Sexual conflict and cryptic female mate choice in the Coelopidae

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

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May 2005

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Abstract

This thesis describes a collection of experiments investigating sexual conflict and cryptic female choice in the Coelopidae. An experiment examining the effect of the αβ inversion karyotype in *C. frigida* revealed that male karyotype has no direct influence on male willingness to mate or other mating interactions, however, male size and karyotype have an influence over the female rejection response. There appears to be no clear genetic effect of female karyotype on female rejection behaviour, which leaves the question as to why αα females are mated with more often, unanswered. The extent to which convenience polyandry occurs in *C. frigida* was also examined through the measurement of female re-mating frequency in wild populations. Females were found to mate hundreds of times in their lifetimes, making *C. frigida* an extremely promiscuous species with great potential for post-copulatory sexual selection to occur in this species. Evidence was found for cryptic female choice as females may have the ability to select sperm from within the ejaculate of a single heterokaryotypic male to produce fitter heterokaryotypic offspring. Time interval between copulations and the order of polyandrous copulations with males of different chromosomal karyotypes were found to interact in their effects on P_2 values. The mating systems of 5 Australasian coelopids were examined and a comparative analysis of sexually antagonistic co-evolution in 13 coelopids was carried out. Female-mediated sexual conflict appears to have played a role in increasing male size and variation in male size, though large male reluctance to mate may mask evidence for antagonistic co-evolution. ISSRs were found to be a very useful tool in determining genetic variation in *C. frigida* and the possible future use of this technique in paternity analysis is discussed.

Contents

Chapter 1. Introduction to sexual conflict and cryptic female mate choice

1.1 Sexual selection and sexual conflict

In his book 'On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life', Darwin (1859) proposed that sexual selection "...depends not on a struggle for existence, but on a struggle between males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring." A more modern interpretation of this may be that sexual selection acts to refine secondary sexual characteristics, such as morphological differences between males and females. However, due to our recent awareness of the processes driving sexual selection, a more operational definition seems appropriate: sexual selection is best defined as a difference in fecundity as a result of heritable differences in access to mates (Arnold & Wade 1984; Arnold 1994). The concept of sexual selection is governed by the theoretical hypothesis that if there is heritable variation in the traits that influence the capacity to acquire mates, then there should be selection for those traits that grant superior mating success (Ridley 1996). It is for this reason that these traits will proliferate and become more widespread over time.

Darwin proposed two forms of sexual selection: (i) male-male competition or 'intrasexual' selection, whereby the individuals (usually males) that outcompete others for access to partners, are selected for; and (ii) mate choice or 'intersexual' selection, whereby individuals of one sex (also usually males) evolve traits that attract members of the opposite sex to mate with them. The existence of two forms of sexual selection is a result of the asymmetries in the factors that regulate the lifetime reproductive success of males and females. Intra and intersexual selection are not mutually exclusive, however, and it is often the case that male competition is driven by the preferences shown by females (Halliday 1978), particularly in lek-breeding species (Balmford et al. 1992). Extricating the different forces of selection acting through male-male competition and female choice has proved to be very challenging. This has been highlighted recently by empirical studies focussing on the relative contributions of post-copulatory forms of sexual selection (e.g. Eberhard 1996, 1997; Simmons et al. 1996; Alexander et al. 1997; Danielsson 2001; Birkhead & Pizzari 2002).

Huxley (1938) was one of the first biologists to make the observation that "...among polygynous species, the variance in male reproductive success is likely to be greater than the variance in female reproductive success". This observation was further strengthened by Bateman a decade later, who proposed that female fertilisation was limited by factors such as egg production, milk production and nutrient supply to the foetus, whereas male fertility was not constrained by the production of sperm, but in the number of available mates (Bateman 1948). From this came Bateman's Principle, which states, "...the sex that invests more in the production of offspring becomes a 'resource' for which members of the less parental sex compete" (Bateman 1948). Bateman demonstrated with *Drosophila melanogaster*, that male mating success tends to be more variable than female mating success, resulting in stronger selection on males to maximise matings, which then generates differences in the selective pressures to which each sex is subjected. This confirmed the hypothesis that sexual selection acts more strongly on the sex that expends the least time and energy in rearing offspring (Trivers 1972) - in the majority of cases, this is the male. This results in a fundamental imbalance between the investment in offspring by males and females, whereby females frequently invest greatly in a small number of energy and nutrient-rich gametes and males invest a relatively small amount in each of a substantial number of energetically poor gametes, generating conflict between the sexes (Parker et al. 1972).

Sexual conflict may also develop from the constraint on reproductive success of one sex by another - any restriction in reproductive success caused by one sex will result in a counter-adaptation by the other sex to overcome the restriction (Birkhead 2000a). Conflict between the sexes can therefore raise the costs of mating because it can lead to the evolution of sexually antagonistic traits i.e. characteristics which increase the fitness of one sex at a cost to the other (Rice 1998). There are many traits, whether morphological, behavioural or physiological, that have the potential to co-evolve antagonistically (e.g. Chapman et al. 1995; Holland & Rice 1999; Johnstone & Keller 2000; Andrés & Arnqvist 2001; Hayashi & Kawata 2001; Arnqvist & Rowe 2002; Rowe & Arnqvist 2002; Laird et al. 2004). In a series of experiments carried out to determine how the genotypes of the sexes affect female oviposition and remating rates in the housefly *Musca domestica*, Andrés and Arnqvist (2001) found that the co-evolution of male seminal signals, and female receptors for those signals, is sexually antagonistic. Female *M. domestica* were also found to resist seminal signals with which they have co-evolved. A number of studies have

also focussed on the sexually antagonistic co-evolution of overt male and female armaments, for example, clasping and anti-clasping structures (e.g. Arnqvist 1989; Thornhill & Sauer 1991; Arnqvist & Rowe 1995; Sakaluk et al. 1995; Rowe & Arnqvist 2002). Arnqvist and Rowe analysed the effects of the evolution of male and female morphological armaments on the evolution of several behavioural traits, such as female mating rate, in the Gerridae (water striders) (Arnqvist & Rowe 2002; Rowe & Arnqvist 2002). They discovered that changes in the relative armament of the sexes were strongly associated with evolutionary changes in the outcome of sexually antagonistic interactions. They also identified the evolutionary trajectory describing the route along which the evolution of male and female arms levels is roughly balanced. However, they also showed that species might evolve off this path, resulting in one sex gaining a relative advantage over the other. The disadvantaged sex would then evolve to overcome the limitations imposed on its reproductive success. The results of a study carried out by Laird, Gwynne and Andrade (2002) suggested the exciting idea that the extreme repeated matings seen in the Australian scaly cricket, *Ornebius aperta*, may have evolved as a counter-adaptation to female-imposed limits on copulation, whereby the female quickly removes the spermatophore left by the male. These three examples of sexually antagonistic co-evolution give an indication of the huge diversity of traits that are able to co-evolve in an attempt to further the interests of one sex over the other.

Constraints on reproductive success may also occur after copulation through two processes: (i) post-copulatory male competition, or sperm competition; and (ii) post-copulatory female choice, or sperm choice, also termed cryptic female choice. These mechanisms, among others, will now be discussed within the context of inter and intrasexual selection.

1.2 Intrasexual selection and male choice

1.2.1 Male-male competition

Unlike natural selection, sexual selection does not select for adaptations in individuals that allow them to live more successfully in their environment, but enhances traits involved in mate acquirement. One of these traits is male weaponry. There are four major reasons why males of many species may have developed weaponry: firstly, they have evolved weapons to defend themselves against predators. Secondly, weapons may

have evolved to defend themselves against attack by other males of their kind. Thirdly, weapons may indicate relative strength and fighting capability. Lastly, weapons may indicate sexual vigour and quality.

In his enlightening book, *Sexual Selection*, Andersson (1994) suggests that 'among explanations for sexually dimorphic horns, antlers, tusks and spurs, the empirical support is strongest for the idea that they have evolved and are favoured in males as weapons in contests over females'. The number of studies which show that males compete with other males to gain matings with females is huge, as is the evidence that male weaponry, in particular, is a trait that has come under sexual selection through male-male competition (e.g. Clutton-Brock et al. 1977; Harvey et al. 1978; Maynard Smith & Brown 1986). A classic example of this can be seen in a study involving the reindeer, *Rangifer tarandus* – out of 713 matches between males of differing antler size, males with smaller pairs of antlers withdrew 90% of the time, and the males with larger antlers went on to successfully copulate with the associated females (Barrette & Vandal 1990). A number of weapon types, such as antlers, horns and mandibles, have probably evolved because of their effectiveness in attacking and defending during combat (Geist 1966) and in many species, males will fight fiercely until the opposition in seriously injured or dead (e.g. Geist 1974; Wilkinson & Shank 1977; Hamilton 1979). This type of serious physical combat is costly (Maynard Smith 1976), so many species use ritualised displays and direct or indirect trials of strength as a means of indicating and assessing fighting ability (Parker 1974). An example of this type of contest can be seen in the common toad, *Bufo bufo*. Males of this species assess the size, an indicator of fighting ability, of opponents by the pitch of their croaks. Smaller males produce higher-pitched croaks and larger males produce lower-pitched croaks. Males will then use this information to determine whether or not they should attack another male that is mounted on a female, and attempt to take over the female (Davies & Halliday 1978).

Male size is a morphological trait subject to many different selection pressures, including selection by male-male competition, as demonstrated above. Individuals that are unsuccessful in direct competitions due to their small size may have to employ some kind of alternative strategy to gain matings. A good example of this are the different strategies used by male bees of the species *Centris pallida*. Alcock, Jones and Buchmann (1977) found that the smallest males, which are often one third of the weight of the largest males,

had a different strategy to gain matings than their larger competitors. Large males patrol areas from which newly eclosed virgin females will emerge. When the male finds an emerging female, he digs her up and copulates with her, frequently having to fend off other males which attempt to dislodge him. Smaller males are unable to win such competitions with larger males, so have adopted a 'hovering' strategy, whereby they try to mate with females that have emerged unnoticed by the larger males. This strategy is by no means as successful as the larger males' strategy in terms of fertilisation success, but it ensures that smaller males get some matings. Both discrete and continuous polymorphisms in male size may also result in different mating strategies that are evolutionarily stable strategies (ESS) (Maynard Smith & Parker 1976; Enquist & Leimar 1983; Grafen 1987), whereby both male types gain equally in fertilisation success. This phenomenon was reviewed by Andersson (1994), who suggested that the processes of 'endurance rivalry' and 'scramble competition' should be included in the categorisation of mechanisms involved in competition for mates. Endurance rivalry, a term coined by Andersson (1994), is based on the principle that large males win more contests than smaller males i.e. they can exhibit greater endurance in fights due to their larger size and strength. In scramble competitions, smaller males can gain more matings because they have shorter development times and emerge earlier than larger males. In addition, they are more agile, a benefit during scrambles, and often engage in 'sneaky matings' with females (e.g. Gross 1985). Both of these mechanisms are present in many species where the alternative mating strategies implemented by males are evolutionarily stable (e.g. Parker 1970a; Alcock et al. 1977; Brockman et al. 1979; Cade 1979; Hamilton 1979; Thornhill 1981; Perill et al. 1982; Cade & Wyatt 1984; Gross 1985; Shuster & Wade 1991).

A less common way in which males may compete with each other is through infanticide, where males will kill any offspring fathered by another male before mating with the mother. This occurs in several species (e.g. Breden & Hausfater 1990; Pontier & Natoli 1999). An example of this can be seen in African lions (*Panthera leo*) - when a new male takes over a pride he will kill any cubs fathered by the previous pride leader (e.g. Grinnel & McComb 1996).

The mechanisms of male-male competition outlined above deal with pre-copulatory ways in which a male might try to maximise his reproductive success. In the next section, post-copulatory competition between males will be examined.

1.2.2 Sperm competition

Bateman's seminal experiments using *D. melanogaster*, demonstrated that the reproductive success of males has a positive association with the number of partners he copulates with, whereas female reproductive success does not (Bateman 1948). However, females frequently copulate with more than one male in many species, whether it is by choice or coercion. Parker (1970) was the first biologist to really comprehend that if more than one male mates with the same female in the same reproductive cycle, then sperm may compete to fertilise the eggs available. He termed this phenomenon 'sperm competition' (Parker 1970a; but see Winge 1937), and defined it as "...the competition between ejaculates of different males for the fertilisation of a female's eggs". This definition has since been updated and extended to 'the competition between the sperm of two or more males for the fertilisation of a given set of ova', to take into account organisms which disperse their gametes externally (Wigby & Chapman 2004). Theoretically, sperm competition could arise within individual ejaculates, though from the perspective of a male's own genetic interest, this seems of less benefit than when in competition with another unrelated male (see Parker & Begon 1993; Manning & Chamberlain 1994). There have been relatively few studies carried out in the field of intra-ejaculate competition to date. In addition to identifying the importance of fertilisation success rather than focussing on the importance of acquiring a mate, Parker also introduced the idea that sexual selection does not stop after copulation but continues to the moment of fertilisation (Parker 1970a). Selective pressures from sperm competition will therefore promote traits that increase the ability of males to successfully fertilise more eggs. The adaptations that have evolved to promote one male's sperm over another are diverse, but can generally be split into two categories, though they are not mutually exclusive (Parker & Simmons 1991):

① Examples of adaptations that allow males to avoid direct competition between sperm:

Prevention of female remating

A classic example of a species in which males influence female remating is *Drosophila melanogaster*. The ejaculate of males contains toxic accessory gland products that not only reduce female lifespan and increase sperm storage, but also delay female remating (Chapman et al. 1995). The transference of anti-aphrodisiac chemicals to females

during mating is also seen in other species; for instance, male butterflies of the species *Pieris napi* pass methyl-salicylate (MeS) to females, which make them unattractive to other males and therefore less likely to re-mate (Andersson et al. 2000). Since last male sperm precedence is an important factor in gaining fertilisations in many species, males deter female re-mating to increase the chance that their sperm will have precedence over any sperm stored from previous matings. If females are reluctant to re-mate, the last male to mate with her will most likely fertilise the majority of the eggs available. Sperm precedence will be discussed more fully in Chapter 8. Another method used by males to prevent females from remating is mate guarding. This behaviour is thought to have evolved as a male adaptation to avoid sperm competition (Thornhill & Alcock 1983; Simmons 2001) particularly in species where last male sperm precedence is common (e.g. McLain 1989). An example of this is the prolonged mate guarding exhibited by males during copulation in the sugarcane rootstalk borer weevil, *Diaprepes abbreviatus*. Harari and co-workers discovered that both males and females are less likely to re-mate when prolonged mate guarding occurs (Harari et al. 2003).

Mating plugs

In some species, males are able to 'plug' the female's reproductive tract or sperm store, sealing in any rival males' sperm or preventing the sperm from being stored. This is seen in species such as the mite, *Macrocheles muscaedomesticae* (Yasui 1994), the grasshopper, *Eyprepocnemis plorans* (Lopezleon et al. 1993) and the ghost spider crab, *Inachus phalangium* (Diesel 1990). In the latter case, the male initially transfers seminal fluid containing no sperm into the female's spermathecae. This coagulates, sealing in any sperm already present, and the male then transfers his sperm to the female.

Sperm displacement and removal

Another method of controlling sperm priority by avoiding direct competition is by the removal or displacement of rival males' sperm (e.g. Waage 1979; Siva-Jothy 1987; Parker & Simmons 1991; Gage 1992; Roderick et al. 2003). During copulation, males of some calopterygid damselflies displace other males' sperm already stored in the females' spermathecae - this happens in the damselfly *Ischnura graellsii* (Cordero & Miller 1992), for example. A fascinating alternative to this strategy is found in another species of

damselfly, *Calopteryx haemorrhoidalis*. In this species the male stimulates the female's vaginal sensilla (which control spermathecal sperm release) with his aedeagus, resulting in sperm ejection (Córdoba-Aguilar 1999, 2002).

② Examples of adaptations that elevate male fertilisation success when sperm are in direct competition with each other:

Sperm number

It is thought that transferring large numbers of sperm to a female is an advantage in sperm competition as it gives a male greater proportional representation in the sperm available for fertilisation (Parker 1982, 1984, 1995). It is also believed that males' fertilisation success increases with sperm number relative to those of other males (Parker et al. 1990; Cook et al. 1997; reviewed in Birkhead & Møller 1998). Quantitative competition may take place via the dilution of rivals' ejaculates by depositing a greater number of sperm than has already been stored, as seen in the beetle *Tenebrio molitor* (Gage 1992), among other species (e.g. Simmons 1987). Furthermore, when the risk of sperm competition is high, males are predicted to transfer more sperm to the female, either in a single ejaculate containing many sperm or by repeated copulations with the same female (e.g. Baker & Bellis 1989, 1993; Simmons et al. 1993; Otronen 1994a; Evans & Magurran 1999). In the fly *Dryomyza anilis*, males repeatedly copulate with females during oviposition (Otronen 1990). It has been suggested that this may have evolved in connection with males trying to secure their paternity, as females can discharge sperm at any point during mating (Otronen 1994a). Selection, through sperm competition, may act on males to maximise the number of sperm they produce, within the limits of their reproductive budget. This can be accomplished by reducing sperm size, resulting in a trade-off between sperm number and size (Parker 1982). The relationship between sperm size and number has been investigated in a number of species, with conflicting results. A trade-off between ejaculate sperm number and sperm size has been found in a cross-species study of *Drosophila* carried out by Pitnick (1996), however no such association between sperm size and number has been found in other species such as the red flour beetle, *Tribolium castaneum* (Arnaud et al. 2001). The role played by sperm size in sperm competition will be discussed in more detail in the next section.

Sperm size and form

Sexual selection via sperm competition is thought to be an influential factor in the evolution of sperm size variation (Roldan et al. 1992). Increased or decreased sperm size could be favoured by sperm competition if it was to result in the production of extra-competitive sperm. It is generally believed that larger sperm may be more competitive and fertilise more eggs than smaller sperm. There are three possible reasons why larger sperm may be more successful than smaller sperm. Firstly, larger sperm have greater reserves of energy so can live for longer inside the female than shorter sperm. There is, so far, no evidence to support this idea (see Birkhead 2000a). Secondly, larger sperm are more effective at avoiding or overcoming female counter-adaptations than smaller sperm. There is support for this idea from a number of studies (e.g. Dybas & Dybas 1981), the most well known of which being the comparative study on passerines, carried out by Briskie and Montgomerie (1993). Sperm length in these species ranges from around 50 μm to 300 μm and this study found that the longer sperm were found in species where sperm competition is intense. More importantly, the length of sperm was found to be positively associated with the length of females' sperm storage tubules, indicating that these two factors may have co-evolved through selection acting on females through sperm competition. The final reason that larger sperm may be more successful than smaller sperm is that sperm with longer flagella, and consequently size, are capable of moving faster than sperm with shorter flagella, therefore winning the 'race' to fertilise the egg. Several studies have shown that faster sperm win more fertilisations than slower sperm (e.g. LaMunyon & Ward 1998), but there is no indication that sperm with longer flagella can swim any faster than sperm with shorter flagella.

In addition to variation in sperm size, sperm morphology may also vary considerably within an ejaculate. Sperm polymorphism has been studied for many years in the Lepidoptera, and it has been suggested that males may influence sperm competition by producing a non-fertilising anucleate form of sperm (apyrene sperm), in addition to fertilising nucelate sperm (eupyrene sperm). Apyrene sperm has been widely shown to delay female remating in some Lepidopteran species by acting as a 'cheap-filler', deceiving the female by indicating to her that she has more fertilising sperm within her than she actually does (Cook & Wedell 1999). It has also been demonstrated that apyrene

sperm can interact directly with rival sperm, with detrimental affects to both forms (Silberglied et al. 1984).

Sperm competition is thought to be prevalent throughout the animal kingdom, though the full extent to which it occurs is currently unknown. It is obvious however, that sperm competition has the potential to shape an enormous range of sexual traits in males and females, including morphological, physiological and behavioural characters.

1.2.3 Male mate choice

In some species the cost of mating can potentially be very high for males. A good example of this is found in the golden orb-web spider, *Nephila plumipes*, where females frequently cannibalise males either before or during copulation (Elgar 1989). There are also considerable costs to mating for males in species where males provide females with nuptial food gifts (e.g. Reinhold 1999), such as in the scorpionfly *Panorpa cognata* (e.g. Engqvist & Sauer 2001). Males may discriminate between females by means of their potential fertilisation success or fecundity as indicated by factors such as body size, condition (e.g. Bonduriansky 2001), age (Simmons et al. 1994), survivorship (e.g. Dunn et al. 2001) and virginity (Simmons 2001; Herberstein et al. 2002). In cases where mating has costs for males, both the hazards involved in sperm competition and its intensity can also influence male mating decisions (Parker 1998; Bonduriansky 2001; Simmons 2001). Since males do not pass only a single gamete to females during copulation, the costs associated with mating are not through individual gametes in males, but though the entire ejaculate (Dewsbury 1982). Males may therefore alter the number of gametes allocated to a particular mating depending on the female's potential fertilisation success (Pitnick & Markow 1994). Indeed, variation in male gametic strategy as a result of varying female fecundity has been demonstrated in a number of species, including the cricket *Gryllodes supplicans* (Gage & Barnard 1996) and the Indian meal moth, *Plodia interpunctella* (Gage 1998).

1.3 Intersexual selection and cryptic female choice

1.3.1 Female mate choice

Female choice is the foundation on which theories concerning the evolution of elaborate male traits via intersexual selection are built (Darwin 1871; Andersson 1994;

reviewed by Kokko et al. 2002a). Females of many species seem to prefer to mate with males that display extreme characteristics such as extravagant plumage and ornaments, bright colours and conspicuous calls; all of which appear to be detrimental to male survival. Though male-male competition for females has been demonstrated in a number of species, providing evidence for female choice has proved to be more challenging. This is because it is often difficult to differentiate between the effects of female choice and male-male competition, from a female perspective. However, recent studies in this area have managed to show that female choice exists and that female mating preferences influence the evolution of male secondary sexual characteristics (reviewed by Andersson 1994).

The importance of mating biases outside the traditional concept of 'choice' has been recognised in recent years (e.g. Kokko et al. 2002a). It is now acknowledged that females may resist mating, and copulate only with males that overcome female reluctance to mate (e.g. Holland & Rice 1998; Blanckenhorn et al. 2000; Gavrilets et al. 2001). Additionally, female choice may be cryptic, taking place during or after mating and creating a fertilisation bias that favours particular males (e.g. Eberhard 1996; Edvardsson & Arnqvist 2000; Tallamy et al. 2002). These will be considered in more detail later in this chapter.

1.3.2 Evolution of female choice

Though it seems obvious why males might have evolved characteristics that make females more likely to choose them when females are choosy, the reasons behind the evolution and maintenance of female preferences are less clear. Female mate choice may have evolved by choosing some males over others as a means for females to increase their immediate fecundity through receiving direct benefits or eliminating some kind of cost (e.g. Hoelzer 1989; Price et al. 1993; Kirkpatrick 1996; Iwasa & Pomiankowski 1999). As a result, the female is able to increase the number of offspring she produces in her lifetime; hence the choice spreads via direct selection. This idea is further explained by the acquisition of resources hypothesis, which works on the principle that females will prefer males that are good providers of resources such as food or territory. This is a direct effect of selection on female preferences and can be relatively straightforward to demonstrate. A classic example of this type of female choice is in the case of hanging flies, *Hylobittacus apicalis*, where males provide females with food in the form of an insect. There is a direct positive association between female preference and the size of the insect provided by the

male, which is due to a direct effect of nutrition on the number of eggs a female is able to lay (Thornhill 1976, 1983). However, this system is open to abuse when males pretend to provide nuptial gifts for females to trick them into mating, as in the case of the dance fly, *Empis snoddyi* (Sadowski et al. 1999). The maintenance of variation in fitness between males can be easily understood, since stochastic environmental variation, such as food availability, can produce differences in the direct benefits that individual males offer females (see Kokko et al. 2002a).

Females of some species, however, do not appear to gain any apparent direct benefits from being choosy, and it is the processes resulting in this form of female choice which have puzzled biologists for decades (e.g. Kirkpatrick & Ryan 1991; Andersson 1994). When there seem to be no direct benefits, female choice can only have spread through the indirect benefits associated with producing offspring with increased lifetime reproductive success. There are currently two hypotheses that have been put forward to explain the evolution of female choice through indirect sexual selection: the 'good genes' hypothesis and the 'Fisherian mechanism' hypothesis (reviewed in Kirkpatrick & Ryan 1991; Andersson 1994).

'Good genes' hypothesis

The good genes hypothesis proposes that females will prefer traits exhibited by males, such as elaborate ornaments or colourful plumage, which indicate good health and good genetic quality. The female can then pass on the 'good' genes to her offspring along with the associated trait. However, the preferred trait must be one that only authentically 'good' males can display, or inferior quality males will be able to imitate the trait, which would make this system of female choice costly to females. It is from this concept that Zahavi's handicap principle was first suggested (Zahavi 1975, 1977). The principle is based on the theory that females must have honest signals of male quality from males in the form of costly traits (or handicaps). Since the costs of these traits are so high, only males of the best quality will be able to display them so females can make an informed decision on whether she should mate with a particular male or not. However, there has been some criticism directed at Zahavi's proposals (e.g. Maynard Smith 1976; Davis & O'Donald 1976). At the heart of this criticism is the idea that, though the offspring of a 'good' male will have inherited his 'good genes', they will also have reduced fitness as a

result of inheriting the male's handicap. To counteract this, the male bearing the handicap must have some kind of advantage in terms of fitness over other males. In addition to this, the preference for the handicap must be strong and the fitness associated with the handicap must be highly heritable. Another problem with the 'good genes' model is that if all individuals have good genes because all of the 'poor' genes have been selected out, then female choice would cease to exist, as there would be no benefits to being choosy since all males would be of the same quality. To combat this there needs to be something constantly creating variation in fitness between individuals. There have been two main hypotheses put forward to overcome this problem: (i) that variation in fitness is maintained by high levels of mutation and variation caused by environmental factors, and (ii) that variation in fitness is maintained because of interactions with co-evolving enemies, such as parasites. In the case of male traits indicating parasite load, a good argument can be made for genetic variation being maintained via the Red Queen Hypothesis (van Valen 1973), where selection for increased parasite resistance can maintain variation. The study carried out by Hamilton and Zuk involving the co-evolution of parasites and hosts, and the effect of this relationship on maintaining genetic diversity and female choice, supports this theory (Hamilton & Zuk 1982). The Hamilton-Zuk hypothesis was constructed in 1982 as a result of a study looking at traits exhibited by male passerines to indicate their parasite load to females (Hamilton & Zuk 1982). The Hamilton-Zuk hypothesis proposes that the traits exhibited to indicate parasite load must be condition-dependent, so males with a greater resistance to parasites are healthier and therefore more able to produce exaggerated traits. In addition, parasite susceptibility should be positively associated with ornamentation in general, for example, species with the potential for lots of parasites should be brighter than species with fewer parasites, as they have no need to display as an indication of their parasite load. Hamilton and Zuk studied the male plumage of 109 different passerine species and found that there was a positive relationship between bright, colourful plumage and parasite load, confirming that male handicaps are condition-dependent in terms of indicating parasite load. These results were further supported by a comprehensive study carried out by Møller (1990), who tested the assumptions made by Hamilton and Zuk using the barn swallow, *Hirundo rustica* and the mite *Ornithonyssus bursa*, a common parasite of barn swallows.

It has been difficult to prove the existence of the 'good genes' hypothesis of female choice because the fitness of offspring must be measured, which is subject to much

variation as a consequence of environmental conditions and other factors. However, several studies appear to provide evidence that it may exist. A good example of this is given by the work carried out by Petrie and co-workers on the peacock, *Pavo cristatus*. They mated females with randomly chosen males with varying numbers of eyespots then determined the fitness of the progeny produced. It was found that offspring fathered by males with a greater number of eyespots weighed more and had a higher chance of survival that offspring produced by males with less eyespots (Petrie et al. 1991; Petrie 1994). Another study, carried out on the dung fly *Sepsis cynipsea*, tested the good genes hypothesis using male body size as a possible indicator of 'good genes' (Blanckenhorn et al. 1998). It revealed that large body size is a heritable trait, associated with high fitness and consequently could be indicative of good genes.

Fisherian mechanism of sexual selection: 'sexy sons'

This hypothesis was proposed by Fisher (1930) and rests on the concept that female choice has, in some cases, co-evolved with a trait exaggeration. Initially female preference may evolve because the preferred trait is favoured by natural selection and as a result, any offspring produced are liable to inherit that advantageous trait. Males with the preferred trait are then at an even greater fitness advantage through natural and sexual selection. Female choice for particular traits may also spread from correlations between genes that make male offspring more attractive to female and female preference genes. Males with the preferred trait will become fitter through the increasing female preference for the trait, so the trait will continue to increase in frequency. In addition, the male offspring (or 'sexy sons') possessing the preferred trait will also increase in frequency. These mechanisms continue increasing in frequency, forming a 'feedback loop' - this process is termed 'Fisher's runaway hypothesis'. The runaway process eventually comes to an end when there is no genetic variation for additional trait or preference exaggeration left, or when the viability costs of the trait are in equilibrium with the mating benefits.

Fisher's runaway hypothesis of sexual selection, however, does appear to be less convincing when looked at in the context of the costs associated with female choosiness, such as the time and energy spent on discriminating among males (e.g. Pomiankowski 1988). Some work has recently been carried out to determine the costs associated with mate choice (e.g. Kokko et al. 2002b), particularly from the perspective of sexually

antagonistic or 'chase-away' models of the evolution of female mate choice (e.g. Holland & Rice 1998). These will be reviewed in more depth later in the chapter. There is another facet of this argument too - when female choosiness is directly advantageous, a female preference for a male trait will evolve, and the mechanisms associated with the male trait will follow (e.g. Lande 1981).

Around fifty years after Fisher proposed the runaway hypothesis, two biologists, Lande and Kirkpatrick, both presented mathematical models of the runaway process (Lande 1981; Kirkpatrick 1982). The Kirkpatrick-Lande model proposes that alleles for female choice lead females to selectively choose males with elaborate traits. Females having the allele responsible for that choice are distinct from 'wild-type' females, which have a more indiscriminate pattern of mate choice, resulting in choosy and non-choosy females. Assortative mating then produces an association between choice and the alleles responsible for the elaborate trait, while at the same time leaving alleles in non-choosy females to become associated with males that display traits that are not elaborate. This system then progresses in a similar way to the runaway process, whereby males with elaborate traits increase in frequency until the trait reaches fixation, even if the trait has a negative effect on male fitness and the mean fitness of the population is reduced. This is because the males with elaborate traits attain all the matings from choosy females and half of the matings from 'wild-type' non-choosy females, with non-elaborate males getting most of their matings from 'wild-type' females (Lande 1981; Kirkpatrick 1982).

A model constructed by Nichols and Butlin (1989) looked at whether the runaway process and the genetic correlation between female choice genes and the genes determining the male trait needed for the process to work, would break down in finite populations. They discovered that female preferences are ineffective at producing the required genetic correlation; as a result the runaway process only works when viability selection is comparatively weak. They concluded that mutations and random genetic drift are able to overcome such weak correlations, so female preferences must evolve via some other process.

Major criticism has recently been aimed at the use of the Fisherian hypothesis in describing the evolution of female choice, as it has been suggested that there may be no critical distinction between Fisherian and good genes processes (Eshel et al. 2000; Kokko

et al. 2002a,b). Kokko and colleagues (2002a) point out that the Fisherian mechanism of sexual selection is typically thought of as being synonymous with the runaway process. They also highlight the fact that the Fisherian mechanism suggests that females should prefer more vigorous males and that attractiveness might therefore be a sign of viability (Zahavi 1975, Jennions et al. 2001), giving rise to the divergence of the Fisherian mechanism of sexual selection from the 'good genes' hypothesis. In one of their celebrated papers, Kirkpatrick and Ryan (1991) emphasise that because preferences often happen to be correlated with male traits when both have a heritable genetic basis, 'sexy sons' should be considered as a vital piece of any realistic model of female choice through indirect benefits. Though this idea has been around for many years, there is still a dichotomous view of indirect benefits in the literature (Kokko et al. 2002a). Three important papers have recently been published dealing with this problem (Eshel et al. 2002; Kokko et al. 2002a,b) and they have all emphasised that attractive male signals always serve as indicators of other fitness components that they are genetically correlated with. In addition, they remind us that Fisher's original model (1930) centred on the belief that indirect selection on mating bias results from genetic correlations between the bias and total offspring fitness, including any 'sexy sons' benefit present as result of the inheritance of the attractive signal. From this, is can be understood that genes that are 'good' (i.e. they enable sons to achieve high mating success) are conceptually no different from genes that enhance offspring survival (Kokko et al. 2002a). It has been suggested that the use of the Fisher-Zahavi model (Eshel et al. 2000) should be adopted to describe the processes of mating-bias evolution by indirect benefits (Kokko et al. 2002a). This idea is currently under discussion, and no definitive answers have yet been found.

In addition to direct and indirect selection on mating preferences being responsible for the spread and maintenance of female choice, there are two alternative models which have recently had a great deal of attention: the sensory exploitation hypothesis and the 'chase-away' sexual selection hypothesis.

Sensory exploitation hypothesis

Selection operating directly on the sensory system of organisms, for reasons unconnected with mate choice, may be responsible for the generation of mating preferences. Females can, in some cases, prefer males with traits that they have a pre-

existing bias for i.e. the female preference for a male trait is a pleiotropic effect of sensory evolution which has evolved for other reasons (e.g. Kirkpatrick 1982; Endler 1990; Ryan & Keddy-Hector 1992; Ryan & Rand 1993). A classic example that demonstrates the pre-existence of female biases is a study carried out on species of *Xiphophorus*, commonly known as swordtail fish, where females have a preference for males with swords. Phylogenetic analyses carried out on these species showed that the females of ancestral species preferred males with swords, even though the corresponding males did not possess them (Basolo 1990; but see Meyer et al. 1994; Rosenthal & Evans 1998; Sherman & Reeve 1999). Another good example of this can be seen in the case of the common grackle, *Quiscalus quiscula.* Males of this species sing only one type of song, though females have been found to prefer multiple songs (Searcy 1992). Phylogenetic mapping was carried out in conjunction with behavioural observations, the results of which suggested two factors: (i) that the single-song condition displayed by males is a derived state and (ii) that the female preference for multiple songs may be a functional preference (Gray & Hagelin 1996).

From the idea of pre-existing sensory preferences in females emerges the 'sensory exploitation hypothesis', whereby males evolve traits to exploit the pre-existing preferences exhibited by females (Ryan 1990). In the water mite, *Neumania papillator*, males vibrate their legs to waft pheromones towards prospective mates. The females of this species react to these vibrations as if they were caused by prey. It is only after the male has deposited his spermatophore that the female recognises the male. It is thought that the vibrations made by the males have evolved as a response to the females pre-existing behaviour; this could therefore be a good example of the sensory exploitation hypothesis is action (Proctor 1991, 1992). The sensory exploitation hypothesis differs from the good genes and runaway hypotheses of sexual selection in one fundamental way: it assumes that male traits and female preferences for those traits have not co-evolved, but that the preference has existed long before the trait has developed. However, even when a male trait has developed to exploit a pre-existing sensory bias in females, indirect selection on the female preference for the trait may arise due to the benefits they receive from the production of sexier sons. Such a signal may then become genetically correlated with other traits that increase fitness. An example of this can be seen in the fiddler crab *Uca beebei*, where males build pillars from mud near their burrows. The presence of these pillars appears to increase male mating success, as they are thought to appeal to the female anti-

predator response (Christy et al. 2002). Additionally, the ability to build mud pillars is condition-dependent, which suggests that females who exhibit a preference for males that build pillars will gain further indirect benefits if the condition is heritable (Backwell et al. 1995).

'Chase-away' sexual selection hypothesis

It has recently been suggested that sexual conflict over mating may result in the evolution of mate choice (Arnqvist & Rowe 1995; Rice 1996, 1998; Rice & Holland 1997; Holland & Rice 1998, 1999; Gavrilets et al. 2001). As discussed earlier in this chapter, sexual antagonism and the conflicts that it can produce are fundamental to our understanding of reproduction and mating systems. Sexually antagonistic, co-evolving adaptations are an area of particular interest (e.g. Trivers 1972; Parker 1984; Arnqvist & Rowe 1995; Rice 1996; Arnqvist & Nilsson 2000). There are two ways in which the fitness of a choosy female may be manipulated by sexual antagonism: (i) the optimal expression of a trait may differ between the sexes (e.g. Rice & Chippindale 2001). However, the evolution of these traits may be relatively gradual (Lande 1980; Rhen 2000), resulting in a trade-off by females between the production of high-quality sons and lower-quality daughters. This situation is an indirect cost of sexual antagonism. (ii) In multiply mating species, males often benefit from increasing the reproductive rate of their mates, even if this is detrimental to the female's lifetime reproductive output (e.g. Chapman et al. 1995). This situation is a direct cost of sexual antagonism, and has been mentioned earlier in this chapter in the context of sexually antagonistic co-evolution of traits. An example of this type of direct effect is the reduction in female lifespan induced by male seminal products in *Drosophila melanogaster* (Chapman et al. 1995; Civetta & Clark 2000).

The first conceptual model of sexual selection leading to the evolution of exaggerated male display characters based on antagonistic co-evolution between the sexes was described by Holland and Rice (1998). This process was termed the 'chase-away' model of sexual selection, and was prompted by work carried out in three fields of research: intersexual conflict, sensory exploitation and female resistance to sensory stimulation. The model works on the premise that males will evolve an initial rudimentary trait that increases their attractiveness to females as a result of a pre-existing sensory bias in females. Females will then mate with these attractive males in a sub-optimal manner

which counter-selects females to evolve resistance to the male trait. This, in turn, makes males evolve even more exaggerated traits to overcome female resistance. Cyclical antagonistic co-evolution then follows (see Figure 1.1).

The main distinction given between the chase-away model and previous models is that the co-evolution between the male trait and female preference is opposing rather than reinforcing (Holland & Rice 1998). However, it has been argued that the chase-away model may be synonymous with other direct-benefit models, since the effort expended by males to encourage females to mate with them could be the reason why elaborate male traits have evolved (Rosenthal & Servedio 1999; Rice & Holland 1999).

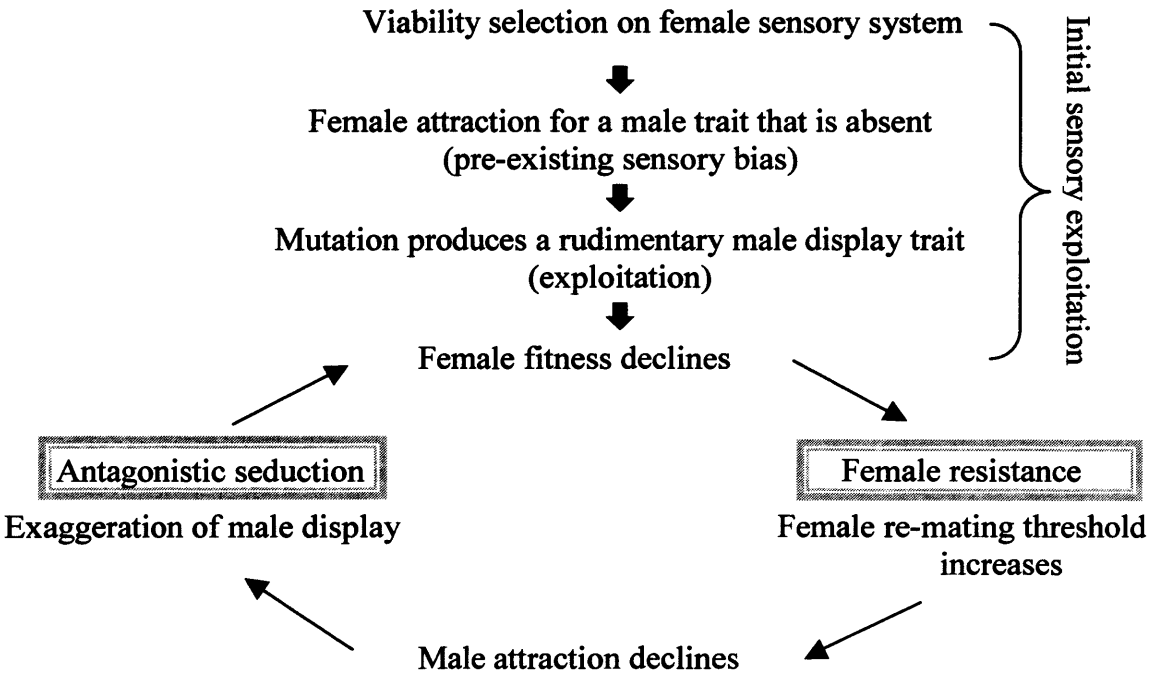

Figure 1.1 The chase-away sexual selection model for the evolution of elaborate male display traits (re-drawn from Holland & Rice 1998).

More recently, the first formal quantitative genetic model of the co-evolution of female mate choice and a male display trait under sexual conflict was constructed (Gavrilets et al. 2001). This model showed that mate choice can be generated as a side-effect of females evolving to reduce the direct costs of mating, and that differences in the interests of the sexes may be a much more general originator of female mate choice than previously thought (e.g. Holland & Rice 1998). A criticism aimed at models such as this is that they focus solely on direct costs and have disregarded the fact that mating itself may be costly to females (Kokko et al. 2002a). Kokko and colleagues (2002a) believe that direct and indirect benefits must be taken into account equally in both sexually antagonistic co-evolution and conventional female choice scenarios, because indirect benefits arise automatically when male traits are heritable. The evolution of female mate choice via sexual conflict is particularly interesting in the case of species such as seaweed flies, where females are subject to male harassment, because in any system where males gain from mating but females do not, antagonistic co-evolution between female perception and male signals can result in the evolution of costly female choice (e.g. Gavrilets et al. 2001).

1.3.3 Cryptic female mate choice

Females are known to be able to differentiate between sperm from a different species and sperm from their own, through signals present in seminal fluid (Markow 1997). Females can also differentiate between the sperm of males of their own species through seminal signals, preventing them from producing inbred offspring, for example (reviewed in Clark et al. 1999). From these findings, it would then seem logical that females may benefit from having a degree of control over the outcome of copulations with two or more partners. The term 'cryptic female choice' was first introduced by Thornhill (1983), and describes the mechanism of post-copulatory sexual selection where females bias sperm use in favour of particular males (Eberhard 1996). Unlike the Darwinian view, whereby female choice is thought to occur before copulation has taken place, and measured only by the success of males in copulation with females, the mechanisms believed to be involved in cryptic female choice are put into play during or after copulation has taken place. There are certain conditions under which females would appear to benefit from controlling fertilisation, in particular when males are able to coerce females into unwanted copulations (e.g. Clutton-Brock & Parker 1995; Gowaty & Buschaus 1998), as happens in the calopterygid damselfly *Calopteryx haemorrhoidales haemorrhoidales* (Cordero 1999).

However, in any polyandrous mating system where females mate with more than one male, there is the potential for cryptic female choice. Post-mating female choice can occur in two contexts: (i) when females favour the use of sperm from preferred mates for fertilising eggs; and (ii) when they direct nurture towards zygotes fertilised by superior mates. The former, results from females' ability to reduce or eliminate completely the fertilising potential of ejaculates from inferior males through a number of processes.

Eberhard (1996, 1997) reviewed the numerous ways in which females are able to control the fertilisation of their eggs. These range from the ability of females to discard sperm from males, as in the case of the fly, *Dryomyza anilis* (Otronen 1990, Otronen & Siva-Jothy 1991), through to the female being able to change the morphology of their reproductive tract to impede sperm transfer, as is seen in the golden orb-weaving spider, *Nephila clavipes* (Christenson 1990). According to Eberhard (1996), there are upwards of twenty mechanisms in total, with more undoubtedly to be discovered. These processes are not mutually exclusive, and are often used simultaneously (see Table 1.1).

Cryptic female choice has recently been demonstrated in a number of species (e.g. Johnson et al. 1999; Edvardsson & Arnqvist 2000; Ward 2000; Burger et al. 2003; Thiel & Hinojosa 2003). A good example of this can be seen in a study using the spider *Opopaea fosuma* (Burger et al. 2003). Burger and colleagues found that the anterior wall of the female's spermatheca is sclerotised and possesses a cone-shaped hole in its upper part. One sclerite, serving as a point of muscle attachment, bears a nail-like structure that gets pressed into the hole of the spermatheca when the muscles contract, locking the uterus externus. Simultaneously, the copulatory orifice becomes enlarged. They suggest that the resulting suction may lead to previously deposited sperm being drawn from the spermatheca and dumped. However, sperm dumping or ejection may not automatically be evidence of cryptic female choice, unless females differentially eject sperm (Eberhard 1996), and results of this kind could be open to interpretation.

There is a major hurdle that must be overcome for the unequivocal demonstration of the existence of cryptic female choice. Once two or more males have inseminated a female, there is potential for two processes to determine which male is successful in fertilisation: (i) the male-mediated process of sperm competition and (ii) the female-mediated process of cryptic female choice. It is difficult to disentangle the effects of these processes, and it has

been suggested that the only successful method is to control for the effect of the opposing process (Birkhead 1998). Birkhead's (1998) discussion of the criteria necessary to demonstrate that females are able coordinate their use of sperm to bias paternity in favour of some males over others, resulted in a flurry of comments and criticisms (e.g. Eberhard 2000; Kempenaers et al. 2000; Pitnick & Brown 2000), highlighting the contention and controversy that exists in this field. The three criteria outlined by Birkhead for demonstrating cryptic female choice are centred on experiments where two males mate with a single female (Birkhead 1998). Firstly, he proposed that it is necessary that there is variance in the proportion of offspring fathered by the second male to mate (P_2 values). Secondly, that some of this variance must be shown to be attributable to the males; and thirdly, that some of the variance must be shown to be attributable to the female. It has been argued that these criteria cannot provide conclusive evidence for or against sperm competition since the data he presented relate to only a narrow range of possible female effects, and that he introduces a misleading dichotomy between the two processes of sperm competition and cryptic female choice (Eberhard 2000). These opinions are also supported by Pitnick and Brown (2000) who also believe that direct evidence of cryptic female choice will ultimately require either experimental manipulation of the recognised criteria of choice, or identification of the mechanisms that females use to affect their choice. In response to these criticisms, Birkhead (2000b) suggested a three-stage protocol for research in the study of sperm competition: (i) that a more general definition of cryptic female choice needs to be used; (ii) that the mechanisms responsible for cryptic female choice need to be established; and (iii) that an attempt needs to be made to determine whether the mechanisms are 'active' or 'passive'.

In a cross-species study carried by Eberhard (1994) looking at courtship during copulation and the implications this has for cryptic female choice, he suggests that it may be possible to use the male's behaviour during copulation as a conservative assay to infer cryptic female choice. In summary, the reason he gives for this is that in species where female choice occurs, males of that species will be selected to attempt to influence the female's choice by inducing her to perform the processes that will increase his chance of fathering her offspring. The role of copulatory courtship in demonstrating cryptic female choice will be discussed in greater depth later in this chapter, using the red flour beetle, *Tribolium castaneum*, as an example.

Table 1.1 Examples of mechanisms of cryptic female choice in insects and arachnids, with an illustrative species for each (from Eberhard 1997).

Mechanism	Illustrative Species	Source
Discard sperm of current male	*Dryomyza anilis* (Diptera)	Otronen 1990; Otronen & Siva-Jothy 1991
Discard sperm from previous male	*Paraphlebia quinta* (Odonata)	Gonzalez-Soriano & Cordoba-Aguilar 2003
Prevent complete intromission	*Anastrepha suspensa* (Diptera)	Webb et al. 1984
Failure to transport sperm to storage	*Neriene litigiosa* (Araneae)	Watson 1991
Female remating with another male	*Centris pallida* (Hymenoptera)	Alcock & Buchmann 1985
Reduced rate of oviposition	*Harpobittacus nigricornis* (Mecoptera)	Thornhill 1983
Premature forceful termination of copulation by female	*Zorotypus barberi* (Zoraptera)	Choe 1995
Impede plugging of reproductive tract	*Agelena limbata* (Araneae)	Masumoto 1991, 1993
Impede plug removal by male	*Phidippus johnsoni* (Araneae)	Jackson 1980
Removal of spermatophore before sperm transfer is complete	*Gryllus bimaculatus* (Orthoptera)	Simmons 1986
Biased use of stored sperm	*Chorthippus parallelus* (Orthoptera)	Bella et al. 1992
Removal of a previous male's sperm to a site where the current male can manipulate them	*Metaplastes ornatus* (Orthoptera)	von Helversen & von Helversen 1991
Increased difficulty in sperm transfer due to morphological changes	*Nephila clavipes* (Araneae)	Christenson 1990
Resistance to male manipulations that result in the discharge of his spermatophore	*Bothriurus flavidus* (Scorpiones)	Peretti 1996
Less investment in each offspring	*Requena verticalis* (Orthoptera)	Gwynne 1986

There still remains much confusion surrounding the appropriate methods to use to demonstrate cryptic female choice, and this situation appears set to continue for the foreseeable future. This field of research is still in its early stages, but it remains exciting and important despite the differences of opinion among biologists.

1.4 Empirical examples

The following empirical examples illustrate some of the work that has been carried out in the fields of mate choice and post-copulatory sexual selection. These examples have been selected in particular because they are some of the most studied species in this area and they also parallel the studies carried out on coelopids in many ways.

1.4.1 The red flour beetle, *Tribolium castaneum*

The red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae) is one of the principal global pests of cereal products such as flour (Sokoloff 1972). It is also used as a model system for studies of copulatory behaviour (e.g. Wojcik 1969; Lewis & Iannini 1995; Edvardsson & Arnqvist 2000; Nilsson et al. 2002; Pai & Yan 2002), sperm movement and competition (e.g. Schlager 1960; Wool & Bergstrom 1979; Lewis & Austad 1990, 1994; Bloch Qazi et al. 1996, 1998; Lewis & Jutkiewicz 1998; Edvardsson & Arnqvist 2000; Arnaud et al. 2001; Bloch Qazi 2003) and cryptic female choice (e.g. Bloch Qazi et al. 1998; Edvardsson & Arnqvist 2000; Bloch Qazi 2003). The mating system of *T. castaneum* is polygamous, with both males and females mating multiply. During copulation, sperm is quickly transferred to the female via a spermatophore (Bloch Qazi et al. 1996).

