Maintenance of a female-limited colour polymorphism in the crab spider *Synema globosum* (Araneae: Thomisidae)

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Abstract

The occurrence of multiple genetically and phenotypically distinct forms in a single interbreeding population, known as polymorphism, represents a long-standing puzzle in evolutionary biology. Several mechanisms, both selective and stochastic, have been proposed to account for the maintenance of such diversity. Nevertheless, although our knowledge about how these mechanisms might operate has increased substantially in recent years, the specific role that they play in the maintenance of polymorphisms in natural populations remains to be determined. In particular, negative frequency-dependent selection, where rare morphs have a fitness advantage over common morphs, has frequently been suggested to explain the occurrence of genetic variation, but conclusive evidence for its importance in natural populations has yet to be obtained. In this thesis, I investigated the maintenance of a female-limited colour polymorphism in the crab spider *Synema globosum*, and the role that negative frequency-dependent selection could have in this process. First, I described the natural history of *S. globosum*, which was previously unknown. Then, I looked at the nature of the colour polymorphism and investigated its mode of inheritence. Finally, I carried out a series of experiments to examine the potential role of negative frequency-dependent selection resulting from interactions with prey, and between potential mates, in the maintenance of the polymorphism. *S. globosum* was shown to be an abundant species in the study area and, apart from its striking polymorphism, it exhibits several interesting characteristics that would make

it a useful model species for studies of behaviour, ecology, and evolution. The female morphs were shown to be discrete and genetically determined. No evidence for directional selection favouring one particular morph, or for geographic variation in selection, was found. Field experiments revealed an effect of a previous negative experience with a particular colour morph on subsequent responses of honeybees (*Apis mellifera*), one of *S. globosum's* main prey, to the presence of spiders on flowers. This result provided support for the hypothesis that such interactions generate negative frequency-dependent selection. Similarly, mating experiments provided some evidence that reduced harassment of less frequently encountered female morphs by male *S. globosum* could also generate negative frequency-dependent selection. This work adds to a growing body research that has increased our understanding on the mechanisms that maintain diversity in nature and establishes the basis for future studies to investigate the exact ecological explanation for the observed phenotypic variation in *S. globosum.*

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Chapter 1: General introduction

Polymorphism occurs when two or more genetically and phenotypically different forms, known as morphs, can be found in a single interbreeding population, with the rarest one occurring at frequencies that are too high to be maintained by mutation alone (Ford 1945). It has long represented an evolutionary puzzle, because alternative forms are expected to vary in fitness, and those with lower fitness should be eliminated by natural selection (Darwin 1883; Fisher 1930). The fact that different morphs do indeed persist alongside one another in many populations (Brockmann 2001; Bond 2007; Gray and McKinnon 2007; Mitchell-Olds et al. 2007; Kunte 2009), remains a central problem in evolutionary biology.

While non-selective processes have been invoked to explain the maintenance of polymorphisms (e.g. the fitness differences among morphs could be negligible), in many cases, it is thought that local selective processes, with or without gene flow among populations, must be involved (King and Lawson 1995). Several selective mechanisms, such as disruptive selection and heterozygote advantage, are likely to play a role, but frequency-dependent selection is perhaps the most commonly cited explanation for observed polymorphisms (Brockmann 2001; Gray and McKinnon 2007; Mitchell-Olds et al. 2007). Negative frequency-dependent selection (NFDS), in which a rare morph has a fitness advantage over common morphs, can account for the existence of different morphs at stable frequencies in a population (Clarke and Odonald 1964; Ayala and Campbell 1974) and has been proposed to explain polymorphisms in a number of contexts (Hori 1993; Fincke 2004; Sinervo and Calsbeek 2006; McKillup and McKillup 2008; Hampton et al. 2009; Koskella and Lively 2009). Evidence of NFDS has been observed both in laboratory (Kojima and Tobari 1969; Maskell et al. 1977; Anderson and Brown 1984; Cooper 1984; Singh and Chatterjee 1989; Gigord et al. 2001; Fitzpatrick et al. 2007; Koskella and Lively 2009) and natural conditions (Reid 1987; Hori 1993; Svensson et al. 2005; Olendorf et al. 2006; Bleay et al. 2007; McKillup and McKillup 2008; Takahashi and Watanabe 2010). Nevertheless, considerable uncertainty exists about the relative importance of this and other mechanisms in the maintenance of genetic and phenotypic diversity in real populations.

There are some genetic polymorphisms that do not affect phenotypic traits. They occur in non-coding areas of the genome, and have been used as markers for studies in population genetics, evolution and medicine (Hacia et al. 1999; Jorde et al. 2000; Syvänen 2001; Williamson et al. 2007). Polymorphisms that do affect phenotypic traits are not always apparent to the observer, such as some of those involving behaviour and resistance to parasites or diseases (Thornhill 1979; Field and Keller 1993; Kirkup and Riley 2004; Duncan and Little 2007; Laine and Tellier 2008). In contrast, conspicuous polymorphisms, particularly those involving colouration, are easy to score, and their study has been central in attempts to understand the mechanisms that could be maintaining genetic and phenotypic variation in populations. Colouration is known to serve an adaptive function in processes such as thermoregulation (Quartau and Borges 1997; Phifer-Rixey et al. 2008), attraction of mates (Nielsen and Watt 2000), avoidance of predators (Hoese et al. 2006; Skelhorn et al. 2010), and attraction of prey (Hauber 2002; Heiling et al. 2005; Bush et al. 2008). This strongly suggests that the maintenance of conspicuous colour polymorphisms is influenced by selection, and NFDS in particular has often been assumed to play a key role.

Many species of insect, mollusc, arachnid and crustacean display conspicuous and easily-measured polymorphic colour traits. Such invertebrates are typically easier to manipulate than vertebrates, both in the field and in the laboratory, and it is relatively easy to get large sample sizes. As a result, many of the most detailed case-studies of the potential influence of NFDS on traits come from the study of colour polymorphic invertebrates. However, in spiders, where there are several well-known colour polymorphic species (Galiano 1981; Oxford 1983; Gunnarsson 1987; Galiano 1990; Clark and Uetz 1992; Oxford and Gillespie 1996; Tso et al. 2002; Bonte and Maelfait 2004; Oxford 2009; Gawryszewski and Motta 2012), mechanisms involved in the maintenance of polymorphism have seldom been investigated. This is despite the fact that spiders represent ideal systems to explore the role that NFDS and other diversifying mechanisms have in the maintenance of genetic variation.

1.1 Negative frequency-dependent selection

The idea that the fitness of an organism is affected by the relative frequencies of the genotypes in a population was first described by Fisher (1930), suggesting that an inverse relation between the two could maintain stable polymorphisms. This concept was later formalized by other researchers, such as Li (1955), Wright (1956), and Lewontin (1958), who developed mathematical models to describe the mechanism.

Evidence that the fitness of a morph depends on its frequency relative to the frequencies of the other morphs was first found by Wright and Dobzhansky (1946) in an experimental population of *Drosophila pseudoobscura*. Three different gene arrangements can be found in the third chromosome of this species, and their frequencies were observed to fluctuate over the year in natural populations. Wright and Dobzhansky set up an experimental population with known frequencies of the different genotypes and controlled environmental conditions. They found that the observed changes in frequencies of the phenotypes at different temperatures fitted the predictions of a model where the fitness of the homozygotes decreases as their frequencies increase, while the fitness of the heterozygotes remains constant. However, Wright and Dobzhansky considered this hypothesis to be an “extreme” one. Since then, there have been several laboratory studies where evidence for NFDS has been found in populations of *Drosophila*, with morph frequencies fluctuating in a manner that is predictable based on the known effects of frequency on fitness (Levene et al. 1954; Kojima and Tobari 1969; Anderson and Brown 1984; Singh and Chatterjee 1989). A correlation between fitness and frequency has also been found in laboratory studies in crustaceans (Maskell et al. 1977; Duncan and Little 2007), land snails (Tucker 1991), and water snails (Koskella and Lively 2009). This correlation has been found in natural populations as well, and is the commonest form of evidence supporting NFDS in the wild (Reid 1987; Gross 1991; Seehausen and Schluter 2004; Svensson et al. 2005; Olendorf et al. 2006; Bleay et al. 2007; McKillup and McKillup 2008; Takahashi and Watanabe 2010). A few studies have also demonstrated oscillations in morph frequencies over time that can be explained by NFDS (Hori 1993; Sinervo and Lively 1996). However, direct evidence for NFDS in the wild is generally scarce because the best way to test for it is to manipulate the frequencies of different morphs in a population, and to obtain reliable measures of fitness from individuals of each morph, both of which pose considerable practical challenges. Even though these complications should be easier to overcome in laboratory conditions, a comprehensive experimental test of the hypothesis with real organisms is yet to be published, and the most unequivocal demonstration of NFDS acting to maintain polymorphism comes from a study involving selection by real predators (birds) on polymorphic computer-simulated prey (Bond and Kamil 1998, 2002).

Although we do not have definitive evidence that NFDS maintains diversity in wild populations, there is a growing body of research which demonstrates the potential for various ecological interactions to generate NFDS. Trophic interactions between natural enemies and their prey/hosts, sexual interactions between conspecifics, and interactions among competitors have all been documented as having frequency-dependent effects on the fitness of morphs in natural populations (Brockmann 2001; Sinervo and Calsbeek 2006). Our ability to study these effects depends partly on the salience of the polymorphic traits concerned to the observer.

1.2 Male-female interactions

Sexual interactions between males and females may lead to NFDS and, as a consequence, maintain balanced polymorphisms in populations. In particular, and for obvious reasons, it has frequently been assumed that sexual interactions are implicated in the maintenance of sex-limited polymorphisms, where one sex (usually the female) exhibits conspicuous variation in colour, while the other is mono-morphic. Negative frequency-dependent sexual selection has also, however, been identified in species in which polymorphism occurs in both sexes. There are, broadly speaking, two kinds of hypothesized explanations involving NFDS and sex in the maintenance of diversity which correspond to two different kinds of sexual interaction: sexual conflict and mate choice (Brockmann 2001).

1.2.1 Sexual conflict

Sexual conflict occurs when males and females have different interests in the outcome of sexual encounters, and this can result in adaptations that counteract each other. One way in which such conflict may lead to NFDS stems from harassment of females by males. If a female receives a significant number of unwanted mating attempts by males, this can generate costs for her in terms of time, energy, fecundity, foraging, longevity and predation risk (Arnqvist 1989; Odendaal et al. 1989; Krupa and Sih 1993; Rowe 1994; Stone 1995; Clutton-Brock and Langley 1997; Jormalainen et al. 2001). In order to avoid these costs, females can evolve alternative strategies that may have a fitness advantage depending on the frequency of either the other female strategies or of the males in the population (i.e. the sex ratio).

NFDS caused by male harassment of females has been extensively researched in damselflies (Kunte 2009). In this group, there are several species that show a female-limited colour polymorphism, with two or more discrete morphs, at least one of which is easily distinguished from the male (known as the gynomorph or heteromorph) and at least one of which resembles the male (the andromorph) (Johnson 1975). Male harassment has been shown to decrease the fitness of females in several damselfly species (Sirot and Brockmann 2001; Svensson et al. 2005; Bots et al. 2009; Takahashi and Watanabe 2010), leading to discussion of the role of selection in the maintenance of the observed polymorphism. More specifically, two hypotheses are commonly suggested to explain how NFDS might be involved: the learned mate recognition hypothesis (LMR) and the male mimicry hypothesis (MM).

The LMR hypothesis posits that males will prefer to mate with females of the morph that they encounter more frequently in the population, because they will become more efficient at recognizing them as potential mates (Miller and Fincke 1999; Fincke 2004). Consequently, females of the common morph are predicted to suffer higher levels of male harassment than females of the rare morph, and have lower fitness as a result. This hypothesis thus suggests that andromorphs exist simply because they are different from heteromorphs, and not because there is any special advantage in resembling a male. Evidence that males prefer to mate with the most common female morph has been found in choice experiments in the laboratory (Van Gossum et al. 1999). Similarly, in natural populations, choice and no-choice experiments with sequential presentations, as well as observations of natural mating events, have shown that males typically choose to mate with the common morph (Fincke 1994; Miller and Fincke 1999; Takahashi and Watanabe 2009, 2010). Additionally, a reduction in fitness, measured as a decrease in egg production and excretion of faeces by the common, more harassed morph, has been observed in natural populations of *Ischnura senegalensis* (Takahashi and Watanabe 2010). It has also been observed, both in the laboratory and in the field, that past experience with a particular female morph will bias the male's preference towards that morph (Miller and Fincke 1999; Van Gossum et al. 2001). Using observations of morph frequencies, levels of harassment and morph fitness, two studies have found a strong correspondence between the predictions of simple models based on the type of frequency dependence likely to result from LMR and morph dynamics in natural populations over several years (Svensson et al. 2005; Takahashi and Watanabe 2010). The conclusion of these studies was that simple NFDS resulting from increased harassment of a common female morph can explain the indefinite persistence of polymorphisms in at least some damselfly populations.

LMR could also occur in some species of colour polymorphic butterflies, where males have been observed to show a preference for the common female morph (Cook et al. 1994; Nielsen and Watt 2000; Kemp and Macedonia 2007). There is, however, no evidence that male harassment reduces the fitness in these species, and in general our understanding of the different mechanisms which may be involved in the maintenance of the observed morphological diversity in butterflies is less well-developed than in the better-known damselfly systems.

In contrast to the LMR hypothesis, the MM hypothesis proposes that andromorph females gain a fitness advantage by mimicking the appearance and/or behaviour of males. There are several versions of the MM hypothesis discussed in the context of damselfly polymorphism (Johnson 1975; Robertson 1985; Hinnekint 1987; Sherratt 2001), but those which involve NFDS all assume that frequency dependence in female morph fitness arises because andromorph mimicry is most effective when heteromorphs are relatively common, such that it benefits males to focus their attention on heteromorphs rather than waste their efforts on individuals which may turn out to be males. The key difference from the LMR hypothesis is that, because mimicry is the source of frequency dependence, there is no equivalent frequency-dependent advantage for heteromorphs when they are rare – they are assumed always to be readily identifiable as mates. Instead, the MM hypothesis assumes that there is some frequency-independent cost borne by the andromorphs: either they are more likely to be attacked by predators than heteromorphs because they are less cryptic (Robertson 1985), or they are more likely than heteromorphs to be encountered by males, because they are more conspicuous or behave more like males (Sherratt 2001).

Several studies have found evidence which appears to support the MM hypothesis (Sirot et al. 2003; Rivera and Sánchez-Guillén 2007; Hammers and Van Gossum 2008; Van Gossum et al. 2011), but in some cases the reported data are also consistent with the LMR hypothesis (Ting et al. 2009). Hence, the need for predictions which are unique to one or other hypothesis, and experimental studies which can test those predictions, has been highlighted (Sherratt 2001; Van Gossum and Sherratt 2008). Because LMR is based on males learning to recognize the common female morph as a mate, this morph should always receive a higher number of mating attempts. In contrast, the MM hypothesis does not predict that the common morph will always receive more mating attempts, but instead it predicts (uniquely) that andromorphs should be less harassed when they are rare relative to males, and hence that under equilibrium conditions andromorphs should be relatively more common in populations where the sex-ratio is male biased (Sherratt 2001; Van Gossum and Sherratt 2008). Although both of these predictions have some empirical support (Hinnekint 1987; Cordero 1992; Forbes et al. 1995; Hammers and Van Gossum 2008), the problem with testing them in real populations is that female morph frequency may correlate with sex ratio in the wild (e.g. Hammers and Van Gossum 2008), making it difficult to disentangle cause and effect. A more fruitful line of enquiry may be to examine male behaviour in more detail. There are two studies where andromorph females have been observed to behave similarly to males and males have responded to them as if they were other males, suggesting that they might actually be fooled by andromorphs (Andrés et al. 2002; Sirot et al. 2003).

Even though both the LMR and the MM hypotheses are plausible explanations for the patterns observed in natural populations of polymorphic damselflies, each with some empirical support, and notwithstanding the widespread perception that NFDS is likely to be central to explaining sex-limited polymorphism (see e.g Van Gossum and Sherratt 2008), there are several plausible alternative hypotheses which do not involve frequency dependence at all. One of these proposes that andromorphs will have an advantage at high population densities by mimicking males, and this advantage will be offset by the risk of not mating at all at low densities (Hinnekint 1987). Very few studies have considered this hypothesis, and no supportive evidence has been found (Cordero-Rivera and Egido-Pérez 1998). An alternative hypothesis suggests that andromorphs will benefit from avoiding interspecific matings, while paying the cost of higher vulnerability to predation (Johnson 1975). However, it is not clear how andromorphs would be more efficient than heteromorphs at avoiding interspecific matings, data supporting this hypothesis are lacking, and the trade-off would have to be perfectly balanced for polymorphism to persist at equilibrium. Abiotic factors could also play a role in the maintenance of the polymorphism. Morph frequencies have been observed to vary across geographical ranges where climatic conditions differ (Van Gossum et al. 2007; Hammers and Van Gossum 2008; Gosden et al. 2011), and it has been found that ambient temperature affects mass and protein content of female morphs differently (Bots et al. 2009). It has also been observed that spatiotemporal patterns of morph frequencies do not always correlate with estimates of male harassment (Van Gossum et al. 2007; Hammers and Van Gossum 2008; Iserbyt et al. 2010). It is thus plausible that different morphs are at a selective advantage in different populations, and that gene flow among those populations maintains diversity in each. However, this hypothesis has not been well explored in damselflies, or other species in which there are sex-limited polymorphisms, and much of what we know about the potential for climatic selection to maintain diversity comes from a rather different example of an invertebrate colour polymorphism: that seen in the land snails of the genus *Cepaea* (Cook 1998; Cameron and Pokryszko 2008), which is discussed later in this review.

1.2.2 Mate choice

Mate choice could lead to NFDS, and consequently to the maintenance of balanced polymorphisms, when either females or males prefer to mate with a rare morph of the opposite sex. An obvious possible advantage of mating with a type of the opposite sex that is not encountered frequently in the population would be to reduce genetic incompatibility from mating with an individual that is genetically similar, or to avoid mating with the same individual more than once (Tregenza and Wedell 2000; Zeh and Zeh 2003). When females choose males, and they prefer to mate with males of a type that has not been encountered before, then the rare male morph in the population will have a mating advantage over the common morph; this is called the rare male effect (Knoppien 1985).

The rare male effect has been predominantly studied in guppies (*Poecilia reticulata*) within the vertebrates (Hughes et al. 1999; Zajitschek et al. 2006; Hampton et al. 2009), and in *Drosophila* within the invertebrates (Ehrman 1972; Pruzan and Ehrman 1974; Spiess and Schwer 1978; Anderson and Brown 1984; Singh and Chatterjee 1989; Depiereux et al. 1990; Terzić et al. 1996; Singh and Som 2001; Som and Singh 2005), and it has been found that at least in some circumstances females do prefer to mate with uncommon males. The rare male effect has also been observed to occur in the two-spotted ladybird, *Adalia bipunctata.* This species shows a polymorphism in the colour and pattern of the elytra and the pronotum that can range from red to almost completely black, and the frequencies of the morphs vary geographically (Creed 1975). Females of this species have shown a preference to mate with the rare male morph in the population both in field and laboratory conditions (Muggleton 1979; Majerus et al. 1982).

Another invertebrate species in which a rare male effect has been found is the African monarch butterfly, *Danaus crhysippus,* which presents a colour polymorphism with two common morphs that have either nut-brown or orange wings. Smith (1975) observed in wild populations that the orange male morph had a mating advantage lasting 3 to 4 months, which was lost as their frequency increased. The mating advantage of both morphs fluctuated through the duration of the study (26 months) with each morph being favoured when rare; however, density-dependent selection could not be ruled out as an explanation for the observed polymorphism, because density and frequency covaried in the study population (Smith 1975), and density-dependent selection is another mechanism that can potentially maintain genetic polymorphisms (Roughgarden 1971; Moorcroft et al. 1996; Sinervo et al. 2000).

Although the rare male effect is quite well supported in some systems, just as in other scenarios in which NFDS generated by sexual interactions might explain the continued persistence of polymorphism, frequency independent abiotic factors have also been implicated. In several *Drosophila* species, there is clinal variation in pigmentation correlated with latitude, altitude, humidity, and temperature (David et al. 1985; Capy et al. 1988; Munjal et al. 1997; Hollocher et al. 2000; Brisson et al. 2005; Pool and Aquadro 2007; Parkash et al. 2008; Rajpurohit et al. 2008; Parkash et al. 2011). A similar correlation has also been found in the ladybird *Adalia bipunctata*, and the variation in colour observed has been suggested to be a result of gene flow among populations experiencing different selection on melanisation for thermoregulation (Brakefield 1984; de Jong and Brakefield 1998).

1.3 Male-male interactions

When individuals in a population are competing for the same resources, genetically-determined alternative strategies to exploit those resources can arise. In nature, males of different species have been observed to adopt alternative mating strategies when competing for females (Gross 1985; Maekawa and Onozato 1986; Gross 1991; Shuster and Wade 1991; Bleay et al. 2007). Theoretically, these alternative strategies can be maintained by NFDS when an individual's fitness is affected by the frequencies of neighboring morphs with which it is competing in its social environment (Gadgil 1972; Maynard Smith 1982; Sinervo and Lively 1996). In invertebrates, the only species where alternative strategies that are known to be genetic in origin have been observed is the marine isopod *Paracerceis sculpta*. However, the polymorphism does not involve colouration, and no formal test of NFDS has been made (Shuster and Wade 1991).

Genetically-determined alternative male mating strategies have been more widely studied in vertebrates, predominantly in birds, fish and lizards (Gross 1984, 1985; Maekawa and Onozato 1986; Sinervo and Lively 1996; Tuttle 2003; Bleay et al. 2007; Formica and Tuttle 2009). Nevertheless, examples providing evidence for NFDS as a mechanism for their maintenance are scarce, and most are of behavioural polymorphisms that are not associated with colour (Gross 1985, 1991).The only study that evaluates NFDS in the maintenance of alternative male mating strategies associated with colour is in the side-blotched lizard (*Uta stansburiana*), where males show three different throat colours. Male morph frequencies have been observed to oscillate in a manner consistent with NFDS in natural populations (Sinervo and Lively 1996), and it has been empirically demonstrated, by manipulating morph frequencies in the field, that a particular morph has a higher mating success when it is rare relative to its antagonistic morph (Bleay et al. 2007). However, even in this well-studied example, some doubt remains about the importance of NFDS, since not all the variance in morph fitness over time and space is satisfactorily explained by frequency (Bleay et al. 2007). Interestingly, evidence for a mechanism analogous to heterozygote advantage in the maintenance of colour polymorphisms has been found in females of other species of lizards with alternative reproductive strategies (Calsbeek et al. 2009; Vercken et al. 2010), highlighting the potential role for other mechanisms in such cases.

1.4 Effects of predators on prey

Interactions between predators and prey have been the focus of many studies of conspicuous polymorphisms. It has long been thought that prey colouration may reflect an evolutionary response to the foraging strategies and cognitive characteristics of predators. Clarke (1962a) proposed a negative frequency-dependent mechanism, involving differential predation of various prey types, which was able to account for conspicuous polymorphisms. He termed this mechanism apostatic selection. He hypothesised that if a predator consumes disproportionately more of a common prey type because it encounters it more frequently, and overlooks a rare type, then the frequency of the common type will decrease, and the frequency of the rare type will increase. Eventually a point will be reached at which the once rare prey type is the more common of the two, and the predator will start to consume disproportionately more of this type. Intuitively, the long-term consequence of such negatively frequency-dependent behavior by the predator will be the stable coexistence of the two prey types. Clarke's hypothesis was given weight by his studies of two polymorphic snail species of the genus *Cepaea, C. nemoralis and C. hortensis*, in which he provided evidence consistent with frequency-dependent predation of the morphs by song thrushes (*Turdus philomelos*) (Clarke 1962b).

Apostatic selection is generated by a pattern of prey consumption which can be characterized by a sigmoid or “Type III” functional response (Holling 1965). Such a response by predators to changing prey frequency is thought to arise from the presence in the predator of a “search image”, which results in prey “switching”. Switching, in the general sense, refers to the tendency of predators to change food sources as their frequencies vary, focusing on the most abundant prey type available, but switching to an alternative type when it becomes relatively more common (Murdoch 1969). The idea of the search image provides an explanation for switching. It was proposed by Tinbergen (1960) after observing the patterns of insectivorous birds preying on different species of cryptic caterpillars on pine trees. In Tinbergen’s study, the birds showed a frequency-dependent consumption of caterpillars, as well as a delay of two to three days for the incorporation of new species in their diet. Tinbergen hypothethised that the birds needed a certain number of chance encounters with novel prey to be able to form a search image for them. It has been demonstrated that the formation of a search image is a result of selective attention after a sequential exposure to a particular stimulus (Croze 1970; Dawkins 1971; Bond and Riley 1991; Blough 1992; Reid and Shettleworth 1992; Langley 1996; Bond and Kamil 1999; Dukas and Kamil 2001; Bond and Kamil 2002). A predator forming a search image will focus on certain features of a frequently encountered prey type that enable it to detect the prey more efficiently, but this focus will interfere with the detection of other types of prey which lack the appropriate features (Kamil and Bond 2006). When the more common prey type becomes rare, “perceptual switching” is predicted to occur (Bond 2007) as a new search image is formed after a series of consecutive detections of what is now the most abundant prey type. This change in search image is what produces the actual switch in predation levels on different prey types.

Apostatic selection has primarily been studied in the context of colour polymorphisms in invertebrates, where the main agent of selection has been assumed to be predation by birds. The fact that birds are easily trained to perform specific tasks in experimental conditions, and that they prey upon colour polymorphic invertebrates with low mobility (e.g. snails), facilitates the study of patterns that are consistent with apostatic selection. In order to demonstrate that apostatic selection occurs, and is capable of maintaining balanced polymorphisms, it is first necessary to establish that predators that feed on polymorphic prey show perceptual switching. This has been demonstrated in laboratory free-choice experiments such as the one carried out by Bond (1983), in which he presented different types of grain on two kinds of background where they were either cryptic or conspicuous to pigeons. The pigeons showed a preference for the more common grain on the cryptic background. The effect was lost when the grains were conspicuous. The response rate was reduced as the relative proportions of grain types became equal, which Bond explained could indicate a decrease in searching efficiency owing to repeated switching from one grain type to another. Other laboratory free-choice experiments have supported the occurrence of perceptual switching (Cooper 1984; Tucker 1991; Reid and Shettleworth 1992; Cooper and Allen 1994). Further evidence in favour of the formation of a search image has been obtained from studies of operant conditioning, using captive birds and artificial prey, where the sequence of prey encounters has been controlled (Dawkins 1971; Pietrewicz and Kamil 1979). Operant conditioning studies have also demonstrated that if a bird experiences sequential presentations of the same prey type, it will detect that prey type more efficiently over time; in contrast, there will be a reduction in the efficiency of detection when presented with a novel prey type or a sequence of mixed prey types (Blough 1989; Langley 1996; Bond and Kamil 1999). In more realistic conditions, wild birds presented with different artificial prey at varying frequencies in natural surroundings have been shown to attack the more common prey type disproportionately, with this effect being stronger on more complex backgrounds and at low prey densities (Allen 1972; Allen 1976; Cooper 1984). Similar results have been obtained from experiments with natural prey in semi-natural conditions, using fish (Murdoch et al. 1975; Maskell et al. 1977; Jormalainen et al. 1995) and birds (Allen 1988; Tucker 1991).

It has thus been demonstrated, in laboratory conditions, that vertebrate predators will disproportionally attack prey they encounter more frequently, and that prey switching can happen as a result of the formation of a search image. This, however, does not prove that natural polymorphisms are maintained through apostatic selection. Predator-prey dynamics where prey frequencies fluctuate over time as a result of perceptual switching, allowing the coexistence of different types, must be tested. Using a “virtual ecology” approach, Bond and Kamil (1998, 2002) not only showed that apostatic selection happens, but that it can also promote phenotypic diversity. They created a digital moth population modelled on the genus *Catocala* with three discrete morphs in equal numbers and exposed them to predation by blue jays (*Cyanocitta cristata*). After 50 generations, the frequencies of all three morphs reached an oscillatory equilibrium that was independent of their initial numbers, and was maintained by apostatic selection alone. To test if apostatic selection could also promote phenotypic diversity, digital moth phenotypes were specified by genomes that were subject to mutation in each generation, starting with a monomorphic population. Experimental lineages were compared with two control lineages, one that was left to evolve by drift alone, and a second one that was under frequency-independent directional selection for crypsis. In both the experimental line and the frequency-independent control, moths developed a higher level of crypsis. However, only in the frequency-dependent line was an increase in phenotypic diversity observed.

Bond and Kamil's (1998, 2002) experiments provide an elegant demonstration of the potential for apostatic selection to promote polymorphism, but equivalent data from natural populations of prey are lacking. As a result, while apostatic selection is often identified as the most plausible explanation for observed conspicuous polymorphism in invertebrates, we have little direct support for this view. There are only a couple of studies in natural populations that in fact test for apostatic selection, both on the mangrove snail *Littoraria filosa*. Reid (1987) manipulated the morph frequencies of *L. filosa* on individual bushes of *Avicennia eucalyptifolia*, and found that the disappearance of yellow and brown shells was frequency-dependent, each morph being favoured when rare. Reid ruled-out the influence of climatic factors since he found no difference in morph frequencies between sunny and shaded trees, or among seasons. Similar results were obtained in more recent experiments with the same species (McKillup and McKillup 2008), with the disappearance of the different morphs being attributed to predation by crabs. Even though these results show that negative frequency-dependent predation happens in natural populations, they are still not sufficient to conclude that apostatic selection is occurring. There were no observations of the interactions between the prey and the predators, and it is not therefore known if the resulting pattern was a consequence of perceptual switching or a different mechanism. Furthermore, the long-term dynamical consequences of the observed changes in morph frequency in *L. filosa* are not known. Thus, these studies are still a long way from proving that apostatic selection maintains prey polymorphism at equilibrium. Unfortunately, the level of experimental control required makes it very difficult for more definitive experiments to be done with natural populations. Moreover, the expected dynamics of morph frequencies under apostatic selection vary depending on a number of factors, including the size of predator and prey populations, the behavioral characteristics of the predator, the heterogeneity of the habitat, prey density and their spatial distribution (Allen et al. 1998; Merilaita et al. 1999; Weale et al. 2000; Merilaita et al. 2001; Shigemiya 2004; Merilaita 2006; Endler and Rojas 2009).

Studies in several species of snails have shown that apostatic selection might not play a central role in the maintenance of polymorphism. For example, the shell polymorphism in the marine snails of the genus *Littoraria* has been attributed to adaptation to local environmental conditions and spatially varying selection for the ability to thermoregulate (Merkt and Ellison 1998; Phifer-Rixey et al. 2008). Similarly, shell variation in several species of land snails is thought to be the result of climatic selection (Abdel-Rehim 1983; Hazel and Johnson 1990; Slotow and Ward 1997; Cazzaniga et al. 2005; Johnson 2011). Even in the land snails of the genus *Cepaea*, which are perhaps the most extensively studied polymorphic taxa, and which inspired early accounts of apostatic selection (Clarke 1962a), studies have shown that there are frequency-independent mechanisms that are sufficient to explain the polymorphism even in the absence of NFDS. These factors include drift, founder effects and differentiation in refugia leading to area effects, and migration combined with geographically variable selection pressures, such as those associated with climate and predation (Goodhart 1962; Cain and Currey 1963; Goodhart 1963; Carter 1967; Jones et al. 1977; Chang and Emlen 1993; Wilson 1996; Cook 1998; Cook and Pettitt 1998; Cowie and Jones 1998; Davison and Clarke 2000; Cameron 2001; Bellido et al. 2002; Cook 2005). Although apostatic selection has not been directly tested in natural populations of *Cepaea*, the conclusion that frequency-independent forces are more a plausible explanation for the persistence of the observed polymorphism are based on inconsistencies between morph frequency patterns observed in some areas and those expected if apostatic selection was operating (Cain and Currey 1963; Carter 1967; Cook and Pettitt 1998). The current consensus is, therefore, that apostatic selection is of minor importance in this system.

In summary, empirical work on natural populations does not, as yet, support the idea that apostatic selection plays a major role in the maintenance of polymorphisms. Nevertheless, studies that test for apostatic selection in natural conditions are very scarce. Furthermore, our understanding of apostatic selection comes almost exclusively from studies of vertebrate predators, despite the fact that invertebrates, with very different sensory and nervous systems, may be important agents of selection in some systems. Clearly, more detailed field experiments are required. However, theoretical and some empirical studies have shown that the potency of apostatic selection as a driver of diversity is highly sensitive to variation in ecological factors such as prey and predator densities, dispersal, habitat heterogeneity, and the spatial distribution of prey (Greenwood 1969; Cook and Kenyon 1991; Allen et al. 1998; Merilaita et al. 1999; Weale et al. 2000; Shigemiya 2004; Merilaita 2006; Endler and Rojas 2009; Merilaita and Ruxton 2009). The effect of apostatic selection can be weakened or even eliminated if one or more of these factors are manipulated. Consequently, it seems likely that in many systems apostatic selection cannot explain polymorphism on its own.

1.5 Other interactions

1.5.1 Host-Parasite interactions

Interactions between parasites and their hosts can lead to NFDS, and hence have the potential to maintain polymorphisms, although in most examples, these polymorphisms are not apparent to the observer. If some degree of genetic matching is necessary for a parasite to infect a host, then hosts with rare genotypes will suffer fewer infections (Hamilton 1980, 1993). As the fitness of common hosts decreases, so will their frequency, and the frequency of rare hosts will increase. Following the Red Queen model of co-evolution, parasites will evolve to counteract this adaptation, and, after a certain period, parasite genotypes that are best able to infect the hosts that were initially rare will be selected for (Decaestecker et al. 2007). This will generate an advantage for rare genotypes that could potentially maintain variation in a population (Tellier and Brown 2007).

While there is some empirical support for the idea that frequency-dependent host-parasite interactions promote cryptic genetic polymorphisms in invertebrates (Dybdahl and Lively 1998; Lively and Dybdahl 2000; Decaestecker et al. 2007; Duncan and Little 2007; Wolinska and Spaak 2009; King et al. 2011), impacts on conspicuous phenotypes are not well documented (but see Langley et al. 2006). The only example where the effect of parasitism on the maintenance of colour polymorphisms has been studied in natural populations is in the marine snail *Littoraria filosa*, which shows variation in shell colour. It has been observed that the parasitoid sarcophagid fly *Sarcophaga megafilosa* selects for crypsis in populations of *L. filosa* by attacking a higher proportion of snails that do not match their background (McKillup and McKillup 2002). However, when the frequencies of *L. filosa* morphs were manipulated, *S. megafilosa* showed a bias for a particular morph when it was rare (McKillup and McKillup 2008). This pattern would produce positive frequency-dependent selection and thus would more likely lead to the fixation of the common morph than the persistence of the polymorphism. In the absence of any other published examples of this sort, we therefore conclude that , while it is technically possible that frequency-dependent fitness effects of parasites might maintain conspicuous polymorphisms in their hosts, no strong evidence exists that this is the case.

1.5.2 Effects of prey on predators

Studies of predator-prey interactions leading to NFDS have focused almost exclusively on the effect that predators have on prey populations (see above). The possibility of prey affecting the frequencies of morphs in predator populations has received far less consideration (Paulson 1973; Hori 1993; Roulin and Wink 2004; Arcos 2007). If a predator's main prey can discriminate between predator morphs, it might learn to avoid the predator morph that it encounters more frequently by associating it with a potential attack. Predators of the morph that is avoided by prey are expected to catch fewer prey and feed less often, which will affect their fitness and cause their frequency to decrease relative to rare morphs that are not as easily recognised by the prey. Such frequency dependence could maintain a balanced polymorphism in exactly the same way as was originally predicted when predators forage preferentially for common prey morphs.

