correlated to both mean incubation temperature ($r^2 = 0.62$, $F_{1,29} = 48.09$, $p < 0.001$) and that during the middle third ($r^2 = 0.51$, $F_{1,29} = 29.97$, $p < 0.001$). The relationship between incubation period and mean incubation temperature is shown in Fig. 5.

4. Discussion

4.1. Basic nest parameters

The basic nest parameters in the study nests are comparable with those described previously for this site (Broderick and Godley, 1996; Godley et al., 2001; Margaritoulis et al., in press). Females nesting in the Mediterranean are smaller than most populations

(Dodd, 1988) and those nesting in Cyprus are the smallest in the Mediterranean (Broderick and Godley, 1996; Margaritoulis et al., in press). It is likely that small body size is a factor leading to the small clutches deposited at this site that are at the extreme low end of the global range of 85–105 reviewed by Dodd (1988). Although we inferred clutch size from a posthatching egg count, any associated error is unlikely to exert a systematic bias to the results of this study. Equally, although this sample of nests had a mean depth slightly shallower than that of the control dataloggers, it appears that the control sites, were on average, adequate to describe the prevailing seasonal change in sand temperature.

4.2. Seasonal profile in temperature at nest depth

It is clear from our results (Fig. 1b and c) that there is a close linkage between prevailing air temperature and sand temperature at nest depth and temperature within nests as has been found in other studies (Maloney et al., 1990; Hays et al., 1999). These relationships can be useful for predictive purposes. However, it is likely that sand colour and relative reflectance will mean that any such relationship is beach-specific, even when meteorological data are comparable (Hays et al., 1995, 2001). However, the difference between the two control sites demonstrated here (Fig. 1a) highlights the need for multiple control sites to account for any intra-beach variability when control site temperatures are used in a predictive manner. The difference in control site temperatures in this study was not important as these data were only used to illustrate seasonal changes and both sites showed very similar seasonal profiles. In the calculation of metabolic heating, differences between absolute values of temperature in the nest and at control sites were controlled for.

4.3. Intra-nest temperatures

Mean temperatures throughout incubation and during the thermosensitive period are above 29.0°C (approximate pivotal temperature) in all nests, often approaching the upper critical limit for incubating eggs of 35°C (Ackerman, 1997). Indeed, it is likely that the decline in hatch success with increasing mean incubation temperature demonstrated here (Fig. 2) is a manifestation of critical effects. From a TSD perspective, these data are suggestive of nests that would produce an extremely high proportion of females, consistent with the estimates generated by Godley et al. (2001).

Although few nests were studied 1996–1998, these earlier data appear similar to those of 1999. Nests were selected at random on given study nights but deployment dates were selected so as to optimise resources and monitor nests through as much of the season as possible. In 1999, the main season of study, this was attained with monitoring being carried out from the first clutch to be laid (nest 9), with constant monitoring of between 1 and 15 nests until nest 23 hatched on 27th August, with only 4 of 49 nests hatching after this date, all within 14 days. The composite nest profile (Fig. 1b and c) can, therefore be relied upon as representative.

Diel temperature fluctuations are expected in such shallow nests in contrast to the deeper nests of the green turtle (*C. mydas*) at the same site (Broderick et al., 2000) that

show no regular diel temperature variation. The levels and patterns of diel variation are comparable with nests of the same species studied in Australia (Maloney et al., 1990), South Africa (Maxwell et al., 1988), Cyprus and Turkey (Kaska et al., 1998), and the USA (Hanson et al., 1998).

Direct measurement of temperature in a large sample of nests is a step forward from inferential studies based solely on control site temperatures. However, one criticism that could be levelled at studies such as this is that by recording temperatures from only one datalogger, we may not be collecting data that are typical for the clutch. This criticism has been highlighted by the work of two studies to date by the placement of dataloggers at the top, middle and bottom of the clutch. Hanson et al. (1998) studied three such nests of the loggerhead turtle in the USA, finding a mean of 0.9°C within the nest range, with mean temperatures in the middle of the clutch during the middle third of incubation being 0.4°C and 0.9°C above the top and bottom of the clutch, respectively. Hanson et al. (1998) suggested, therefore, that the middle dataloggers seemed reasonable for predicting temperature of most eggs but that it might slightly overestimate temperature of some at the periphery of the clutch, a factor that is only likely to be of significance for nests incubating within the transitional range of temperatures. Comparable results were found in eight loggerhead turtle nests in the Mediterranean studied by Kaska et al. (1998), which also showed a mean intra-nest range of 0.9°C. In addition, although not yet expressly investigated, metabolic heating is likely to be greatest in the centre of the clutch, so estimates herein should be considered maximal values.

4.4. Metabolic heating

Metabolic heating was described in all but one of the 1999 study nests, and was in general confined to the latter part of incubation as has been found in previous studies of the same species in South Africa (Maxwell et al., 1988). However, in the South African study, the magnitude of metabolic heating was greater, possibly as much as 3°C. It may be that the small clutch volume of Mediterranean loggerhead turtles leads to less metabolic heating. It has been suggested that metabolic heating will be insignificant concerning TSD unless greater than 1°C (Mrosovsky and Yntema, 1980). If prevailing sand temperatures used for predicting nest temperatures are close to the pivotal temperature, then the metabolic heating may significantly affect calculations. Within the population under study, the mean difference of 0.2°C is not likely to be significant. A far more important source of potential error of such predictions would be the intra-nest thermal range.

In this study, we cite field incubation durations, i.e. duration from laying to emergence, which is the period it takes for hatchlings to complete the hatching process and ascend through the sand to the surface. This will in fact differ from the true incubation duration, i.e. laying to hatching, by the order of several days. Although Godfrey and Mrosovsky (1997) estimate this lag as some 4.1 days for loggerhead turtles in the USA, we do not know what the duration is in Mediterranean loggerhead turtles. Assuming it is comparable, although slight changes in the estimated timings of the thermosensitive period will result, the total difference to mean metabolic heating calculations will be negligible.

The systematic positive difference between mean nest temperature and mean control temperature (Fig. 1b) is undoubtedly indicative of metabolic heating. The difference only evolves after the first clutches have reached mid to late incubation, the period when, as we have demonstrated, metabolic heating begins to rise (Fig. 4).