Sperm competition studies in this species have shown that the relative fertilisation success of males is very variable (Lewis & Austad 1990, 1994; Wade et al. 1994; Bloch Qazi et al. 1996). Lewis and Austad (1990) examined the sources of variation in sperm precedence in *T. castaneum* and found that 17.8% of the total variation seen could be attributed to consistent differences between pairs of males. They also re-examined the published literature in this field and discovered that there was a high degree of intraspecific variation in sperm precedence. This variation has been subsequently attributed to the role played by females in sperm storage, and there is compelling evidence that females actively

move sperm from the site of deposition (bursa copulatrix) into sperm storage areas (spermathecae) (Bloch Qazi et al. 1998).

A number of studies have demonstrated that females exhibit pre-copulatory mate choice via a pheromone, 4-8 dimethyl decanal, which acts as a sexual attractant as well as an aggregation pheromone (Boake 1985, 1986; Obeng-Ofori & Coaker 1990; Lewis & Austad 1994). Post-copulatory female choice in this species has also been investigated (e.g. Bloch Qazi et al. 1998; Edvardsson & Arnqvist 2000). Edvardsson and Arnqvist's (2000) study suggests that females are the cause of the positive relationship found between male copulatory courtship behaviour and relative fertilisation success seen in this species. They compared the importance of copulatory courtship behaviour for relative fertilisation success in normal *T. castaneum* males, with that shown by males in which the tarsi of one or two legs had been removed (during copulation with the female, the male rubs the lateral edges of the female's elytra with his tarsi). It was found that treated males performed the rubbing behaviour, but they did not reach the target site of stimulation for the female, resulting in no positive relationship. This indicates that it is the female perception of the male behaviour, not the male behaviour *per se*, that determines the fate of sperm, consequently implying that the courtship behaviour exhibited by male *T. castaneum* is under selection via cryptic female choice. Eberhard (1996) interpreted male copulatory behaviour as a form of copulatory courtship used to stimulate the female into increasing the paternity success of the male. This idea has been reinforced by a number of studies where male copulatory behaviour has been associated with fertilisation success. (e.g. Otronen 1994a; Otronen & Siva-Jothy 1991; Watson 1991, 1998; Eberhard 1996; Arnqvist & Danielsson 1999; Edvardsson & Arnqvist 2000). Female copulatory behaviour has also been found to be a predicting factor in sperm fate: female quiescence during copulation in *T. castaneum* may provide information about the number of sperm transferred (Bloch Qazi 2003).

As mentioned earlier in this chapter, direct empirical evidence for cryptic female choice is limited and controversial (e.g. Birkhead 1998; Telford & Jennions 1998). It has been suggested that the best evidence for female-mediated post-mating paternity biases (i.e. cryptic female choice) comes from studies of copulatory behaviour (Eberhard 1996), such as those carried out using *T. castaneum*. It has also been suggested that examining the interactions between sperm use and copulatory behaviour may not present conclusive

evidence for cryptic female choice, but it is certainly valuable in revealing how this mechanism operates (e.g. Birkhead 1998, 2000; Eberhard 2000; Simmons 2001; Bloch Qazi 2003).

1.4.2 The yellow dung fly, *Scathophaga stercoraria*

The yellow dung fly, *Scathophaga stercoraria*, is one of the most intensively studied insect species in terms of sexual selection and sexual conflict, particularly in the field of post-copulatory sexual selection (e.g. Parker 1970b; Simmons & Parker 1992; Ward 1993, 1998, 2000; Otronen et al. 1997; Hosken et al. 2002; Ward et al. 2002). Females of this species visit cowpats, their oviposition source, and at this point, are ready for males to mate with them (Parker 1970b). Males mount females almost immediately, and take them to the surrounding grass to copulate, returning them to the cowpat in order to oviposit (Parker 1971). However, at any point during copulation, another male may attempt to dislodge the mounted male (Parker 1970 b). If the challenging male is larger than the mounted male, a successful 'take-over' may ensue. Sigurjónsdóttir and Parker (1981) found that 30% of pairs were subjected to successful take-overs. Copulation lasts for approximately forty minutes, and males remain mounted on the female while she oviposits, which may take an additional twenty minutes (Parker 1970b).

Depending on factors such as the size of the oviposition source and the number of males in the vicinity, males adopt different strategies in order to maximise their mating success. In areas with a lower density of males, larger males may establish territories around cowpats, defending them from smaller males through direct male-male combat. In areas where there is a high density of males, males compete for females through scramble competition (Borgia 1980, 1982).

S. stercoraria have long been thought of as an excellent subject on which to study the processes of male-male competition and female choice (Ward 2000), and they have become a model system for the study of sperm competition (e.g. Parker 1970b; Parker et al. 1990; Simmons & Parker 1992; Hosken & Ward 2000; Hosken & Ward 2001; Hosken et al. 2003). Last male precedence is found in this species, with approximately 80% of the offspring produced being fathered by the last male to mate (Parker 1970b). This percentage

is strongly associated with the duration of copulation of the last male to mate (Ward et al. 2002). Sperm competition takes the form of ejaculate displacement, where males use their own ejaculate to displace rival males' ejaculates (Parker & Simmons 1991). The direct displacement model proposed by Parker and Simmons (1991), where males were thought to ejaculate directly into females' sperm stores in a process solely under male control, was later found to be incorrect (Hosken & Ward 2000). Instead, the use of histological techniques demonstrated that sperm displacement was indirect and female-aided. Hosken and colleagues (2001, 2002) also discovered that there is an additive genetic component to sperm competitiveness in *S. stercoraria*, but that genetic similarity does not have an appreciable effect.

It has been suggested that female *S. stercoraria* may be able to bias sperm use towards particular males (Ward 1993; but see Simmons et al. 1996). Several recent studies have shown that cryptic female choice may be present in this species (e.g. Hellriegel & Bernasconi 2000; Ward 2000). The study of female-mediated differential sperm storage carried out by Hellriegel and Bernasconi (2000) presented evidence for differential storage of sperm from different males during copulation. It also showed that female muscular contractions were able to affect the storage and separation of ejaculates from competing males. Additionally, Ward (2000) convincingly demonstrated that females preferentially use the sperm of males of a certain genotype at the phosphoglucomutase (PGM) locus.

These empirical examples show the complexity of the processes involved in sexual selection and sexual conflict, in particular the entangled effects of the post-copulatory mechanisms of sperm competition and cryptic female choice on fertilisation success.

Chapter 2. Seaweed flies (Coelopidae)

2.1 Introduction

This chapter will present an overview of the biology of the Coelopidae, focussing primarily on *Coelopa frigida*, which is the main species studied throughout this thesis. Chapters dealing with other members of the family contain more detailed descriptions of their biology.

C. frigida is a sexually dimorphic species with male wing lengths ranging from around 2.0 mm to 8.0 mm and female wing lengths ranging from around 2.5 mm to 6.0 mm (Remmert 1957). Both sexes have a flattened body that is brown or black in colour, with females being generally darker in colour than males. Leg colouration tends to be of a lighter brown in both sexes (Aldrich 1929; Mayhew 1939). In profile, the face of *C. frigida* is deeply hollowed and the antennae are relatively small (Hendel 1928; Aldrich 1929).

2.2 Taxonomy and phylogeny of the Coelopidae

The Coelopidae (Diptera), commonly known as 'seaweed' or 'kelp' flies, are a small family of acalyptrate flies belonging to the super-family Sciomyzoidea. There are 28 known species divided into two sub-families, the Lopinae and the Coelopinae (McAlpine 1991). The diagnostic features of the Coelopidae family are described by Séguy (1934) and Hennig (1937). The huge variation in intra- and inter-specific morphology has caused a great deal of dispute between biologists trying to classify the various species (e.g. Haliday 1838; Zetterstedt 1847; Stenhammar 1854; Aldrich 1929; Hennig 1937; McAlpine 1991; Meier & Wiegmann 2002; Laamanen et al. 2003).

Phylogenetic studies of the Coelopidae using only morphological features have proved to be difficult due to the variation found between and within species (Meir & Wiegmann 2002). Only two real attempts have been made to create a phylogeny of the Coelopidae. The first was proposed by McAlpine (1991) and was based solely on morphological characteristics. He suggested that the Coelopidae phylogeny was basically dichotomous, with the sub-family Lopinae comprising one branch and the sub-family

Coelopinae comprising the second. The Lopinae branch consists of only one genus, *Lopa*, and the other is made up of the remainder of the genera. More recently a phylogenetic analysis of the Coelopidae based on DNA sequence data, in addition to morphological data, has been carried out (Meier & Wiegmann 2002). When a cladogram was constructed from morphological characters alone, it was found to closely resemble McAlpine's suggested phylogeny. However, once the DNA sequence data had been added into the analysis, the relationships between species were found to differ slightly from McAlpine's phylogeny.

2.2.1 Taxonomic history of *Coelopa frigida*

Fabricius (1805) was the first to name *Coelopa frigida*, but due to the extent of the morphological variability within this species, it was given several other names over the subsequent 50 years. Haliday was the first to allocate discrete species names to the three different size morphologies: *Coelopa gravis*, *Coelopa simplex* and *Coelopa parvula* (Haliday 1838), though this was later contested my Aldrich (1929) who believed that *C. gravis* and *C. parvula* were the same species, and thereafter assigned to them the name of *C. nebularum*. The Scandinavian species of *C. frigida* were first described by Zetterstedt in 1847. He suggested that the smallest morph be called *C. nitidula* (Zetterstedt 1847). The largest morph was named *C. eximia* seven years later by Stenhammar (1854). Thus, up to 1854, before Aldrich's revision in 1929, there were no less than five different species names. This classification was completely transformed in 1937 by Hennig, who suggested that the five distinct species of *Coelopa* were, in fact, only different morphs of the same species, *C. frigida* (Hennig 1937). This remained the popular view and was substantiated with an experiment, whereby all the different morphs previously described were attained from the same culture of *C. frigida* by simply altering the conditions in which they were kept, for example, by varying the quantity of seaweed provided for the development of larvae (Remmert 1959). In the case of the species *C. frigida* and *C. nebularum* (Aldrich 1929), the former found on Atlantic coastlines and the latter on Pacific coastlines, Hennig proposed that *C. nebularum* was only a part of a species complex, with *C. nebularum* being a sub-species of *C. frigida* (Hennig 1937). This idea was not acknowledged however, and they were regarded as discrete species until recently, when a study carried out in Denmark revealed that there is no post-zygotic reproductive isolation in the *C.frigida/nebularum* complex, and very little evidence for any pre-zygotic reproductive isolation mechanisms

(Laamanen et al. 2003). Since the majority of species concepts depend on reproductive isolation as the decisive factor in allocating separate species status, this study suggests that *C. frigida* and *C. nebularum* may be the same species, if morphologically very variable. However, the debate continues.

2.3 Geographic distribution of the Coelopidae

The Coelopidae are widely distributed throughout the world's coastal countries with the exception of South America, where none have been found to date, though members of the closely related Helcomizidae are present (Malloch 1933; C.P.M. Ramirez, personal communication). There also appears to be a scarcity of coelopids on tropical coastlines, with the majority found on temperate coasts, which corresponds with patterns of macroalgae distribution (McAlpine 1991). There are only three species that inhabit sub-tropical and tropical coastlines - *Dasycoleopa australis*, which is found on the east coast of Australia and two *Coelopa* species, *Coelopa ursina* and *Coelopa alluaudi*, which are distributed throughout the Indian and west Pacific Oceans. All other species are found on the temperate coastlines of the North Pacific and Atlantic Oceans (McAlpine 1991). The eighteen species of Coelopidae found on Australian and New Zealand coasts, ten of them endemic, comprises more than half of all the described species of coelopids so far, suggesting that Australasia is the 'centre of diversity' of the Coelopidae (McAlpine 1991).

C. frigida has been found throughout the shores of the North Atlantic and North Sea, including the eastern coast of Canada and the U.S.A, Iceland, Britain, Faroe Islands, Norway and Spitzbergen. It has also been found around the coastlines of the Skagerrak, Kattegat and Baltic Sea (Crean 1997).

2.4 Habitat and ecology of *Coelopa frigida*

Like all other members of the Coelopidae family, *C. frigida* reside and reproduce in a coastal 'wrack-bed' habitat. 'Wrack-beds' (Backlund 1945) are formed from supratidal deposits of beach-cast wrack. Macroalgae genera such as *Laminaria* and *Fucus* are torn from the intertidal areas of rocky shorelines or offshore seaweed beds and kelp forests, and through wave and tidal action, are deposited in masses just above the tide line. The peak times for wrack depositions are after a storm or a spring tide (Dobson 1974a,b). Wrack-

beds do not randomly form on any rocky shore that may have seaweed growing on it or in the sea nearby, but tend to form recurrently at particular sites on the coast where the local currents and topography cause the detached weed to accumulate (Phillips & Arthur 1994). The macroalgal composition of wrack-beds at a given site is thought to be very inconsistent and depending very much on the weather conditions at the time (Dobson 1974a).

If the wrack is cast upon the shore above high tide level, the wrack-bed may stay there without being disturbed for a number of days, weeks or even months (Egglishaw 1965). Bacterial action quickly leads to a breakdown of the algal material and an anaerobic layer is formed during the process of decomposition (Phillips & Arthur 1994). The next step in the succession is colonisation - initially, the wrack-bed is colonised by a numerically rich but species poor macro-invertebrate fauna, dominated by dipteran larvae (Backlund 1945; Bunt 1955; Remmert 1960; Griffiths & Stenton-Dozey 1981; Griffiths et al. 1983; Lavoie 1985), amphipods (Backlund 1945; Egglishaw 1965) and nematodes (Egglishaw 1965). Stranded wrack may provide a sizeable component of the diet of many supralittoral isopods, amphipods and dipterans (Stenton-Dozey & Griffiths 1980) and the huge numbers of these organisms present can form a considerable biomass (Griffiths & Stenton-Dozey 1981; Griffiths et al. 1983).

Stranded seaweed and debris can provide refuge and food for both aquatic and terrestrial organisms. It is a rich but ephemeral food and oviposition source, subject to tidal conditions - beach-cast wrack may be suspended by wave action at any time. It is also a limiting resource due to rapid algal decay, consumption and other environmental disturbances responsible for it's degradation. Another aspect of its volatility as a habitat is the range of temperature and salinity that a wrackbed can be subjected to. The variation in these factors depends largely on exposure to the sun and precipitation (Blanche 1992). The core temperatures of wrackbeds have been known to attain 50 °C (Phillips & Arthur 1994).

2.4.1 Trophic relationships between wrack-beds, *Coelopa* genera and microbial fauna

The most abundant colonisers of stranded algal material in Northern Europe, including Britain, are the Coelopid flies, *Coelopa frigida* and *Coelopa pilipes*. The feeding activity of the larvae of these species, together with various other dipteran larvae and

annelids, greatly accelerates the decomposition of the wrack-beds in which they are found by fragmenting the seaweed and therefore increasing the surface area exposed to bacterial breakdown (Bunt 1955; Rowell 1969). Larval feeding activity, in combination with bacterial activity, can eventually render seaweed into homogenous 'slurry' (Cullen et al. 1987).

Coelopids are thought not to breed successfully on any substrate other than the decomposing seaweed found on beaches (Egglishaw 1960; Dobson 1974a; Cullen et al. 1987) though there have been several reports of Coelopids having been found elsewhere (Oldroyd 1954; Egglishaw 1961) and in some cases, possibly feeding on the nectar from flowering plants and shrubs (Rebelo 1987; McAlpine 1991). In addition, there is anecdotal evidence that the larvae of some coelopid species may live successfully on decomposing carcasses, one case of which involved the body of a man found in the sea near the Swedish island of Oland on June 4th 1966. The larvae of *C. frigida* were found inside the body, which had come from a Finnish ship sunk in the Baltic Sea on January 14th 1966, suggesting that the body had, at some point, been washed up onshore[1]. If this story is true, then the Coelopidae could play a valuable role in the field of forensic entomology.

The reliance of coelopids on seaweed has been investigated to some extent and it has been found that seaweed, and the microorganisms living on it, provide a dual role in the nutritional requirements of Coelopids (Cullen et al. 1987). Coelopids have a saprophagous mode of nutrition whereby they ingest the bacteria involved in the process of decomposition, such as *Bacillus* and *Staphylococcus*, among others. The colonisation of the gut by bacteria takes place as the newly hatched larvae feed on the decomposing matter produced on the surface of the decaying seaweed, though it is currently unknown whether the bacteria are then digested or maintained in the gut by the larvae (Cullen et al. 1987). They also feed on the sugars released by degraded cells, in particular mannitol, and also require a minimum of 6% concentration of sodium chloride in addition to an, as yet, unidentified secondary metabolite contained within algal cells (Rowell 1969; Cullen et al. 1987). Adult nutrition and resource allocation has not yet been examined closely in *C. frigida* and nuptial feeding in this species has not been found (Pitafi 1991).

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2.4.2 Life History

Coelopids have a characteristically holometabolous life-cycle, whereby larvae hatch from eggs, go through three instar stages and a pre-pupal instar stage, pupate and then eclose as fully grown adult flies. Coelopids typically mate and lay their eggs deep within the wrack-bed, particularly in areas of well-decomposed algae with a high level of bacteria present and where the temperature is relatively high. Egg clutches normally contain approximately 70 to 120 eggs, though occasionally these are dispersed rather than laid in distinct clutches (Thompson 1951; Burnet 1960; Surver & Bender 1970). Females have been found to lay approximately 300 eggs in a lifetime, though this depends greatly on the condition of the female (Hill 1996; Pitafi 1991). The larvae hatch and feed, progressing through the instar stages of development. When the time comes to pupate, the larvae make their way upwards to drier areas of the wrackbed, occasionally onto pieces of driftwood or other debris. There they will pupate, sometimes creating 'rafts' of pupae, and finally eclose as adults. Coelopids become sexually mature approximately 18 hours after eclosion.

The entire life-cycle depends a great deal on the ambient temperature – lower temperatures result in slower development, while the contrary is true for higher temperatures (Thompson 1951). Previous studies suggest that the entire lifecycle may be completed in approximately eight to twenty-four days, depending on ambient temperature (Mayhew 1939; Aziz 1975). Under a laboratory culture temperature of 24°C, the full life-cycle of *C. frigida*, up to the point of eclosion, takes approximately 10 to 14 days (see Table 2.1). The most unpredictable stage of development, in terms of time, is the third larval instar. This stage can take anything from three to fifteen days to complete depending on environmental conditions such as food availability and humidity. It is thought that the variation in life-cycle length associated with changes in temperature may be due to the effect of temperature on the third instar developmental stage (Mayhew 1939). A study carried out by Mayhew (1939) demonstrated the marked effect of temperature on egg-to-adult development time (see Figure 2.1). Though the dataset for this experiment was small, a strong negative correlation can be seen between temperature and time taken to develop. This is supported by personal observation. It has been shown that the approximate development time of *C. frigida* in the wild, from a wrack-bed at St Mary's Island in the northeast of England, is 21 days, though this number may vary greatly depending on environmental conditions (Dobson 1974b). Variation in egg-to-adult development time

depends also on the size and sex of the fly. Females and smaller males develop more rapidly than larger males, and usually eclose one or two days earlier than their larger siblings (Day et al. 1980; personal observation).

Table 2.1 Duration of each developmental stage of *C. frigida* at 24°C (the pre-pupal instar stage is included in the pupa stage) (data from Thompson 1951).

Egg	First instar	Second instar	Third instar	Pupa	Total
20.6 hours	22.3 hours	23.8 hours	96.6 hours	94.9 hours	11.47 days

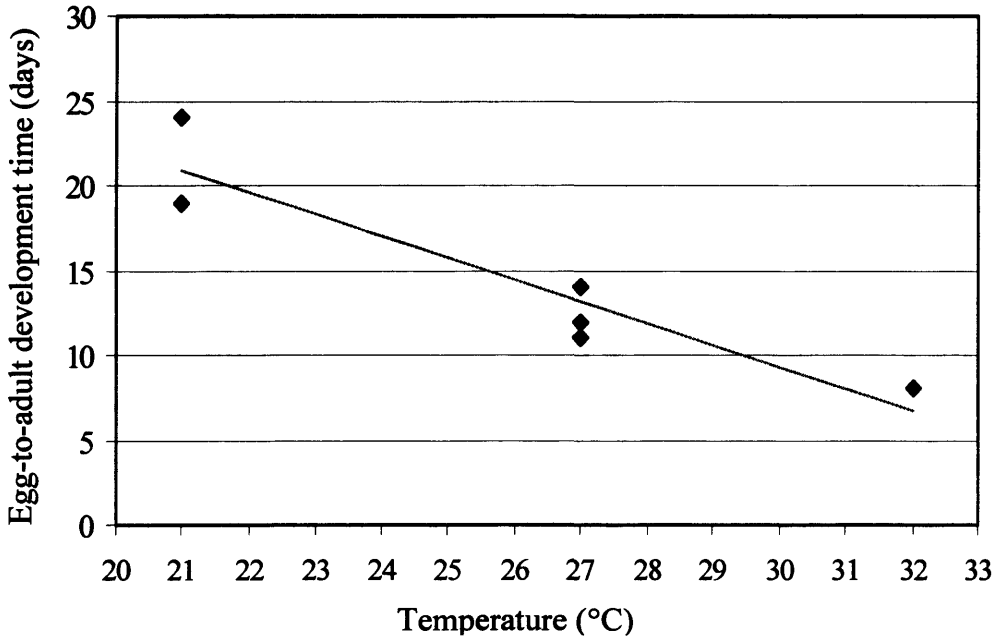


Figure 2.1 The effect of temperature on egg-to-adult development time for *C. frigida* with a linear line of best fit added (re-drawn from Mayhew 1939).

2.4.3 Population dynamics

Due to the instability of abiotic factors in the wrack-bed habitat in tidal areas, populations of *C. frigida* in Britain experience great fluctuations in numbers and may be subject to severe bottlenecks as a result of particularly high tides or storms. Attempts have been made to estimate the population size of *C. frigida* colonies using techniques such as mark-release-recapture, but with limited success (Dobson 1974b). Larval population size, determined via cores, was used by Burnet (1960, 1961) to calculate the frequency of lethal genes in a population of this species. It was found that the frequency was extremely high, suggesting that the actual population size far exceeds the effective population size. This is further supported by studies carried out using the frequency of rare alleles in populations of *C. frigida* to determine the effective population size (Butlin & Day 1989; Gilburn 1992). Further discussion involving population estimates and the movement of adult *C. frigida* is given in Chapter 5.

As mentioned earlier in this chapter, female *C. frigida* prefer to oviposit within areas of the wrack-bed where there is a relatively high temperature caused by active decomposition. This preference leads to an uneven distribution of larvae, with these "hot spots" being the sites of the highest larval density, whereas there are fewer larvae in colder areas (Burnet 1961; Phillips & Arthur 1994). However, it has been found that there is a more even distribution of larvae when larval density is very high (Burnet 1961; Butlin 1983). This could be as a result of an accelerated rate of decay, due to high levels of larval activity, which may generate more heat, hence the more homogenous distribution of larvae. The density of larvae within a wrack-bed is an important determining factor in the successful development and viability of larvae (Butlin 1983). The survival of larvae at the first instar stage of development depends greatly on high larval density. This is because the larvae are reliant on a well-decomposed environment to enable them to ingest enough bacteria for successful growth, which is facilitated by an elevated rate of larval activity. However, the benefits of high larval density are negated when the larvae reach the third instar stage, whereby high levels of intraspecific competition have a detrimental effect on viability (Butlin 1983; Leggett et al. 1996). A strong negative association has been found between the proportion of third instar *Coelopa* larvae present in a wrack-bed and temperature (Phillips & Arthur 1994), which suggests that third instar larvae may migrate

short distances at this stage, perhaps to avoid intraspecific competition and to pupate in drier regions.

2.4.4 Predation and parasitism

Due to the considerable abundance of coelopid larvae and other organisms colonising wrack-beds, beached wrack is an important foraging resource for shore birds (Brown & McLachlan 1990). Species commonly found feeding on coelopid larvae in Britain include the common starling (*Sturnus vulgaris*), gull species (*Larus spp.*) crow species (*Corvus spp.*), the common wren (*Troglodytes troglodytes*) (Davis & Baldridge 1980), rock pipit (*Anophus spinoletta*), purple sandpiper (*Calidris maritima*), turnstone (*Arenaria interpres*), redshank (*Tringa totanus*) and sanderling (*Calidris alba*) (A. Gilburn, personal communication). Outside Britain, other vertebrates use coelopid larvae as a food source, for example, the Pedra Branca skink (*Niveoscincus palfreymani*) (Brothers et al. 2003). Staphylinid beetles are also predators of larvae within wrack-beds and have been found to parasitise coelopid pupae (Backlund 1945), which are also parasitised by Hymenopteran families such as the Ichneumonidae (Ardö 1957; Egglishaw 1958). Parasitism of coelopids may be higher in areas of desiccated seaweed than in deep, moist wrack-beds, suggesting that the parasites may be less well adapted to a coastal environment (Heitland 1988). Additionally, wild coelopid populations are usually infested with the phoretic mite *Thinoseius fucicola*, which in excessive numbers can be damaging to laboratory cultures (Butlin 1983). The extent to which coelopid populations are affected by predation and parasitism is currently unknown, though in populations of extremely high density, it could prove minimal.

2.5 The genetics of *Coelopa frigida*

The first studies carried out on the genetics of *C. frigida* concerned the isolation of recessive mutations, from natural populations, affecting phenotypic traits such as eye colour (Thompson 1951). These were followed by investigations into the distribution of recessive autosomal embryonic lethals in wild populations (as described earlier in this chapter in terms of effective population size); the nature of the damage caused by these lethals and estimations of the pre-adult viability of heterozygotes for these lethals (Burnet

1961, 1962). A study carried out by Collins (1978) showed that approximately 34% of the loci determining enzyme production are polymorphic in *C. frigida*, which suggests that genetic variation in this species is fairly typical in relation to other Diptera. More recent studies looking at the intraspecific molecular variation in British populations have also been carried out (MacDonald & Brookfield 2002; this thesis). These are discussed in further detail in Chapter 9.

A remarkable proportion of the *C. frigida* genome, around a quarter, is contained within eight polymorphic chromosomal inversion systems (Philip 1958, 1966). There is one pair of dot chromosomes and five pairs of metacentric chromosomes, though the inversions are only found on chromosomes I to III (Aziz 1975). The largest inversion system, the αβ system, consists of three overlapping inversions and two forms exist that are subject to segregation in wild populations, the α and β forms. The αβ system is located on chromosome I, and it constitutes approximately 10% of the genome (Aziz 1975; Day et al. 1982). The αβ chromosome I inversion system has been found in all *C. frigida* populations studied so far (Butlin 1983; Day et al. 1983; Gilburn 1992; Gilburn & Day 1994a) and may also be present in the closely related species, *Coelopa nebularum* (Dunn et al. 1999). It is thought that this inversion system may be maintained via heterosis, whereby homokaryotypes are less fit than heterokaryotypes due to an accumulation of recessive deleterious mutations (Butlin 1983; Butlin & Day 1984; Butlin et al. 1984). The accumulation of recessive deleterious mutations is a result of a lack of recombination within the inversion region in heterokaryotypes (Butlin 1983). The occurrence of heterosis in *C. frigida* will be considered further in Chapter 8.

The presence of the αβ inversion system in *C. frigida* has a great effect on many life-history traits, in particular the rate of egg-to-adult development (Day et al. 1980; Butlin 1983), size (Butlin et al. 1982a), longevity and viability (Butlin & Day 1989). These effects are reviewed in Table 2.2. Though the inversion system influences both sexes similarly, the effects are more prominent in males (Day et al. 1980).

Table 2.2 The effect of the αβ inversion system on life-history traits (adapted from Crean 1997).

αβ inversion karyotype	Rate of larval development	Adult size	Adult longevity	Egg-to-adult viability
αα	slow	large	long	very low
αβ	intermediate	intermediate	intermediate	high
ββ	fast	small	short	Low

2.6 The mating system of *Coelopa frigida*

2.6.1 Mating behaviour

The mating behaviour of *C. frigida* has been studied comprehensively in the laboratory using controlled mating trials (e.g. Day et al. 1990; reviewed in Day & Gilburn 1997). Unlike many other Diptera, *C. frigida* does not exhibit any elaborate courtship behaviours such as the visual and auditory displays given by numerous *Drosophila* genera (e.g. Spieth 1952; Shorey 1962; Ewing & Bennet-Clark 1968). This is not unexpected considering the dark and complex environment of the wrack-bed habitat (Day et al 1990). Video recordings of mating trials have shown that both males and females appear to move around randomly until they come within a distance of approximately 2 cm of each other, whereby the male will often attempt to mount the female (Day et al. 1990). Males sometimes attempt to mount other males, but dismount immediately, and males are around 20 times more likely to mount a female than another male, indicating a degree of gender recognition (Day et al. 1990).

Once a male has successfully mounted a female, a pre-mating struggle typically ensues with three possible conclusions to the mount. Firstly, the female can physically remove the male. This is achieved by the display of three rejection responses by the female, which can occur either in succession or simultaneously: (i) the female curls her abdomen downwards to prevent the male from engaging genitalia; (ii) the female kicks the

male using her metathoracic legs and (iii) the female shakes her entire body vigorously in an attempt to dislodge the male. Secondly, the male can reject the female and dismount without any pre-mating struggle instigated by the female. A male may reject a female on the basis of a number of factors; for example, males are more likely to reject small females rather than large ones (Pitafi et al. 1990, 1994). Males are also less likely to reject fully gravid females than females that have already laid some of their eggs (Pitafi et al. 1995). Thirdly, the male can overcome any resistance and engage genitalia with the female. Copulation duration can range from 30 seconds to more than 10 minutes. Females usually terminate copulations by resuming rejection responses, whereby the male will either dismount or overcome them to copulate again (personal observation). Though males often copulate with a female more than once during a single mount (Shuker 1998), this is not considered to be mate guarding (e.g. Westneat 1994) due to the relatively brief duration of encounters and the fact that males do not remain with the female until oviposition, as is seen in the yellow dungfly, *Scathophaga stercoraria* (Parker 1970b).

Since males and females both exert a certain amount of mate choice, as discussed later in this chapter, trials in which the male dismounts while the female is exhibiting a rejection response such as abdomen curling, could introduce uncertainty into whether the result of the trial is due to male rejection of the female or vice versa. In the majority of cases in this thesis, this situation has been categorised as female rejection of the male, since the female is displaying rejection behaviour. Where this is not the case, the distinction has been made where necessary.

2.6.2 Sexual selection and mate choice

The majority of studies carried out on the mating preferences of *C. frigida* have focussed on female choice (e.g. Butlin et al. 1982b; Butlin 1983; Day & Butlin 1987; Engelhard et al. 1989; Gilburn 1992; Gilburn & Day 1994; Crean et al. 2000), though more recent studies have examined male choice (e.g. Dunn et al. 1999; Dunn et al. 2001). The first detailed studies of female mate choice used paternity inference to determine whether females preferentially mate with larger males rather than smaller males (Butlin et al 1982a; Butlin 1983). In half of the trials the larger males were monomorphic for the *Adh*-B allele and in the other half, the larger males were monomorphic for the *Adh*-D allele. The results showed that the larger of two males fathered the majority of offspring, independent of *Adh*

genotype. In contrast, later experiments suggested that there may be a small male advantage in mass-mated wild populations (Butlin 1983; Day et al. 1987; Foster 1989). There is also a great deal of variation found in the willingness of males to mount females (e.g. Dunn et al. 1999). These conflicting results generated some confusion, but they may not have been an accurate assessment of female mating preference, as they did not take into account the possibility of mixed paternity and multiple mating (Foster 1989). In response to this, further experiments were carried out using mating trials in which one wild female was placed with three males (Engelhard et al. 1989). These revealed that female preferences vary with female *Adh* genotype, implying that the αβ chromosome I inversion system influences the pattern of female choice and the preferred male trait (Engelhard et al 1989). One criticism that has been aimed at these experiments is that they did not control for the effects of male-male competition and male mate choice (Day & Gilburn 1997). Competition between males has been seen in *C. frigida*, whereby larger males are able to displace smaller males that have already mounted a female, in mating trials involving a single female and multiple males (Day et al. 1990; personal observation). However, the extent to which male-male competition operates as a selective force in *C. frigida* is currently unknown.

The introduction of 'single-mount' experiments, involving pairs of virgin flies of a similar age, has helped somewhat to unravel the complexities of female choice in this species (e.g. Gilburn 1992; Gilburn et al. 1992; Crean 1997; Shuker 1998). This method was used in a number of experiments measuring female acceptance rates (the proportion of mounts resulting in an acceptance) in wild populations of *C. frigida* (Gilburn et al. 1992; Gilburn et al. 1993; Gilburn et al 1996; Gilburn & Day 1994a). From these data the strength of female preference was estimated from the regression coefficients of acceptance rate on male size (Day & Gilburn 1997). It was found that in every population studied, females with αβ and ββ karyotypes preferred large males, though the strength of preference varied more in females with the αα karyotype, with a preference for small males seen occasionally. In addition, female preference was also found to vary with the tidal range of the habitat in which populations lived, with the preferences of αα and αβ females being stronger in non-tidal than in tidal populations. No such association with tidal conditions was found in ββ females (Gilburn & Day 1994a; Day & Gilburn 1997). The relationship between female preference and the chromosome I αβ inversion system

indicates that there is a genetic basis to variation in female behaviour. This was confirmed by a study of the inheritance of female mating behaviour in *C. frigida* undertaken by Gilburn and Day (1994c), which determined that the genes responsible for female mate preferences and female receptivity are co-inherited with the αβ inversion on chromosome I.

The selective forces driving the evolution of female mating preferences in *C. frigida* are not immediately clear. In many species, preferences have spread and are maintained in populations because they bring obvious direct benefits to choosy females, for example, female choice for males offering the greatest nuptial gifts in hanging flies (*Hylobittacus apicalis*). The ability of females to produce eggs in this species depends on their nutritional status (Thornhill 1976), so it is easy to understand how and why the female preference for males bringing the largest gifts has spread. However, female *C. frigida* do not appear to gain anything from males except genetic material (Pitafi 1991; Pitafi et al. 1994). It is possible that female choice may be advantageous in this species because it reduces the costs females incur in mating, for example, by lowering the probability of infection by parasites (see Borgia & Collis 1989). Alternatively, females may gain direct benefits in term of increased fecundity. These possibilities have not yet been investigated in any depth in *C. frigida.*

Alternatively, the evolution of female choice in *C. frigida* may have developed in response to indirect sexual selection (Gilburn et al. 1993; Gilburn & Day 1994a). As discussed in Chapter 1, the two main hypotheses put forward to explain the evolution of female choice in species where there are no direct benefits are (i) the 'good genes' model of sexual selection (alternatively known as the viability indicator mechanism of sexual selection), where the genes which increase fitness confer improved viability and are consequently favoured by natural selection; and (ii) the Fisherian mechanism of sexual selection, where females select males which are fitter because they are attractive to females, so they then produce 'sexy sons' through sexual selection. Both hypotheses have been examined in *C. frigida*, and both appear to contribute to the evolution and maintenance of female preference for large males in different ways (Gilburn et al. 1993; Gilburn & Day 1994a). The effect of the chromosome I αβ inversion system is one of the primary determinants of male body size (Day et al. 1980) and therefore, also the major determinant of viability between karyotypes (Butlin et al. 1984; Butlin & Day 1985a).

Because of these differences in viability, females with the αα or ββ homokaryotypes could mate disassortatively to produce heterokaryotypic offspring with increased viability. Large male size could therefore act as a viability indicator to those females whose offspring would subsequently gain from greater viability (Day & Gilburn 1997). When a model was constructed to establish the potential effects of the good genes mechanism, via heterosis, on the evolution of female preferences, it was found that when the force of indirect sexual selection acts alone on mating preference, only disassortative mating preferences will evolve (Gilburn & Day 1996). It was also found that the good genes model of sexual selection bore more advantages than the Fisherian mechanism of sexual selection under the majority of conditions. However, empirical studies looking at the relative contributions of both hypotheses have found that the effectiveness of each mechanism depends greatly on ecological factors such as tidal range and food stability. In populations where there is very little tidal variation and the availability of food is stable, females were found to prefer large males and produce 'sexy sons' via the Fisherian mechanism. On the contrary, females from tidal populations that underwent regular genetic bottlenecks seemed to mate disassortatively with regards to size and karyotype, thus producing a greater proportion of more viable heterokaryotypic offspring (Gilburn & Day 1994a). Although there is evidence for both mechanisms working in *C. frigida* populations, it remains problematic to understand how these mechanisms explain all of the mating patterns seen in females of this species (Day & Gilburn 1997). In particular, the mating preferences exhibited by αα homokaryotypes: under the good genes mechanisms, these females would be expected to prefer small males, which is rarely found in natural populations. Under the Fisherian mechanism, sexual selection would be predicted to make the inversion monomorphic, which does not seem to have happened in any of the populations studied so far. An in depth investigation into variation in female mate preferences in wild and laboratory populations of *C. frigida* carried out by Crean (1997) challenged the theory that there are different mechanisms of indirect sexual selection working in different populations. Instead she proposed that αα females show a range of preferences rather than distinct assortative or disassortative mating patterns. Furthermore, she introduced doubt into the idea that female preferences are genetically determined by discovering that they become unstable after several generations of inbreeding (see also Gilburn & Day 1994c).

These conundrums have opened the door to other potential ways in which female preferences are driven, for example, the sensory exploitation mechanism, whereby female choice may have arisen in some ecological context rather than through mate choice i.e. pre-existing female preferences. Though this may be the case in *C. frigida*, it is only through phylogenetic studies that this can be determined. Now that a phylogeny of the Coelopidae has been constructed (Meier & Wiegmann 2002), it may prove to be extremely interesting to investigate hypotheses such as sexual selection for sensory exploitation in this family.

Though the majority of experiments have dealt with female preferences, there is a smaller body of work concerning male mating preferences in *C. frigida*. The grounds for male choice are somewhat more transparent than in female choice. Research has shown that, in *C. frigida*, the number of male mount attempts steadily increases with female size and that the number of males dismounting females decreases sharply with female size (Pitafi et al. 1995, but see Dunn et al. 1999). This may be because there is a strong positive association between female size and lifetime fecundity (Butlin & Day 1985b; Pitafi et al. 1995). Reproductive success is also known to be higher in larger females (Pitafi et al. 1995). In addition, males also demonstrate a preference for gravid females with distended abdomens over females that have just laid eggs and have less distended abdomens (Pitafi et al. 1990; Pitafi 1991; Pitafi et al. 1995). These results suggest that males may be discriminating between females on the basis of female fertility, which is supported by the finding that males do not have an exhaustive supply of sperm (Pitafi et al. 1994). There is, however, some controversy surrounding the association (or lack of) between female size and male choice, as a recent study suggests that the willingness of males to mount females is not associated with size in a number of coelopid species, including *C. frigida* (Dunn et al. 1999). The debate continues as to whether male choice exists in this species.

2.6.3 Sexual conflict

Until recently, the majority of behavioural studies involving coelopids, in particular *C. frigida*, focussed primarily on mating preferences, as discussed above. These studies mainly examined the preference for large males exhibited by females and the forces of sexual selection associated with this preference (e.g. Gilburn et al. 1992; Gilburn & Day 1994). However, though these studies produced a wealth of interesting and useful results, it became apparent that concentrating solely on female choice was limiting the scope of

investigations. The results of recent studies looking at an alternative to the female choice explanation for the evolution of female mating behaviours that result in non-random mating patterns with respect to particular male characters, have placed the work carried out in this area on coelopids into the context of sexual conflict (e.g. Crean & Gilburn 1998; Shuker 1998; Crean et al. 2000; Shuker & Day 2001, 2002). The reason for this change in perspective was the discovery that sexual selection for large male size may occur as a side effect of sexual conflict (Crean & Gilburn 1998).

Male coelopids harass females into mating with them, with females being generally reluctant to mate, which results in a pre-mating struggle (Day et al. 1990). The absence of any pre-mating courtship behaviour and the presence of a pre-mating struggle in *C. frigida* suggest immediately that there is an inherent conflict of interests between the males and females of this species. The pre-mating struggle implies that males are trying to maximise the number of females with which they mate, whereas females are reluctant to mate and may attempt to stop unwanted copulations. Indeed, experiments in which females' antennae were masked suggest that females are normally in 'rejection mode' (Foster 1989; Day et al. 1990). However, the pre-mating struggle, compounded with the absence of any pre-mating courtship behaviour, could result in a dual interpretation of the outcomes of mating events: females may somehow be assessing the ability of males to overcome their rejection responses and from that, exercising choice over which males they mate with. Conversely, males may be coercing females into unwanted copulations, whereby females accept matings if the cost of rejecting the male outweighs the costs associated with accepting the male. This phenomenon is known as 'convenience polyandry' (Thornhill & Alcock 1983). However, a study carried out using *Coelopa nebularum* found that the choice of rejection strategy used by females is inconsistent; different strategies have similar success rates; and that the response to a mount is not influenced by the size of a male encountered immediately beforehand (Weall & Gilburn 2000). This indicates that female behaviour is not due to adaptive mate choice or mate assessment, but that females are simply attempting to avoid the costs of mating with all males (Weall & Gilburn 2000). Experiments conducted by Shuker (1998) provide additional evidence that females attempt to avoid mating if possible. He also suggests that if harassment levels are high enough, mating with a male reduces harassment and may become energetically more efficient. Convenience polyandry may be an important determinant of female mating frequency in

wild populations and its role in the mating system of *C. frigida* will be examined in greater detail in Chapter 5.

2.7 The aims of this thesis

The work presented in this thesis stems from unanswered questions arising from previous studies carried out on male and female mating preferences and sexual conflict in coelopids. This work has naturally become divided into two sections over the course of the research: pre-copulatory mechanisms of sexual conflict and post-copulatory mechanisms of sexual conflict, primarily focussing on the potential for cryptic female mate choice in *C. frigida.*

The aim of the first experiment examining pre-copulatory mechanisms of sexual conflict attempts to establish whether male *C. frigida* of differing size and karyotype have different mating strategies and whether females vary their rejection responses with male and female size and karyotype. It also tries to determine why females with the αα homokaryotype appear to mate more often than females of other karyotypes. This continues work initiated by Shuker (1998).

An experiment was constructed and carried out to reveal whether males exhibit plasticity in mating preferences for increased female survivorship as has been previously demonstrated in a closely related species, *Gluma musgravei* (Dun 2001; Dunn et al. 2001), in environments where oviposition substrates are scarce; with a corresponding increase in male preference for female gravidity, as has been found previously in *C. frigida* (Pitafi et al. 1995), when there is an abundance of oviposition substrate. Though this experiment was completed and the data analysed, the results were ambiguous and puzzling. The reason for these results became clear when a very recent study showed that the association between male mating preference and longevity was significantly dependent on the type of seaweed used in the experiment. Flies kept in the presence of *Laminaria*, a common oviposition substrate and constituent of wrack beds, showed a hugely increased mortality rate along with an increase in overall male mating activity compared to flies kept in the presence of *Fucus* (Meader, unpublished results), the macroalgae used in this study. It was therefore decided that this experiment would be omitted from the thesis, and will be repeated, taking into account this new information, at a later date.

The main aim of the second set of experiments is to elucidate and quantify female re-mating frequency in a wild population of *C. frigida* as a means of determining the extent to which convenience polyandry plays a role in this species' mating system, and hence indicate the potential for post-copulatory mechanisms of sexual selection. It also looks at temporal and spatial variation in sex ratio and population size of *C. frigida* in a natural wrack bed habitat, in addition to male mating behaviour overnight and differences between the mating behaviour of laboratory-reared and wild-collected flies.

The mating behaviour of a number of other members of the Coelopidae family will also be determined and this data will be added to what is already known about mating behaviour in coelopids. This extended dataset will then be used in a comparative study of sexually antagonistic co-evolution in the Coelopidae, which opens a new avenue of research in the field of coelopid biology.

In response to calls for post-copulatory mechanisms of sexual selection and hence sexual conflict, to be examined in the Coelopidae (e.g. Shuker 1998), the potential for cryptic female mate choice after copulation with a single male will be examined in addition to the effect of the αβ inversion system and inter-copulation interval on post-copulatory sexual selection in *C. frigida*.

The penultimate chapter describes a preliminary study of genetic variation in British populations of *C. frigida* using a recently introduced molecular technique that may prove invaluable in future studies of post-copulatory sexual selection in the Coelopidae, through methods such as parentage analysis.

Chapter 3. General materials and methods

3.1 Introduction

This chapter outlines the standard experimental techniques used during the project. Any additional methods for individual experiments will be given in the appropriate chapters.

3.2 Collection of *Coelopa frigida* larvae from the wild

Samples of wild flies for culture were collected from natural populations of larvae in the manner of Dobson (1973). Wrack-beds were located on the shore and the top layer removed to expose the larvae underneath. Handfuls of these were then scooped up from random sites around the wrack-bed to avoid aggregation of kin, and placed into standard perspex culture cages (20cm x 31.5cm x 25.5cm). Ventilated hardboard lids were placed over the entrance of the cages before being transported back to Leicester.

3.3 Collection and treatment of seaweed

Fresh minced seaweed was used as a substrate for oviposition, and as a source of food and moisture. This was collected approximately every three months from the intertidal zone of South Landing, Flamborough Head, Humberside (Ordinance Survey Map Reference: TA 255708). Collections took place at low tide when the seaweed was not submerged. Seaweed flies do not oviposit on fresh, living seaweed this far down the shore, and parasites here are absent. The macroalgae harvested consisted of *Fucus serratus* and *Fucus vesiculosus*. The seaweed was put into black plastic sacks and transported to the laboratory, where it was stored at -20°C until required. Sacks of seaweed were removed from the freezer 24 hours prior to use, and allowed to defrost in a refrigerator at 5°C before mincing. Defrosting in the refrigerator seemed to minimise the growth of fungal spores and kept the seaweed fresher than if it was defrosted at room temperature. Seaweed was minced using a Monarch E4612 industrial mincing machine (Fields & Plimblett, Oldham). Minced seaweed was usually used immediately, but on occasion was used up to two days after mincing. In these cases, it was stored at 5°C. Mincing increases the surface area of the

seaweed (Crean 1997), which promotes the proliferation of bacteria and other microbes, on which the larvae are known to feed (Cullen et al. 1987).

3.4 Laboratory culture and stock maintenance of *C. frigida*

Wild populations

Minced seaweed was frequently added to cages containing wild collections of larvae to provide additional sustenance. These cages were kept under quarantine conditions to prevent the proliferation of *Thinoseius fucicola*, a phoretic mite that infests natural populations. This was achieved by placing the cages in a water bath containing a dilute solution of household detergent. *T. fucicola* can have a damaging effect when found in high densities in laboratory cultures of seaweed flies (Butlin 1983; Crean 1997). The larvae were allowed to develop to pupae and then eclose within the population cages. As *C. frigida* usually occurs sympatrically with *Coelopa pilipes* in Britain, the adults were sorted into species and the *C. pilipes* discarded. This was carried out using light CO_2 anaesthesia.

Laboratory stock lines

Stocks of flies were maintained in perspex culture cages (20cm x 31.5cm x 25.5cm) covered by lids taped down tightly with masking tape. The lids were custom-made from hardboard with access holes, surrounded by a sleeve of fine gauge nylon netting to provide air exchange and to prevent flies from escaping during collection. The netting was knotted when not in use. A wad of absorbent cotton wool was placed over the access hole, on top of the netting, to prevent mould spores from entering the cage, whilst still allowing the exchange of air. The cages were half-filled with freshly minced seaweed (approximately 1.5-1.8 kg). During the summer months, a fungicide – a 20% solution of p-hydroxybenzoic acid methyl ester (Sigma Chemical Co., Dorset) in absolute ethanol (Sigma Chemical Co., Dorset) - was added to the seaweed in an attempt to prevent over-colonisation of fungal spores. This fungicide is known to be non-toxic but it was not always successful at combating mould in the cages, particularly in the height of summer, when spore numbers were at their highest. It was also found that the alcohol used as the solvent tended to make the seaweed degrade slightly and produce more mucus, which had a negative effect on the numbers of flies produced. Stock cages were kept in a constant temperature-controlled

room at a temperature of 25°C, approximately 60% relative humidity, with a 12 hour light-dark cycle.

To set up a cage for culture, approximately 20 males and 20 females were put into a cage prepared as above. The flies were allowed to mate and the females to oviposit. When the larvae were at the second or third instar stage, the surviving adults were removed. The larval density within the cage was closely monitored and if it became too high half of the larvae were removed and used to set up another culture. Additional seaweed was then added to both cages to reduce larval competition.

Cages in which the flies had finished eclosing were frozen at -20°C for several days before being thoroughly cleaned to prevent any contamination of future cultures.

3.5 Collection, treatment and handling of adult *C. frigida*

Collection

The collection of flies was carried out using an electric vacuum pump (Model D/351VM/13, Compton Compressors, Dawson McDonald & Dawson Ltd., Ashbourne, Derbyshire) attached to a 260 ml collecting bottle. When the bottle was considered full (approximately 200 flies), a piece of absorbent cotton wool (Wilkinson, Worksop, Nottinghamshire) soaked in 5% sucrose solution (Sigma Chemical Co., Dorset) was placed inside as a food source, and the bottle corked using a foam bung. Flies were collected within 18 hours of eclosion to ensure virginity, as adults are known to reach sexual maturity approximately 20 hours after eclosion (Thompson 1951).