Evidence for NFDS on predator morphs by prey is scant (Hori 1993), but there is clear potential in some systems. For example, some spiders show conspicuous variation in body colour and pattern (Théry and Casas 2009), and attack prey, such as bees, which are known to be able to discriminate colours (Chittka and Menzel 1992; Chittka 1996; Hempel de Ibarra et al. 2002; Giurfa 2004; Dyer and Neumeyer 2005; Srinivasan 2010; Dyer et al. 2011). Studies have shown that spider colouration affects the behavior of some prey species in such a way that spider fitness is likely to be affected (Hauber 2002; Tso et al. 2004; Heiling et al. 2005; Tso et al. 2006; Ings and Chittka 2008; Herberstein et al. 2009; Llandres et al. 2011). Therefore, spiders are model systems in which the idea that NFDS can be generated by prey and maintain balanced polymorphisms in predators can be tested.

1.6 Polymorphism in spiders

Most studies that have investigated colour variation in spiders have concentrated on species with forms which choose their backgrounds in relation to their colour, and use colouration to appear cryptic or to attract prey (Théry and Casas 2002; Heiling et al. 2003; Heiling et al. 2005; Brechbühl et al. 2010; Defrize et al. 2010). Nevertheless, most of these species are not truly polymorphic, but rather they have the ability to change colouration behaviourally, usually to match their background (Théry and Casas 2002; Heiling et al. 2005). Genetic colour polymorphisms in spiders have been most extensively studied in the candy-stripe spider (*Enoplognatha ovata*) and the Hawaiian happy-face spider (*Theridion grallator*), both species belonging to the family Theridiidae. In the case of *E. ovata,* individuals can show one of three discrete colour morphs: plain yellow abdomen (var. *lineata* Clerck), yellow abdomen with two dorsolateral carmine stripes (var. *redimita* Clerck), and yellow abdomen with the dorsal surface entirely carmine (var. *ovata* Clerck) (Oxford 1983). This species also shows variation in the number of black spots in the dorsolateral surface of the abdomen. Data from observations and manipulations of morph frequencies, as well as a comparison between morphological and allozyme variation, have provided evidence for both genetic drift and selection acting to maintain the polymorphism (Oxford 1985; Oxford and Shaw 1986; Reillo and Wise 1988; Oxford 1989; Oxford and Reillo 1993; Oxford and Gunnarsson 2006). Oxford (2005) proposed a model of intermittent selection which explains how drift and selection can act together to maintain a balanced polymorphism. In this model, there is only weak selection when morph frequencies are within certain limits and, due to small population sizes, drift overrides selection in this neutral area of parameter space. If drift causes morph frequencies to move outside these limits, strong selection will push them back. There is some evidence that seems to be in accordance with this model (Oxford 2005), but its predictions have not been formally tested and the selective force is unknown.

T. grallator is an endemic Hawaiian species that occurs in four islands (Gillespie and Oxford 1998). It shows an exuberant polymorphism consisting of a yellow morph and a number of morphs with different superimposed patterns on the yellow background. The yellow morph is the most common one in the studied populations in all the islands (Gillespie and Tabashnik

1990). A lack of temporal and spatial variation in morph frequencies as well as comparisons between morphological and allozyme differentiation suggest that selection is operating to maintain the polymorphism (Gillespie and Tabashnik 1990; Gillespie and Oxford 1998). An inverse relationship between morph frequency and time spent at a subsite along with evidence for rare-male advantage suggest the occurrence of NFDS (Gillespie and Tabashnik 1990; Gillespie and Oxford 1998). Gillespie and Oxford (1998) propose that NFDS caused by bird predation fits the ecological characteristics of this species. Nevertheless, this prediction is yet to be tested. Other less well studied species of colour polymorphic spiders appear to show a correlation between colour and environmental factors such as substrate and temperature (Gunnarsson 1987; Bonte and Maelfait 2004). Thus, although colour polymorphic spider species represent good models for the study of the maintenance of genetic variation, they have been surprisingly neglected in this regard. Therefore, the main aim of this investigation is to contribute to the existing knowledge of the mechanisms that might be playing a role in the maintenance of conspicuous polymorphisms using *Synema globosum* (Fabricus 1775), a member of the Thomisidae family that shows three discrete colour morphs, as a model system.

1.7 Objectives

The specific objectives of each chapter are the following: Chapter 2 aims to provide a description of the natural history of *S. globosum* based on observations carried out in the field and in the laboratory. In Chapter 3, I investigate the nature of the polymorphism, aiming to determine whether the observed colour morphs are discrete, and if there are any differences among them in life-history components. In Chapter 4, I carried out a breeding experiment with the objective of establishing whether the colour polymorphism in *S. globosum* has a genetic origin. Chapter 5 examines the interactions between *S. globosum* and one of its main prey, the honeybee, in order to determine if directional selection or NFDS influence the maintenance of the polymorphism. Finally, in Chapter 6 I investitage the interactions between females and males aiming to establish if males have a preference for a particular female colour morph and if NFDS generated by mate choice plays a role in the maintenance of the polymorphism.

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Chapter 2: Description of the natural history of S*ynema globosum*

2.1 Introduction

Although *S. globosum* is known to be a relatively abundant species among spiders in some areas of Europe and Asia (Szymkowiak 2008; Pérez-Guerrero et al. 2009; Yilmaz et al. 2009; Benhadi-Marin et al. 2011), very little is known about its biology. It appears to be an excellent model to investigate colour polymorphism, and could also be a good potential model for studies of some aspects of behaviour such as foraging and agonistic behaviour, but before this potential can be realised, it is necessary to have at least a basic knowledge of its natural history. An understanding of the basic natural history of a species is essential if the impacts that interactions with the biotic and abiotic environment have on fitness are to be unravelled.

S. globosum belongs to the family Thomisidae, members of which are commonly known as crab spiders because of the first two pairs of legs that extend laterally and their tendency to move sideways in a similar way to crabs. The Thomisidae family comprises over 2000 species in 174 genera, which are distributed worldwide (Platnick 2012). They possess short and wide bodies with particularly large anterior pairs of legs. Crab spiders are ambush sit-and-wait predators that can be found foraging on flowers, the bark of trees or on the ground (Punzo 2007). They wait until prey are in close proximity and use their large forelimbs to seize them in a rapid movement. Subsequently, they will bite them and inject a highly toxic venom that will paralyse and kill them within a few minutes (Foelix 1996; Schmalhofer and Casey 1999).

Crab spiders show a great variation in colour, ranging from bright white and yellow to duller shades of brown (Foelix 1996). Some species that forage on flowers appear to match the colour of the flower on which they sit remarkably well, such that both predators and prey can be deceived (Théry and Casas 2002). Some flower-dwelling species have the ability to change colour in relation to the background on which they are found (Thery 2007), and other species use colouration to resemble flower signals and attract prey (Heiling et al. 2003; Herberstein et al. 2009). Crab spiders have been observed to be generalist predators preying on a variety of insects and some spiders (Morse 1981, 1983; Novo et al. 2010).

Several crab spider species exhibit extreme sexual dimorphism, with males being considerably smaller than females (Legrand and Morse 2000). They belong to the Entelegynae, which possess complex female genital structures (Foelix 1996). Courtship in crab spiders is of the simplest type, with no elaborated displays or rituals (Foelix 1996). However, males of some species of the genus *Xysticus* will bind the females with threads of silk prior to mating (Bristowe 1958). Agonistic behaviour and mate-guarding by males has also been observed to occur (Dodson and Beck 1993). Reproduction takes place in late spring in temperate regions, and most species overwinter as subadults (Punzo 2007).

S. globosum has been observed to occur in Southern Europe, Asia and Northern Africa (Preston-Mafham 1998). It possesses a black cephalothorax with pale brown colouration in the ocular area, with a white, yellow, or orange/red colouration in the abdomen surrounding a characteristic black marking (Jones 1983). It has been found to dwell on flower heads, tall vegetation, and bushes (Roberts 1995). In my Portuguese field site, adults conspicuously occupy flowers of many different species and can be seen attacking flower-visiting invertebrates. This predatory behaviour has been shown to have different effects on the foraging behaviour of solitary bees (*Eucera notata*) and honeybees (*Apis mellifera*) Reader et al. (2006). Honeybees were found to be less likely to visit flowers harbouring individuals of four different species of crab spiders, of which *S. globosum* was the most common, while no such effect was observed in *E. notata*. These results demonstrate that the presence of *S. globosum* on flowers can have a significant impact on the foraging behaviour of pollinators and that it can vary among species. Such impact can have important implications on the fitness of spiders, pollinators, and plants. Since *S. globosum* was the most abundant species of crab spider used in the study by Reader et al. 2006, it is likely to have a greater effect on honeybee foraging behaviour than the less common species of crab spiders found in the study area.

S. globosum has been found to exhibit sexual size dimorphism to some extent, and the larger females show a lower tendency than the smaller males to disperse using bridges of silk (Corcobado et al. 2010). Almost nothing else has been published about *S. globosum*'s behaviour or ecology, but one study has considered the striking colour polymorphism that is displayed by adult females. Théry and Casas (2009) showed that the observed colours of the three morphs (red, yellow and white) are produced by different types of pigment granules in the epithelial cells of the abdomen. Unpublished data collected by the undergraduate students of the University of Nottingham at my study area showed that males of *S. globosum* exhibit agonistic behaviour towards each other, and that spiders might have certain preference for particular types of flowers. Although these findings come from studies with relatively small sample sizes, they provide some insight into some basic aspects of *S. globosum's* behaviour. These studies remain the only ones to have considered the biology of the species in any detail and almost nothing is known about its habitat preferences, its reproductive biology, or the morphological differences among individuals of different sexes and ages.

In contrast to *S. globosum*, a large amount of information exists on the biology of the crab spider *Misumena vatia. M. vatia* has similar foraging habits to *S. globosum* as it also occurs on flowers and captures similar types of prey. Its natural history has been described in detail (Morse 2007), and it has been used as a model system to address important questions on foraging theory such as how animals make decisions on where to forage and what to consume, and what are the fitness advantages of these decisions as well as the factors that constrain them. *M. vatia* is a generalist predator that forages primarily on three species of flowering plants in the study area where it has been mostly studied: common milkweed (*Asclepias syriaca*), goldenrod (*Solidago juncea*), and pasture rose (*Rosa carolina*) (Morse 1981). It has been observed to feed on a variety of prey (Morse 1981, 1983), but it consumes bumblebees (*Bombus* spp.) and honeybees (*A. mellifera*) more frequently than other prey. Predation by *M. vatia* appears to have a significant impact on the fitness of honeybees and some species of bumblebees as these have been observed to respond negatively to the presence of the spiders by avoiding patches of flowers where spiders occur (Dukas and Morse 2003, 2005). Although *M. vatia* represents a good model to investigate several aspects of behaviour and ecology because it is a species that is easy to observe in the wild, it can only be found at very low densities (Morse 2007). Therefore, its convenience as a model system and its ecological importance are both limited in comparison with *S. globosum*, which is a more abundant species that can be used to address similar questions.

2.1.1 Objectives

The aim of this chapter is to provide a description of the natural history of *S. globosum.* First, I carried out a survey to determine the constitution of the spider community at my field site, and the relative abundance of *S. globosum* in the different habitats where it is found. A second survey was carried out to determine the frequency of *S. globosum* on different species of flowers where it commonly occurs. The objective of this survey was to investigate whether *S. globosum* is generalist in its habitat preferences or if it specialises in hunting on particular flower species, as is the case of *M. vatia*. Laboratory and field observations were carried out to describe its morphology, development, and mating and reproductive behaviour. Finally, a third survey was carried out to establish the extent to which adults of *S. globosum* are faithful to particular flowers or patches of habitat.

2.2 Materials and methods

2.2.1 Study area

All surveys and observations were carried out at the Quinta de São Pedro Study Centre and surrounding areas located in the municipality of Almada in the South of Lisbon, Portugal (38°38’19’’ N, 9°11’50’’ W; Figure 2.1). The Quinta de São Pedro Study Centre consists of a total area of 4 ha that comprises a set of buildings that provide accommodation and study facilities, and open areas of meadow, shrubland, and woodland. The vegetation consists mainly of a variety of annual and perennial flowering plants in the meadow, areas of garrigue, pine and eucalyptus woodland, as well as areas of native macchia and introduced acacia woodland. Patches of ornamental flowering plants can also be found. Areas surrounding the Quinta de São Pedro Study Centre, where observations were also carried out, consisted of open meadow, shrubland and woodland with a similar type of vegetation as the study centre. All areas are found at an altitude of approximately 50 m above sea level with a maximum average temperature of 33°C, a minimum average temperature of 13°C, and an annual precipitation of 715.9 mm (Anonymous, 2008).

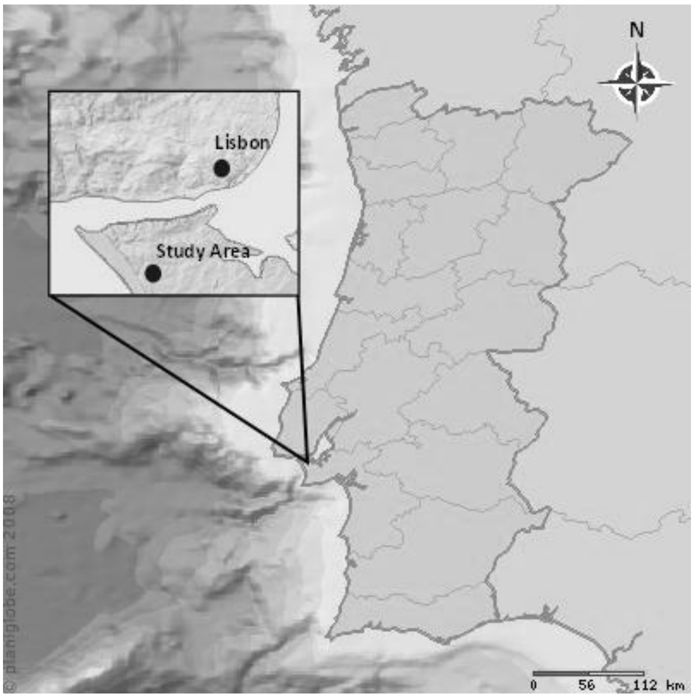

Figure 2.1. Geographic location of the study area within Portugal. The open rectangle shows its location relative to Lisbon (Anonymous, 2012).

2.2.2 Spider community survey (Survey 1)

To investigate the composition of the spider community in the study area, a systematic search of spiders was carried out in January and February 2009 over a period of 17 days. Five different types of vegetation were selected to survey: sage-leaf rockrose (*Cistus salvifolius* L.), gum cistus (*Cistus ladanifer* L.), french lavender (*Lavandula stoechas* L.), thicket (comprised of various species of shrubs and trees), and meadow (composed mainly of grasses). Mature or nearly mature *S. globosum* have commonly been found on flowers of *C. salvifolius, C. ladanifer* and *L. stoechas* at the field site from March to May. However, the time of the year when the survey was carried out was outside of the flowering season for these and most other plants at the field site, when only subadult *S. globosum* were seen, and their habitat preferences were unknown.

Two different sites were surveyed: the Quinta de Sao Pedro Study Centre (Site 1) and a large open area of meadow, scrubland and woodland located opposite to the study centre (Site 2) (Figure 2.2). For all vegetation types, except the meadow, an area of bushes and trees was randomly selected, delimited, every bush within that area was numbered, and bushes were randomly selected for sampling. In the case of the meadow, a plot of 10 m x 10 m was laid out with 10 m transects every 1 m traced as a grid. Each transect within the plot was numbered, and transects were randomly selected for sampling.

Figure 2.2. Location of the sites where the spider community survey was carried out (1 and 2), and where the survey of *S. globosum* on different flower species was carried out (1, 2, and 3). Hybrid satellite image and map taken from Google Earth.

Four different sampling methods were used to collect the spiders: sweeping, beating, cryptic search, and pitfall traps. Each bush, tree and transect was sampled for a maximum of 15 minutes (sweeping, beating and searching) or with one, two, or three pitfall traps per bush, tree or transect. For the sweeping method, a sweep net was swept from side to side through the bushes, trees, and grass. The beating method consisted of beating the branches of bushes and trees with a stick and placing a cloth beating tray underneath to collect the material that fell off the plants. This method was not used in the meadow as there were no plants with branches to beat. For the cryptic method, a systematic search by hand was carried out through bushes, trees, and the ground around them. In the case of the meadow, a hand search of the ground was made every two metres along the transects. The pitfall traps consisted of plastic cups (7 cm in diameter) with a few drops of dish soap diluted in a small amount of water to prevent spiders from climbing out. One, two or three plastic cups were buried in the ground around a bush, tree or every 3 m along a transect in the meadow. The plastic cup was buried such that the rim was level with the soil surface. The number of pitfall traps for each sample depended on the size of the bush or tree, and on the time that it took to place them. The pitfall traps were left for 48 hours, after which they were collected and spiders were removed.

Six repetitions were made for each sampling method in each type of vegetation in both sites. Sampling methods and types of vegetation were selected in a random sequence. Visits to Sites 1 and 2 were made in the morning or afternoon, with the order being alternated each day to reduce any effect of time. Collected spiders were brought to the laboratory and separated into morphospecies. Due to time constraints, only some morphospecies could be identified to family level. However, the morphospecies classification approach has been shown to be effective for difficult to identify and species-rich taxa such as spiders and other invertebrates (Oliver and Beattie 1996; Krell 2004). Specimens were preserved in Perspex tubes with 70 % alcohol.

2.2.3 Occurrence of *S. globosum* on different flower species (Survey 2)

To determine the frequency of adult or nearly adult *S. globosum* on the different species of flowers where they are commonly found, a survey was carried out in March and April of 2009 over a period of 22 days. Eight different species of flowers where *S. globosum* had previously been observed to occur were selected to survey: purple viper's bugloss (*Echium plantagineum* L.*)*, cornflower marigold (*Chrysanthemum segetum* L.*),* marguerite daisy *(Euryops abrotanifolius* L.*),* calla lily *(Zantedeschica aethiopica* L.*)*, French lavender (*L. stoechas)*, sage-leaved cistus (*C. salvifolius),* gum cistus *(C. ladanifer),* and yellow lupin *(Lupinus luteus* L.*). L. stoechas* produces flowers in the form of inflorescences, the flowers are considerably smaller than spiders, and an inflorescence never harbours more than one spider (except when a male is looking to mate with a female). Therefore, each inflorescence of *L. stoechas* was treated as an individual flower. The survey was carried out at three different sites: Sites 1 and 2 (as above), and a second open field about 300 m South-East from the study centre with similar vegetation (Site 3) (Figure 2.2).

Locations were selected where target flower species were abundant and several species occurred together. However, not all species occurred in all sites, thus not all species were surveyed in each site. Due to its large size and the quantity of target flower species present, the meadow in Site 1 was surveyed using 4 x 4 m plots with transects every 1 m traced as a grid. Flower species or individual plots, locations, and groups of bushes or individual plots were selected randomly. Visits to Sites 1 and 2 alternated between morning and afternoon as above. Due to Site 3 having a considerably smaller area than Sites 1 and 2, the survey was carried out in one day. All flowers in a group of bushes or a plot were searched systematically by hand. Flowers with and without spiders were counted, and flowers where a spider was found were marked with sticky tape and the spider was brought to the laboratory. Spiders were weighed with a digital balance and the length of the cephalothorax from front to back was measured under a stereo microscope using an eye-piece graticule. The spiders were then returned to the flower where they were found.

Data on the sex ratios observed in 2010 in Sites 1 and 2, and in 2011 in all three sites, were obtained from additional less intensive surveys carried out to determine the frequencies of the three colour morphs in different locations and years (see section 3.2.3.).

<u>2.2.4 Observations of reproductive biology, development, and behaviour</u>

To investigate the mating behaviour of *S. globosum*, laboratory observations were made during mating experiments that were carried out to investigate the role of mate choice in the maintenance of the polymorphism (see Chapter 6). Males were introduced in petri dishes containing either one or two females and were observed for 10-15 minutes or until a mating occurred (see Section 6.2). Observations on the reproductive biology and development of *S. globosum* were obtained from females that mated in the mating experiments, and females collected from the field that looked gravid and were considered to be likely to produce eggs in the following days. These females were measured prior to laying eggs following the same methodology as in the previous section. The females produced egg sacs at the Quinta de São Pedro Study Centre and were subsequently transported to the United Kingdom. Females were fed twice a week. Their offspring were reared in the laboratory at the University of Nottingham to determine the mode of inheritance of the polymorphism (see Section 4.2). Second instar spiderlings from the second clutches produced by these females were measured (from the anterior end of the cephalothorax to the posterior end of the abdomen) and weighed in the same way as adult spiders.

To determine the extent to which adult spiders are faithful to particular flowers and habitat patches over a period of several days, observations were made on all spiders found in two 10 m x 10 m plots built in the meadow and on two bushes of *L. stoechas* in Site 1 (Survey 3). Spiders collected in the plots and in *L. stoechas* were marked with three dots of enamel paint on the cephalothorax behind the eyes (each spider had a unique combination of colours), and they were weighed and measured. Afterwards, they were released on the flower where they were found. The plots and bushes were checked two to three times a day over a period of 11 days. If a new spider was found, it was marked, weighed, measured and released on the flower where it was found.

Descriptions of agonistic behaviour between males and kleptoparasitism of females by males were made from casual observations during the process of collecting the data mentioned above.

2.2.5 Statistical analyses

To estimate the diversity of spider species in the study sites, the Simpson's Index of Diversity and the Shannon-Wiener Index were calculated (Magurran 2004). Generalised linear models (GLMs) with the appropriate error structures (Gaussian, Quasipoisson, or Binomial) were used to establish whether site, type of vegetation, sampling method or an interaction between any of them had an effect on species richness (number of species) or on abundance (number of individuals) in Survey 1; to test the effect of mother cephalothorax length on sum of size of all offspring per clutch and total clutch mass; and to test the effect of sex (female or male) on the proportion of spiders found at the beginning that were missing at the end of Survey 3, and of spiders that were found during the survey that were not found at the start. Generalised linear mixed models (GLMMs) with the appropriate error structures (Gaussian, Poisson, or Binomial) and mother of clutch as a random effect, were used to examine the effect of mother size on different components of reproduction, as well as the effect of some components of reproduction on other components of reproduction (specifications of each analysis are provided with the results). Wilcoxon rank-sum tests were used to investigate differences between females and males in size, mass, the time they spent in a habitat patch and on a given flower, and in the number of flowers they occupied. Chi-squared tests were conducted to test for differences in the frequencies of individuals of *S. globosum* in different types of vegetation, with different sampling methods, on different species of flowers, and to test for differences in sex ratio in different types of flowers and whether the sex ratio differed from 0.5. For GLMs and GLMMs, the significance of each term was assessed by backward deletion from a saturated model using the appropriate test statistic (Likelihood ratio, F-ratio, or Chi-squared) following Crawley (2007) and Zuur et al. (2009). Statistics are presented for comparisons of models after each term was removed. All analyses were conducted in R 2.12.2 (The R Development Core Team 2011).

2.3 Results

2.3.1 Spider community survey (Survey 1)

A total of 684 spiders were collected, which were separated into 151 morphospecies. Ninety-nine of these morphospecies could be identified to family level, and a total of eighteen families were identified. Spider diversity in the study area was high (Simpson's Index of Diversity: D = 0.972, Shannon-Wiener Index: H = 4.198; see Figure 2.3). From the species that could be identified to family, the most species rich families were the orb-weaving spiders (Araneidae), with 27 different morphospecies, the comb-footed spiders (Theridiidae) with 13 morphospecies, and the crab spiders (Thomisidae) with 10 different morphospecies.

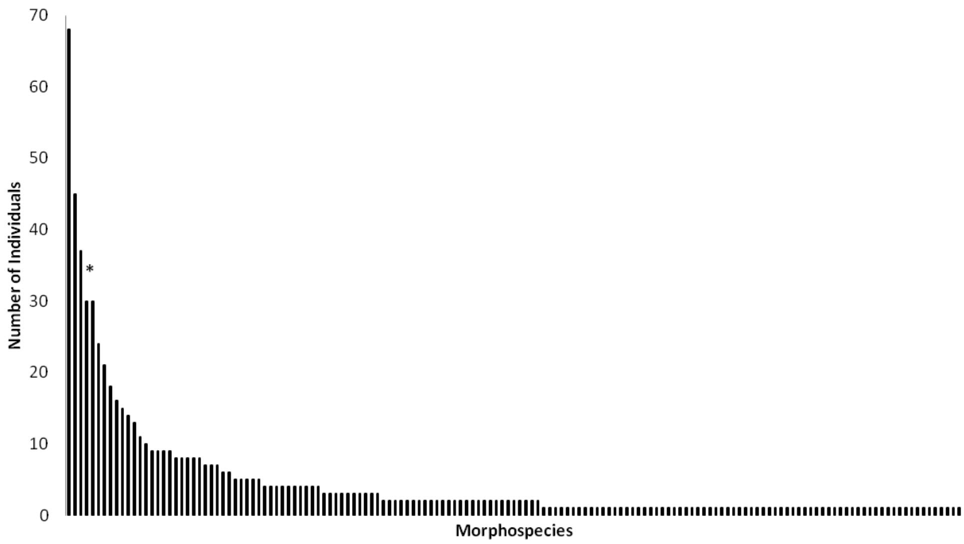

Figure 2.3. Total number of individuals collected from each morphospecies across all samples at Sites 1 and 2, in rank order from the most abundant (left) to the least abundant (right). *S. globosum* is indicated with a star.

The most abundant morphospecies, with 68 individuals collected in total, belongs to an unidentified family. It was most commonly found in *C. salvifolius* and in the thicket with the cryptic and pitfall trap sampling methods. Individuals collected of this morphospecies were mature spiders, with brown legs, a dark brown cephalothrax with a light brown line along the middle, and a speckled light and dark brown abdomen. They were small spiders of approximately 4 mm in length. The second most abundant morphospecies, with 45 individuals collected, belongs to the family of the running crab spiders (Philodromidae). All individuals of this morphospecies were immature and they were most commonly found in *C. salvifolius* with the beating and cryptic methods. These were small in size (approximately 4 mm in length), with brown legs, a light brown cephalothorax with dark brown lines along the sides, and a light brown abdomen with dark brown and grey markings. The third most abundant morphospecies, with 37 individuals collected, was a member of the Theridiidae. It was most commonly found in *L. stoechas* with the cryptic method. Individuals of this morphospecies were very small (approximately 2 mm in length) with a brown cephalothorax and legs with dark brown spots, and a white abdomen with dark brown lines along the middle and the sides. They built small webs inside the bracts of inflorescences of *L. stoechas*. *S. globosum* was the fourth most abundant morphospecies, with 30 individuals collected across all samples (Figure 2.3).

There were significant main effects of sampling method and type of vegetation on the number of spider morphospecies that were collected, whereas there was no main effect of site (Table 2.1). The interactions between site and type of vegetation, site and sampling method, and among site, type of vegetation and sampling method, were also significant (Table 2.1). Overall, *C. salvifolius* was the most species-rich vegetation type, and more morphospecies were collected with the cryptic search method (Figure 2.4a and b). At Site 1, the vegetation type where the highest number of morphospecies was collected was *C. salvifolius*, and the most efficient sampling methods in this vegetation type were cryptic search and sweeping (Figure 2.4a). In *C. ladanifer* and in *L. stoechas* at this site, fewer morphospecies were collected with the sweeping method, and the most efficient sampling methods were beating, cryptic search and pitfall traps for *C. ladanifer* and cryptic search for *L. stoechas* (Figure 2.4a). No substantial differences in the number of morphospecies were evident among the sampling methods for Meadow and Thicket at Site 1. At Site 2, more morphospecies were collected in *C. salvifolius* and in the Meadow than in the other three vegetation types (Figure 2.4b). In *C. salvifolius* the most efficient sampling method was cryptic search, in the Thicket the most efficient methods were cryptic search and pitfall traps, and in the other three types of vegetation a similar number of species was collected with all sampling methods (Figure 2.4b).

There were no significant main effects of site, vegetation or sampling method on the total abundance of spiders (i.e. number of individuals collected per sample; Table 2.1), but there was a significant interaction between site and vegetation type. A higher number of individuals were found in the Thicket in Site 1 than in Site 2 (Figure 2.4c and d).

Significantly more individuals of *S. globosum* were found in *C. ladanifer* and *L. stoechas* than other vegetation types (Chi-squared: $X^2 = 60.727$, df = 4, $P < 0.001$), and a significantly higher number of individuals were collected with the beating and the cryptic search methods ($X^2 = 26.310$, df = 3, $P < 0.001$; Figure 2.5). No individuals of *S. globosum* were found in the meadow, and none were collected with pitfall traps.

Table 2.1. Results of general linear models (GLMs) with quasipoisson error structures testing the effects of site, sampling method, type of vegetation and their interactions on the number of spider morphospecies (richness) and number of individuals (abundance) collected.

	Species richness			Abundance		
	X^2	df	*P*	X^2	df	*P*
Site	2.337	1	0.206	2.495	1	0.399
Method	70.609	3	**< 0.001**	1.219	3	0.945
Vegetation	64.897	4	**< 0.001**	25.703	4	0.118
Site x Method	13.009	3	**0.013**	0.315	3	0.992
Site x Vegetation	32.43	4	**< 0.001**	76.645	4	**0.001**
Method x Vegetation	21.612	11	0.073	11.118	11	0.987
Site x Method x Vegetation	23.405	11	**0.031**	13.67	11	0.975

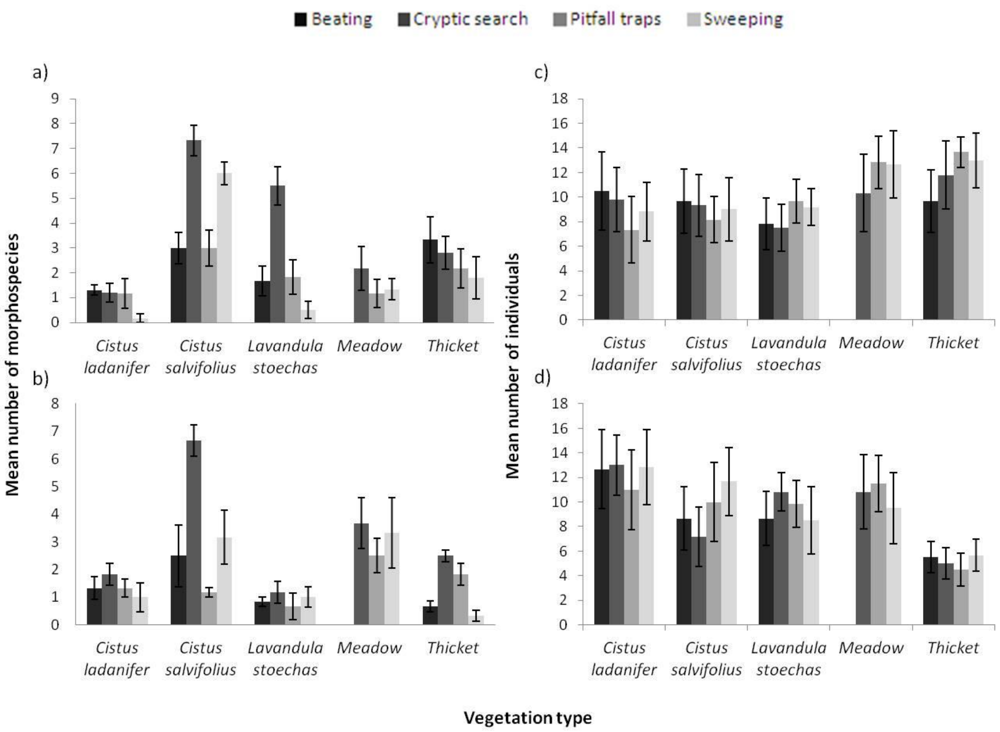

Figure 2.4. Mean number of morphospecies (± S.E.) collected per sample at a) Site 1 and b) Site 2, and mean number of individuals (± S.E.) collected per sample at c) Site 1 and d) Site 2 in five different vegetation types using four different sampling methods.

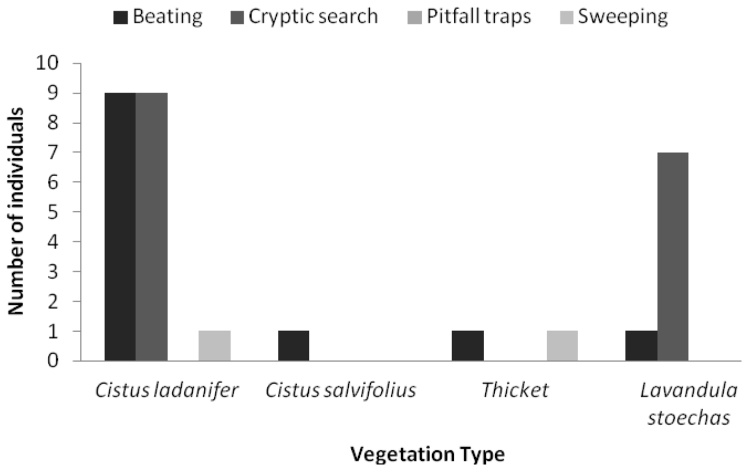

Figure 2.5. Total number of individuals of *S. globosum* found in bushes of *C. ladanifer*, *C. salvifolius*, and *L. stoechas*, and in thickets, at Sites 1 and 2 (combined) using four different sampling techniques. Meadows are not included in the figure because no individuals of *S. globosum* were found in this vegetation type.

2.3.2 Occurrence of *S. globosum* on different flower species (Survey 2)

In the second survey, which was designed to investigate the abundance of *S. globosum* on different types of flowers, there was a significant effect of flower species on the probability of finding an individual of *S. globosum* on a flower at Site 1 (Chi-squared: $X^2 = 88.321$, df = 6, $P < 0.001$), at Site 2 ($X^2 = 253.849$, df = 3, $P < 0.001$), and at Site 3 ($X^2 = 19.150$, df = 2, $P < 0.001$). At Site 1, the flower species with the highest probability of finding an individual of *S. globosum* was *Z. aethiopica*, followed by *C. segetum* (Figure 2.6a). No spiders were found on *L. luteus* at this site, and they were also scarce on *C. salvifolius* and *E. abrotanifolius*. Spiders were similarly scarce on *C. salvifolius* at Site 2, but the flower species with the highest probability of finding an individual of

S. globosum at this site was *L. luteus* (Figure 2.6b). Finally, at Site 3 spiders were most common on *L. stoechas*, and less common on *C. salvifolius* and *E. plantagineum* (Figure 2.6c).