4.5. Relationship between nest temperature and incubation period

Although incubation period is a crude measure of developmental rate (Georges et al., 1994), it is a parameter for which data are readily collected in a low-tech manner giving meaningful integrated information regarding the nest environment. It is clear that incubation period is largely influenced by temperature as has been previously described (Ackerman, 1997), and incorporating the data from Kaska et al. (1998) has allowed us to describe this relationship for Mediterranean loggerhead turtles in a more rigorous manner than using our data alone. In the 23 study nests at Alagadi, there was little difference in the amount of variance in incubation period accounted for by the significant relationships with mean incubation temperature and mean temperature during the middle third of incubation. Worthy of note, however, is the fact that there is a closer co-relationship between the overall mean incubation temperature than that of the middle third (r^2 of 0.62 versus 0.51). Although the thermal environment in the middle third is that which will dictate the sex of the offspring, the incubation duration is an integration of the speed of development throughout incubation. These results mirror those for green turtles nesting at Alagadi (Broderick et al., 2000).

4.6. Estimating nest temperatures throughout the Mediterranean

As yet, no overall estimation of loggerhead hatchling sex ratio has been undertaken in the Mediterranean region, although Godley et al. (2001) have suggested that based on data regarding incubation durations in Cyprus and available published data on incubation durations, sex ratio of hatchling production is likely to be female-biased. The biology of loggerhead turtles in the Mediterranean has recently been reviewed (Margaritoulis et al., in press) and these authors presented many data on mean incubation durations over many seasons at several key sites subject to monitoring in the region. The incubation durations cited in Margaritoulis et al. (in press) and additional published and unpublished data are given in Table 3. On available nesting figures, these beaches constitute a large proportion of nesting activity in the region, representing approximately 10%, 85% and 50% of totals for Cyprus, Greece and Turkey, respectively. It should be noted that data from Egypt and Israel are from clutches transplanted to hatcheries and are the only data available.

Given that we have demonstrated a clear link between incubation temperature and incubation duration in this regional population (Fig. 5), it is possible to use this relationship to estimate mean incubation temperatures for the studies cited in Table 3. Estimated mean incubation temperatures for these studies are plotted in Fig. 6.

At the present, no pivotal incubation period for Mediterranean loggerheads has been generated from laboratory work, with the only study carried out in the region (Kaska et

Table 3
Mean incubation durations for sites subjected to monitoring around the Mediterranean

Country, area	Mean number of nests per season	Number of seasons monitored	Range of annual mean incubation durations	Literature source
Cyprus				
Alagadi	60	7	47.3–48.7	Margaritoulis et al., in press
Greece				
Bay of Chania	115	2	53.3–54.3	Margaritoulis et al., in press
Kefalonia	29	1	54.9	Margaritoulis et al., in press
Kyparissia Bay	581	3	48.2–55.5	Margaritoulis et al., in press
Lakonikos Bay	192	5	52.1–59.3	Margaritoulis et al., in press
Rethymno	387	3	51.7–55.2	Margaritoulis et al., in press
Rhodes	11	2	49.0–55.0	Margaritoulis et al., in press
Zakynthos	1286	4	57.4–61.9	Margaritoulis et al., in press
Libya				
	Large but not quantified	1	55.0	Margaritoulis et al., in press
Turkey				
Akyatan	15	1	52.0	Margaritoulis et al., in press
Anamur	191	1	51.3	Margaritoulis et al., in press
Dalyan	165	1	59.3	Margaritoulis et al., in press
Fethiye	124	2	55.0–56.9	Margaritoulis et al., in press
Göksu	65	2	54.8–57.0	Van Pigglen, 1993; Peters and Verhoeven, 1992
Kizilot	107	1	59.6	Kaska, 1993
Patara	53	1	60.0	Kaska, 1993
Egypt				
Sinai Peninsula[a]	Small but not quantified	1	49.3	Clarke et al., 2000; Clarke, pers. comm.
Israel				
Whole coastline[a]	30	6	52.7	Kuller, 1999; Kuller, pers. comm.

[a] Represents data from hatcheries where no other data are available.

al., 1998) suggesting that the pivotal temperature was just below 29°C. This is similar to the values of 29.2°C for Brazil (Marcovaldi et al., 1997) and 29.0°C for the USA (Mrosovsky, 1994). As a point of reference, if we assume that the pivotal temperature is 29.0°C, it can be seen in Fig. 6, that because the mean temperature experienced by nests incubating throughout the Mediterranean is likely to be well above 29.0°C at most sites in most years, that female-biased sex ratios must be common. However, it would appear that at least in some years and some sites (Zakynthos and Lakonikos Bay (Greece), Dalyan, Kizilot and Patara (Turkey)), mean temperatures may be just below 29°C; suggestive of balanced or even slightly male-biased hatchling sex ratios.

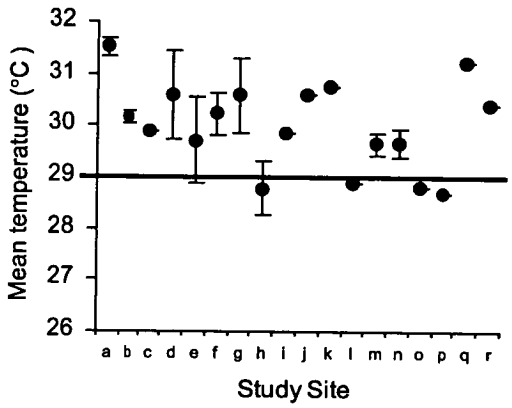

Fig. 6. Estimated mean nest temperatures throughout the Mediterranean as calculated using the equation derived in Fig. 5 and the data in Table 3. Bars represent range of values when data were present for several seasons. (Key: Cyprus: (a) Alagadi; Greece: (b) Bay of Chania; (c) Kefalonia; (d) Kyparissia Bay; (e) Lakonikos Bay; (f) Rethymno; (g) Rhodes; (h) Zakynthos; Libya: (i) not specified; Turkey: (j) Akyatan; (k) Anamur; (l) Dalyan; (m) Fethiye; (n) Göksu; (o) Kizilot; (p) Patara; Egypt: (q) Sinai Peninsula; Israel: (r) whole coastline.)

This may be analogous to the situation in Brazil where, although most sites are largely female-producing, some key sites may have conditions that are biased toward the production of male hatchlings (Baptistotte et al., 1999). Although subject to limited geographical sampling and possible artefact as a result of transplantation (see Table 3), it would appear that sites such as Cyprus, Israel, Egypt and Libya are likely to produce hatchlings with highly female-biased sex ratios. The data are not yet available to make a detailed comparison of expected sex ratios among sites or among years but it would appear that with data already collected by workers in the region, a plausible estimate of hatchling sex ratio for the Mediterranean may be attainable once a clearly defined pivotal temperature for the Mediterranean population is generated by laboratory and/or field-based studies.