Collection bottles containing flies were labelled with culture name and date, then put into a refrigerator at 5°C to slow down their metabolism and prevent them from copulating. Flies were kept under these conditions until required, though in most cases this period was for no more than 14 days.

Flies were transferred between collection bottles, mating chambers and microcentrifuge tubes via a manual aspirator whenever possible.

Carbon dioxide anaesthesia

In some instances, flies were required to be anaesthetised before experiments. This was carried out using carbon dioxide gas (BOC gases, Nottingham). A forked rubber tube leading from a CO_2 gas cylinder was fitted with a hollow metal needle and a gas pad (Strand Scientific, Sandiacre, Nottingham). The needle was inserted into the collection bottle and gas was passed through until the flies were unconscious. This usually took less than a minute. Once the flies were unconscious the foam bung was removed and the flies tapped out onto the gas pad, which had a slow, steady stream of CO_2 passing through it.

Flies could then be manipulated as required, but were never kept under anaesthesia for more than 5 minutes at a time, as CO_2 has been known to reduce, and in some cases, stop oviposition in a closely related species of seaweed fly, *Coelopa pilipes*, when left under prolonged anaesthesia. (A. Gilburn, personal communication). Etherisation was never used as a method of anaesthesia as it has been known to affect mate choice in the fruit fly, *Drosophila melanogaster* (Joachim & Curtsinger 1990).

3.6 Sex determination of flies

Male and female flies were usually stored separately. The determination of sex was done under CO_2 anaesthesia. The sexes are morphologically quite distinct with females having a more pointed, shiny abdomen than males, with paired cerci at the end, whereas males have conspicuous darkened external genitalia.

3.7 Determination of size

In the laboratory flies were measured using a dissection microscope (Model G26, Leica) fitted with an eyepiece graticule. In the field flies were measured using an electronic digital calliper (Model AK962EV, Sealey Professional Tools, Bury St. Edmunds, Suffolk) under x10 magnification provided by a hand lens (Opticron model, Alana Technology, Shropshire). When measurements from both pieces of equipment were compared, it was found that there was no significant difference between them, although the callipers were more accurate, measuring to the nearest 0.01mm rather than the 0.1mm resolution of the dissection microscope.

The traditional method of measuring seaweed fly size is to measure wing length, as this has been found to correlate well with the size of other morphological characters (Butlin 1983; Day et al. 1990; Dunn 2001), and it's measurement is highly repeatable (Shuker 1998). This was measured as a straight line between the two supra-alar bristles at the point of articulation with the thorax (Day et al. 1990) and the distal margin of the wing. Either wing could be measured, as no consistent difference between left and right wing sizes has been found within seaweed flies (Butlin 1983). Measurements were taken at a magnification of x15, which were then converted into millimetres (15 eye piece units = 1 mm).

3.8 Containers used in the culture of flies and in experiments

Several plastic, glass and perspex containers were used in experiments and in the culture and maintenance of fly stocks (see Table 3.1). Custom-made hardboard lids were used in conjunction with culture cages, as discussed previously. Bottles were stoppered using foam bungs and the entrances of powder-rounds were blocked using non-absorbent cellulose wadding (Robinson Ltd., Chesterfield). Plastic lids were provided with the small pots (diluvials) and these were pierced with holes to allow ventilation.

3.9 Derivation of inbred isokaryotypic lines

Inbred isokaryotypic lines were created from wild flies collected from St Mary's Island on the northeast coast of England (Ordinance Survey Ref: NZ352753). Larvae were collected and reared in the standard way, as discussed earlier in the chapter. Once eclosed, pairs of virgin adult flies from the wild cages were placed into individual pots containing minced seaweed and left to mate for approximately 72 hours under standard culture conditions. Flies were generally paired with individuals of a similar size, i.e. small males with small females and large males with large females. The pots were then checked for the presence of eggs and larvae and the adults removed. Any pots with no eggs or larvae were discarded at this stage. The *Adh* genotype of the adults was then determined using starch gel electrophoresis (see following section).

The larvae of desirable pairings were kept and put into individual powder rounds filled with minced seaweed, to allow them to finish development. The remaining larvae

were discarded. The most desirable pairings were any homogenotypic crosses, for example, *Adh*-BB ♀ x *Adh*-BB ♂. The progeny of these pairing were guaranteed to be isogenic, and isokaryotypic for the αβ inversion system (see subsequent section for an explanation). Isokaryotypic lines were also established from *Adh*-BD x BB and *Adh*-BD x DD pairings. In these instances, the larvae from each cross were pooled with larvae of the same cross and reared to adulthood in culture cages. These were then paired with each other to establish further BB (termed B1) and DD (termed D1) lines. This method occasionally had to be repeated for another generation or two in order to establish lines. All lines were produced within a maximum of five generations.

Table 3.1 Container specifications of those used in fly stock culture, maintenance and experiments.

Container	Dimensions	Manufacturer
Culture cage	20 cm x 31.5 cm x 25.5 cm	North West Plastics Ltd, Manchester, UK.
Powder-round (glass)	Height: 15 cm Diameter: 7.5 cm Volume: 550 ml	Unknown
Small pot (Diluvial)	Height: 5.5 cm Diameter: 3 cm Volume: 30 ml	Fisher Scientific UK Ltd, Loughborough, UK.
Bottle	260 ml	Stewart Plastics Ltd, Croydon, UK.
Microcentrifuge tube	1.5 ml	Fisher Scientific UK Ltd, Loughborough, UK.

3.10 Use of starch gel electrophoresis to determine αβ-chromosomal inversion karyotype

The genotype at the locus of the alcohol dehydrogenase allozyme in *C. frigida* can be used to ascertain the αβ chromosomal inversion karyotype of individual flies (Butlin

1982a). This is possible because of the complete linkage disequilibrium existing between the α and β forms of the inversion and the *Adh*-B and *Adh*-D alleles respectively (Day et al. 1982). There is also no recombination between the two forms. It has proved much quicker to type individuals using this method rather than karyotyping them using polytene chromosome preparations (Day & Gilburn 1997). The *Adh* genotypes of the flies can be assayed using starch gel electrophoresis. Flies that were to be used in this technique were frozen at -20°C immediately after use in experiments. This was to ensure that the *Adh* allozymes did not degrade excessively.

Porcelain spotting tiles (11.8 cm x 9.2 cm) with 12 wells each (diameter of wells 2.4 cm), which had been kept at a temperature of -20°C, were laid out in trays of ice. Each well corresponded to an individual fly. A small, inexact quantity of carborundum powder (Fisher Scientific UK Ltd., Loughborough, Leicestershire) was put into each well along with one drop of distilled water. Individual flies were then put into each well and homogenised using a solid glass rod to release the intracellular *Adh*. A small disc (diameter: 6 mm) of chromatography paper (Whatman 3MM) was then placed into each well to absorb the resulting homogenate. These discs were then transferred to a 24-tooth metal comb, which was used to insert the discs into a prepared 12 % starch gel (18.5 cm x 10 cm). Starch gels were prepared using hydrolysed potato starch (Sigma Chemical Co., Dorset) in a 1:1 mixture of Trisborate (TBE) buffer (see Table 3.2.) and distilled water. Each gel usually carried three lines of 24 inserts, allowing the assay of genotypes for 72 individuals.

Horizontal electrophoresis was carried out in custom-made perspex gel tanks (30 cm x 38 cm) containing TBE buffer of the same concentration as was used in the preparation of gels. Gels were run at 55mA (350V) for approximately 120 minutes using a BIO-RAD power pack (model 300). Ice packs were placed on top of the gels during electrophoresis to act as a cooling system. Once electrophoresis was complete, the gels were removed from the tanks and stained for alcohol dehydrogenase using an agar overlay stain (see Table 3.3.). When the agar overlay had solidified, the gels were transferred to an oven and incubated at 40°C for approximately 1 hour until the banding patterns had developed. The bands were then scored for *Adh* genotype.

Table 3.2 Constituents of Trisborate (TBE) buffer made up with distilled water.

Constituent	Final Concentration	Quantity added/ 2000 ml distilled water	Manufacturer
Tris (hydroxymethyl) aminomethane	0.17 M	41.174 g	Sigma Chemical Co., Dorset, UK.
Ethylenediaminetetraacetic acid (EDTA)	0.002 M	1.481 g	Sigma Chemical Co., Dorset.
Boric Acid (to adjust pH to 8.7)	0.05 M	6.183 g	Sigma Chemical Co., Dorset.

Table 3.3 Constituents of alcohol dehydrogenase stain overlay.

Constituent	Volume	Manufacturer
Tris-HCl buffer (0.1M Tris adjusted to pH 8.6 with 10M HCl)	10 ml	Sigma Chemical Co., Dorset, UK.
Isopropanol (anhydrous)	6 ml	Sigma Chemical Co., Dorset.
MTT (3-[Dimethylthiazol-2-yl]-2,5-diphenylteterazolium bromide) (10 mg/ 1 ml distilled water)	1.5 ml	Duchefa, Haarlem, Netherlands.
NAD (Nicotinamide adenine dinucleotide free acid) (1.5 mg/ 1 ml distilled water)	10 ml	Melford Laboratories Ltd., Chelsworth, Ipswich, Suffolk.
PMS (N-Methyldibenzopyrazine methyl sulfate salt) (2 mg/ 1 ml distilled water)	0.5 ml	Sigma Chemical Co., Dorset.
Bacteriological agar (2% w/v)	20ml	Sigma Chemical Co., Dorset.

3.11 Standard mating trials

All mating trials were carried out under the same conditions, unless stated otherwise, using virgin flies that were stored at 5°C for no longer than 5 days. Flies were lightly anaesthetised using CO_2 and the males put separately into small individual pots containing minced seaweed. Females were put into bottles in groups of approximately 20, with a ball of cotton wool soaked in 5% sucrose solution. Both sexes were then left for 48 hours at 25°C under a 12-hour light:dark regime. This provided a period of sex deprivation for both sexes, which facilitates willingness to mate (Gilburn 1992; Crean 1997). Males were stored individually to prevent them mounting each other, which results in a subsequent overall decrease in mount rate (Crean 1997). Females were not given access to seaweed during sex deprivation, as it acts as a cue for oviposition. Females were required to be gravid during experiments, as males have been found to prefer gravid females in some species of Coelopids (Pitafi 1991). After being sorted for the sex deprivation phase, flies were handled without anaesthesia.

Mating trials and behavioural observations were carried out in a mating chamber at room temperature (approximately 20 °C). This consisted of a 50 mm petri dish turned upside down, inside a culture cage resting on its side. The culture cage acted as a trap for any flies that escaped during the trials. Since adult seaweed flies are positively phototactic, a lamp was placed behind the cage to aid in catching any escapees. Generally three chambers were set up at any one time, with the additional chambers acting as acclimatisation areas for flies yet to be used in the actual experiment.

At the beginning of the trial, one female and one male fly were aspirated into the mating chamber. Each pair of flies was observed carefully and their behaviour noted. Examples of measurements and observations made include the time taken for the male to mount the female; the total time the male spent mounted on the female; pre-mating struggle duration; female rejection responses and the outcome of the trial. All trials were carried out over a period of 5 minutes, unless explicitly stated. When a male fly failed to mount a female within the 5-minute period allocated, the trail was scored as a non-mount. Exact details of behavioural observations carried out in each experiment are given in the Methods section for each chapter.

3.12 Statistical analyses

Particular statistical procedures carried out on data are described in the relevant chapters. General statistical analyses were carried out using the statistical packages SPSS 11.5 (SPSS Inc.) and Minitab 14 (Minitab Inc.). Additional computer packages used during analyses are detailed in specific chapters.

Chapter 4. The effect of chromosome I αβ inversion karyotype on mating behaviour in *Coelopa frigida*

4.1 Introduction

A number of studies have been carried out in recent years examining the nature of mating interactions in coelopids, with several of them focussing on the effect of factors such as body size and mating experience on such interactions in an attempt to elucidate the mechanisms involved in sexual conflict (e.g. Shuker 1998; Dunn 2001). It has been demonstrated that male size has a much greater influence on mating interactions than female size (e.g. Gilburn 1992; Shuker 1998). A positive association between male size and mating success has also been found in all of the coelopid species studied so far (e.g. Dunn et al. 1999). However, the impact of male and female αβ inversion karyotype has been investigated only briefly to date (Shuker 1998). As discussed in Chapter 2, *C. frigida* has a chromosome I αβ inversion system which is extremely large and comprises of three overlapping polymorphic inversions (Crocker & Day 1987; Day & Butlin 1987). Natural populations segregate for two forms: α and β, therefore forming αα and ββ homokaryotypes and αβ heterokaryotypes. The effects of the inversion system are inherited in a basic Mendelian way. The impact of the αβ inversion system on development and physiology are marked, especially in its effects on egg-to-adult viability (Collins 1978; Butlin 1983; Butlin et al. 1984; Butlin & Day 1985b; Foster 1989; Butlin & Day 1989; Leggett et al. 1996; Gilburn et al. 1996). The inversion system also influences the rate of larval development, adult longevity and adult size (Day et al. 1980; Butlin 1983). Karyotype, and its association with size, has a profound effect on the mating behaviour of *C. frigida.* An example of this can be seen in female mating behaviour, whereby α-homokaryotypic females have been found to be have greater mating success than β-homokaryotypes and αβ-heterokaryotypes (Gilburn et al. 1992).

Male size has been found, in various studies, to have a strongly negative association with male willingness to mate in *C. frigida* (Butlin et al. 1982, Gilburn & Day 1994a,b,c, Day et al. 1996, Dunn 2001). Since sexual selection favours large males, it is possible that this has forced males to increase in size over and above their naturally selected optimum, resulting in a reduction in male vigour (Dunn et al. 1999). There is conflicting evidence

concerning the association between male size and the rapidity of males to mount females in *C. frigida*, with some studies having found that larger males mount more rapidly (e.g. Butlin 1983), other studies having found the opposite (e.g. Gilburn 1992) and some studies finding no association at all (e.g. Shuker 1998). The study carried out by Shuker (1998) failed to find any association between size and mating interaction components such as copulation duration, mount rate, time to mount or rejection behaviours. He also found that the karyotypes of both sexes were not associated with mating interactions. However, the results of Shuker's study should perhaps be interpreted with care, since the isokaryotypic lines used in the mating trials were inbred and it has been found that inbreeding in laboratory strains of *C. frigida* may result in a loss of preference and an alteration in mating behaviour (Gilburn & Day 1994a; Crean 1997). Because of this body of conflicting evidence concerning the interactions between male size, willingness to mate and mating success, it would be highly informative to re-examine the mating interactions of *C. frigida* in an attempt to clarify these effects. The first aim of this study therefore, is to carry out such a re-examination. In addition, previous studies have suggested that male karyotype may have an effect on male behaviour due to sexual selection acting on different karyotypes to different degrees (Dunn et al. 1999). This is because the inversion system helps to maintain genetic variation in male size, resulting in the evolution of different size classes. In a simulation run by Gilburn (unpublished), differences in the interactions between genes determining size and other genes fixed on one form of the inversion were found to be due to differences in the strength of natural selection acting against large male size between the two inversion karyotypes, $\alpha\alpha$ and $\beta\beta$. The costs associated with being large, for example having to spend more time foraging (Ghiselin 1974), would vary between homokaryotypes and this, along with sexual selection for large male size, would result in two karyotype-associated size classes. It is therefore possible that α and β forms of the inversion may evolve separately. It has been suggested that the negative association between male willingness to mate and size could be due to genetic differences between the size classes of males rather than as a size effect *per se* (Dunn et al. 1999). The relative importance of male inversion karyotype and male size on the willingness of males to mate will also be evaluated in this study in order to separate the effects of these two factors.

As mentioned earlier, $\alpha\alpha$ females have been found to have greater mating success than those with the $\beta\beta$ or $\alpha\beta$ karyotypes (Gilburn & Day 1992; Day & Gilburn 1997),

though the factors that are responsible for generating this effect are, so far, unknown. The second aim of this study is therefore to determine whether there is a genetic effect on female behaviour that results in this phenomenon. As an integral part of this, the function and relative success of the different female rejection responses will be investigated within the context of the size and karyotype of both sexes. Female rejection response intensity and how it varies with female mating experience and karyotype has previously been studied by Shuker (1998), who categorised female rejection intensity into three classes: (i) high-intensity rejection, (ii) low-intensity rejection and (iii) passive. He found no association between female karyotype and these classes of behaviours or the karyotype effect on female mating success shown by previous studies. However, perhaps unsurprisingly, rejection intensity was found to be significantly associated with the outcome of mounts, with females showing high-intensity rejection responses being more successful at rejecting males than females showing low-intensity or passive rejection responses. No attempt has yet been made to look at whether the actual rejection responses exhibited by females vary depending on the size or karyotype of the male mounted, or whether females of different sizes or karyotypes show differences in the types of rejection response the use. It may be the case that females with the αα karyotype have a less successful rejection strategy than ββ or αβ karyotype females, resulting in a greater number of copulations.

This study will attempt to clarify and disentangle the effects of size and karyotype on a range of mating behaviours in *C. frigida*. It is hoped that this will lead to a greater knowledge of mating interactions in this species, and consequently, further our understanding of the mechanisms associated with sexual selection and sexual conflict in coelopids.

4.2 Materials and methods

The flies used in this experiment were collected as larvae from St Mary's Island (Tyne & Wear, North-East England, UK (Ordinance Survey Ref: NZ352753)) in November 2001, and were reared and maintained under standard laboratory conditions (see Chapter 3). Virgin flies from the first and second generations were used and the flies were stored for no longer than 7 days at 4°C before use.

The experiment was carried out using the protocol for standard laboratory mating trials as outlined in Chapter 3. Both males and females were left for 48 hours sex-deprivation at 25°C before the experiment was carried out - males in individual pots of seaweed and females in communal bottles with sucrose in groups of approximately 20 individuals.

One female and one male fly were aspirated into a mating chamber at a time. As soon as the male entered the chamber the trial began. The following observations were documented (all those involving time were recorded in seconds using a stopwatch). Shortened variable labels are given in parentheses:

① Whether the male mounted the female or not
② Time taken for the male to mount the female (time to mount)
③ Total time spent mounted (total mount time)
④ The outcome of the mount: a) male successfully kicked or shaken off by the female; b) female rejects male via abdomen curling*; c) male rejects female without copulating; d) the pair copulate.
⑤ Pre-mating struggle duration (struggle duration)
⑥ Rejection behaviours exhibited by the female during pre-mating struggle: a) abdominal curling b) shaking and c) kicking, and the durations of these behaviours
⑦ Whether the female is struggling at the point of male dismount
⑧ Copulation duration

* Males dismounting while females are showing the abdomen curling rejection response have been included as a separate category within mount outcomes, as the females are not forcibly removing the males.

When a male fly failed to mount a given female within a period of five minutes, the trail was scored as a non-mount.

Flies were subsequently stored individually in 1.5 ml microcentrifuge tubes at -20°C until their wing lengths could be measured. The *Adh* genotypes of the flies were then determined by starch-gel electrophoresis (see Chapter 3).

4.2.1 Statistical analyses

The following variables were log-transformed prior to analysis: male and female sizes, time to mount, total mount time, copulation duration, struggle duration and the durations of female rejection responses. This was necessary as the variances of these data sets were larger than their means and normalisation was required before parametric tests could be carried out. Log-transforming the data also removed the dependence of the variance upon the mean (Fowler et al. 1999). This procedure follows earlier work on similar variables (Dunn et al. 1999). All other variables were recorded as binary data (1 = behaviour occurred, 0 = behaviour did not occur) or categorical data.

4.3 Results

4.3.1 Descriptive statistics

A summary of mean values for each continuous variable measured during the experiment is given in Table 4.1. The male mounted the female in 371 out of 522 mate trials (71.1%). The outcomes of these mounts are presented in Figure 4.1. Females showed the abdomen curling rejection response in 265 out of 371 mounts (71.4%); the shaking response in 201 out of 371 mounts (54.2%) and the kicking response in 226 out of 371 mounts (60.9%). The success rate of each rejection response strategy is given in Table 4.2. The percentage of mounts where females exhibited different combinations of these responses is shown in Figure 4.2.

Mean values for each continuous variable, grouped by male and female karyotype, are given in Tables 4.3 and 4.4. The percentages of mounts ending in each outcome, grouped by female and male karyotype, are presented in Figures 4.3 and 4.4. The percentages of mounts where females exhibited each type of rejection response, grouped by male and female karyotype, are shown in Tables 4.5 and 4.6. The number of mounts where females showed each combination of rejection behaviours, grouped by male and female karyotype, are given in Tables 4.7 and 4.8. The success rates of each of the female rejection strategies, grouped by female karyotype, are given in Table 4.9.

Table 4.1 Mean male size (mm), female size (mm) and behaviour durations (secs) with standard errors given in parentheses.

Variable	n	Mean (S.E.)
Male size	512	6.20 (0.03)
Female size	508	5.58 (0.02)
Time to mount	372	86.52 (4.31)
Total mount time	372	65.23 (3.59)
Struggle duration	358	25.41 (2.01)
Abdomen curling duration	265	29.50 (2.43)
Shaking duration	200	8.43 (0.77)
Kicking duration	225	9.84 (0.79)
Copulation duration	172	91.23 (4.61)

Table 4.2 The success rate of each rejection response (i.e. the percentage of mounts where the male was successfully rejected by the female for each rejection response) with the number of trials where the male was rejected for each response (n_1) given in parentheses. The total number of mounts where the female showed the rejection response is also given (n_2).

Rejection response	% (n_1)	n_2
Abdomen curling	37.36 (99)	265
Shaking	58.71 (118)	201
Kicking	52.65 (119)	226

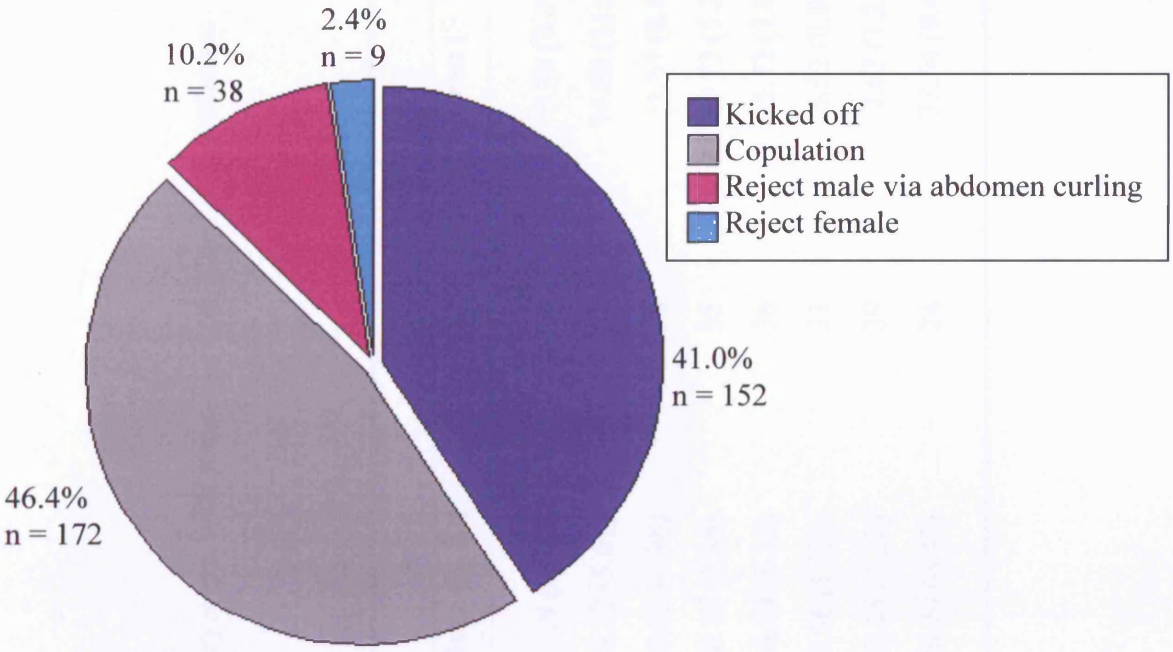


Figure 4.1 Pie chart showing the percentage of mounts ending in each outcome.

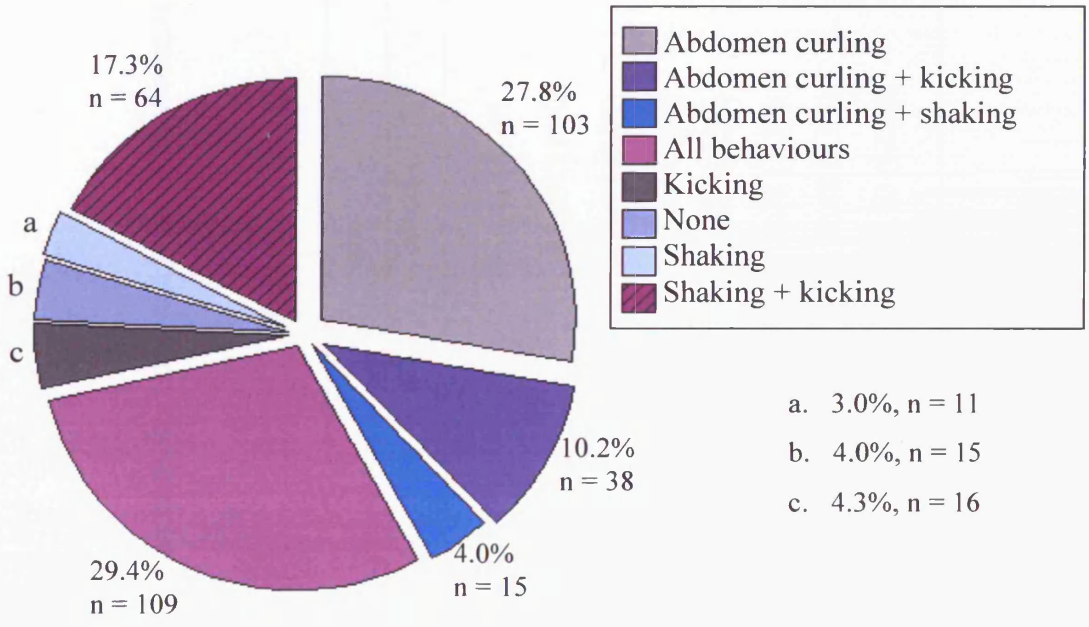


Figure 4.2 Pie chart showing the percentage of mounts where females exhibited different combinations of rejection responses.

Table 4.3 Mean male size (mm) and behaviour durations (secs) grouped by male karyotype, with standard errors given in parentheses.

	αα		αβ		ββ	
Variable	**n**	**Mean (S. E.)**	**n**	**Mean (S. E.)**	**n**	**Mean (S. E.)**
Male size	82	6.41 (0.09)	293	6.29 (0.04)	79	5.59 (0.07)
Time to mount	55	87.00 (11.40)	216	84.32 (5.61)	58	94.60 (11.30)
Total mount time	55	72.80 (9.06)	216	68.61 (4.98)	58	51.57 (8.43)
Struggle duration	55	29.98 (4.69)	208	26.75 (2.85)	55	19.73 (5.25)
Abdomen curling duration	46	34.30 (5.36)	151	30.40 (3.38)	36	25.72 (7.83)
Shaking duration	28	8.75 (1.30)	115	9.56 (1.25)	33	5.52 (0.80)
Kicking duration	33	12.88 (2.03)	131	10.24 (1.18)	39	7.67 (1.23)
Copulation duration	24	95.50 (12.50)	102	96.70 (6.42)	24	77.54 (9.44)

Table 4.4 Mean female size (mm) and behaviour durations (secs) grouped by female karyotype with standard errors given in parentheses.

	αα		αβ		ββ	
Variable	**n**	**Mean (S. E.)**	**n**	**Mean (S. E.)**	**n**	**Mean (S. E.)**
Female size	75	5.56 (0.04)	254	5.62 (0.02)	132	5.56(0.03)
Time to mount	62	65.19 (8.10)	185	93.99 (6.52)	80	90.04 (9.49)
Total mount time	62	76.40 (8.32)	185	61.79 (5.03)	80	57.15 (7.78)
Struggle duration	62	21.79 (2.93)	179	29.04 (3.54)	73	22.77 (3.26)
Abdomen curling duration	43	24.07 (3.92)	137	32.45 (4.15)	50	29.94 (4.25)
Shaking duration	34	9.56 (1.34)	104	8.91 (1.30)	38	6.97 (1.33)
Kicking duration	42	13.86 (1.79)	112	10.44 (1.30)	45	6.07 (1.11)
Copulation duration	41	85.76 (8.18)	73	87.16 (5.40)	32	100.50 (15.30)

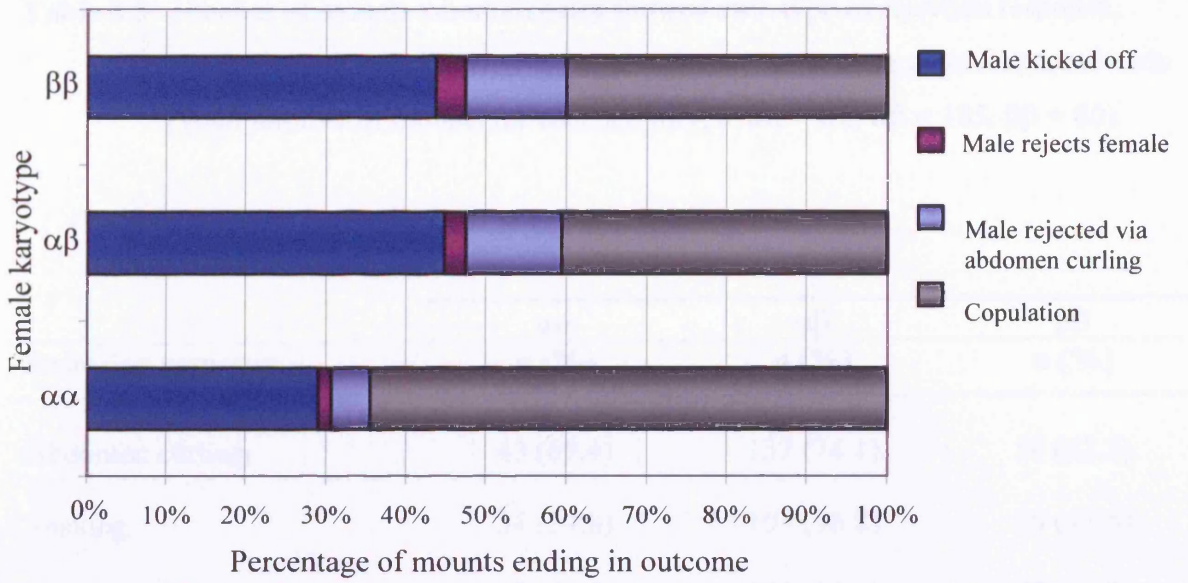


Figure 4.3 Percentage of mounts ending in each possible outcome, grouped by female karyotype. (Number of mounts for each karyotype: αα = 62, αβ = 185, ββ = 80)

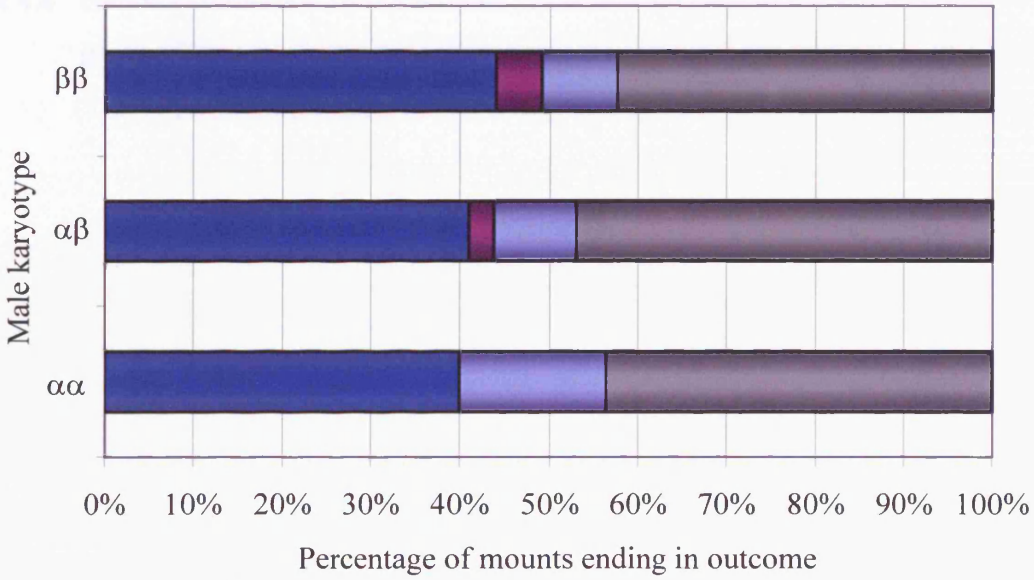


Figure 4.4 Percentage of mounts ending in each possible outcome, grouped by male karyotype. (Number of mounts for each karyotype: αα = 55, αβ = 216, ββ = 58). Key is as for previous figure.

Table 4.5 Number of mounts where females showed each type of rejection response, grouped by female karyotype. Percentages of mounts are given in parentheses. (Total number of mounts for each karyotype: αα = 62, αβ = 185, ββ = 80).

	αα	αβ	ββ
Rejection response	**n (%)**	**n (%)**	**n (%)**
Abdomen curling	43 (69.4)	137 (74.1)	50 (62.5)
Shaking	34 (54.8)	105 (56.8)	38 (47.5)
Kicking	42 (67.7)	113 (61.1)	45 (56.3)

Table 4.6 Number of mounts where females showed each type of rejection response, grouped by male karyotype. Percentages of mounts are given in parentheses. (Total number of mounts for each karyotype: αα = 55, αβ = 216, ββ = 58).

	αα	αβ	ββ
Rejection response	**n (%)**	**n (%)**	**n (%)**
Abdomen curling	46 (83.6)	150 (69.4)	37 (63.8)
Shaking	28 (50.9)	115 (53.2)	34 (58.6)
Kicking	33 (60.0)	131 (60.7)	40 (69.0)

Table 4.7 Number of mounts where females showed each combination of rejection behaviours, grouped by female karyotype. Percentages of mounts are given in parentheses. (Total number of mounts for each karyotype: αα = 62, αβ = 185, ββ = 80).

	αα	αβ	ββ
Combination	**n (%)**	**n (%)**	**n (%)**
Abdomen curling	12 (19.4)	50 (27.0)	26 (32.5)
Abdomen curling + kicking	12 (19.4)	16 (8.7)	6 (7.5)
Abdomen curling + shaking	5 (8.1)	7 (3.8)	1 (1.3)
All behaviours	14 (22.6)	64 (34.6)	17 (21.3)
Kicking	4 (6.5)	8 (4.3)	3 (3.8)
Shaking	3 (4.8)	6 (3.2)	1 (1.3)
Shaking + kicking	12 (19.4)	26 (14.1)	19 (23.8)
None	0 (0.0)	8 (4.3)	7 (8.8)

Table 4.8 Number of mounts where females showed each combination of rejection behaviours, grouped by male karyotype. Percentages of mounts are given in parentheses. (Total number of mounts for each karyotype: αα = 55, αβ = 216, ββ = 58).

	αα	αβ	ββ
Combination	**n (%)**	**n (%)**	**n (%)**
Abdomen curling	13 (23.6)	65 (30.1)	12 (20.7)
Abdomen curling + kicking	11 (20.0)	20 (9.3)	5 (8.6)
Abdomen curling + shaking	6 (10.9)	5 (2.3)	2 (3.5)
All behaviours	16 (29.1)	60 (27.8)	18 (31.0)
Kicking	3 (5.5)	8 (3.7)	4 (6.9)
Shaking	3 (5.5)	4 (1.9)	1 (1.7)
Shaking + kicking	3 (5.5)	44 (20.4)	13 (22.4)
None	0 (0.0)	10 (4.6)	3 (5.2)

Table 4.9 The success rate of each rejection response (i.e. the percentage of mounts where the male was successfully rejected by the female for each rejection response), grouped by female karyotype. The total number of mounts where the female showed the rejection response(n), for each karyotype, is given in parentheses.

	αα	αβ	ββ
Rejection response	**% (n)**	**% (n)**	**% (n)**
Abdomen curling	32.6 (43)	43.1 (137)	36.0 (50)
Shaking	41.2 (34)	61.0 (105)	76.3 (38)
Kicking	40.5 (42)	55.8 (113)	66.7 (45)

4.3.2 Male willingness to mate

Mount attempts may be used as an indicator of male willingness to mate. A binary logistic regression analysis confirmed that there was a considerable significant negative association between the willingness of male to mate and size of males in *C. frigida* (χ^2_1 = 21.439, $p < 0.001$). Further analyses showed that though male karyotype was strongly associated with male size (ANOVA, $F_{2,453} = 34.04$, $p < 0.001$) it had no association with willingness to mate once the effect of size had been removed (Binary logistic regression, $\chi^2_2 = 1.421, p = 0.491$) (see Table 4.10).

Table 4.10 Percentage of trials in which males mounted females, grouped by male karyotype ('n' denotes the number of mounts with the total number of trials given in parentheses).

Male karyotype	**n**	**%**
αα	55 (82)	67.1
αβ	216 (293)	73.7
ββ	58 (79)	73.4

Female size was not associated with male willingness to mate (χ^2_1 =1.231, p = 0.267). However, female karyotype was found to be significantly associated with male willingness to mate (χ^2_2 = 12.475, p = 0.006), with αα female being most likely to be mounted by males, followed by αβ females then ββ females (see Table 4.11).

Table 4.11 Percentage of trials in which males mounted females, grouped by female karyotype ('n' denotes the number of mounts with the total number of trials given in parentheses).

Female karyotype	n	%
αα	62 (75)	82.7
αβ	185 (255)	72.6
ββ	80 (132)	60.6

4.3.3 Time taken to mount

The time taken for a male to mount a female was not associated with male size (Regression analysis, $F_{1,362}$ = 2.67, p = 0.103) or female size (Regression analysis, $F_{1,362}$ = 1.37, p = 0.243). When the data was analysed for female karyotype, a trend was present, although this not significant (ANOVA, $F_{2,326}$ = 2.80, p = 0.062): the time taken for males to mount females with the αα karyotype was slightly longer than that taken to mount females with the ββ or αβ karyotypes. There were no significant differences between the times taken by each male karyotype to mount a female (ANOVA, $F_{2,328}$ = 0.35, p = 0.708).

4.3.4 Total mount time

There was a significant positive association found between male size and total mount time (Regression analysis, $F_{1,362}$ = 5.9, $p < 0.05$). As with the results for the time taken to

mount, there was no association between the total mount time and female size (Regression analysis, $F_{1,362} = 0.72$, $p = 0.395$). Female karyotype (ANOVA, $F_{2,326} = 2.89$, $p = 0.057$) and male karyotype (ANOVA, $F_{2328,} = 2.69$, $p = 0.069$) were not significantly associated with total mount time though they both showed definite trends whereby αα males mounted females for longer, and ββ females were mounted for longer, than αβ individuals.

4.3.5 Copulation

Male size was shown not to have any significant association with the whether copulation occurred or not (Binary logistic regression, $\chi^2_1 = 0.354$, $p = 0.552$). This was also the case for male karyotype and copulation occurrence (Binary logistic regression, χ^2_2=0.823, p=0.799).

Binary logistic regression analyses also revealed that female size was not associated with copulation occurrence ($\chi^2_1 = 0.681$, $p = 0.409$). However, there was a highly significant association between copulation occurrence and female karyotype (Binary logistic regression, $\chi^2_2 = 13.904$, $p < 0.01$) - females with the αα homokaryotype were copulated with more often than ββ or αβ females (see Table 4.12).

Table 4.12 Percentage of mounts ending in copulation, grouped by female karyotype ('n' denotes the number of mounts ending in copulation, with the total number of mounts given in parentheses).

Female karyotype	n	%
αα	40 (62)	64.5
αβ	74 (185)	40.0
ββ	32 (80)	40.0

4.3.6 Copulation duration

There were no associations between copulation duration and male size (Regression analysis, $F_{1,166} = 0.14$, $p = 0.708$), male karyotype (ANOVA, $F_{2,149} = 0.55$, $p = 0.578$), female size (Regression analysis, $F_{1,168} = 0.02$, $p = 0.875$) or female karyotype (ANOVA, $F_{2,145} = 0.06$, $p = 0.943$).

4.3.7 Associations between mount outcomes, size and karyotype

Female size and karyotype

Binary logistic regressions showed that there were no significant associations between mount outcomes and female size or karyotype (see Table 4.13).

Table 4.13 Results of binary logistic regression analyses of mount outcomes with female size and female karyotype.

	Female size		Female karyotype	
Mount outcome	χ^2_1	p	χ^2_1	p
Male kicked off by female	0.221	0.638	1.119	0.290
Male rejects female	1.651	0.199	0.106	0.744
Male rejected via abdomen curling	0.772	0.379	0.373	0.794

Male size and karyotype

A strong significant negative association was found between male size and the frequency of males being kicked off by females (Binary logistic regression, $\chi^2_1 = 12.948, p < 0.001$) whereby smaller males are kicked off more frequently than larger males. There was also a significant positive association found between male size and the frequency of males being rejected by female abdomen curling (Binary logistic regression, $\chi^2_1 = 12.948, p < 0.001$) i.e. larger males dismounted as a result of female abdomen curling more often than smaller males. There was no significant association found between male size and the frequency of male rejection of females (Binary logistic regression, $\chi^2_1 = 3.377, p = 0.066$). No significant associations were found between mount outcomes and male karyotype as shown in Table 4.14.

Table 4.14 Results of binary logistic regression analyses of mount outcomes with male karyotype.

Mount outcome	χ^2_2	p
Male kicked off by female	0.319	0.853
Male rejects female	4.079	0.130
Male rejected via female abdomen curling	2.340	0.310

4.3.8 Associations between female rejection response, karyotype and size

Binary logistic regression analyses showed that there were no significant associations between the rejection responses exhibited by females and female karyotype (see Table 4.15). This was also the case for male karyotype, with the exception of abdomen

curling, which was significantly associated with male karyotype - females use the abdomen curling rejection response more often with αα males (see Tables 4.15 and 4.16).

Table 4.15 Results of binary logistic regression analyses of female rejection responses, female karyotype and male karyotype (male karyotype statistics are given in **bold** type).

Female rejection response	χ^2_2	*p*
Abdomen curling	2.614	0.106
	6.417	**< 0.05**
Shaking	1.315	0.251
	0.757	**0.685**
Kicking	0.033	0.855
	1.488	**0.475**

Table 4.16 Percentage of mounts females showed the abdomen curling rejection response, grouped by male karyotype ('n' denotes the number of mounts where females abdomen curled, with the total number of mounts given in parentheses).

Male karyotype	n	%
αα	46 (55)	83.6
αβ	150 (216)	69.4
ββ	37 (58)	63.79

Further analyses uncovered significant associations between male size and all of the female rejection responses, whereby females are less likely to kick or shake, but are more likely to abdomen curl, with larger males. There were no significant associations between female size and any of the rejection responses, with the exception of kicking (see Table 4.17), whereby larger females are more likely to use the kicking response.

Table 4.17 Binary logistic regression analyses results of male and female size and female rejection responses. The slopes of significant regressions are also presented with standard errors in parentheses.

	Female size			Male size		
Female rejection response	χ^2_1	p	**Slope (S.E.)**	χ^2_1	p	**Slope (S.E.)**
Abdomen curling	1.580	0.209	-	30.217	< 0.001	13.221 (2.551)
Kicking	4.143	< 0.05	7.928 (2.336)	36.801	< 0.001	-13.558 (2.336)
Shaking	1.901	0.168	-	28.631	< 0.001	-11.720 (2.242)

4.3.9 Associations between struggle duration, size and karyotype

A regression analysis revealed a strongly significant positive association between struggle duration and male size ($F_{1,34\,8}$= 16.25, $p < 0.05$) as shown in Figure 4.5.

A significant difference was found between the struggle durations of αα and ββ karyotype males, though no significant difference was found between the struggle durations of αα and αβ males, and ββ and αβ males (ANOVA, $F_{2,316}$=3.4,p<0.05 with Tukey's *post hoc* test). Males with the αα karyotype were involved in longer struggles than those with the ββ karyotype (see Figure 4.6).

There were no significant associations found between struggle duration and female size (Regression analysis, $F_{1,348}$=1.03, p=0.310). There was also no significant differences

found between the struggle durations exhibited by the different female karyotypes (ANOVA, $F_{2,312}$=0.63, p=0.531).

4.3.10 Associations between female rejection response duration, size and karyotype

Female abdomen curling response

There was a highly significant positive association between the duration of the female abdomen curling response and male size (Regression analysis, $F_{1,258} = 11.76$, $p < 0.05$) as shown in Figure 4.7. Analyses also revealed significant differences between the durations of female abdomen curling with certain karyotypes of male. Females abdomen curled for longer with αα males than they did with ββ males, though there were no significant differences in abdomen curling durations between αα and αβ males, and ββ and αβ males (ANOVA, $F_{2,232}$=3.73, p<0.005, with Tukey's *post hoc* test) as shown in Figure 4.8. No significant association was found between female abdomen curling duration and female size (Regression analysis, $F_{1,258} = 0.15$, $p = 0.702$). There were also no significant differences found between the abdomen curling durations of the different female karyotypes (ANOVA, $F_{2,229} = 0.51$, $p = 0.602$).

Female shaking response

There was no significant relationship found between female shaking duration and male size (Regression analysis, $F_{1,194} = 0.02$, $p = 0.891$). A trend was apparent in the association between shaking duration and female size, which suggested that as female size increases, shaking duration also increases, though this was not significant (Regression analysis, $F_{1,193} = 3.25$, $p = 0.073$). No significant differences were found between the female shaking durations of the different male karyotypes (ANOVA, $F_{2,175} = 2.19$, $p = 0.115$) or female karyotypes (ANOVA, $F_{2,175} = 1.94$, $p = 0.147$).

Female kicking response

Regressions analysis revealed no significant association between male size and female kicking duration ($F_{1,219}$=1.46, p=0.228). However, a strong association was present

between female size and kicking duration (Regression analysis, $F_{1,218} = 4.74$, $p < 0.05$) as shown in Figure 4.9. A trend was apparent when female kicking duration was analysed for each male karyotype - females appeared to kick for longer with αα karyotype males than with ββ karyotype males. This trend was not statistically significant however (ANOVA, $F_{2,202}=2.54$, $p=0.081$). Strong significant differences were found between the kicking durations of αα and ββ females, and between the kicking durations of αβ and ββ females (ANOVA, $F_{2,198}=8.3$, $p<0.05$, Tukey's *post hoc* test) (see Figure 4.10).

4.3.11 Combinations of female rejection responses

A multinomial logistic regression showed that the combination of rejection responses exhibited by females was not significantly associated with female size ($\chi^2_7 = 10.141$, $p = 0.181$). However, there was a highly significant positive association between the combination of rejection responses exhibited by females and male size (Multinomial logistic regression, $\chi^2_7 = 68.638$, $p < 0.001$) as shown in Table 4.18.

Table 4.18 Rejection response combinations significantly associated with male size (with slopes, standard errors in parentheses and p-values).

Rejection response combination	Slope (S.E.)	*p*
Abdomen curling	24.69 (3.88)	< 0.001
Abdomen curling + kicking	14.12 (4.53)	< 0.01
Abdomen curling + shaking	17.18 (6.31)	< 0.01
All	9.89 (3.56)	< 0.01
None	19.44 (6.21)	< 0.01

Multinomial regression analyses were also used to determine whether there were any associations between the combination of female rejection responses and karyotypes. A highly significant association was found with female karyotype ($\chi^2_{14} = 29.569$, $p < 0.01$). When the table of parameter estimates was examined more closely, the significance was found to be produced by a negative association between females with the ββ homokaryotype and the exhibition of 'all' of the rejection responses (slope = -1.01, S.E. = 0.41, $p < 0.05$). There was also a significant association found with male karyotype ($\chi^2_{14} = 28.245$, $p < 0.05$) as shown in Table 4.19.

Table 4.19 Rejection response combinations significantly associated with the male αα homokaryotype (with slopes, standard errors in parentheses and p-values).

Rejection response combination	**Slope (S.E.)**	***p***
Abdomen curling + kicking	2.09 (0.71)	< 0.01
Abdomen curling + shaking	2.87 (0.85)	< 0.01
All	1.36 (0.66)	< 0.05
Shaking	2.40 (0.97)	< 0.05

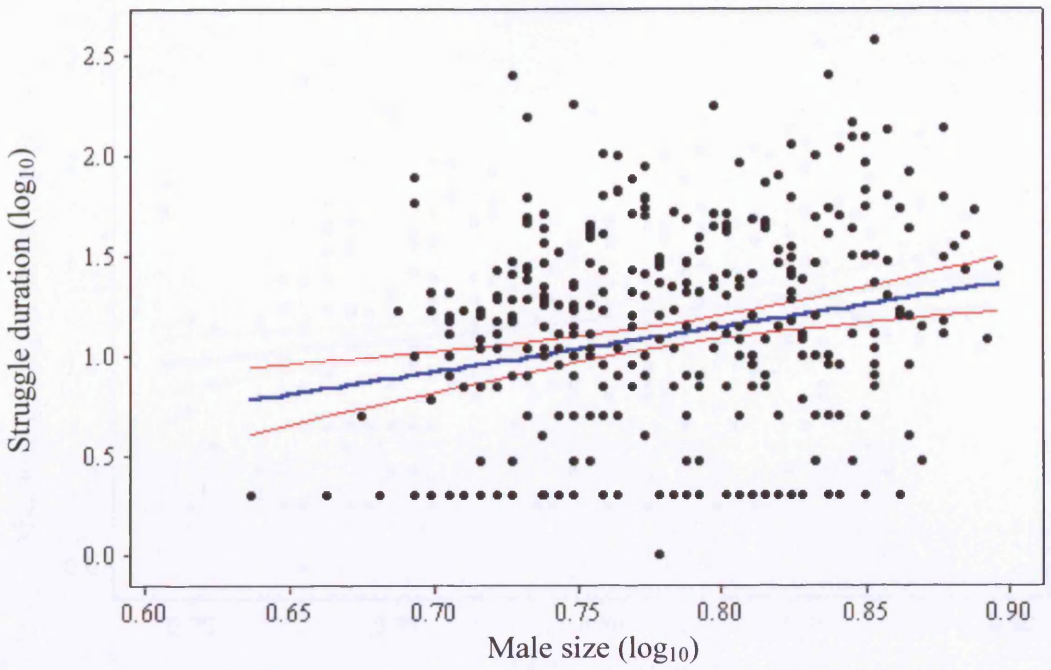


Figure 4.5 Fitted line plot showing the positive association between struggle duration (secs) and male size (mm), with 95% C.I. shown in red.