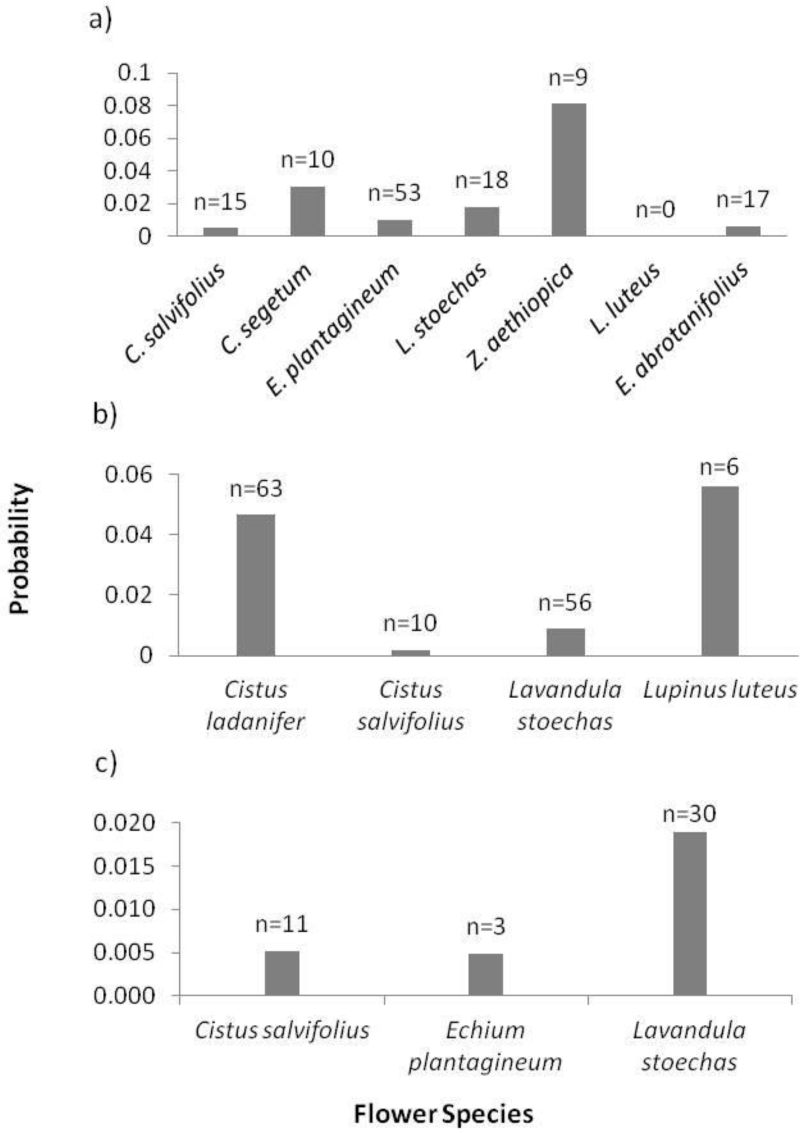

Figure 2.6. Probability of finding an individual of *S. globosum* on different flower species at a) Site 1, b) Site 2, and c) Site 3. The total number of flowers with spiders for each flower species is indicated above each bar.

The sex ratio observed in *S. globosum* across all sites in Survey 2 was 0.306, which is significantly female-biased (Chi-squared test: $X^2 = 20.803$, df = 1, $P < 0.001$). Flower species did not have an effect on the sex ratio at any of the surveyed sites (Site 1: $X^2 = 5.512$, df = 5, $P = 0.357$; Site 2: $X^2 = 4.339$, df = 3, $P = 0.227$; Site 3: $X^2 = 5.968$, df = 2, $P = 0.051$).

2.3.3 Observations on the natural history of *S. globosum*

Morphology

S. globosum possesses a black cephalothorax with yellowish brown colouration in the frontal part encompassing the ocular area. In the females, the dorsal and lateral sides of the abdomen have either red, yellow or white colouration surrounding a characteristic black pattern (Figure 2.7a, b, and c). Males have a much larger abdominal black pattern and smaller coloured areas on the lateral sides of the abdomen which are always white (Figure 2.7d). The first and second pairs of legs are black with yellowish brown rings at the beginning of each of the last three segments of the leg (tibia, metatarsus, and tarsus) in both males and females. The second and third pairs of legs in females have a much ligther colouration, lacking a large amount of pigment making them appear almost translucent. In males, these pairs of legs have a larger area of black colouration. There was no significant difference in the cephalothorax length of females and males (Wilcoxon rank-sum test: W = 3834.5, P = 0.063; Figure 2.8a). However, females were significantly heavier than males (W = 0.946, P = 0.002; Figure 2.8b).

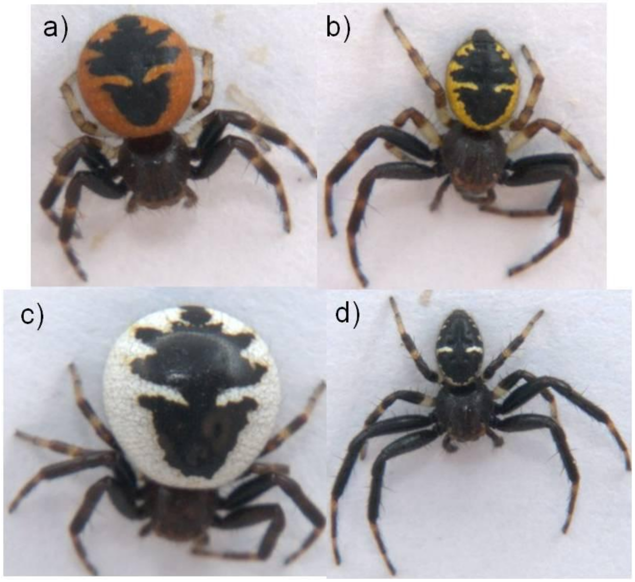

Figure 2.7. a) Red female, b) yellow female, c) white female, and d) male *S. globosum.* All are adult spiders.

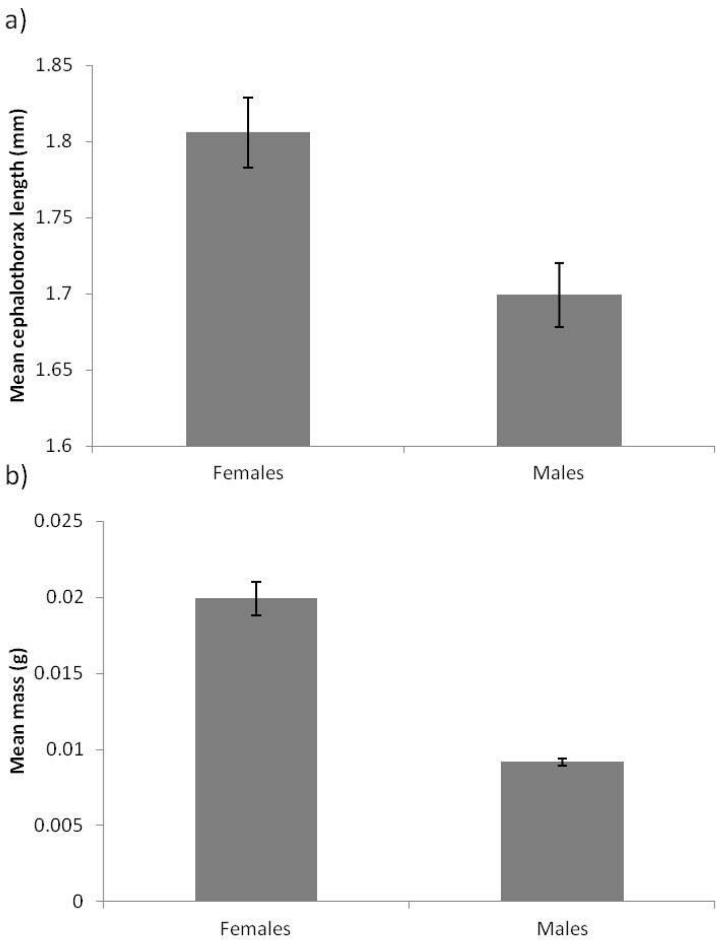

Figure 2.8. a) Mean cephalothorax length (± S.E.) and b) mean mass (± S.E.) of females (n = 227) and males (n = 126) collected from the field.

Newly emerged spiderlings have translucent light brown cephalothoraxes and legs, and dark grey abdomens (Figure 2.9a). Second instar spiderlings have a light brown cephalothorax, light brown legs with a darker colouration towards the distal segments, and a lighter brown or beige abdomen with dark brown markings (Figure 2.9b). Second instar spiderlings from second clutches measured on average 3.906 mm (± 0.019) and had an average wet mass of 0.0006 g (± 5.790e-06). Subsequent instars developed the black colouration in the cephalothorax and legs, and the black marking in the abdomen surrounded by cream or beige colouration (Figure 2.9c). The cream or beige colouration in the abdomen eventually developed to red, yellow or white in females, or just white in males, with the black area becoming larger. Red colouration developed earlier than yellow and white colouration in laboratory conditions. In the laboratory, spiders took approximately fourteen months to reach maturity. Nevertheless, since immature individuals were only seen very rarely during the reproductive season in the field, the natural life cycle of *S. globosum* is most likely annual. This supposition is further supported by the fact that only immature individuals were seen during a brief survey carried out in October 2008 and during the spider community survey carried out in January and February 2009.

Figure 2.9. a) Newly hatched, b) second instar, and c) intermediate instar juvenile *S. globosum*.

Reproduction

Pooling data across all surveys carried out in all years in all sites, the sex ratio of *S. globosum* was found to be significantly female-biased (0.233; Chi-squared: $X^2 = 99.993$, df = 1, $P < 0.001$). It was not possible to obtain the operational or primary sex ratio for the study population because of difficulty in distinguishing mature from immature females in the field, and because of deaths of juvenile spiders reared in the laboratory before they reached a developmental stage at which it was possible to determine their sex (but see Section 4.3).

Females and males could be seen in pairs from the end of March to mid May. When an exuvia was found on a flower where a female and a male had been seen together, the male was no longer on the flower while the female remained. Since crab spiders belong to the Entelegynae (see Section 2.1), and most likely exhibit first-male sperm priority (Christenson & Cohn, 1988; Watson, 1991; Bukowski & Christenson, 1997), these observations suggest that a male guards a female that is about to undergo her final moult to ensure that he is the first one to mate with her. On several occasions two males were seen with one female on the same flower, and in all such cases males were engaging in agonistic behaviour. On the 11 days that marked spiders were observed, some females were found with two or three different males on different occasions. Overall, these observations suggest that at least some of the time males compete for access to reproductively receptive females.

During staged matings in petri dishes in the laboratory, once a male entered a petri dish with a female (which was introduced at least two hours beforehand), the male usually started to follow the silk lines laid by the female until he found her. The male would then either try to seize the female with his forelimbs or walk away from her. If a male tried to seize a female, the female would either raise her anterior legs and attack him by tossing them aggressively towards him or contract her legs and allow the male to seize her. If the female attacked the male, the male would either keep trying to seize her, or run away. If the male was successful in seizing the female, he began to cover her with a few strands of silk. He then positioned himself with the cephalothorax under the female's abdomen in order to insert the palps in her genital opening while vibrating the abdomen rapidly (Figure 2.10). Copulation lasted, on average, 29.58 ± 2.66 minutes (n = 13), and successful matings lasted on average 31.14 ± 2.69 minutes, from the entry of males to the dish to the end of copulation. Only a few mating events were observed in the field. They took place on the flower occupied by the female, and all of them exhibited the same patterns as matings in the laboratory. Usually if the female was not receptive and the male insisted on trying to mate, she would either attack the male or leave the flower. If the female attacked the male and he insisted on mating, she would leave the flower. Sometimes the male would leave the flower after an attack from the female. Sexual cannibalism was observed only once in the laboratory, but it has also been observed in the field (Tom Reader, personal communication).

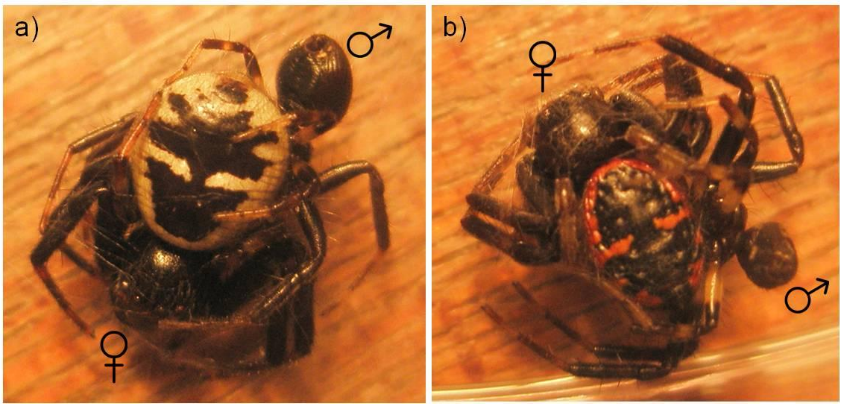

Figure 2.10. Male *S. globosum* copulating with a) a yellow and b) a red female inside a petri dish in the laboratory.

Females were first seen with egg sacs in the field in mid May. However, females captured in early spring started laying eggs in the laboratory in late April. Thus, it is possible that females started producing eggs earlier than mid May in the field but were not found. Females produced a single white egg sac of an irregular dish shape covered with soft layers of silk mesh (Figure 2.11). Females attached the egg sac to the flower where they were last seen foraging. Females and egg sacs remained inside completely closed shelters made by females attaching the petals of a flower together with silk. Some females with egg sacs were found inside senescing flowers (Figure 2.12).

Figure 2.11. Yellow female *S. globosum* with egg sac inside a petri dish.

Figure 2.12. Female *S. globosum* with egg sac inside senescing flowers of a) *C. coronarium* and b) *L. stoechas*.

In laboratory conditions, some females were able to produce up to three clutches. Females (n = 33) produced a mean of 1.972 (± 0.123) clutches. Almost half of the females in the laboratory (47.2 %) produced a second clutch, while only a quarter (25 %) produced a third clutch. Females with spiderlings were first seen in early June in the field and mothers stayed in close proximity for a few days. Females with egg sacs were observed to feed occasionally both in the field and in the laboratory. There was a significant effect of number of clutch on time to hatching (Table 2.2). Offspring from third clutches took significantly more time to hatch than offspring from first and second clutches (Figure 2.13). However, no significant effects of maternal cephalothorax length or its interaction with number of clutch were found on time to hatching (Table 2.2.) There were no main effects of maternal cephalothorax length or number of clutch on clutch size, and the interaction between these main effects was also not significant (Table 2.2.).

Table 2.2. Results of GLMMs with mother of clutch as a random effect testing the effects of maternal cephalothorax length (CL), number of clutch and the interaction between them on time to hatching (Poisson error structure) and clutch size (Gaussian error structure).

	Time to hatching			Clutch size		
	X^2	df	P	LR	df	P
Maternal CL	2.149	1	0.143	0.017	5	0.896
Number of clutch	57.447	2	**< 0.001**	5.562	3	0.062
Maternal CL x number of clutch	0.749	2	0.687	0.007	6	0.997

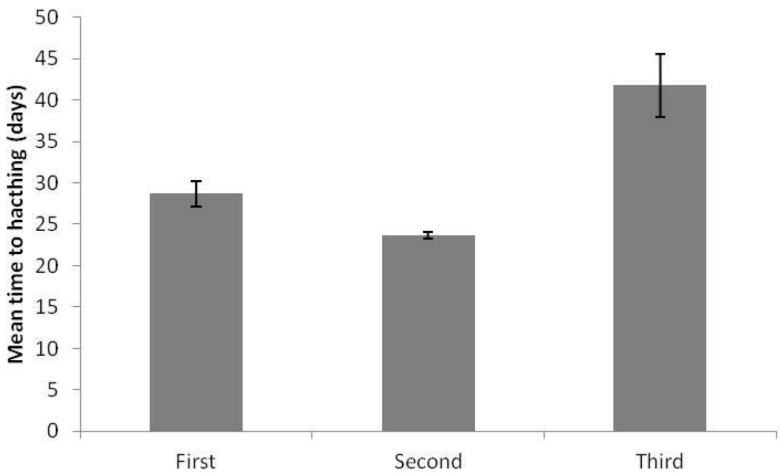

Figure 2.13. Mean number of days (± S.E.) from egg production to hatching of offspring from first (n = 18), second (n = 18) and third (n = 8) clutches produced by females of *S. globosum* in the laboratory.

A significant negative correlation was found between clutch size and offspring size ($t = -2.125$, $df = 20$, $P = 0.046$). Larger clutches produced smaller offspring than smaller clutches (Figure 2.14). There was no significant effect of mother cephalothorax length on individual offspring size, individual offspring mass, sum of size of all offspring per clutch, total clutch mass, or the probability that a spiderling would survive until the end of the breeding experiment (Tables 2.3 and 2.4). However, there was a marginally significant effect of clutch size and a significant effect of number of clutch on the probability of survival of offspring (Table 2.4). Across clutches, offspring that hatched from larger clutches had a lower probability of survival than offspring that hatched from smaller clutches, and offspring from third clutches had a lower probability of survival than offspring from first and second clutches (Figure 2.15). The interaction among mother cephalothorax length, clutch size, and number of clutch also had a significant effect on the probability of survival of offspring (Table 2.4). In first clutches, offspring that hatched from smaller clutches produced by larger mothers had a higher probability of survival (Figure 2.15a). In second clutches, offspring from both large and small clutches produced by larger mothers had a lower probability of survival (Figure 2.15b). Finally, in third clutches, offspring hatched from larger clutches produced by larger mothers had a higher probability of survival (Figure 2.15c). Significant effects of individual offspring size (GLMM with mother of clutch as a random effect and a binomial error structure: $X^2 = 10.777$, $df = 1$, $P = 0.001$) and of individual offspring mass ($X^2 = 13.384$, $df = 1$, $P < 0.001$) were also found on the probability of survival of offspring: larger and heavier offspring had a higher probability of survival than smaller lighter offspring (Figure 2.16).

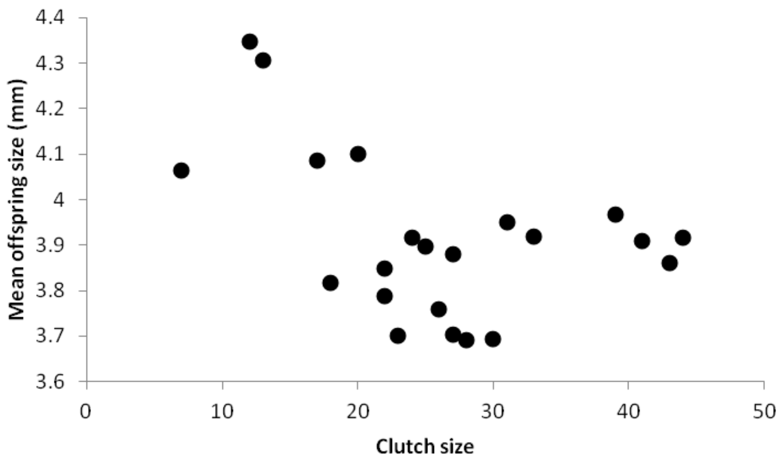

Figure 2.14. Relationship between clutch size and mean offspring size (n = 22) of second clutches laid by females in the laboratory.

Table 2.3. Results of a GLMM with Gaussian error structure and mother of clutch as a random effect testing the effect of mother cephalothorax length (CL) on individual offspring size and individual offspring mass; and results of GLM with Gaussian error structure testing the effect of mother cephalothorax length (CL) on sum of the sizes of all offspring per clutch and total clutch mass.

	Mother CL		
		GLMM	
	LR	df	P
Individual offspring size	0.001	2	0.975
Individual offspring mass	0.236	2	0.627
		GLM	
	X^2	df	P
Sum of offspring size	-8.793	1	0.947
Total clutch mass	< 0.001	1	0.907

Table 2.4. Results of GLMM with mother of clutch as a random effect and a binomial error structure testing the effect of mother cephalothorax length (CL), clutch size, number of clutch, and the interactions among them on the probability that a spiderling would survive until the end of the breeding experiment.

	Probability of survival		
	X^2	df	P
Mother CL	1.232	1	0.267
Clutch size	3.844	1	**0.050**
Number of clutch	653.99	2	**< 0.001**
Mother CL x clutch size	0.826	1	0.364
Mother CL x number of clutch	0.018	2	0.991
Clutch size x number of clutch	5.327	2	0.070
Mother CL x clutch size x number of clutch	19.589	2	**< 0.001**

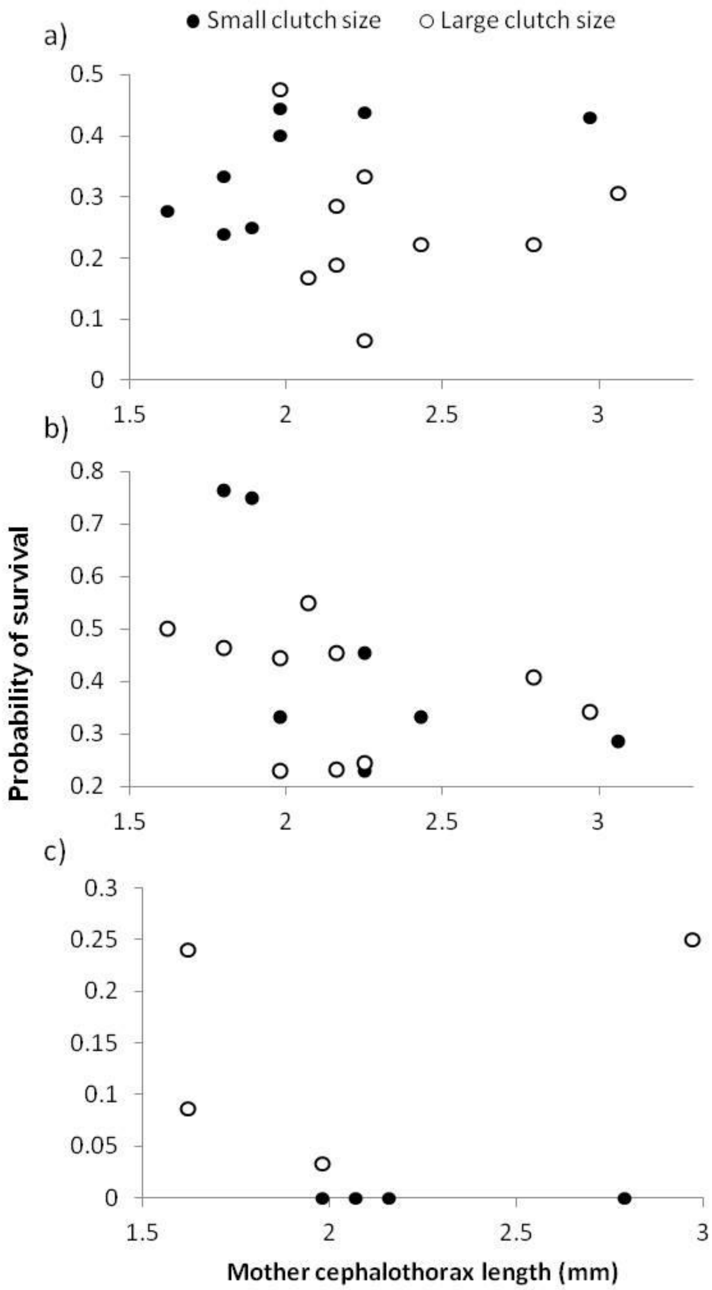

Figure 2.15. Relationship between mother cephalothorax length and probability of survival of spiderlings from the time they hatched until the end of the breeding experiment from a) first (n = 17), b) second (n = 18), and c) third (n = 8) clutches laid by females in the laboratory. Clutch size was fitted as a covariate in the analysis (see Table 2.4), but to make the patterns clearer here, clutches are simply divided into large (first half of ranked values) and small (second half of ranked values).

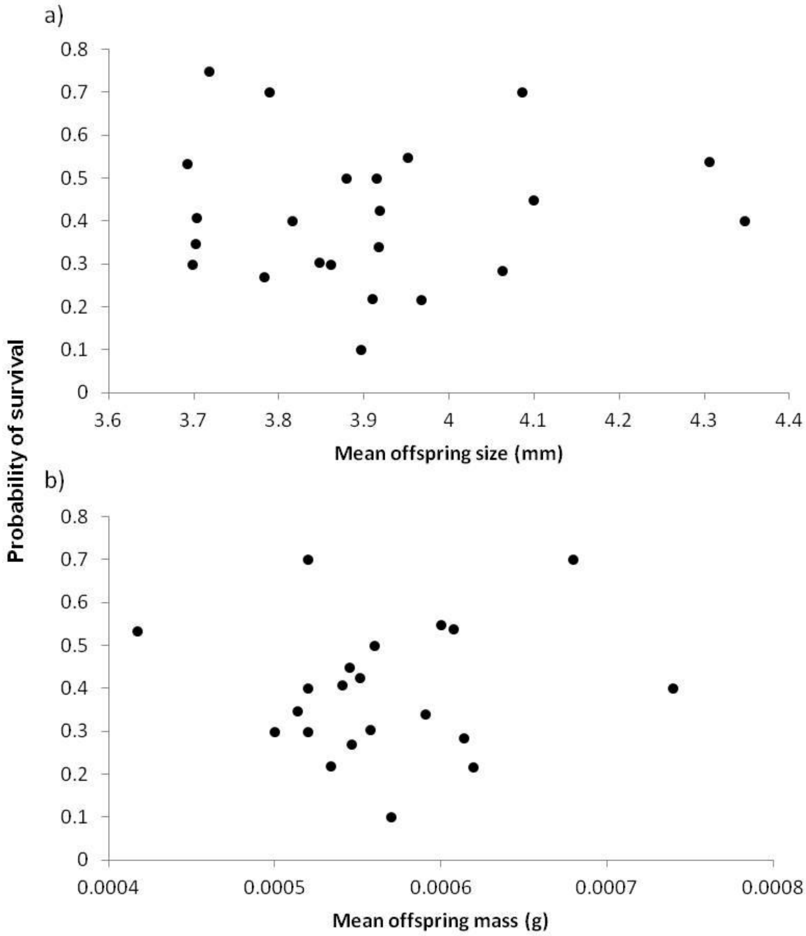

Figure 2.16. Relationship between probability of survival of offspring until the end of the breeding experiment from second clutches laid by females in the laboratory and a) mean offspring size (n = 23) and b) mean offspring mass (n = 21) per clutch.

Foraging behaviour

In addition to the target flower species considered in the surveys described above, *S. globosum* was observed on almost all flowering herbs and small woody plants at the study area (note that only a couple of flowering trees were checked, and no individuals of *S. globosum* were seen on them). A complete list of the flower species where individuals of *S. globosum* were observed is provided in Table 2.5.

Table 2.5. Flower species where individuals of *S. globosum* were observed during the four years of this investigation.

Black mustard (*Brassica nigra* L.)
Calla lily (*Zantedeschica aethiopica* L.)
Cape daisy (*Osteospermum sp.* L.)
Chamomile (*Anthemis sp.* L.)
Cornflower marigold (*Chrysantemum segetum* L.)
Corn poppy (*Papaver rhoeas* L.)
Crown daisy (*Chrysantemum coronarium* L.)
Daisy (*Bellis perennis* L.)
Field bindweed (*Convolvulus arvensis* L.)
French lavender (*Lavandula stoechas* L.)
Gladiolus (*Gladiolus imbricatus* L.)
Gum cistus (*Cistus ladanifer* L.)
Hairy fleabane (*Inula oculus-christi* L.)
Lace-leaf euryops (*Euryops abrotanifolius* L.)
Milk thistle (*Galactites tomentosa* l.)
Purple viper's bugloss (*Echium plantagineum* L.)
Rockrose (*Cistus crispus* L.)
Sage-leaved cistus (*Cistus salvifolius* L.)
Tuberous hawkbit (*Leontodon tuberosus* L.)
Yellow lupine (*Lupinus luteus* L.)

In the field, adult spiders were commonly oberved feeding on honeybees (*Apis mellifera*), other types of bees, several types of flies, and other spiders. They were also, less frequently, observed to feed on ants, beetles, and some types of insect larvae. A female was found feeding on another female of *S. globosum* on one occasion, which suggests that cannibalism occurs but it appears to be rare. Spiders could be found on top of flowers completely exposed or inside of self-made shelters of folded petals attached with silk (Figure 2.17). Spiders were found at an average density of 1.591 ± 0.308 per m^2 in the meadow at Site 1 in Surveys 2 and 3 combined. In laboratory conditions, early instar *S. globosum* fed on fruit flies (*Drosophila melanogaster*) reared in the laboratory and purchased from Blades Biological Ltd., and hatchling black field crickets (*Gryllus bimaculatus*) purchased from

Livefoods Direct. Later instar and adult spiders fed on different species of hoverflies and blowflies caught in the field, as well as houseflies (*Musca domestica*) purchased from Dartfrog.

Figure 2.17. *S. globosum* self-made shelter of folded petals attached with silk on a flower of *E. abrotanifolius*.

Results of the monitoring of marked spiders revealed that, across all patches, 50 % of spiders that were found at the beginning of the observation period were missing at the end. Similarly, 51.8 % of spiders found during the survey had not been found at the beginning. The proportion of individuals found during the survey that were not found at the beginning was significantly higher for males (GLM with binomial error structure: X^2 = 93.027, df = 1, P = 0.003; Figure 2.18a). However, no significant difference was found between females and males in the proportion of individuals found at the beginning that were missing at the end (X^2 = 45.470, df = 1, P = 0.619; Figure 2.18b). Spiders spent an average of 76.154 hours (± 8.049) on a particular flower and occupied 1.711 different flowers (± 0.149) over the period of 11 days. Females were found to occupy, on average, a significantly higher number of flowers during the survey than males (Wilcoxon rank-sum test: W = 614.5, P = 0.031; Figure 2.19). Males spent significantly more time on a given flower than females (W = 283, P = 0.005; Figure 2.20a). However, no significant difference was found between females and males in the time that they spent in a habitat patch (W = 527.5, P = 0.510; Figure 2.20b).

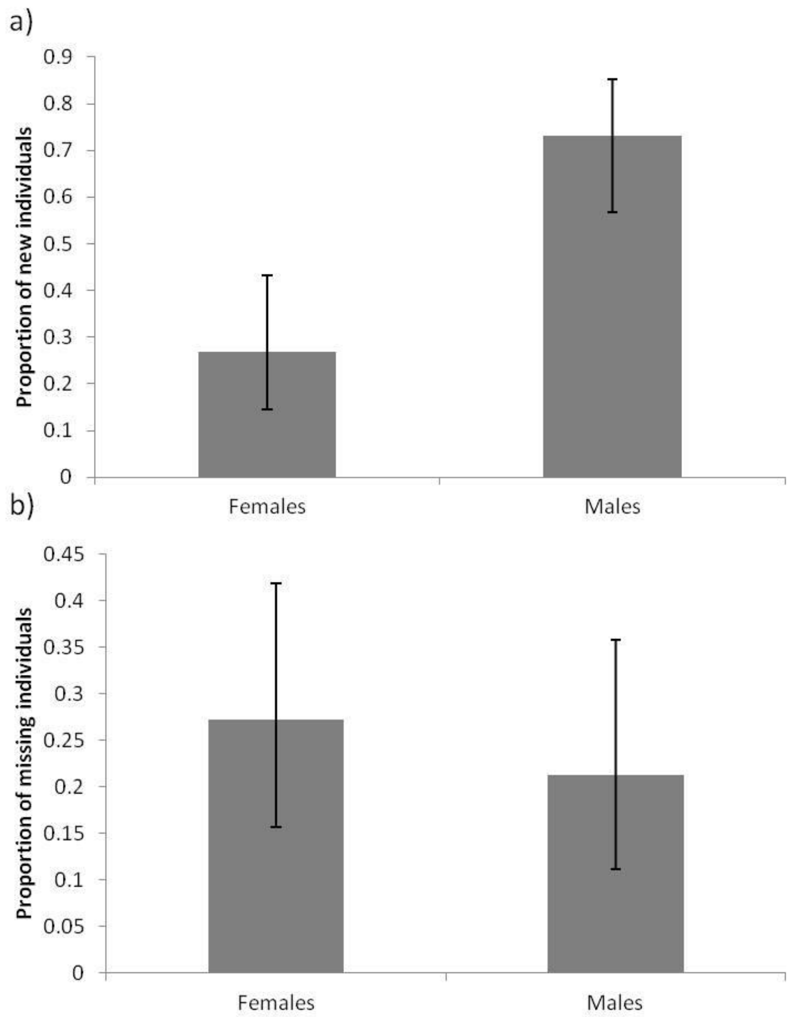

Figure 2.18. Proportion of a) females (n = 11) and males (n = 30) found during the survey which were not found at the beginning and b) females (n = 9) and males (n = 7) found at the beginning that were missing at the end of the survey. Error bars are 95 % confidence intervals calculated using the binomial distribution.

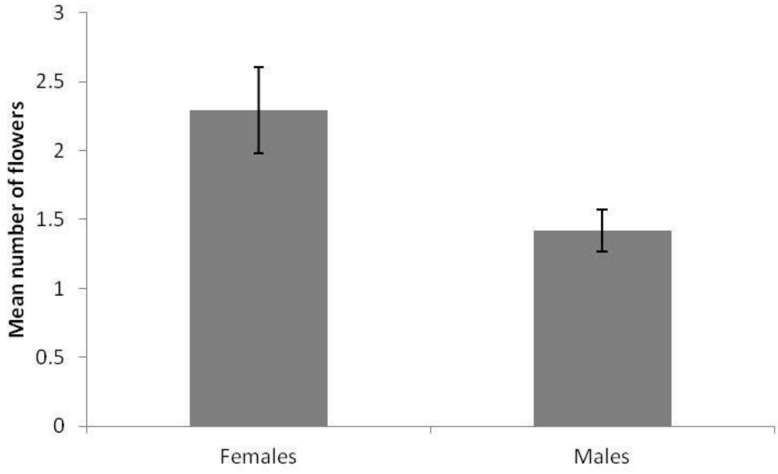

Figure 2.19. Mean number of flowers (± S.E.) occupied by females (n = 31) and males (n = 43) in the field.

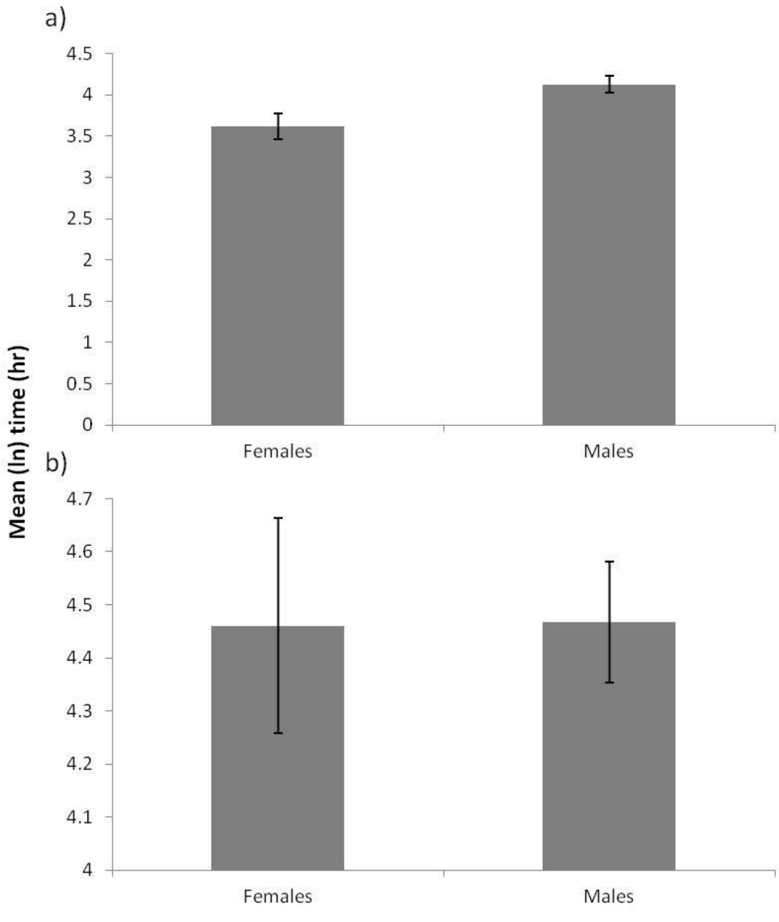

Figure 2.20. Mean natural log of time (± S.E.) spent by females (n = 31) and males (n = 43) a) on a particular flower and b) in a habitat patch in the field.

Aggressive behaviour

S. globosum was observed to show aggressive behaviour towards conspecifics in a number of situations. Females showed aggressive displays towards other females when they encountered each other on the same flower. Both females faced each other and slowly waved the first two legs from one side up and downwards repeatedly in no consistent or fixed sequence, often alternating the movement with the first two legs from the other side. This behaviour continued until one of the females retreated or the two females came into full contact with each other, in which case, one of them retreated and left the flower. A female was seen feeding on another female on one occasion, which presumably was the result of such an encounter. Unreceptive females behaved aggressively towards males that attempted to mate with them (see above). Males also exhibited agonistic behaviour towards other males in the presence of a female. Often a male searching for a mate would encounter another male that was guarding a female on a flower. During an encounter, males moved the front legs in a similar manner to females (see above), and at the same time vibrated their abdomens rapidly. Males alternated this behaviour with raising the first pair of legs, coming into close proximity to the other male, and slowly touching its raised legs, and then backing away. When backing away from each other, males would occasionally walk around the flower and adjacent leaves. All behavioural patterns were repeated until one of the males retreated and left the flower. Males were never seen coming into full contact during encounters.