Acknowledgements

This work was undertaken as part of expeditions supported by: British Association of Tortoise Keepers, British Chelonia Group, British Ecological Society, Carnegie Trust, Cross Trust, European Commission (DG1B/1A), Institute of Biology, Glasgow Natural History Society, Glasgow University Court, MEDASSET UK, People's Trust for Endangered Species, North of England Zoological Society, and Zebra Foundation. The Department of Environmental Protection and the Society for the Protection of Turtles gave logistic support in Northern Cyprus. Zeev Kuller and Mike Clarke provided unpublished data. Earlier drafts of the manuscript benefited from the constructive comments of Matthew Godfrey and Nicholas Mrosovsky. *[SS]*

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Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites

Stuart A. West, Sarah E. Reece and Andrew F. Read

'Survival of the fittest' is usually interpreted to mean that natural selection favours genes that maximize their transmission to the next generation. Here, we discuss recent applications of this principle to the study of gametocyte sex ratios in malaria and other apicomplexan parasites. Sex ratios matter because they are an important determinant of fitness and transmission success – and hence of disease epidemiology and evolution. Moreover, inbreeding rates can be estimated from gametocyte sex ratios. The sex ratio is also an excellent model trait for testing the validity of important components of what is being marketed as 'Darwinian medicine'.

The study of offspring SEX RATIOS (see Glossary) has provided one of the greatest success stories for the evolutionary (ADAPTATIONIST or Darwinian) approach to biology[1–3]. Theoretical models that predict the best or UNBEATABLE SEX RATIO for a given situation can be constructed relatively easily. These models have been remarkably successful in explaining why the sex ratio is approximately 1:1 in many species, as well as when it should deviate from that. Indeed, the close, sometimes quantitative, fit between theoretical predictions and observational or experimental data gives sex ratio theory a predictive power almost comparable to that of the 'hard' sciences of chemistry and physics[4].

This evolutionary approach to sex ratio differs from the mechanistic approach described elsewhere[5,6]. It attempts to explain and predict the sex ratio in terms of what would be favoured by natural selection; that is, why has natural selection favoured the sex ratios that we observe in nature? By contrast, a mechanistic approach attempts to explain how certain sex ratios are achieved by describing sex-determining mechanisms. On their own, neither approach provides a complete account of sex ratios. Moreover, each can assist the other. Knowledge of the evolutionary forces shaping sex ratio evolution can suggest, for instance, what kind of environmental cues might influence sex determination, and also explain when natural selection favours variable sex ratios.

Sex ratio theory applied to apicomplexans

This section introduces the predictions that theoretical models make for apicomplexan sex ratios. We use the term 'sex ratio' to refer to the GAMETOCYTE SEX RATIO, which we define as the proportion of gametocytes that are male. The relevant natural history for those unfamiliar with the group is given in Box 1. What is the unbeatable sex ratio if there is only one distinct clonal lineage per host (hereafter referred to as clone or genotype) and total INBREEDING (selfing)? In this case, the unbeatable sex ratio will be the one that maximizes successful transmission (i.e. number of zygotes)[7]. Given that one male gametocyte can produce several gametes that could fertilize the gametes produced by several female gametocytes, the best sex ratio will therefore be very female biased. Specifically, if c is the mean number of viable gametes released by a male gametocyte then the best sex ratio to produce will be $1 \div (1 + c)$.

However, if there is a chance that some OUTCROSSING will occur, then a less-female-biased sex ratio will be favoured by natural selection[7]. This is because mutant clones that produce more males will be present at a higher frequency among the mating males and hence obtain a disproportionate share of the mates (and hence make a greater genetic contribution to the next generation). In the extreme, with no inbreeding, a sex ratio of 0.5 (50% males) will be favoured, because at this point, FITNESS through males will balance fitness obtained through females. (This is the reason that a sex ratio of 50% males dominates in humans and other populations with negligible inbreeding[8].)

Glossary

Sex ratio: The proportion of individuals that are male.
Adaptationist approach: The attempt to explain trait values in terms of the process of adaptation.
Unbeatable sex ratio: The sex ratio with the highest fitness (which can therefore not be beaten by any other sex ratio) for a given set of conditions. This sex ratio is often termed an evolutionary stable strategy: a population of individuals playing this strategy cannot be invaded by a mutant that produces a different sex ratio.
Gametocyte sex ratio: The proportion of gametocytes that are male (microgametocytes).
Inbreeding: Mating between related gametes. The level of inbreeding is defined as Wright's coefficient of inbreeding (F), the probability that two homologous genes in two mating gametes are identical by descent.
Outcrossing: Mating between unrelated gametes.
Fitness: Genetic representation in future generations.
Darwinian medicine: The application of the adaptationist approach to matters of medical importance.

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http://parasites.trends.com 1471-4922/01/$ – see front matter PII: S1471-4922(01)02058-X

Box 1. Cast

The Apicomplexa form a large and cosmopolitan phylum that consists entirely of parasites, including a number of species of medical and veterinary importance, and can be split into five taxonomic groups[a].

Adeleorins (subclass Coccidiasina, suborder Adeleorina)
These are one-host parasites of invertebrates or vertebrates, or two-host parasites that alternately infect haematophagous (blood feeding) invertebrates and vertebrates (e.g. *Cyrilia*, *Desseria*, *Haemogregarina* and *Hepatozoon*).

Eimeriorins (subclass Coccidiasina, suborder Eimeriorina)
These are often called the coccidia (although this term is also used to include the adeleorins) and are a diverse group that include one-host parasites of invertebrates, two-host parasites of invertebrates, one-host parasites of vertebrates and two-host parasites of vertebrates (e.g. *Cryptosporidium*, *Cyclospora*, *Eimeria*, *Isospora*, *Neospora*, *Sarcosystis* and *Toxoplasma*).

Gregarines (subclass Gregarinasina)
These are generally one-host parasites of invertebrates (e.g. *Gregarina*, *Lankesteria* and *Mattesia*).

Haemospororins (subclass Coccidiasina, suborder Haemospororina)
These are often known as the malaria parasites and are two-host parasites of blood-feeding dipteran flies and various tetrapod vertebrates (e.g. *Haemoproteus*, *Leucocytozoon* and *Plasmodium*).

Piroplasms (subclass Piroplasmasina)
These are two-host parasites infecting ticks and vertebrates (e.g. *Babesia* and *Theileria*).

We are concerned only with the three groups in which sexually dimorphic stages have been observed, the adeleorins, eimeriorins and haemospororins (although our ideas would apply to any species in which dimorphic sexual stages can be found). The basic life history of all these species involves an alteration of sexual and asexual reproduction, and the features relevant to understanding their sex allocation can be summarized as follows[a]. Haploid infective stages called sporozoites infect host tissues and form feeding stages called trophozoites. These stages undergo a period of asexual proliferation and become multicelled stages called meronts (or schizonts). These rupture to produce merozoites, some or all of which transform into sexual stages, which are termed gametocytes for the haemospororins and eimeriorins, and gamonts for the gregarines, adeleorins and piroplasms[b]. For the purposes of consistency we refer to gamonts as gametocytes.