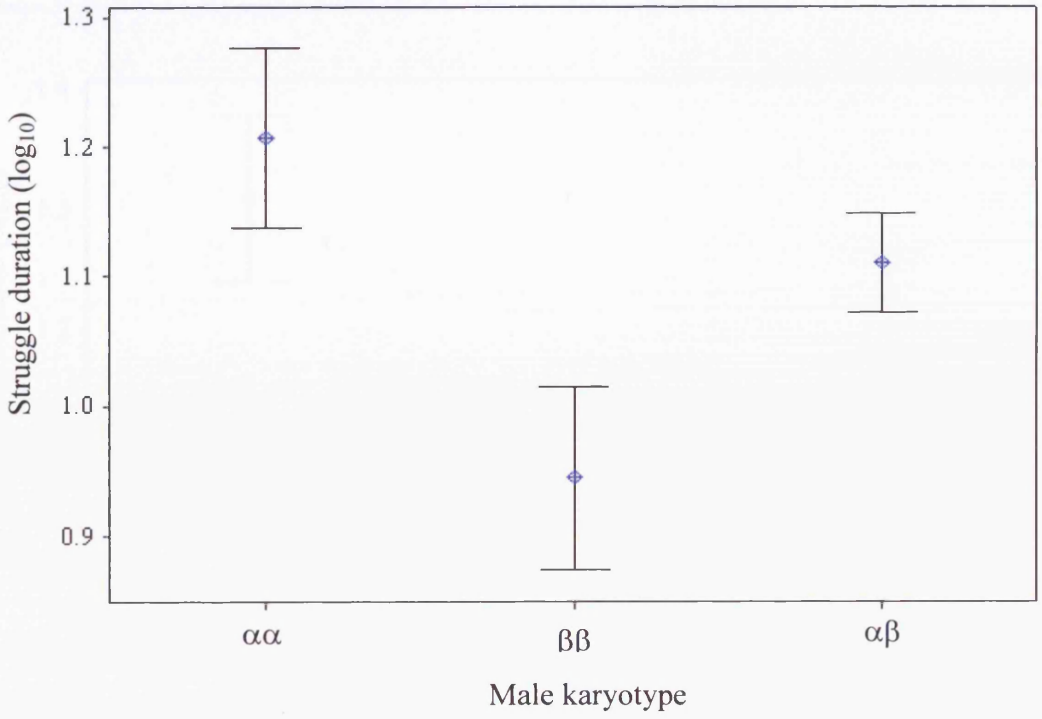


Figure 4.6 Interval plot of struggle duration (secs) for each male karyotype (with standard errors).

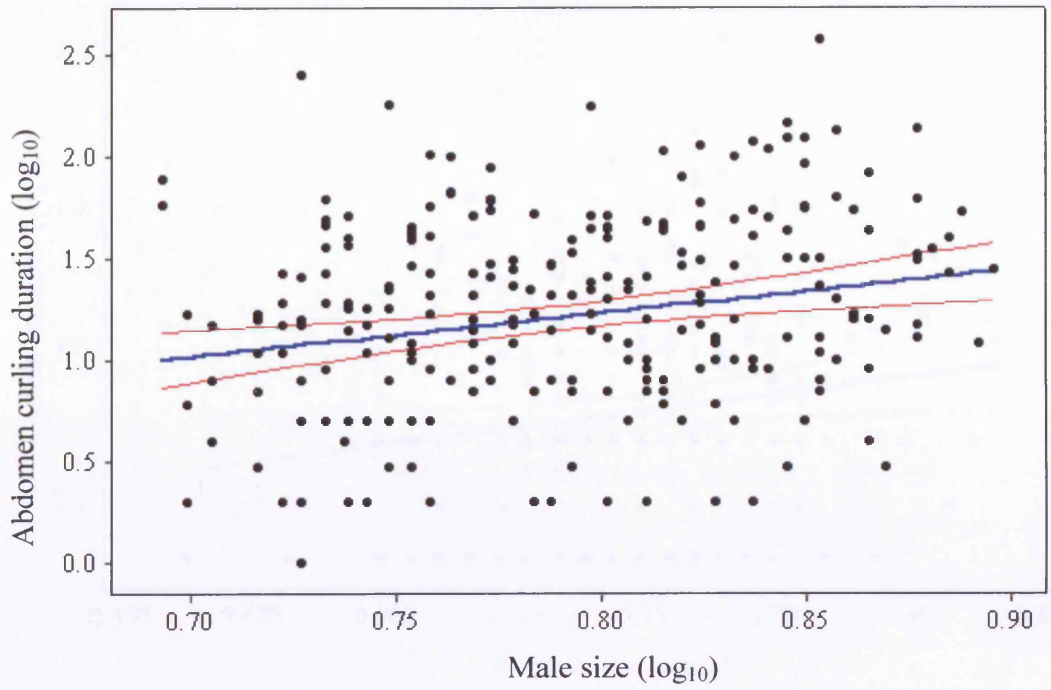


Figure 4.7 Fitted line plot showing the positive association between abdomen curling duration (secs) and male size, with 95% C.I. shown in red.

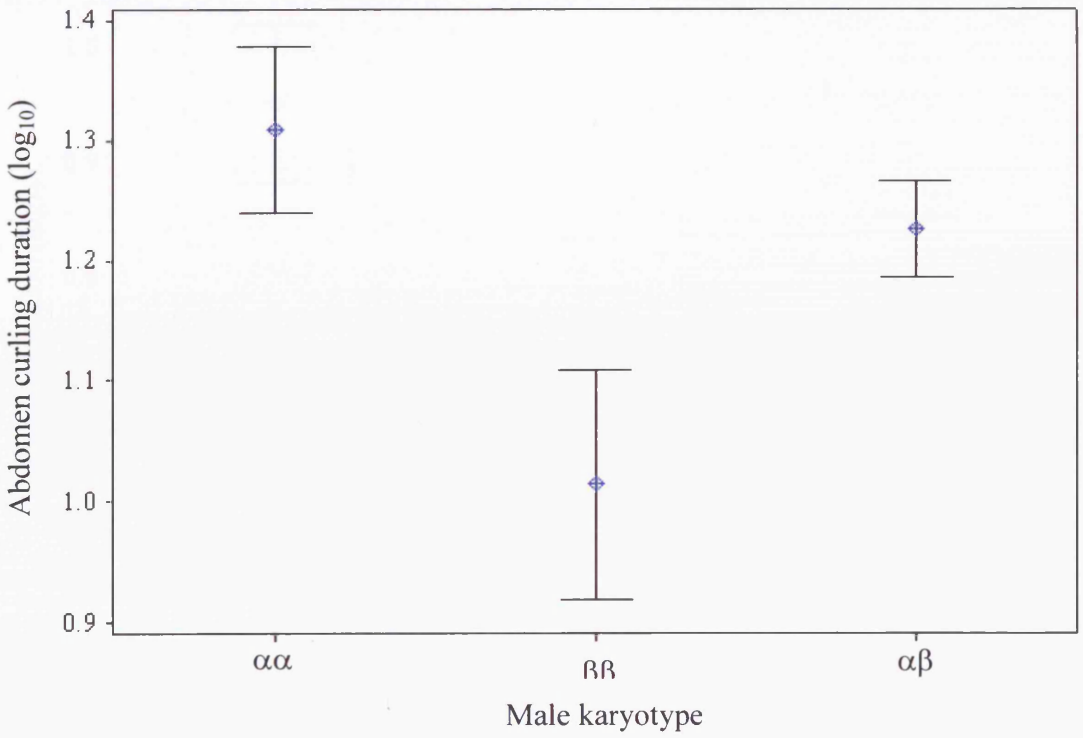


Figure 4.8 Interval plot of abdomen curling duration (secs) for each male karyotype (with standard errors).

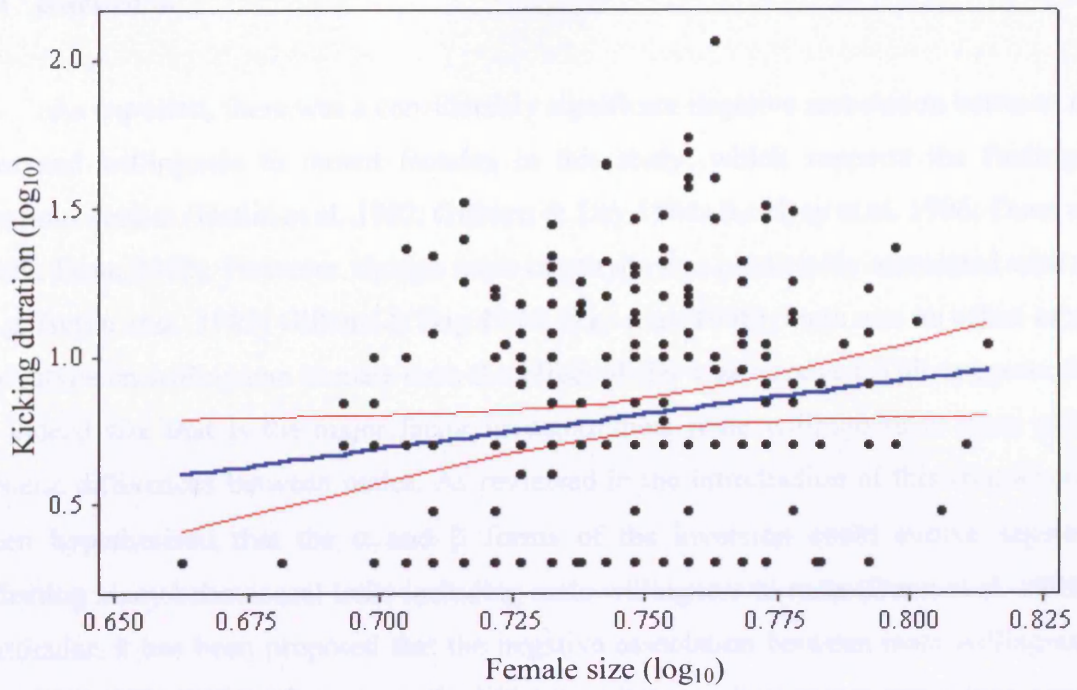


Figure 4.9 Fitted line plot showing the positive association between kicking duration (secs) and female size (mm), with 95% C.I. shown in red.

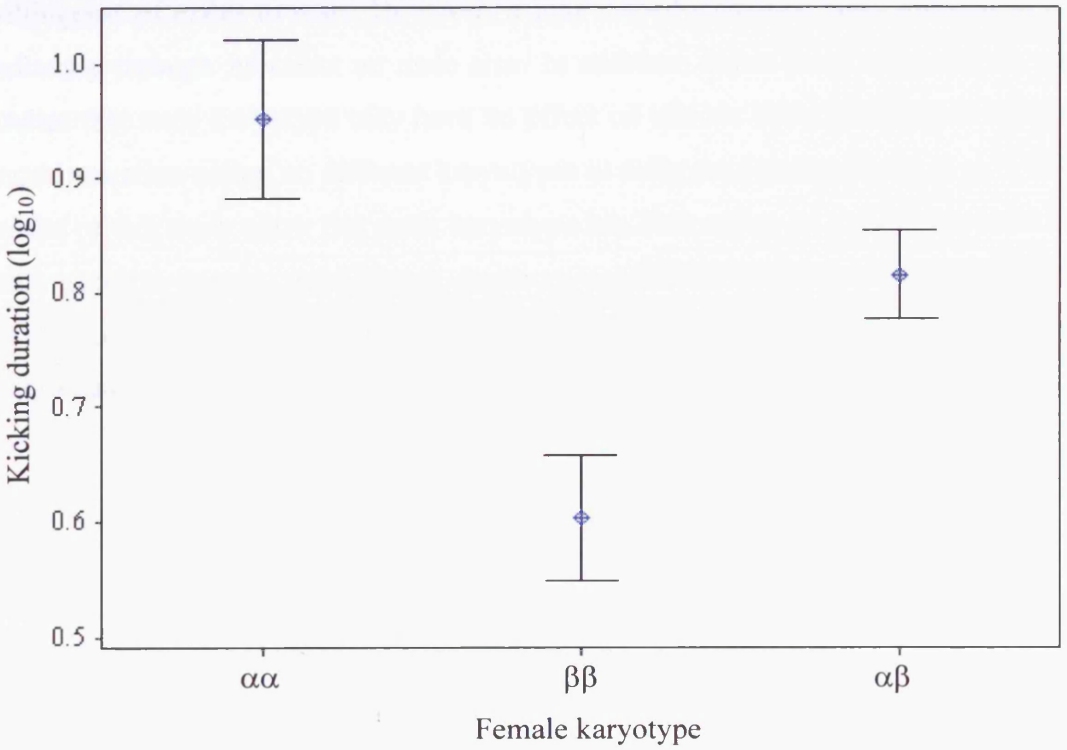


Figure 4.10 Interval plot of kicking durations (secs) for each female karyotype (with standard errors).

4.4 Discussion

As expected, there was a considerably significant negative association between male size and willingness to mount females in this study, which supports the findings of previous studies (Butlin et al. 1982; Gilburn & Day 1994a,b,c; Day et al. 1996; Dunn et al. 1999; Dunn 2002). However, though male karyotype was predictably associated with size, (e.g. Butlin et al. 1982; Gilburn & Day 1994; Day et al. 1996), there was no effect of male karyotype on willingness to mate once the effect of size was removed. This suggests that it is indeed size that is the major factor in determining male willingness to mate and not genetic differences between males. As reviewed in the introduction of this chapter, it has been hypothesised that the α and β forms of the inversion could evolve separately, affecting many behavioural traits including male willingness to mate (Dunn et al. 1999). In particular, it has been proposed that the negative association between male willingness to mate and size could be due to genetic differences between karyotypes rather than purely a size effect. This study challenges this theory, indicating that though the inversion system present in *C. frigida* may maintain the considerable variance seen in male size (e.g. Butlin et al. 1982; Wilcockson et al. 1995; Day et al. 1996), it is not directly involved in the willingness of males to mate. However, it may indeed influence male willingness to mate indirectly through its effect on male size. In addition, it has been suggested in previous studies that male karyotype may have an effect on various male behavioural traits due to sexual selection acting on different karyotypes to different degrees (Dunn et al. 1999). The results of this study show that male karyotype has little effect on male behaviours such as willingness to mount, total mount duration, copulation duration and mount outcomes, which is in agreement with the findings of Shuker (1998). In summary, there is no genetic determinant of male behaviour, discounting that of size. There is also no evidence that males have different reproductive strategies on the basis of male karyotype, though males adopting different reproductive strategies as a result of size cannot be ruled out (see Dunn et al. 1999).

There was no evidence of male mate choice on the basis of female size or karyotype, which supports recent findings (Dunn et al. 1999). This indicates that alternative mechanisms may be responsible for variation in male willingness to mount and mate with females. An investigation into male mating preferences carried out using *Gluma musgravei*, an Australian species of coelopid, found that males preferentially mounted

females with higher future longevity, which may have evolved as a result of females frequently having to survive for long periods after mating until a suitable oviposition site becomes available (Dunn et al 2001). Some evidence for a male preference for female fecundity has also been found in *C. frigida* (Pitafi et al. 1995). As outlined in the aims section of this thesis, experiments have been carried out looking at the plasticity of male preferences for female longevity and fecundity in *C. frigida*, but in light of recent findings, these experiments need to be repeated to take into account the effect of seaweed-type on mating behaviour. Once this has been completed, it will hopefully lead to a greater understanding of male mating preferences in *C. frigida.*

Somewhat surprisingly, copulation occurrence, which is taken as a measure of female willingness to mate by some coelopid researchers (e.g. Gilburn & Day 1996; Crean et al. 2000), was not associated with male size or karyotype. This conflicts with the results of many previous studies that suggest large male size is a preferred trait of female *C. frigida* (e.g. Butlin et al. 1982; Gilburn et al. 1992, 1993, 1996; Crean 1997; Day & Gilburn 1997) or that larger males are more able to resist the female rejection response and therefore have greater reproductive success (Crean & Gilburn 1998). The reasons for this unusual result are unclear, but the huge variation in the strength of female preference for large males (or large male advantage) is known to be such that it is not feasible to predict with certainty that a particular study of mating patterns in *C. frigida* will reveal a large male mating advantage (Shuker 1998). This intra-specific variation in mating patterns creates an added element of complexity when viewing this type of result within the context of sexual selection (Jennions & Petrie 1997; see Shuker 1998; Kokko 2002a).

Female size was a found not to be related to copulation occurrence in this study; however, there was a highly significant association with female karyotype - females with the $\alpha\alpha$ karyotype were copulated with more often than those with the $\alpha\beta$ or $\beta\beta$ karyotypes. It could be hypothesised that this result is due to the $\alpha\alpha$ females being more willing to mate with males; consequently they exhibit (i) a relatively less intense rejection response or (ii) less effective rejection strategies, than $\alpha\beta$ and $\beta\beta$ females. This does not seem to be the case, however, as there was no significant association between the rejection behaviours exhibited by females and female karyotype i.e. there were no differences between the

rejection responses used by females of different karyotypes. Female size was also not a determining factor in which rejection responses were used by females.

Interestingly, male size and karyotype appeared to have a much greater influence on female rejection responses, for example, females used the abdomen curling rejection response more often, and the shaking and kicking response less often, with larger αα males than with smaller αβ or ββ males. This may go some way to explain the result that larger males dismounted females as a result of abdomen curling more frequently than smaller males, though this effect was not as a result of male karyotype. It may also explain why struggle duration was found to be positively associated with male size and the αα karyotype - it may be that the development of this strategy on the females' part could be as an energy expenditure reduction measure, as it is may be less energetically expensive to abdomen curl rather than shake or kick, therefore they can persist for longer with this behaviour. In addition, larger males may be able to overcome kicking a shaking more easily than smaller males, leaving abdomen curling as the most effective strategy against larger males. There was also a positive association between female kicking duration and the size and karyotype of females, with αα females kicking for the longest duration. The association seen with size may be an effect of karyotype on female size, though this seems unlikely since female karyotype was not found to be associated with female size in this study. Why larger females kick for longer is puzzling, particularly when there was no association found between the shaking rejection response, size or karyotype of either sex.

Abdomen curling was the most commonly used rejection response, occurring in over 70% of trials in which the male mounted the female, though a combination of all responses was the most frequently used strategy. Kicking was the next most often used response, followed by shaking. It therefore appears remarkable that the abdomen curling response was the least successful at rejecting males, with a success rate of just over 34% and the shaking response the most successful with a success rate of nearly 59%. It might be expected that females would use the shaking response most often, since the chance of rejecting the male is greater using this method, however, this response is almost certainly the most energetically expensive to perform which may deter female from using this behaviour unless necessary.

The interactions between the rejection responses exhibited by females, size and karyotype appear more complex than was initially anticipated. There appears to be no clear genetic effect of female karyotype on female rejection behaviour, which leaves the question as to why αα females are mated with more often, unanswered. This study has uncovered some intriguing, if perplexing, results on this subject and it is clear that further work is needed to determine the exact nature of these relationships.

Chapter 5. Convenience Polyandry in *Coelopa frigida*

5.1 Introduction

As discussed in Chapter 1, asymmetry in the primary mating interests and energetic investment of males and females into producing progeny results in sexual conflict. A seminal study carried out by Bateman (1948) using *Drosophila*, highlighted the role played by mating frequency in reproductive success. Bateman found convincing evidence that male *Drosophila*, a polyandrous species of insect, increased their reproductive success with repeated copulations, whereas females did not receive any benefits from multiple copulations. However, it has subsequently been found in many insect species, that a high remating frequency in females actually decreases their fitness (reviewed by Arnqvist & Nilsson 2000). These findings suggest that there are two factors leading to conflict. Firstly, males benefit from an increased mating frequency in terms of fitness, with very little additional energetic costs involved. Secondly, females do not receive these fitness benefits, but instead incur costs in terms of energy and increased predation risk, for example (Arnqvist in Choe & Crespi 1997). This inequality in the costs and benefits associated with remating frequency results in conflict over which sex controls the mating decision. Consequently, this implies that females should avoid superfluous copulations, and males should attempt to mate at every opportunity (Rivera & Andrés 2002).

Female rejection responses to male mounting attempts are exhibited in many insect taxa, including the Coelopidae (Day et al. 1990; reviewed by Day & Gilburn 1997), Gerridae (Arnqvist 1989; Rowe 1992), *Scathophaga* spp. (Parker 1970; Borgia 1981), *Musca* spp. (Tobin & Stoffolano 1973) and the Coccinellidae (de Jong et al. 1998). The intensity of resistance may fluctuate in some species as a result of varying ecological conditions, for example, female sexual resistance in gerrids decreases with an increase in population density, as a result of an increase in male harassment (Arnqvist 1992; Rowe 1992). Females can therefore alter their mating rate in response to changes in factors such as increased male harassment, to reduce the costs associated with performing rejection responses. When faced with increased male harassment, females will increase the frequency at which they mate in order to avoid these costs. When males are able to coerce females into accepting unwanted copulations by increasing the likely costs of rejection beyond the costs of mating, this is termed 'convenience polyandry' (Thornhill & Alcock

1983). There are a number of insect taxa in which this mating system is present, such as the coelopids (Crean & Gilburn 1998), megachilid bees (Alcock et al. 1977), calopterygid damselflies (Rivera & Andrés 2002) and gerrids (Rubenstein 1984; Wilcox 1984; Arnqvist 1988, 1989; Rowe 1992). However, the occurrence of convenience polyandry is controversial in some cases (see Fincke et al. in Choe & Crespi 1997, for example) and its frequency among insects is still relatively unknown (Rivera & Andrés 2002).

The existence of a vigorous pre-mating struggle in *C. frigida*, whereby the female displays several rejection responses to male mounting attempts, is indicative of a mating system based on convenience polyandry (Thornhill & Alcock 1983; Rowe et al. 1994). This is further supported by the absence of pre-copulatory courtship (Day et al. 1990), as these behaviours in some species have been used in past studies to reject the idea of convenience polyandry on the basis of a lack of sexual coercion (Fincke in Choe & Crespi 1997). Population densities in *C. frigida* can vary considerably, both temporally and spatially, and can often be extremely high (Day & Gilburn 1997). High-density populations of some species exhibit an increased male harassment rate (Arnqvist 1992), and this is also the case in *C. frigida*. Though copulation is likely to have costs in *C. frigida* (see Watson et al. 1998), females appear to copulate many more times than is necessary to fertilise their eggs. However, it is not currently known exactly how many times a female is mounted by different males, or how many times they copulate in their lifetime. In order to estimate the degree of convenience polyandry present in a population, the number of lifetime matings of individual females needs to be estimated. Indeed, there is a considerable lack of information on the remating frequencies of insects generally, including those with mating systems based on convenience polyandry.

One of the aims of this study was to ascertain the sex ratio (primary and operational sex ratios (OSR) combined) of flies within a wrack-bed. This was to establish whether females actively avoid wrack-beds when they are not ovipositing in order to evade harassment by males and reduce their number of superfluous copulations. Wrack-beds are the site of oviposition in this species, so females must visit them in order to lay their eggs. This gives males a good opportunity to harass females for copulations while they are congregating on the wrack-beds to oviposit. In two species of water strider that have a convenience polyandry-based mating system, *Gerris elongates* and *Halobates robustus*, males are habitually found seeking females in areas where females oviposit (Hayashi 1985;

Foster & Treherne 1982; Arnqvist in Choe & Crespi 1997). If females wanted to evade harassment by males and reduce their number of superfluous copulations, they would be expected to avoid wrack-beds when not ovipositing. Previous studies suggest that, under most conditions, adult flies are found most frequently on wrack-beds or on smaller patches of seaweed nearby (Dobson 1974a). Scattered individuals can be found on rocks and vegetation in the vicinity of the wrack-beds on the shore (Dobson 1974a). However, groups of adult *C. frigida* have been known to migrate away from the main colony site to new wrack-beds (Egglishaw 1961). The sex of these groups of flies is unknown, but they must contain females for re-colonisation. Another study suggests that adult flies remain close to the beach, but have been known to move one or two miles inland (Oldroyd 1954). Miall (1903) reported that *C.frigida* adults have been seen visiting flowers inland. The sex ratio of the flies observed in these studies was not noted. Identifying any variation in the sex ratio of *C. frigida* due to temporal and ecological factors was also one of the aims of this study.

The primary objective of this study was to determine how often males will mount and mate with females in a wild population of *C. frigida*, and from this, estimate the degree of convenience polyandry in this species. A working hypothesis was proposed that females would show extreme convenience polyandry as a result of male harassment in a high-density population. In addition, an experiment was constructed to determine whether polyandry results in increased female reproductive success compared to monogamy. A direct comparison was also carried out between the mating behaviours of wild and laboratory-bred flies to determine whether laboratory-based mating trials are an acceptable imitation of natural mating behaviour.

5.2 Experiment 1: Temporal and spatial variation in sex ratio and population size

5.2.1 Materials and methods

5.2.1.1 Estimation of sex ratio of *C. frigida* in the wild

The study sites

This study was carried out at St Mary's Island (Tyne & Wear, North-East England, UK (Ordinance Survey Ref: NZ352753)) between the 8th and 15th June 2002. Three contrasting study sites were chosen on the basis of the age of the wrack-bed present

(determined by the stage of larval development of flies and the level of vegetative decay), as shown in Figure 5.1.

Site A was located in the upper zone of the shore in an area of sand. The seaweed present, which was comprised mostly of *Fucus* species, was relatively old. The upper layer of seaweed was extremely desiccated by weathering, and the layer underneath was well decomposed. The wrack-bed was relatively shallow with a maximum depth of 12cm. The majority of larvae present were in the final developmental instar stage. Dead larvae were also present among the desiccated weed.

Site B was located lower down the shore than Site A, at the upper edge of the inter-tidal zone. The wrack-bed was of an intermediate age and was comprised mostly of *Fucus* and *Laminaria* in roughly equal proportions. The lower layers of the wrack-bed showed signs of decomposition but the seaweed in the upper levels was still intact with very little evidence of decomposition, though larvae at an intermediate stage of development were present. At the beginning of the study the maximum depth of the wrack-bed was approximately 60cm.

Site C showed a gradient from fresh seaweed, which had been deposited only 2 or 3 days previously, to seaweed that had been present for 4 or 5. The wrack-bed was a mixture of *Fucus* and *Laminaria* species, with *Fucus* being the predominant type of seaweed present. The depth of the wrack-bed ranged from approximately 20cm to approximately 50cm.

Sex ratio determination

Sex ratios of *C. frigida* were determined using a revised version of the kick-sampling technique (Ausden 1996), whereby a perspex cage was placed over the wrack-bed and the seaweed underneath disturbed, causing the adult flies to fly up into the cage. The lid of the cage was then moved over the entrance, trapping the flies inside. The flies present were then aspirated into bottles according to sex. As several species of fly were present, any flies that were not *C. frigida* were discarded at this stage. Counts were made of both sexes. This method of sampling was deemed the most effective, as conventional insect traps have been found to be unsuccessful in capturing adult *C. frigida* (Dobson 1974a). Release-

recapture estimates are also ineffective, as marked individuals have been shown not to mix evenly throughout the population (Dobson 1974a).

Each day of the study was partitioned into 7 collection periods of one hour. The sites and sampling periods were organised in a randomised block design for equal sampling as shown in Table 5.1.

5.2.1.2 Statistical analyses

Prior to testing for any deviations from a 50:50 sex ratio, the data were adjusted using Yates' Correction for Continuity, as there were only two categories in each distribution: male and female. In addition, the Bonferoni correction was also applied when examining the sex ratio of individual samples. The sex ratio data were also checked for normality.

Table 5.1 Randomised block design of sampling periods and sites for each day of the study.

	Date				
Sampling period	**10/06/02**	**11/06/02**	**12/06/02**	**13/06/02**	**14/06/02**
08:30-09:30	Site A	Site B	Site C	Site A	Site B
09:40-10:40	Site C	Site A	Site B	Site C	Site A
10:50-11:50	Site B	Site C	Site A	Site B	Site C
14:00-15:00	Site A	Site B	Site C	Site A	Site B
15:10-16:10	Site C	Site A	Site B	Site C	Site A
16:20-17:20	Site B	Site C	Site A	Site B	Site C
17:30-18:30	Site A	Site B	Site C	Site A	Site B

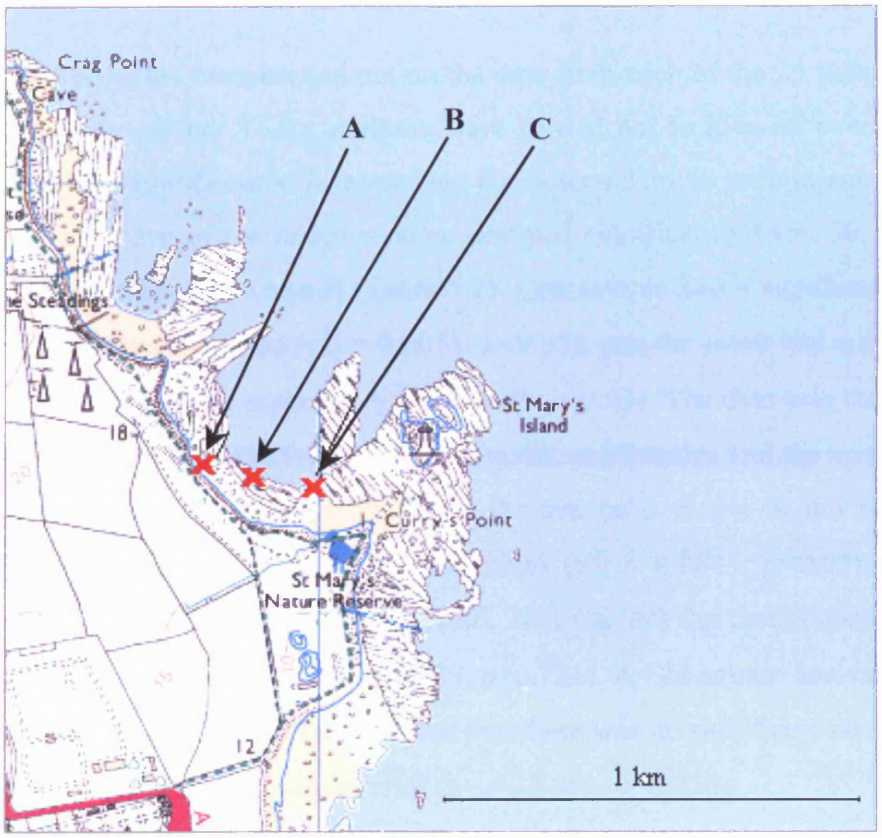


Figure 5.1 Map of St Mary's Island and surrounding area, showing the locations of the three sites (A, B and C) chosen for the study. [Image reproduced with the kind permission of Ordinance Survey]

5.2.2 Results

5.2.2.1 Temporal and spatial variation in sex ratio and population size in a wild *C. frigida* population

Site-dependent variation in sex ratio

Chi-square analyses were carried out on the data from each of the 25 samples of flies taken over the study period. These analyses were carried out to give an overall view of whether the sex ratio significantly differed from the expected 50:50 ratio in any samples. It was found that only two of the samples taken deviated significantly from 50:50. Both of these samples were taken from site B (Table 5.2). One sample had a significantly greater number of females than expected ($\chi^2_1 = 7.5031$, $p<0.05$), and the other had a significantly greater number of males than expected ($\chi^2_1 = 4.1495$, $p<0.05$). The data was then analysed for each site separately using the total number of males and females and the mean expected frequencies (see Table 5.3). It was found that the sex ratio at site A did significantly deviate from the expected ratio of males to females ($\chi^2_1 = 4.2645$, $p<0.05$), with there being a greater number of females than expected. This was not the case, however, for site B ($\chi^2_1=2.7190$, $p=0.889$) or site C ($\chi^2_1= 0.0429$, $p=0.724$). A Chi-square analysis was then performed on the data as a whole. This showed that there was no significant deviation from the expected 50:50 male-female ratio overall ($\chi^2_{24}=37.859$, $p=0.830$).

Site-dependent variation in population size

A One-way ANOVA showed that there were significant differences between the total number of flies found at each of the sites (ANOVA, $F_{2,67} = 0.95$, $p<0.001$) as shown in Figure 5.2. A *post hoc* test confirmed that there was a significant difference between the number of flies found at all sites (Tukey's Test).

5.2.2.2 Temporal variation in the number of males and females present at each site

Variation in the number of females present

At site A, the number of female flies remained relatively constant, with a small degree of fluctuation around an average of 35 flies throughout the sampling period (see

Figure 5.3). At site C, there was an initial increase in the number of females present between the 10th and 11th of June. This increase reached a peak at 10:50 on the 11th and then began to decline again until 9:40 on the 13th, when a slight increase occurred. The largest fluctuations in the number of females occurred at site B. Numbers were relatively high at the beginning of the sampling period then decreased sharply between the 10th and 11th. There then followed a series of sharp increases and decreases, with the number of females present ranging between approximately 70 and 120.

Variation in the number of males present

At site A the number of males showed a similar pattern of fluctuation to the number of females (see Figure 5.4), with no discernable pattern. Site C showed more fluctuation in numbers with an increase between the 11th and 12th, but again no discernable pattern was present. Site B, again showed the greatest amount of fluctuation in numbers with a sudden decrease in the number of males present between the 10th and 11th of June. A sharp increase then followed, which was temporarily halted between the 12th and the beginning of the 13th, but continued dramatically throughout the 13th to reach 150 flies at 17:30.

5.2.2.3 Does the tidal cycle affect the number of flies present at the three study sites?

The times of high and low tides during the study period were recorded, and intermediate times calculated from these, which were categorised using an ordinal scale (see Figure 5.5). The time periods between tidal heights were then derived from this scale, producing categories corresponding to the tidal heights on any given day (see Figure 5.6).

When the data was viewed graphically (see Figure 5.7), there was little apparent association between tidal height and the number of flies present, although at site B, there seemed to be a greater number of flies present at high tide in comparison with low tide.

Regression analyses confirmed that there was no significant association between tide height and the number of flies present at each of the three sites: A (R^2= 0.0%, p=0.638), B (R^2= 0.0%, p= 0.543) and C (R^2 = 0.0%, p=0.449).

Table 5.2 Chi-square analyses (with Yates' Correction) for each sample showing actual and expected numbers of males and females present at each site (significant deviations from a 50:50 sex ratio are marked **).

Site	Number of Males	Number of Females	Expected male:female ratio	χ^2_1	χ^2_1 with Yates' Correction
A	30	41	35.5	0.8521	0.7042
A	26	32	29.0	0.3103	0.2155
C	59	52	55.5	0.2207	0.1621
B	107	152	129.5	3.9092	3.7374
A	41	31	36.0	0.6944	0.5625
C	58	49	53.5	0.3785	0.2990
B	83	110	96.5	1.8886	1.7513
A	27	37	32.0	0.7812	0.6328
B	55	105	80.0	7.8125	7.5031**
C	47	76	61.5	3.4187	3.1869
B	145	99	122.0	4.3360	4.1495**
A	16	24	20.0	0.8000	0.6125
C	74	65	69.5	0.2913	0.2302
B	95	119	107.0	1.3457	1.2359
C	58	59	58.5	0.0042	0.0000
B	79	72	75.5	0.1622	0.1192
A	20	31	25.5	1.1862	0.9803
B	82	109	95.5	1.9083	1.7696
A	26	39	32.5	1.3000	1.1076
C	71	57	64.0	0.7656	0.6601
B	94	83	88.5	0.3418	0.2824
A	38	22	30.0	2.1333	1.8750
C	49	65	57.0	1.1228	0.9868
B	151	114	132.5	2.5830	2.4452
A	11	26	18.5	3.0405	2.6486

Table 5.3 Chi-square analyses (with Yates' Correction) testing for differences in sex ratio for each site. Results that are significant at $P < 0.05$ are marked**.

Site	Number of Males	Number of Females	χ^2_1
A	235	283	4.2645**
B	891	936	2.7190
C	416	423	0.0429

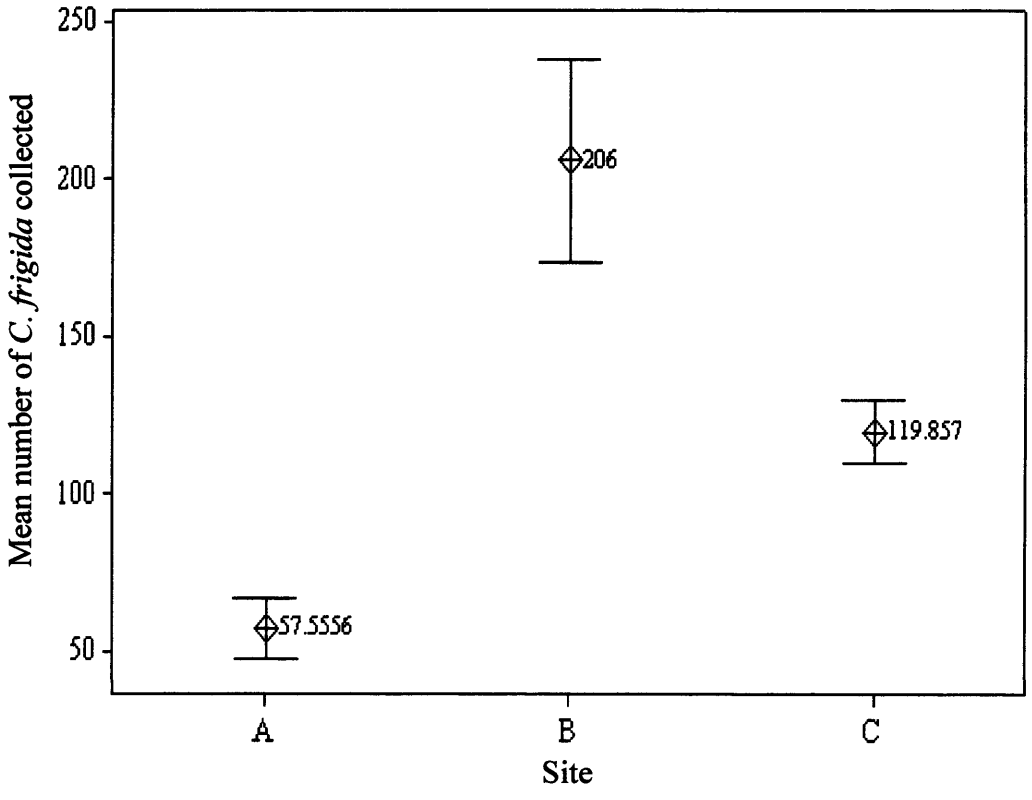


Figure 5.2 The mean total number of *C. frigida* flies (male and female) collected at each site (with 95% C. I.).

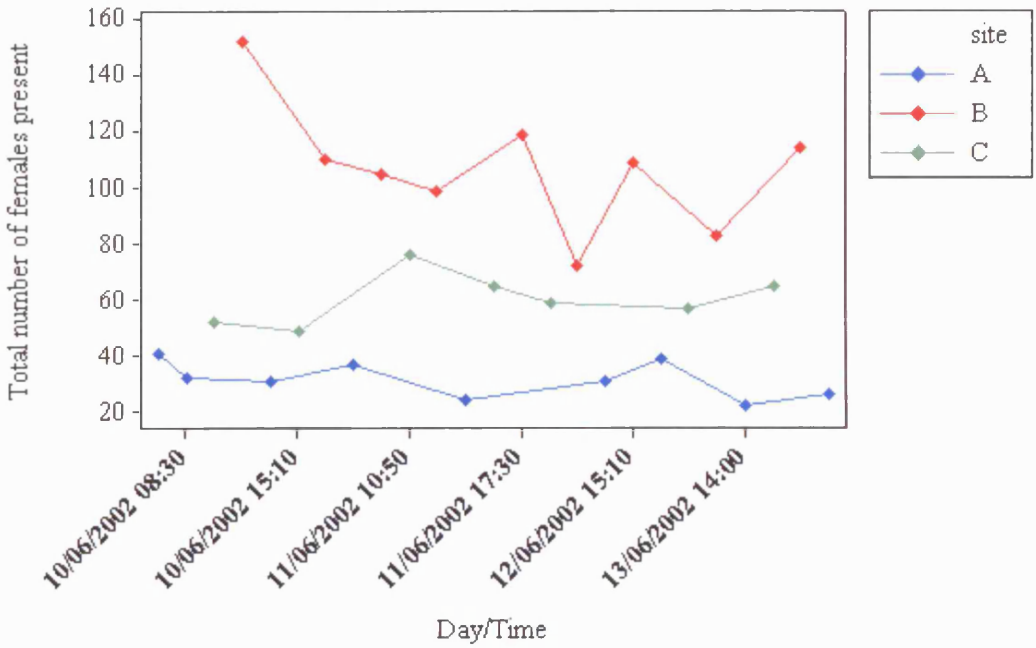


Figure 5.3 Time series plot showing the total number of female *C. frigida* present at each site.

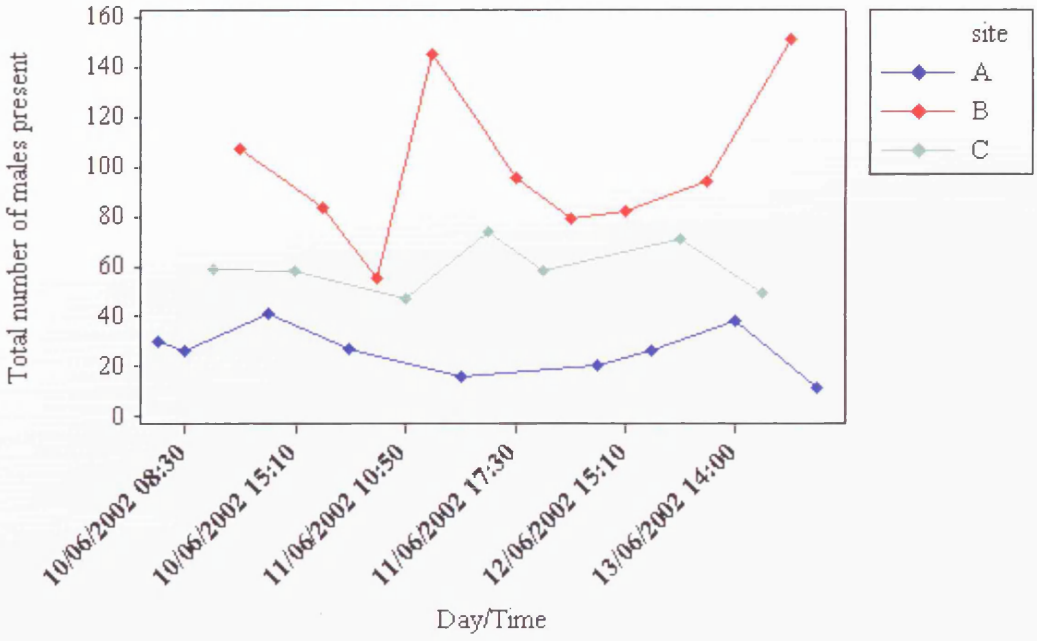


Figure 5.4 Time series plot showing the total number of male *C. frigida* present at each site.

Table 5.5 Ordinal scale of tidal heights and their corresponding times of the day for the study period (9th June – 14th June).

	Tidal height at times during the day								
Date	**High 1**	**HH/L**	**H/L**	**H/LL**	**Low**	**LL/H**	**L/H**	**L/HH**	**High 2**
09/06/2002	16:04	17:31	18:59	20:26	21:54	23:11	00:49	03:16	03:45
10/06/2002	16:35	18:07	19:38	21:10	22:40	00:12	01:43	03:15	04:45
11/06/2002	17:15	18:50	20:20	21:55	23:25	00:00	02:30	04:05	05:35
12/06/2002	18:15	19:45	21:15	22:45	00:15	01:45	03:15	04:45	06:15
13/06/2002	19:25	20:52	22:18	23:45	01:10	02:37	04:03	05:30	06:55
14/06/2002	20:25	21:49	23:12	00:36	02:00	03:24	04:47	06:11	07:35

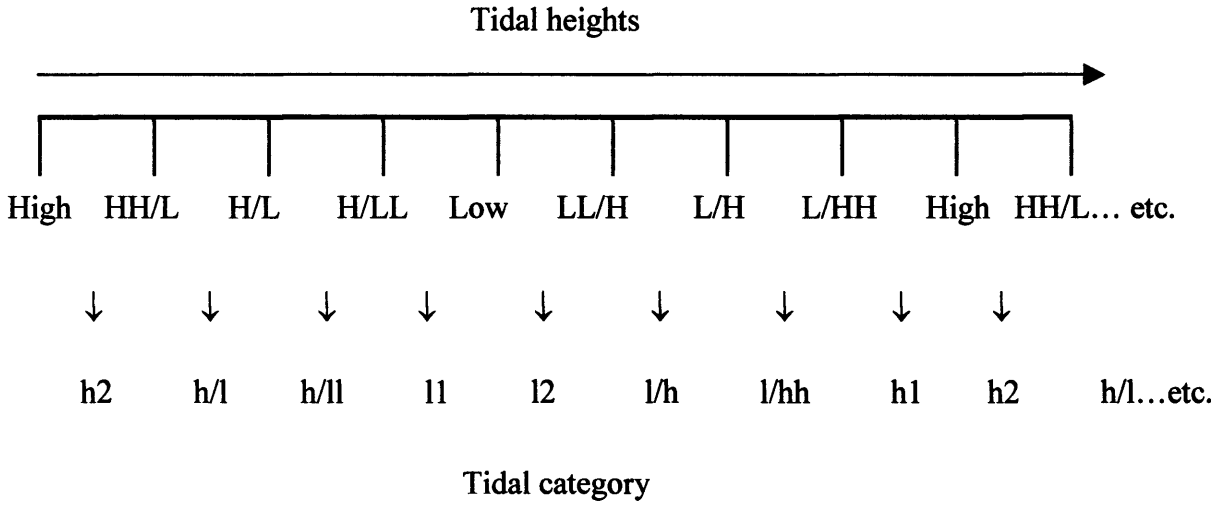


Figure 5.6 Diagram showing the derivation of tidal height categories from the ordinal scale of tidal height.

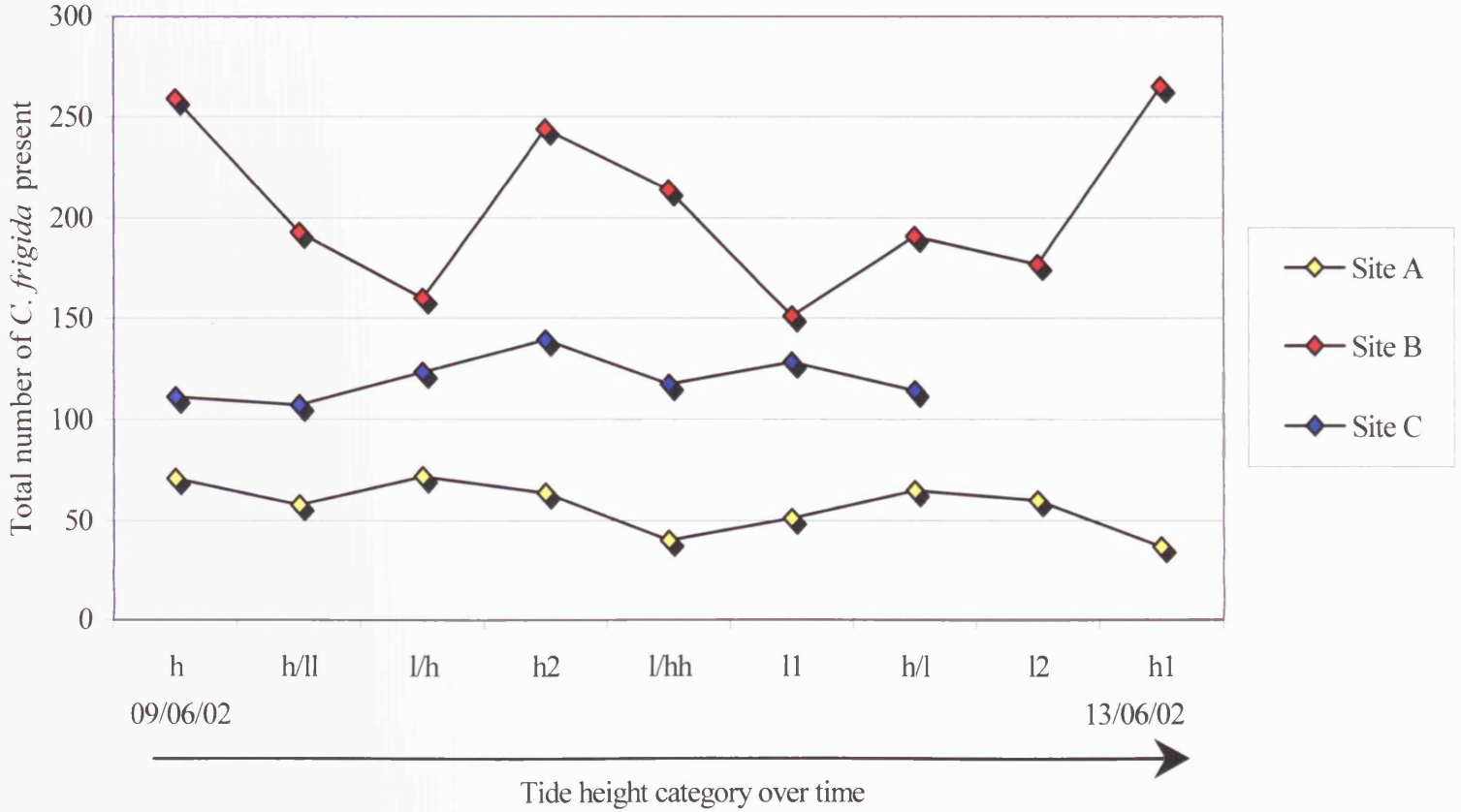


Figure 5.7 The total number of *C. frigida* caught at each site for each tide height category.