Kleptoparasitism

Kleptoparasitism occurs in *S. globosum*, but it appears to be rare. Males were observed eating the dead prey of a female on two occasions, one in the field and one in the laboratory. On the first occasion, a male arrived on a flower of *C. ladanifer* where a female was hiding inside a folded petal. When the female perceived the presence of the male, she came out from hiding and displayed an aggressive posture towards the male by raising her first pairs of legs. The male then retreated to the underside of the flower where a dead bee, which was presumably killed by the female, was attached with silk. When the male came into contact with the bee, he started to feed on it. When the male had finished feeding, he left the flower. The second time kleptoparasitism was observed was during a staged mating in the laboratory. A male was introduced in a petri dish with a female that had been previously fed with a fly whose corpse had not been removed. When the male came into contact with the dead fly, he started feeding on it.

2.4 Discussion

The results presented here show that the spider community in the study area has a high diversity, with *S. globosum* being one of the most abundant species. *S. globosum* was found to be a very common and conspicuous predator on flowering plants with very generalist preferences for foraging sites and types of prey. Results also revealed possible trade-offs in reproductive investment, differences between females and males in flower occupancy times, male-male competition for mates, cannibalism, and kleptoparasitism. Altogether, these results provide a description of some important aspects of *S. globosum's* natural history which were previously unreported.

2.4.1. Spider community composition

The high diversity of the spider community found in the study area is in line with previous studies that have assessed the spider richness and composition of spider communities in Mediterranean scrubland and forests in Portugal (Cardoso et al. 2008a; Cardoso et al. 2008b; Cardoso et al. 2009). The abundance of *S. globosum* found in this survey is also consistent with previous studies where it has been reported to be a relatively common species in some parts of Asia and Europe, including Portugal (Szymkowiak 2008; Pérez-Guerrero et al. 2009; Yilmaz et al. 2009; Benhadi-Marin et al. 2011). These previous studies were carried out during spring and summer months, in contrast to the present study which was carried out in winter months. Therefore, *S. globosum* appears to be consistently abundant throughout the year.

In winter surveys, *S. globosum* was most commonly found in shrubs of *C. ladanifer* and bushes of *L. stoechas*. These two plant species were also found to have a high proportion of individuals of *S. globosum* during the spring season when both plants produced flowers. It thus appears that during the winter, when plants with flowers are rare in the study area, *S. globosum* juveniles forage on the plant species which later produce flowers that are suitable foraging sites for adults. Most individuals of *S. globosum* were collected with the beating and cryptic methods and no individual was caught with pitfall traps, which suggests that *S. globosum* do not wander on the ground, at least during this season, and no individuals were observed on the ground in spring either.

The number of spider species collected in the survey was affected by the type of vegetation. Most morphospecies were found on *C. salvifolius*. This may be explained by the fact that *C. salvifolius* bushes are very dense. It has been found that higher spider densities and species richness can be found in vegetation with greater density (Balfour and Rypstra 1998; Raizer and Amaral 2001). This in turn is explained by the fact that high density vegetation has greater structural complexity, and hence more available niches (Macarthur and Macarthur 1961; Kelaher 2003).

The sampling method employed also had an effect on the number of spider morphospecies that were found. Most species were collected with the cryptic search method. This contrasts with several studies by Cardoso et al. (2008a, 2008b, 2009), which also assessed the species richness and composition of spider communities in Mediterranean scrubland and forests in Portugal. These studies found that a similar total number of species were collected with each method used. Nevertheless, they found that sampling methods differed significantly in other measures of diversity and abundance such as the number of species and individuals per sample and the number of unique species per method. The authors concluded that, in comparison to other variables that could possibly affect number of species and of individuals collected, sampling method had the strongest effect, and they stressed the importance of employing a variety of sampling methods when assessing species richness and abundance. The results of my study support this conclusion.

Not only did sampling method and vegetation type each affect spider morphospecies richness independently, but also there was a significant interaction between the two factors. Some sampling methods were more efficient in some types of vegetation than in others, which would be expected since all types of vegetation differ greatly in their characteristics. For example, cryptic searching was particularly effective for *C. salvifolius*, perhaps because these compact bushes are harder to beat or sweep a net through. The number of morphospecies collected was also affected by the interactions between site and type of vegetation, site and sampling method, and among site, type of vegetation and sampling method. The interaction between site and type of vegetation also had an effect on the number of individuals collected. These results most likely reflect the differences between vegetation types at the two sites. For example, the meadow at Site 2 was located in a more open and disturbed area than the meadow in Site 1, which was located within the Quinta de São Pedro Study Centre. The meadows also differed in some of the plant species found in the area where they were located. The inconsistency in the efficiency of the different sampling methods between the two sites further supports the use of multiple sampling methods when assessing spider (and other arthropods) community composition.

2.4.2 Habitat preferences of *S. globosum*

There was a significant effect of flower species on the probability of finding an individual of *S. globosum* on a flower of the species that were surveyed. This suggests that the spiders have a preference for particular species. However, in Site 1 and Site 2, the species of flowers with the highest probability of finding a spider were those that were surveyed at locations where they did not occur along with any of the other species that were surveyed. Therefore, spiders might have been more common on these because of a lack of alternative choices of flowers. Additionally, some flower species differed considerably in their characteristics. For example, flowers of *Z. aethiopica* (a large ornamental lily) are much larger and found at lower densities than *L. luteus* (European yellow lupin, which has many medium-sized flowers per inflorescence). Therefore, spiders might be found at equivalent densities per unit area and yet appear to be at different frequencies on flowers of different species.

The choice of flower as a foraging site might depend on several factors associated with prey rather than directly with the characteristics of the flower as is the case of *M. vatia* (Morse and Fritz 1982; Morse 1988). These factors may include prey visitation rates, success rates of previous attacks, the frequency of close encounters with prey, and length of prey visits. In the case of *M. vatia*, it has been shown that the most important factor for choice of foraging site is frequency of prey visits (Morse and Fritz 1982; Morse 1988). Since *S. globosum* exhibits similar foraging behaviour to *M. vatia*, it is possible that it relies on similar cues to choose a foraging site. However, further studies are required to be able to corroborate this idea. Alternatively, spiders might choose a flower to forage in relation to the way prey and/or predators perceive them on that type of flower. Some flower-dwelling crab spiders appear cryptic to both prey and predators on the flowers they occupy (Théry and Casas 2002), and others use colour contrasts to attract prey (Heiling et al. 2003; Herberstein et al. 2009). Because females of *S. globosum* show three different colour morphs, it is possible that each morph chooses a different type of flower relative to its colour, although this does not seem to be the case (see Section 3.3.3).

Overall, notwithstanding the possible flower preferences evident in the survey results, the main conclusion of this exercise must be that *S. globosum* is indeed very generalist in both its habitat and prey choice. Over-wintering juvelines are found off the ground in most vegetation types, while sub-adults and adults in spring forage on most types of flower-visiting arthropods, and on flowers of all different shapes and colours.

2.4.3 Morphology

This study is consistent with a previous report (Corcobado et al. 2010) that *S. globosum* shows a moderate degree of sexual size dimorphism: females were larger than males, but not significantly so, and twice as heavy. Some other crab spider species display a more pronounced sexual size dimorphism. For example, in *M. vatia* females are twice as big as males and can weigh up to a hundred times more (Legrand and Morse 2000). *S. globosum's* sexual dimorphism is more comparable to crab spider species of genera such as

Xysticus, *Coriarchne, Ozyptila*, and *Tmarus* (Legrand and Morse 2000). The extreme sexual dimorphism in *M. vatia* has been attributed to selection for smaller male size resulting from scramble competition, where smaller males are more successful at finding females in low density populations (Legrand and Morse 2000). Since population densities of *S. globosum* in this study (1.96 ± 0.39 individuals/m^2) were much higher than those reported for *M. vatia* (0.0219 individuals/m^2; Anderson and Morse 2001), and male-male contests appear to be a lot more common than in *M. vatia*, males of *S. globosum* probably find females frequently and selection for smaller male size is less important.

2.4.4 Reproductive investment

Under laboratory conditions, females of *S. globosum* were able to produce up to three egg clutches. However, they may lay fewer clutches in the field since they might not feed as often or might be constrained by the length of the season. Such is the case of *M. vatia*, where females in natural conditions have been observed to lay one single egg sac in their lifetime, but are able to lay a second clutch in the field after being provisioned in the laboratory with a richer diet than they would obtain in the field (Morse 1994). Time to hatching and probability of survival of offspring did not differ significantly between first and second clutches, but did between the first two clutches and the third one. Spiderlings from third clutches took longer to hatch, and had a lower probability of surviving until the end of the breeding experiment. It is possible that third clutches were significantly less successful than first and second clutches because females were not remated in the interval between the production of each clutch and thus retention of viable sperm might be a limiting factor.

No significant effect of mother size was found on individual offspring size, individual offspring mass, sum of all offspring per clutch, total clutch mass, or clutch size. However, a significant negative correlation was found between clutch size and offspring size, which suggests that there is a trade-off between the two traits in *S. globosum*. Nevertheless, a trade-off between offspring size and number, predicted by life-history theory (Roff 2002), has been found to vary among populations or over time in spiders (Simpson 1993; Brown et al. 2003). Therefore, the occurrence of this trade-off has been proposed to depend on a number of constraining factors such as food limitation and/or species-specific offspring size (Brown et al. 2003). Furthermore, the analysis presented here is based on only second instar spiderlings from second clutches, and data from newly hatched spiderlings (or eggs) from all clutches would be necessary to obtain a more accurate description of maternal investment in offspring in this species.

The probability of survival of offspring was found to be affected by clutch size. Offspring from larger clutches had a lower probability of survival than offspring from smaller clutches. This might represent a trade-off between the number of offspring produced and the quantity of resources allocated to each offspring (Smith and Fretwell 1974). This is further supported by the negative correlation found between clutch size and offspring size, since larger clutches produced smaller offspring. Moreover, the probability of survival of offspring was also found to be affected by offspring size and mass: larger heavier offspring had a higher probability of survival than smaller lighter offspring. In general, larger offspring have higher fitness (Roff 1992), and this pattern in spiders could be explained by a higher tolerance to starvation, a lower vulnerability to parasitism, and/or a higher ability of prey capture by larger offspring (Tanaka 1992, 1995; Walker et al. 2003; Iida 2005). The interaction among mother cephalothorax length, clutch size, and number of clutch also had a significant effect on the probability of survival of offspring. This might indicate a difference in allocation of resources to first, second, and third clutches by mothers of different sizes.

2.4.5 Behaviour in the field

Changes in the proportions of spiders present in habitat patches that were monitored do not suggest that *S. globosum* is particularly faithful to specific patches. Half of the individuals that were found at the beginning of the survey, across patches, were missing at the end, and, of the total number of spiders observed, half were individuals that were not found at the beginning of the survey. However, missing spiders do not necessarily indicate departures from the study area. Since the presence of spiders was only recorded on flowers, individuals that were moving about on other parts of the vegetation were therefore missed. Missing spiders might also indicate mortality.

Spiders spent an average of 76.15 hours on a particular flower and occupied a mean number of 1.71 flowers over the period of 11 days, which suggests that

S. globosum is faithful to particular flowers to some extent. There were significant differences between females and males in the behaviour of spiders on flowers. A higher proportion of males found during the survey were not observed at the start of the survey (about 70 % of all individuals), but a similar proportion of females and males that were found at the beginning were missing by the end of the survey. A higher proportion of new males observed in patches is likely to be a result of a higher rate of movement in order to find mates (Kotiaho et al. 1998; Schütz and Taborsky 2003). Increased mobility in male spiders as a result of mate-searching has been found to lead to higher mortality rates (Vollrath and Parker 1992), which can be a consequence of increased vulnerability to predators (Kotiaho et al. 1998). This increased mortality could account for the observed female bias in sex ratios of *S. globosum* in the field (Vollrath and Parker 1992). However, it would first be necessary to obtain primary sex ratios. Males were also found to spend significantly more time than females on a given flower, and occupied fewer flowers than females. Since males of *S. globosum* were observed mate-guarding females until these underwent what I have assumed to be their final moult, most males probably remained on a flower with a female for a considerable period. In contrast, females could be moving more among flowers in order to find optimal sites to forage. These patterns can be comparable with those observed in *M. vatia*, where females move frequently among umbels and, with less frequency, between flower stems to find high quality foraging sites (Morse and Fritz 1982). However, adult *M. vatia* females do not move long distances and usually remain in the same plant where they subsequently lay eggs (Morse and Fritz 1982; Morse 1993). In contrast, half the females of *S. globosum* that were found at the beginning of the survey were missing at the end. Nevertheless, these disappearances could be due to other reasons such as mortality.

2.4.6 Conclusion

Knowledge of the basic natural history of *S. globosum* is essential for more sophisticated studies of the behaviour, ecology and evolution of this species. The information in this chapter is by no means comprehensive, but it does allow the identification of aspects of *S. globosum*'s morphology and behaviour which are of relevance in later chapters. In particular, several aspects of the species's natural history might have important implications for the maintenance of its female colour polymorphism. The type of prey they consume might influence the presence of alternative morphs independently of the type of flower where they encounter the spiders. For example, negative frequency-dependent selection on morphs may occur if prey can learn to associate a colour morph with an attack and subsequently avoid that particular morph (see Chapter 5). Mate-guarding, multiple mating attempts, and kleptoparasitism by males can represent costs to females that could generate mechanisms that favour the maintenance of different morphs (see Chapter 6).

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Chapter 3: Nature of the colour polymorphism

3.1 Introduction

Information about the nature of a polymorphism, such as the way the polymorphic trait varies among individuals and in time and space, can help us to understand the factors determining its occurrence. Colour variation among individuals in a population can be discrete, which means that it can be categorised into separate groups, or it can be continuous. If a colour polymorphism is adaptive, whether it is discrete or continuous will have different implications for its function. Discrete colour morphs are usually genetically determined and have been observed to be associated with sexual selection, avoidance of predators, and alternative behavioural strategies (Clarke and Murray 1962; Seehausen et al. 1999; Brooks 2002; Roulin et al. 2003; Bleay et al. 2007; McKillup and McKillup 2008; Formica and Tuttle 2009; Dijkstra et al. 2010; Hurtado-Gonzales et al. 2010; Takahashi et al. 2010). Although also sometimes associated with these interactions, continuous colour morphs have typically been found to be condition-dependent and hence to change with health condition or with the environment (Hill and Montgomerie 1994; Fitze et al. 2003).

In the case of genetic colour polymorphisms, different morphs in a population might vary in the way their fitness is affected by different factors of the environment, which could either lead to the fixation of one morph through consistent directional selection, in which case the polymorphism would only be transient (Mitchell-Olds et al. 2007), or to the maintenance of multiple morphs through balancing or fluctuating selection. A fitness advantage for one particular morph has been observed in the Alpine leaf beetle (*Oreina gloriosa*), where individuals that belong to the predominant morph in the population suffer significantly less predation than the rare morph (Borer et al. 2010). A fitness advantage can also occur through sexual selection, as is the case in the wolf spider *Schizocosa ocreata*, where males with larger tufts of bristles on the first pair of legs are more successful at mating and have a lower risk of postmating cannibalism (Persons and Uetz 2005). A balanced polymorphism can be maintained if different morphs employ alternative strategies that result in equal fitness. For example, females of the tawny owl (*Strix aluco*) adopt alternative reproductive strategies that are correlated with plumage colour (Roulin et al. 2003). Females with grey plumage breed less often than reddish-brown females, but produce offspring of higher quality. Similarly, polymorphisms can be maintained when morphs experience spatial or temporal variation in selection coupled with gene flow. We would expect such fluctuating or spatially heterogeneous selection to be reflected in the distribution of morphs across time and space, with the fittest morph(s) in particular locations or time periods becoming relatively more frequent. Therefore, variation of morph frequencies at different spatial and temporal scales provides important information about the mechanisms that might be involved in the maintenance of a polymorphism.

When looking at the distributions of morph frequencies in space, if the distributions do not differ from a random distribution, it is possible that stochastic processes, such as genetic drift, are responsible for the polymorphism. On the other hand, if the distribution of morph frequencies is not random, it indicates that a selective agent is most likely in operation. However, non-random patterns can also result from genetic drift or founder effects with limited gene flow between populations (Slatkin 1987). If the selective agent is one or several climatic factors, or other factors which are correlated with climate, then geographic variation of a polymorphic trait will be parallel to the geographic variation of the climatic factors (Endler 1986). A correlation between morph frequencies and climatic factors such as temperature, humidity, and other factors associated with latitude and altitude has been found in several polymorphic species of ladybirds, *Drosophila*, damselflies and snails (David et al. 1985; Munjal et al. 1997; de Jong and Brakefield 1998; Bellido et al. 2002; Parsonage and Hughes 2002; Brisson et al. 2005; Hammers and Van Gossum 2008; Phifer-Rixey et al. 2008).

Spatial variation of morph frequencies at smaller scales can reveal patterns of local adaptation not only to climatic factors, but also to biotic factors such as predation or sexual interactions (Thompson 2005; Van Gossum et al. 2007; Gosden and Svensson 2008). For example, a correlation between morph frequencies and local habitat characteristics, such as type and colour of background on which individuals are found, has been observed to occur and is usually attributed to visual selection from predators (Lees and Stewart 1987; Hazel and Johnson 1990; Ekendahl 1995; Slotow and Ward 1997; Bonte and Maelfait 2004; Hargeby et al. 2004; Todd et al. 2006; Forsman et al. 2011; Johnson 2011). The most notable cases where such correlation has been found are the peppered moth (*Biston betularia*) and the land snails of the genus *Cepaea*. It has been observed that a higher frequency of darker morphs of the peppered moth are associated with areas with more pollution, which produce darker backgrounds (Kettlewell 1973; Cook and Turner 2008). In the case of the *Cepaea* snails, brown and pink unbanded individuals are associated with a brown and uniform background of dead leaves, yellow banded individuals are associated with mixed green backgrounds, and intermediate frequencies of morphs are found in intermediate backgrounds (Cain and Sheppard 1950, 1954; Greenwood 1974). However, in the *Cepaea* snails, a discrepancy in the variation of morph frequencies and visual selection has been found in some populations, where morph frequencies have been observed to show sharp differences across uniform habitats (Cain and Currey 1963; Carter 1968; Jones 1973). These “area effects” are thought to be a result of founder effects (Goodhart 1963) and/or differentiation in refugia (Cameron et al. 1980; Davison and Clarke 2000).

Temporal variation in morph frequencies can also be associated with changes in climate. For example, morph frequencies have been found to vary seasonally (Chang and Emlen 1993; Parkash et al. 2011), as well as over lager temporal scales of several years, in relation to climate (Cowie and Jones 1998; Cameron and Pokryszko 2008). Seasonal changes in morph frequencies that are not associated with climatic factors have been observed to be caused by migration (Smith et al. 1997). Other patterns of variation of morph frequencies over time can result from other types of selection, such as a pattern of oscillation around unity which is consistent with models of NFDS generated by different ecological interactions (Hori 1993; Svensson et al.

2005; Takahashi et al. 2010). Temporal stability of these fluctuations, if less than expected by drift, and directional trends in morph frequencies are indicators that selection is operating (Endler 1986). Stability suggests a stable equilibrium that can be caused by stabilizing selection (e.g. NFDS) or a balance between selection and meiotic drive, gene flow, mutation, or genetic drift (Dhondt et al. 1979; Endler 1986; Cook 1998; Oxford 2005; Hartl and Clark 2007; Vishalakshi and Singh 2009). As well as looking at the spatial and temporal distribution of phenotypes, supporting evidence for the type of mechanism that might be operating to maintain a polymorphism can also be obtained by comparing the genetic or visible differentiation in a polymorphic trait among populations with the differentiation of a set of neutral molecular loci (Whitlock 2008). If the measure of differentiation of a trait (Q_{ST}) is significantly higher than the measure of differentiation of the neutral loci (F_{ST}), then the variation would be expected to be a result of adaptation to local environmental conditions (Lande 1992; Spitze 1993; Lynch and Walsh 1998). On the other hand, if Q_{ST} is significantly lower than F_{ST}, it would indicate that global selection is acting on similar allele frequencies among populations. Finally, if Q_{ST} equals F_{ST}, the differentiation of the trait would not deviate from that expected from genetic drift and thus it could be considered as neutral. This comparison approach has been employed to investigate whether selection is operating to maintain variation in a number of species (Bowcock et al. 1991; King and Lawson 1995; Long and Singh 1995; Gillespie and Oxford 1998; Hoffman et al. 2006; Oxford and Gunnarsson 2006; Abbott et al. 2008).

3.1.1 Objectives

In summary then, knowledge of how genetically determined traits vary among individuals (whether variation is continuous or discrete), and among populations at different spatial and temporal scales, is fundamental when investigating the mechanisms that maintain polymorphisms. In this chapter, therefore, my aim was to characterise quantitatively colour variation among individuals of female *S. globosum*, and to look for non-random spatial and temporal patterns in the frequencies of morphs. To establish whether the colour polymorphism in *S. globosum* is discrete or continuous, the reflectance spectra of individuals of each morph were measured. To check for possible fitness differences among morphs, and to look for other more cryptic traits which might correlate with colour, differences in behaviour on flowers, reproductive output and morphology among morphs were examined. Then, to investigate the distribution of morph frequencies in time and space, three surveys were conducted at different spatial scales. First, morph frequencies in the flower species where *S. globosum* is most commonly found were recorded to establish whether there is a correlation between colour morph and type of flower. Second, morph frequencies at different sites in the study area were recorded across three years. Finally, the regional distribution of morph frequencies in the Iberian Peninsula was obtained from an online database of photographs of *S. globosum*.

3.2 Materials and methods

3.2.1 Colour measurements

To evaluate if the three colour morphs of *S. globosum* are discrete morphs, reflectance spectra from the coloured area of the abdomen of the spiders were measured and analysed in the context of honeybee (*Apis mellifera*) and crab spider vision. These two visual systems were used because of their relevance in the evaluation of the possible mechanisms maintaining the polymorphism considered in this study (see also Chapters 5 and 6). Females of *S. globosum* were collected at the Quinta de São Pedro Study Centre and surrounding areas in spring of 2011 and spring of 2012. Spiders collected in 2011 were transported live back to the University of Nottingham for measurement. Spiders collected in 2012 were measured at the Quinta de São Pedro Study Centre. The spiders were gassed with CO_2 for a few minutes in order to immobilise them and take the measurements of the reflectance spectra.

Reflectance spectra were measured using an Ocean Optics USB2000 spectrometer connected to a miniature deuterium halogen light source (DT-Mini-2-GS) and a custom made bifurcated reflection probe (ZFQ-13666) with two 100µm fibres. The fibre-optic probe was positioned at 45° to the surface of the abdomen of the spiders at a standardised distance of 3 mm. The spectrometer was attached to a PC running Spectra Suite Spectroscopy Software (Ocean Optics, Inc. 2008). All measurements were carried out in a dark room and generated relative to a white diffuse reflectance standard

(Ocean Optics WS-1) and a dark standard (light source turned off, specimen covered with black cloth). The probe was positioned at three different locations near the coloured part of the body of each spider, and the average of the spectra recorded was used for the analyses. Each time the probe was re-positioned, ten scans of the spectrum, with an integration time of 250 ms, were taken and averaged to reduce noise from the spectrometer (following Llandres and Rodríguez-Gironés 2011).

To evaluate how honeybees would perceive the different spider colours, photoreceptor excitation estimates were calculated using the colour hexagon model (Chittka and Menzel 1992; Chittka 1996; Chittka and Kevan 2005). To obtain the excitation values (E) of each of the three photoreceptors (blue, green, and UV) of honeybees, the sensitivity factor of each photoreceptor (R_i) was calculated using the formula:

$$R_i = \frac{1}{\int_{700}^{300} I_B(\lambda) \cdot S(\lambda) \cdot D(\lambda) \cdot d(\lambda)} \qquad (1)$$

where $I_B(\lambda)$ is the spectral reflectance of the environmental background to which receptors are adapted, in the case of honeybees this is typically green foliage (Chittka et al. 1994), and the green leaf spectrum provided by Chittka and Kevan (2005) was used; $S(\lambda)$ is the spectral sensitivity function of the photoreceptor (Peitsch et al. 1992), and $D(\lambda)$ is the illumination spectrum for which the standard function D65 for open habitats is employed (provided by (Wyszecki and Stiles 1982), $d\lambda$ is the wavelength step size. Subsequently, the relative quantum catch absorbed by each spectral photoreceptor type (P_i) was calculated using the formula:

$$P_i = R_i \int_{700}^{300} I_S(\lambda) S(\lambda) \cdot D(\lambda) \cdot d(\lambda) \qquad (2)$$

where $I_S(\lambda)$ is the spectral reflectance function of the stimulus (the spider colour). The excitation value of each honeybee photoreceptor, E_{UV}, E_{Blue} and E_{Green}, was then calculated with the formula:

$$E_i = \frac{P_i}{P_i + 1} \qquad (3)$$

To determine the position of the colour of spiders in the honeybee colour space, the values of E_{UV}, E_{Blue} and E_{Green} were used to calculate the coordinates in the honeybee colour hexagon using the following equations:

$$x = \sqrt{3}/2 \cdot (E_{Green} - E_{UV}) \qquad (4)$$

$$y = E_{Blue} - 0.5(E_{Green} + E_{UV}) \qquad (5)$$

The colour difference among red, yellow, and white spiders was then calculated by the Euclidian distance between them in the colour hexagon:

$$\Delta St = \sqrt{(x_{red} - x_{yellow})^2 + (y_{red} - y_{yellow})^2} \qquad (6)$$

where x and y are the coordinates of the hexagon calculated by equations (4) and (5).

To evaluate if crab spiders can discriminate among the colour morphs of *S. globosum*, measurements of the reflectance spectra of each morph were analysed for differences in the wavelengths at which the photoreceptors in the visual system of crab spiders have the highest sensitivity. Two classes of photoreceptors have been found to be present in the visual system of the crab spider *Misumena vatia*, one sensitive in the UV region of the spectrum (around 340 nm) and one sensitive in the green region (around 520 nm) (Defrize et al. 2011). Reflectance intensities of the morphs of *S. globosum* at these wavelengths were used for the analysis.

3.2.2 Differences in life history traits among morphs

Morphology

To determine if there are any differences among morphs in size and body mass, data obtained from the survey carried out to investigate the abundance of *S. globosum* in different types of flowers were used (Survey 2, see Section 2.2.3). Spiders were weighed with a digital balance and the length of the cephalothorax from front to back was measured under a stereo microscope using an eye-piece graticule.

Behaviour in the field

Data on the time spiders spend on a flower, the time they spend in a habitat patch, and the number of flowers they occupy were obtained from spiders that were marked and monitored over a period of 11 days (Survey 3, see Section 2.2.4) to look for differences among morphs. Habitat patches were defined as 10 m x 10 m plots in the meadow of the Quinta de São Pedro study centre or bushes of *L. stoechas* in the same location.

Reproduction

To search for any differences among morphs in components of reproduction, data were obtained from spiders used to determine the genetics of the polymorphism that were collected from the field and maintained in the laboratory, where they produced offspring (breeding experiment, see Chapter 4). The offspring of these females were reared in the laboratory for approximately 15 months. Not all spiders reached sexual maturity after this time. Data on the components of reproduction obtained from these females were: number of clutches laid before death, time from egg sac production to hatching, clutch size, offspring size and mass, clutch size, clutch mass, and number of surviving offspring until the end of the breeding experiment. The spiderlings from the second clutches produced by these females were measured (from the anterior end of the cephalothorax to the posterior end of the abdomen) and weighed in the same way as adult spiders.

<u>3.2.3 Spatial and temporal distribution of morphs at a local scale</u>

All surveys were carried out at the Quinta de São Pedro Study Centre and surrounding areas (see Section 2.2.1. for description). To determine the frequencies of the three colour morphs in different types of flowers and in different locations in the study area, data from the survey carried out to determine the abundance of *S. globosum* in different types of flowers were used (see Section 2.2.3 for detailed methodology). The survey was carried out in March and April of 2009 over a period of 22 days, and the following species of flowers were surveyed: purple viper's bugloss (*Echium plantagineum)*, cornflower marigold (*Chrysanthemum segetum),* marguerite daisy *(Euryops abrotanifolius),* calla lily *(Zantedeschica aethiopica)*, lavender (*Lavandula stoechas)*, sage-leaved cistus (*Cistus salvifolius),* gum cistus *(Cistus ladanifer),* yellow lupin *(Lupinus luteus)*, and crown daisy (*Chrysantemum coronarium*). The survey was carried out in the following three sites: the Quinta de Sao Pedro Study Centre (Site 1), a large open field of meadow, scrubland and woodland located opposite to the study centre (Site 2), and a second open field about 300 m South-East from the study centre with similar vegetation (Site 3) (Figure 2.2).

Additional, less intensive surveys were carried out in 2010 and 2011. These surveys consisted of recording all individuals observed in areas where flowers on which *S. globosum* is often seen. The survey of 2010 was conducted over a period of two days in May in Sites 1 and 2. The survey of 2011 was conducted over a period of nine days in May in Sites 1, 2, and 3.

3.2.4 Regional distribution of morphs

To investigate the regional distribution of *S. globosum* colour morphs, data on morph frequencies across the Iberian Peninsula were obtained from the online database Biodiversidad Virtual (Anonymous, 2009). Biodiversidad Virtual is a Spanish collaborative project that collects online data on the biodiversity of a number of taxonomic groups through digital photographs. Membership of Biodiversidad Virtual is open to the general public, and members submit digital photographs of animals, plants and fungi which are identified by specialised taxonomists and published on the website. Information provided with each photograph includes: the date when the photograph was taken, the type of habitat where the individual was found, province or district, and country. Data from a total of 394 photographs of female *S. globosum* were collected. The longitude and latitude for the centroid of the province where each individual was photographed was established. Values of 18 climatic variables (Table 3.1) and of altitude for these locations were obtained from WorldClim Version 1.4 (Release 3), a free global climate dataset available online (Hijmans et al. 2009), to investigate any effect of climate on the distribution of the different morphs.

Table 3.1. Climatic variables used in the analysis of the distribution of *S. globosum* colour morphs across the Iberian Peninsula. Taken from WorldClim Version 1.4 (Release 3) (Hijmans et al. 2009).

Annual Mean Temperature
Mean Diurnal Range (Mean of monthly (max. temp. – min. temp.)
Isothermality (Mean diurnal range/Temperature annual range) (*100)
Temperature Seasonality (Standard deviation * 100)
Max. Temperature of Warmest Month
Min. Temperature of Coldest Month
Mean Temperature of Wettest Quarter
Mean Temperature of Driest Quarter
Mean Temperature of Warmest Quarter
Mean Temperature of Coldest Quarter
Annual Precipitation
Precipitation of Wettest Month
Precipitation of Driest Month
Precipitation Seasonality (Coefficient of variation)
Precipitation of Wettest Quarter
Precipitation of Driest Quarter
Precipitation of Warmest Quarter
Precipitation of Coldest Quarter

3.2.5 Statistical analyses

One-way analyses of variance (ANOVAs) and Kruskal-Wallis tests were used to investigate any differences among *S. globosum* colour morphs in the sensitivity of honeybee and *M. vatia* photorecepors, and to test for differences among morphs in morphology (cephalothorax length and body mass) and behaviour in the field. Generalised linear models (GLMs) and generalised linear mixed models (GLMMs), with the appropriate error structures (Gaussian, Poisson, Quasipoisson, or Binomial) and mother of clutch as a random effect in the case of mixed models, were conducted to examine differences among morphs in the following components of reproduction: time to hatching of offspring, clutch size, offspring size and mass, sum of size of all offspring per clutch, total clutch mass, and probability of survival of offspring from time of hatching to the end of the breeding experiment. Chi-squared tests were used to analyse differences among morphs in number of clutches produced in the laboratory, differences in morph frequencies in different flower types, among sites and years, and between the Iberian Peninsula and my study area. To test for an effect of altitude, latitude, longitude or any of the 18 climatic variables on the distribution of *S. globosum* morphs across the Iberian Peninsula, a multinomial log-linear model was used. For GLMs, GLMMs, and the multinomial log-linear model, the significance of each term was assessed by backward deletion from a saturated model using the appropriate test statistic (Likelihood ratio, F-ratio, or Chi-squared) following Crawley (2007) and Zuur et al (2009). Statistics are presented for comparisons of models after each term was removed. Spider cephalothorax length and time spent on a particular flower were Box-Cox transformed to satisfy the assumption of a normal distribution. All analyses were conducted in R 2.12.2 (The R Development Core Team 2011).

3.3 Results

3.3.1 Colour variation among morphs

Red, yellow, and white females collected and measured in 2011 and 2012 had markedly different reflectance spectra (Figure 3.1). Red spiders showed substantial reflectance of light between 500 and 550 nm in wavelength and above, while yellow spiders did so between 350 and 450 nm and above, and white spiders between 300 and 350 nm and above. The three colour morphs of *S. globosum* appear quite close in the honeybee colour hexagon, with some overlap (Figure 3.2). However, the mean colour contrasts among the three colour morphs were all higher than 0.05 colour hexagon units (Table 3.2), which is the threshold considered necessary for colour discrimination in the visual system of the honeybee (Théry and Casas 2002). Moreover, excitation values were all significantly different among spider morphs for all honeybee photoreceptors in both years (Table 3.3 and Figure 3.3). Likewise, significant differences were found among spider morphs in the relative reflectance for the wavelengths at which the photoreceptors of *M. vatia* are most sensitive (Table 3.4 and Figure 3.4).

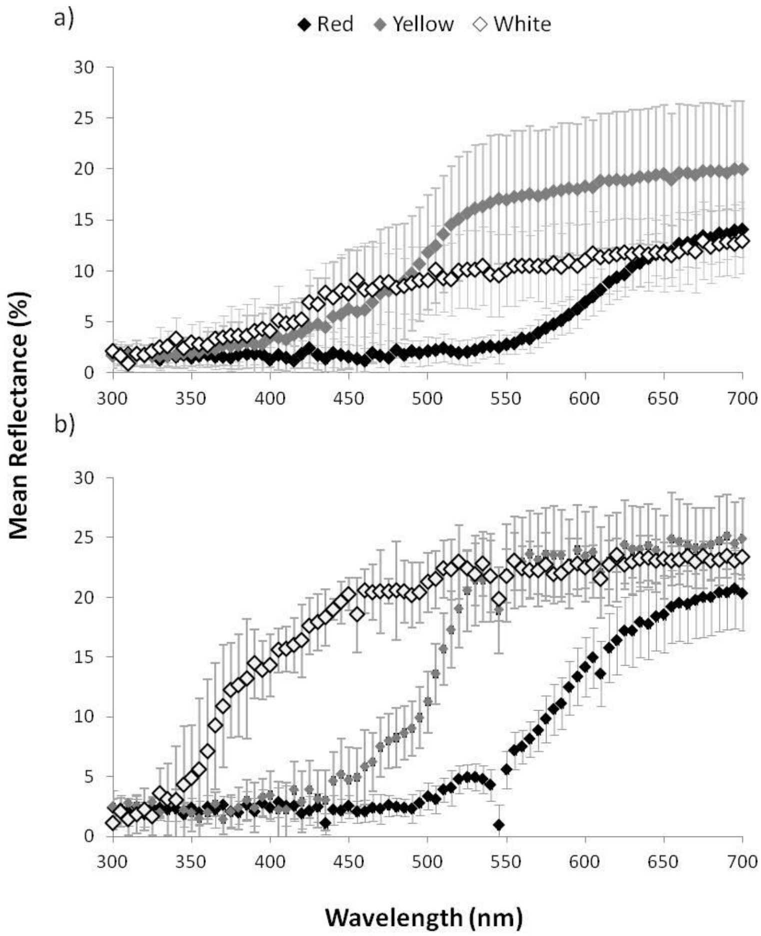

Figure 3.1. Mean reflectance spectrum values for a) red (n = 22), yellow (n = 27), and white (n = 6) spiders measured in 2011 and b) red (n = 38), yellow (n = 43), and white (n = 3) spiders meausred in 2012. Reflectance values were averaged from ten spectra taken at each of three locations on the coloured part of the body for each individual. Error bars represent standard deviations.