'Male' gametocytes (microgametocytes) rupture to release a number of male gametes (~4–8 in haemospororins[c] and ~5–1000 in eimeriorins[d]), whereas 'female' gametocytes (macrogametocytes) give rise to a single female gamete. The male gamete fertilizes the larger female gamete to form a diploid zygote. The diploid zygote undergoes meiosis, including genetic recombination involving random segregation of chromosomes and crossing-over of homologous regions of DNA, which restores the haploid state. This immature oocyst then divides mitotically to form spores containing the infective stages (sporozoites), which initiate the period of asexual proliferation once again.

Fundamental to applying sex ratio theory to these groups is the possibility that inbreeding can occur[c]. In haemospororins and adeleorins, this arises because fertilization occurs in the blood meal of the invertebrate host, so mating will commonly occur between the gametes from parasites infecting a single vertebrate host. When few parasite genotypes infect a host, this will lead to inbreeding (for example, when single clones self-fertilize). In eimeriorin species, fertilization occurs in the intestines of the host and so, if few genotypes (clones) infect a host, there will be large amounts of inbreeding. In addition, the extent of inbreeding might be increased further in some eimeriorin species because: (1) sexual development and fertilization occur on a very localized scale in a small portion of the intestinal cells of infected hosts[e,f], and (2) male gametes only disperse a short distance to fertilize female gametes[g,h]. Consequently, if different genotypes infect different areas of the intestine, they will not cross fertilize, in which case, high levels of selfing would occur even when the host is infected by many genotypes[i].

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The idea that the sex ratio favoured by natural selection depends on inbreeding rates can be quantified theoretically, and the unbeatable sex ratio (r^*; defined as the sex ratio strategy with the highest fitness) can be shown (Fig. 1) to be related to the inbreeding rate by a pleasingly simple equation (Eqn 1):

$$r^* = (1 - F) \div 2 \quad [1]$$

where F is Wright's coefficient of inbreeding (the probability that two homologous genes in two mating gametes are identical by descent). Eqn 1 is remarkably robust, and can be shown to hold with very general models as well as those specifically based on the life history of parasites such as *Plasmodium*[7,9–11]. The way in which the population is structured at various levels (e.g. host, house, village

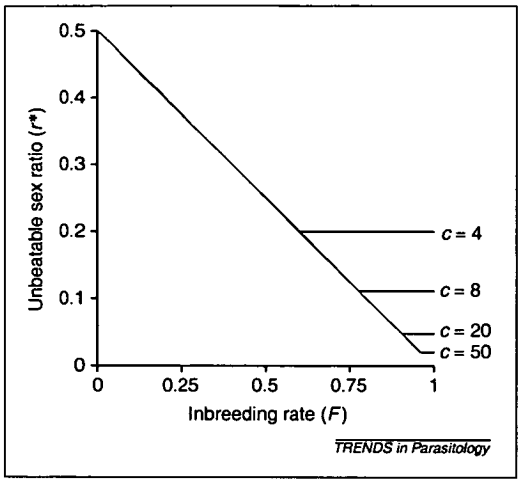

Fig. 1. The unbeatable sex ratio (r^*) plotted against the inbreeding rate (F). When the rate of inbreeding is high, the extent of female bias in the sex ratio is constrained by the need to produce enough male gametes to fertilize the female gametes. This constraint is determined by c, the mean number of viable gametes released by a male gametocyte[7].

and region) can affect F, but Eqn 1 holds nonetheless (S. Nee *et al.*, unpublished). However, a lower limit is put on the expected sex ratio of $1 + (1 + c)$ by the need to produce enough male gametes to fertilize the female gametes, as described above (c is the mean number of viable gametes released by a male gametocyte).

For multicellular organisms, Eqn 1 is one of the best-verified predictions of evolutionary theory, with substantial quantitative support coming from a wide range of organisms, including wasps, ants, beetles, mites, spiders, snakes and a variety of flowering plants[1,3,12–14].

Predictions

The theory described above make several testable predictions for the sex ratios of haemospororin and eimeriorin parasites. (1) The sex ratio should be 0.5 or female biased, and never male biased. (2) The extent of female bias observed in the sex ratio (r) of a population or species should be related to the inbreeding rate (F) by Eqn 1. (3) Across species and populations, the sex ratio should be negatively related to correlates of the inbreeding rate. For example, higher gametocyte prevalence (that is, the proportion of hosts that are infectious) is likely to lead to lower levels of inbreeding, and so the sex ratio should be positively correlated with gametocyte prevalence[11].

These three predictions require that natural selection act merely on the average sex ratio produced in a population or species. However, conditional adjustment of the offspring sex ratio in response to local conditions has been observed in many organisms (especially ants, bees and parasitic wasps, whose haplodiploid sex-determining mechanism readily enables a mother to control the sex of her offspring[1–3]), and malaria parasites have been shown conditionally to alter another aspect of reproduction (the commitment to asexual or sexual stages) in response to environmental conditions[15–17]. If apicomplexan parasites can detect the likelihood with which they will inbreed and adjust their sex ratio accordingly then we would make one more prediction. (4) More female-biased sex ratios should occur during infections in which there is a higher probability of inbreeding (i.e. fewer clones)[7,18]. This predicts variation in the sex ratio: (1) across infections with different numbers of genotypes producing gametocytes, and (2) within infections over time as the number of genotypes producing gametocytes changes.

This final prediction provides a clear example of the need to understand both the mechanistic and the evolutionary approach, a point that we shall return to when discussing future directions. The evolutionary approach predicts when sex ratios should be adjusted conditionally (Why?) and in what direction. However, an understanding of mechanism is required to determine whether this shift in sex ratio is expected (if sex ratios are genetically fixed and cannot be adjusted conditionally then we should not expect shifts) and, if so, in response to what cues (How?). Consequently, an understanding from both approaches is required to interpret sex ratio data fully.

Supporting evidence

Several recent studies of apicomplexan sex ratios have provided support for these predictions. First, the sex ratios observed in natural populations of haemospororin and eimeriorins are generally female biased (Fig. 2). Second, for cases in which we have sex ratio data and direct genetic estimates of the selfing rate, they are in quantitative agreement. (1) The mean sex ratio observed (0.18) in human malaria infections in Papua New Guinea predicted an inbreeding rate of 0.64–1.0, which was confirmed by molecular genetic analyses that showed an inbreeding rate of 0.9 (Refs 19,20). (2) Molecular genetic analyses of *Toxoplasma gondii* show extremely high levels of inbreeding[21,22] and, as expected, *T. gondii* sex ratios are extremely female biased (mean sex ratio of 0.02–0.06)[23].