5.3 Experiment 2: Mating behaviour of *Coelopa frigida* in the wild and in the laboratory

5.3.1 Materials and methods

5.3.1.1 Mating behaviour of *Coelopa frigida* in the wild

This study was carried out at St Mary's Island (Tyne & Wear, South-East England, UK (Ordinance Survey Ref: NZ352753)) between 2nd and 5th June 2003. Randomly selected *C. frigida* adults were collected from a wrack-bed using the kick-sampling technique as outlined in the previous section. Male and female flies were aspirated into collection bottles after capture for immediate use in mating trials. Pairs of flies were aspirated into the mating chamber. The time taken for each male to mount the female was determined up to a maximum period of five minutes. When the male failed to mount a female within five minutes, the trail was scored as a non-mount. Measurements were taken of the following components of the mating interactions:

① Whether the male mounted the female or not
② Time taken for the male to mount the female (time to mount)
③ Total time spent mounted (total mount time)
④ The outcome of the mount: a) male successfully kicked or shaken off by the female; b) male dismounts whilst the female is abdomen curling; c) male dismounts passive female without copulating; d) the pair copulate
⑤ Pre-mating struggle duration (struggle duration)
⑥ Rejection behaviours exhibited by the female during pre-mating struggle: a) abdominal curling b) shaking and c) kicking, and the duration of these behaviours
⑦ Copulation duration

5.3.1.2 Mating behaviour of *C. frigida* in the laboratory

Data from mating pairs kept individually in the presence of seaweed for 48 hours prior to the experiment were used for this section of the study. The methods used were the same as those described in Chapter 4, with the modification stated above.

Continuous data were log-transformed prior to analyses to improve normality.

5.3.2 Results

5.3.2.1 Comparisons between the mating behaviours exhibited by wild collected flies and virgin laboratory-bred flies

Descriptive statistics

Mean values for each of the mating behaviour component durations for both wild-collected flies and virgin laboratory-reared flies are given in Table 5.4. The data concerning the remaining components are given in the details of the corresponding analyses.

Table 5.4 Mean values for each of the mating behaviour component durations with standard errors in parentheses. All measurements are in seconds.

	Laboratory-reared flies (n = 522)	**Wild-collected flies (n = 164)**
Variable	**Mean (S.E.)**	**Mean (S.E.)**
Time taken to mount female	86.52 (4.31)	103.21 (9.91)
Total mount duration	65.23 (3.59)	59.18 (9.39)
Total struggle duration	25.41 (2.01)	26.06 (5.16)
Female abdomen-curl duration	29.50 (2.43)	32.94 (7.44)
Female shaking duration	8.43 (0.77)	10.02 (1.54)
Female kicking duration	9.84 (0.79)	7.76 (1.16)
Copulation duration	91.23 (4.61)	109.1 (11.2)

Male willingness to mate

Two Proportions Analysis showed that there was a significant difference in the proportion of lab-bred males and wild males willing to mount females (lab-bred males = 71.1% [370 out of 522] mounted; wild males = 44.5% [73 out of 164] mounted; $Z = 6.09$, $p < 0.05$). Virgin lab-bred males appear to be more willing to mate than wild collected males, as shown in Figure 5.8.

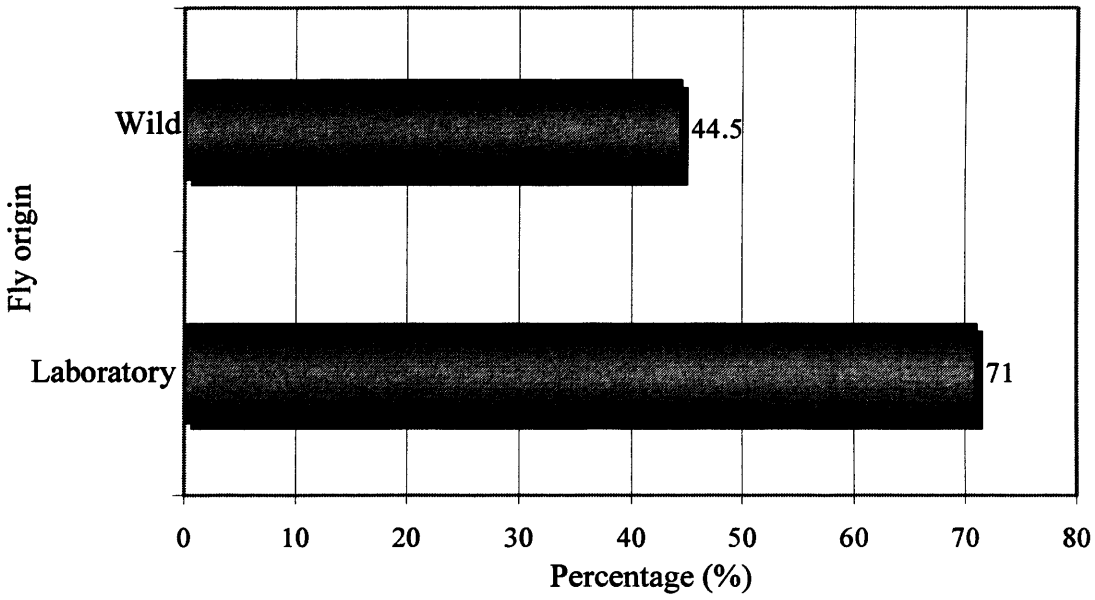


Figure 5.8 Percentage of virgin lab-bred males and wild collected males attempting to mount females.

Time taken for males to mount females

When the data were analysed, no significant difference was found between the time taken by wild and virgin lab-bred males to mount females (ANOVA, $F_{1,443} = 0.01$, $p = 0.941$).

Mean duration spent by males mounted on females

A One-way ANOVA revealed that there was a significant difference between the mean duration spent mounted on females by lab-bred and wild collected males ($F_{1,443} = 9.77, p < 0.05$), with lab-bred males spending a greater length of time mounted on females (Figure 5.9).

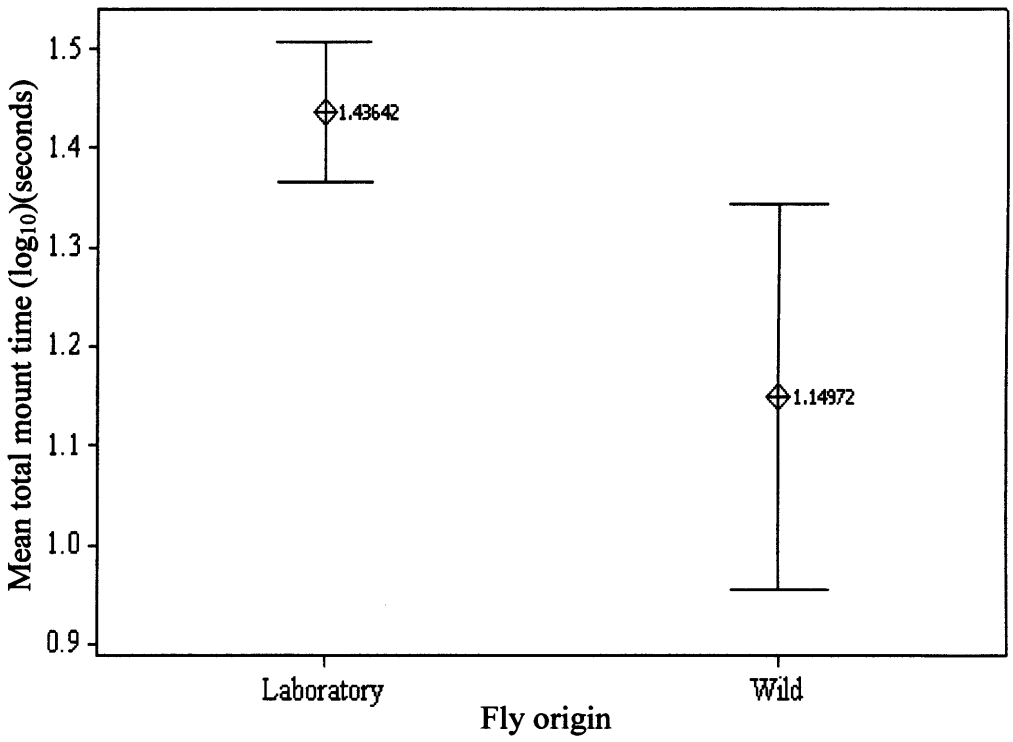


Figure 5.9 Mean duration spent mounted on females by wild and lab-bred males (with 95% C.I.).

Female willingness to mate: the number of mating trials resulting in copulation

There was found to be no significant difference in the proportion of mating trials that resulted in copulation, between wild flies and lab-bred flies (Two Proportions Analysis; lab-bred flies = 46.6% [173 out of 371] of pairs copulated; wild flies = 38.4% [28 out of 73] of pairs copulated; $Z = 1.32, p = 0.186$).

The rejection responses exhibited by females (see Table 5.5)

(i) Abdomen curling

A highly significant difference was found between the proportion of lab-bred and wild females exhibiting the abdomen curling rejection response. Lab-bred females were more likely to exhibit abdomen curling.

(ii) Shaking

No significant difference was revealed between the proportion of lab-bred and wild females exhibiting the shaking rejection response.

(iii) Kicking

Again, no significant difference was found between the proportion of lab-bred and wild females exhibiting the kicking rejection response.

Table 5.5 The percentages of females exhibiting each rejection response for both groups (laboratory and wild) with the results of Two Proportions Analyses carried out for each response (significant result marked **).

Rejection response	**% of virgin lab-reared females exhibiting behaviour**	**% of wild collected females exhibiting behaviour**	**Z**	***p***
Abdomen- curling	71.4	49.3	3.51	< 0.05**
Shaking	54.2	62.9	1.37	0.170
Kicking	60.8	52.1	1.37	0.172

Durations of individual female rejection responses and total struggle duration

One-way ANOVA revealed no significant differences between the duration of each female rejection response: abdomen curling ($F_{1,300} = 0.92$, $p = 0.339$), shaking ($F_{1,243} = 3.06$, $p = 0.081$) and kicking ($F_{1,262} = 0.05$, $p = 0.822$). There was also no significant difference between the mean struggle durations of lab-bred and wild pairs (ANOVA, $F_{1,407} = 0, p = 0.951$).

Females exhibiting struggling behaviour at point of male dismount

There was a highly significant difference between the proportion of lab-bred and wild females that exhibited struggling behaviour at the point of male dismount (Two Proportions Analysis, lab-bred females = 91.4% [339 out of 371]; wild females = 69.4% [50 out of 72]; $Z = 3.9, p < 0.05$).

Copulation duration

There was no significant difference between the mean copulation duration of lab-bred and wild collected pairs (ANOVA; $F_{1,199} = 2.11, p = 0.148$).

Mount outcomes (see Figure 5.10)

(i) Male kicked or shaken off by female

A significant difference was found between the proportion of lab-bred male flies and wild males successfully rejected by females (Two Proportions Analysis; lab-bred males = 41.0% [152 out of 371]; wild males = 28.8%; $Z = 2.07, p < 0.05$). Wild collected females seem to reject males significantly less than lab-bred females.

(ii) Male dismounts as a result of female abdomen curling

A significant difference was also found between the proportion of lab-bred and wild collected males that dismounted as a result of female abdomen curling (Two Proportions

Analysis; lab-bred males = 10.2% [38 out of 371]; wild males = 2.7% [2 out of 73]; Z = 3.03, $p < 0.05$).

(iii) Male rejects female

Statistical analyses of the data revealed that there was a highly significant difference between the proportion of lab-bred and wild males that rejected females (Two Proportions Analysis; lab-bred males = 2.4% [9 out of 371]; wild males = 30.1% [22 out of 73]; Z = 5.10, $p < 0.05$).

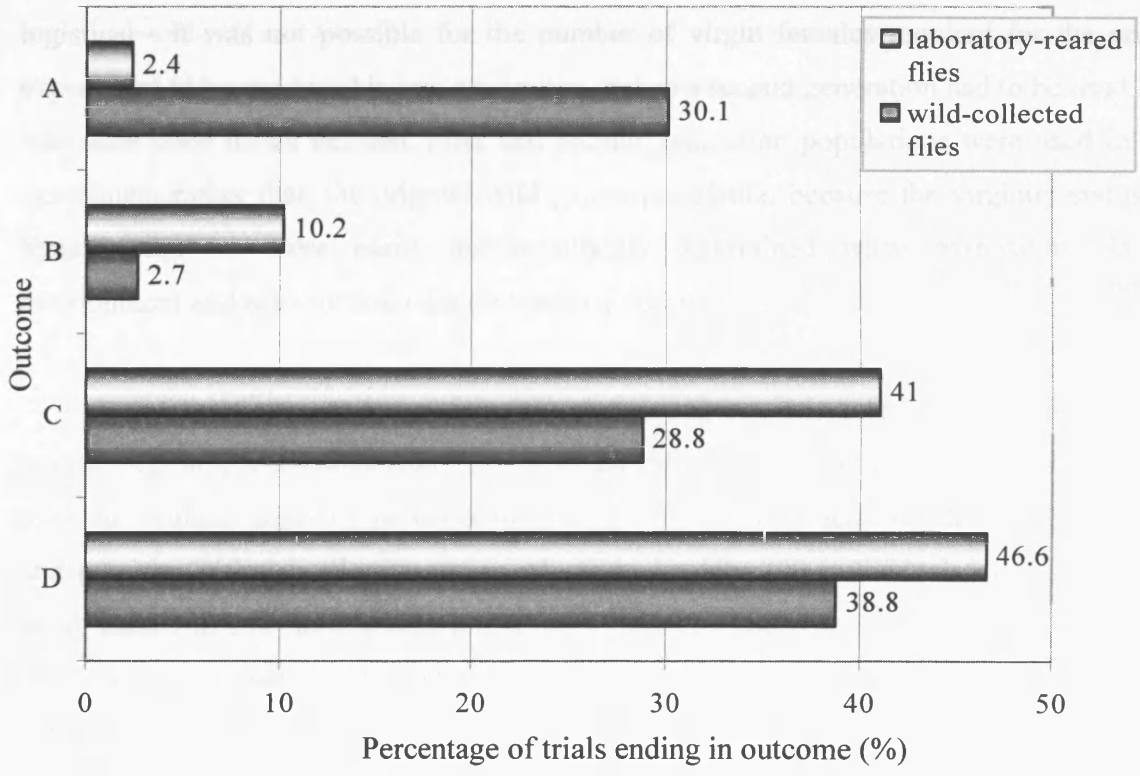


Figure 5.10 Percentage of trials ending in A: male dismounts after rejecting the female; B: male dismounts as a result of female abdomen-curling; C: male is successfully rejected by female; and D: mount results in a copulation

5.4 Experiment 3: Male mating behaviour overnight

5.4.1 Materials and methods

The flies used in this experiment were collected as larvae from St Mary's Island (Tyne & Wear, South-East England, UK (Ordinance Survey Ref: NZ352753)) in April 2004. The larvae were reared to adulthood under standard laboratory conditions (see Chapter 3) under an 11hr light:13hr dark light regime, and the adults allowed to reproduce to create a first generation population. A second-generation population was then bred from the first generation. The populations were maintained at large sizes to prevent any changes to natural behaviour caused by the effects of inbreeding. The flies from the first generation were used in session one of the experiment, and the subsequent second generation was used in session two. The reason for the experiment being carried out in two sessions, using two different populations, rather than in a single session using one population, was purely logistical - it was not possible for the number of virgin females required for the entire experiment to be produced in one generation, hence a second generation had to be used at a later date once it had eclosed. First and second generation populations were used in the experiment rather than the original wild parent population because the virginity status of females can be more easily and accurately determined when oviposition, larval development and eclosion are under laboratory control.

Individuals were collected from population cages within a few hours of eclosion to ensure virginity, and immediately sorted under light CO_2 anaesthesia (flies were handled after this without anaesthesia, using mouth aspirators). Males and females were separated and put into 260ml bottles in groups of approximately 200 individuals. Balls of cotton wool soaked in 5% sucrose solution were provided in each bottle. The males chosen for this experiment were of a similar size to each other, and of an intermediate size in comparison to the population as a whole, to remove any variation in behaviour due to size. The bottles containing the sorted flies were then kept at 5°C until required. Flies were stored in this manner for a maximum of seven days before use. Males were placed into individual pots (30ml diluvials) containing freshly minced seaweed and kept at 25°C (11hr light:13hr dark regime) for a period of 48 hours prior to the experiment. Approximately 24 hours before the experiment, the females were sorted into groups of 20 individuals, which were kept in bottles with sucrose solution-soaked cotton wool under the same conditions as the individual males.

As mentioned earlier, the experiment was carried over two separate sessions - the second session simply being a continuation of the experiment on a different night, and having exactly the same methods as were used in session one. Session one contained males 1 to 5, and sessions two contained males 6 to 10. The following methods were duplicated in session two.

A few minutes before 'lights off' in the 13hr light-dark cycle, which began at 19:00, five males were aspirated individually into separate 550ml powder rounds containing freshly minced seaweed covered by a fine-gauge nylon mesh and stoppered with cotton wool. The nylon mesh was used to create a barrier between the seaweed and the flies to prevent female oviposition on the seaweed, and also to facilitate the collection of flies. The males were then left undisturbed to acclimatise to their surroundings, and at 20:00 twenty virgin females were added to each powder round. This was carried out under red light, using a Safelight red filter 1 (Kodak) attached to an electronic lamp (model KL1500), which the flies are unable to detect, therefore maintaining the 'dark' conditions and removing the possible stimulating effects of light. All subsequent phases of the experiment that were carried out during the 'lights off' period were performed under red light. Noise and vibration were also kept to minimum to avoid any unnecessary disturbance to the males' natural nocturnal behaviour.

Four hours later (see Figure 5.11), at approximately 00:00, each male was removed and put into a new powder round containing seaweed covered by mesh. These powder rounds also contained 20 virgin females, which had been put there at least 30 minutes prior to the male, for acclimatisation. The males were transferred to the new powder rounds as quickly and smoothly as possible to keep disturbance to a minimum. The males were transferred to new powder rounds rather than the females being replaced with new females because it was deemed that female removal would create more disturbance. All of the females used in this experiment were removed from the powder rounds immediately after the removal of the males and placed separately into individual pots of minced seaweed, where they were left to oviposit under standard laboratory conditions. The males were left undisturbed for a period of four hours until 04:00, when they were again removed from the powder rounds and placed in new powder rounds containing seaweed and 20 more virgin females. At 08:00, the males were removed from the powder rounds for the final time and then frozen at -20°C in individual 1.5ml microcentrifuge tubes. The males were stored at

this temperature until their karyotypes could be determined using standard starch gel electrophoresis techniques. Genotyping was carried out to ensure all of the males had the same αβ inversion karyotype, eliminating variation in behaviour due to karyotype.

The females were left in the individual pots for approximately four days and the presence of larvae was used as an indicator that the female had mated with the male.

5.4.2 Results

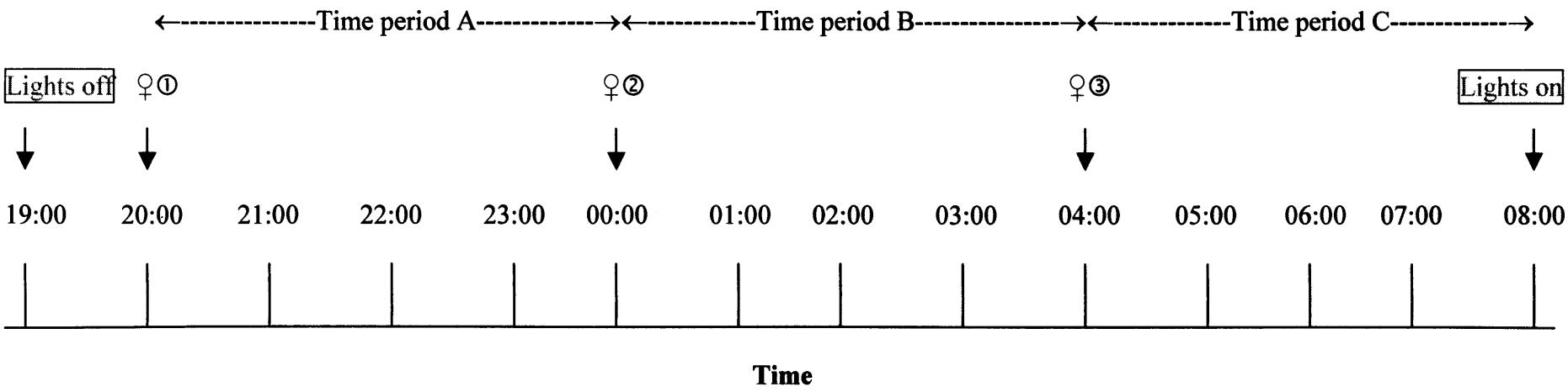
The proportion of females that produced larvae was calculated for each of the males for each time period (Table 5.6).

The medians of the proportions of fertilised females from session 1 (median = 0.800, n = 15) and session 2 (median = 0.800, n = 15) were found to be equal (Mann-Whitney *U*-test, W = 223.5; $p = 0.7217$), indicating that there was no significant difference between the proportions of females fertilised by the males from session 1 compared to those in session 2.

It was found that there was a significant difference between the proportions of fertilised females between the three time periods (median of A = 0.875, median of B = 0.700, median of C = 0.825; n = 10; overall number of observations = 30) (Kruskal-Wallis test, H = 12.20, d.f. = 2, $p < 0.01$). It is clear from an examination of the distribution of the ranks of the proportions (see Figure 5.12), and the proportions themselves (see Figure 5.13), that the main difference lie between time period B and the other two time periods. The mating activity levels are lower during time period B than in the other two time periods.

Further analyses revealed that there was no significant difference between the proportions of females fertilised by each male (Kruskal-Wallis test, H = 8.76, d.f. = 9, $p = 0.460$).

The assay carried out to determine the genotype of the males used in the study revealed that all males had the αβ inversion karyotype.



♀① - First group of females added to the powder rounds
♀② - Males introduced to second group of females
♀③ - Males introduced to third group of females

Figure 5.11 Time series diagram outlining the stages of the experiment.

Table 5.6 Proportion of females fertilised by each male in each time period (two females died before the egg-laying phase and these were discounted from the study).

	Trial Number	Time Period	Male Number	Proportion of fertilised females
Session 1	1	A	1	0.90
	2	B	1	0.70
	3	C	1	0.85
	4	A	2	1.00
	5	B	2	0.70
	6	C	2	0.80
	7	A	3	1.00
	8	B	3	0.75
	9	C	3	0.85
	10	A	4	0.70
	11	B	4	0.40
	12	C	4	0.60
	13	A	5	0.80
	14	B	5	0.65
	15	C	5	0.95
Session 2	16	A	6	0.75
	17	B	6	0.70
	18	C	6	0.80
	19	A	7	0.0
	20	B	7	0.75
	21	C	7	0.80
	22	A	8	1.00
	23	B	8	0.70
	24	C	8	0.85
	25	A	9	0.85
	26	B	9	0.80
	27	C	9	0.75
	28	A	10	0.75
	29	B	10	0.70
	30	C	10	0.95

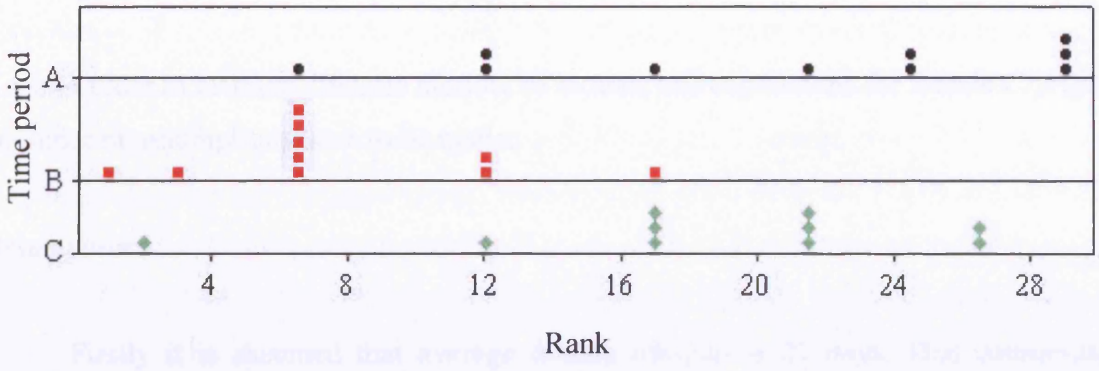


Figure 5.12 Dotplots showing the distributions of ranks for the proportion of females fertilised by males for each of the three time periods, A, B and C.

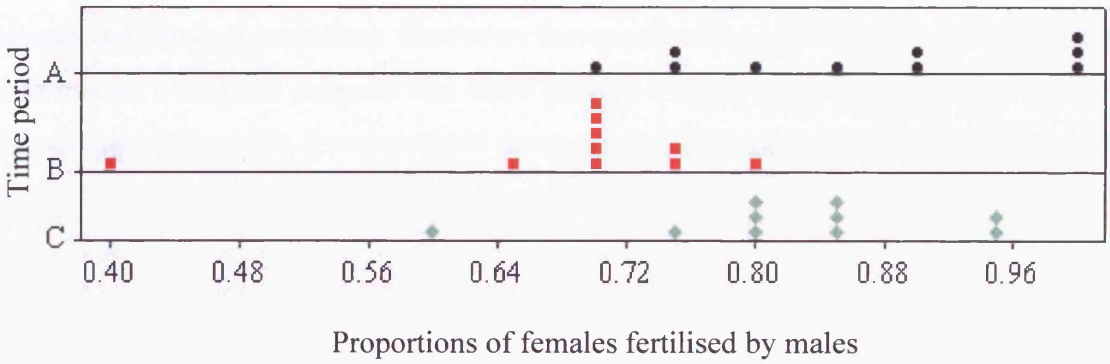


Figure 5.13 Dotplots showing the distributions of the proportion of females fertilised by males for each of the three time periods, A, B and C.

5.5 Female lifetime re-mating frequency

5.5.1 Assumptions made before estimating female lifetime re-mating frequency

In order to estimate lifetime number of mounts and copulations for female *C. frigida*, a number of assumptions have to be made:

Assumption 1

Firstly it is assumed that average female lifespan is 21 days. This conservative estimate of lifespan is based upon previous studies of longevity (Burnet 1960; Dobson 1974b; Collins 1978), which revealed an average lifespan in the wild of 3-4 weeks.

Assumption 2

Secondly it has been assumed that there is no variation in the mount and mating rate exhibited by males during daylight hours. Unlike other Dipterans, such as *Drosophila*, which show clear bimodal morning and evening peaks in activity (Helfrich-Förster 1998), *C. frigida* displays a continuous level of activity throughout the day, when seaweed is present (personal observation). However, the experiment carried out to determine night-time mating behaviour suggests that there may be a slight reduction in mating activity in the middle of the night. It was decided that a conservative estimate of male mating rate at night of 50% of the daytime rate would be used.

Assumption 3

The final assumption is that males come into contact with females with the same frequency in the mating chamber during the experiment, as they do within the wrackbed. Population density within the wrack bed was so high that searching males were likely to be almost continuously meeting females. Observation of male flies in the presence of seaweed in the laboratory has revealed they spend about 80% of their time searching for females (Meader & Gilburn, unpublished results). A similar level of male searching behaviour is seen in mating chambers (Day & Gilburn 1997). Thus the use of the mating chamber appears to be a good simulation of the situation within a very high-density wild population.

5.5.2 Calculation of female lifetime re-mating frequency

During the mating trials involving wild-collected flies, 73 male mount attempts were observed within a total observation time of 581 minutes (total time until mount of trials in which the male mounted plus total observation period of trials in which the male did not mount). This suggests that wild males will mount a female every 7.95 minutes. It was found that females managed to successfully resist copulation in 31.50% of pre-mating struggles. The males terminated another 30.14% of mating attempts by dismounting the female after she had ceased resisting. The remaining 38.36% of male mating attempts resulted in a copulation. Assuming a constant rate of male mounting activity throughout the day and a 50% reduction in male mounting rate during the night, females will be mounted over 135 times in a period of 24 hours.

Assuming a female lifespan of 21 days, a female will be mounted approximately 2850 times in a lifetime. From this, we can assume that females mate with around 1090 males in a lifetime, if 38.36% of mounts result in a copulation. In addition, males regularly copulate with a female more than once in the same mount (Shuker 1998), which could result in this figure being doubled in some instances.

5.6 Experiment 4: Female reproductive success under polyandrous vs. monogamous mating regimes

5.6.1 Materials and methods

Wild *C. frigida* larvae were collected from St Mary's Island, Tyne & Wear, Southeast England, UK in March 2003. The resulting flies were maintained in the laboratory under standard laboratory conditions. Males and females were sex-deprived for 48 hours prior to the experiment, and were a maximum of one week old.

Virgin females were randomly allocated to two groups: the first group were each mated with a single randomly selected virgin male (monogamous group) and the second group were each mated with five randomly selected virgin males consecutively (polyandrous group) under standard mating trial conditions. Males of a similar size were chosen to control for any effects of male size on the results. Following mating, the females were aspirated into individual pots containing fresh minced seaweed to oviposit. The pots

were left at 25°C (12 hour light:dark regime) for between 3 and 5 days or until any larvae present were approximately 5mm in length. Earlier manipulation of larvae would have resulted in injury to the delicate first instar larvae.

5.6.1.1 Heat extraction of larvae

The number of larvae in each pot was determined by a method of heat extraction. The apparatus used is shown in Figure 5.14. For each pot the following method was utilised:

The seaweed, containing the larvae, was tipped into a glass funnel (diameter 12 cm) so that it lay on top of a medium-gauge wire mesh. Water was then poured on top of the seaweed until the level was approximately 3 cm beneath the rim of the funnel. An electric lamp (D.Z. Industries Co. Ltd.) was then switched on and positioned roughly 10cm above the funnel, and the light directed onto the water within. Larvae could be collected in 2 ways: firstly, some larvae floated to the water's surface and hung there upside-down, breathing through the respiratory organs located at their posterior end. These were collected immediately by using a pair of insect forceps. Secondly, the remainder of the larvae tried to burrow downwards through the seaweed, away from the heat and light emitted by the lamp, whereby they passed through the wire mesh and fell down the rubber tubing, getting trapped where the rubber tubing was clamped shut to allow no water to pass through into the beaker.

The apparatus was left for approximately 30 minutes to ensure that all the larvae have passed through the mesh, with the seaweed being disturbed several times throughout that period, to allow any larvae that were trapped to pass through. After 30 minutes, the clamp closing off the rubber tubing was released and the water drained out into the beaker, carrying with it the larvae.

Since the offspring were counted at the larval stage, the problem of the results being affected by flies dying before they reached adulthood was avoided. A better method may have been to count the number of fertilised eggs in each pot, but this proved impossible as *C. frigida* lay their eggs in clumps which are very difficult to separate without causing damage.

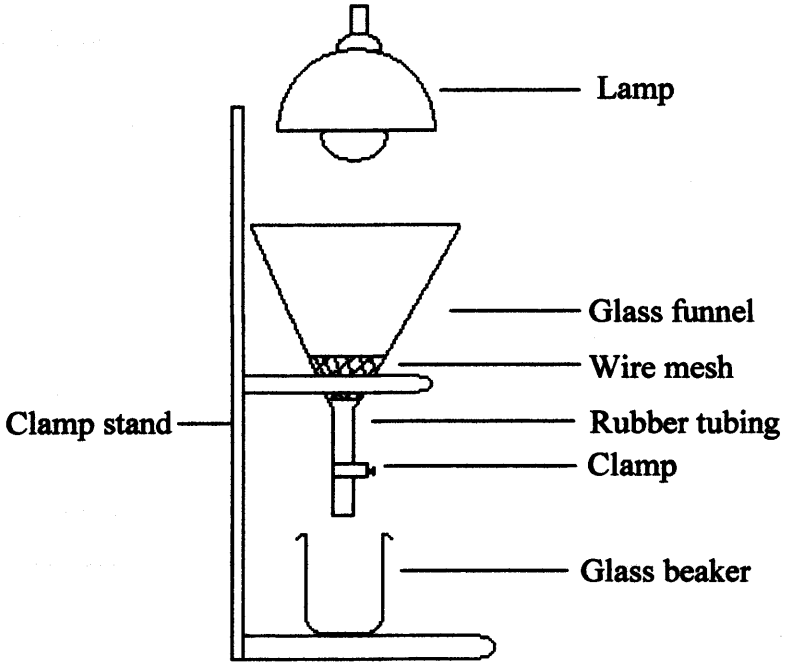


Figure 5.14 Apparatus set-up used for the heat extraction of larvae from seaweed.

The number of larvae produced and female size were both log-transformed to improve the normality of their distributions.

5.6.2 Results

The descriptive statistics for this experiment are presented in Table 5.7. A Two Samples T-test confirmed that there was no significant difference in mean female size between the two mating regimes ($T = 0.48$, $p = 0.649$). However, a significant difference was found between the mean numbers of larvae produced by females in each of the regimes, with a significantly greater number of larvae being produced by polyandrous females compared with monogamous females (Two Samples T-test, $T = -2.66$, $p < 0.05$). This result is shown by Figure 5.15. The number of larvae produced by females was found not to be associated with female size (Regression analysis, $F_{1,9} = 1.40$, $p = 0.271$).

Table 5.7 Mean female wing length and mean number of larvae produced for monogamous and polyandrous mating regimes. Standard errors are given in parentheses.

	Monogamous		Polyandrous	
	n	Mean (S.E.)	n	Mean (S.E.)
Number of larvae produced	5	68.80 (4.60)	5	87.20 (5.25)
Female wing length (mm)	5	5.88 (0.15)	5	5.74 (0.27)

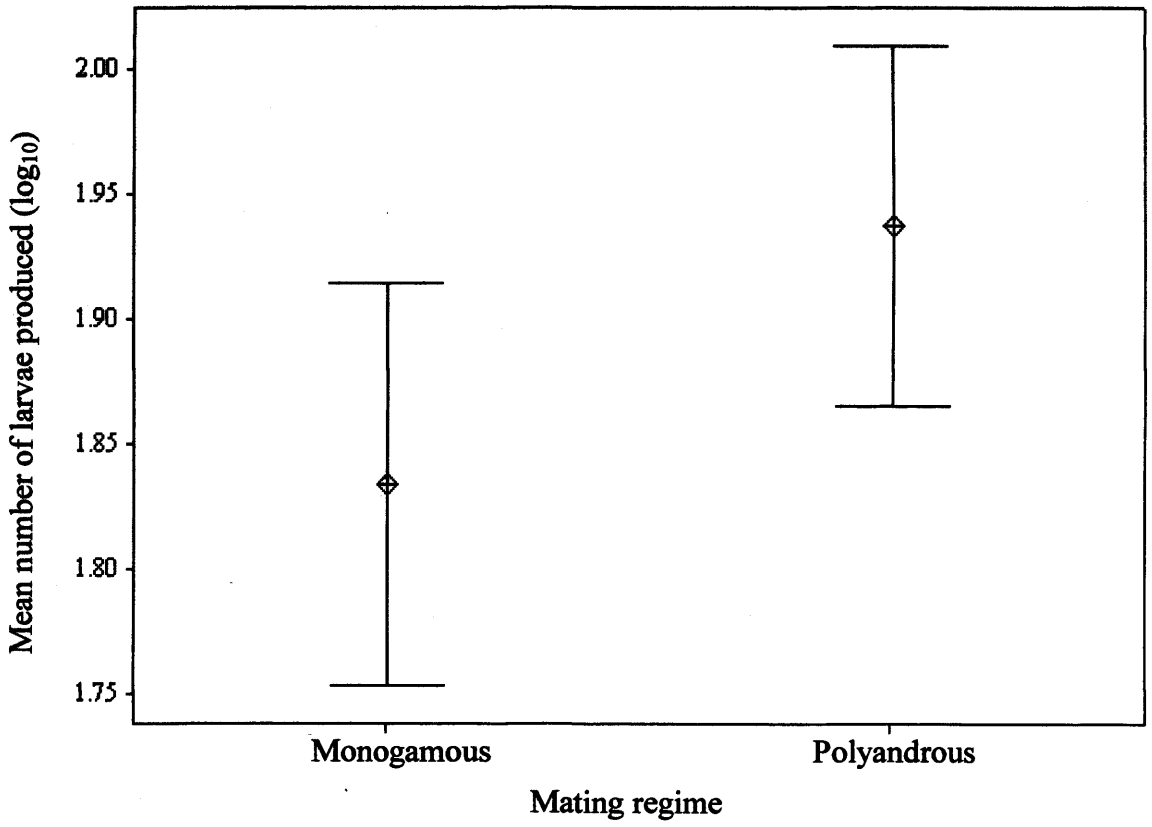


Figure 5.15 Interval plot showing the mean number of larvae produced for both mating regimes (with 95% C.I.).

5.7 Discussion

The analyses carried out on the data collected from each of the three sites showed that there was no deviation from a 50:50 sex ratio even when factors such as site, tide level and time of day were taken into consideration. There were two instances where the ratio deviated from 50:50 at site B, but there appeared to be no obvious reason or pattern to this deviation and it was most likely caused by natural random fluctuations in population numbers.

This constancy in sex ratio suggests that females do not avoid wrack-beds to reduce the level of harassment by males when they have already laid their egg clutches, indicating that there may be additional benefits in remaining close to the wrack-bed which outweigh the costs of continuous male attention. These benefits may include shelter, a source of nutrients and moisture, and 'safety in numbers'.

The overall number of flies present differed greatly between the three sites suggesting that age, macro-algal composition and position of wrack-beds are major factors in determining the degree of colonisation and the population densities found there. Site A, which was farthest up the beach and consisted of the oldest wrack had the least number of flies living in or near it. This was probably due to the deficiency of a suitable substrate on which females could lay their eggs, and hence produce healthy larvae. Larvae require a considerable quantity of decomposing wrack in order to develop sufficiently and this site did not provide this resource. The wrack at this site was very dry resulting in a low level of bacterial activity, which the larvae rely upon as a source of nutrients. Further evidence of this was the presence of dead, dehydrated larvae. Parasitism of coelopids is also higher in areas of desiccated seaweed than it is in deep, moist wrack-beds (Heitland 1988), and this may have played a role in the low number of flies found there. It may have been the case that the wrack-bed at this site had previously been a good-quality oviposition site, but that weathering, bacterial and larval activity had rendered it exhausted of resources by the time the experiment had begun. Site B had the greatest number of flies inhabiting it in relation to the other sites. The wrack-bed at this site was of intermediate age and was the deepest of those found at each of the three sites. There was a relatively large mass of decomposing seaweed present, providing an ideal habitat for *C. frigida* larvae to develop in. This wrack-

bed was also composed of approximately equal proportions of *Fucus* and *Laminaria* species, whereas the other two wrack-beds were mainly composed of *Fucus*. It has previously been suggested that *C. frigida* shows a preference for *Laminaria* as an oviposition substrate (Butlin 1983). The results of this experiment appear to support this finding. The wrack-bed at site C showed a gradient of age from fresh seaweed through to seaweed at a transitional stage of decomposition. The number of flies found at this site was intermediate between those found at the other two sites suggesting that, though it provided a source of decomposed seaweed suitable for healthy larval development, it was not as large or of such an ideal composition as that found at site B. This was most likely due to the presence of a proportion of fresh seaweed, which had not yet reached the stage of decomposition or stability suitable for larvae to thrive upon.

The results of this experiment suggest that there was no apparent temporal variation in numbers of flies present on a wrack-bed, nor with position within the tidal cycle. Fluctuations in population numbers naturally occurred but with no obvious pattern.

As a result of the mating trials carried out using wild-collected flies, one major difference found between the behaviours exhibited by wild-caught, multiply-mated males and virgin laboratory-bred males was in the total time they spent mounted on a female. Virgin lab-bred males spent a significantly longer time mounted than wild-caught males. This difference in behaviour may be due to lab-reared males being kept in a state of sexual deprivation before being used in experiments. This deprivation may indicate that females are in short supply and therefore they stay mounted for longer when they eventually do get an opportunity to mate. Multiple copulations are also present in virgin pairings, with females frequently being inseminated several times before the males dismount (Shuker 1998, personal observation). In contrast, non-virgin males collected from a wrack-bed seem to recognise that there is a huge number of females present with which to mate, and therefore quickly move onto another female when they are rejected.

It might be expected that other behaviours would vary between virgin and non-virgin males, for example struggle duration and mating duration. This is not the case however. The higher frequency of female rejection by wild males suggests that there are a greater proportion of females that may be less desirable to males in the wild. This effect is likely to be the result of males avoiding less gravid females. Male mate choice for fecund females

has previously been identified in *C. frigida* (Pitafi 1991; Pitafi et al. 1995) and was found to be an important factor in determining whether a male will dismount a female. Another potentially important factor is the female's age. Male mate choice for high female survivorship has been found in another coelopid, *Gluma musgravei* (Dunn et al. 2001). It is likely that males will come into contact with a relatively large proportion of older females in the wild, some of which might be post-reproductive, whereas in the laboratory, males are typically offered young, gravid females. The reason for this aversion to older females could be due to their shortened longevity and consequently, future egg-laying potential. The presence of a male preference for long-lived and gravid females suggests that the volume of sperm and other ejaculate components may be a limiting factor in male fitness in the extremely high-density populations. This is particularly likely to be the case if males are mating with a new female every 30 minutes during daylight hours, as found in this study. In addition, it has been suggested that females may complete their reproductive activity in the first half of their lives if oviposition conditions are favourable (Shuker 1998), though the evidence for this is not conclusive. This phenomenon may also explain male reluctance to mate with older females.

There was an obvious difference in the proportion of males successfully rejected by virgin lab-bred females and non-virgin wild females, with lab-bred females successfully rejecting more males. It could be argued, that this difference is not due to lab-bred females being better at rejecting males, but that wild males may be more resistant to the female's struggling behaviours, although this seems unlikely as virgin lab-bred males would be expected to be particularly keen to mate. When the data were examined for any differences in the female rejection responses exhibited by the two types of fly, either in frequency or intensity, no differences were found except in the case of abdomen curling. This behaviour appears to be more prevalently exhibited by virgin lab-bred females than in wild-collected non-virgin females, which may explain the greater proportion of virgin males being successfully rejected. This, in turn, could suggest that abdomen curling is the most effective method of female rejection response and that males that are subjected to abdomen curling, are less resistant overall than males that are not subjected to it i.e. non-virgin males are not subjected to abdomen curling as much as virgin males and therefore appear to resist female rejection with less difficulty. From this observation, it may be suggested that the effect is not due to differences in male resistance ability, but to female rejection response behaviour.

It is interesting to note that there was no difference between the copulation durations of laboratory-reared pairs and wild-collected pairs. In high-density populations, such as those in which the flies collected at St Mary's Island lived, it might be expected that females would accept matings of a longer duration before rejecting the male by struggling. This is because there may be benefits to females associated with carrying a male, such as a reduction in harassment by other males. Though this aspect of coelopid mating interactions has not yet been investigated, it has been found in *Aquarius remegis*, a water strider with a similar mating system to that of *C. frigida*, that females benefit from an increased ability to forage when carrying a male, as a result of a reduction in harassment from other males (Wilcox 1984). The occurrence of female preference for longer copulation duration associated with a high-density population has also been reported in several other species of gerrid (Arnqvist 1992; Rowe 1992; Erlandsson 1992). However, the lack of a difference in copulation duration found in this study of *C. frigida* between flies taken directly from a high-density population and flies bred in the laboratory, suggests that this may not be the case in this species. This finding may be because the benefits to a male from mating with as many different females as possible in a high-density population, where females are an unlimited resource, may outweigh the benefits of increased copulation duration as a form of 'mate guarding'. Indeed, the relative briefness of coelopid copulation durations supports this. However, the fact that laboratory-bred females are found to show struggling behaviour at the point of male dismount more often than in wild-collected females, suggests that laboratory-bred females may prefer copulations of a shorter duration compared to wild-collected females. This result appears to support the concept that females might benefit from longer copulation durations in high-density populations. Until further investigation is carried out into the costs and benefits of longer and shorter copulation durations in *C. frigida*, the reasons behind these findings will remain unresolved.

There was no difference between the willingness to mate of virgin laboratory-bred females and non-virgin wild-collected females, suggesting that willingness to mate does not alter depending on whether the female has been previously mated or not. This supports the results of previous studies in which female mating experience did not appear to influence future female mating behaviour (Shuker 1998). This result was not duplicated for male willingness to mate as virgin males were nearly one and a half times more likely to mount a female than non-virgin males. Increased willingness could be due to the period of sex-deprivation preceding the experiment for virgin males. This conditioning may override

any preferences present in the males, for example, a mating preference for larger, more fecund females (Pitafi et al. 1990; 1995), which would otherwise have an effect on the type of females a male may choose to mate with.

The high level of male mount attempts in wild-collected flies indicates that the 48 hours of sex deprivation carried out as standard practice before mating trials in the laboratory (Day & Gilburn 1997), may not facilitate male mounting behaviour. This period may act only as a maturation phase, as in laboratory mating trials, flies are collected as virgins soon after eclosion and may need some time to become sexually mature. In addition, the differences found between the mating behaviours exhibited by wild and laboratory-bred flies may have some implications for the validity of the results from future laboratory mating trials. In order to get a realistic view of mating interactions in *C. frigida*, it would be valuable to repeat some of the experiments carried out in the past, using wild-caught flies rather than first or second generation laboratory-reared flies. Whether the results of these experiments would differ from those already recorded, may be of great interest.

It is perhaps not surprising that the number of larvae produced by females mated once and females mated with a number of different males should differ, as this has been found to happen in many Dipteran species (reviewed in Ridley 1988). This result suggests that female *C. frigida* may need more than one mating to fertilise their entire egg load. A similar result has been found previously in *C. frigida* (Pitafi 1991). Indeed, several monogamously mated females were seen to lay unfertilised egg clutches, which was not seen in the polyandrous females. Ridley's (1988) comprehensive review of mating frequency and fecundity in 67 species of insect showed that repeated mating increased female fertilisation success in nearly all of the naturally polyandrous species studies. Female *C. frigida* mate many times more than is needed to fertilise their entire egg load, which suggests that multiple-mating in this species has an additional function. A criticism that could be directed at this experiment is that it did not control for any possible karyotype effects, so it could be worthwhile to repeat the experiment with males and females of known karyotypes.

As predicted, females may typically copulate with many hundreds of males in their lifetime within a population of such high density that males have effectively continual

access to females. The actual number of copulations a female might receive in her lifetime may exceed this however, as multiple copulations within a single mount do occur in some instances (Shuker 1998; personal observation). This re-mating frequency is in extreme excess of what is required to fertilise their egg loads, indicating that convenience polyandry is indeed present in this species. This is further compounded by the surprising result that females do not avoid wrack-beds in order to reduce male harassment. When this number of copulations is compared to those seen in other invertebrates (see Table 5.8), it is clear that *C. frigida* is an extraordinarily promiscuous species.

The elevated level of female remating frequency in *C. frigida* also suggests that the mating system may be under 'male control'. Ridley (1990) presents an example of a mating system based on convenience polyandry in the megachilid bee, *Anthidium maculosum.* Females of this species mate with males repeatedly in order to avoid the costs of wasting time that could be spent foraging (Alcock et al. 1977). In addition, copulation duration is comparatively short and this is also thought to be a factor in the control of this mating system. *C. frigida* has a very similar mating system to *A. maculosum*, has a relatively short mating duration and female remating frequencies are very high. These elements combined present strong evidence that the mating system in *C. frigida* is also under male control.

In summary, this series of experiments show that male harassment can result in extreme convenience polyandry in *C. frigida*, whereby females mate many hundreds of times more than is required to assure total fertilisation of their eggs. This, in turn, indicates that there exists huge potential for sperm competition (Parker 1970) and cryptic female choice to operate within this species (Thornhill 1983; Eberhard 1996).