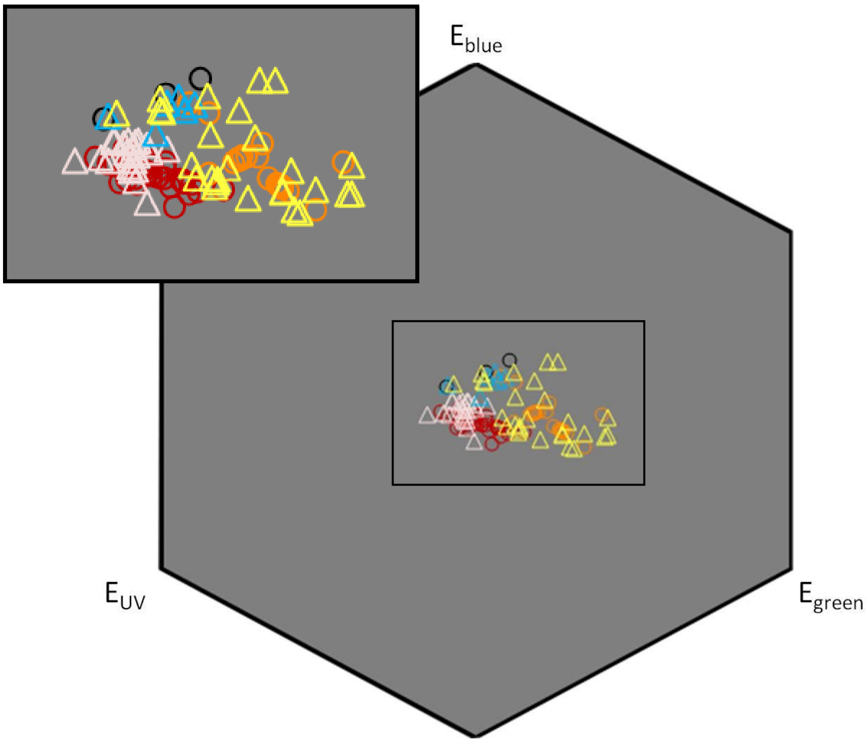

Figure 3.2. Colour loci of red (pale pink triangles, n = 22), yellow (yellow triangles, n = 27), and white (blue triangles, n = 6) female *S. globosum* in the honeybee colour hexagon measured in 2011; and red (red circles, n = 38), yellow (orange circles, n = 43), and white (black circles, n = 3) females measured in 2012. The small window indicates the actual position of colours in the hexagon, shown in detail in the enlarged window.

Table 3.2. Mean Euclidian distance given in honeybee colour hexagon units (± S.E.) between individuals of red and yellow, red and white, and yellow and white morphs of *S. globosum* measured in 2011 and 2012.

Distance between	2011	2012
Red and yellow	0.242 ± 0.022	0.210 ± 0.012
Red and white	0.133 ± 0.013	0.177 ± 0.036
Yellow and white	0.191 ± 0.047	0.189 ± 0.081

Table 3.3. Results of one-way analyses of variance (ANOVAs) and Kruskal-Wallis tests analysing differences in the excitation values of the honeybee photoreceptors (UV, blue and green) among colour morphs of *S. globosum* from individuals measured in 2011 and 2012.

Photoreceptors	2011			2012		
	F	df	P	X^2	df	P
UV	6.69	2,52	**0.003**	17.099	2	**< 0.001**
Blue	21.86	2,52	**< 0.001**	60.021	2	**< 0.001**
Green	111.9	2,52	**< 0.001**	63.745	2	**< 0.001**

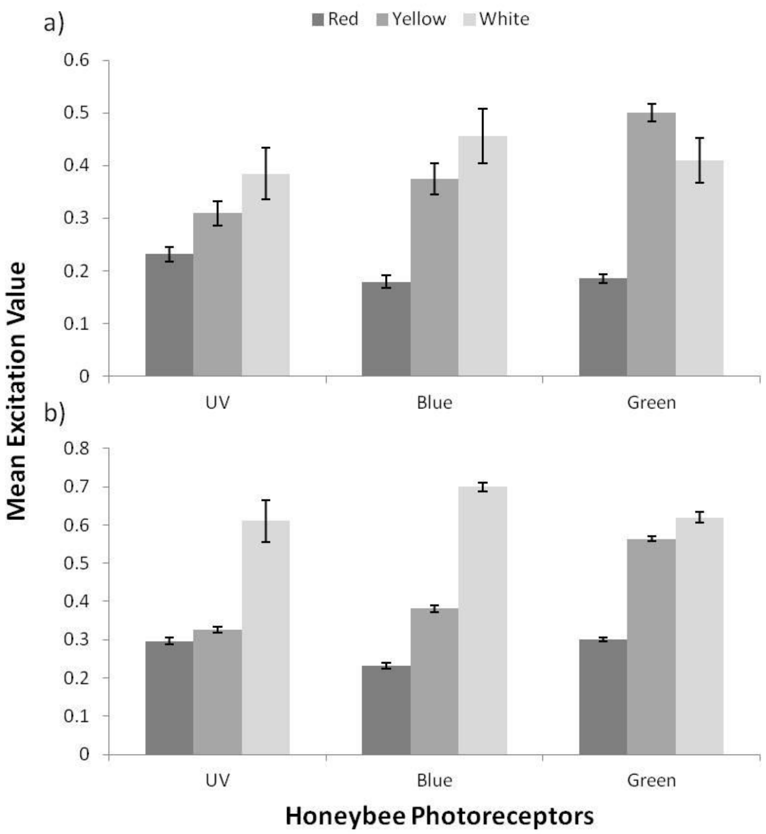

Figure 3.3. Mean excitation values (± S.E.) of the three photoreceptors of the honeybee (UV, blue and green) for a) red (n = 22), yellow (n = 27), and white (n = 6) females of *S. globosum* measured in 2011 and b) red (n =38), yellow (n = 43) and white (n = 3) females measured in 2012.

Table 3.4 Results of Kruskal-Wallis tests analysing differences among morphs of *S. globosum* measured in 2011 and 2012 in the relative reflectance of the wavelengths at which the photoreceptors of *M. vatia* have the highest sensitivity.

Wavelength (nm)	2011			2012		
	X^2	df	P	X^2	df	P
340	16.214	2	**< 0.001**	20.056	2	**< 0.001**
520	44.1	2	**< 0.001**	62.493	2	**< 0.001**

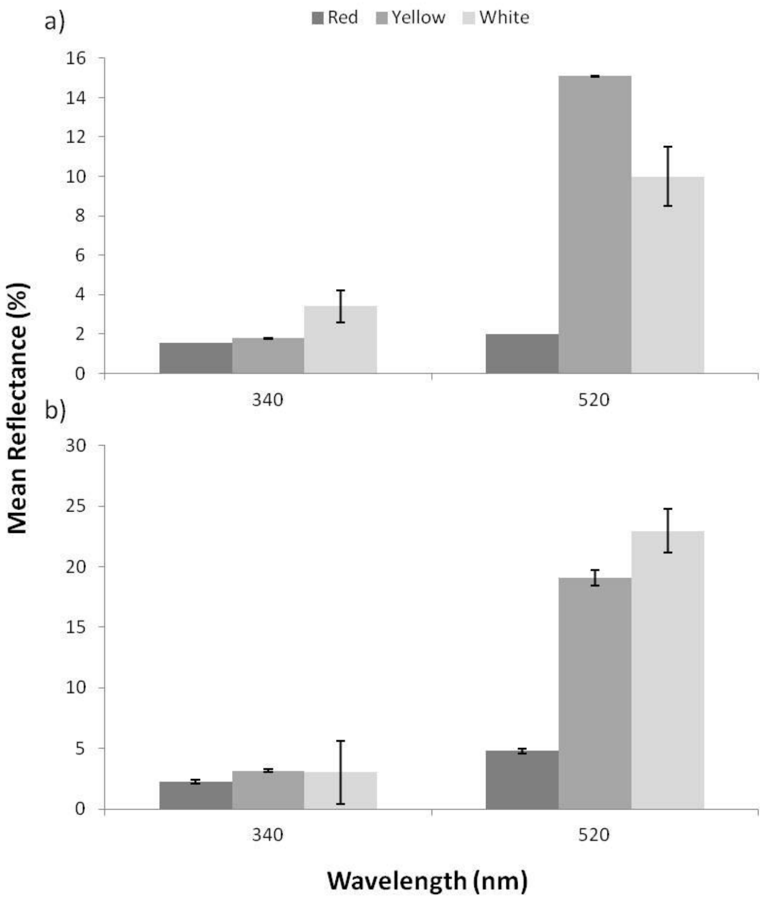

Figure 3.4. Mean reflectance (± S.E.) at the wavelengths to which the photoreceptors of *M. vatia* are most sensitive for a) red (n = 22), yellow (n = 27), and white (n = 6) females of *S. globosum* measured in 2011 and b) red (n =38), yellow (n = 43) and white (n = 3) females measured in 2012.

3.3.2 Differences in life history traits among morphs

Morphology

A significant difference was found in cephalothorax length among red, yellow and white females (One-way ANOVA: $F = 3.802_{2,180}$, $P = 0.024$). White females had larger cephalothoraxes than red and yellow females (Figure 3.5). However, no significant difference in body mass was found among morphs (Kruskal-Wallis test: $X^2 = 2.527$, df = 2, $P = 0.283$) (Figure 3.6).

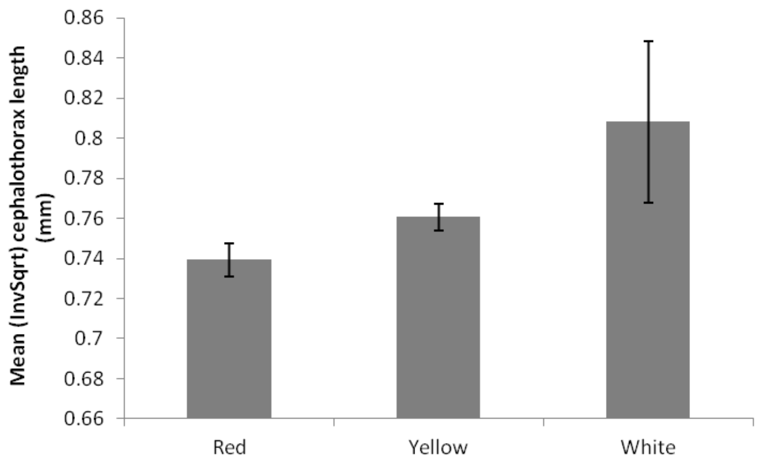

Figure 3.5. Mean inverse square root cephalothorax length (± S.E.) of red (n = 68), yellow (n = 109), and white (n = 6) females of *S. globosum* collected from the field in 2009.

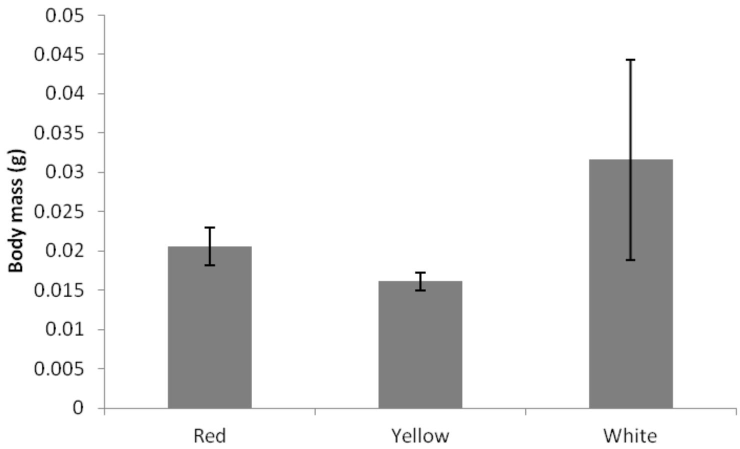

Figure 3.6. Mean body mass (± S.E.) of red (n = 68), yellow (n = 109), and white (n = 6) females of *S. globosum* collected from the field in 2009.

Behaviour in the field

A significant difference among colour morphs was found in the time a spider spent on a given flower (One-way ANOVA: $F = 4.488_{2,28}$, $P = 0.020$; Figure 3.7a). Yellow females spent significantly more time on a particular flower than red or white females. However, no significant difference among morphs was found in the time a spider spent in a particular patch (Kruskal-Wallis test: $X^2 = 2.899$, df = 2, $P = 0.235$; Figure 3.7b). Likewise, no significant difference among morphs was found in the number of flowers that a spider occuppied over the duration of the observations ($X^2 = 0.719$, df = 2, $P = 0.698$; Figure 3.8).

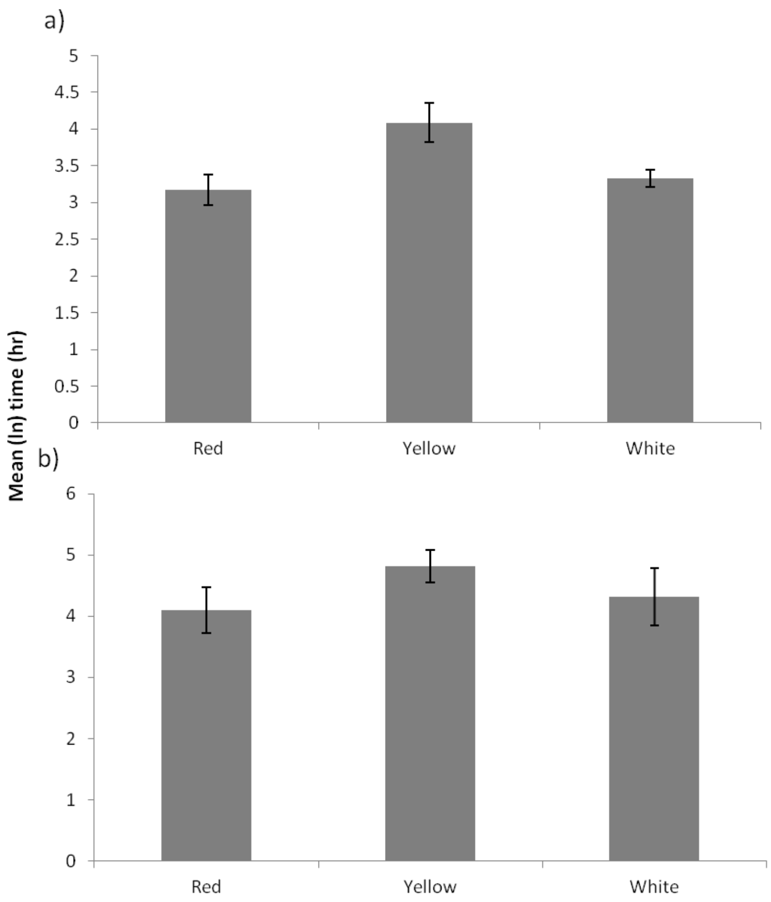

Figure 3.7. Mean natural log of time (± S.E.) spent on a) a particular flower and b) a habitat patch for red (n = 11), yellow (n = 14), and white (n = 6) female *S. globosum* over a period of 11 days.

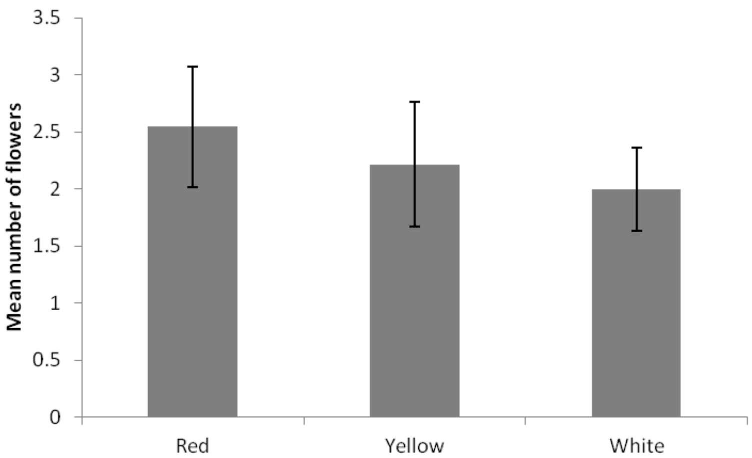

Figure 3.8. Mean number of flowers (± S.E.) occupied by individuals of red (n = 11), yellow (n = 14), and white (n = 6) *S. globosum* over a period of 11 days.

Reproduction

No significant difference was found among morphs in the proportion of females that produced one, two or three clutches in the laboratory from the time they were collected from the field to the time of death (Chi-squared test: $X^2 = 2.365$, df = 4, P = 0.669; Figure 3.9).

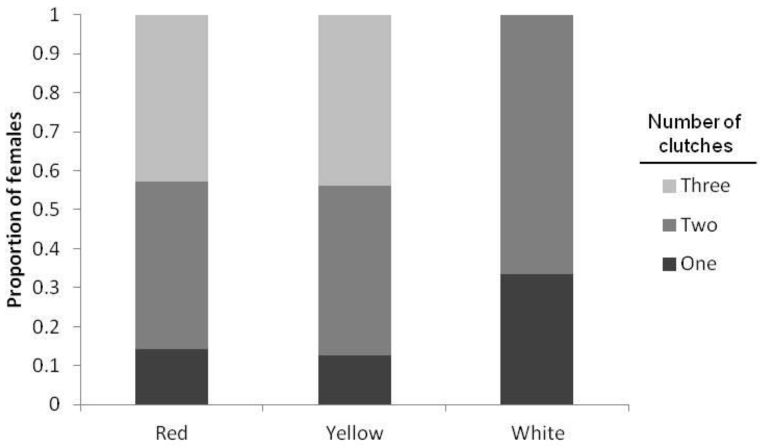

Figure 3.9. Proportion of females of red (n = 14), yellow (n =16), and white (n = 3) morphs that produced only one, only two, and exactly three clutches of eggs in the laboratory.

A significant effect of number of clutch (first or second) was found on the number of days spiderlings took to hatch (GLMM: $X^2 = 5.165$, df = 1, P = 0.023). Spiderlings from first clutches took longer to hatch than spiderlings from second clutches (Figure 3.10). However, there was no significant effect of mother colour morph ($X^2 = 0.715$, df = 2, P = 0.700) or its interaction with number of clutch ($X^2 = 3.101$, df = 2, P = 0.212). Similarly, there was no significant main effect of mother colour morph on clutch size from second clutches or across clutches, individual offspring size, individual offspring mass, sum of size of all offspring, total clutch mass, or probability of survival of offspring from second clutches and across all clutches (Table 3.5). Likewise, no significant main effect of mother chephalothorax length was found on any of the variables mentioned earlier. Furthermore, the interaction between mother colour morph and mother cephalothorax length was not significant for any of the response variables (Table 3.5).

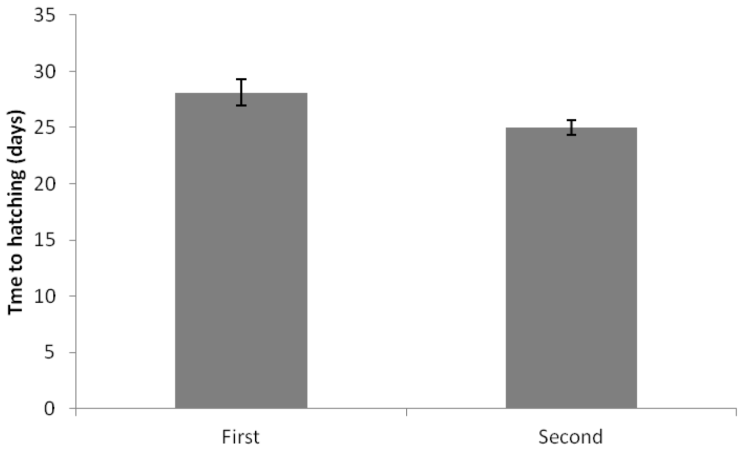

Figure 3.10. Mean number of days (± S.E.) from egg production to hatching of spiderlings for first (n = 32) and second (n = 26) clutches laid by females in the laboratory.

Table 3.5. Results of GLMs and GLMMs testing for an effect of mother colour morph, mother cephalothorax length (CL), and the interaction between them on clutch size, individual offspring size, individual offspring mass, sum of size of all offspring per clutch, total clutch mass, and probability of survival from time of hatching to the end of the breeding experiment. All data analysed were from second clutches, and also across clutches in the case of clutch size and probability of survival.

Response variable	Mother morph			Mother CL			Mother morph x mother size		
	GLM (error structure: Quasipoisson)								
	X^2	df	P	X^2	df	P	X^2	df	P
Clutch size (2nd clutch)	13.574	2	0.186	0.006	1	0.971	18.39	2	0.081
Clutch size (across clutches)	23.388	2	0.350	2.183	1	0.667	31.612	2	0.273
	GLMM (error structure: Gaussian, random effect: mother of clutch)								
	LR	df	P	LR	df	P	LR	df	P
Individual offspring size (2nd clutch)	3.402	2	0.183	0.001	2	0.975	4.371	2	0.112
Individual offspring mass (2nd clutch)	0.383	2	0.826	0.236	2	0.627	1.560	2	0.458
	GLM (error structure: Gaussian)								
	X^2	df	P	X^2	df	P	X^2	df	P
Sum of offspring size (2nd clutch)	7045.4	2	0.146	8.793	1	0.947	7312.9	2	0.113
Total clutch mass (2nd clutch)	< 0.001	2	0.132	< 0.001	1	0.907	< 0.001	2	0.350
	GLMM (error structure: binomial, random effect: mother of clutch)								
	X^2	df	P	X^2	df	P	X^2	df	P
Probability of survival (2nd clutch)	0.738	2	0.691	3.604	1	0.058	1.646	2	0.439
Probability of survival (across clutches)	1.745	2	0.418	1.259	1	0.262	0.257	2	0.879

3.3.3 Spatial and temporal distribution of morphs at a local scale

No effect of flower species or flower colour was found on the proportions of red, yellow or white females observed on the flowers in any of the three sites that were surveyed (Table 3.6 and Figures 3.11 and 3.12). In all sites and years, red and yellow spiders were found at approximately equal frequencies, while white spiders were found at much lower frequencies than red and yellow spiders (total frequencies: red: 0.440, yellow: 0.527, and white: 0.032). A significant difference in morph frequencies was found among years across the two sites that were sampled in all years (Sites 1 and 2; Chi-squared test: $X^2 = 14.773$, df = 4, P = 0.005). A posteriori analyses revealed a significant difference between the two sites in 2010 ($X^2 = 7.410$, df = 2, P = 0.025). In Site 2, a lower frequency of red spiders and a higher frequency of white spiders were observed than in Site 1 (Figure 3.13). However, there was no significant difference in the frequencies of individuals of the three morphs found in these two sites in 2009 (X^2 = 4.859, df = 2, P = 0.088) or in 2011 (X^2 = 0.503, df = 2, P = 0.778). No significant difference was found among Site 1, 2, and 3 across the two years when all three sites were sampled (X^2 = 5.129, df = 4, P = 0.274; Figure 3.13).

Table 3.6. Results of Chi-squared tests for differences in the proportions of red, yellow, and white females of *S. globosum* found on flowers in Sites 1, 2, and 3.

Site	Flower Species			Flower Colour		
	X^2	df	*P*	X^2	df	*P*
1	3.406	5	0.638	1.367	2	0.505
2	5.238	6	0.514	5.075	4	0.280
3	1.403	2	0.496	0.007	1	0.936

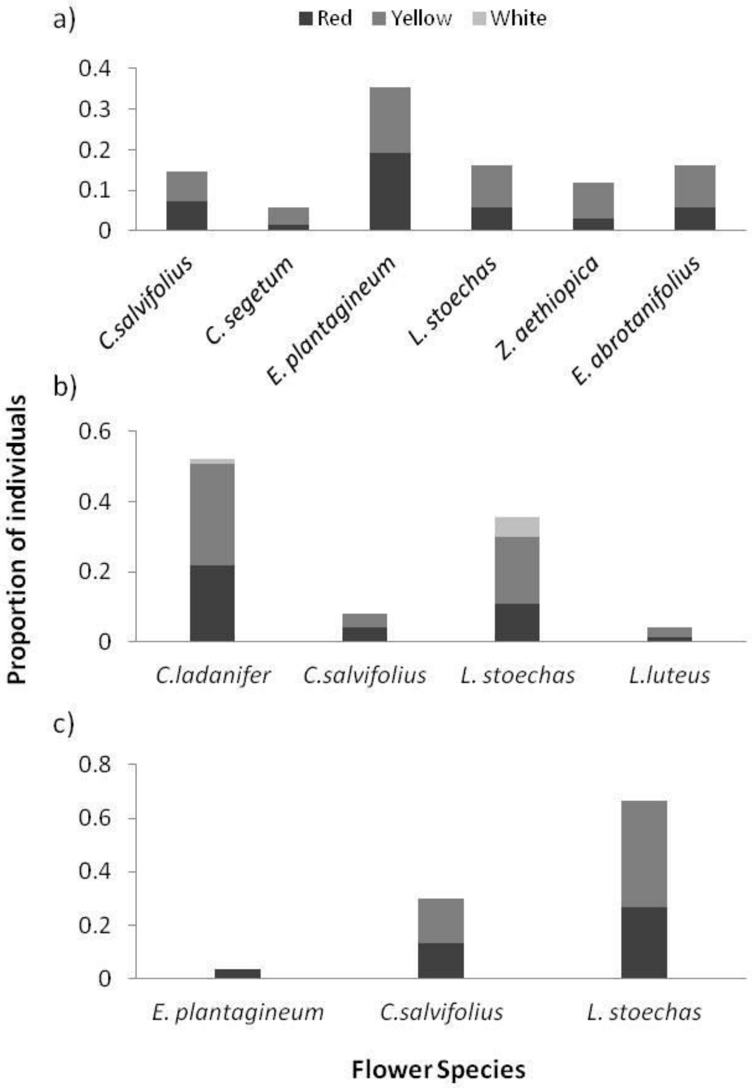

Figure 3.11. Proportion of individuals of red, yellow and white morphs of *S. globosum* found on different species of flowers at a) Site 1, b) Site 2, and c) Site 3.

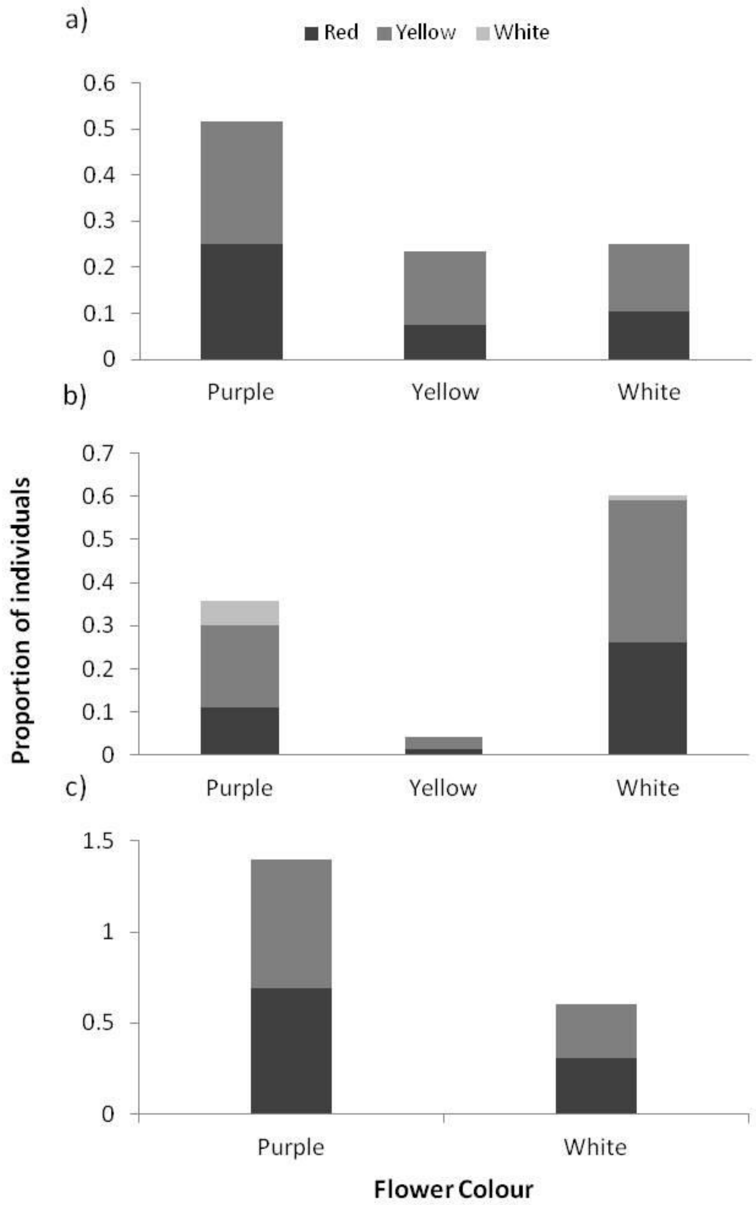

Figure 3.12. Proportion of individuals of red, yellow and white morphs of *S. globosum* found on flowers of different colours at a) Site 1, b) Site 2, and c) Site 3.

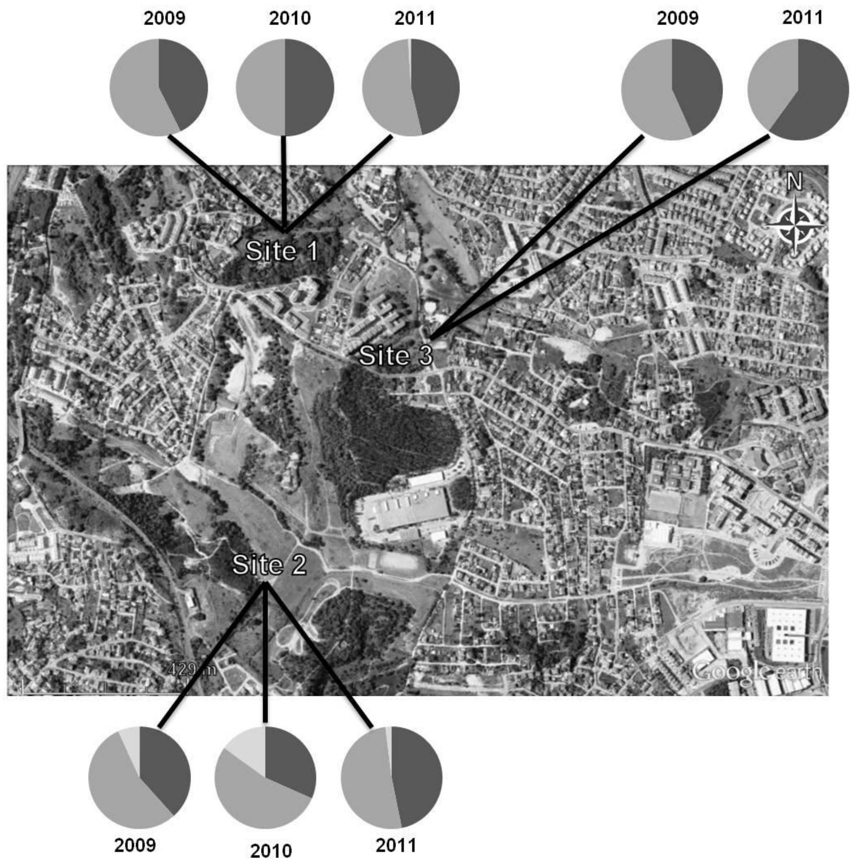

Figure 3.13. Morph frequencies of red (dark grey), yellow (grey), and white (light grey) morphs of *S. globosum* in Site 1 in 2009, in 2010, and in 2011 (red = 97, yellow = 114, white = 1); in Site 2 in 2009, in 2010, and in 2011 (red = 143, yellow = 177, white = 18); and in Site 3 in 2009, and in 2011 (red = 22, yellow = 23, white = 0).

3.3.4 Regional distribution of morphs

There was no effect of altitude, latitude, longitude, or any of the 18 climatic variables on the distribution of morph frequencies of red, yellow and white females of *S. globosum* obtained from photographs taken across the Iberian Peninsula (Table 3.7). All three morphs showed a similar distribution in this region (Figure 3.14). However, a significant difference was found in the frequencies of the three morphs between the Iberian Peninsula as a whole and my study area (across all sites and years) (Chi-squared test: $X^2 = 6.069$, df = 2, $P = 0.048$). The data collected from the photographs taken in the Iberian

Peninsula showed a slightly higher proportion of white females than I recorded at my study area (Figure 3.15). Nevertheless, the frequencies are broadly similar, with red and yellow females being approximately equally common and white females being much rarer.

Table 3.7. Results of multinomial log-linear model testing the effects of altitude, latitude, longitude, and 18 climatic variables on the distribution of the frequencies of red, yellow, and white females of *S. globosum* across the Iberian Peninsula.

Term	X^2	df	*P*
Altitude	0.054	2	0.974
Latitude	1.869	2	0.393
Longitude	0.851	2	0.653
Annual Mean Temperature	0.033	2	0.984
Mean Diurnal Range	5.865	2	0.053
Isothermality	0.004	2	0.998
Temperature Seasonality	2.380	2	0.304
Max. Temperature of Warmest Month	0.374	2	0.830
Min. Temperature of Coldest Month	2.843	2	0.241
Mean Temperature of Wettest Quarter	3.287	2	0.193
Mean Temperature of Driest Quarter	0.575	2	0.750
Mean Temperature of Warmest Quarter	3.313	2	0.191
Mean Temperature of Coldest Quarter	2.258	2	0.323
Annual Precipitation	2.029	2	0.363
Precipitation of Wettest Month	1.400	2	0.496
Precipitation of Driest Month	2.201	2	0.333
Precipitation Seasonality	0.918	2	0.632
Precipitation of Wettest Quarter	2.029	2	0.363
Precipitation of Driest Quarter	3.342	2	0.188
Precipitation of Warmest Quarter	1.742	2	0.419
Precipitation of Coldest Quarter	4.004	2	0.135

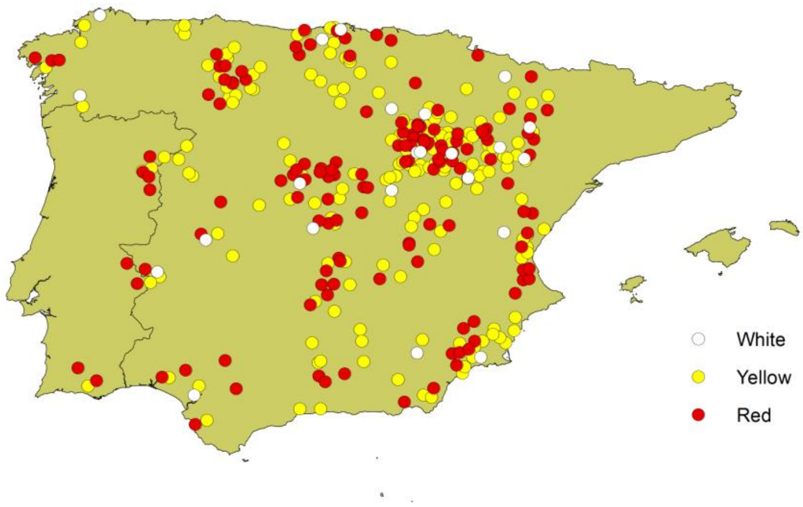

Figure 3.14. Distribution of red (n = 137), yellow (n = 185), and white (n = 22) females of *S. globosum* across the Iberian Peninsula.