Third, variation in the sex ratio across species and populations are consistent with sex ratio theory. The mean sex ratio (0.22) observed in human malaria in Cameroon is less female biased than that observed in Papua New Guinea (0.18), as predicted by a greater potential for outcrossing in Cameroon, where transmission intensity, and hence the number of clones per host, is greater[24]. Across *Leucocytozoon* populations in birds, more-female-biased sex ratios are observed for populations in which gametocyte prevalence is lower, as would be predicted by the fact that inbreeding rates are likely to be higher in these populations (Fig. 3)[11]. In addition, the sex ratios of eimeriorin species are more female biased and less variable than those of haemospororin species (Fig. 2), which is expected because eimeriorin species mate on a very local scale within intestinal tissue, and so inbreeding rates are expected to be consistently high, even when multiple genotypes infect a host (Box 1)[23].

Fourth, there is evidence that some apicomplexan species show conditional sex ratio strategies,

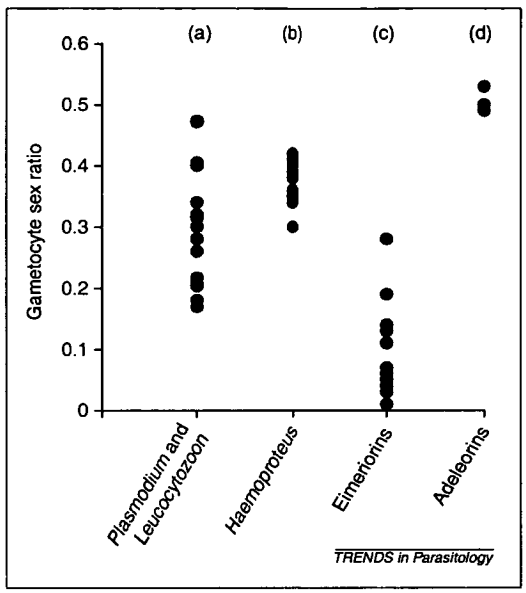

Fig. 2. The mean sex ratios observed in populations of: (a) *Plasmodium* and *Leucocytozoon*; (b) *Haemoproteus*; (c) Eimeriorins (*Eimeria, Isospora, Sarcocystis, Schellackia* and *Toxoplasma*); (d) Adeleorins (*Cyrilia, Desseria, Haemogregarina* and *Hepatozoon*). Data are from Refs 7,11,18,23,24,29,30 and 54.

producing less female-biased sex ratios in hosts infected with more clones, when inbreeding rates will be lower. Studies on lizard[18,25] and rodent (L.H. Taylor, PhD thesis, University of Edinburgh, 1997) malarias have shown a positive relationship between gametocyte densities and the sex ratio, both across and within infections (Fig. 4). This relationship is predicted if gametocyte densities are higher in infections containing more clones, and there is some evidence for this[26,27].

More support: species with syzygy

Some apicomplexans (adeleorins, gregarines and piroplasms) infringe a necessary assumption of Eqn 1 in such a way that unbiased sex ratios are predicted. 'Syzygy' occurs when a single male gametocyte and a single female gametocyte pair together physically or in close proximity, either in host cells or in the lumen of host organs, just before gametogenesis[28]. The crucial consequence of syzygy is that gametes from a single male gametocyte can only fertilize the gamete from a single female gametocyte (excess gametes die). Consequently, the reproductive success of a parasite is always maximized by ensuring there are enough male gametocytes to form pairs with female gametocytes, and so a sex ratio of 0.5 (50% males) is favoured[23]. Data from four species of adeleorins support this prediction (Fig. 2)[23]. Unfortunately, sexual dimorphism of the gametocyte stage at the light microscope level is rare in adeleorins, and is completely absent from piroplasms and gregarines, which also have syzygy. Further work looking for sexually dimorphic species in these groups, and measuring their sex ratios, would be extremely useful.

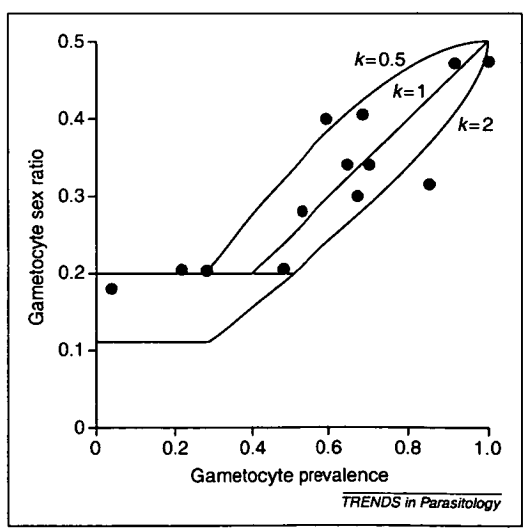

Fig. 3. The observed relationship between the sex ratio and gametocyte prevalence across populations of *Leucocytozoon* and *Plasmodium* parasites. This positive relationship is predicted because when fewer hosts are infectious transmission rates will be lower and mixed infections rarer, and so the rate of inbreeding will be higher. The solid lines show the predicted relationships for various degrees of parasite genotype (clone) aggregation. The variable *k* represents the aggregation parameter from the negative binomial equation[11].

Unpredictable sex ratios and fertility insurance

The above data provide strong support for the application of sex ratio theory to apicomplexans, but some data are contradictory. (1) Across *Haemoproteus* populations in birds, there is no evidence of the predicted relationship between sex ratio and gametocyte prevalence[29] that was observed across *Leucocytozoon* populations (Fig. 3)[11]. (2) Data from within populations of human malaria, some lizard malarias and *Haemoproteus* fail to show the relationship between gametocyte density and sex ratio[24,29,30] that was observed in other lizard and rodent malarias (Fig. 4)[18,25] (L.H. Taylor, op. cit.). (3) Data from *Haemoproteus* of lizards show no consistent trend towards female-biased sex ratios and possibly even some male-biased sex ratios[31]. (4) Data from several *Plasmodium* species show that estimates of the sex ratio taken at different stages from the same infection can be extremely different[30,32,33] (L.H. Taylor, op. cit.).