Table 5.8 Lifetime remating frequencies for females measured in different invertebrate species

Species	Female remating frequency	Notes	Source
Coelopa frigida	1090		This study
Yellow dung fly (*Scathophaga stercoraria*)	10 - 12	Thought to mate once per clutch in the wild	Gibbons (1987)
Scorpionfly (*Panorpa vulgaris*)	1.3±0.8 – 5.5±1.2		Sauer et al. (1999)
Monarch butterfly (*Danaus plexippus*)	0 - 15 (mean=6, median=5)		Oberhauser (1997)
Rattlebox moth (*Utetheisa ornatrix*)	[1] Up to 13 times. [2] On average 11 times but can mate up to 23 times	Number of colla in sperm receptacle counted	[1]Lamunyon (1994) [2]Bezzerides & Eisner (2002)
Bumble bee (*Bombus terrestris*)	1	Presence of plug	Schmid-Hempel & Schmid-Hempel (2000)
Honey bee (*Apis mellifera*)	[1]2-9 [2] mean =12.48	Queens: 1-3 mating flights with several drones*	[1]Franck et al. (2002) [2]Strassmann (2001) *Estoup et al. (1994)
Harvester ant spp.	Mean = 6.76		Strassmann (2001)

Table 5.8 continued. Lifetime remating frequencies for females measured in different invertebrate species

Species	Female remating frequency	Notes	Source
Stingless bee spp.	Mean = 1.06		Strassmann (2001)
Polistine wasp spp.	Mean = 1.01		Strassmann (2001)
Vespula spp.	Mean = 3.68		Strassmann (2001)
Clithon retropictus (a freshwater snail)	0 - 91	Number increases with age but can live up to 20 years	Shigemiya & Kato (2001)
Melon fly (*Bactrocera cucurbitae*)	3±1.7 – 5.3±2.9		Miyatake 1996
Screwworm fly (*Cochliomyia hominivorax*)	1	Ensures screwworm eradication program successful	US Embassy, San Jose, Costa Rica
Black Widow spider (*Latrodectus hesperus*)	1	Retains sperm for future egg-laying	Phillips& Comus (1999)
Mosquito (*Anopheles culex*)	1	Stores sperm	Spielman& D'Antonio (2002)

Table 5.8 continued. Lifetime remating frequencies for females measured in different invertebrate species

Species	Female remating frequency	Notes	Source
Tsetse fly (*Glossina* spp)	1	Stores sperm	Kettle (1990)
Olive fruit fly (*Bactrocera oleae*)	'several times'		Christenson et al. (1960)
Blue crab (*Callinectes sapidus*)	1	Called 'terminal mote'	Takass (2000)
Alfalfa leafcutter bee (*Megachile pacifica Panzer*)	1		Hobbs (1967)
Damselfly (*Ischnura graellsii*)	0 – 9		Rivera & Pérez (1998)
Damselfly (*Ischnura pumilio*)	0 - 4		Rivera & Abad (1999)
Decorated crickets (*Gryllodes sigillatus*)	Mate on average 9.2 ± 1.48 times (range=1 –25), with 7.28 ± 0.98 different males (range=1-15)		Sakaluk et al. (2002)

Chapter 6. Mating behaviour and sexual selection in several species of seaweed fly

6.1 Introduction

In addition to *Coelopa frigida*, the mating systems of several other members of the Coelopidae have been studied in the contexts of sexual selection, sexual conflict and mating preferences (Crean & Gilburn 1998; Dunn et al. 1999; Crean et al. 2000; Weall & Gilburn 2000). The species studied to date are *Coelopa nebularum* (Dunn et al. 1999; Crean et al. 2000; Weall & Gilburn 2000), *Coelopa ursina* (Crean & Gilburn 1998; Dunn et al. 1999), *Coelopa pilipes* (Dunn et al. 1999; Crean et al. 2000), *Coelopa vanduzeei* (Dunn et al. 1999; Crean et al. 2000), *Gluma musgravei* and *Gluma nitida* (Crean et al. 2000; Dunn et al. 2001). The mating systems of all of these species are based on sexual conflict, with vigorous pre-mating struggles ensuing once males have mounted. Females show similar behavioural rejection responses to being mounted by males: kicking upwards with the hind legs, shaking the body and curling their abdomens away from the male to prevent genital contact (e.g. Crean & Gilburn 1998; Crean et al. 2000). All *Coelopa* females curl their abdomens downwards (Day et al. 1990; Crean & Gilburn 1998; Crean et al. 2000), with the exception of *C. pilipes*, which curl their abdomens upwards (Crean et al. 2000). Both of the *Gluma* species studied so far appear to be able to curl their abdomens in both directions (Crean et al. 2000). All of these species are sexually dimorphic, with male size being more variable than female size. Mean male size is greater than female size in all species, with the exception of *G. musgravei* (Crean et al. 2000), and there is also a large male mating advantage in all species (Crean & Gilburn 1998; Crean et al. 2000). In addition, female mate choice for large male size has been found in all species, with particularly strong preferences for large males being expressed by the two species of *Gluma* (Crean et al. 2000). It has been suggested that female choice for large males may occur as a side-effect of the evolution of the female rejection response, which may have evolved as a measure to avoid all unnecessary copulations due to the costs associated with mating (Crean & Gilburn 1998). This phenomenon is known as the female reluctance hypothesis (Arnqvist 1992; Rowe et al. 1994). Alternative modes of sexual selection acting in *C. frigida* are discussed in Chapter 2.

Factors affecting male choice and willingness to mate have also been studied in coelopids other than *C. frigida*, though primarily in the *Coelopa* genus (Dunn et al. 1999). A study examining the results of nearly 2000 individual mating trials using *C. ursina*, *C. nebularum*, *C. pilipes* and *C. vanduzeei*, in addition to *C. frigida*, showed that there is no association between male willingness to mate and female size in any of these species (Dunn et al. 1999). The same result was found in *C. frigida* in this thesis (Chapter 4). These results contradict previous findings of male mate choice for large females in *C. frigida* (Pitafi et al. 1990). Dunn and co-workers (1999) also discovered a negative association between male size and willingness to mate in *C. frigida*, *C. nebularum* and *C. ursina*, though this was not present in *C. pilipes* and *C. vanduzeei*. This effect is thought to be as a result of greater intraspecific variation in male size in the former three species (Dunn et al. 1999).

In order to enhance the current data sets gathered on the mating systems of coelopids, the major aim of this study was to make collections and measurements of wild individuals of an additional five coelopid species - *Chaetocoelopa sydneyensis*, *Chaetocoelopa littoralis*, *Gluma keyzeri*, *This canus* and *Amma blanchae* - and observe their mating behaviours. From this data, analyses could then be carried out to determine whether sexual conflict and mate choice exists in these species. Due to the marked effects of sexual size dimorphism, and size in general, on the selective forces operating in the species of coelopids studied so far, it would be extremely interesting and informative to determine whether such forces act in coelopids where there is no sexual size dimorphism. In particular, the potentially monomorphic coelopids, *This canus* and *Amma blanchae*. A further purpose for gathering additional data is the potential for expanding the scope of future research into sexual selection and other areas using these species (see Chapter 7). This is certainly the case in studies involving phylogenetic analyses, where the comprehensiveness of datasets is often of great importance.

6.2 Materials and methods

Observations of wild collected flies were made of the following Australasian species: *Chaetocoleopa littoralis, Chaetocoelopa sydneyensis, Gluma keyzeri, This canus* and *Amma blanchae*.

6.2.1 Choice of study sites

Potential collection sites were identified using information from previous seaweed fly collections made by McAlpine (1991), Blanche (1992) and from personal communications (A. Gilburn and D. Dunn). Many sites visited lacked populations of coelopids. This was particularly the case in Australia, highlighting the ephemeral nature of Australian coelopid populations. The lack of coelopids was due to the scarcity of suitable oviposition substrates. This was partly due to anthropogenic interference in the form of mechanical seaweed-clearing, which occurred on beaches used for recreation.

Location A: Kaikoura, South Island, New Zealand (geographic coordinates: NZ26031, 42° 25' S, 175° 33' E)

Populations of *C. littoralis* were evident at several sites around the Kaikoura peninsula, and collections were made at sites 1 and 2 (see Figure 6.1). Two females of *Baeopterus philpotii* were also collected, but this species was excluded from the study as no additional individuals could be found. Site 1 was a small area of shoreline that was completely covered by deposits of macroalgae, with a depth of no less than 50 cm at any point. The seaweed at the water's edge appeared to have been deposited within only a day or two of the visit to the site. Further up the shore the seaweed was several days old and decomposition was evident. The population density at this site was relatively high in comparison with the other sites that were visited. Site 2 was an unusual site as there was no seaweed present in the immediate vicinity except for living seaweed that was growing in the intertidal zone. In addition to this, the majority of the flies found here were inside a large water drainage pipe located at the very top of the shore. The flies appeared to be at rest and located within the first 5m of the pipe's entrance. The remainder of the flies were sparsely scattered around nearby boulders. The lack of activity may have been due to the low ambient temperature, which did not exceed 9°C during the collections. Mating trials were carried out at the George Knox Research Laboratory within the Edward Percival Field Station, Kaikoura, South Island, New Zealand.

Location B: Forrester's Beach, New South Wales, Australia (geographic coordinates: NSW19186 33° 24' S, 151°28' E) (see Figure 6.2 and Figure 6.4)

T. canus adults were collected at this location in moderate numbers. There were no wrack-beds present at this location, and flies were found inhabiting small pieces of dried seaweed ('wrack strings') widely scattered around the sandy shore, with distances of up to 50 metres between them.

Location C: Asling's Beach, Twofold Bay, New South Wales, Australia (geographic coordinates: NSW17194 37° 03' S, 149° 54' E)(see Figure 6.4)

T. canus were collected at this location in moderate numbers. There were no wrack-beds present, and flies were found inhabiting 'wrack strings' sparsely scattered along the sandy shore. The seaweed on this shore had been mechanically cleared several days previously.

Location D: Quarantine Bay, New South Wales, Australia (geographic coordinates: NSW47849 37° 04' S, 149° 53' E)(see Figure 6.3 and Figure 6.4)

This location was approximately 5 km south of location C. Two species of seaweed fly were collected from this location: *Amma blanchae* and *Chaetocoelopa sydneyensis*. The shoreline here was a mixture of rock and sand with small areas of wrack-bed present, comprised mainly of brown macro-algae. When disturbed, many *C. sydneyensis* adults became airborne in a swarm, but settled on the kelp again within 30 seconds. *A. blanchae* adults were found in moderate numbers, scattered around the rocks near the upper edge of the shore. The population appeared to be relatively fragmented and sparsely distributed.

Location E: Victor Harbour, South Australia, Australia (geographic coordinates: SA68562 35° 33' S, 138° 37' E)(see Figure 6.4)

Adults of *C. sydneyensis* and *Gluma keyzeri* were collected at this location. The shoreline here was a mixture of sand and gravel, with a relatively large amount of macroalgae deposited on the strandline. Collection was slightly hampered due to strong winds and rain.

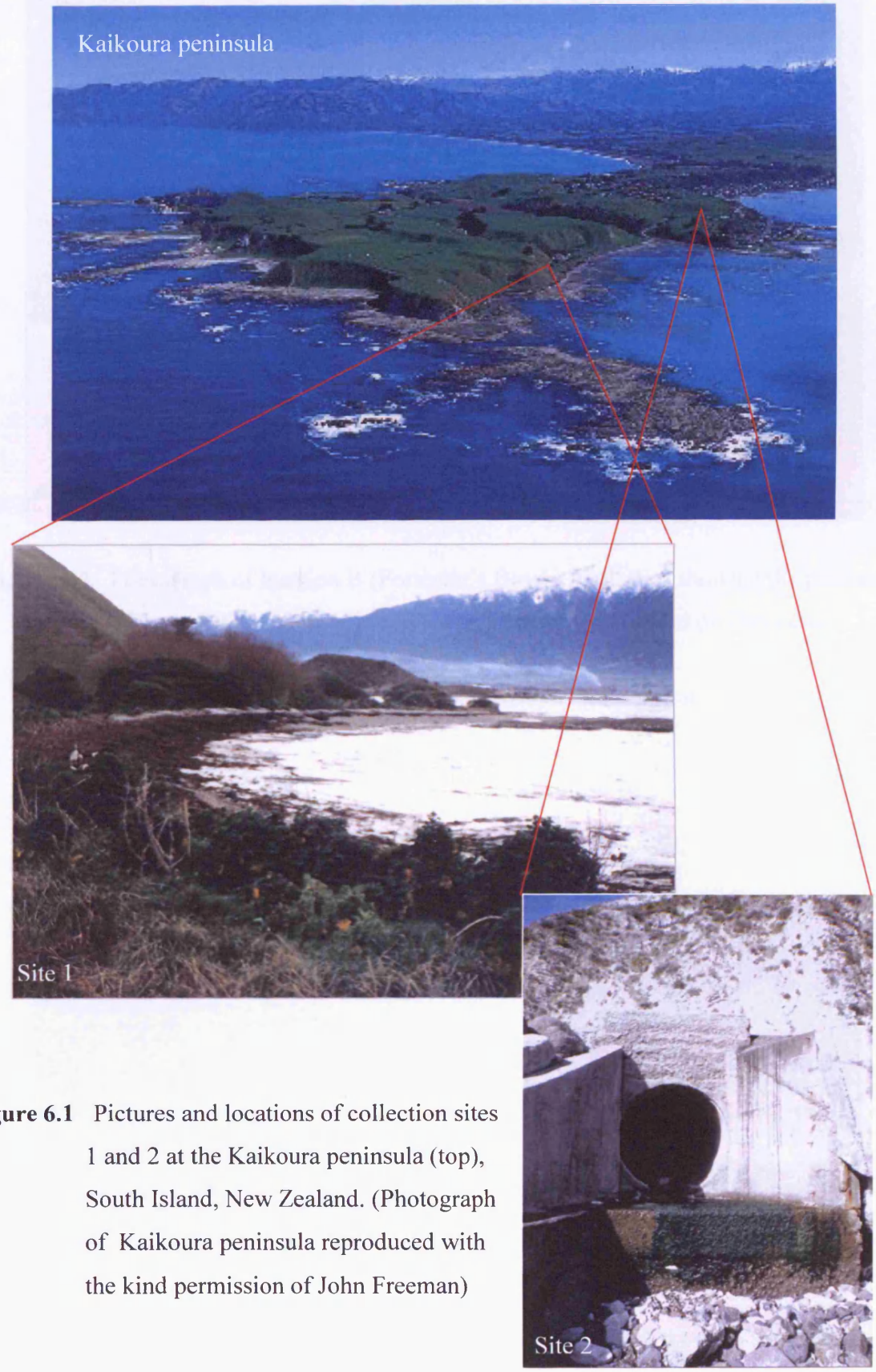


Figure 6.1 Pictures and locations of collection sites 1 and 2 at the Kaikoura peninsula (top), South Island, New Zealand. (Photograph of Kaikoura peninsula reproduced with the kind permission of John Freeman)

Figure 6.2 Photograph of location B (Forrester's Beach, Australia) showing the presence of the small pieces of seaweed ('wrack strings') inhabited by *This canus*.

Figure 6.3 Photograph of location D (Quarantine Bay, Australia) showing the mixture of rock and sand that makes up the shore.

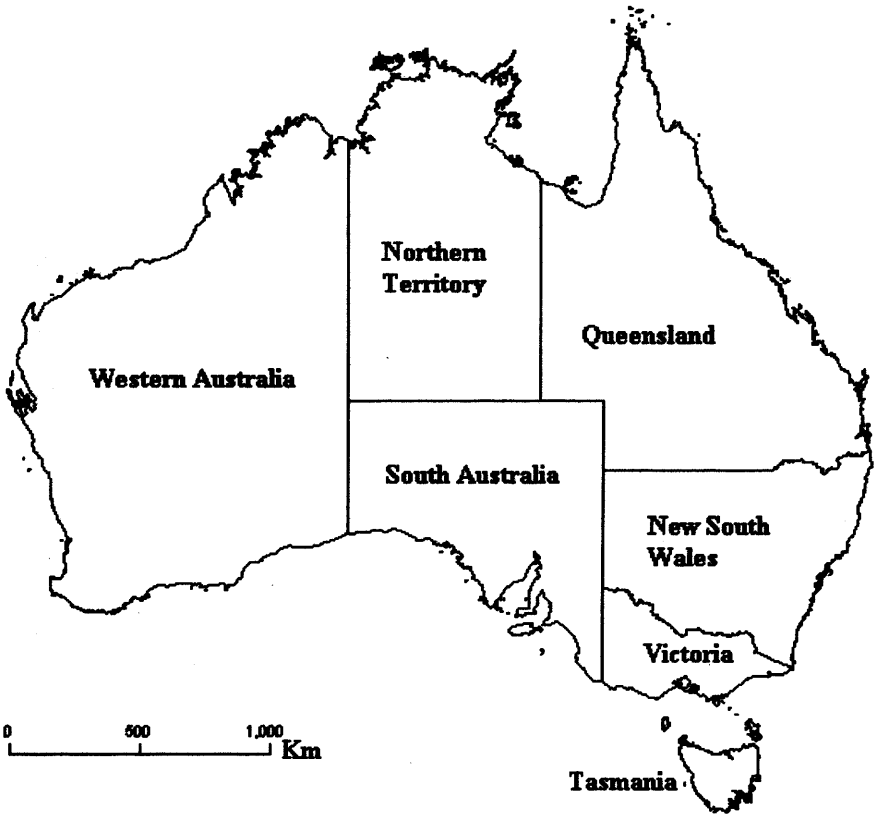


Figure 6.4 Map of Australia showing the locations of collection sites B, C, D and E.

- Location B – Forrester's Beach, New South Wales, Australia
- Locations C and D – Asling's Beach, Twofold Bay and Quarantine Bay, New South Wales, Australia
- Location E – Victor Harbour, South Australia, Australia

6.2.2 Identification of species

New Zealand species were identified using several sources (Harrison 1959; Wise 1973; Winterbottom & Gregson 1981; McAlpine 1991). Prior to the collection of the Australian species, a visit was made to the Entomology Department at the Australian Museum in Sydney, where D. McAlpine provided an extensive collection of mounted specimens for examination and measurement. This examination confirmed the identification of the New Zealand species was correct.

6.2.3 Collection method, measurement and storage of flies

Adult flies of the following species were collected using the revised kick-sampling technique outlined in Chapter 5: *Amma blanchae*, *This canus*, *Chaetocoelopa sydneyensis*, *Chaetocoleopa littoralis* and *Gluma keyzeri*. There was one exception to this at site 1, location A (Kaikoura peninsula), where the flies were collected by mouth aspirator. Additional *T. canus* were collected from location B (Forrester's Beach), by D. McAlpine. These flies were added to those collected by kick-sampling.

Flies were sorted according to sex immediately after collection and put into plastic collection bottles sealed with foam bungs. Masking tape was used to secure the bungs in place for transit. A small ball of cotton wool soaked in 5% sucrose solution was added to the bottles. Mating trials were carried out within 12 hours of collection. Once the flies had been used in mate trials they were frozen, and their wing-lengths measured using digital callipers (see Chapter 3).

6.2.4 Mating trials

Standard mating trials were carried out on all five species (see Chapter 3). However, the trials carried out using *T. canus* and *A. blanchae* were continued for 10 minutes rather than the standard 5 minutes. This was due to the reluctance of the males of these species to mount the females within the five-minute observation period. However, the time taken by the male to mount the female in these species was only recorded for the first 5 minutes of the trial. This ensured consistency with the other species. The males were not kept for the standard 48 hours of sex deprivation in the presence of seaweed prior to the trials. This was

due to difficulties in acquiring fresh seaweed and storing the flies in the field. Instead, males and females were put into same-sex communal bottles with 5 % sucrose solution until trials could be carried out (a maximum of 24 hours).

The behavioural variables measured using *C. littoralis*, *C. sydneyensis* and *G. keyzeri* were:

① Whether the male mounted the female or not within the 5 minute observation period
② Time taken for male to mount the female
③ Total duration of mount
④ Trial outcome: (i) male successfully rejected by the female; (ii) male rejects female or (iii) mount results in copulation
⑤ Total struggle duration
⑥ Female rejection responses and their durations
⑦ Total copulation duration
⑧ Whether the female exhibited struggling behaviour at the point of male dismount

The behavioural variables measured using *T. canus* and *A. blanchae* were as above, with the exception that the 'total struggle duration' measurement was deemed to be inappropriate, as the females did not show continuous struggling behaviour before the result of the mount was resolved. This term was replaced by 'period before mount outcome' and was a measurement of the time taken between the male mounting the female and the point at which copulation began or the male dismounted. The total duration of kicking behaviour was noted however, and this included any kicking that took place during copulation.

6.2.5 Statistical analyses and transformations

The continuous variables in all of the data sets were logarithmically transformed to normalize their distributions prior to use in parametric analyses. This procedure follows earlier work on similar variables (Dunn et al. 1999).

6.3 Results

6.3.1 Descriptions of mating behaviour

Chaetocoelopa littoralis

In *C. littoralis*, the male approaches the female and mounts with no preliminary courtship behaviour. The female, in most cases, then shows a combination of three main rejection responses: (1) the female curls her abdomen upwards to prevent the male making genital contact; (2) the female curls her abdomen downwards to prevent the male making genital contact; and (3) the female kicks the male using her hind legs. The female usually curls her abdomen downwards briefly in the first instance, and then curls her abdomen upwards. This upward motion appears to be the predominant behaviour, although the angle of the upwards position is less extreme than that seen in *C. sydneyensis* (see below). Brief periods of kicking are interspersed throughout the struggle, with momentary episodes of downward abdomen curling. Premating struggles end in three ways: (1) the female successfully rejects the male; (2) the pair copulate; or (3) the male rejects the female and voluntarily dismounts. Females occasionally struggle after copulation has occurred, during which the male dismounts

Chaetocoelopa sydneyensis

This species appeared to have very similar mating behaviour to that of *C. littoralis*, with one main exception – none of the females observed displayed any downwards curling of the abdomen. Instead, there were only two main rejection responses exhibited by the females: (1) severe upward curling of the abdomen, sometimes even the entire body, almost like a 'headstand', whereby the hind legs were completely straight; and (2) kicking of the male using the hind legs. Females immediately curled their abdomens upwards in response to being mounted by a male, and showed brief periods of kicking, while in this posture. Females of this species also exhibited some struggling behaviour, in the form of upwards abdomen curling, after copulation had occurred, which usually coincided with the males' dismount.

Gluma keyzeri

G. keyzeri males also mounted females with no preliminary courtship behaviour. Again, females usually attempted to reject the male by exhibiting four main rejection responses: (1) vigorous shaking of the body; (2) upward abdomen curling; (3) downward abdomen curling; and (4) kicking the male with the hind legs. Females usually performed all four of the rejection responses within a single mount episode. Female *G. keyzeri* occasionally showed rejection responses after copulation.

Amma blanchae

A. blanchae displayed markedly different mating behaviour compared to the previous species discussed here. Though there was no preliminary courtship display prior to the male mounting the female, once the female was mounted, there was very little struggling behaviour evident, except for sporadic kicking of the male with the hind legs. This appeared to be an ineffective rejection response unless the male attempting to copulate with the female was particularly small. Smaller males appeared to be rejected relatively quickly, usually within approximately 30 seconds of mounting. If the male successfully withstood this period of kicking, copulation seemed to occur in almost every case. Females sometimes kicked the male during copulation, though this did not appear to disturb the male or disrupt copulation. Females exhibited no rejection responses at the point at which the male dismounted. The position of the male while mounted on the female appeared to be different from other coelopid species studied (with the exception of *T. canus*). The males' position was relatively far back on the female, with the prothoracic legs balanced on the thorax of the female rather than on the front of the head near the antennae (see Figure 6.5), as found in other species (Day et al. 1990).

This canus

T. canus appeared to have very similar mating behaviour to *A. blanchae*.

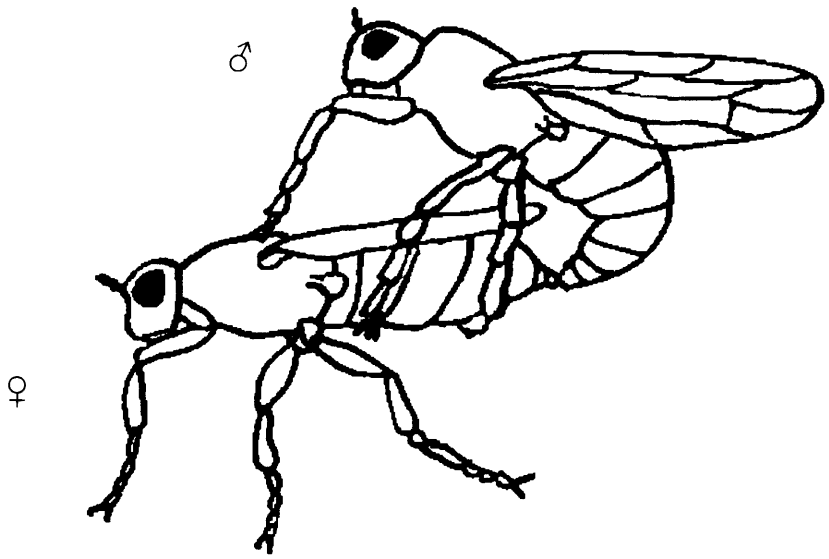


Figure 6.5 Diagram (lateral view) of the relative positions of male and female *A. blanchae* (and *T. canus*) during copulation. The male's prothoracic legs are located in the region of the female's thorax. In the case of smaller males, the legs often do not touch the surface the female is on during the mount (Not to scale).

6.3.2 Descriptive statistics

Adult size

The mean wing lengths of males and females, and their associated variables, for all five species are presented in Table 6.1. Males were typically larger than females in all species except *T. canus* and *A. blanchae*, where the mean female size was larger. The sexual size dimorphism of each species was found to be significant in all species with the exception of *A. blanchae*. Female size was more variable than male size in all species except *G. keyzeri*, where male size was more variable.

Mating interactions

The mean durations of mating behaviours for each species can be seen in Table 6.2. The percentage of trials having a particular outcome, for each species, is presented in Figure 6.6. The number of trials in which the male mounted the female, for each species, is as follows: *C. littoralis* = 24 out of 81 trials (29.63%); *C. sydneyensis* = 70 out of 161 trials (43.48%); *G. keyzeri* = 12 out of 21 trials (57.14%); *T. canus* = 8 out of 124 trials (6.45%); and *A. blanchae* = 7 out of 63 trials (11.11%).

The number of trials in which the female exhibited struggling behaviour at the point of male dismount is as follows: *C. littoralis* = 7 out of 24 trials (29.17%); *C. sydneyensis* = 24 out of 52 trials (46.15%); and *G. keyzeri* = 7 out of 12 (58.33%). *A. blanchae* and *T. canus* females showed no struggling behaviour at the point of male dismount.

6.3.3 Effect of size on mating interactions

Time taken to mount

Regression analyses showed that the time taken for a male to mount a female was not significantly associated with male or female size in any of the species studied (see Table 6.3).

Total mount duration

Regression analyses also revealed a significant positive association between male and female size and total mount duration for all species, with the exception of male size for *T. canus* and *A. blanchae*, which showed no significant association with total mount duration (see Table 6.4).

Total struggle duration

Further regression analyses showed that there was no significant association between male and female size and total struggle duration in any of the five species studied (see Table 6.5).

(i) Upwards abdomen curling duration

No significant association was found between male size and upwards abdomen curling duration in *C. littoralis* (regression analysis, $F_{1,13} = 1.96, p = 0.187$); *C. sydneyensis* (regression analysis, $F_{1,51} = 1.92, p = 0.171$) or *G. keyzeri* (regression analysis, $F_{1,9} = 0.500$, $p = 0.500$). Additional regression analyses revealed that this was also the case for female size (*C. littoralis*, $F_{1,13} = 0.021, p = 0.880$; *C. sydneyensis*, $F_{1,49} = 0.064, p = 0.807$; and *G. keyzeri*, $F_{1,9} = 0.135, p = 0.725$).

(ii) Downwards abdomen curling duration

In *G. keyzeri*, the was no significant association found between male and female size, and downwards abdomen curling duration (regression analyses; male size, $F_{1,5} = 0.564, p = 0.495$; female size, $F_{1,5} = 1.397, p = 0.304$). Female size was found to have no significant association with downwards abdomen curling duration in *C. littoralis* (regression analysis, $F_{1,11} = 1.998$, $p = 0.189$), however a trend towards a significant positive association was apparent between male size and downwards abdomen curling duration (regression analysis, $F_{1,11} = 4.67, p = 0.056$).

(iii) Kicking duration

No significant associations were found between male and female size, and kicking duration in any of the species with the exception of male size in *C. sydneyensis*, where there was a positive association with kicking duration (see Table 6.6).

(iv) Shaking duration

G. keyzeri was the only species that exhibited shaking behaviour as a rejection response. The duration of this behaviour was found to have no significant association with male or female size (regression analyses; male size, $F_{1,7} = 0.074$, $p = 0.795$; and female, $F_{1,7} = 0.011, p = 0.919$).

Copulation duration

None of the species studied showed a significant association between male and female size and copulation duration (see Table 6.7).

6.3.4 Adult size and sexual selection

Male willingness to mate

Binary logistic regression analyses revealed a highly significant negative association between the willingness of males to mount females and male size, and a highly significant positive association with female size, in most of the study species (see Table 6.8). There were a number of exceptions however - male size was found to have no significant association with male willingness to mate in *T. canus*, and female size was found to have no significant association with male willingness to mate in *T. canus* and *C. littoralis*.

Mating success

There was a large male mating advantage in all species of coelopids in the study apart from *T. canus* (see Table 6.9). Male mating success was positively associated with female size in all species except in the case of the two *Chaetocoleopa* species.

6.3.5 Mount outcomes

Male successfully rejected by female

Binary logistic regression analyses revealed that females successfully rejected larger males less often than smaller males in *C. sydneyensis*, *A. blanchae* and *T. canus*. Further analyses also indicated that larger females, of the species *C. littoralis*, *A. blanchae* and *T. canus*, were more successful at rejecting males than smaller females (see Table 6.10).

Male rejection of female

There was no significant association found between male size and female rejection by males in the study species, with the exception of *C. littoralis* and *G. keyzeri*, in which

larger males appeared to reject females less often than smaller males. There was a strong significant negative association between female size and the number of mounts resulting in the male rejecting the female in all of the species (see Table 6.10).

6.3.6 Female struggling at point of male dismount

A significant negative association between male size and whether the female struggled at the point of male dismount was found in *C. sydneyensis*. Further analyses showed a significant positive association between female size and whether the female struggled at the point of male dismount in *C. littoralis*. No other significant associations were found between male or female size and this variable in any of the remaining species (see Table 6.11).

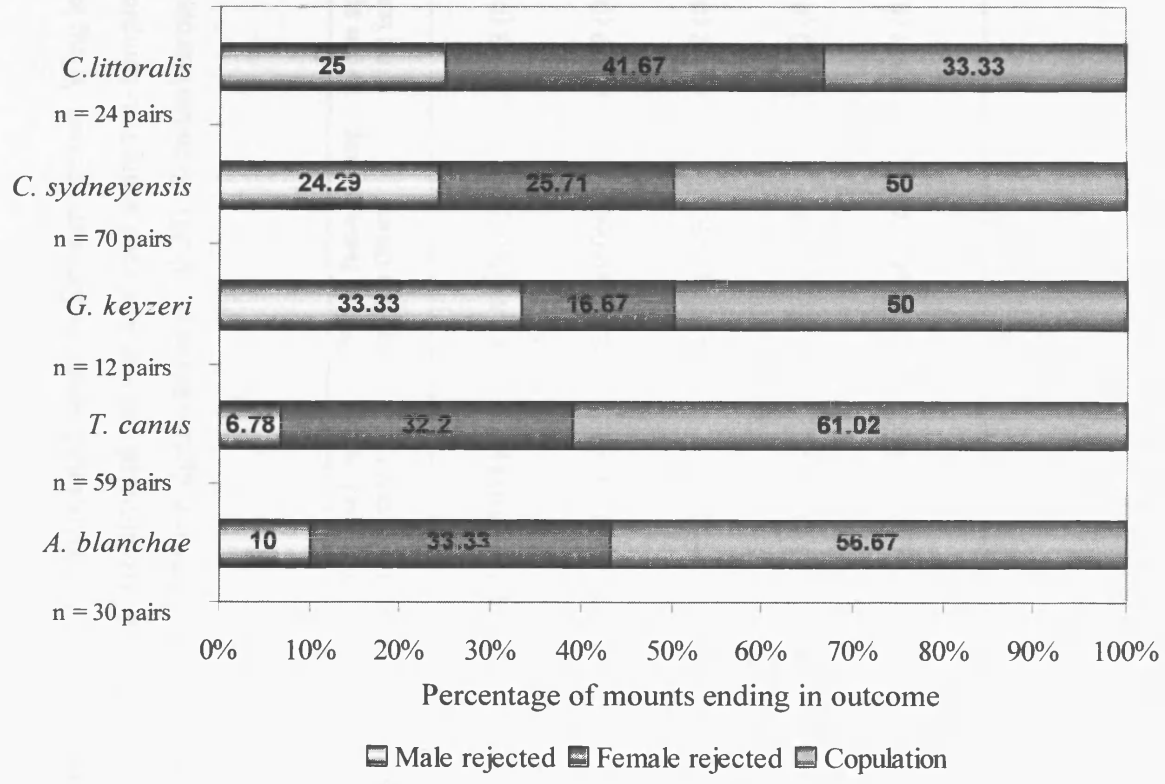


Figure 6.6 Percentage of mounts ending in each outcome, for each of the five species (bar labels are given in percentages).

Table 6.1 Mean wing lengths and associated variables for each species. Wing length ratio was calculated by dividing male wing length female wing length. Sexual size dimorphism was analysed using two-samples t-tests carried out on logarithmically transformed wing length data. Results marked *** are significant at $p < 0.001$. Standard errors are given in parentheses.

Species	Mean male wing length (mm)	Mean female wing length (mm)	Mean species wing length (mm)	Wing length ratio	CV male wing length (%)	CV female wing length (%)	Sexual size dimorphism
C. littoralis (n = 81♂; 81♀)	6.700 (0.074)	4.247 (0.085)	5.473 (0.122)	1.577	9.953	18.007	T = 21.100***
C. sydneyensis (n = 161♂; 157♀)	6.402 (0.057)	5.678 (0.060)	6.039 (0.045)	1.127	11.257	13.126	T = 8.660***
G. keyzeri (n = 21♂; 21♀)	4.939 (0.158)	4.046 (0.082)	4.492 (0.112)	1.220	14.679	9.231	T = 4.920***
T. canus (n = 123♂; 123♀)	2.313 (0.037)	3.227 (0.065)	2.770 (0.047)	0.716	17.757	22.231	T = -11.620***
A. blanchae (n = 63♂; 61♀)	2.484 (0.052)	2.608 (0.056)	2.544 (0.038)	0.952	16.452	16.627	T = -1.520, $p = 0.132$

Table 6.2 Mean values of mating behaviours for each species. All times are in seconds. * In the case of *T. canus* and *A. blanchae*, kicking duration is analogous with struggle duration, as this was the only rejection response exhibited by females (n = number of trials).

	C. littoralis		*C. sydneyensis*		*G. keyzeri*		*T. canus*		*A. blanchae*	
Behaviour	**Mean (SE)**	**n**	**Mean (SE)**	**n**	**Mean (SE)**	**n**	**Mean (SE)**	**n**	**Mean (SE)**	**n**
Time to mount	145.2 (16.7)	24	182.6 (9.1)	70	199.5 (20.5)	12	274.0 (6.8)	59	261.0 (14.4)	7
Total mount duration	125.5 (30.5)	24	165.1 (16.2)	70	229.0 (49.3)	12	91.9 (10.5)	59	114.5 (17.7)	30
Total struggle duration*	119.1 (22.7)	14	107.3 (7.1)	52	228.8 (38.6)	10	20.7 (2.0)	40	23.9 (2.5)	20
Struggle duration accept	143.0 (32.1)	8	109.7 (9.3)	35	281.0 (38.2)	6	20.9 (2.0)	36	23.6 (2.7)	17
Struggle duration reject	87.2 (29.0)	6	102.6 (10.4)	17	150.5 (64.4)	4	18.75 (9.6)	4	25.7 (7.8)	3
Rejection behaviour duration										
(i) abdomen-curl up	110.9 (21.9)	14	135.0 (32.8)	52	126.1 (23.9)	10				
(ii) abdomen-curl down	8.3 (1.9)	14			18.1 (6.5)	10				
(iii) kicking	6.0 (1.3)	14	17.4 (1.9)	52	13.1 (2.1)	10	20.7 (2.0)	40	23.9 (2.5)	20
(iv) shaking					22.3 (7.5)	9				
Copulation duration	156.3 (25.9)	8	167.8 (10.7)	35	215.0 (25.2)	6	128.4 (8.6)	36	154.6 (15.7)	17

Table 6.3 Results of regression analyses of male and female size on time taken to mount for each species (d.f. = 1, n = number of trials).

	Male				Female			
Species	**n**	**Regression coefficient (S.E.)**	**F**	***p***	**n**	**Regression coefficient (S.E.)**	**F**	***p***
C. littoralis	23	0.580 (1.073)	0.287	0.597	23	-0.045 (1.447)	0.148	0.704
C. sydneyensis	69	0.296 (0.525)	0.194	0.661	67	0.859 (0.556)	2.380	0.154
G. keyzeri	11	-0.431 (1.289)	0.215	0.653	11	-3.442 (2.286)	2.305	0.160
A. blanchae	5	1.067 (0.229)	0.340	0.584	6	-0.438 (0.950)	0.210	0.664
T. canus	7	0.074 (0.136)	0.290	0.607	7	0.019 (0.072)	0.070	0.798

Table 6.4 Results of regression analyses of male and female size on total mount duration for each species (d.f. = 1, n = number of trials).

	Male				Female			
Species	**n**	**Regression coefficient (S.E.)**	**F**	***p***	**n**	**Regression coefficient (S.E.)**	**F**	***p***
C. littoralis	23	9.223 (2.574)	12.844	< 0.05	23	5.872 (1.236)	22.531	< 0.001
C. sydneyensis	69	4.327 (1.638)	6.945	< 0.05	67	4.643 (1.765)	6.942	< 0.05
G. keyzeri	9	12.439 (4.025)	0.007	< 0.05	9	21.384 (8.646)	0.112	< 0.05
A. blanchae	29	1.241 (1.787)	0.482	0.493	29	7.027 (1.236)	32.338	< 0.001

Table 6.5 Results of regression analyses of male and female size on total struggle duration for each species. * In the case of *A. blanchae* and *T. canus*, total struggle duration was analogous with total kicking duration, since this was the only struggling behaviour exhibited by these species (d.f. = 1, n = number of trials).

	Male				Female			
Species	**n**	**Regression coefficient (S.E.)**	**F**	***p***	**n**	**Regression coefficient (S.E.)**	**F**	***p***
C. littoralis	13	-2.651 (1.619)	2.680	0.127	13	-0.449 (1.447)	0.101	0.762
C. sydneyensis	51	0.673 (0.689)	0.953	0.334	49	-0.526 (1.250)	0.185	0.676
G. keyzeri	9	0.860 (4.077)	0.049	0.838	9	2.541 (7.594)	0.113	0.747
*A. blanchae**	19	-0.154 (0.446)	0.129	0.734	19	1.884 (1.478)	1.630	0.218
*T. canus**	39	-0.766 (0.848)	0.824	0.372	39	0.605 (1.100)	0.300	0.586

Table 6.6 Results of regression analyses of male and female size on kicking duration for each species (d.f. = 1, n = number of trials).

	Male				Female			
Species	**n**	**Regression coefficient (S.E.)**	**F**	***p***	**n**	**Regression coefficient (S.E.)**	**F**	***p***
C. littoralis	9	-0.668 (0.946)	0.501	0.500	9	-0.131 (0.723)	0.030	0.861
C. sydneyensis	46	1.908 (0.837)	5.200	< 0.05	44	0.004 (1.537)	0.002	0.988
G. keyzeri	8	0.544 (1.538)	0.136	0.734	8	3.414 (2.820)	1.477	0.265
A. blanchae	19	-0.154 (0.446)	0.129	0.734	19	1.884 (1.478)	1.630	0.218
T. canus	39	-0.766 (0.848)	0.824	0.372	39	0.605 (1.100)	0.300	0.586

Table 6.7 Results of regression analyses of male and female size on copulation duration for each species (d.f. = 1, n = number of trials).

	Male				Female			
Species	**n**	**Regression coefficient (S.E.)**	**F**	***p***	**n**	**Regression coefficient (S.E.)**	**F**	***p***
C. littoralis	7	-0.559 (1.436)	0.154	0.710	7	1.583 (0.750)	4.450	0.079
C. sydneyensis	34	0.210 (0.916)	0.050	0.820	32	-1.474 (1.158)	1.620	0.212
G. keyzeri	5	2.167 (1.763)	1.514	0.286	5	-4.937 (3.396)	2.110	0.220
A. blanchae	16	-3.331 (2.387)	1.955	0.183	16	-0.026 (2.145)	0.001	0.990
T. canus	35	0.308 (0.649)	0.236	0.638	35	1.261 (0.801)	2.480	0.125

Table 6.8 Results of binary logistic regression analyses of male and female size on whether the male mounted the female or not (an indicator of male willingness to mate) within the trial period. (d.f. = 1, n = number of trials)

	Male				Female			
Species	**n**	**Regression coefficient (S.E.)**	χ^2_1	*p*	**n**	**Regression coefficient (S.E)**	χ^2_1	*p*
C. littoralis	81	-14.649 (6.141)	6.190	< 0.05	81	2.776 (3.253)	0.728	0.394
C. sydneyensis	161	-8.694 (3.325)	7.245	< 0.05	161	21.522 (3.949)	41.499	< 0.001
G. keyzeri	21	-3240.457 (2707.340)	28.682	< 0.001	21	100.255 (47.910)	14.204	< 0.001
A. blanchae	63	-29.441 (15.160)	7.196	< 0.05	63	15.823 (8.235)	5.076	< 0.05
T. canus	124	-7.337 (4.643)	2.598	0.107	124	-0.599 (3.362)	0.031	0.860

Table 6.9 Results of binary logistic regression analyses of male and female size on whether copulation was the mating outcome (d.f. = 1).

	Male				Female			
Species	**n**	**Regression coefficient**	χ^2_1	*p*	**n**	**Regression coefficient**	χ^2_1	*p*
C. littoralis	24	16.619 (14.322)	4.926	< 0.05	24	7.040 (4.429)	2.840	0.092
C. sydneyensis	70	26.589 (6.802)	24.631	< 0.001	70	7.685 (9.115)	1.788	0.181
G. keyzeri	21	50.601 (27.787)	6.436	< 0.05	21	67.290 (39.578)	4.316	< 0.05
A. blanchae	30	61.755 (24.992)	16.207	< 0.001	30	18.524 (8.136)	9.637	< 0.05
T. canus	59	8.141 (4.564)	3.489	0.062	59	15.008 (3.472)	35.544	< 0.001

Table 6.10 Results of binary logistic regression analyses of male and female size on mount outcomes. Results in black type are the mounts that ended in the male being successfully rejected by the female. Results in **bold** type are mounts whereby the male rejected the female then dismounted (d.f. = 1, n = number of trials).

	Male				Female			
Species	**n**	**Regression coefficient**	χ^2_1	*p*	**n**	**Regression coefficient**	χ^2_1	*p*
C. littoralis	24	5.318 (7.623)	0.479	0.489	24	15.447 (7.208)	7.731	< 0.05
	24	**-38.543 (21.433)**	**10.037**	**< 0.05**	**24**	**-28.948 (11.583)**	**19.376**	**< 0.001**
C. sydneyensis	70	-22.891 (6.507)	17.298	< 0.001	70	8.507 (6.711)	1.952	0.152
	70	**-5.960 (4.726)**	**1.612**	**0.204**	**70**	**-12.881 (5.133)**	**7.029**	**< 0.05**
G. keyzeri	12	-6.435 (14.047)	0.212	0.645	12	-5.419 (27.326)	0.039	0.843
	12	**-87.589 (54.059)**	**10.813**	**< 0.05**	**12**	**-94.592 (86.978)**	**10.813**	**< 0.05**
A. blanchae	30	-235.699 (60.099)	19.505	< 0.001	30	120.890 (76.297)	13.898	< 0.001
	30	**1.062 (4.637)**	**0.054**	**0.816**	**30**	**-13.380 (73.131)**	**31.576**	**< 0.001**
T. canus	59	-80.906 (36.509)	17.546	< 0.001	59	581.506 (639.477)	24.847	< 0.001
	59	**-0.717 (4.327)**	**0.028**	**0.868**	**59**	**-67.857 (54.872)**	**69.662**	**< 0.001**

Table 6.11 Results of binary logistic regression analyses of male and female size on the presence of female struggling behaviour at the point of male dismount (d.f. = 1, n = number of trials).

	Male				Female			
Species	**n**	**Regression coefficient**	χ^2_1	***p***	**n**	**Regression coefficient**	χ^2_1	***p***
C. littoralis	24	8.342 (7.393)	1.285	0.257	24	9.770 (5.112)	4.564	< 0.05
C. sydneyensis	52	-20.463 (6.793)	12.346	< 0.001	52	7.685 (9.115)	0.723	0.395
G. keyzeri	12	1.776 (13.407)	0.018	0.895	12	23.540 (27.867)	0.762	0.383

6.4 Discussion

This study is the first to describe the mating behaviours of five members of the Coelopidae not yet examined in terms of mating interactions. There were no obvious pre-mating courtship behaviours seen in any of the species, consistent with all other coelopids studied to date (e.g. Day et al. 1990; Crean & Gilburn 1998). In all of the species, except for *T. canus* and *A. blanchae*, mounting generally elicited an immediate rejection response by females, suggesting that the mating systems of these species is based upon sexual conflict (Day & Gilburn 1997; Crean & Gilburn 1998). As anticipated, *G. keyzeri* had a very similar mating system to that described in other *Gluma* species (Crean et al. 2000), with both upwards and downwards abdomen curling being used as a female rejection response, along with shaking and kicking behaviours. Interestingly, *C. littoralis* showed similar rejection responses to those shown by the *Gluma* species, though females did not exhibit any shaking behaviour, only abdomen curling (in both directions) and kicking. The behaviour of *C. sydneyensis*, though similar to *C. littoralis*, also showed parallels with that of *C. pilipes*, with both of these species demonstrating upwards abdomen curling only, which was particularly extreme in *C. sydneyensis*. The similarity of the female rejection behaviours in the *Chaetocoelopa* and *Gluma* species is perhaps not surprising, since they are thought to be more closely related to each other than both species are to *Coelopa* species (McAlpine 1991; Meier & Wiegmann 2002). The mating behaviour of *T. canus* and *A. blanchae* was considerably different to that of any of the other coelopid species. Females showed very little struggling in response to being mounted, with only occasional bouts of kicking behaviour, which appeared to be ineffective at removing males unless they were particularly small. This lack of a vigorous pre-mating struggle suggests that sexual conflict may not operate so obviously in these species. The position of the male while mounted was also different to that seen in other coelopids – males appeared to be positioned further back on females, with the prothoracic legs resting on the females' thorax rather than on the antennae area, as seen in *C. frigida* (Day et al. 1990). This has interesting implications, since females may receive stimuli from males through their antennae, which are rubbed by males' prothoracic legs during mounting and copulation, as is seen in *C. frigida* (Day et al. 1990). The exact function of this stimulation is unknown.

Sexual dimorphism was apparent in all of the species studied, except for *A. blanchae*, the first species of coelopid found to be sexually monomorphic. Males were on

average larger than females in all species apart from *T. canus* and *A. blanchae*, where females were generally larger or of the same size as males. Contrary to previous findings in other coelopids (e.g. Crean et al. 2000), female size was more variable than male size in all of the species studied except *G. keyzeri*. A possible explanation for this is some kind of sampling bias during collection, as this study only measured the wing lengths of wild-caught adult flies. It may be useful to rear wild-collected larvae of these species to determine whether the relative variability in size shown by the subsequent adults produced concurs with the findings of this study.

The analyses of the data gathered during this study reveal both intriguing and, at times, confusing results. Male size was found to be negatively associated with male willingness to mate (as measured by male willingness to mount (e.g. Dunn et al. 1999)) in all species, with the exception of *T. canus*. However, males appeared to show a preference for large females in all but two of the species (*T. canus* and *C. littoralis*), which corresponds to one previous study of *C. frigida* (Pitafi et al. 1990), but is completely at odds with a major study carried out on five species of *Coelopa* which found no association between female size and willingness of males to mate (Dunn et al. 1999). The results of the current study suggest that the males of some coelopid species may exhibit mate choice for large female size, which is known to be positively associated with fecundity (Butlin & Day 1985b). It may be that in populations subject to disturbance and a frequent lack of suitable oviposition substrates, males may exercise choice for more fecund females to enhance their reproductive success when oviposition substrates are available. This may be a legitimate reason for the findings of this study, as the species exhibiting male choice for larger females were found on shores where suitable sources of seaweed for oviposition were ephemeral and scattered. A large male mating advantage was found in all of the species studied, except in *T. canus*, which has also been found previously in at least six other coelopids (e.g. Gilburn et al. 1992; Crean et al. 2000). Male mating success was found to be positively associated with female size in all of the species, with the exception of the two *Chaetocoelopa* species. Though this finding does not appear to fit with the female reluctance hypothesis, the presence of a large male mating advantage found in several of these species may help to explain this result (Crean et al. 2000). Associations found between mating success and female size may also be obscured by variation in size. These results may therefore be tentatively interpreted as being consistent with the female

reluctance hypothesis, whereby the large male mating advantage is a side effect of selection to reduce female willingness to mate (Arnqvist 1992; Rowe et al. 1994).

Larger females were found to be more successful at rejecting males in *C. littoralis*, *A. blanchae* and *T. canus*. This has also been found in a previous study of *C. ursina* (Crean & Gilburn 1998), though no such association has been found in *C. frigida* (Chapter 4). The ability of larger females to successfully reject males in *A. blanchae* and *T. canus* is perhaps not an unexpected result, as females are generally of the same size as males, if not larger. In addition, the females of these species appeared to be able to successfully reject smaller males only. This association was also found in *C. littoralis*, though not in the other species studied. This is somewhat surprising as a strong significant negative association between male size and the frequency of males being kicked off by females has previously been found in a number of coelopids, including *C. ursina* (Crean & Gilburn 1998) and *C. frigida* (Chapter 4). This may suggest that sexual selection favouring large male size has occurred as a means to overcome female rejection responses. However, studies of *C. nebularum* have found that females take longer to reject larger males than smaller males, supporting the idea that large male size confers an advantage in being able to withstand female rejection responses for longer (Crean et al. 2000; Weall & Gilburn 2000), but no association was found between struggling duration and male or female size in any of the species studied here.

The manner of rejection response exhibited by females was not associated with male or female size in any of the species, though females show a trend towards abdomen curling for longer with larger males in *C. littoralis*, and kick for longer with larger males in *C. sydneyensis*. Previous studies of female rejection responses have found that females may be more likely to adopt an abdomen curling response with larger males (Chapter 4; Weall & Gilburn 2000), and a kicking response with smaller males (Weall & Gilburn 2000). If females tailor their rejection responses depending on the type of male that has mounted, is seems unlikely that females are attempting to in some way assess the male, as females would be predicted to perform a standard rejection response with all males irrespective of their size. Instead, it would suggest that females use rejection responses simply as a method of rejecting any male that mounts. The results of this study are therefore inconclusive in this aspect and do not appear to point obviously towards one strategy or the other.