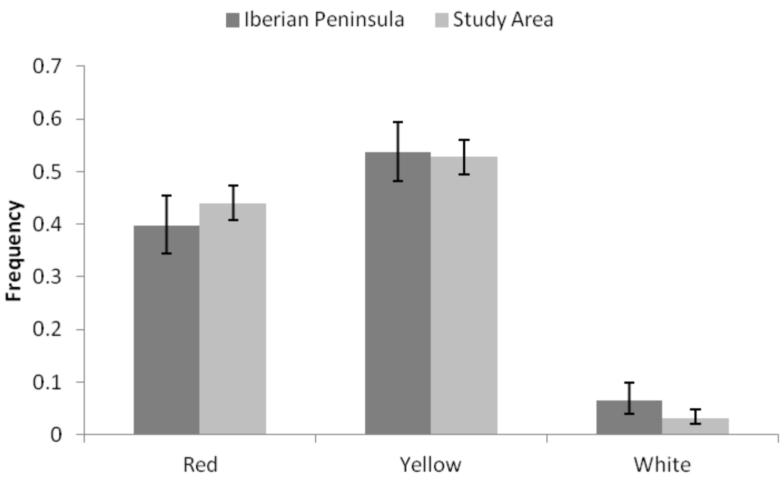

Figure 3.15. Frequencies of red, yellow, and white females of *S. globosum* obtained from photographs from the Iberian Peninsula and from surveys carried out in the study area (across all sites and years). Error bars are 95 % confidence intervals calculated using the binomial distribution.

3.4 Discussion

The results presented here on the nature of the polymorphism of *S. globosum* showed that the red, yellow, and white morphs appear to be discrete based on their spectral properties and how they are perceived by honeybee and crab spider vision. There was limited evidence that colour morphs differed in morphology and ecology: there was significant variation among morphs in size and the time they spent on a particular flower, but there was no such variation in components of reproduction. Morphs also showed weak variation in frequency in space, at an intermediate spatial scale (from site to site), and in time (across years). However, morphs did not vary significantly in the frequencies in which they occurred on different flower species and colours, or at a regional scale. These results provide important information that contributes to our understanding of the origin and maintenance of the polymorphism in this species.

3.4.1 Colour variation among morphs

Red, yellow, and white morphs of *S. globosum* differed in the regions of the spectrum at which they showed substantial reflectance in both years that they were measured. The differences in reflectance spectra among morphs were found to be detectable by the visual system of the honeybee and the crab spider *M. vatia*. Honeybees might play an important role in the maintenance of the polymorphism of *S. globosum* (see Chapter 5), since spiders have frequently been observed to prey on them and honeybees have been shown to be able to discriminate colours (Chittka and Menzel 1992; Chittka 1996; Hempel de Ibarra et al. 2002; Giurfa 2004; Dyer and Neumeyer 2005; Srinivasan 2010; Dyer et al. 2011). Similarly, if we assume that the visual system of crab spiders is reasonably consistent across the family, we can infer from the results presented here that male *S. globosum* will be able to discriminate among females of different colours. This suggests the possibility of mate choice as a factor contributing to the maintenance of the polymorphism (see Chapter 6).

Although honeybees and crab spiders should be able to detect the difference between the three female morphs of *S. globosum*, some details of their visual systems argue against a role for either sexual selection or selection by prey in the evolution of the polymorphism. The long-wave photoreceptors of honeybees and crab spiders have the highest sensitivity at wavelengths of 535 nm and 520 nm respectively, and hence the ability of the two species to be able to perceive red colour on females (which reflects light only above ~550 nm) as distinct from black, which is the colour of the rest of the cephalothorax, may be limited. Consequently, the evolution of red colour from a hypothetical black ancestral state in *S. globosum* as a result of a selective pressure from honeybees or mate choice may be intuitively implausible. Nevertheless, it has been argued that honeybees can actually perceive red colour since the spectral range covered by the long-wave photoreceptor (535 nm) has an extended tail reaching longer wavelengths, and approaches zero only at around 650 nm (Chittka and Waser 1997). Since the spectral range of the long-wave receptor of *M. vatia* extends to similar wavelengths as the long-wave receptor in the honeybee (see Defrize et al., 2011), it could thus be possible that crab spiders are also able to see red colour on females as distinct from black. Further empirical studies are necessary to clarify the situation. Even if bees and spiders cannot distinguish it from black, red colour in females may still be adaptive, perhaps if yellow or white pigments are ancestral, and selection favoured a modification to red over a change to black because the transition was either more easily achieved or less costly. Alternatively, the evolution of the red morph could implicate other forms of selection pressure, such as that resulting from predation by vertebrate predators, which can see red clearly (e.g. birds; Bowmaker et al. 1997). Exploring these ideas in future studies would help with the understanding of the maintenance of this polymorphism.

3.4.2 Differences in life history traits among morphs

Morphology

A significant difference in size was found among *S. globosum* colour morphs. White females had a significantly larger cephalothorax than red and yellow females. This difference in size could be explained by a faster development of individuals of this morph than individuals of the yellow and red morphs. At the time of year when the survey was carried out, not all spiders had reached maturity and it was not always possible to differentiate between juveniles and adults. Therefore, it is possible that red and yellow females were at an earlier developmental stage than white females when they were measured and thus have a smaller size. If this was the case, a faster development of white females could be explained in the context of negative frequency-dependent selection generated by prey (e.g. honeybees). If honeybees learn to avoid spider colour morphs that they encounter more frequently on flowers, then, since the white morph of *S. globosum* occurs at much lower frequencies than the red and yellow morphs, white females would be expected to have a higher probability of capturing prey. Spiders that feed on a greater quantity of prey items have been observed to develop in shorter time (Mayntz et al. 2003). A faster developmental rate might result in an earlier onset of reproduction which could represent a fitness advantage (Roff 1992). However, proper tests quantifying developmental rates of the different morphs are necessary to establish any variation among them.

Behaviour in the field

Yellow females remained for significantly longer on a given flower than red or white females (on average, 3.5 times longer than red and white females). Remaining for longer periods on a particular flower might result from the adoption of alternative foraging strategies. Prey capture has been found to be an important factor affecting the time that individuals of the crab spider *M. vatia* spend on a given flower (Morse 2000). Individuals of *M. vatia* that captured prey remained significantly longer on a flower than those that did not catch prey. Consequently, yellow *S. globosum* might remain longer on a particular flower if they capture prey more frequently than red and white females. More detailed information about *S. globosum's* foraging behaviour, diet composition, and the way they are perceived by prey is necessary to establish whether the differences observed are in fact indicative of alternative foraging strategies adopted by females of different morphs.

Reproduction

No difference among morphs was found in the components of reproduction measured in this study in laboratory conditions. This could suggest that the three morphs have similar fitness. However, in natural conditions females might be limited by the availability of prey or the duration of the season (Morse 1994), and therefore not be able to produce more than one or two clutches of eggs. If white females in fact develop faster than red and yellow females, they might be able to produce egg sacs earlier and thus have the possibility to produce more than one clutch in a given season. Although these results provide some information about particular components of reproduction of the different morphs, complete measures of fitness in natural conditions were not available in this study. If any adaptive advantage to one particular colour morph over the others is to be confirmed, the effect of colour on other aspects of reproduction (ideally total lifetime reproductive success) and survival (from egg to adult) should be quantified.

3.4.3 Spatial and temporal variation in morph frequencies

No significant effect of flower species or flower colour was found on the proportion of red, yellow, or white females of *S. globosum* observed on different types of flowers. These results do not support the role of a selection pressure at the microhabitat level in the maintenance of the colour polymorphism. Nevertheless, there are other variables that could have an effect in the variation of microhabitat choice among colour morphs, such as quantity of light (Hazel and Johnson 1990) or other abiotic variables that might not select for colour directly (Slotow and Ward 1997).

Morph frequencies showed some weak variation among sites and years. In 2010 in Site 2, red spiders were found at lower frequencies and white spiders were found at higher frequencies than in Site 1 in the same year. Morph frequencies in 2010 in Site 2 also differed significantly from frequencies observed in 2009 and 2011 in the same site. Since the regional distribution of morph frequencies was not affected by any of the climatic variables considered in this study, it seems unlikely that the difference in morph frequencies found at a local spatial scale among years could be explained by changes in climate. A more plausible explanation could be the occurrence of highly localised frequency-dependent and/or density-dependent selective regimes influenced by sexual selection (Gosden and Svensson 2008) and/or predation pressure (Sherratt 2006). In the case of *S. globosum*, frequency-dependent selection could also be driven by avoidance of spiders by prey (see Chapter 5). Differences in morph frequencies among sites and years could also be a result of limited dispersal of spiderlings, which would cause some clustering of morphs in each site. Hence, the increased abundance of white spiders at Site 2 in 2010 could simply be the result of one or two successful broods of spiderlings from a single white mother remaining close to the maternal nest site. However, the stability of morph frequencies among sites, and within sites in 2009 and 2011, and at a regional scale, suggests that whatever selective pressures are acting on the polymorphism of *S. globosum* do not vary greatly at a local or a regional scale.

3.4.4 Conclusion

The results reported in this chapter do not provide strong evidence in favour of any particular mechanism operating to maintain the colour polymorphism in *S. globosum*. However, some of the observed patterns suggest the occurrence of certain mechanisms. Evidence supporting the discrimination of the colour morphs by the visual systems of honeybees and crab spiders makes it plausible that interactions with prey and mates could generate NFDS mechanisms that influence the maintenance of the polymorphism. Although the distribution of morph frequencies at a regional scale were not affected by factors related to climate, and some variation could be observed at a local scale across years, the overall spatial and temporal stability of morph frequencies indicate that some selective agent is likely to be in operation, which could be acting in combination with limited gene flow. The larger size of the rare white morph could indicate an advantage resulting from negative frequency-dependent selection generated by prey, and the longer time spent on a particular flower by the yellow morph could indicate the occurrence of alternative foraging strategies that could balance fitness costs and maintain a stable polymorphism. However, the influence of stochastic processes such as genetic drift cannot be dismissed and it is possible that both selective and stochastic processes are acting to maintain the polymorphism (Cook 1998; Oxford 2005). The results obtained here, although not conclusive, hint at mechanisms likely to be operating to maintain the colour polymorphism in *S. glbosoum* and that require further research to be able to determine their actual role in this process.

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Chapter 4: The heritability of the colour polymorphism

4.1 Introduction

The presence of different visible forms (morphs) in a population can be a result of genetic variation among individuals (Hedrick 2000). Alternatively, if variation in a phenotypic trait is not genetically determined, it may result from differences among developmental stages (Shine et al. 2000), or as a response to variation in health, condition or the environment (Brakefield and Larsen 1984; Hill and Montgomerie 1994; Tanaka 2004). Therefore, if the occurrence of a polymorphism in a population is to be explained, it is essential first to establish its origin. In the case of genetically-determined polymorphisms, knowledge about their genetic control, and related inferences about allele frequencies in natural populations, can be used to identify mechanisms which may be acting to maintain them (Hedrick 2000).

Conspicuous polymorphisms, such as those involving colouration, offer the possibility to infer the genetic mechanism by which they are controlled without the need of molecular techniques. This can be accomplished through breeding experiments where crosses of individuals of known phenotypes are carried out. This approach has been employed to determine the genetic control of polymorphisms in a number of systems from different taxonomic groups which have contributed to the understanding of the maintenance of genetic diversity (Clarke and Sheppard 1973; Oxford and Gillespie 1996; Seehausen et al. 1999; Roulin and Dijkstra 2003). One of the best known examples is the peppered moth *Biston betularia*, which exhibits light, intermediate, and dark colour morphs. In this species, higher frequencies of the dark morph have been found to be associated with areas with more pollution which produce darker backgrounds, and this has been explained as a result of a selection pressure by avian predators (Kettlewell 1973; Cook and Turner 2008). The polymorphism in the peppered moth has been shown to be controlled by multiple alleles at a single locus, where the allele of the dark morph shows complete dominance over the intermediate and light morphs, and several alleles of the intermediate morphs show an approximate hierarchy of incomplete dominance over the light morph (Kettlewell 1973).

Colour polymorphisms in some species have been found to be controlled by more complex genetic mechanisms. A notable example is the land snail *Cepaea nemoralis,* which is polymorphic for several elements of shell colouration. A number of classical genetic studies on this polymorphism have revealed that the different colour patterns are controlled by a supergene of tightly linked loci consisting of two or more alleles (Jones et al. 1977). Knowledge about the genetic control of the polymorphism in *C. nemoralis,* along with studies on the variation in morph frequencies observed in natural populations and molecular analyses of allele frequencies, have indicated that the polymorphism is most likely maintained by multiple mechanisms, both stochastic and selective, operating simultaneously (Jones et al. 1977; Cook 1998; Davison and Clarke 2000; Bellido et al. 2002) (see Section 1.4).

Female-limited colour polymorphisms, like that observed in *S. globosum*, have been most extensively studied in damselflies, and the mode of inheritance has been investigated in five species: *Ischnura graellsii, Ischnura elegans, Ischnura damula, Ischnura demorsa,* and *Ceriagrion tenellum*. These species show two or three female colour morphs, one which resembles the male, and one or two different morphs that are easily distinguished from the male. Crosses in the laboratory showed that the polymorphism in all species is controlled by one autosomal locus with two or three alleles, which have the same hierarchical dominance in *I. graellsii and I. elegans* but not in *I. damula, I. demorsa*, and *C. tenellum* (Johnson 1964, 1966; Cordero 1990; Andres and Cordero 1999; Sanchez-Guillen et al. 2005). Sánchez-Guillén et al. (2005) suggest that these differences in hierarchical dominance indicate alternative ancestral scenarios for the polymorphic locus in these species. The most common explanation for the maintenance of female-limited colour polymorphisms in damselflies is negative frequency-dependent selection generated by harassment of females by males (see Section 2.1.2).

Female-limited colour polymorphisms have also been found to be controlled by more complex genetic mechanisms. For example, females of the brown anole lizard (*Anolis sagrei*) show three different dorsal colour patterns, and the genetic model that best fits the data obtained from a breeding study is a two-locus model with recessive epistasis (Calsbeek et al. 2009). In this model, one allele is dominant at the first locus, but two copies of the recessive allele at the second locus override the expression of the non-dominant phenotype and produce the dominant phenotype. The polymorphism has been proposed to be maintained by temporal variation in selection with the protection of alleles through a two-locus inheritance mechanism which is analogous to heterozygote advantage (Calsbeek et al. 2009).

Although breeding studies have been useful in identifying the genetic control of conspicuous polymorphisms in many model systems, which has been fundamental to explaining the maintenance of such diversity, the characterisation of genetic mechanisms at a molecular level can yield further insights into the evolutionary biology of polymorphisms. Considerable progress has been made in this area in recent years, which has provided a more complete understanding of the processes involved in the maintenance of genetic variation (Mitchell-Olds et al. 2007; Kronforst et al. 2012). For example, the wing-pattern polymorphism in the mimetic butterfly *Heliconius numata* is known to be controlled by multiple alleles in a single supergene locus (Brown and Benson 1974). Using fine-scale genetic mapping and positional cloning, Joron et al. (2011) showed that the different colour morphs in *H. numata* are associated with different rearrangements of the genome at the supergene locus. These genomic rearrangements locally limit recombination and gene flow, allowing the persistence of multiple phenotypes (Joron et al. 2011).

4.1.1. Objectives

Since establishing the origin of phenotypic variation in a population is crucial for investigating the mechanisms that maintain diversity, the aim of this chapter is to determine if the colour polymorphism observed in *S. globosum* is genetic in origin. In order to accomplish this, a breeding experiment was carried out in the laboratory at the University of Nottingham using females collected from the field at the study area in Portugal. The ultimate objective of the experiment was if possible to determine the mode of inheritance of the polymorphism, but logistical constraints and the biology of the system (most notably that males do not express the trait in question) meant that a formal consideration of competing models of inheritance was not possible.

4.2. Materials and methods

Adult females of *S. globosum* were collected from the field at the Quinta de São Pedro Study Centre and surrounding areas in April 2009 (see Section 2.2.1. for description of study area). Females were kept in the laboratory at the Quinta de São Pedro Study Centre, and housed individually in petri dishes with a small piece of moist cotton wool to maintain humidity. Each female was fed twice a week a hoverfly, housefly, or blowfly caught in the field twice a week. Red (n = 14), yellow (n = 16), and white (n = 3) females that produced egg sacs were transported to the United Kingdom and kept in the laboratory at the University of Nottingham. Some of these females were used in mating experiments that were carried out to investigate the role of mate choice in the maintenance of the polymorphism (see Chapter 6). Therefore, some females used in this breeding study were mated with known males in the laboratory, and some mated with unknown males in the field. Nevertheless, since males of *S. globosum* do not show colour variation, it can be assumed that females were randomly selected with respect to genotype of males they mated with. Females were kept at a constant temperature of 22°C, and were housed and fed in the same way as in the Quinta de São Pedro Study Centre. However, females with egg sacs did not feed as often as they did prior to laying egg sacs (casual observation).

The moment spiderlings were observed to have hatched from the egg sacs, they were removed from the petri dish and placed in separate 7 ml Perspex tubes. A piece of cotton wool was used to cover the tubes, and was moistened regularly to maintain humidity. Newly hatched spiderlings were fed on *Drosophila melanogaster* flies reared in the laboratory and purchased from Blades Biological, Ltd. Early and intermediate instar spiders were fed on black field crickets (*Gryllus bimaculatus*) purchased from Livefoods Direct, and houseflies (*Musca domestica*) purchased from Dartfrog. Late instar and adult spiders were fed on different species of hoverflies and blowflies caught in the field as well as houseflies (*M. domestica*) purchased from Dartfrog. Spiders were fed twice a week. Mothers were kept in petri dishes and fed regularly until death. Dead spiders were preserved in 7 ml Perspex tubes with 70 % alcohol. Females were able to produce up to three clutches of eggs. Due to time constraints, only second instar spiderlings from 23 second clutches were measured and weighed. The length of spiderlings was measured from the anterior end of the cephalothorax to the posterior end of the abdomen under a stereo microscope using an eye-piece graticule. They were weighed using a digital balance. Once spiders started to show signs of development of adult colouration (i.e. showing variation in colour from a previous uniform beige/cream colouration across all spiders), or sexual maturity in the case of the males (i.e. swelling of the palps), sex and colouration were recorded approximately every month until the end of the breeding experiment. Owing to time limitations and deaths of spiders approximately fifteen months after the first spiderlings hatched, the breeding experiment was ended. Surviving spiders were killed by freezing them.

4.2.1 Statistical analyses

Chi-squared tests were conducted to investigate differences between the sex ratio observed in spiders reared in the laboratory and the one observed in spiders in the field, and to test for differences between morph frequencies observed in the laboratory and those observed in the field. Generalised linear mixed models (GLMMs) with binomial error structures and mother of clutch as a random effect were used to test the effect of maternal colour morph on the probability of a daughter having red, yellow, or white colouration. The significance of each term was assessed by backward deletion from a saturated model using a Chi-squared test statistic following Crawley (2007) and Zuur et al. (2009). Statistics are presented for comparisons of models after each term was removed. Data were pooled across clutches. All analyses were conducted in R 2.12.2 (The R Development Core Team 2011).

4.3 Results

A total of 1864 offspring hatched from 68 broods produced by 33 females in the laboratory. Spiderlings took approximately fourteen months to reach maturity. Of the offspring that hatched, 28.9 % survived until the end of the experiment. Only 12.8 % could be scored as a male, or a female of one of the three colour morphs. Red daughters started to become distinctly red approximately six months before it was possible to determine the phenotype of yellow or white daughters. Most yellow and white daughters showed a beige/cream colouration before developing a distinct yellow or white colour. Females that could not be assigned to a phenotype had beige, cream, gray, pink, or almost completely black colouration. The sex ratio obtained from the spiders that could be scored as female or male did not differ significantly from 0.5 (Chi-squared test: $X^2 = 0.406$, df = 1, P = 0.524), and differed significantly from the female-biased sex ratio observed in the field (comparison with data presented in Section 2.3.3: $X^2 = 71.258$, df = 1, P < 0.001).

Morph frequencies of daughters for which the phenotype could be determined differed significantly from those of females observed in the field ($X^2 = 96.353$, df = 2, P < 0.001). Red and white spiders were significantly more common in the laboratory than in the field, and yellow spiders were rarer (Figure 4.1). A significant effect of mother colour morph was found on the probability of a daughter having red or white colouration: daughters had a higher probability of having red colouration if they were produced by red mothers, and had a higher probability of having white colouration if they were produced by white mothers (Table 4.1 and Figure 4.2). Although yellow offspring were more common amongst the offspring of yellow mothers than amongst the offspring of other mothers, there was no significant effect of maternal colour on the probability that a daughter would be yellow.

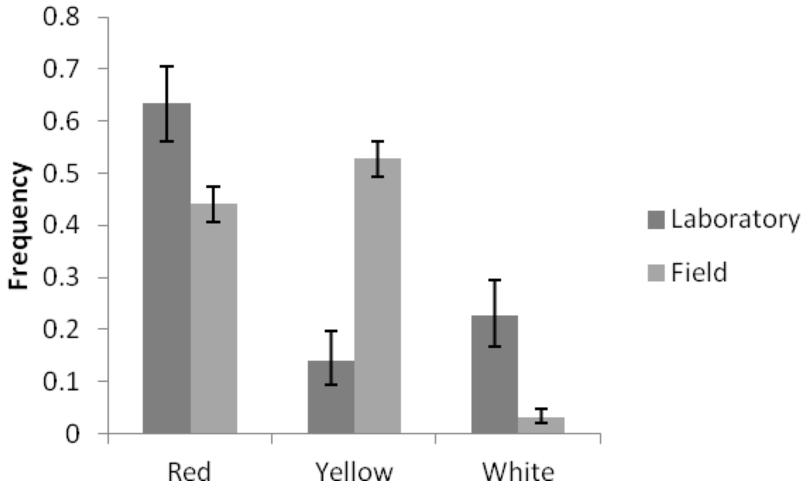

Figure 4.1. Frequencies of red, yellow, and white female *S. globosum* either reared in the laboratory from egg sacs produced by females collected from the field, or observed in the field. Error bars are 95 % confidence intervals calculated using the binomial distribution.

Table 4.1. Results of GLMMs with binomial errors testing the effect of maternal colour morph on the probability of a daughter having red, yellow, or white colouration. A separate model was fitted for each offspring colour.

Offspring colour	X^2	df	P
Red	22.519	2	**< 0.001**
Yellow	5.367	2	0.068
White	8.721	2	**0.013**

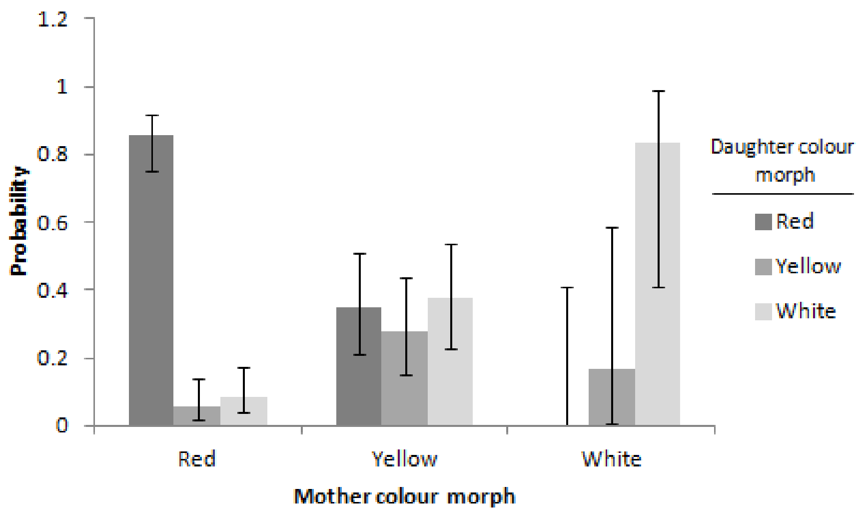

Figure 4.2. Probability of red, yellow, and white mothers producing red, yellow, or white daughters, across clutches, in the laboratory. Error bars are 95 % confidence intervals calculated using the binomial distribution.

4.4 Discussion

The results presented in this chapter provide evidence supporting a genetic origin of the colour polymorphism in *S. globosum*. Nevertheless, owing to the high mortality rate of spiders reared in the laboratory and the amount of time that they took to develop adult colouration, only a small percentage of females could be sexed and classified as belonging to one of the three colour morphs. Therefore, it was not possible to determine with any certainty whether one of a number of candidate genetic models would fit the data. Nevertheless, the genetic models that were considered, and the reasons why they were thought not to be valid, are discussed here.

The sex ratio of the spiders that were able to be classified as female or male did not differ significantly from 0.5, but differed significantly from the female-biased sex ratio observed in the field. This could be a result of the difficulty of determining the sex of most spiders. However, if the sex ratio observed in the sample of spiders that were scored is representative of the primary sex ratio in *S. globosum*, then it would suggest that males have a higher mortality rate, or are more difficult to sample, than females in the field. A higher mortality rate of males could be explained by a higher vulnerability to predators resulting from a higher rate of movement due to mate-searching (Vollrath and Parker 1992; Kotiaho et al. 1998; Schütz and Taborsky 2003). Indeed, male *S. globosum* appear to move more frequently than females (see Section 2.4.5). Higher mortality of males could also result from a lower resistance to cold temperatures, which would lead to a decrease in the number of males over winter (Gunnarsson 1987), or from the presence of male-killing bacteria in the population (Goodacre et al. 2006; Duron et al. 2008). However, since the search of spiders was mostly concentrated on flowers during surveys of populations of *S. globosum*, the possibility of overlooking individuals moving about in other parts of the vegetation cannot be discarded.

There was also a significant difference between the laboratory and the field in the observed frequencies of *S. globosum* colour morphs. Red and white females were more common in the laboratory, and yellow females were rarer. The overall pattern differed in that red and white females were more common than yellow females in the laboratory, while yellow females were slightly more common than red females and much more common than white females in the field. One possible explanation for the observed difference is variation in the time to develop adult colouration. Red females developed adult colouration approximately six months earlier than yellow or white females. Therefore, if yellow females took longer than white females to develop adult colouration in the conditions under which they were reared, it is possible that yellow females died or were killed (when the experiment was ended) before their colouration could be determined. Such differences in rates of development in colouration have been observed in the colour polymorphic Theridiid spiders *Enoplognatha ovata* and *Theridion grallator* (Oxford 1983; Oxford and Gillespie 1996). In these species, some of the colour morphs observed in populations of adult individuals develop later than other morphs. Another possible explanation is that the populations of *S. globosum* sampled in the field are not found in Hardy-Weinberg equilibrium (Hedrick 2000). However, it would be necessary to determine the mode of inheritance of the polymorphism in order to establish if this is the case.

Maternal colour morph had a significant effect on the probability of daughters having red or white colouration. Daughters had a higher probability of having red colouration if they were produced by red mothers, and a higher probability of having white colouration if they were produced by white mothers. These results strongly suggest that the three colour morphs observed in *S. globosum* are genetically inherited. Late development of adult colouration in yellow daughters (mentioned above) could account for the non-significant effect of mother colour morph on the probability of daughters having yellow colouration.

Two genetic models were considered to explain the mode of inheritance of the colour polymorphism in *S. globosum*. The first model was that female colour is determined by three alleles at a single autosomal locus with a hierarchy of dominance. This genetic model has been found to fit the phenotype frequencies obtained from breeding studies and observed in the field in the female-limited colour polymorphic damselflies *I. graellsii, C. tenellum,* and *I. elegans* (Cordero 1990; Andres and Cordero 1999; Sanchez-Guillen et al. 2005). Assuming that the daughters of spiders in the present experiment which it was possible to assign a phenotype are an unbiased sample, however, this model is a very poor fit for the observed colour morph frequencies in the F1 generation, regardless of which dominance relationship among alleles is assumed (results not shown).

The second genetic model considered was a model of two alleles at a single autosomal locus where the red allele (R) has incomplete dominance over the white allele (W), and thus heterozygous females (RW) show yellow colouration. This model was also a very poor fit to the data. A modification of the model was also considered, in which yellow females are assumed to take longer to develop adult colouration and may look white before becoming yellow, and therefore a proportion (p) of the heterozygous daughters are assumed to be incorrectly classified as white. A later development of colouration in a morph that results in immature individuals showing the same colour, but adult individuals showing different colours has been observed in lizards (Carpenter 1995; Sinervo and Zamudio 2001). This modified model with p = 0.5 was a fairly good fit to the data obtained from spiders reared in the laboratory, but the morph frequencies observed in the field deviated from those expected under Hardy-Weinberg equilibrium. Additionally, morph frequencies obtained from one survey carried out early in the season where immature individuals were abundant (see Section 2.2.3), did not show a higher frequency of white females. Moreover, white spiders were never observed to change to yellow colouration during development in the laboratory. Almost all spiders reared in the laboratory showed a cream/beige colouration before becoming red, yellow, or white, with the exception of some females which showed very pale yellow colouration before becoming white.

4.4.1 Conclusion

The results obtained from the breeding study provide strong evidence for a genetic origin of the colour polymorphism in *S. globosum*. The apparent longer developmental rate of yellow colouration (at least in the conditions of this study), and the high mortality of spiders in the laboratory, did not allow to determine a model for the genetic control of the polymorphism. More breeding studies are necessary to establish accurately the developmental rates of the different morphs and the mode of inheritance of the polymorphism. Nevertheless, establishing that the polymorphism in *S. globosum* is genetic in origin allows for the investigation of the role of particular selective mechanisms in the maintenance of the polymorphism in the subsequent two chapters.

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Chapter 5: Effects of *Synema globosum's* colour morphs on honeybee behaviour

5.1 Introduction

To be able to determine the mechanism or mechanisms that maintain a polymorphism it is essential to know the effect(s), if any, that the phenotypic differences among morphs have on fitness. Such effects can be a result of interspecific and/or intraspecific interactions and can lead to the fixation of one morph, if the polymorphism is transient, or to balancing mechanisms such as negative frequency-dependent selection (NFDS) (see Chapter 1). Because of the characteristics of the study system in this investigation (see Chapter 2), interactions between *S. globosum* and one of its main prey, the honeybee (*Apis mellifera* L.), are of particular interest in this context.

5.1.1 Frequency-independent selection by prey

The effect of a prey's behaviour towards one of its main predators can potentially lead to directional, frequency-independent selection if it causes one morph to have a significantly higher fitness than the alternative morphs (see Section 3.1). In the case of *S. globosum*, if honeybees show lower aversion towards a particular female morph, then that morph will presumably have a higher probability of successfully attacking honeybees, and have increased survival and/or fecundity as a result. Assuming that this selection is more potent than drift, it will eventually lead to fixation of the genotype which codes for the less aversive morph (Bell 1997). However, the process of fixation may be slow, and polymorphism may be observed during transition (Mitchell-Olds et al. 2007).

There is an alternative scenario in which directional selection could actively maintain the evolution of phenotypic diversity in a population. The response of predators towards their prey could depend on the local environment, so that the different morphs are more successful at catching prey in different microhabitats or at different times (e.g. if they forage on different flower species). If offspring are more likely to be found in habitats inhabited by their parents, a selection mosaic might exist which favours the persistence of the polymorphism in the population as a whole (Kondrashov and Mina 1986; Forde et al. 2004). Thus, although there is no evidence for microhabitat specialisation in *S. globosum* morphs (see Section 3.3.3), directional selection, which may or may not vary with habitat type, could still have an important effect on the maintenance of the polymorphism.

5.1.2 Frequency-dependent selection by prey

Although few empirical studies exist, NFDS of predator traits caused by prey has the potential to promote the maintenance of polymorphisms (see Section 1.5.2; Paulson 1973; Hori 1993; Roulin and Wink 2004; Arcos 2007). Perhaps the best evidence supporting the idea that prey can have an effect on the fitness of predator morphs in a frequency-dependent manner has been obtained in a study with the scale-eating cichlid fish *Perissodus microlepis* (Hori, 1993). Populations of *P. microlepis* show a genetically determined polymorphism for handedness. Right-handed individuals always attack fish from the left side to snatch several scales off their flank to feed, while left-handed individuals always attack from the right side. Frequencies of the two morphs oscillated around unity in a period of 11 years. This is explained by an increase of the prey's guard on the side from which they are more frequently attacked, causing the most abundant morph to feed less often. The commonest handedness morph among breeding pairs was shown to be the opposite to the most common morph in the whole population, suggesting that the rare morph has a reproductive advantage over the common morph. Thus, this study demonstrates the potential of the effects of prey on predators to lead to NFDS and possibly maintain balanced polymorphisms, but very few other studies have considered the possibility, and none have actually tested for it (Paulson 1973; Roulin and Wink 2004; Arcos 2007).

In theory, NFDS could occur in any system in which prey are able to distinguish between predator morphs, associate the polymorphic trait with a potential attack, and avoid the morph that is encountered more frequently. *S. globosum* is an ideal candidate for such a system, because one of their main prey species is the honeybee (see Section 2.3.3), which is known to be able to discriminate colours (Chittka and Menzel 1992; Chittka 1996; Hempel de Ibarra et al. 2002; Giurfa 2004; Dyer and Neumeyer 2005; Srinivasan 2010; Dyer et al. 2011), appears to be able to discriminate among *S. globosum* colour morphs (see Section 3.3.1), shows associative learning (Menzel 1993; Giurfa 2007; Srinivasan 2010), and has been observed to associate colour with a negative experience (Avarguès-Weber et al. 2010).

The response of honeybees to spiders that are sit-and-wait predators has been widely investigated. Honeybees show a negative response towards the presence of spiders by avoiding flowers or orb-webs (Dukas 2001; Dukas and Morse 2003; Tso et al. 2004; Dukas and Morse 2005; Reader et al. 2006). Therefore, some spiders have evolved body colouration and patterns to appear camouflaged or to attract prey by exploiting flower signals (Hauber 2002; Théry and Casas 2002; Heiling et al. 2003; Heiling et al. 2005; Defrize et al. 2010). Spiders of different colour morphs have been observed to be perceived in different ways by honeybees. One example is the giant wood orb-weaving spider *Nephila pilipes* (formally *N. maculata*) which has two colour morphs, one that is brightly coloured (typical) and another that is totally dark (melanic) (Tso et al. 2002). Tso et al. (2004) compared prey interception rates and colour contrasts between the background and the body surface of the two morphs of *N. pilipes* in the visual colour space of the honeybee (*Apis mellifera*). They found that individuals of the melanic morph intercept significantly fewer prey than the typical morph. Colour contrast calculations showed that the brightly coloured areas of the typical morph produce visual signals similar to some food resources, while the outline of the melanic morph's body was significantly clearer to the honeybees. Therefore, the difference in prey capture can be explained by the difference in the honeybee's perception of the two colour morphs. However, it is not known to what degree, if any, this effect influences the maintenance of polymorphism in this species.

An association between perceived predation risk and (simulated) spider presence has been observed in bumblebees (*Bombus terrestris dalmatinus*) in a study which used electromechanical devices to mimic spider attacks (Ings and Chittka 2008). Bumblebees were exposed to visual cues (coloured plastic spiders) that were either cryptic or conspicuous, and received a simulated predation attempt by being held by electromechanical pincers for two seconds. Previous to the predation attempts, bumblebees visited flowers harbouring both types of cue at rates close to random. After a number of predation attempts, the rate of visits to these flowers fell exponentially, but did not differ between flowers with cryptic and conspicuous cues. However, bumblebees that experienced a predation attempt from cryptic artificial spiders showed a decrease in the speed of inspection of all flowers, which represented a change in the resolution of a speed-accuracy trade-off balancing predation risk and foraging time. These results show that bees can associate predation risk with the presence of a spider and learn to avoid it. Moreover, bees responded differently to different artificial spider colour morphs since bees that experienced attacks from cryptic morphs were slower to inspect any flower they visited than bees that experienced attacks from conspicuous morphs. Nevertheless, in this case there would not be a difference in the effect of bee responses on fitness between spider morphs because, although bees that were attacked by cryptic spiders adjusted their inspection speed, all bees were equally good at learning to avoid flowers with conspicuous and cryptic spiders. Additionally, the effect of experience with one type of cue on the response of bumblebees towards the same or the other type of cue was not investigated. Therefore it is not known whether bumblebees would more effectively avoid the type of cue that they encounter more frequently, and hence have the potential to generate NFDS on predator morphs.