One factor that might play an important role in explaining these discrepancies is 'fertility insurance'[33–35]. Consider a situation in which there is a danger that female gametes might not encounter any male gametes. This could occur for a number of non-exclusive reasons, including: (1) small blood meals and/or low gametocyte densities lead to an appreciable chance that a blood meal contains no male gametocytes; (2) high mortality of male gametocytes or gametes; (3) low mobility of gametes. Under these conditions, natural selection would favour a less-female-biased sex ratio in order to increase the frequency of female gametes being fertilized[32,35]. Fertility insurance can easily be incorporated into sex ratio models and quantitative predictions made for the favoured sex ratio[35]. Importantly, the logic of this idea has already been supported from data on highly inbred parasitic wasps in which brood sizes are small and the production of extra males is favoured as an insurance against the possibility that all the males might die during development[36–39].

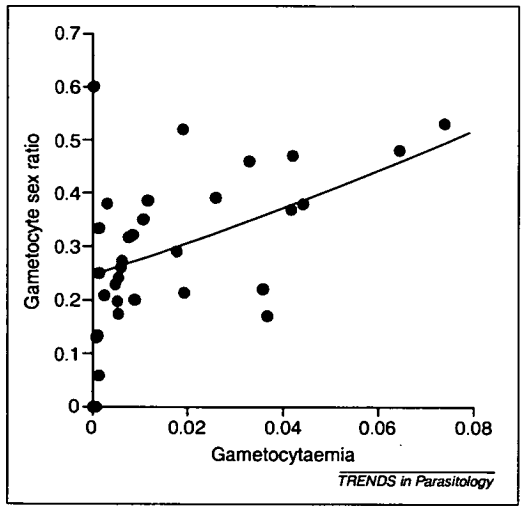

Fig. 4. The observed relationship between the sex ratio and gametocytaemia (proportion of red blood cells containing gametocytes) in the lizard malaria *Plasmodium tropiduri*. This positive relationship is predicted when a lower gametocyte density reflects fewer clones per host, and the parasites are adjusting their sex ratio conditionally based on this higher probability of inbreeding[18].

Fertility insurance is not an alternative to (or in conflict with) the sex ratio theory described above. Fertility insurance should be regarded as an additional factor that can be incorporated into more complicated sex ratio models [indeed, the lower limit of $1 + (1 + c)$ discussed above reflects the simplest possible case of fertility insurance][35]. Specifically, fertility insurance can only have an effect on the sex ratio when female-biased sex ratios are being favoured by inbreeding – in this case, fertility insurance favours a less-female-biased sex ratio. In a population with no inbreeding, the sex ratio favoured would be 0.5, regardless of the probability that female gametes encounter male gametes.

An enhanced sex ratio model that incorporates fertility insurance could explain some of the data that apparently contradict the predictions of Eqn 1 (Refs 33–35). For example, if fertility insurance was particularly important for *Haemoproteus* species then we would not expect to observe extremely female-biased sex ratios or a relationship between sex ratio and gametocyte prevalence (and indeed we do not). This might be due to small vector size, leading to low numbers of gametocytes in a blood meal, and/or the concentration of blood by their vectors, reducing gamete mobility[34]. Similar ideas could be applied to lizard malaria parasites with small vectors. Clearly, much further work is required on the dynamics of mating within vectors to test this possibility.

Fertility insurance could also explain why sex ratios are variable within infections[33–35]. For example, less-female-biased sex ratios would be predicted at times when immune pressure reduces gamete survival or mobility[33]. This could provide an explanation for the observations in at least some *Plasmodium* infections that the sex ratio becomes less female biased during the course of infection, correlating with an increase in immune pressure[32,33], and, across infections, when the sex ratio is negatively correlated with gametocyte density[5]. Detailed discussion of this idea applied to *Plasmodium* is provided in an accompanying paper[5], in which it is suggested that the hormone that induces red blood cell production (erythropoietin) is used as a cue of host immune status[33]. Clearly, this is another area in which there is considerable scope for further work[40]. For example, is gametocyte or gamete survival and/or mobility reduced sufficiently to make fertility insurance important? Why should erythropoietin be more informative than an assay of more direct cues such as effector molecules? Are there other, more direct, environmental cues that *Plasmodium* responds to and, if so, how do they interact to determine the sex ratio?

Future directions

We hope that this article has emphasized the enormous potential for future research on apicomplexan sex ratios. There are six particularly important areas. First, direct molecular genetic estimates of population structure and inbreeding rates open up several exciting avenues[20,41–46]. (1) Do the mean sex ratios of populations correspond quantitatively to the mean inbreeding rate as predicted by Eqn 1? Such data would be extremely useful from populations in which the inbreeding rate (F) is considerably lower than 1.0, because lower confidence intervals will be placed on estimates of F from sex ratio data owing to the need to produce enough male gametes to fertilize the female gametes (Fig. 1). (2) Across populations and species, is the sex ratio positively correlated with the inbreeding rate? (3) Measures of clonal diversity across individual hosts within a population would allow testing for conditional sex ratio shifts in response to the likelihood of inbreeding. Usefully, molecular data are accumulating rapidly on many *Plasmodium* populations and so, in many cases, all that is required is the relatively easy task of collecting sex ratio data. (4) Measures of both sex ratios and relative abundance of different genotypes within single hosts will allow testing for conditional sex ratio shifts in response to relative abundance within a host. Theory predicts that genotypes that are producing more gametocytes will experience a higher level of inbreeding and should produce more-female-biased sex ratios; genotypes producing relatively few gametocytes will experience a lower level of inbreeding and should produce less-female-biased sex ratios. There is evidence for such sex ratio shifts in parasitic wasps[47].

Second, several areas of basic biology need further investigation, especially those suggested by the possible importance of the fertility insurance hypothesis[33–35]. In particular, very little is known about: (1) parasite–vector interactions – in many cases, the vector has not even been identified[34], still less have there been estimates of how many gametocytes there are in a blood meal or the likelihood of female gametes being fertilized[35]; (2) the consequences of the vertebrate immune response for gametocyte and gamete survival and mobility[33]; (3) the extent to which gametocyte aggregation in the blood[48]

Box 2. What do sex ratios tell us about the inbreeding rate of protozoan parasites?

The rate of inbreeding and extent (or not) of clonality in protozoan parasites, especially *Plasmodium*, has been the subject of much debate[a–d]. This controversy has arisen in part because much previous evidence has come from indirect genetic measures such as linkage disequilibrium (non-independent segregation of alleles at pairs of loci), which are open to multiple explanations and cannot be used easily to estimate the inbreeding rate quantitatively[a,e]. More recently, molecular genetic analyses of the diploid products of sex (oocysts) have been used to provide direct estimates of the inbreeding rate[f]. However, this method is extremely laborious and expensive, and so has only been applied to two populations[f,g]. In addition, interpretation of this data is also proving problematic – estimates of the inbreeding coefficient from human malaria in Papua New Guinea vary from 0.48 to 0.90, depending upon how one accounts for the existence of null alleles in the data[e]. Also, this method could not be applied to all populations – the prevalence of infection in mosquitoes from low-transmission areas (often <1 in 1000) makes this method impractical[h].