An unexpected association between male and female size and copulation duration was found in all species, with the exception of *T. canus* and *A. blanchae* whereby an association between copulation duration and female size only was found. These results are in contrast with those found in *C. nebularum* (Weall & Gilburn 2000) and *C. frigida* (Chapter 4), where copulation duration was independent of male or female size. If males and females actively prefer to mate with larger partners, it might be expected that they will mate with them for longer to maximise their reproductive success. It appears that this may be the case in the majority of species studied here.

Earlier studies have found no association between male or female size and the frequency of female rejection by males (Dunn et al 1999; Chapter 4). However, this study suggests that males are more likely to reject smaller females in all of the species studied. This is perhaps due to the same reasons proposed earlier for the result that the males from some species prefer to mate with larger females. An additional surprising result was that smaller males in two of the species studied (*C. littoralis* and *G. keyzeri*) appeared more likely to reject females than larger males. This could suggest the possible presence of a small male mating advantage in these species, leading to smaller males being more choosy over the females they mate with, though this hypothesis seems highly unlikely in the face of the earlier finding that large males appear to have a mating advantage in these species. This result may therefore be erroneous, and further mating trials need to be carried out to clarify this.

This study has uncovered a wealth of interesting and conflicting results that cannot help but to add further fuel to the fire of controversy surrounding the presence of pre-copulatory male and female choice in the Coelopidae. Though some may regard the results presented here as only preliminary findings in these species, it is clear that by increasing the volume and scope of the data available, more robust conclusions can eventually be drawn concerning sexual conflict and mating systems in coelopids. In addition, the path has now been partially cleared to enable comparisons of mating behaviours between species within the Coelopidae to be carried out. The exciting possibilities of this expansion in the field of coelopid research will be investigated in the following chapter.

Chapter 7. Sexually antagonistic co-evolution in the Coelopidae

7.1 Introduction

Sexually antagonistic co-evolution, as a result of sexual conflict, has been studied in numerous species, encompassing a diverse range of traits that may co-evolve antagonistically to promote the interests of one sex over the other (see Chapter 1). These empirical studies can be grouped into three categories (Rowe & Arnqvist 2002). Firstly, the opposing interests of male and females, within the context of mating interactions, have been studied from an economic perspective. An example of this can be seen in the study of the costs of mating in female *Drosophila melanogaster*, carried out by Chapman and co-workers, in which they found that these costs were mediated by male accessory gland products (Chapman et al. 1995). Secondly, studies in which the evolution of species has been experimentally altered have demonstrated the possible short-term effects of sexually antagonistic interactions. In an experiment carried out using *D. melanogaster*, a naturally promiscuous species, Holland and Rice discovered that by forcing a population to evolve a monogamous mating system, males evolved to be less harmful to their mates and females evolved to be less resistant to male harm than in the promiscuous control group (Holland & Rice 1999). This study indicates that the experimental removal of sexual selection may reverse intersexual antagonistic co-evolution. However, it has been argued that the effective population size of the monogamous population used in this experiment was less than the control population, so the results may be due to other factors such as inbreeding (Wigby & Chapman 2004). In an experiment that manipulated the intensity of sexual conflict in *D. melanogaster* by altering the adult sex ratio, females from high sexual conflict populations lived significantly longer in the presence of males than females from low conflict populations (Wigby & Chapman 2004). This study provides evidence that sexual conflict can be experimentally manipulated causing females to adapt to alterations in the intensity of sexual conflict by evolving increased or decreased resistance to male-induced harm. Thirdly, many studies have been carried out to examine the function of traits and behaviours that appear to have evolved as a result of sexual conflict (e.g. Arnqvist & Rowe 1995; Sakaluk et al 1995; Laird et al. 2004). This is an area on which the study of sexual conflict in coelopids has been particularly focussed, both in previous studies (e.g. Crean & Gilburn 1998; Crean et al. 2000; Shuker & Day 2001) and in this thesis.

As highlighted by Rowe and Arnqvist (2002), though these approaches provide evidence for the presence of sexually antagonistic co-evolution of male and female traits, they do not shed light on the role played by sexual conflict in the diversification of mating systems in the natural world. In order for this to be investigated, data gathered from empirical studies, involving many related species, needs to be analysed using comparative techniques within a phylogenetic framework (e.g. Felsenstein 1985; Harvey & Pagel 1991; Blomberg et al. 2003). Phylogenies are central to virtually all comparisons among species as they help to detect independent evolutionary events, which are key to any statistical analyses that need to be used for this purpose (Harvey & Pagel 1991). Comparisons between the traits or behaviours shown by contemporary forms of species, without considering their phylogenetic lineages, can result in misleading correlations and potential confounding effects. It may also mask interesting patterns in the data, since large numbers of significant associations can be found if species are used as independent data points, which are unlikely to be informative (Harvey & Pagel 1991). Early studies using the comparative method did not incorporate any phylogenetic information into the comparison of, typically, two phenotypes across a range of species. A classic example of this approach is the association found between population density and body size in mammals (Damuth 1981). However, these methods received considerable criticism in a seminal paper written by Felsenstein (1985). He pointed out a serious statistical flaw in these studies: species are part of a hierarchically structured phylogeny, and therefore cannot be regarded, for statistical purposes, as points drawn independently from the same distribution (Felsenstein 1985). Since the majority of correlation, regression and contingency table statistics, including non-parametric methods, assume that data points are drawn independently from a common distribution, the direct use of these tests on species with a branching phylogeny will cause an amplification in the significance of results.

A number of scientists working in the field of comparative biology had noticed the problem of non-independence prior to the publication of Felsenstein's paper (e.g. Clutton-Brock & Harvey 1977; Baker & Parker 1979; Ridley 1983). They attempted to deal with this challenge in a variety of different ways, for example, by using nested analyses of variance to find the taxonomic level accounting for the majority of variation and then using this as the units of further statistical analyses (Clutton-Brock & Harvey 1977; Harvey & Clutton-Brock 1985). However, using higher nodes, such as genera or families, results in a reduction in the number of degrees of freedom and any information from lower taxonomic

levels is lost (Harvey & Pagel 1991). Alternatively, parsimony was used by some to detect the number of times particular traits or behaviours had evolved independently on a phylogeny, and from this, tests were carried out to determine whether the occurrence of changes in two characters were independent (e.g. Ridley 1983). Two problems are apparent with this use of parsimony: firstly, a monophyletic arrangement of taxa in the Linnean classification system needs to be assumed when the phylogenetic relationship between groups is unknown. This classification system is not completely monophyletic, however, and lacks the detail required to show the complete structure of the phylogeny (Felsenstein 1985). Secondly, the use of parsimony in comparative analyses can result in statistical errors and biased results (reviewed in Felsenstein 1983).

All of the methods mentioned above make a distinction between the variation associated with phylogeny and the variation that is independent of phylogeny (Harvey & Pagel 1991). Felsenstein introduced a technique combining all variation into one model, termed the 'pairwise independent comparisons method' (Felsenstein 1985). This works on the premise that pairwise differences or 'contrasts' between species are independent of each other and that by segregating the variation correctly, all of it can be used (Harvey & Pagel 1991). The independent comparisons method compares pairs of species or higher nodes that share a common ancestor, usually using the Brownian Motion Model as a statistical model of character change (Felsenstein 1985), though this is not always the case (see Garland et al. 1992). The role played by the Brownian Motion Model will be discussed in greater detail in the methods section of this chapter. Though alternative methods have been constructed since Felsenstein's method was introduced, and improvements on the original model have been made, the independent comparisons approach is now widely accepted as being one of the most reliable techniques in this area (Garland et al. 1992; Garland & Diaz-Uriarte 1999). Various studies have verified its reliability, both analytically and through computer simulations (Grafen 1989; Martins & Garland 1991), even when there is phylogenetic uncertainty (Garland & Diaz-Uriarte 1999).

As discussed in the previous chapter, all coelopid species studied to date, with the exception of *Amma blanchae* and *This canus*, exhibit pre-mating struggling as a response to male mounting (Day & Gilburn 1997; Crean & Gilburn 1998) and all show a large male mating advantage, with the exception of *T. canus* (e.g. Gilburn et al. 1992; Crean et al.

2000), which are indicators that the mating systems of these species are based on sexual conflict. In addition to this, it is also thought that sexual selection for large male size may have developed as a side-effect of sexual conflict, promoted by the rejection responses exhibited by females when mounted by a male (e.g. Crean & Gilburn 1998). The existence of two coelopid species, *T. canus* and *A. blanchae*, which do not appear to share these features, presents a good opportunity for investigating the importance of sexual conflict in the evolution and diversification of coelopid mating systems in the natural world. This is further enhanced by the addition of the behavioural data collected for the study described in the previous chapter, which increases the current dataset on mating behaviour in coelopids to a total of thirteen species.

Female resistance can result in a pre-mating struggle and is often associated with sexual selection for male characteristics that increase their ability to overcome female resistance (Rowe et al. 1994; Arnqvist & Rowe 1995; Arnqvist & Rowe 2002). Some researchers believe that female rejection responses are screening mechanisms that enable females to gain indirect benefits from exhibiting mate choice (Eberhard 1996: Eberhard & Cordero 2003). For such a mechanism to explain the evolution of female resistance behaviour, the indirect benefits from mate choice generated by the screening process must outweigh any decrease in fitness gained by costly matings (Chapman et al. 2003). Due to the controversy surrounding the idea that sexual selection can occur as a side-effect of sexual conflict (e.g. Brooks & Jennions; Getty 1999; Rosenthal & Servedio 1999), the first aim of this study was to measure the strength of sexual selection for large male size in the additional five species of coelopid and to determine the success rate of female resistance across all thirteen species. This was then used to test the mate assessment hypothesis (or screening hypothesis) as an explanation for the evolution of female resistance behaviour. The mate assessment hypothesis proposes that female resistance solely exists to generate sexual selection, thereby predicting that the strength of sexual selection for large male size will be strongest in those species in which females are best able to resist male mating attempts. In other words, the proportion of pre-mating struggles ending in rejection of the male should be positively associated with the strength of sexual selection for large male size. However, previous studies carried out with coelopids have failed to find any evidence for the mate assessment hypothesis (Weall & Gilburn 2000).

Once the mate assessment hypothesis can be ruled out as an explanation for the evolution of female resistance behaviour, the next aim of this study was to test whether there is any evidence for the antagonistic co-evolution of male and female behaviours and traits that are thought to be associated with sexual conflict, across a variety of coelopid species. This was achieved by implementing a comparative approach to analysing the data available. There are a number of interspecific hypotheses that can be constructed from what is already known about the traits involved in sexual conflict. Since large male size is thought to be a trait which is acted on by sexual selection as a result of sexual conflict, and which also confers a mating advantage, it might be expected that in species where there are relatively low levels of sexual conflict, there will be less selective pressure on males to increase their size. Instead, males would be relatively smaller. In addition, there may be a lesser degree of sexual dimorphism than is found in species where sexual conflict is high. Another interesting hypothesis is that in species where male harassment of females is infrequent and males are more likely to exert a greater degree of mate choice over females, there may be selective pressure on females to increase their size and therefore overall fecundity. This could increase the likelihood of such females successfully fertilising their entire egg load, since males of some coelopid species have been found to prefer larger, more fecund females (e.g. Pitafi 1991). An increase in female size may therefore be expected with a decrease in male harassment intensity. A corresponding decrease in pre-mating struggle duration, or the absence of a pre-mating struggle altogether, may also be expected in these cases, as would a decrease in the number of males that are rejected by females before copulation takes place.

7.2 Materials and methods

7.2.1 Data collection

The behavioural data for this study came from three sources: the behavioural and size data for *C. frigida* were initially collected for a study described briefly in the aims section of this thesis (see Chapter 3). These data were gathered from standard mating trials, carried out as in Chapter 4, using male and female *C. frigida* that had been kept in the presence of seaweed for 48 hours prior to the experiment. This was done in order to control for the effect of seaweed on mating behaviour. This data set was augmented by the addition of data provided by D. Dunn (Dunn 2001). The behavioural and size data for

Coelopa pilipes, *Coelopa nebularum*, *Coelopa vanduzeei*, *Coelopa ursina*, *Dasycoelopa australis*, *Gluma musgravei* and *Gluma nitida*, were also derived from data provided by D. Dunn (Dunn 2001). The behavioural and size datasets on *Amma blanchae*, *This canus*, *Chaetocoelopa sydneyensis*, *Chaetocoleopa littoralis* and *Gluma keyzeri* were recorded during the previous study (see Chapter 6). The sources of the collections made for each species are given in Table 7.1. The data provided by D. Dunn were gathered from fly collections made in 1996 and 1997. The variables used in this study were (for each species):

1. Mean male wing length (mm)
2. Coefficient of variation (CV) of male wing length
3. Mean female wing length (mm)
4. Mean species wing length (mm)
5. Sexual size dimorphism (male wing length/female wing length)
6. Pre-mating struggle duration of trials where the female successfully rejected the male (secs)
7. Pre-mating struggle duration of trials where the female did not reject the male (secs)
8. Proportion of trials where the male mounted the female
9. Proportion of mounts that resulted in copulation
10. Proportion of mounts where the female successfully rejected the male
11. Proportion of mounts where the male rejected the female
12. Strength of sexual selection (logistic regression co-efficient of male wing length against whether the male mounted the female or not) (Gilburn et al. 1992; Day & Gilburn 1997; Crean & Gilburn 1998; Crean et al. 2000).

Variables 6 to 12 were chosen because they have been found to be linked in some way with sexual conflict in previous studies. In addition, they can be used as an indication of female or male-mediated sexual conflict, for example, the struggle duration of trials where the female successfully rejected or accepted the male can be used as a measure of female-mediated sexual conflict, as can the proportion of males successfully rejected by females. The proportion of trials where the male mounted the female can be used as a measure of the intensity of harassment, and therefore male-mediated sexual conflict and the proportion of trials where the male rejected the female can be used as an indicator of

male choice. The proportion of mounts resulting in copulation can be used to indicate which sex has the most controlling influence over the conflict. Various direct and derived measures of male and female size were also incorporated into analyses (variables 1 to 5).

Table 7.1 The source of collections made of each species used in the study.

Species	Source
Coelopa frigida	Longhoughton and St Mary's Island, England, U.K.
Coelopa pilipes	Longhoughton, England, U.K.
Coelopa nebularum	Roads End, Kodiak Island, Alaska, U.S.A.
Coelopa vanduzeei	Asilomar and Van Damme, California, U.S.A.
Coelopa ursina	Capetown, South Africa
Gluma musgravei	Mallacoota Bay, Victoria, Australia
Gluma nitida	Eaglehawk Neck, Tasmania and Victor Harbour, South Australia
Gluma keyzeri	Victor Harbour, South Australia
Chaetocoelopa sydneyensis	Victor Harbour, South Australia and Quarantine Bay, New South Wales, Australia
Chaetocoelopa littoralis	Kaikoura, South Island, New Zealand
This canus	Forrester's Beach, New South Wales and Asling's Beach, Twofold Bay, New South Wales, Australia
Amma blanchae	Quarantine Bay, New South Wales, Australia
Dasycoelopa australis	Various, New South Wales, Australia

7.2.2 Statistical treatment of behavioural and size data

The measurements of wing length were log-transformed before use in the calculation of independent contrasts. This was to ensure that the different lineages were equally likely to make the same proportional change in size (Felsenstein 1985). The pre-

mating struggle durations were also log-transformed to increase the normality of their distributions and improve linearity. The data used to generate the independent contrasts are given in Appendix 7.1.

7.2.3 Treatment of phylogenetic data

The phylogenetic data used in this study were taken from the phylogeny constructed by Meier and Wiegmann (2002), which used a data matrix consisting of morphological and DNA sequence characters (see Figure 7.1). This phylogeny was then transformed into the following treefile format for use in subsequent analyses (see Table 7.2 for explanation of species abbreviations):

((Amma,This),(Pili,((((Chaes,Chael),Dasy),(Glumk,(Glum,Niti))),(Vand,(Ursi,(Nebu, Frid))))));

Table 7.2 Abbreviations of each species' name for use in generating independent contrasts.

Abbreviation	Species	Abbreviation	Species
Amma	*A. blanchae*	Niti	*G. nitida*
This	*T. canus*	Vand	*C. vanduzeei*
Pili	*C. pilipes*	Ursi	*C. ursina*
Chaes	*C. sydneyensis*	Nebu	*C. nebularum*
Chael	*C. littoralis*	Frid	*C. frigida*
Dasy	*D. australis*	-	-
Glumk	*G. keyzeri*	-	-
Glum	*G. musgravei*	-	-

Before the treefile could be used in any computations however, branch lengths had to be added. Since no branch lengths had been calculated for the Coelopidae phylogeny, they were set arbitrarily following the method described by Grafen (1989), where the depth of the node equals one less than the number of species descended from it. This method removes the effect of any taxonomic rankings. Once the node "heights" had been assigned, the length of the path segments, or branch lengths, could be added (see Figure 7.2). These branch lengths were then added to the treefile, as shown below, ready for use in the computation of independent contrasts:

((Amma:1.00,This:1.00):11.00,(Pili:10.00,((((Chaes:1.00,Chael:1.00):1.00,Dasy:2.00):3.00,(Glumk:2.00,(Glum:1.00,Niti:1.00):1.00):3.00):4.00,(Vand:3.00,(Ursi:2.00,(Nebu:1.00,Frid:1.00):1.00):1.00):6.00):1.00):2.00);

7.2.4 Generation of independent contrasts

As discussed in the introduction of this chapter, before any cross-species analyses can be carried out, the possible effects of phylogeny have to be removed, as species cannot be directly treated as independent data points due to their hierarchical structure. Instead, the independent comparisons method is used to generate pairwise differences between species, which are independent of each other (Felsenstein 1985). These pairwise differences are called 'independent contrasts'. Felsenstein's method of pairwise independent comparisons is based on the Brownian Motion Model (Edwards & Cavalli-Sforza 1964; Cavalli-Sforza & Edwards 1967), which describes the random wanderings of a variable along a continuous dimension. Continuous variables, such as wing length or struggle duration, can be thought of as discrete variables in which the distance between units becomes vanishingly small, and movements backwards and forwards can be just as likely as each other (Harvey & Pagel 1991). Following Harvey and Pagel's (1991) description of the theory behind the 'random walk' process, the position along a scale after an arbitrary length of time is the sum of all of the proceeding steps. If this discrete process

of a random walk is transferred to continuous time, the result is the Brownian Motion Model.

The Brownian Motion Model is implemented in the CONTRAST module of the PHYLIP computer program package (Felsenstein 1993), which is used to generate the independent contrasts required for the appropriate statistical analyses of the data set. CONTRAST implements the contrasts calculation described by Felsenstein (1985). It reads in a continuous characters dataset along with a treefile. The reason that character differences between pairs of adjacent tips are independent is that each character is evolving via Brownian Motion, which is independent in each lineage i.e. the difference between the characters of species *w* and species *x* depends solely on events that take place in the branches connecting these two species, and is completely independent of the difference between the characters of species *y* and *z*, caused by events on the branches connecting *y* and *z* (Felsenstein 1985).

Before any statistical analyses could be carried out using independent contrasts, the contrasts had to be standardised. This was to ensure that they received equal weighting in subsequent correlation and regression analyses (Garland et al. 1992). CONTRAST standardises the independent contrasts by scaling them with their expected standard deviations (i.e. the square root of the sum of their branch lengths), which are derived using the Brownian Motion Model. This translates the branch lengths into units of expected evolutionary change (Felsenstein 1985). A further check was made to verify whether the contrasts, continuous variables or branch lengths required further transformation before analyses, by plotting the absolute value of each standardised independent contrast versus it's standard deviation (Garland et al. 1991; Garland et al. 1992). A significant linear trend indicates that the contrasts are not adequately standardised, however, in this case the standardised independent contrasts showed no significant linear trend, so were suitable for use in standard statistical analyses.

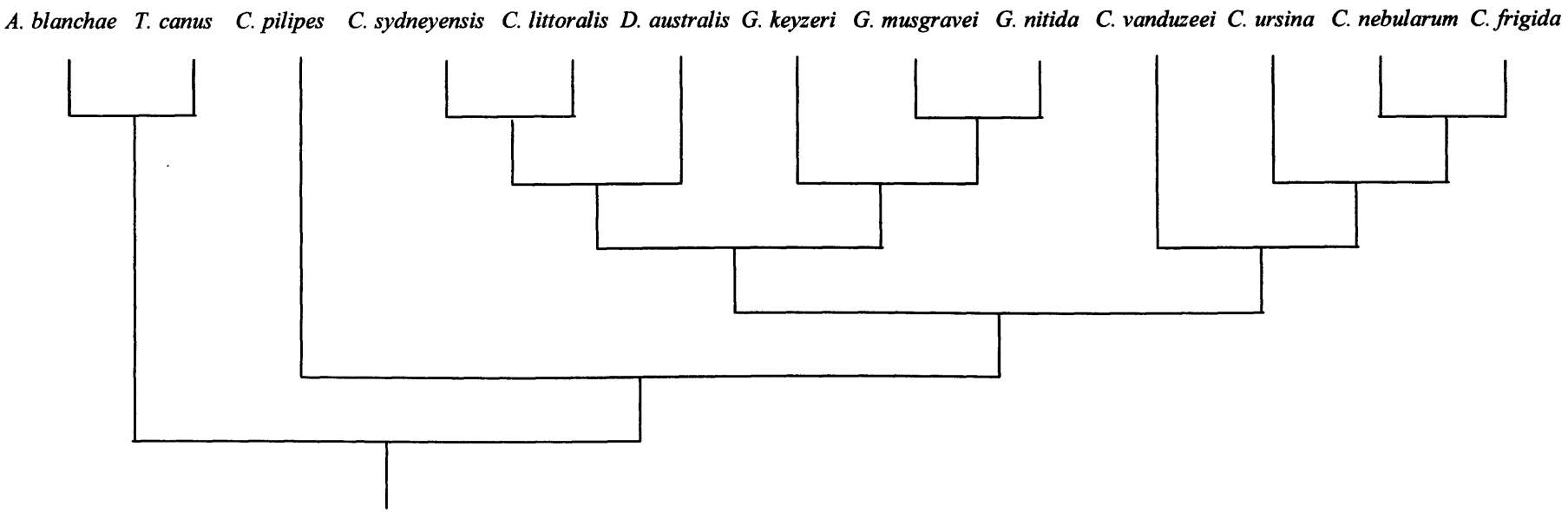


Figure 7.1 Phylogeny of the Coelopidae species used in this study, based on morphological and DNA sequence data (redrawn from Meier & Wiegmann 2002).

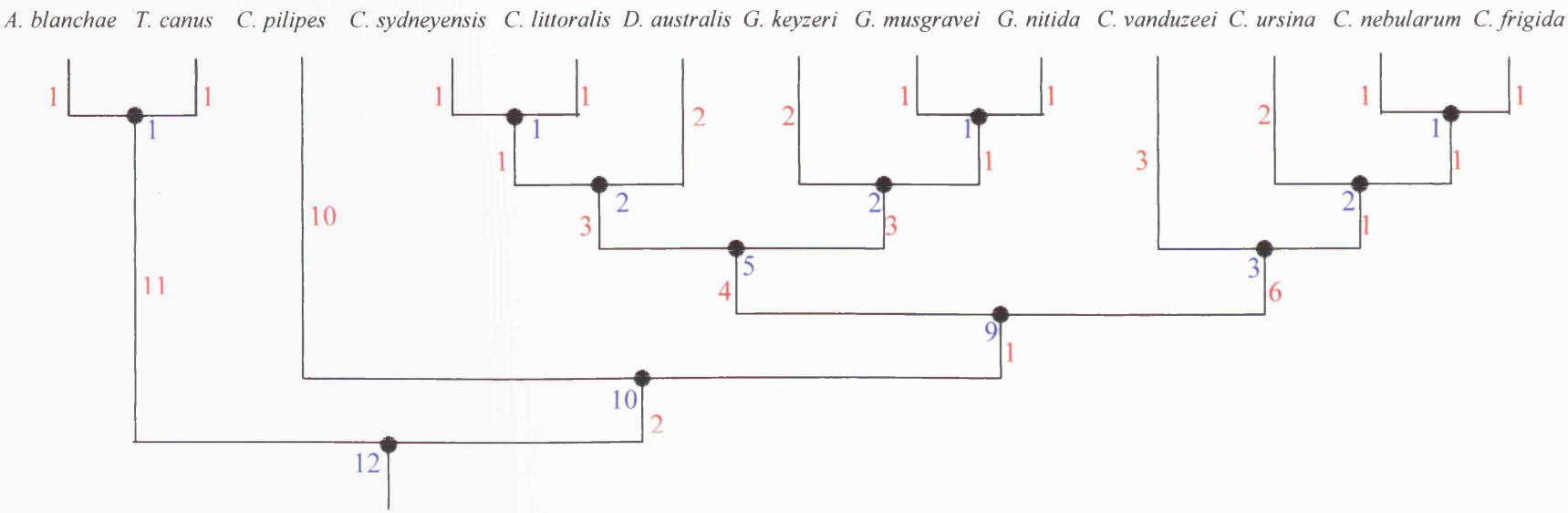


Figure 7.2 Coelopidae phylogeny showing the allocation of node (●) 'heights' (highlighted in blue) and corresponding branch lengths (highlighted in red) using the method proposed by Grafen (1989). Phylogeny redrawn from Meier & Wiegmann 2002.

7.2.5 How independent contrasts relate to the Coelopidae tree

Each independent contrast generated by CONTRAST is associated with an interior node of the Coelopidae tree i.e. there are 13 species included in the study, therefore 12 contrasts per variable are generated. Contrasts are read in the order left to right and top to bottom. More accurately, they represent a 'pre-order tree transversal': this shows all the contrasts in the left sub-tree, then all in the right sub-tree and then the sub-tree at the bottom. For each sub-tree, this is repeated (J. Felsenstein, personal communication).

Example:

For the tree **(((A,B),(C,D)),((E,F),(G,H));** the contrasts are read for A-B, then C-D, then AB-CD, then E-F, then G-H, then EF-GH, then finally, ABCD-EFGH. This is demonstrated in Figure 7.3.

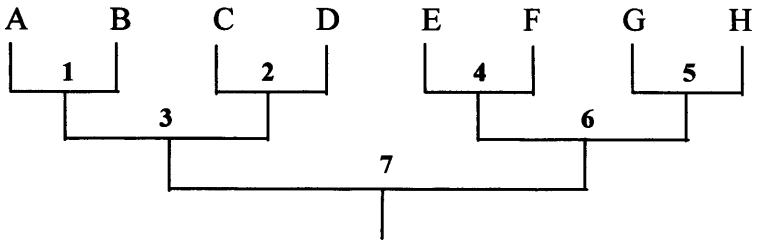


Figure 7.3 Tree showing the order in which independent contrasts are read, for species A to H.

The same criteria can be applied to the Coelopidae tree (see Figure 7.4). The independent contrasts generated by CONTRAST for the variables measured in this study are given in Appendix 7.2.

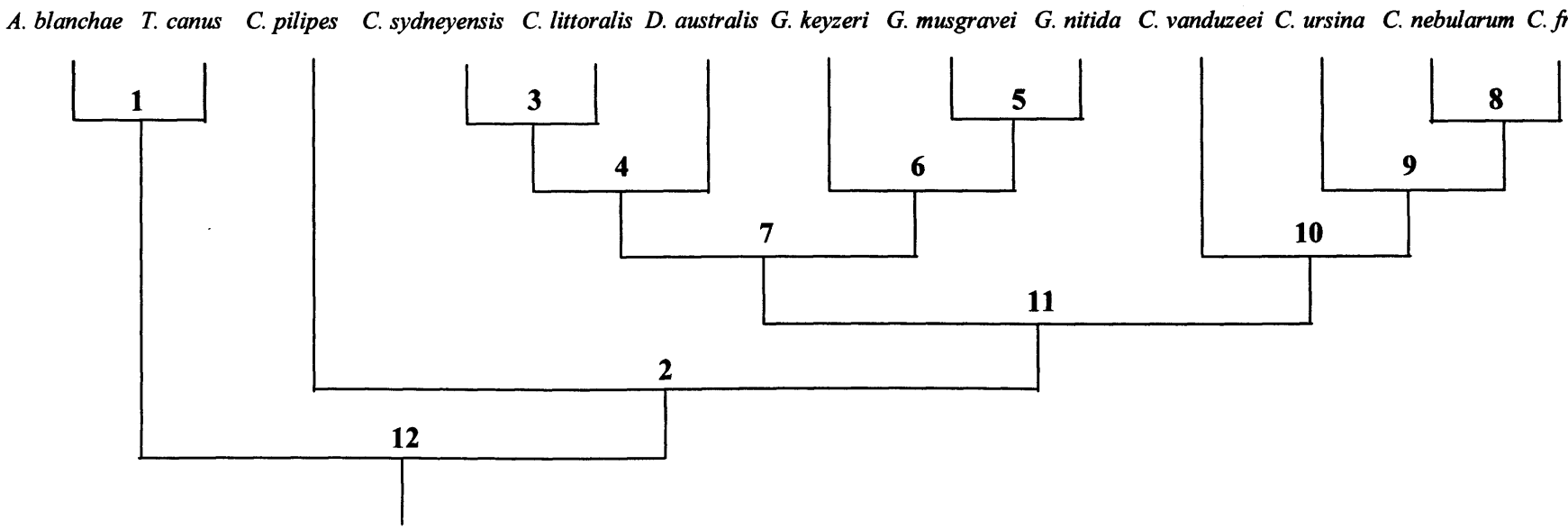


Figure 7.4 Coelopidae tree showing the 'pre-order tree transversal' order in which independent contrasts between nodes are read (node numbers are in **bold** type).

7.2.6 Statistical treatment of independent contrasts

Initially it was deemed that parametric statistics should not be used to analyse trends between and across species using the independent contrasts, because there was no guarantee that the assumptions of these tests could be met. Though the contrasts could be regarded as being independent, parametric statistics assume that they will all have equal variances. It has been suggested that the Brownian motion model doesn't ensure this, as it depends on the accuracy of the model at describing the process of evolutionary change (Harvey & Pagel 1991). However, the standardised residuals around the regression lines for the independent contrasts were checked for homogeneity of variance and a normal distribution. The distribution of residuals was generally normal with approximately 95% of the observations within 2 standard deviations of the mean. This confirmed that there was homogeneity of the variance among the residuals. It was subsequently decided that the use of parametric statistics was appropriate for this study.

There are restrictions placed on the methods than can be used to regress independent contrasts against each other because of the uncertainty associated with determining the sign of independent contrasts (Garland et al. 1992). This uncertainty is due to the way in which independent contrasts are calculated - the values of the variables involved are subtracted, with the direction of the subtraction being arbitrary (Garland et al. 1992). To overcome this, any regression analyses performed using the independent contrasts must be forced through the origin (Garland et al. 1992; Grafen 1992).

7.3 Results

7.3.1 Testing the mate assessment hypothesis

As predicted, there was no significant association between female rejection success rate (i.e. the proportion of mounts where the female successfully rejected the male) and the strength of sexual selection, across species (Regression analysis, $F_{1,12} = 1.20$, $p = 0.741$). Therefore female rejection success does not determine the strength of sexual selection and the mate assessment hypothesis can be ruled out as an explanation for the evolution of female resistance behaviour.

7.3.2 Characterisation of variation in behavioural traits

To identify which combinations of variables explain the largest amount of variation in the dataset, principal component analysis was applied to the independent contrasts of the behavioural variables (Rencher 1995; Rowe & Arnqvist 2002). This yielded 3 principal components (PCs). The percentage of the variance in the dataset accounted for by these components, with corresponding eigenvalues, is presented in Table 7.3.

Table 7.3 Eigenvalues, percentage of variance accounted for by each component and the cumulative percentage of variance.

Component	Eigenvalue (λ)	% of variance	Cumulative %
1	2.355	33.647	33.647
2	1.959	27.987	61.634
3	1.653	23.610	85.244

The component loadings for each variable are shown in Table 7.4 and the ordination of the variables along PC1, 2 and 3 loadings are shown in Figure 7.5. From Table 7.4 and Figure 7.5, it can be seen that the variables linked with female-mediated sexual conflict are positively associated with PC1. The variables indicating male harassment intensity, i.e. 'proportion mounted', and male mate choice, i.e. 'proportion of females rejected', are linked with male-mediated sexual conflict, with the former being positively associated with PC3 and the latter being negatively associated with PC3. The variables associated with PC2 appear to already have been explained by PC1 with the exception of 'proportion copulated', which could tentatively be categorised as a variable indicating male control, since it is probable that males control whether copulation takes place. This appears to be positively associated with PC2. These relationships are clarified further in Figure 7.6.

Table 7.4 Component loadings for each variable.

	Component loadings		
Variable	**PC1**	**PC2**	**PC3**
Struggle duration reject	0.896	0.238	0.050
Struggle duration accept	0.722	0.510	-0.402
Proportion of males rejected	0.552	-0.801	-0.155
Proportion of females rejected	-0.388	0.231	-0.759
Proportion mounted	0.208	-0.207	0.817
Proportion copulated	-0.476	0.726	0.448
Strength of sexual selection	0.552	0.614	0.144

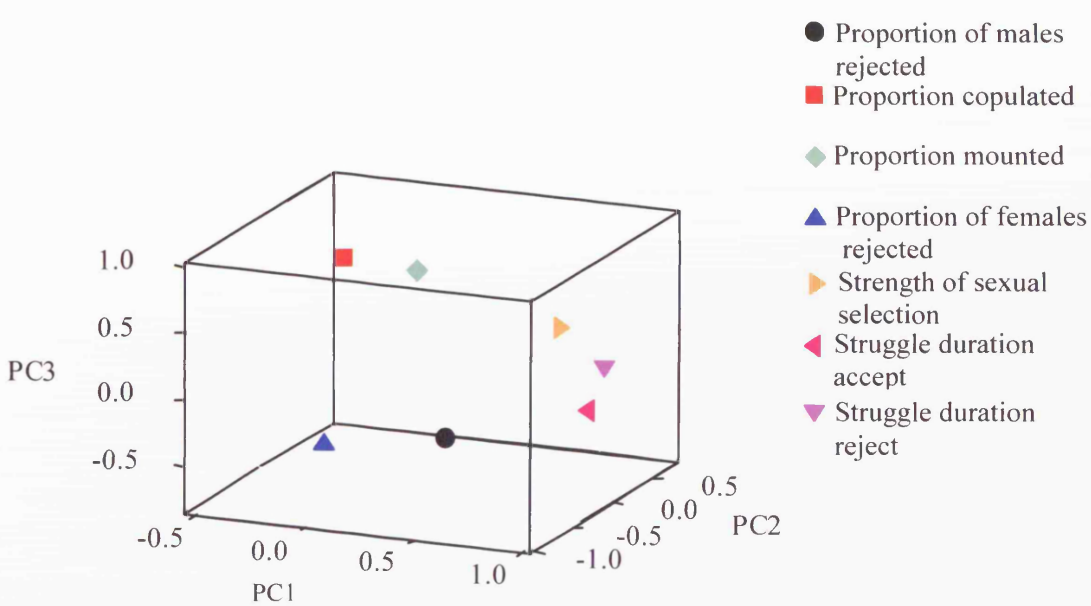


Figure 7.5 Position of variables along PC1, 2 and 3 loadings axes.

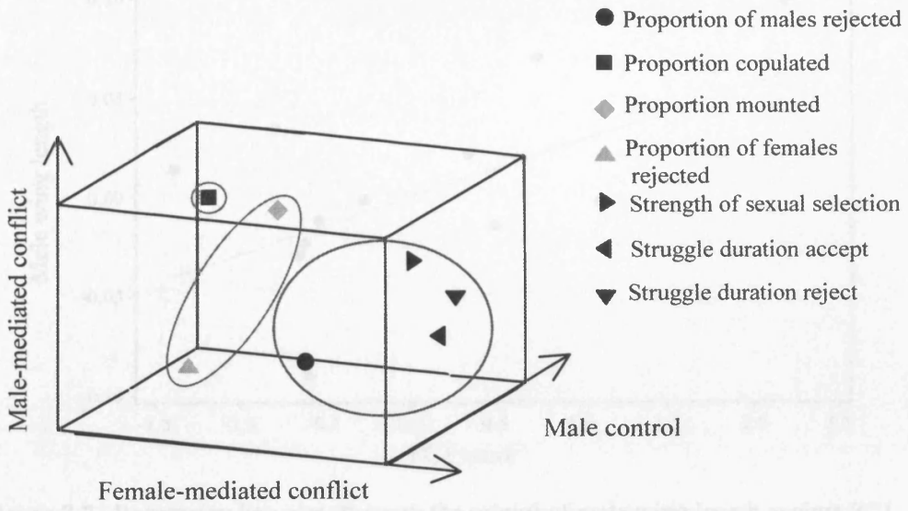


Figure 7.6 Ordination of variables along axes of increasing conflict or control. Variables associated with female-mediated conflict are black, those associated with male-mediated conflict are grey and those associated with male control are blue. Red rings have been added to highlight variable groupings.

7.3.3 Correlated evolution of sexual conflict and size variables

Regression analyses (forced through the origin) were carried out with the principal component scores against several different measures of size for which independent contrasts had been generated:

There were no significant associations between male wing length and PC2 ($F_{1,12}$ = 0.596, p = 0.456) or PC3 ($F_{1,12}$ = 0.863, p = 0.373). However, a significant positive association was found between male wing length and PC1 ($F_{1,12}$ = 2.355, $p < 0.05$) as shown in Figure 7.7.

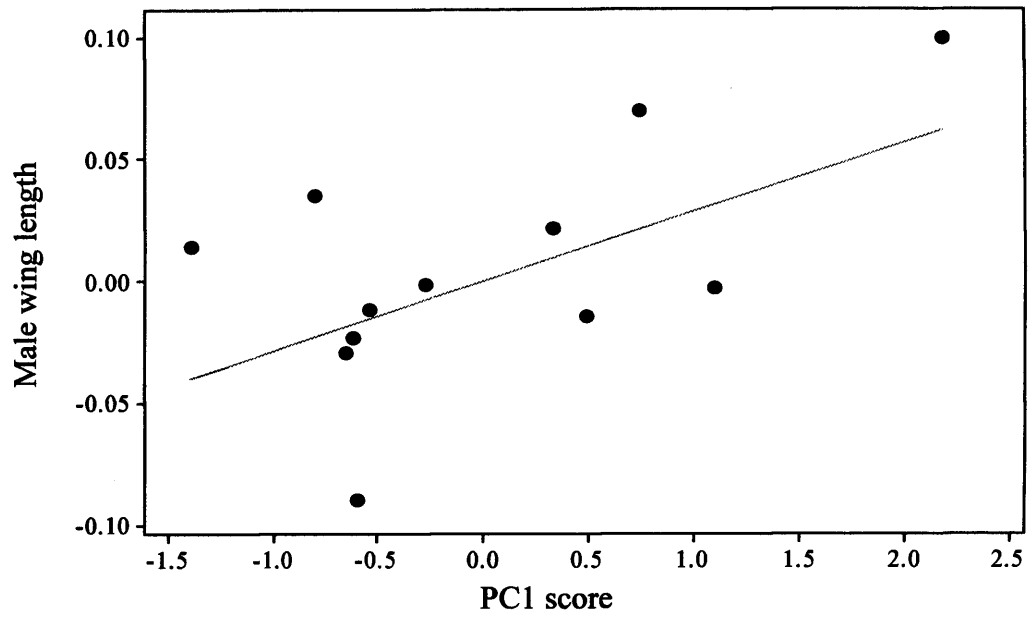


Figure 7.7 Regression line plot (through the origin) of male wing length against PC1 score (female-mediated sexual conflict).

Further analyses revealed that there were no significant associations between the CV of male wing length and PC2 ($F_{1,12} = 0.008$, $p = 0.930$) or PC3 ($F_{1,12} = 0.341$, $p = 0.571$). However, a significant positive association was found between the CV of male wing length and PC1 ($F_{1,12} = 8.465$, $p < 0.05$) as shown in Figure 7.8.

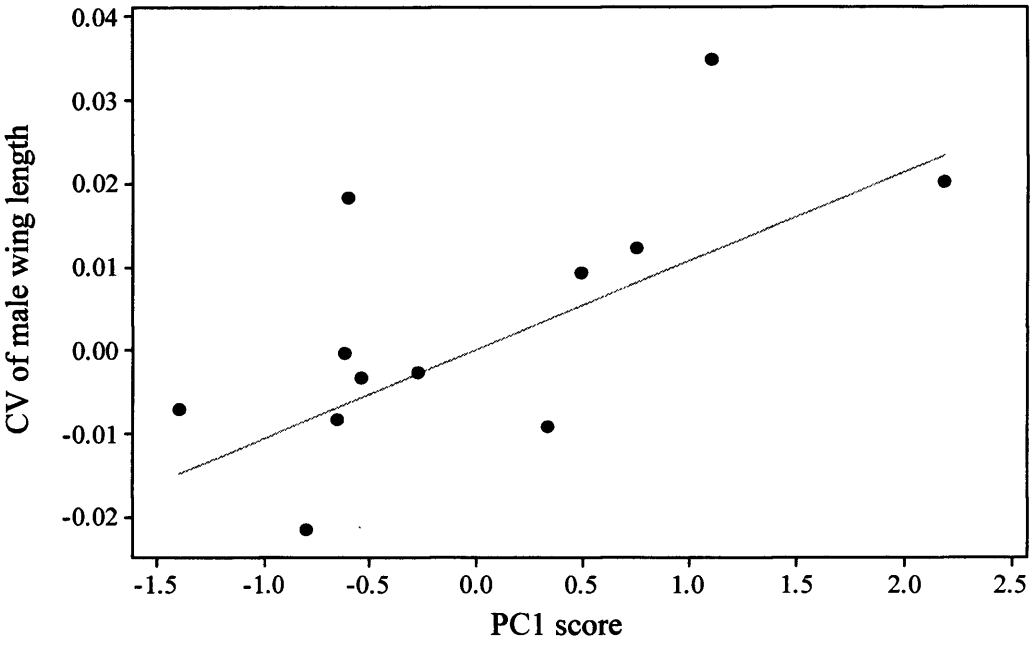


Figure 7.8 Regression line plot (through the origin) of CV of male wing length against PC1 score (female-mediated sexual conflict).

There were no significant associations found between female wing length and the principal component scores or between mean wing length and the principal component scores (see Table 7.5).

Table 7.5 Results of regression analyses of female and mean wing lengths against principal component scores.

Variable	Component	$F_{1,12}$	p
Female wing length	1	1.366	0.267
	2	3.437	0.091
	3	0.403	0.539
Mean wing length	1	3.917	0.073
	2	2.211	0.165
	3	0.071	0.795

No significant associations were found between sexual size dimorphism and PC1 ($F_{1,12}$ = 0.511, p = 0.490) and PC2 ($F_{1,12}$ = 0.820, p = 0.384). There was, however, a significant negative association between sexual size dimorphism and PC3 ($F_{1,12}$ = -2.333, p < 0.05) as shown in Figure 7.9.

In summary, there appear to be two major forces at work: as female-mediated sexual conflict increases there is a significant increase in male size and variation in male size, but with increasing male-mediated conflict, there is a significant decrease in sexual size dimorphism.

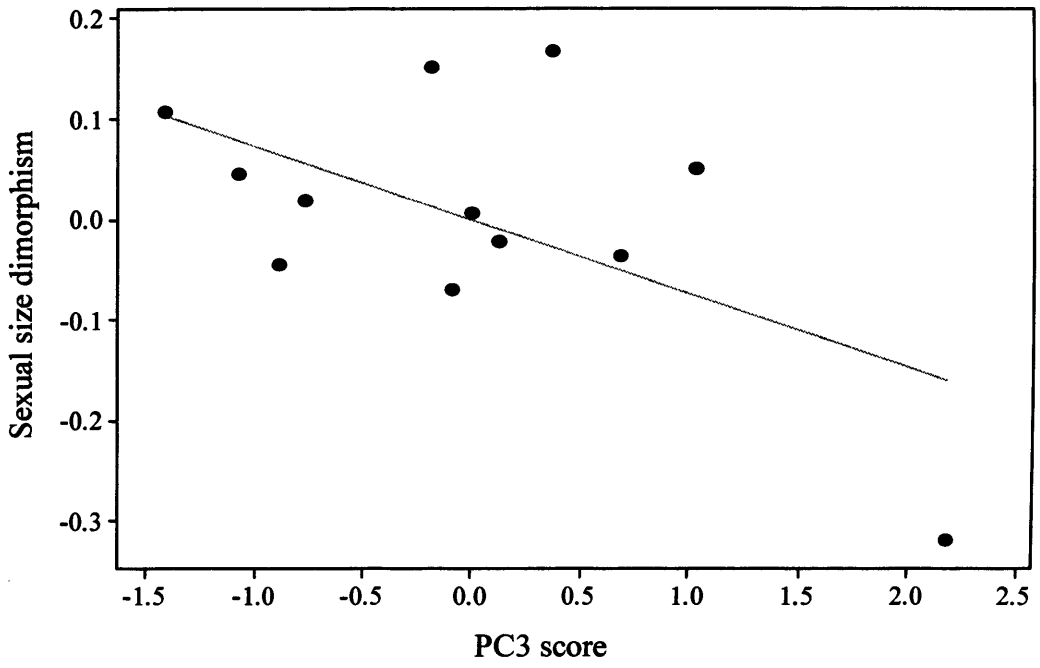


Figure 7.9 Regression line plot (through the origin) of sexual size dimorphism against PC3 score (male-mediated sexual conflict).

7.3.4 Co-evolution of male harassment rate, female resistance rate and struggle duration

A multiple regression model (forced through the origin) revealed no associations between male harassment rate (proportion of trials where the male mounted the female), female rejection success rate and pre-mating struggle duration (for trials where the female accepted the male and trials where the female rejected the male) ($F_{3,12} = 0.622$, $p = 0.619$).

7.4 Discussion

The mate assessment hypothesis proposes that the sole benefit of female resistance is to generate indirect sexual selection. In addition, female resistance is not expected to occur in order to avoid the possible costs associated with male harassment. Consequently, the mate assessment hypothesis makes the prediction that the ability of females to successfully resist male mating attempts will determine the level of sexual selection within a species, as this is the only reason that females will attempt to reject males. This study revealed that there was no positive association found between female rejection success rate and the strength of sexual selection across species of coelopids, as predicted, which suggests that the ability of females to resist male mating attempts does not determine the level of sexual selection for large male size within coelopids. These results indicate that the evolution of female resistance behaviour, and therefore sexual selection for large male size, is not due to mate assessment.

The alternative explanation for the evolution of the pre-mating struggle and sexual selection for large male size in coelopids is that they occur as a side-effect of sexual conflict (Crean & Gilburn 1998) through a co-evolutionary arms race between the sexes over the outcome of pre-mating struggles. Sexual conflict theory predicts that any benefit to the female gained via indirect sexual selection is negligible relative to the costs associated with unwanted copulations (Chapman et al. 2003). Pre-mating struggles will evolve through sexual conflict if the costs of mating outweigh the costs associated with rejection of males, plus any costs of indirect sexual selection generated as a side-effect of sexual conflict. However, this study showed no evidence for the co-evolution of male harassment and female resistance behaviours, which is a somewhat surprising result. However, a number of intriguing relationships have been uncovered which, in part, may help to explain this outcome. First of all, female-mediated sexual conflict appears to have played a role in increasing male size and variation in male size. Female resistance to male mounting attempts therefore influence male size rather than male behaviour. Secondly, there is evidence of a trade-off between sexual size dimorphism and male-mediated sexual conflict i.e. male harassment rate. This may be obscuring the presence of sexually antagonistic co-evolution if large male size reduces male harassment rate - willingness to mount and mate with females is known to decrease with increasing male size in a number of coelopid species (e.g. Dunn et al. 1999). In a recent study looking at the antagonistic co-

evolution of relative armaments of the sexes and the outcome of sexually antagonistic interactions in water striders (Gerridae), Arnqvist & Rowe (2002) found that sexually antagonistic co-evolution can be hidden by the continuous adaptation and counter-adaptation of traits. They also found that the average co-evolutionary trajectory which describes the evolutionary path along which the levels of arms in the two sexes may evolve, is to a great extent balanced and that if co-evolution follows this path without deviation, the effects of the arms race on interactions may be masked (see Figure 7.10).

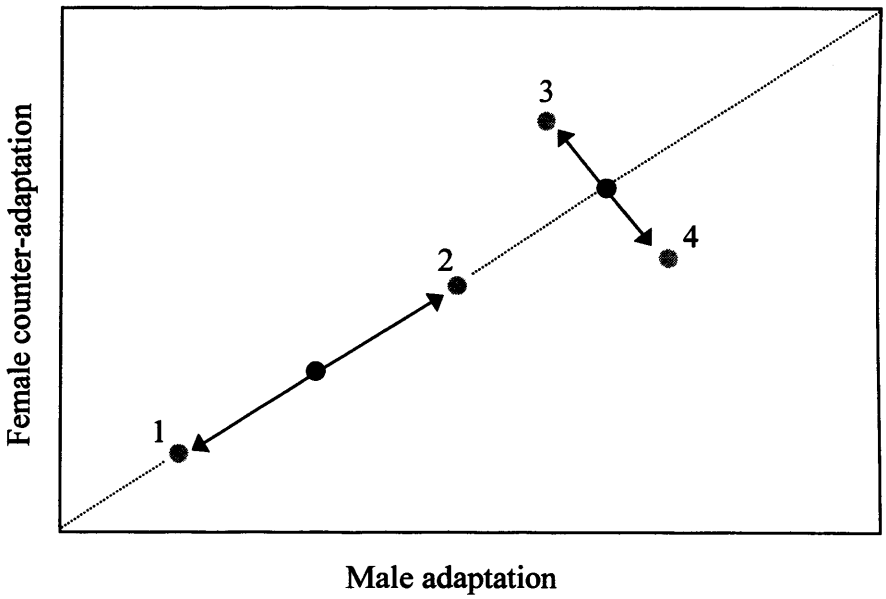


Figure 7.10 Sexually antagonistic co-evolution is expected to be hidden because adaptations in one sex should be balanced by counter-adaptation in the other. Mutual and matched de-escalation (1) or escalation (2) of adaptations in the sexes should not therefore affect the outcome of antagonistic interactions. However, when this arms race is not balanced perfectly, co-evolution will lead to either females (3) or males (4) gaining a relative advantage in conflict (from Arnqvist & Rowe 2002).