Although it did not involve simulated or real predation, a study by Avarguès-Weber *et al*. (2010) showed that honeybees can associate a negative experience with a specific colour and learn to avoid it. Honeybees were trained to feed from a rewarding or a non-rewarding solution associated with a particular colour. Rewarding solutions consisted of sucrose and non-rewarding solutions consisted of either water or quinine. In a test of discrimination between dissimilar colours associated with the rewarding and non-rewarding solutions, honeybees always chose the colour associated with the sucrose solution. However, when given a choice of a colour associated with one of the non-rewarding solutions or a colour associated with a novel stimulus (a colour that was not used in the training phase), honeybees avoided the colour associated with the non-rewarding solution only when that solution contained quinine, and not when it was plain water. Thus, it is possible that bees can learn to avoid a particular colour after a recent negative experience associated with that colour. Indeed, bumblebees (*Bombus spp*.) have been observed to avoid common colour morphs in populations of unrewarding polymorphic flowers (Smithson and Macnair 1997; Gigord et al. 2001). The favoured explanation for this behaviour is that rare morphs are preferred because bumblebees have learned that the more frequently encountered morphs offer no reward (Smithson 2001). The avoidance of particular flower colours is not a result of an aversive stimulus - it is the result of a cost of visiting unrewarding flowers - but nevertheless these studies show that bees can respond in a negative-frequency dependent fashion with respect to colour.

5.1.3. Objectives

To investigate if interactions between *S. globosum* and one of its main prey, the honeybee, could lead to directional or frequency-dependent selection on female *S. globosum* morphs which might influence the maintenance of the colour polymorphism, two experiments were carried out. Experiment 1 tested whether wild honeybees from a population naturally exposed to crab spider predation responded differently to the different colour morphs of *S. globosum* on different types of flowers. If honeybees showed a higher propensity to visit flowers that harbour a particular spider colour morph in this experiment, it could suggest that directional selection is operating in favour of that morph and/or be a consequence of an interaction between the frequencies of female morphs present in the population from which honeybees were tested and honeybee behaviour. Additionally, if honeybee responses to a particular spider morph depend on the species of flower where the spider is found, this could be indicative of habitat-specific directional selection. Experiment 2 tested for an effect of a recent previous negative experience with a particular colour morph of *S. globosum* on the subsequent response of honeybees to spiders of the same or a different morph. In this experiment, a decrease in the propensity to visit a flower with a particular morph caused by a recent negative experience with that morph would be consistent with the idea that NFDS could maintain the polymorphism in *S. globosum*.

5.2 Materials and methods

All experiments were carried out in meadows and open woodland near the Quinta de São Pedro Study Centre (see Section 2.2.1 for description).

5.2.1 Experiment 1: Response of honeybees to spider colour morphs

To investigate if there was an effect of spider colour on the foraging behaviour of honeybees, flowers of sage-leaf rockrose (*Cistus salvifolium*), purple viper's bugloss (*Echium plantagineum*), and lavender (*Lavandula stoechas*) harbouring spiders were offered to honeybees. These are flower species on which *S. globosum* is commonly found at the study site (see Section 2.3.3). The following four spider treatments were used for each flower species: red female, yellow female, male, and control (N = 100 presentations per combination of flower species and spider treatment). White spiders were excluded from all experiments because they were found at such low frequencies that it was not possible to obtain proper sample sizes. Spiders were collected from different flowering plants in the area, and killed by leaving them in a Perspex tube filled with CO_2 for approximately one hour. Observations were carried out in April 2010 from 0930 to 1700 over a period of seven days. Ten spiders of each type were collected every day, and were used for all observations that were carried out on that day. Flour mixed with water was used to glue the spiders to the flowers. This type of glue was used because it appeared to have the least effect on honeybee behaviour compared to other types of glue that were tested prior to the experiment. A spider was glued to a petal, or inflorescence in the case of the lavender, where it was completely visible for honeybees. The control treatment consisted of the flower with only a drop of glue on it. Treatments were performed in blocks of time, with each combination of flower species and treatment being applied once in each block. Spiders and treatment-flower combinations were chosen randomly within blocks.

A flower of one of the three species with one of the four spider treatments was offered to a wild honeybee that was foraging on the same species of flower at the study site. The flower was held with a pair of tweezers and placed next to the flower where the honeybee was feeding. If the honeybee did not approach it, the flower was repositioned until the honeybee did approach it. When offered a flower, honeybees were observed and one of three responses was recorded (following Duffield et al. 1993; Higginson and Barnard 2004):

Accepted – the honeybee alighted on the flower and started probing for nectar.

Rejected – the honeybee inspected the flower hovering in close proximity, sometimes touching the flower with its antennae or legs, but did not alight.

Ignored - the honeybee approached the flower but changed its course without pausing or making any physical contact with it.

5.2.2 Experiment 2: Effect of spider colour associated with an attack on honeybee responses

To test if honeybees respond to spider colour after previously experiencing an attack associated with that colour, an experiment was conducted using flowers of *C. salvifolius* harbouring a spider. *C. salvifolius* was used in this experiment because it was the flower species with the highest probability of acceptance by honeybees (see results of Experiment 1 below, Section 5.3.1). Female spiders were glued to the flowers in the same way as in Experiment 1. A flower with a glued spider was attached with sticky tape to a stick measuring 60 cm in length, to minimise disturbance from the human observer to the honeybees. To simulate an attack from the spider, a teasing needle from a dissection kit attached with sticky tape to another stick of the same length was used. A flower with a spider on it was offered to a honeybee (Offer 1), followed by a second flower with a different spider on it (Offer 2). The following four female spider treatments were used for each pair of flowers: red followed by yellow, yellow followed by red, red followed by red, and yellow followed by yellow (n = 40 pairs of flowers per treatment). All observations were conducted in April 2011 between 0930 and 1700 over a period of four days. Ten spiders of each type were collected every day, and were used for all observations that were carried out that day. Spiders and treatments were assigned at random.

Two people conducted each trial. One person offered the first flower to a honeybee foraging on *C. salvifolius* repeatedly until it was accepted, or until ten rejections had been observed. When/if the flower was accepted, the same person prodded the honeybee with the teasing needle with enough strength to simulate an attack from a spider, but not too much to injure the honeybee. Rejections were recorded using the same criteria as in Experiment 1. Two types of acceptance were considered for this experiment: if the honeybee landed on the flower, but did not probe it (“accept land”), and if the honeybee landed and probed the flower (“accept feed”). For Offer 1, both people observed the honeybee and counted the number of times the flower was rejected before an acceptance. If the honeybee rejected the flower ten times, a different honeybee was chosen. After the honeybee was prodded, it was followed on its foraging flight, and the second person offered the second flower (Offer 2). Honeybees always flew away after they were prodded, but they generally stayed in the same patch of *C. salvifolius* bushes, so honeybees were lost from sight only very occasionally. Again, for Offer 2, both observers counted the number of rejections, until there were ten rejections or until the honeybee accepted the flower. An index of rejection speed was established to score the speed of the honeybee when it rejected the flower. The rejection speed index was: 1 for slow, 2 for fast, and 3 for very fast. The person who offered the second flower to the honeybee scored the rejection speed. This was to determine whether honeybees adjusted the time to reject a flower based on the perceived danger.

5.2.3 Statistical analyses

Honeybee responses in Experiment 1 were analysed using a generalised linear mixed model (GLMM) with a binomial error structure and block as a random effect. Binomial responses were: accepted vs. not accepted (reject + ignore), accepted vs. rejected (with ignored flowers excluded from the analysis), and inspected (accept + reject) vs. not inspected (ignore). Differences between the spider treatments were analysed by comparing a model containing all four treatments with a model where the three spider treatments were collapsed into one and contrasted with the control.

For Experiment 2, the differences in number of rejections of flowers by honeybees in Offer 1 between those harbouring red and yellow spiders, and among days, were analysed using a non-parametric two-way analysis of variance (ANOVA). Honeybee responses to spider treatments in Offer 2 were analysed using generalised linear models (GLMs) with binomial error structures. Three types of binary response variable were used: a) whether the honeybee accepted the flower (regardless of whether it fed: "accept feed and accept land"), b) whether it accepted *and* fed on the flower ("accept feed"), and c) whether the number of rejections of Offer 2 was higher than the number of rejections of Offer 1. For the first two of these types of variables, two separate models were fitted. Firstly, only the first attempt to offer a flower to a honeybee in Offer 2 was considered (described as "without rejections" in the results), and secondly all attempts to offer the flower (a maximum of ten) were considered ("with rejections"). Day was included as a fixed factor to account for changes in honeybee behaviour over time. For the GLMM and all GLMs, the significance of each term was assessed by backward deletion from a saturated model using a Chi-squared test statistic following Crawley (2007) and Zuur et al. (2009). Statistics are presented for comparisons of models after each term was removed. A chi-squared test was used to calculate the difference in the rejection speeds of the honeybees receiving different treatments. All analyses were conducted in R 2.12.2 (The R Development Core Team 2011).

5.3 Results

5.3.1 Experiment 1: Response of honeybees to spider colour morphs

Significant effects of spider presence and flower species were found on the probabilities that a honeybee would accept and inspect a flower for all response variables (Figures 5.1-5.3 and Table 5.1). There was a higher probability of a honeybee landing on and inspecting a flower without a spider than a flower with either a red or yellow female, or a male spider, and honeybees were more likely to inspect and accept a flower of *C. salvifolius* than a flower of *E. plantagineum* or an inflorescence of *L. stoechas* (Table 5.1). No significant differences were found among spider treatments: the effect of collapsing the spider treatments together in the statistical model was not significant for any of the response variables: accepted vs. not accepted (X^2 = 2.718, df = 6, P = 0.843), accepted vs. rejected (X^2 = 3.477, df = 6, P = 0.747), and inspected vs. not inspected (X^2 = 6.484, df = 6, P = 0.371). The interaction between spider type and flower species was not significant for any of the response variables (Table 5.1). Hence, the negative effect of the presence of a spider on bee behaviour was similar on all flower species.

Table 5.1. Results of GLMMs testing the effects of spider treatment, flower species, and the interaction between them on honeybee responses to flowers offered in the field.

Response	Spider			Flower			Spider x Flower		
	X^2	df	P	X^2	df	P	X^2	df	P
Accepted vs. not accepted	26.869	1	**<0.001**	119.21	2	**<0.001**	1.392	2	0.500
Accepted vs. rejected	17.77	1	**<0.001**	77.606	2	**<0.001**	1.640	2	0.440
Inspected vs. not inspected	8.957	1	**0.003**	48.01	2	**<0.001**	3.174	2	0.205

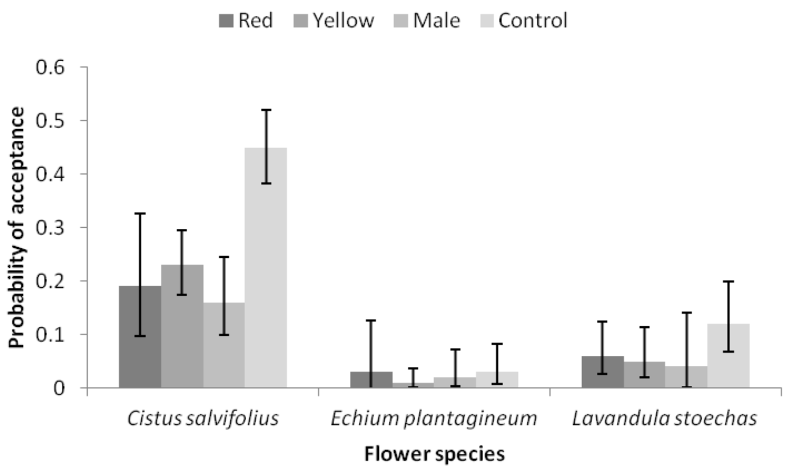

Figure 5.1. Probability of acceptance by honeybees of all flowers of *C. salvifolius, E. plantagineum,* and inflorescences of *L. stoechas*, regardless of whether they were inspected. Flowers harboured a red or yellow female spider, or a male spider, or no spider (control). Error bars are 95 % confidence intervals calculated using the binomial distribution.

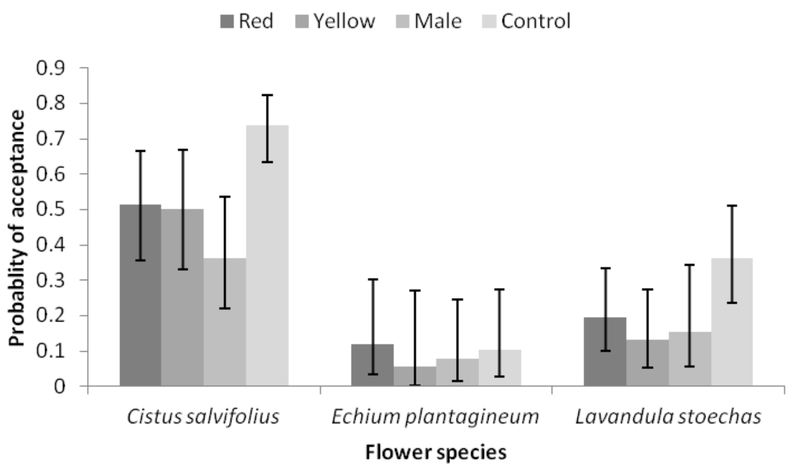

Figure 5.2. Probability of acceptance by honeybees of those flowers of *C. salvifolius, E. plantagineum,* and inflorescences of *L. stoechas*, which were inspected. Flowers harboured either a red or yellow female spider, or a male spider, or no spider (control). Error bars are 95 % confidence intervals calculated using the binomial distribution.

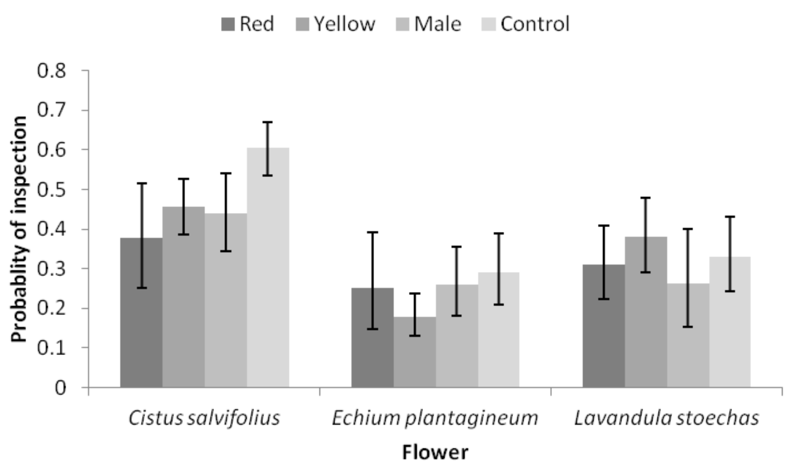

Figure 3. Probability of inspection (including acceptances and rejections) by honeybees of all flowers of *C. salvifolius, E. plantagineum,* and inflorescences of *L. stoechas*. Flowers harboured a red or yellow female spider, or a male spider, or no spider (control). Error bars are 95 % confidence intervals calculated using the binomial distribution.

5.3.2 Experiment 2: Effect of spider colour associated with an attack on honeybee responses

Consistent with the results of Experiment 1, no significant effect of spider colour was found on the number of times that honeybees rejected flowers in Offer 1 (two-way non-parametric ANOVA: H = 0.40, df = 1 P = 0.529) (Figure 5.4). In addition, no significant effect of day (H = 3.43, df = 3, P = 0.331) and no significant interaction between spider colour and day (H = 7.46, df = 3, P = 0.060) were found on the number of times that honeybees rejected flowers in Offer 1, although there was a tendency for flowers harbouring yellow spiders to be rejected more earlier in the study.

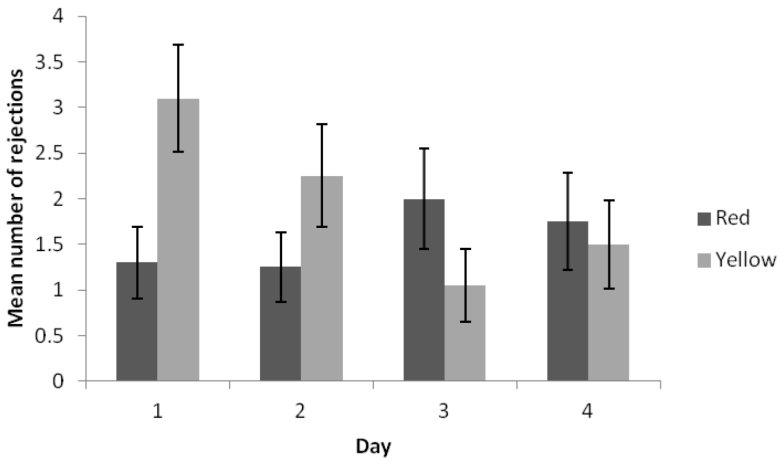

Figure 5.4. Mean number of rejections (± S.E.) of flowers that harboured red and yellow spiders in Offer 1 for each of the four days on which offers were made.

There was a significant effect of the treatment applied to the first flower to be offered (Offer 1) on the proportion of Offer 2 flowers that were accepted and probed on the first occasion that they were encountered by honeybees (analysis “without rejections” - see Table 5.2). If the flower in Offer 1 carried a red spider, a lower proportion of honeybees accepted and fed on flowers in Offer 2 than if the flower in Offer 1 harboured a yellow spider, regardless of spider colour in Offer 2 (Figure 5.5). No such effect was observed, however, on the probability of a honeybee accepting and feeding on a flower, when all attempts to offer the flower were considered (“with rejections”). Similarly, no overall effect of the treatment in Offer 1 was found on the probability of a honeybee landing on a flower, whether it fed or not (“accept feed and accept land”), considering either just the first attempt to offer the flower (“without rejections”), or all attempts (“with rejections”) (see Table 5.2).

Table 5.2. Results of GLMs testing the effects in Experiment 2 of the spider treatment in Offer 1 and the spider treatment in Offer 2, and day, on honeybee responses to flowers in Offer 2. All main effects and significant interactions are shown; interactions that were not significant for any response variable are not shown.

	Accept feed with rejections			Accept feed without rejections			Accept feed and accept land with rejections			Accept feed and accept land without rejections			Difference in number of rejections		
	X^2	df	P	X^2	df	P	X^2	df	P	X^2	df	P	X^2	df	P
Offer 1	0.008	1	0.928	4.361	1	**0.037**	0.280	1	0.597	3.747	1	0.053	0.826	1	0.364
Offer 2	0.479	1	0.489	0.466	1	0.495	0.144	1	0.704	1.038	1	0.308	0.232	1	0.63
Day	0.973	1	0.324	0.170	1	0.68	0.395	1	0.530	0.016	1	0.900	0.541	1	0.462
Offer 1:Offer 2	0.234	1	0.629	2.955	1	0.086	1.207	1	0.272	0.188	1	0.665	6.712	1	**0.01**
Offer 1: Day	1.238	1	0.266	6	1	**0.014**	5.530	1	**0.019**	9.433	1	**0.002**	2.216	1	0.137

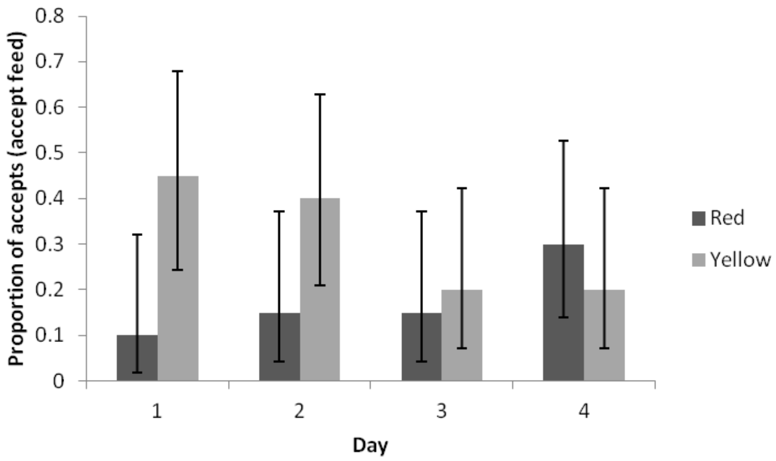

Figure 5.5. Proportion of honeybees which accepted and fed on flowers on the first occasion they were offered to them in Offer 2, after experiencing a previous attack associated with either a red or a yellow spider in Offer 1, for each of the four days on which offers were made. Error bars are 95 % confidence intervals calculated using the binomial distribution.

Over the four days of the duration of the experiment, there was no overall significant change in the proportion of honeybees landing or feeding on flowers in Offer 2 (Table 5.2). There was, however, an interaction between day and the treatment honeybees had received in Offer 1. The proportion of honeybees that accepted and fed on a flower in Offer 2 after experiencing a red spider in Offer 1 increased over time, but it decreased in cases where bees had experienced a yellow spider in Offer 1 (Figure 5.5). A similar pattern was observed for the probability of a honeybee landing on a flower, whether it fed or not ("accept feed and accept land") considering either just the first attempt to offer a flower ("without rejections") or all attempts ("with rejections") (see Table 5.2). The interaction between the effect of treatment in Offer 1 and day on the probability that honeybees accepted and fed on a flower in Offer 2 was not significant when considering all attempts to offer flowers to honeybees (“with rejections”; Table 5.2).

There were no main effects of the spider treatment in Offer 2, or of day, on any aspect of honeybee behaviour towards flowers presented in Offer 2 (Table 5.2). Similarly, the interaction between the effects of spider treatment in Offer 1 and the spider treatment in Offer 2 on the probability of a honeybee accepting flowers in Offer 2 was not significant, whether or not all attempts to offer a flower in Offer 2 were considered (Figure 5.6). However, crucially, there was a significant effect of the interaction between the effects of the spider treatments in Offer 1 and Offer 2 on the change in the number of rejections of flowers by honeybees between Offer 1 and Offer 2. Honeybees were more likely to reject the flower in Offer 2 more often if it harboured a spider of the same colour morph as the spider which they encountered in Offer 1 (Table 5.2 and Figure 5.7).

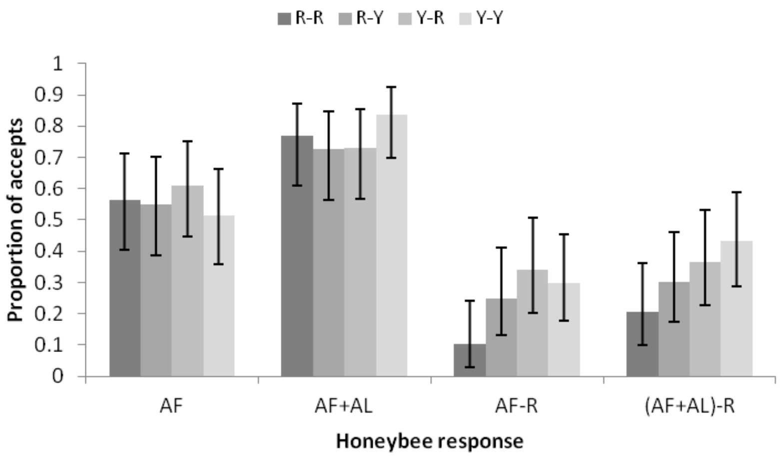

Figure 5.6. The effect of spider treatments in Offer 1 and Offer 2 on the proportion of flowers accepted by honeybees that were offered to them in Offer 2. Responses are: accept feed (AF), accept feed and accept land (AF+AL), accept feed without rejections (AF-R), and accept feed and accept land without rejections ([AF+AL]-R). Spider treatments were as follows: red in Offer 1 followed by red in Offer 2 (R-R), red followed by yellow (R-Y), yellow followed by red (Y-R), and yellow followed by yellow (Y-Y). Error bars are 95 % confidence intervals calculated using the binomial distribution.

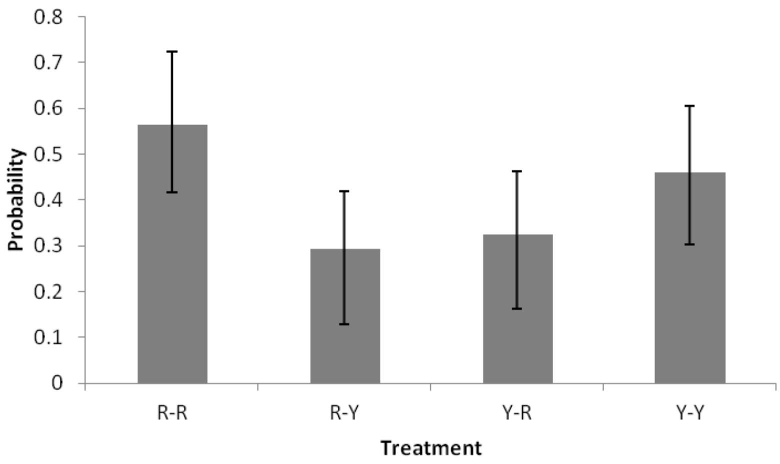

Figure 5.7. The effect of spider treatments in Offer 1 and Offer 2 on the change in the number of rejections by honeybees of flowers offered to them between Offer 1 and Offer 2. The y axis shows the probability of honeybees to reject a higher number of times a flower in Offer 2 than in Offer 1, after experiencing a simulated attack from a spider in Offer 1 for each of the experimental treatments. Spider treatments were as follows: red in Offer 1 followed by red in Offer 2 (R-R), red followed by yellow (R-Y), yellow followed by red (Y-R), and yellow followed by yellow (Y-Y). Error bars are 95 % confidence intervals calculated using the binomial distribution.

Although honeybees tended to reject flowers harbouring a particular colour of spider in Offer 2 more quickly if they had previously experienced an attack associated with a different spider colour, the effect of the combination of treatments received in Offer 1 and Offer 2 on the frequency with which honeybees rejected flowers carrying spiders in Offer 2 at different speeds was not significant (Chi-squared: X^2 = 11.802, df = 6, P = 0.067; Figure 5.8).

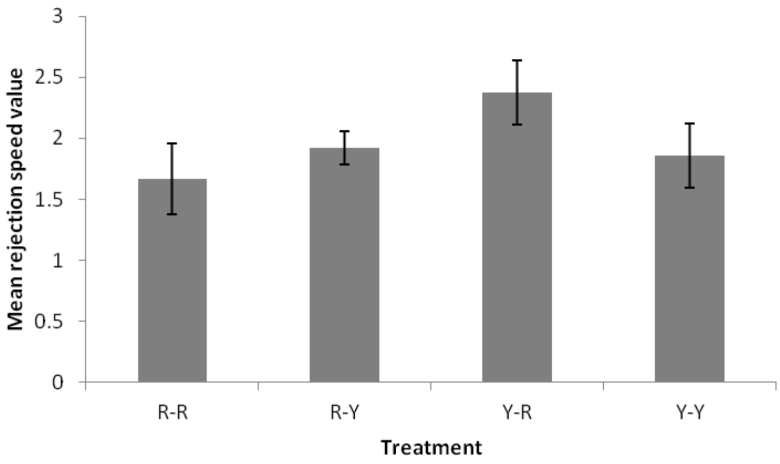

Figure 5.8. Mean rejection speed (± S.E.) (1 = slow, 2 = fast, 3 = very fast) of honeybees when rejecting flowers in Offer 2 in each of the experimental spider treatments: red in Offer 1 followed by red in Offer 2 (R-R), red followed by yellow (R-Y), yellow followed by red (Y-R), and yellow followed by yellow (Y-Y).

5.4 Discussion

The results of these experiments show no evidence that wild-caught honeybees discriminate between the different colour morphs of *S. globosum* on different species of flowers. However, there was evidence that honeybees were able to learn to avoid spiders of a colour morph from which they had recently experienced an attack. These results have important implications for the understanding of the mechanisms that might operate to maintain the polymorphism in *S. globosum*.

5.4.1 Frequency-independent selection

The presence of spiders on flowers had significant effects on the responses of honeybees. Honeybees avoided flowers where an individual of *S. globosum* was present, no matter if it was a red female, a yellow female, or a male.

These results support previous findings where honeybees have been observed to avoid flowers and even whole patches of flowers that harbour spiders or cues associated with their presence (Dukas 2001; Dukas and Morse 2003, 2005; Robertson and Maguire 2005; Reader et al. 2006). However, honeybees did not discriminate among spider types in Experiment 1, or between female spider colour morphs at the start of Experiment 2. Furthermore, there was no effect of flower species on the tendency of honeybees to avoid spiders, or on their tendency not to discriminate among spider types.

These results are not consistent with directional selection favouring one *S. globosum* morph, or habitat-specific selection on morphs. Combined with the results obtained in Chapter 3, where no evidence was found that *S. globosum* morphs favour particular flower types, this suggests that the polymorphism in *S. globosum* is not transient (Mitchell-Olds et al, 2007) and is not maintained by gene flow between sub-populations of spiders which are adapted to different flower-specific niches (King and Lawson 1995). Nevertheless, there may be other forms of niche-specific selection operating in this system, such as bees being better able to avoid certain colours of spider in relation to levels of light or other environmental factors. It is therefore impossible to rule out spatial (selection mosaics) or temporal variation in selection affecting the persistence of the polymorphism. Such processes have been observed to occur in other polymorphic species such as the marine snails of the genus *Littoraria,* where certain morphs are found at higher frequencies in different parts of mangrove trees and at different positions at different times of the year (Parsonage and Hughes 2002).

The results suggest that, when foraging at a site like the one studied here, where red and yellow female *S. globosum* are roughly equal in frequency, honeybee beahviour does not have strong effects on the relative fitness of these different colour morphs. This is not strongly indicative of NFDS, but could be consistent with it, if the observed ratio of red to yellow females is close to equilibrium in this population. Different patterns of fitness that are consistent with NFDS have been observed in polymorphic damselflies and lizards, both in natural populations that vary in morph frequencies, and in populations where morph frequencies have been manipulated (Van Gossum et al. 1999; Sinervo et al. 2001; Bleay et al. 2007). However, the studied populations of each of these species had a higher frequency of one the morphs, and thus there is no information on morph fitness from populations where morph frequencies are approximately equal. Nevertheless, two studies in damselflies showed that observations of morph frequencies and measurements of morph fitness agree with simple genetic models that predict NFDS dynamics, where similar frequencies result in approximately equal morph fitnesses (Svensson et al. 2005; Takahashi et al. 2010).

<u>5.4.2 Learning and the potential for frequency-dependent selection</u>

Honeybees rejected flowers in Offer 2 relatively more often if they harboured a spider of the same colour morph as a spider from which they received a simulated attack in Offer 1. Although experiments of associative learning in honeybees have shown that it will take a number of experiences with a particular stimulus for a honeybee to learn to associate it with another stimulus (Zhang et al. 1996), honeybees in this experiment appear to have learned to associate spider colour with an attack quick enough to show a change in response after just one aversive experience. These results are consistent with previous studies of bees where they have been observed to avoid a colour that they have associated with a negative experience (Avarguès-Weber et al. 2010) and to behave in a negative frequency-dependent way with respect to colour (Smithson and Macnair 1997; Gigord et al. 2001). Based on these findings, it is plausible that in a population of *S. globosum* where one female morph is found at a higher frequency than the other morphs, honeybees would experience attacks from spiders of this morph more often, and would avoid them more than the rarer morphs. In the light of this, further supporting evidence could be obtained from studies where honeybees are exposed to flower patches with varying *S. globosum* morph frequencies. This is the first time that evidence supporting a role for NFDS caused by prey behaviour that could help to maintain a colour polymorphism in a predator has been provided. Taken together with findings of other studies of this kind (Hori 1993; Arcos 2007), this suggests that predator-prey interactions may play a more general role in the maintenance of morphological and behavioural diversity than would otherwise have been thought.

Honeybees did not show a significant difference in the speed to reject a flower between any of the experimental treatments. However, the measurement of rejection speed was very crude and under the subjectivity of the observer. Therefore, a more accurate assessment of the honeybee's rejection speed, which could be obtained from video recordings of honeybee behaviour, is necessary to corroborate the results of this study.

It is important to note that we do not know what effect, if any, the ability of honeybees to learn to avoid spiders of a particular colour as demonstrated in Experiment 2 has on the fitness of *S. globosum* morphs in wild populations. The assumption that honeybees are one of the main prey of *S. globosum* is based on casual observations in the field, where I have often seen females feeding on them. Therefore, although the results in the present study support the idea that the female-limited polymorphism in *S. globosum* could be maintained by NFDS generated by honeybees, data on the composition of *S. globosum's* diet and on how much do honeybees actually affect its fitness are necessary to determine the relevance of this mechanism.

5.4.3 Differences in learnt responses to red and yellow spiders

Although wild-caught honeybees did not discriminate between spider morphs in either experiment, in Experiment 2 there was some evidence that honeybees that had previously experienced a simulated attack from a red spider were less likely to accept and feed on flowers in Offer 2 than those that had previously experienced a simulated attack from a yellow spider, regardless of what spider morph the flower in Offer 2 harboured. This result could be explained by the different ways in which red and yellow spiders reflect light and the different chromatic contrasts of the spiders and the flowers. Yellow colouration in spiders has been shown to reflect UV light to some extent (Tso et al. 2004; Heiling et al. 2005; Théry and Casas 2009), and reflectance spectra measurements of S. *globosum* have shown that such is the case for yellow females (see Section 3.3.1). Some spiders have been observed to use UV reflection to produce visual signals similar to those produced by flowers to attract honeybees (Heiling et al. 2003; Tso et al. 2004; Herberstein et al. 2009). Because UV-containing signals may more typically be used as cues associated with positive stimuli (i.e. food), honeybees might not be as efficient at learning to associate them with danger. Consequently, after a honeybee has experienced an attack from a red spider, it might be relatively more cautious about feeding on a flower with anything that might resemble a spider than would be the case after an experience with a yellow spider.

This effect of spider colour in Offer 1 on honeybee behaviour in Offer 2 reduced in magnitude over the four day period, until by day four the difference between the effects of experiences with red and yellow spiders in Offer 1 was small and in the opposite direction (Figure 5.5). The change over time might be owing to the use of the same population of honeybees during the experiment. It has been observed that honeybees show patch fidelity (Osborne and Williams 2001; Slaa et al. 2003; Franzén et al. 2009), and hence individual honeybees may have been exposed to spider treatments more than once over the four days. Therefore, it is possible that honeybees eventually learned to associate yellow colour with predation risk, thus reducing the probability that they would accept a second flower with a spider after experiencing an attack from a yellow spider.

5.4.4 Conclusion

The findings in the present chapter contribute to our understanding of how the presence of predators might affect the foraging behaviour of their prey, and how these predator-prey interactions might influence the maintenance of polymorphism in a population of predators. However, clearly further research is needed to understand the general importance of this mechanism in the maintenance of polymorphisms in *S. globosum* and other similar species, and its relative importance compared with other diversifying mechanisms.

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Chapter 6: Effects of *Synema globosum's* colour morphs on male mate choice

6.1 Introduction

Since *S. globosum* exhibits a female-limited polymorphism, it is logical to consider sexual interactions as one of the factors which may contribute to its maintenance. Alternatively, sexual selection may be purifying, and thus counteract any mechanisms which are promoting the polymorphism. In this chapter, I look for evidence of sexual behaviour which might lead to either frequency-dependent or directional sexual selection on female colour in *S. globosum*.