Sex ratio data provide a relatively cheap and easy indirect method with which to estimate the population structure of a species[i]. Specifically, the inbreeding rate (F) can be estimated from the observed sex ratio (r) using Eqn I:

$$F = 1 - 2r \qquad [I]$$

The validity of this approach is supported by the fact that, in cases for which we have molecular genetic and sex ratio data, they are in agreement[f,i,j].

A major advantage of the sex ratio approach is that sex ratio data are relatively easy to obtain and so can be collected from many species and populations, allowing generalizations to be made. The sex ratio data from haemospororin species show a range of sex ratios from extremely female biased to 50% males, suggesting that the rate of inbreeding varies enormously between populations and species, from highly inbred to highly outbred (see Fig. 2 in main text), depending upon transmission rates[k] (see Fig. 3 in main text). By contrast, the data from eimeriorin species are highly female biased, predicting consistently high inbreeding rates (see Fig. 2 in main text), as expected from the fact that mating occurs on a very local scale within the intestine[j] (see Box 1 in main text).

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reduces the need for fertility insurance; (4) cues that might allow parasites to shift their sex ratios conditionally; (5) the mean number of viable gametes released by a male gametocyte (c). In *Plasmodium mexicanum*, a lizard malaria, the modal value of c is around 2 (Ref. 25), and so the maximum female bias that could be favoured by natural selection is 0.33, which happens to be that observed. Similarly low values of c across *Haemoproteus* species could explain their lack of sex ratio variation in response to a correlate of inbreeding[29], and the variation in C as a result of immune-induced mortality could explain variation in the sex ratio during infections[33,35].

Third, almost nothing is known about mechanism of sex determination within the Apicomplexa[6,33,49,50], despite its fundamental importance for determining the extent to which parasites can adjust their sex ratio in response to environmental conditions (as opposed to having genetically fixed strategies) and whether male and female gametocytes are equally costly to produce[49]. Is sex determination a response to one or more environmental cues that are related to inbreeding rates and/or the need for fertility insurance? How linked are the cellular and molecular mechanisms involved in sex determination to those in commitment to asexual or sexual stages[15,49,51,52] (e.g. 'stressful' conditions might favour production of gametocytes[15–17] and the need for fertility insurance[33])?

Fourth, more sex ratio data are required from species in which syzygy occurs; a sex ratio of 0.5 is then predicted irrespective of the inbreeding rate[23].

Fifth, many fundamental assumptions that can alter the predictions of sex ratio theory need to be tested. Is gametocyte mortality sex biased? Current evidence suggests that it is not[7,53] but this conclusion is based on perilously low sample sizes (seven infections) given the importance of the issue. Are estimates of the sex ratio biased by sampling from hosts with high gametocyte densities and/or by being taken from relatively large blood vessels (vectors typically feed from very small vessels)? How frequently is the sex ratio underestimated from Giemsa-stained blood films owing to male gametocytes being incorrectly identified as immature gametocytes (compared with estimates based on mRNA probes or stage- and sex-specific antibodies)[49]?

Sixth, much of the data and background biology presented in this article are drawn from studies of human and laboratory *Plasmodium*. Expanding research of all forms to a wider range of species might turn up novel aspects of biology, with fundamental consequences for sex allocation, as is the case with

syzygy[23] (e.g. explaining why the sex ratio pattern is so different in *Haemoproteus*[34]).

Conclusions

Existing data suggest that apicomplexan sex ratios are broadly consistent with the predictions of sex ratio theory. In addition to providing an explanation for patterns of variation in the sex ratio per se, this work is important because it provides an excellent model trait[11,23] with which to test the usefulness of 'DARWINIAN MEDICINE' – if we cannot use the adaptationist approach to understand sex ratios then we cannot use it to understand more complex traits that are of direct medical importance, such as virulence. It also provides a relatively easy method for estimating the inbreeding rate in natural populations, a parameter that is currently the subject of much debate (Box 2). However, work on the sex ratios of apicomplexans is still in its infancy compared with studies of other taxa, and there is much to catch up on – even in the realms of human malaria and associated laboratory models.

Acknowledgements
We thank R. Carter, R. Paul, T. Smith and D. Walliker for useful discussion and comments on the manuscript, and to the BBSRC and NERC for funding.

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Malaria sex ratios

Can sex-allocation theory account for the sex ratios of malaria parasites? The sex ratios of many metazoans change in response to environmental cues, often as predicted by theory, providing some of the best evidence that adaptationist thinking actually works[1]. Now, Paul *et al.*[2] have reported that environmental conditions also affect the sex ratio of malaria parasites. This discovery allows a fuller analysis of the adaptive basis of sex allocation in these protozoans. More generally, it provides a model for testing the use of optimality theory when applied to infectious disease research – a key component of the much hyped 'Darwinian Medicine'[3].

Transmission of *Plasmodium* occurs when a mosquito has a bloodmeal containing the dioecious haploid stages known as gametocytes. Gametocytes rupture within the vector, releasing either one female gamete or up to eight male gametes. Zygotes formed following fertilization are briefly diploid before haploid asexual replication is resumed, first in the mosquito and subsequently back in a vertebrate host. Individual haploid lineages are capable of generating gametocytes of both sexes and there is no evidence of sex differences in gametocyte mortality[2,4]. In the peripheral blood of infected humans, birds, lizards and rodents, gametocyte sex ratios are almost always female biased, but are often more variable than expected by chance[2,4–9]. Variability occurs between hosts within a population and during the course of single infections, which led to the suggestion that sex allocation might partially be a response to environmental conditions[4,7,8]. The experiments recently reported in *Science*[2] are the first to demonstrate this response.

Linking erythropoiesis and the sex ratio

Paul *et al.*'s study[2] was triggered by their earlier observations of gametocyte sex ratios in chickens experimentally infected with *Plasmodium gallinaceum*[9]. In lethal infections, the gametocyte sex ratio remained female biased throughout; however, in infections successfully contained by protective immune responses, sex ratios became progressively less female biased[9]. An important symptom of malaria is anaemia, which occurs largely as a result of red blood cell destruction by replicating parasites. Anaemia induces erythropoiesis, the release of reticulocytes (immature red blood cells) into the bloodstream. Among surviving chickens, Paul *et al.*[2] observed that gametocyte sex ratio became less female biased as reticulocyte concentration increased. To determine whether this correlation was causal, Paul *et al.*[2] induced erythropoiesis by exposing chickens to hypoxic conditions or by removing 25% of total blood volume. Both treatments generated less female-biased sex ratios than observed in control infections, even though there were no differences in parasite numbers. However, the shift in sex ratio was not a response to the presence of reticulocytes: the replacement of 20% of the blood volume with reticulocyte-rich blood had no effect on the sex ratio. Thus, erythropoiesis, rather than the inducing anaemia or the resulting reticulocytes, apparently affects the sex ratio.

Erythropoiesis is induced by the endogenous hormone erythropoietin (Epo) and, as demonstrated by numerous top athletes and cyclists, by exogenous Epo. Paul *et al.*[2] found that injections with mouse recombinant Epo shifted gametocyte sex ratios in the rodent malaria *Plasmodium vinckei* closer to 1:1, apparently without any other effects on the parasites. This shift was indistinguishable from the shift induced by hypoxia. Combining these results, Paul *et al.*[2] concluded that the parasite sex ratio is altered as a direct response to Epo concentrations.

Rigorously controlled testing of this conclusion should be possible *in vitro*, at least for *Plasmodium falciparum* – the most virulent of the human malarias and the only malaria parasite that can be reliably cultured. Recent *in vitro* experiments have demonstrated that all the progeny of a sexually committed parental parasite are the same sex[10]. By extending this approach to include a wide variety of Epo treatments, it should be possible to resolve whether sex is irrevocably determined by the parent (facultative sex allocation[1]) or whether the environment directly determines the sex of developing gametocytes (environmental sex determination[11]). In addition, comparisons of gene-expression patterns in otherwise identical parasites induced to develop into different sexes could be used to determine the elusive molecular genetic mechanism that allows individual haploid lineages to produce both males and females.

Adaptive explanations?

Is a shift of gametocyte sex ratio in response to Epo adaptive? Paul *et al.*[2] argue that it is, because they found that reproductive success was greatest when the sex ratio shifted in response to natural Epo levels, but was lower when sex ratio shifts are induced by experimentally altered erythropoiesis.

In single clone infections, where all zygotes will be the result of self fertilizations, the optimal sex ratio is one that results in just enough male gametes to fertilize all the available female gametes. Because one male gametocyte can produce up to eight male gametes, while a female gametocyte produces just one gamete, a sex ratio of up to 8:1 should be favoured. Why should erythropoiesis trigger a shift from female to male sex allocation?

Transmission-blocking immunity is well known in malaria infections. A variety of evidence demonstrates that the viability of male gametes (but not gametocytes) is disproportionately reduced by vertebrate effector mechanisms acting in the midgut of the mosquito after a bloodmeal[2,12]. In these circumstances, natural selection should favour a shift from females to males to ensure the number of zygotes is maximized. Paul *et al.*[2] assume that rising Epo concentrations coincide with the period when sex-biased antigamete immunity begins to impact on fertilization success.

Just so stories?

Like the best adaptationist hypotheses – and characteristic of those concerned with sex allocation – Paul *et al.*'s explanation provokes several tractable questions. First, it requires that Epo level is a reliable indicator of the onset of significant reductions in male gamete viability. Why should Epo concentrations be more informative than the effector molecules themselves? Second, antibodies play a key, although not necessarily unique, role in antigamete immunity. Immunological orthodoxy states that from antigen presentation it takes a naïve host about two weeks to mount an effective T-cell-dependent antibody response[13]. However, the sex-ratio shifts observed by Paul *et al.*[2] occurred only a few days after the first gamete antigens could have been presented to the host immune system; therefore, perhaps a T-cell-independent sex-biased effector mechanism is involved? Third, what happens in chronic infections where gametocytes are present for extended periods of time? What happens when gametocytes are produced in semi-immune hosts who are not anaemic?

There might also be other adaptive explanations for Epo-induced sex ratio alterations. For instance, bloodmeals from anaemic hosts might contain fewer red blood cells, and thus fewer gametocytes and gametes; hence, less female-biased

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sex ratios are required to ensure sufficient males enter the mating pool. Lack of data on gamete motility and location efficiency means that we cannot yet incorporate the effect of these parameters into models to predict the gametocyte sex ratio. Nevertheless, comparative work on the sex ratios of *Plasmodium* species, transmitted by vectors that do or do not concentrate blood during feeding might be informative.

Future directions

The discovery that malaria sex ratios are altered in response to environmental cues opens up a range of new experimental options for analysing the evolution of sex ratios in these protozoa. First, experimentally generated sex ratios could be used to directly test the fitness consequences of sex-ratio variation. An unexpected finding, reported in human and lizard malarias[5,6], is that sex ratios closer to 1:1 generate more zygotes than the more female-biased sex ratios that predominate in nature. If this correlation proves to be causal, the assumptions of several current models might be violated. Second, there is no reason to expect that only one environmental cue will influence the malaria sex ratio – identifying other cues would enable testing of other sex-allocation theories. For instance, the role of local mate competition (LMC) (Ref. 14) in shaping malaria sex ratios has been controversial[4,7,8,15,16], but evidence of facultative sex-ratio adjustment in the presence of co-infecting clones would be compelling evidence that LMC plays an important part. Detecting the presence of unrelated clones might seem unlikely, but the specificity of antibody binding to polymorphic epitopes might be easily assayed, even by a single-celled organism. More generally, a combination of environmental and genetic factors might explain the variable sex ratios observed in malaria parasites[2,4–8]. Using animal models it should be possible to experimentally alter factors such as Epo concentrations, the strength of sex-biased antigamete immunity and genetic relatedness within infections to test this proposition.

Much of what is optimistically known as darwinian medicine involves adaptationist arguments[3]. Many hypotheses, particularly those concerned with inherited human disease, will be hard to advance beyond *Just So Stories*. Those concerning infectious diseases might be more vulnerable to quantitative experiments because relevant animal models are often well established. Theories of the evolution of virulence bear much in common with models underpinning sex-ratio evolution[7]. Simple optimality arguments underlie work in both areas but typically these assume population dynamic equilibrium – not an obvious feature of many medically relevant diseases. How successful can virulence theory be? Rigorous testing requires quantitative measures of the fitness tradeoffs associated with different levels of pathogen virulence; however, obtaining such data is a major experimental challenge. The beauty of sex-allocation theory – and possibly a reason for its quantitative success – is that the relevant tradeoffs are usually obvious. If we cannot understand gametocyte sex ratios for malaria, there is little reason to think we can understand more complex phenotypes, such as virulence. Paul *et al.*'s[2] discovery provides a new way to move forward our rudimentary understanding of malaria sex ratios.

Acknowledgements

We thank S. West and R. Paul for comments. S.R. and A.R. are funded by the NERC, BBSRC and Leverhulme Trust.

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