6.1.1 Frequency-independent sexual selection

Sexual interactions can lead to directional selection in favour of one morph. Sexual preferences for one particular morph can arise in several ways (Andersson 1994). For example, if the polymorphic trait is indicative of fitness, then individuals may prefer to mate with individuals of the morph that advertises higher fitness. Additionally or alternatively, runaway sexual selection may occur, where a preference for a particular trait of the opposite sex becomes genetically correlated with that trait. Finally, a pre-existing bias in the sensory system of the choosing sex may generate a preference for a particular morph among potential mates (Andersson 1994). Such mechanisms have been observed to influence mate choice in birds, frogs, and fish (Berglund et al. 1986; Hill 1991; Ryan and Rand 1993). In spiders, directional selection as a result of mate choice has been proposed in the wolf spider *Schizocosa ocreata*, where the size of tufts of bristles on the first pair of legs in the male is influenced by female preference and sexual cannibalism (Persons and Uetz 2005). Evidence for directional sexual selection on behavioural and morphological male traits has also been found in populations of the jumping spider *Habronattus pugillis* (Masta and Maddison 2002). Directional selection would be expected to eliminate the less preferred morphs as they will mate less frequently, leading to the fixation of the preferred morph and hence a monomorphic population (Ridley 2004). In such case, as in the interactions between predators and prey (Chapter 5), an observed polymorphism would only be transient.

Nevertheless, spatial or temporal variation in directional sexual selection on the colour morphs could potentially maintain the polymorphism (Arnqvist 1992; Gamble et al. 2003). This might occur if morphs are particularly attractive in certain kinds of microhabitat, for example females might conceivably be more easily located by males depending on their colour contrasts with the flowers on which they are sitting.

6.1.2 Frequency-dependent sexual selection

A stable polymorphism can be maintained when sexual interactions generate negative frequency-dependent selection (NFDS) as a result of mechanisms such as learned mate recognition (LMR; Miller and Fincke 1999), where males prefer to mate with females of the morph that they encounter more frequently. Damselflies with female-limited colour polymorphisms are the only invertebrate species where LMR has been studied (see Section 1.2.1). Choice and no-choice experiments with and without previous exposure to only one female morph, as well as observations of natural mating events, have detected a preference amongst males for the most common female morph in the population, providing partial support for LMR (Fincke 1994; Miller and Fincke 1999; Van Gossum et al. 1999; Van Gossum et al. 2001; Svensson et al. 2005; Takahashi and Watanabe 2009, 2010; Takahashi et al. 2010). These findings suggest that males can modify the internal template of the characteristics of a potential mate in a frequency-dependent way. Such behavior may allow the male to cue to a potential mate more efficiently in a population where there is more than one form to be recognized as female and female morph frequencies vary considerably over short time periods (Fincke 1994, 2004). In species where mating opportunities are not limited for females, the outcome could be frequency-dependent fitness costs of male harassment for the female morphs, and this mechanism could thus maintain a balanced polymorphism (Miller and Fincke 1999; Fincke 2004).

The polymorphism observed in damselflies has also been proposed to occur by the adoption of alternative female mating strategies, the success of which varies in a partly frequency-dependent manner (Robertson 1985; Sherratt 2001; Sirot et al. 2003). One of the morphs resembles the males morphologically (Iserbyt et al. 2011) and behaviourally (Sirot et al. 2003). It has been hypothesized that females of this morph use mimicry to avoid mating attempts from males when they occur at low frequencies relative to males (see Section 1.2.1). Thus, the possibility that female morphs of *S.*

globosum adopt alternative mating strategies to avoid harassment by males should be considered.

The female-limited colour polymorphism in *S. globosum* is similar in several ways to that seen in polymorphic damselflies, and the hypothesis that LMR maintains polymorphism in *S. globosum* seems plausible. Most importantly, several lines of evidence suggest that female *S. globosum* are exposed to multiple mating attempts by males, and are therefore likely to be subject to costly harassment. First, *S. globosum* can be found at high population densities (1.59 individuals/m^2 – see Section 2.3.3), which are more similar to those of polymorphic damselflies (*Enallagma hageni*: 4.6 individuals/m^2, Xu and Fincke 2011) and *Ischnura ramburi*: 1.02 individuals/m^2, Sirot et al. 2003) than to other crab spiders (*Misumena vatia*: 0.0219 individuals/m^2, Anderson and Morse 2001). In species with relatively high population densities, males are predicted to encounter females frequently enough for male harassment to represent a significant cost to them (Miller and Fincke 1999; Fincke 2004). Indeed, I have observed females of *S. globosum* with different males on different occasions (a maximum of three males in a period of 11 days). I also found two males with a single female at the same time on several occasions, where males were engaging in agonistic behavior (see Section 2.3.3).

A second observation which suggests that female *S. globosum* are likely to be harassed by males is that males often appear to be mate guarding, with the same male staying with a female for several days until she undergoes her final moult (personal observation). Crab spiders belong to the Entelegynae, females of which possess complex genitalia, and are known to exhibit first-male sperm priority (Christenson and Cohn 1988; Watson 1991), presumably as a result of which Entelegynae males have been found to prefer to mate with females in the penultimate instar (prior to sexual maturity) or adult virgins (Dodson and Beck 1993; Morse 2009). It thus seems likely that a *S. globosum* male guards a female to ensure that he is the first one to mate with her. A final observation indicating that male attention is costly for female *S. globosum* is that females often appear to fight off males during mating attempts.

Overall then, fitness may well be reduced in female *S. globosum* by excessive male attention. The costs may be direct (death or injury), or more likely in the form of a decrease in foraging opportunities, which has been observed to occur in other species where females are harassed by males (Krupa and Sih 1993; Stone 1995). A lower foraging rate has been observed to have a significant effect on the reproductive output of the crab spider *M. vatia*, one of the main prey of which is also the honeybee (*Apis mellifera*) (Morse 1983; Morse and Stephens 1996). *M. vatia* females with higher foraging success prior to laying eggs and, consequently, a larger body size, laid larger egg masses which had higher survival than smaller egg masses because they were more efficiently guarded by the larger females (Fritz and Morse 1985; Morse 1988).

6.1.3 Objectives

On the assumption that male harassment probably occurs in *S. globosum*, and may represent a substantial cost to females, two experiments were carried out to evaluate if sexual interactions have the potential to influence the maintenance of the polymorphism in this species. The first experiment (Experiment 1) aimed to investigate if adult males from a population in which red and yellow females are roughly equally common and white females are rare (see Section 3.3.4), have any preference for particular female colour morphs. Any preference expressed in this experiment could be indicative of directional selection favouring one particular morph, and/or a consequence of an interaction between the frequencies of female morphs present in the population from which males were collected and male behaviour. The second experiment (Experiment 2) tested if recent experience with a female of a particular morph had an effect on male mate choice. An increase in preference for females of a particular morph caused by recent experience with that morph (Experiment 2) would be consistent with the predictions of the LMR hypothesis, and hence point to an explanation for the maintenance of the polymorphism in female *S. globosum*. The behaviour of females was also evaluated to determine whether the different morphs adopt alternative mating strategies.

6.2 Materials and Methods

All experiments were carried out in the laboratory at the Quinta de São Pedro Study Centre and surrounding areas.

6.2.1 Experiment 1: Male mate choice

To evaluate if wild-caught adult male *S. globosum* have a preference for a particular female morph, a binary choice experiment was conducted in May and June 2009 over a period of six days between 1200 and 1800. Spiders were collected from different flowering plants at the Quinta de São Pedro Study Centre and surrounding areas, and kept in Perspex tubes in the laboratory. A male was given a choice between two females of different colour morphs. The following treatments for female colour were used: red vs. yellow (N = 18) and white vs. yellow (N = 5). Because of the small sample size of white females it was not possible to do a comparison against red females. Treatments and spiders were chosen at random. Two females were placed at opposite sides of a petri dish separated by a piece of cardboard. Once the females were settled, the cardboard strip was removed and a male was introduced into the petri dish at approximately the same distance from both females. A trial ended when the male started to mate with one of the females or after fifteen minutes. Based on observations of pilot experiments, it was considered that if a male did not manage to mate with a female after fifteen minutes, mating would not occur.

A series of behavioural patterns was recorded for males (Table 6.1) and females (Table 6.2). A preference index was established by scoring behaviours as negative or positive and ranking them according to their perceived significance in the mating process, according to preliminary observations of successful and unsuccessful mating attempts. Negative behaviours were those where spiders appeared to respond in a fearful or aggressive manner, and were considered as rejection of mating. In the case of males, positive behaviours were those where the spiders appeared to be actively interested in mating. Females never appeared to be actively interested in mating, and therefore only performed negative behaviours except when keeping still after being seized by a male and allowing him to mate with them.

Table 6.1. Male behavioural patterns observed during mating experiments, ranked by preference index value.

Behavioural pattern	Preference index value	Description
Attack	-4	Moved the first two pairs of legs in a quick, aggressive fashion towards the female.
Raise legs	-3	Raised the first two pairs of legs facing the female.
Run away	-2	Ran in the opposite direction to the female.
Walk away	-1	Walked away from the female.
Approach	1	Detected the presence of the female and walked towards her. A male was considered to have detected the presence of a female in two possible ways: when the female made a movement and the male oriented its body facing towards her, or when the male followed the female's silk lines until he arrived to where the female was.
Extend legs	2	Slowly extended the first two pairs of legs towards the female, either touching her or not.
Touch	3	Gently touched the female with the first two pairs of legs.
Try to grab	4	Attempted to seize the female with the first two pairs of legs, but was unsuccessful either because the female ran away or because she attacked him.
Mate	5	A male was considered to have mated with a female when he inserted at least one pedipalp into the female's genital opening

Table 6.2. Female behavioural patterns observed during mating experiments, ranked by preference index value.

Behavioural pattern	Preference index value	Description
Grab	-7	Seized the male with the first two pairs of legs.
Try to grab	-6	Attempted to seize the male with the first two pairs of legs.
Attack	-5	Moved the first two pairs of legs in a quick, aggressive fashion towards the male.
Raise legs	-4	Raised the first two pairs of legs facing the male.
Extend legs	-3	Slowly extended the first two pairs of legs towards the male, either touching him or not.
Run away	-2	Ran in the opposite direction of the male.
Walk away	-1	Walked away from the male.
Mate	1	Kept still while being seized by the male and he inserted at least one pedipalp into her genital opening.

6.2.2 Experiment 2: Effect of previous mating experience on male mate choice

A no-choice mating experiment was conducted to determine whether males of *S. globosum* prefer to mate with females of a colour morph that they have recently encountered. All observations were conducted in May 2011 over a period of four days between 0900 and 1700. Each male had two trials with different females, and was randomly assigned to one of the following female colour morph treatments: red followed by yellow, yellow followed by red, red followed by red, and yellow followed by yellow (n = 10 per treatment). White spiders were excluded from this experiment because of the low frequencies at which they were found. Spiders were collected from different flowering plants at the Quinta de São Pedro Study Centre, and kept in Perspex tubes in the laboratory. There was a limited number of females available, so females were sometimes used more than once, but never more than twice. A randomly selected female was placed in a petri dish and allowed to settle for at least two hours. A male was then introduced into the petri dish with the female, and the behaviour of both spiders was recorded. The spiders were observed for ten minutes or until they started to mate. The male was removed from the petri dish after ten minutes or after the mating ended. There was an interval of two days between each mating trial because males tested prior to the experiment did not show an interest in mating again one hour or 24 hours after having previously mated (also observed in *M. vatia*, Morse 2007). The same procedure was followed for the second mating trial. To evaluate the responses of the spiders, the behavioural patterns with the preference index from Experiment 1 were used.

6.2.3 Statistical analyses

A chi-squared test was used for each behavioural pattern for both males and females to analyse the difference in responses with respect to female colour morphs in Experiment 1 and in the first mating trial of Experiment 2. A Wilcoxon rank-sum test was used to compare the general response of males and females with respect to female colour morphs in Experiment 1 and in the first mating trial of Experiment 2 using the sum of preference index values for those behaviours which were observed at least once. The difference in the responses of males between the two mating trials in Experiment 2 was analysed using a generalised linear model (GLM) with a binomial error structure, where the response variable indicated whether the sum of preference index values of the male's behaviour was higher in the second mating trial or not. Similarly, the effect of female colour in the first mating trial on the probability that males would perform each behavioural pattern in the second trial was analysed with a binomial GLM. For the GLMs, the significance of each term was assessed by backward deletion from a saturated model using a Chi-squared test statistic following Crawley (2007) and Zuur et al. (2009). Statistics are presented for comparisons of models after each term was removed. All analyses were conducted in R 2.12.2 (The R Development Core Team 2011).

6.3 Results

6.3.1 Experiment 1: Male mate choice

There was no significant effect of female colour morph on the response of males and females when presented with individuals of the opposite sex. There was no significant difference in the frequency with which males performed any of the observed behavioural patterns in the presence of females of different colour morphs in either choice test treatment (Table 6.3). Likewise, there was no significant difference in the frequency of any of the behavioural patterns performed by females of different colour morphs in the presence of a male (Table 6.4). Furthermore, there was no significant difference in the sum of the preference index values for males in the presence of females of different colour morphs, or for females of different colour morphs in the presence of a male (Table 6.5, and Figures 6.1 and 6.2).

Table 6.3. Results of Chi-squared tests for differences in the frequency with which various male behavioural patterns were observed between female colour morphs in the two treatments in Experiment 1. Tests were not conducted for those behaviours which were not performed at all in the relevant treatments.

	Treatment					
Behavioural Pattern	Red vs. Yellow (n = 18)			White vs. Yellow (n = 5)		
	X^2	df	P	X^2	df	P
Attack	-	-	-	-	-	-
Raise legs	-	-	-	-	-	-
Run away	0.200	1	0.655	0.200	1	0.655
Walk away	0.400	1	0.527	0.333	1	0.564
Approach	0.040	1	0.842	0.667	1	0.414
Extend legs	-	-	-	1	1	0.317
Touch	0.143	1	0.706	0	1	1
Try to grab	0.692	1	0.405	0.333	1	0.564
Mate	1	1	0.317	0	1	1

Table 6.4. Results of Chi-squared tests for differences in the frequency with which various female behavioural patterns were observed between female colour morphs in the two treatments in Experiment 1. Tests were not conducted for those behaviours which were not performed at all in the relevant treatments.

	Treatment					
Behavioural Pattern	Red vs. Yellow (n = 18)			White vs. Yellow (n = 5)		
	X^2	df	P	X^2	df	P
Grab	1	1	0.317	-	-	-
Try to grab	0.333	1	0.564	0.333	1	0.564
Attack	0.667	1	0.414	1	1	0.317
Raise legs	1	1	0.317	-	-	-
Extend legs	-	-	-	1	1	0.317
Run away	1	1	0.317	1	1	0.317
Walk away	1.923	1	0.166	1	1	0.317
Mate	0.500	1	0.480	0	1	1

Table 6.5. Results of Wilcoxon rank-sum tests of differences in the sum of the preference index values for males when offered a choice of two females of different morphs, and for females of two different morphs in the presence of a single male, in the two treatments in Experiment 1.

	Treatment			
	Red vs. Yellow (n = 18)		White vs. Yellow (n = 5)	
	W	*P*	*W*	*P*
Males	187	0.431	18	0.287
Females	126.5	0.260	12.5	1

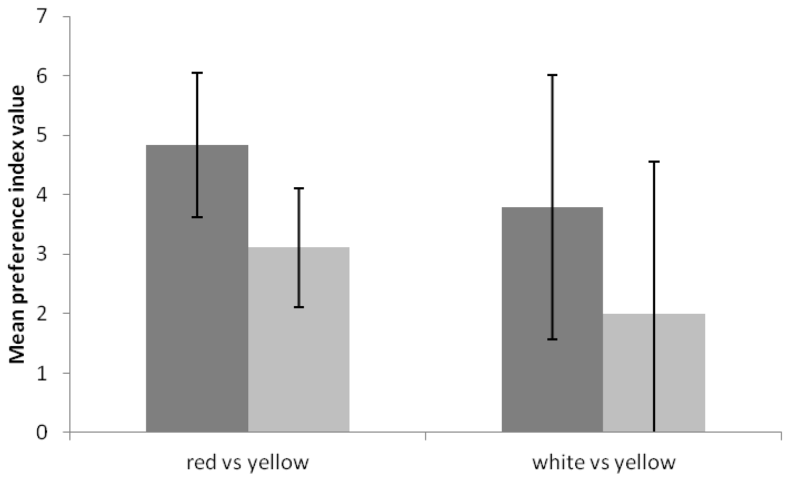

Figure 6.1. Mean sum of the preference index values (± S.E.) for the responses of males to red (dark bar) and yellow (light bar) females in the red vs. yellow treatment and to white (dark bark) and yellow (light bar) females in the white vs. yellow treatment.

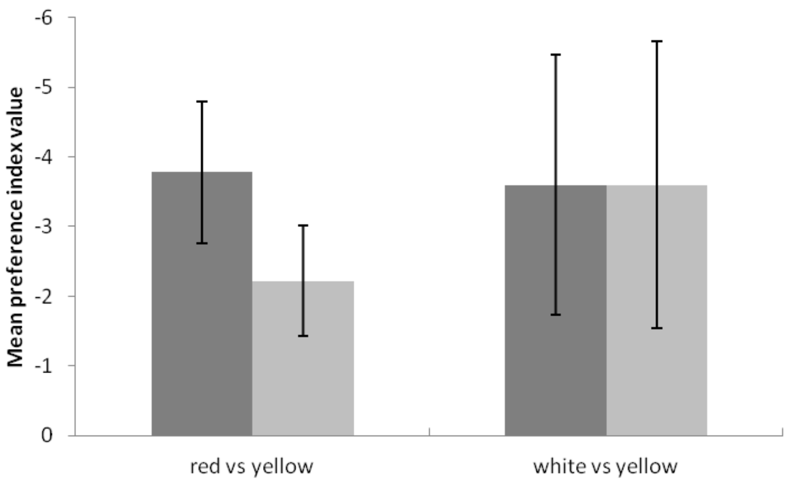

Figure 6.2. Mean sum of the preference index values (± S.E.) in the presence of a male for red (dark bar) and yellow (light bar) females in the red vs. yellow treatment and white (dark bar) and yellow (light bar) females in the white vs. yellow treatments.

6.3.2 Experiment 2: Effect of previous mating experience on male mate choice

Consistent with the results of Experiment 1, there was no significant difference in the frequency of any of the behavioural patterns displayed by males when presented with red and yellow females in the first mating trial (Table 6.6). Additionally, there was no difference in the frequency of any of the behavioural patterns performed by red and yellow females in the presence of a male (Table 6.7). Likewise, in accordance with Experiment 1, there was no significant difference in the sum of the preference index values for males or females with respect to colour morph in the first mating trial (Wilcoxon rank-sum test: males: $W = 168.5$, $P = 0.4$, females: $W = 481.5$, $P = 0.816$). No significant main effect of female colour in the first trial was found on the probability that a male performed any of the behavioural patterns in the second trial (see Table 6.8). There was also no significant main effect of female colour in the second trial on the probability that a male performed any of the behavioural patterns in the second trial. There was, however, a significant interaction between female colour in the first trial and female colour in the second trial on the probability that a male would raise the first two pairs of legs and walk away. Males were more likely to raise the first two pairs of legs and to walk away when presented with a female of a different colour morph from a female with which they had had a previous mating experience (Figure 6.3).

Taking into account all the behaviours performed by the male during the mating trials, males were generally slightly less interested in mating in the second trial of Experiment 2 (percentages of males that had less positive sums of preference index values in the second trial in each treatment: R-R: 30 %, R-Y: 60 %, Y-R: 60 %, and Y-Y: 50 %). There was, however, no significant effect of female colour in either the first trial (GLM: $X^2 = 0$, d.f. = 38, P = 1) or the second trial ($X^2 = 1.777$, d.f. = 39, P = 0.183) on the change in the sum of the preference index values for males between the first and second trials. Importantly, there was also no significant interaction between the effects of female colour in the first trial and female colour in the second trial on the change in the sum of the preference index values for males between the first and second trials ($X^2 = 0.470$, d.f.=37, P = 0.493). No significant difference in the proportion of males for which the sum of the preference index values increased between the first and second trials was observed among the experimental treatments (Figure 6.4).

Table 6.6. Results of Chi-squared tests for differences in male behavioural patterns between female colour morphs in the first mating trial in Experiment 2.

Behavioural Pattern	X^2	df	P
Attack	0	1	1
Raise legs	0.200	1	0.655
Run away	0.200	1	0.655
Walk away	0.286	1	0.593
Approach	0.222	1	0.637
Extend legs	0.143	1	0.706
Touch	0.067	1	0.796
Try to grab	0.077	1	0.782
Mate	0.667	1	0.414

Table 6.7. Results of Chi-squared tests for differences in female behavioural patterns between female colour morphs in the first mating trial in Experiment 2.

Behavioural Pattern	X^2	df	P
Grab	1	1	0.317
Try to grab	0.111	1	0.739
Attack	0.029	1	0.866
Raise legs	0.029	1	0.866
Extend legs	0.067	1	0.796
Run away	0.250	1	0.617
Walk away	1	1	0.317
Mate	0.667	1	0.414

Table 6.8. Results of binomial GLMs testing the effect of female colour morph in the first and second mating trials, and their interaction, on each of the behavioural patterns performed by males in the second trial.

Behavioural Pattern	Trial 1			Trial 2			Trial 1: Trial 2		
	X^2	df	P	X^2	df	P	X^2	df	P
Attack	0	38	1	0	39	1	0	37	1
Raise legs	0.673	38	0.412	2.594	39	0.107	6.393	37	**0.011**
Run away	1.667	38	0.197	1.601	39	0.206	1.596	37	0.206
Walk away	<0.001	38	1	0.4	39	0.199	1.6	37	**0.006**
Approach	0	38	1	<0.001	39	0.999	1.681	37	0.195
Extend legs	3.752	39	0.053	0.158	38	0.691	0.063	37	0.803
Touch	0.418	38	0.518	<0.001	38	0.999	0.423	37	0.516
Try to grab	0.126	38	0.723	0.126	39	0.723	1.108	37	0.292
Mate	0.025	38	0.317	0.025	39	0.317	0.025	37	0.317

Figure 6.3. Proportion of males that raised the first two pairs of legs and walked away from the female for each of the experimental treatments: red in the first trial followed by red in the second trial (R-R), red followed by yellow (R-Y), yellow followed by red (Y-R), and yellow followed by yellow (Y-Y). Error bars are 95 % confidence intervals calculated using the binomial distribution.

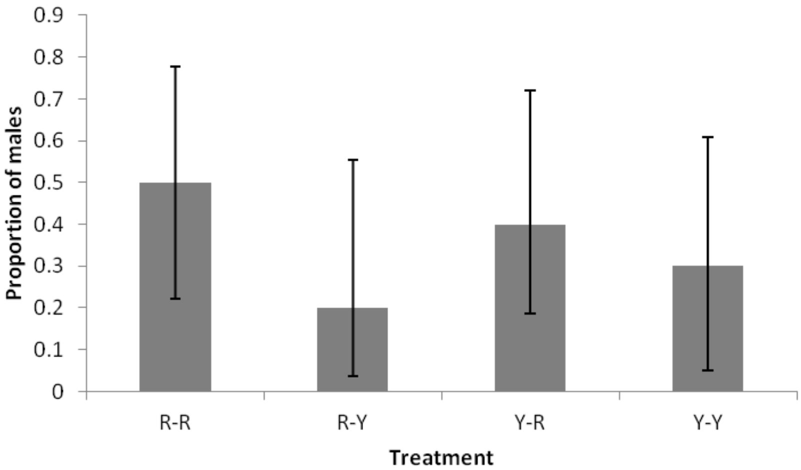

Figure 6.4. Proportion of males that showed an increase in the sum of preference index values in the second mating trial for each of the experimental treatments: red in the first tiral followed by red in the second trial (R-R), red followed by yellow (R-Y), yellow followed by red (Y-R), and yellow followed by yellow (Y-Y). Error bars are 95 % confidence intervals calculated using the binomial distribution.

6.4 Discussion

Males of *S. globosum* did not show a preference to mate with a female of a particular colour morph in either the binary choice or the no choice experiments. Males were not more likely to perform any of the behavioural patterns more frequently, or be generally more or less positive towards females, when presented with a female of a particular colour morph. The same results were obtained for females of different colour morphs in the presence of a male. These results suggest that, in spiders taken from a population in which red and yellow females are roughly equally common, female colouration does not have an effect on a male's choice of a potential mate, and that colouration is not associated with alternative female mating strategies. However, some evidence was found for an effect of female colour on male behaviour towards females of different colours when having a recent experience with a female of a particular colour. Mating behaviour could therefore play a role in the maintenance of polymorphism in *S. globosum.*

These results contrast with some studies on damselflies, where wild-caught males have been observed to show a preference for one female colour morph, both in the laboratory and in the field, in choice and no choice experiments (Fincke 1994; Miller and Fincke 1999; Van Gossum et al. 1999; Van Gossum et al. 2001; Svensson and Abbott 2005; Takahashi and Watanabe 2009, 2010; Takahashi et al. 2010). In all of these studies, the preferred female morph always had a higher frequency in the population than the other morphs, and the observed bias in preference was thus consistent with the

LMR hypothesis. In contrast to these damselfly examples, the *S. globosum* population studied here had roughly equal frequencies of the two common female morphs (red and yellow). In such circumstances, all things being equal, the absence of a detectible preference among male spiders from this population for red versus yellow spiders is also entirely consistent with the LMR hypothesis, which predicts weak selection when morph frequencies are at equilibrium. Having said this, because there are likely to be at least some frequency-independent fitness differences among morphs, such as susceptibility to predators or parasites (Robertson 1985; Losey et al. 1997) or a possible difference in costs of the production of pigments (Graham et al. 1980), it is not possible to know whether the rough equivalence of red and yellow morph frequencies observed in all years of this study (see Section 3.3.4) is close to the equilibrium state under frequency-dependent selection. The same is true when considering the relative frequencies of yellow and white females, and hence it is also inappropriate to interpret the lack of preference for the common yellow morph in the relevant choice trials as evidence against LMR. Perhaps more importantly, the statistical power to test for a preference was especially weak in these trials, because white females were so hard to find. Overall, therefore, the apparent absence of pre-existing mate preferences in wild-caught males really only tells us that there is probably no strong and consistent directional selection on female morphs caused by male behaviour.

Significant effects of a previous recent experience with a female of a particular colour morph were detected on two aspects of the response of males (leg raising and walking away) when presented with a second female. Specifically, males were less likely to perform these behaviours if they had encountered a female of the same colour before. Since leg raising and walking away were considered to be negative behaviours, these results suggest that males are more likely to reject a female of a different colour from one they have encountered recently. This suggestion was not, however, supported when all positive and negative behaviours performed by a male were considered together, using a preference index. Looking at an effect on the general response of males, which includes all scored behaviours, should be more informative about any preference that males might have for a particular female morph, but its usefulness is dependent on the ranking system used (see Table 6.1) being meaningful. While I made a priori decisions about the extent to which observed behaviours were positive or negative based on preliminary observations of mating trials, a degree of arbitrariness and subjectivity could not be avoided in the preparation of the preference score.

For practical reasons and because of the characteristics of the model species, the sequential no-choice experiment in the present study has a different design from those carried out with damselflies, in which males have shown a preference for females of the colour morph that they have experienced recently. In the experiments with damselflies, 9 to 20 males were introduced in an enclosure containing 20 females of either one or another colour morph from the population for two days (Miller and Fincke 1999; Van Gossum et al. 2001). In contrast, in the experiment with *S. globosum*, each male was only exposed once to one female of either morph before his preference was evaluated. It is possible that males need multiple encounters with a female of a particular morph to generate a preference for females of that morph, especially given the statistical noise which was probably generated in the present study by using wild-caught males, each of which is likely to have had a different set of natural encounters with females of different morphs prior to the experiment. Given these limitations, it is most appropriate to conclude overall that the results of Experiment 2 provide some weak support for LMR, but further experiments with larger sample sizes are needed to clarify the role of past experience in determining male behaviour towards female morphs.

If LMR does occur in *S. globosum* and females of the common morph in the population receive significantly more male harassment, it might be costly for them in terms of prey consumption because they might miss foraging opportunities while males attempt to mate with them, but also because females are kleptoparasitised by males (See Section 2.3.3). I have observed on two occasions a male feeding from a prey corpse that had been killed by a nearby female. However, in species where sexual cannibalism occurs, such as some spiders (Kralj-Fiser et al. 2012; Welke and Schneider 2012; Wilder and Rypstra 2012), male attention could actually be beneficial to the female, as she may have more opportunities to feed. Indeed, I have observed one occasion in which a female of *S. globosum* cannibalised a male during a pilot mating trial. Nevertheless, these are all casual observations, and it remains to be seen whether male harassment, kleptoparasitism and sexual cannibalism occur frequently enough to effect female fitness.

If the female-limited colour polymorphism in *S. globosum* is in any way maintained by male mate choice, it is necessary for males to be able to discriminate between female colours. It has been shown that the visual system of the crab spider *M. vatia* possesses the necessary structures for colour vision (Defrize et al. 2011; Insausti et al. 2012), and differences in reflectance spectra among *S. globosum* colour morphs were found to be detectable by *M. vatia's* visual system (see Section 3.3.1) Therefore, it is likely that male *S. globosum* are able to visually discriminate among female colour morphs. Additionally, male spiders can obtain information on characteristics of females, such as identity and developmental stage, through chemical cues from silk lines produced by the female (Roland 1983; Herberstein et al. 2002; Gaskett 2007; Baruffaldi and Costa 2010; Tuni and Berger-Tal 2012). It has been observed that males of the colour polymorphic jumping spider *Myrmarachne plataleoides* show a preference for the silk of females of their own colour morph (Borges et al. 2007), which indicates the possibility of the presence of cues associated with body colour in the silk. Nevertheless, females of the crab spider *M. vatia*, which is more closely related to *S. globosum,* do not leave chemical information in their silk lines, males thus seem to rely exclusively on mechanical cues (Anderson and Morse 2001), and it is not known whether these cues can be associated with body colour.

In the binary choice experiments, males simply seemed to attempt to mate with the female whose presence they noticed first. However, there are some subtle characteristics of females that influence male mate choice in spiders which were not measured in these experiments, such as whether they have mated before or not (Dodson and Beck 1993; Morse 2009; Tuni and Berger-Tal 2012). Since *S. globosum* is likely to exhibit first-sperm male priority (see introduction) it is possible that males have a preference for unmated females.

6.4.1 Conclusion

While it is not yet clear whether sexual interactions play an important role in the maintenance of colour polymorphisms in natural populations, there is a growing body of research on damselflies that has provided important insights into the potential of sexual behaviour to generate NFDS. Studies in different systems are needed to determine the general relevance of such interactions across taxa in the maintenance of phenotypic diversity. Although the present study in *S. globosum* does not provide strong evidence for sexual interactions influencing the maintenance of colour polymorphism, it does suggest that they might be playing a role. Therefore, it would be worth carrying out future research looking at how sexual interactions might generate NFDS and possibly maintain colour polymorphism in *S. globosum,* and how the situation in this system compares with the better known examples in damselflies.

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Chapter 7: General discussion

The main objectives of this investigation were first to establish whether the striking female-limited colour polymorphism observed in *S. globosum* is discrete and has a genetic basis, and second to obtain some insight into the possible mechanisms influencing its maintenance. The results reported here show that the female morphs are indeed discrete and genetically inherited. No evidence for substantial directional selection on morphs was found, and hence the polymorphism does not appear to be transient. This seems to be the case not only in the population studied, but also throughout the Iberian Peninsula, and there is no obvious explanation for the polymorphism involving geographic variation in selection. Given all this, balancing selection within the study population appears likely to play a role in the persistence of the polymorphism. Although not conclusive, my results implicate interactions with prey, and between the sexes, in the generation of negative frequency dependent selection on morphs. This highlights the potential of ecological interactions which have typically been rather neglected in studies of the maintenance of diversity. This investigation prepares the ground for future studies to identify the exact nature of the genetic polymorphism underlying the observed phenotypic variation in *S. globosum*, and the ecological explanation for its existence. Additionally, this study demonstrates the potential that *S. globosum* has as a model system for the study both of morphological diversity, and of other aspects of ecology, behaviour, and evolution.

Several characteristics of *S. globosum's* natural history, such as the type of prey they consume and potential costs for females generated by males, implicate negative frequency-dependent selection (NFDS) in the maintenance of the observed polymorphism. There were several lines of evidence obtained in this investigation that support this idea. First, differences in colouration among morphs of *S. globosum* were found to be detectable by the visual systems of honeybees (*Apis mellifera*) and the crab spider *Misumena vatia*. This makes the occurrence of NFDS as a result of interactions with prey and mates plausible. Second, honeybees were found to avoid a flower relatively more often if it harboured a spider of the same colour morph as a spider from which they received a simulated attack in a previous encounter. Finally, male *S. globosum* were less likely to perform some behaviours upon encountering a female, suggesting a lack of interest in mating, if they had encountered a female of the same colour before.

To confirm the potential of interactions with prey, and between males and females, to promote morphological diversity in female *S. globosum*, it is necessary to determine the impact that avoidance by honeybees and multiple mating attempts by males have on fitness. It would also be desirable to test the effect that spiders of the white morph have on the behaviour of honeybees and males. Since white spiders are so difficult to find in the field, a possible alternative would be to use artificial spider models (Ings and Chittka 2008). Testing the effect of manipulations of spider morph frequencies on the behaviour of honeybees and males in the wild could also provide further supporting evidence for these mechanisms, although this would be a major undertaking.

A possible source of NFDS that was not considered in this investigation but that could be influencing the polymorphism in *S. globosum* is interactions with visual predators with good colour vision (most plausibly birds). Several studies have demonstrated the potential that predation by birds has to generate NFDS mechanisms on colour polymorphic prey (Allen 1972; Allen 1976; Bond 1983; Cooper 1984; Bond and Kamil 1998, 2002), and the occurrence of colour polymorphisms in spiders has been attributed to this mechanism (Gillespie and Oxford 1998). However, NFDS generated by predation by birds has not yet been formally tested in natural populations, and thus it is not clear how it might influence the maintenance of colour polymorphisms. Predation by birds of *S. globosum* does not appear likely to occur frequently; in many hours of watching plants harbouring spiders, birds were never seen gleaning insects from flowers. Nevertheless, without understanding fully the sources of mortality of spiders, it is impossible to rule out a role of selection by predators entirely.

If the mechanism responsible for the maintenance of the polymorphism in *S. globosum* is not NFDS, or if it is not the only mechanism involved, niche differentiation through alternative foraging strategies may play a role. Yellow females spent significantly more time at a given flower than red or white females, but why this might be, and what the consequences are, remains unknown. Alternatively, genetic drift, heterozygote advantage, and density-dependent selection, which have been shown to influence the maintenance of colour polymorphisms in several species (Cook 1998; Sinervo et al. 2000; Bellido et al. 2002; Calsbeek et al. 2009), could also play a role. In reality, it is likely that a combination of a number of mechanisms is responsible for the polymorphism observed in *S. globosum*, and NFDS might only play a minor role, as has been shown to be the case of the best-studied examples of species which exhibit colour polymorphisms, the land snails of the genus *Cepaea* (Jones et al. 1977; Cook 1998; Bellido et al. 2002).

In conclusion, the results reported in this thesis provide evidence supporting NFDS as a mechanism that could be maintaining the colour polymorphism in *S. globosum*.

NFDS generated by interactions with prey, which has been a mechanism that has not received much consideration in explaining polymorphisms (but see Paulson 1973; Hori 1993; Roulin and Wink 2004; Arcos 2007) and had never been tested in invertebrate species before, could be playing an important role in this system, and perhaps may have been overlooked as a diversity-promoting mechanism more generally. Future studies that look at changes in morph frequencies over time and that obtain appropriate measurements of fitness (Thompson et al. 2011) would provide more definitive evidence in favour or against NFDS in this species. The lack of appropriate long-term data-sets represents a general weakness in studies of conspicuous polymorphisms, and it is one of the main reasons why we still do not have any compelling evidence in favour of NFDS maintaining diversity in natural populations, more than 50 years after the idea came to prominence. Even though tracking frequencies of different phenotypes in natural populations over time is expensive and difficult to fund, it may ultimately be the only way to understand more fully the factors influencing diversity in natural populations.

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