It is possible that this is the case in coelopids, whereby neither sex is currently gaining an advantage over the other and that the escalation or de-escalation of female rejection success rate and male harassment rate are in equilibrium, thereby masking any effects of sexually antagonistic co-evolution (Arnqvist & Rowe 2002).

It is clear from the results of this study that, though sexually antagonistic co-evolution is a major factor driving evolution, with the potential to influence a variety of interactions between the sexes (e.g. Rice 1996; Gavrilets et al. 2001), it is an extremely difficult process to provide evidence for. As pointed out by Arnqvist and Rowe (2002), direct empirical evidence for a primary role of arms races in the evolution of sexual interactions in natural systems is scarce due to the undetectable nature of antagonistic interactions as demonstrated by Figure 7.10. Some evidence does exist for the co-evolution of behaviours within a shared mating system and for antagonistic co-evolution in the relative abilities of each sex to control the outcome of pre-mating struggles in water striders (Gerridae)(Rowe & Arnqvist 2002), which have a similar mating system to coelopids. Though this study provides no such definite evidence, the possibility that sexually antagonistic co-evolution exists in coelopids cannot be dismissed. The presence of a trade-off between size and male harassment intensity may be responsible for constraining any effects of antagonistic co-evolution, in addition to the masking effects of adaptive and co-adaptive equilibrium in sexually antagonistic traits.

This study is the first to investigate sexually antagonistic co-evolution in coelopids, and it has yielded a number of intriguing, if perplexing, results. Hopefully it will open up further debate in this field, broaden the field of coelopid research and provide a good platform for further investigation into sexually antagonistic co-evolution in coelopids.

Chapter 8. Post-copulatory sexual selection in *Coelopa frigida*

8.1 Introduction

As discussed in Chapter 1, post-copulatory sexual selection has been found to be an increasingly important force in shaping the evolution of mating systems, particularly in species that are known to mate multiply within a single reproductive cycle (Birkhead & Møller 1998; Simmons 2001; Birkhead & Pizzari 2002). There is great potential for post-copulatory sexual selection in *C. frigida* due to the exceptionally high re-mating frequency exhibited which is a consequence of their convenience polyandry-based mating system, as discussed in Chapter 5. Though studies involving pre-copulatory sexual selection in *C. frigida* are numerous (e.g. Butlin et al. 1982b; Gilburn et al. 1992; Gilburn & Day 1994a, b, c; Crean & Gilburn 1998; Crean et al. 2000; Dunn et al. 1999; this thesis), few studies have yet determined the extent to which post-copulatory sexual selection may play a role in the mating system of this species (but see Day & Gilburn 1997).

Of the two main components of post-copulatory sexual selection, sperm competition (Parker 1970a; Smith 1984; Birkhead & Møller 1998) and cryptic female choice (Thornhill 1983; Eberhard 1996), less is known about the mechanisms underlying cryptic female choice and the field of research is subject to controversy (see Birkhead 1998; Pitnick & Brown 2000; Birkhead 2000). However, several insect taxa have been shown to exhibit cryptic female mate choice (e.g. Thornhill 1983; LaMunyon & Eisner 1993; Bloch Qazi et al. 1998; Hellriegel & Ward 1998; Hellriegel & Bernasconi 2000; Johnson et al. 1999; Edvardsson & Arnqvist 2000; Ward 2000; Tallamy et al. 2002). There are various types of data thought to verify the presence of cryptic female choice (reviewed in Eberhard 1996 and Eberhard 1997). These include data regarding the evolution of male genital morphology (e.g. Eberhard 1985; Danielsson & Askenmo 1999), spermathecal morphology and it's role in female-mediated differential sperm storage (e.g. Ward 1993; Hellriegel & Bernasconi 2000; Burger et al. 2003; Cordoba-Aguilar et al. 2003) and cryptic female choice with respect to the qualities of male seminal products (e.g. LaMunyon & Eisner 1993, reviewed in Eberhard 1996). In addition, it has been suggested that the bias towards certain males achieving paternity over other males due to cryptic female choice may be enhanced by pre-copulatory female choice, whereby both processes

work in concordance to ensure maximum viability of the offspring produced (Eberhard 1996; Lewis & Austad 1994).

Pre-copulatory sexual selection for large male size in *C. frigida* appears to have occurred as a side effect of sexual conflict, with large males gaining an advantage through their greater ability to withstand the female rejection response (Gilburn et al. 1992; Crean & Gilburn 1998; Crean et al. 2000). However, male willingness to mate decreases with increasing male size, which may pose some limitations on the extent to which sexual selection can favour large males (Dunn et al. 1999). In *C. frigida*, there is a great deal of variation in male size, mostly caused and maintained by the αβ inversion system on chromosome I (Butlin et al. 1982a; Gilburn & Day 1994a, b, c; Wilcockson et al. 1995; Day et al. 1996; Dunn et al. 1999).

In *C. frigida*, heterokaryotypes (αβ) have been found to have a greater egg-to-adult viability than homokaryotypes (αα or ββ), particularly under increased larval density (Day et al. 1983; Butlin et al. 1984; Butlin & Day 1985, 1989). In response to this, Gilburn and Day (1996) suggested that homokaryotypic females may have evolved disassortative mating with males of the opposite inversion karyotype in order to produce heterokaryotypic offspring. However, due to the presence of the inversion system on chromosome I and the potential for pre-copulatory choice being severely compromised by coercive male behaviour, there is a high potential for female *C. frigida* to benefit from mating multiply and selecting sperm via post-copulatory cryptic female choice. The inversion system may therefore provide a considerable selective advantage for females to exhibit mate choice on the basis of male karyotype in order to produce fitter heterokaryotypic offspring. Little evidence has yet been found of pre-copulatory female mate choice on the basis of inversion karyotype in *C. frigida*, with the exception of one study which demonstrated that females with the ββ karyotype have a mating preference for αα karyotype males, even when the effect of karyotype on size has been removed (Butlin et al. 1982b). This would suggest that females might be capable of detecting the karyotype of males at some level. A study carried out by Crocker and Day (1987) found that females were more likely to successfully reject males of the same karyotype, and that copulation was more likely to occur in pairings of different karyotypes. However, they also pointed out that females presented with males of the same karyotype, but of a larger size than

themselves, may have difficulty in rejecting them, thereby introducing error into the results.

Despite a large male mating advantage in *C. frigida*, most of the wild populations found in Britain exhibit an excess of heterokaryotypic individuals. This is surprising, since a large male mating advantage would be expected to create a bias towards populations with a greater number of αα homokaryotypes. This suggests that there is indeed a strong heterokaryotypic advantage i.e. heterosis, present in this species (Butlin et al. 1982a). The large number of heterokaryotypic adults in wild populations could be as a result of two systems working simultaneously: firstly, that more heterokaryotypes are able to survive to adulthood than homokaryotypes, due to their increased fitness (Butlin et al. 1984), and secondly, that there is some mechanism at work increasing the numbers of heterokaryotypic offspring produced by females over homokaryotypic offspring e.g. a deficiency in the number of matings between homokaryotypes (Crocker & Day 1987) and cryptic female choice for heterokaryotypic offspring.

In light of this phenomenon, the first major aim of this study was to investigate whether homokaryotypic female *C. frigida* are able to distinguish between the sperm within a single ejaculate produced by a heterokaryotypic male, in order to produce a greater number of heterokaryotypes than homokaryotypes and therefore increase the overall fitness of their offspring.

The second component of post-copulatory sexual selection, sperm competition, has been the most documented to date. Sperm competition has been found to be prevalent in many taxa (Smith 1984; Birkhead & Møller 1992, 1998; Eberhard 1996) and is common in insects (reviewed by Simmons & Siva-Jothy 1998), often being reported in the form of last male sperm precedence. The extent of last male sperm precedence within a species, usually termed the P_2 value, can be defined as the mean proportion of eggs fertilised by the second of two males that mate with a single female (Boorman & Parker 1976). Variation in P_2 values has predominantly been ascribed to sperm competition in many studies (e.g. Birkhead & Hunter 1990; Simmons & Siva-Jothy 1998; Cook et al. 1997). Investigations into variation in P_2 values can reveal the mechanisms influencing sperm precedence and male fertilisation success.

A variety of factors have been found to affect P_2 values in insect taxa. Mating order is considered to be one of the governing features affecting male fertilisation success, particularly in insects, as many taxa exhibit last male sperm precedence, whereby the last male to mate with a female fertilises more than 50% of the eggs (e.g. Parker 1970b, 1984; Gwynne 1984; Ridley 1989; Birkhead & Hunter 1990; Lewis & Austad 1990; Seth et al. 2002; Drnevich 2003). The extent of last male sperm precedence can vary greatly within many species however (reviewed in Lewis & Austad 1990), for example, the P_2 values found in the yellow dung fly, *Scathophaga stercoraria*, can range from 0.23 to 1.00 (Parker 1970b) and from 0.00 to 1.00 in the rove beetle, *Aleochara curtula* (Benken et al. 1999). This variation indicates the presence of other influences on male fertilisation success (reviewed in Simmons 2001), such as the relative body size of the two males (e.g. McLain 1980; Lewis & Austad 1990, 1994; Wedell 1991; Simmons & Parker 1992; LaMunyon & Eisner 1993; Ward 1993; Otronen 1994, 1998; Ueno 1994; Sakaluk & Eggert 1996; Simmons et al. 1996; Bissoondath & Wiklund 1997; Arnqvist & Danielsson 1999) and copulation duration (e.g. Parker 1970b; Dickinson 1986; Sawada 1998; Zhu & Tanaka 2002). The age of the males mated to the female is also known to affect last male sperm precedence in insects, for example, in *Drosophila melanogaster*, last male sperm precedence decreases with increasing female age (Mack et al. 2003). In addition, it has been found in the noctuid moth, *Heliothis virescens*, that the second male to mate is more likely to achieve sperm precedence if it is older than the female's first mate (LaMunyon 2001). The time interval between matings has also been found to have a great effect on male fertilisation success (e.g. Butlin 1983; Radwan 1997; Kraaijeveld-Smit et al. 2002). Simmons' review (2001) highlighted the fact that P_2 values increase with longer intervals between matings in the majority of insects. It is possible that this occurs as a result of sperm loss over time from the females' spermathecae (Tsubaki & Yamagishi 1991; Yamagashi et al. 1992; Colegrave et al. 1995).

Last male sperm precedence has previously been reported in *C. frigida*, with P_2 values increasing with a corresponding increase in time interval between matings (Thompson 1951; Burnet 1960; Butlin 1983). However, no further investigations have been done to look at other factors influencing male reproductive success via sperm competition. In addition to this process, the inversion system on chromosome I affects many traits including egg-to-adult viability via heterosis. Consequently, there is also considerable potential for cryptic female choice to operate on the basis of sperm karyotype,

as discussed above. There is also the possibility that there may be an interaction between cryptic female choice and last male sperm precedence, whereby factors such as the time interval between matings and the karyotype of males and females may affect which process determines the paternity of the offspring produced.

The second chief purpose of this study was therefore to determine whether the time interval between copulations and the chromosome I inversion system had an effect on the processes involved in post-copulatory sexual selection in *C. frigida*, and whether there was an interaction between the processes of last male sperm precedence and cryptic female choice in this species.

8.2 Evidence for cryptic female mate choice after copulation with a single male

The aim of this experiment was to determine whether homokaryotypic females are able to preferentially select sperm of the opposite homokaryotype from a single copulation with a heterokaryotypic male, in order to produce a greater than expected proportion of heterokaryotypic offspring. Under conditions where the success of sperm to fertilise the eggs is random, a 1:1 ratio of homokaryotypic to heterokaryotypic offspring would be expected. If females were to exhibit sperm selection in favour of producing heterokaryotypic offspring, a significantly biased ratio towards heterokaryotypes would be expected.

8.2.1 Materials and methods

Homokaryotypic lines B1 (isokaryotypic for the α form) and D1 (isokaryotypic for the β form) were used in this experiment. These lines were crossed using standard culture techniques, to create heterokaryotypic BD individuals. Males and females were separated under CO_2 anaesthesia after eclosion and placed into same-sex communal bottles with 5% sucrose solution. These bottles were stored at 5°C, for a maximum of 10 days, until required.

The following combinations of mating pairs were put into individual pots containing freshly minced seaweed:

① αβ ♂ x ββ ♀ (experimental cross)

② αβ ♂ x αα ♀ (experimental cross)

③ ββ ♂ x αβ ♀ (control cross)

④ αα ♂ x αβ ♀ (control cross)

In the case of experimental crosses 1 and 2, the female may utilise either α or β sperm for fertilisation of her eggs. The results of the experimental crosses were substantiated by the implementation of two control crosses (control crosses 3 and 4), whereby females are given homokaryotypic males and are therefore not given a choice of sperm karyotype.

The pots were kept under standard culture conditions for 3 to 4 days until the larvae produced had reached a length of approximately 5mm and were sturdy enough not to be damaged when removed from the pots. After removal, the larvae were placed into powder rounds with excess minced seaweed. The excess seaweed ensured that larval competition was kept to a minimum and conditions were optimal for survival and growth (Butlin & Day 1984). Any remaining adult flies were also removed at this stage.

The powder rounds were kept under standard culture conditions until the offspring had developed fully. Offspring were collected within 24 hours of eclosion and stored at -20°C in individual 1.5 ml microcentrifuge tubes to prevent enzyme degradation. Once all of the offspring had eclosed (usually after a period of approximately 4 days), the genotypes of all female progeny were determined by means of starch gel electrophoresis and staining for *Adh* alleles. Only female offspring were included in the study, as they are under a much lower intensity of selection pressure during the larval stages than males (Butlin et al. 1982b). Females are generally similar in size and have a comparable development time to each other. The number of homo- and heterokaryotypic female offspring was recorded.

8.2.1.1 Statistical analyses

Chi-square analyses were used to determine any deviation of the observed frequencies of homo- and heterokaryotypic offspring from their expected frequencies. These were carried out incorporating Yates' Correction for Continuity, as there was only one degree of freedom.

8.2.2 Results

The descriptive statistics for this study are presented in Table 8.1. When chi-square analyses were carried out on the frequencies of heterokaryotypic female offspring produced by each cross, significantly greater than expected frequencies of heterokaryotypic offspring were found in experimental crosses 1 and 2. There were no significant differences found between the observed and expected frequencies of heterokaryotypic offspring for control crosses 3 and 4 (see Table 8.2).

Table 8.1 Mean total of all female offspring and the number of homo- and heterokaryotypic female offspring produced by each cross (standard error of means given in parentheses, n = number of trials per cross).

		All female offspring		**Homokaryotypic female offspring**		**Heterokaryotypic female offspring**	
Cross	**n**	**Total**	**Mean (S.E.)**	**Total**	**Mean (S.E.)**	**Total**	**Mean (S.E.)**
1	13	234	18.0 (3.38)	91	7.0 (2.00)	180	13.9 (3.37)
2	3	54	18.0 (3.06)	30	10.0 (4.36)	59	19.7 (8.74)
3	12	348	29.0 (4.14)	163	13.6 (2.36)	180	15.0 (2.40)
4	5	93	18.6 (4.17)	49	9.8 (1.69)	44	8.8 (2.63)

Table 8.2 Results of chi-square analyses of the frequencies of heterokaryotypic female offspring (with Yates' Correction for Continuity) for each cross. Values marked ** are significant at the 0.01 level.

Cross	Observed frequency	Expected frequency	χ^2_1
1	180	135.5	28.576**
2	59	44.5	8.809**
3	180	171.5	0.746
4	44	46.5	0.172

When the results produced by each experimental cross were compared with their corresponding control cross, significant positive associations were found between the number of heterokaryotypes produced and the experimental crosses. Conversely, significant negative associations were found between the number of heterokaryotypes produced and the control crosses (see Table 8.3). This confirms that the control crosses were successful in acting as controls, and that the proportional differences between the number of heterokaryotypes and homokaryotypes produced by each experimental cross cannot be accounted for by sampling error alone.

Table 8.3 Results of chi-square analyses of the frequencies of heterokaryotypic female offspring (with Yates' Correction for Continuity) for each experimental cross compared with its corresponding control cross. The observed frequencies of heterokaryotypes in relation to the expected frequencies are given in parentheses. Values marked ** are significant at the 0.01 level and those significant at the 0.05 level are marked *.

Experimental cross	x	Control cross	χ^2_1
Cross 1 (O > E)	x	Cross 3 (O < E)	11.565**
Cross 2 (O > E)	x	Cross 4 (O < E)	5.916*

8.3 The effect of an inversion system and inter-copulation interval on post-copulatory sexual selection

This experiment was carried out to determine whether the time interval between mating with two males of different karyotypes has an effect on last male sperm precedence and whether cryptic female choice for heterokaryotypic offspring interacts with last male sperm precedence. The order in which the males mate with the female was altered so cryptic female mate choice could operate either in concert or antagonistically with last male sperm precedence in terms of the resulting karyotypes of the offspring produced i.e. when the second male to mate with the female is of the opposite karyotype in relation to the female, both cryptic female choice and last male sperm precedence would be expected to act in concert towards producing heterokaryotypic offspring. Conversely, when the second male to mate with the female is of the same karyotype as the female, the female would be expected to preferentially select sperm from the first male to mate to produce heterokaryotypic offspring, therefore acting antagonistically with last male sperm precedence.

8.3.1 Materials and methods

Homokaryotypic lines B1 and D1 were used in this experiment. Flies were a maximum of 7 days old, and had been stored at 5°C until 48 hours before the experiment began. Since last male sperm precedence has been found to decline as female age increases (Mack et al. 2003), females of an intermediate age were used. Virgin females from both homokaryotypic lines were individually placed into a 1.5 ml microcentrifuge tube (Fisher Scientific UK. Ltd, Loughborough) with two males consecutively until they mated with each male for up to 5 minutes. One of the males was of the same homokaryotype as the female and the other of the opposite homokaryotype. The males were restricted to mating with the female for a maximum of 5 minutes to limit any chance that variation in fertilisation success was due to copulation duration, though these factors have not been found to be associated to date. Microcentrifuge tubes were used rather than standard mating chambers because they reduced the ability of the females to successfully reject the males and the close proximity of the male to the female appeared to encourage the males to mount the females. The order in which the two males were presented varied between females, as did the time interval between matings, whereby the second male was either

introduced to the female immediately after the first male had finished copulating or after a period of 24 hours. There were eight combinations of crosses in total (see Table 8.4).

In the trails where the second male was introduced immediately after the first, the female was removed from the microcentrifuge tube and put immediately into a single pot containing freshly minced seaweed. In trials where the second male was introduced 24 hours later, the female was removed from the microcentrifuge tube and put into a single pot containing cotton wool soaked in 5% sucrose solution. The reason females were kept with sucrose as a source of nourishment and moisture rather than seaweed is that the seaweed acts as a cue for oviposition (Dunn et al. 2001). The pots containing the females were then left for 24 hours at 25°C under 12hr light-dark conditions in the intervening period between mating with the first male and the second male. When the second mating had taken place, the female was removed and put into a pot containing minced seaweed.

Once females had been put into pots of seaweed after mating with two different males, they were left for approximately three days at 25°C under 12hr light-dark conditions to allow them time to oviposit. The eggs and any larvae present in each pot were subsequently transferred to a powder-round containing excess minced seaweed. The powder-rounds were stoppered using non-absorbent cotton wool and the larvae were left to develop to adulthood under standard culture conditions.

Adult flies were collected within 12 hours of eclosion and siblings were stored together in collection bottles with 5% sucrose solution at a temperature of 4°C until all the flies from each powder-round had finished eclosing.

Female offspring were then separated from males using CO_2 anaesthesia and their genotypes determined using starch gel electrophoresis and staining for *Adh* alleles. Only female offspring were used to determine P_2 values in this study to reduce the strong selective effects associated with size on egg-to-adult survival of males, whereby larger males have a longer development period than smaller males and are therefore more vulnerable to competition and environmental changes than smaller males (Butlin et al. 1982b). The selective pressures are thought to be less in female egg-to-adult development since females are of a similar size and hence have very similar development times.

The proportion of females fathered by the second male to mate (P_2) was then calculated from the genotypes of the offspring. Families that contained less than 10 female offspring were disregarded. In total 42 families were included in the dataset. The inversion karyotypes of 1909 female offspring were determined.

Table 8.4 The eight combinations of crosses used in the study.

Combination	Cross	Time interval between matings
1	αα ♀ x αα ♂ → ββ ♂	Immediate
2	ββ ♀ x ββ ♂ → αα ♂	Immediate
3	αα ♀ x ββ ♂ → αα ♂	Immediate
4	ββ ♀ x αα ♂ → ββ ♂	Immediate
5	αα ♀ x αα ♂ → ββ ♂	24 hours
6	ββ ♀ x ββ ♂ → αα ♂	24 hours
7	αα ♀ x ββ ♂ → αα ♂	24 hours
8	ββ ♀ x αα ♂ → ββ ♂	24 hours

8.3.1.1 Statistical analyses

The data were analysed using general linear models (GLM) of the number of offspring fathered by the two males. An inherent problem encountered with P_2 data is that they are not normally distributed and have unequal variances. The arcsine transformation typically used with proportional data does not solve this problem. Instead, a binomial error distribution was used. Since there was a considerable overdispersion generated, the F-values were adjusted by the overdispersion parameter in all models. Akaike's Information Criterion (AIC) was used to generate models of best fit (Akaike 1987). In both models all terms were retained. A GLM with a normal error distribution was used to model the total number of offspring produced by each female.

8.3.2 Results

The P_2 values obtained for each of the 42 families are shown in Appendix 8.1. The inversion karyotype of the female did not affect P_2 value ($F_{1,34} = 0.00$, $p = 0.99$), nor did the karyotype of the second male ($F_{1,34} = 1.17$, $p = 0.29$). There was also no association between time interval between matings and P_2 value ($F_{1,34} = 0.33$, $p = 0.57$). There were no significant two-way interaction terms. However, there was a significant three-way interaction term between the karyotype of the female, the karyotype of the second male and the time interval between matings ($F_{1,34} = 5.69$, $p < 0.05$), with low P_2 values found when the second male was of the same karyotype as the female and when matings occurred in rapid succession (see Figure 8.1)

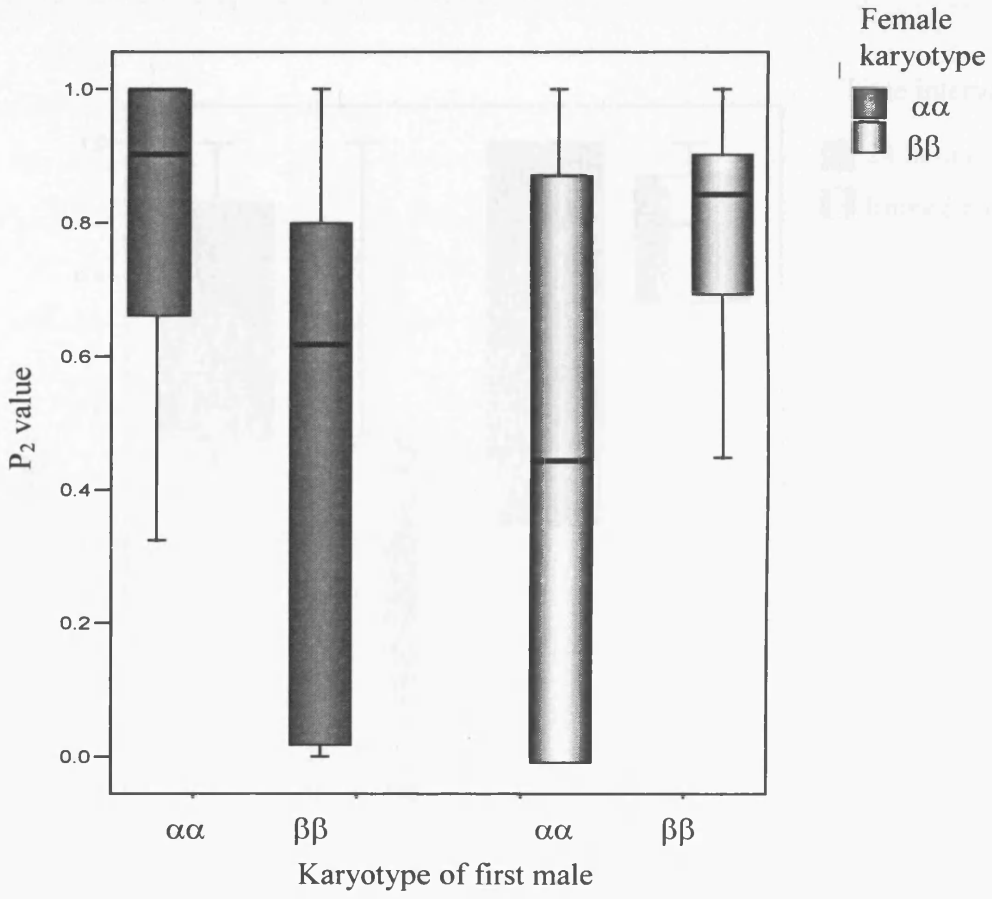


Figure 8.1 A box plot showing the median, quartiles and range of the P_2 values for the two female karyotypes when mated to a male of the same or opposite karyotype first.

As neither female karyotype nor the karyotype of the second male affected P_2 value, the analysis was simplified by pooling the data for the two female homokaryotypes. The data were then analysed using a new independent variable - the order of matings (i.e. whether the second male was of the opposite or same homokaryotype as the female). This model showed that neither the time interval between matings ($F_{1,38} = 0.08$, $p = 0.79$) nor the order of matings ($F_{1,38} = 2.36$, $p = 0.13$) affected P_2 value. However, a significant interaction term ($F_{1,38} = 5.40$, $p < 0.05$) was found between time interval and order of matings, with lower P_2 values found when the second male was of the same homokaryotype as the female and the second mating immediately followed the first. Indeed, P_2 values below 50% (first male sperm precedence) were typically found when the first male was of the opposite karyotype to the female and the second mating immediately followed the first (see Figure 8.2).

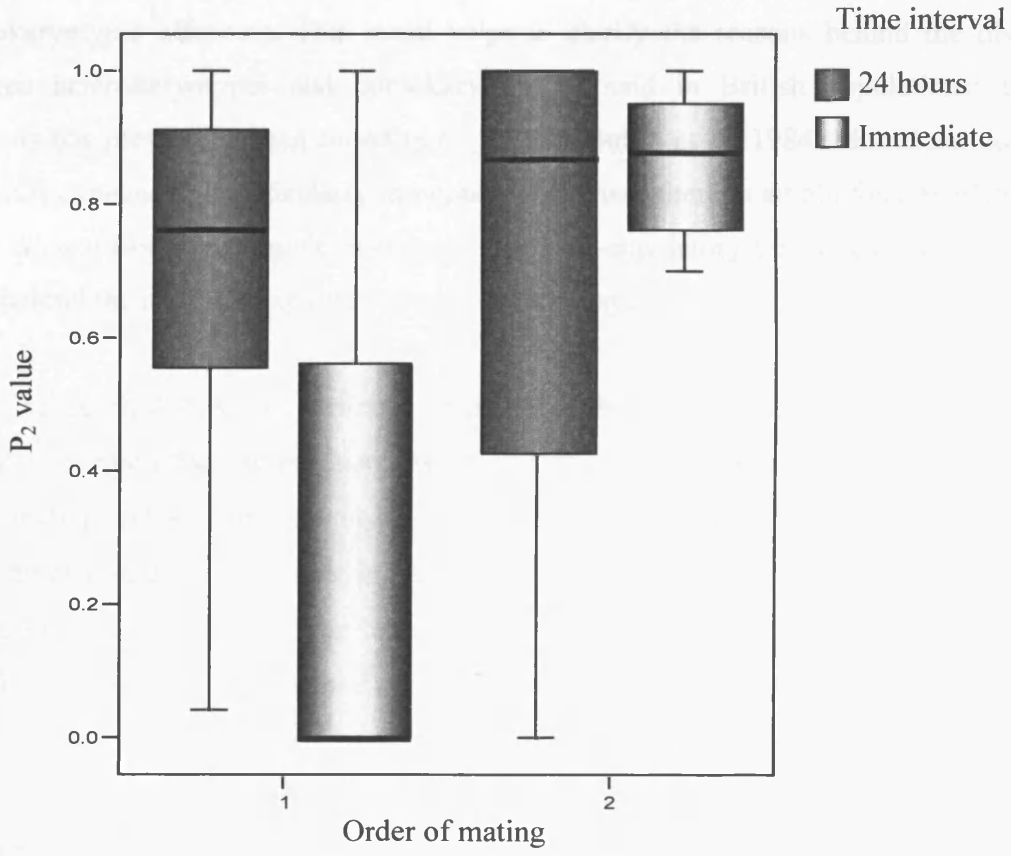


Figure 8.2 A box plot showing the median, quartiles and range of the P_2 values for the offspring of females when mated to a male of the opposite homokaryotype first or second and also separated by the time interval between matings.

The inversion karyotype of the female was not associated with the number of female offspring produced ($F_{1,34} = 0.01$, $p = 0.92$), or the karyotype of the first male to mate $F_{1,38} = 0.08$, $p = 0.79$). This was also the case for the karyotype of the first male to mate and the number of female offspring produced ($F_{1,34} = 0.01$, $p = 0.93$). There was also no association between time interval between matings and number of female offspring produced ($F_{1,34} = 1.01$, $p = 0.32$). In addition, there were no significant interaction terms associated with the number of female offspring.

8.4 Discussion

The experiments carried out for this investigation into post-copulatory sexual selection in *C. frigida* have generated some thought-provoking results. The first study presents clear evidence that females of this species may have the ability to select sperm from within the ejaculate of a single heterokaryotypic male to produce fitter heterokaryotypic offspring. This result helps to clarify the reasons behind the disparity between heterokaryotypes and homokaryotypes found in British populations: though heterosis has previously been found in *C. frigida* (Butlin et al. 1984), this alone does not explain the inequality, particularly in populations where there is ample food available and larval competition is negligible. It appears that post-copulatory female choice may be the force behind the maintenance of the excess of heterozygotes.

One result that was of particular interest was the greater than expected proportion of heterokaryotypic offspring produced by cross 2. It could be assumed that due to the large male mating advantage present in *C. frigida*, αα homokaryotype females might preferentially select sperm carrying the α rather than the β karyotype, leading to the production of a majority of larger αα homokaryotypic offspring. A female preference for large males seems to make sense under Fisherian models of selection, and a correlation between the presence of large male size and female preference for this character has previously been observed in *C. frigida* (Gilburn et al. 1993). However, this preference would lead to an overall decrease in the viability of any progeny (Butlin et al. 1984). The results of the experiment show that females select sperm which make the progeny more viable in natural selective terms, following a 'good genes' model, thereby incurring Fisherian costs (Gilburn & Day 1996). These findings also corroborate research that has

found both Fisherian and 'good genes' models of selection working within different populations of *C. frigida*, with ecological factors being the determining force in the relative contribution of each model to the maintenance of female mating preference (Gilburn & Day 1994b). It was also suggested that in tidal populations, such as those found at St Mary's Island, αα karyotype females show either no mating preference at all or a preference for small males and ββ females show a consistent preference for large males, indicating that the Fisher process may not operate in tidal populations which suffer genetic bottlenecks (Gilburn & Day 1994b).

Female *C. frigida* possess three spermathecae (Hennig 1958), two of which share a common branched duct (McAlpine 1991; Day & Gilburn 1997) and sperm are known to enter and exit via the same route (Foster 1989). The existence of several structures in which to store sperm raises the possibility that females may be able to store different types of sperm from within a single ejaculate and between ejaculates from different males, in different parts of the spermathecae, allowing her to exercise choice in which sperm to use in the fertilisation of her eggs (Ward 1998). The female yellow dung fly, *Scathophaga stercoraria*, also has multiple spermathecae. This has prompted a number of researchers to investigate female-mediated sperm storage and utilisation in this species (e.g. Ward 1993, 1997; Simmons et al. 1996; Otronen et al. 1997; Hellriegel & Ward 1998; Hellriegel & Bernasconi 2000). There was some initial controversy surrounding the early studies in whether the females actually preferentially used the sperm from larger males or if the results were due to the larger males' ability to transfer greater numbers of sperm to the female (see Ward 1993; Simmons et al. 1996). However, further studies have shown that during copulation females are able to store competing males' sperm differentially between the spermathecae, then use muscular activity to move sperm to desired locations (Hellriegel & Bernasconi 2000). Other studies have shown that females play an active role in moving sperm from the site of deposition to the site of storage in other species of insect, such as the red flour beetle, *Tribolium castaneum* (e.g. Bloch Qazi et al. 1998). This body of research creates an opportunity to hypothesise whether similar processes occur in *C. frigida*. Though the morphology of the spermathecae of this species has been examined to a certain extent, further knowledge regarding its potential role in post-copulatory sexual selection in unknown. To remedy this, a study was planned to investigate sperm storage and allocation in *C. frigida* for inclusion in this thesis. Unfortunately, the study had to be

abandoned when the difficulties involved in dissecting out and separating the spermathecae, without mixing the ejaculates of competing males, proved to be impossible.

A question that needs to be addressed in light of the results generated by this study is: why are the offspring produced by the females not all heterokaryotypic? The maintenance of homokaryotypes in the population is probably a consequence of several processes. Firstly, each female was only mated to one male and it is possible that the male did not transfer enough sperm of the preferred karyotype in his ejaculate to fertilise the female's egg load. As a result, the female utilised less desirable sperm to ensure that as many of her eggs were fertilised as possible. In wild populations, females are known to mate many times more than is needed to fertilise all of their eggs (see Chapter 5), so this would not necessarily be a determining factor under natural conditions. Secondly, there may be some benefit to females in producing homokaryotypic offspring as 'bet-hedging', particularly in populations that live in unpredictable environmental conditions. It has been suggested that smaller flies may be less prone to being swept away by high tides (Day et al. 1980). In addition, smaller males develop at a greater rate than larger males, suggesting that ββ homokaryotypes may be at an advantage in gaining access to females before their larger rivals have eclosed (Dunn et al. 1999). Smaller males are also more active and willing to mount females than larger males (Dunn et al. 1999; personal observation). It is possible that large αα homokaryotypic offspring may be produced by Fisherian selection still acting in populations. It could also be the case that the female is not in complete control over the paternity of her offspring and that sperm from the same karyotype as the female may be able to avoid the mechanisms used by the female to select sperm. There is also potential for the asymmetric storage of sperm, due to sperm displacement, to result in the production of homokaryotypic offspring (Hellriegel & Ward 1998). Furthermore, this study suggested that females are able to determine the rate at which different sperm may be stored at a particular location, resulting in a form of sperm precedence.

The second study investigated the effect of time interval between copulations and inversion karyotype on post-copulatory sexual selection in *C. frigida*. Both the time interval between copulations and the order of polyandrous copulations with males of different chromosomal karyotypes interacted in their effects on P_2 value. First male sperm precedence occurs when males mate in rapid succession and the second male has the same inversion karyotype as the female. The consistent finding of last male sperm precedence

when there is an interval of 24 hours between copulations, regardless of the mating order, implies that sperm loss probably occurs from the female within 24 hours of mating, as has been found in other species such as the melon fly, *Bactrocera cucurbitae* (Yamagishi et al. 1992). The loss of sperm from the females' spermathecae between mating with the first and second male may be due to sperm death and degradation, which is known to occur in several species (Cunningham et al. 1971; Yamagishi et al. 1992). The occurrence of last male sperm precedence when there is a 24 hour time interval between matings is not surprising in a species in which females mate many times a day (see Chapter 5), as there is little need to store sperm for long periods of time. It is also possible that *C. frigida* sperm are very short-lived since this species exhibits last male sperm precedence and re-mates frequently, further supporting the idea of sperm loss due to sperm death.

The finding that the pattern of paternity biases varies with the time interval between matings suggests that population density may have a major impact on levels of post-copulatory sexual selection. This, in turn, implies that population density may have a major effect on the evolution of the *C. frigida* mating system, as this will determine the frequency with which females mate and therefore the opportunity for cryptic female choice and sperm competition to operate. At higher population densities, such as many of those found in Britain, the time interval between matings will be relatively short, resulting in greater potential for adaptive paternity biases. In lower density populations last male sperm precedence would be expected to be the main mechanism determining the paternity of offspring. Consequently, adaptive paternity biases would be expected to contribute more to the heterokaryotypic excess in higher density populations. As mentioned in the introduction of this chapter, high-density populations of *C. frigida* show an excess of heterokaryotypic individuals, which has previously been assumed to be associated with increased egg-to-adult viability of heterokaryotypes at higher larval density (Butlin et al. 1982, 1984; Day et al. 1983). This study therefore provides an additional explanation for the association between population density and heterokaryotype excess. The relative importance of these two mechanisms in generating this excess could be determined experimentally since the extent of post-copulatory sexual selection should be dependent on adult density, whereas variation in the relative egg-to-adult variabilities of the different karyotypes should be dependent on larval density.

It has been suggested that laboratory studies of post-copulatory sexual selection in which females are mated with only two males test a simple situation (Simmons 2001), whereas in many species, including *C frigida*, females are known to mate with more than two males. In addition, studies that have used more than two males have found evidence that last male sperm precedence may break down in some species (Zeh & Zeh 1994; Radwan 1997; Drnevich 2003), though this is not always the case (see Radwan 1997). However, last male sperm precedence appears to play a minor role in high-density populations of *C. frigida*, so this criticism maybe of little relevance. The ability to check the karyotype of individuals via the inversion system on chromosome I and staining for *Adh* alleles, allows us to determine the paternity of an individual from a choice of two known homokaryotypic fathers. As yet, there are no alternative ways in which to carry out paternity analyses in *C. frigida* with offspring that may have more than two possible fathers. Paternity analyses using molecular techniques need to be developed in order for studies involving the paternity of offspring produced by females mated to more than two males to be achieved. This topic is discussed in greater detail in the following chapter.

Many studies carried out to determine the patterns of sperm usage in females mated with multiple males make use of the sterile male technique (Knipling 1955; Parker 1970a; Boorman & Parker 1976). Females are mated with two males, one of which has been sterilised. This is usually achieved by irradiation, for example, with gamma rays from ^{60}Co (e.g. Ueno & Itô 1992; Radwan 1997; Rugman-Jones & Eady 2001; Kraaijeveld-Smit et al. 2002). Though sterilised males are still able to fertilise eggs, embryos die in the early stages of development due to an accumulation of lethal mutations (Parker 1970). The offspring that are produced are therefore the progeny of the unsterilised male. Though this technique appears to have been successful in a number of studies, it was not used in this investigation. The presence of the αβ inversion system provided an opportunity to carry out the experiment without the use of radiation, as it acted as a marker for paternity. In addition, there is some controversy over the use of the sterile male technique, particularly in studies involving inter-copulation interval (Rugman-Jones & Eady 2001), due to the possible degradation of sterility over time. Other experiments have used genetic markers (Zhu & Tanaka 2002) and mutants (Brakefield et al. 2001) effectively, so the use of the inversion system was deemed appropriate for this study. In addition to its use as a marker, the inversion system was also the probable source of the post-copulatory sexual selection observed in *C. frigida*.

This chapter attempts to elucidate some of the questions surrounding post-copulatory sexual selection in the highly promiscuous species *C. frigida*. The presence of a heterotic system, linked with the αβ chromosome I inversion, provides an ideal opportunity for the study of post-copulatory mechanisms such as sperm competition and cryptic female choice. The two studies show that females are able to select sperm of the opposite karyotype within and between the ejaculates of males in order to produce a majority of heterokaryotypic offspring. It has also been demonstrated that last male sperm precedence occurs when matings are separated by 24 hours, however in high-density wild populations this mechanism is almost certainly inconsequential and breaks down in the face of the extraordinarily high remating frequency of females. This therefore indicates that cryptic female choice may be the greatest determinant of male fertilisation success in this species. It could be argued that there is the potential for sperm competition, working independently of cryptic female mate choice, to produce the results yielded by these studies and that the competitive ability of sperm may be female karyotype-dependent. Indeed it has been found that the success of a particular males' sperm is female genotype-dependent in *Drosophila* (Clark et al. 1999). Currently there is no way of determining whether this is the case or not in *C. frigida* and the argument will persist while there is contention within the field concerning the experimental demonstration of cryptic female choice. However, it is only with further investigation into the mechanisms that differentiate between male and female-mediated processes of post-copulatory sperm use, that definitive answers will be achieved.

Chapter 9. A preliminary analysis of intraspecific genetic variation in British populations of *Coelopa frigida* using ISSRs

9.1 Introduction

Genealogical inference in natural populations, in the form of parentage analysis, has become one of the major components in the study of behavioural ecology and evolution, with the relatively new field of molecular ecology leading the way in the resolution of the many questions associated with this topic (Hughes 1998). The study of parentage using chromosomal polymorphisms was pioneered in the mid 1970s, using mainly *Drosophila* species (Anderson 1974; Milkman & Zeitler 1974). This was later followed by the development of techniques involving inter- and intraspecific allozyme variation in the 1980s. Allozymes are single-locus polymorphisms, which have often been used in past studies involving mating systems (e.g. Foltz & Hogland 1981; Meagher 1986) and multiple paternity (e.g. Ellstrand 1984). As practical methods were being developed for the study of parentage analysis, statistical techniques were also being constructed (Meagher & Thompson 1986; Meagher & Thompson 1987; Chakraborty et al. 1988). These studies were facilitated by the introduction of DNA fingerprinting, which was used extensively to study paternity in numerous bird species, leading to the modification of many ideas which had previously been formed about their reproductive behaviour (for examples see Birkhead & Møller 1992). More recent studies of paternity involving DNA-based markers such as RFLPs (restriction fragment length polymorphisms) and RAPDs (random amplified polymorphic DNA) have focussed on male mating success and reproductive tactics in species as diverse as the leafcutter ant, *Atta colombica* (Fjerdinstadt et al. 1998); fathead minnow, *Pimephales promelas* (Bessert & Ortí 2001); and the North Atlantic humpback whale, *Megaptera novaeangliae* (Nielsen et al. 2001).

Within the last ten years another type of molecular marker has been used in studies of genetic variation and paternity, predominantly in plant species, which is relatively inexpensive and provides reliable results. Intersimple sequence repeat (ISSR) markers are hypervariable nuclear markers generated from single primer polymerase chain reactions (PCR), where the primer is designed from a di- or tri-nucleotide repeat motif with an anchoring sequence (Gupta et al. 1994; Zietkiewicz et al. 1994). Regions amplified using

these primers represent the nucleotide sequence between two microsatellite priming regions, known as SSRs (simple sequence repeats), located on opposite DNA strands (reviewed in Wolfe et al. 1998). These regions are scattered uniformly throughout the genome, so the probability of amplifying an ISSR sequence using a polymerase enzyme such as *Taq*, is great enough that many polymorphic bands are produced (Tautz & Renz 1984; Condit & Hubbell 1991). Until recently the use of ISSR markers for investigating genetic variation and paternity had been limited to studies of plant populations (e.g. Wolfe et al. 1998; Ge & Sun 1999; Ye et al. 2004). However, a small number of genetic studies carried out in the last few years have used ISSRs in birds such as the snowy plover, *Charadrius alexandrinus* (Gorman 2000) and the comb-crested jacana, *Irediparra gallinacea* (Haig et al. 2003). Even more recently, a study was carried out using ISSRs to look at genetic variability in the tropical tasar silkworm, *Antheraea mylitta* (Chatterjee et al. 2004). Though a previous investigation into the application of ISSRs to insect cell lines had been carried out successfully (Grasela & McIntosh 2003), the study involving *A. mylitta* is one of the only published works using ISSRs in insect species to date.

Though *C. frigida* has been studied extensively in terms of its chromosome I inversion polymorphism system and the influence it has on factors such as egg-to-adult survival, longevity and fecundity (Butlin et al. 1982a, b; Butlin et al. 1984; Butlin & Day 1985), very few studies have focussed on genetic variation in this species and the further use of molecular techniques to carry out analyses of paternity. An investigation into intraspecific molecular variation within *C. frigida* populations in Britain and Sweden was carried out in 2002 using variation in the mitochondrial cytochrome oxidase II gene, which uncovered a clear differentiation at the molecular level between the two countries' populations and also within British populations (MacDonald & Brookfield 2002). However, no studies have yet implemented the use of ISSRs to study the genetic variation in *C. frigida* populations, or used this technique to analyse parentage. Paternity analysis would be an extremely useful tool in the study of factors such as last male sperm precedence, sperm competition and cryptic female choice in this species, as females are known to mate with many different males in the wild (Chapter 5). Current studies of post-copulatory sexual selection have used *Adh* alleles to determine the paternity of offspring (e.g. Chapter 8), but this has severely limited the number of males that can be mated to a single female within an experiment. This is because there are only two alleles that can be

easily used to create homoallelic populations (*Adh*-B and *Adh*-D alleles). A third allele (*Adh*-C) can also be used, but inbred populations are weak and difficult to establish and maintain (personal observation). In addition, this allele is associated with both the α and β forms of the inversion system, whereas the B and D alleles are in linkage disequilibrium with the inversion karyotype (Day et al. 1982). Other alleles (A, E and F) are present in natural populations, but these are relatively rare. This situation creates a maximum of two, perhaps three, males of differing allelic forms for use in experiments that require the paternity of resulting offspring to be known. As discussed in Chapter 8, it has been suggested in the past that laboratory studies in which females mate with two males test only a simple situation (Simmons 2001). Since *C. frigida* is known to mate with more than two males, experiments where many males are mated with a particular female would be of great interest and would mimic natural situations much more accurately than the present situation can. Therefore a reliable method of paternity analysis using molecular techniques should be developed in order to progress in this field of study.

The primary aim of this study therefore, was to assess the performance of ISSRs in detecting intraspecific genetic variation in several British populations of *C. frigida*, with a view to developing this technique further into a method for paternity analysis in this species.

9.2 Materials and methods

9.2.1 Collection and rearing of samples

Wild larvae of *C. frigida* were collected from five locations in the UK (see Chapter 3 for collection method):

① St Mary's Island, Tyne and Wear, England, UK (National Grid Reference: NZ 352 753 GB).

② Belhaven Bay, East Lothian, Scotland, UK (National Grid Reference: NT 657 788 GB).

③ Barns Ness, East Lothian, Scotland, UK (National Grid Reference: NT 719 768 GB).

④ Longhoughton Steel Promontory, Northumberland, England, UK (National Grid Reference: NU 269 155 GB).

⑤ Boulmer Haven, Northumberland, England, UK (National Grid Reference: NU 269 135 GB) (see Figure 9.1).

These were reared under standard laboratory conditions (see Chapter 3), with each population being reared in separate cages. Adult flies were collected on the day of eclosion, frozen immediately and stored individually in 1.5ml microcentrifuge tubes at -20°C until required. Only the first generation of flies were used to maintain as much genetic diversity as possible.

9.2.2 Preparation of flies for DNA isolation and purification

Individuals were sexed then snap frozen individually in liquid nitrogen to remove any phoretic mites present on the cuticle. A hand lens was then used to examine each fly and any mites remaining on the cuticle were removed using forceps. The flies were then snap frozen again and the abdomen of each fly was excised using a scalpel and discarded to reduce the chance of DNA contamination from any material ingested by the fly. Once the abdomens had been removed, the remaining body parts were stored individually in 1.5ml microcentrifuge tubes in liquid nitrogen until needed.

9.2.3 Isolation and purification of genomic DNA

A DNeasy® Tissue Kit (Qiagen Ltd., West Sussex, UK) was used to isolate and purify the genomic DNA from the stored heads and thoraxes prepared as described above. The process, as outlined by the manufacturers, was carried out for each individual fly (see Appendix 9.1). The purified DNA from each fly was kept in individual labelled 1.5 ml microcentrifuge tubes at 5°C until the next stage of the experiment could be carried out.

9.2.4 DNA quantification

To ensure the samples extracted from the flies contained similar quantities of DNA, 5 µl of each sample was run on a 1% agarose gel (see subsequent section for gelling and staining techniques) along with 5µl λ-DNA/*Eco*RI+*Hin*dIII ladder (100 ng/µl) (Fermentas Life Sciences, UK). The concentrations of DNA in each sample were estimated by comparing the bands visually with a band of a known size provided by the ladder (in this case, fragment 21226 bp) (see Figure 9.2). The samples were then diluted accordingly to a concentration of approximately 1 ng/µl and stored at 5°C.

9.2.5 DNA electrophoresis

Two concentrations of agarose gels were used during this study: 1% gels were used to visualise and quantify the DNA after purification, and 1.6% gels were used to visualise the ISSR PCR products (which will be discussed later). The type of gel to be used to visualise DNA is determined by the size of the fragments contained within the sample. Since DNA molecules are negatively charged, they move in an anodal direction i.e. negative to positive, when an electric current is passed through a gel containing them. The rate at which the DNA travels down the gel depends mainly on the molecular weight of the DNA. The smaller the molecular weight of the DNA, the greater the rate at which it travels through the gel. For the visualisation of total genomic DNA, such as that used in the quantification of the DNA samples extracted from the flies, a 1% agarose gel was the most suitable. However, for the products of the ISSR PCR-amplification, a 1.6% agarose gel was the most suitable.

To make a 1% agarose gel (for a 1.6% gel, the appropriate corresponding quantities were used), 1g of multi-purpose molecular grade agarose (Bioline, UK) was mixed with 100 ml of 0.5 x TBE buffer (see Table 9.1) in a 500 ml conical flask. This was then heated for approximately 2 minutes on full power, in a microwave, until the agar had dissolved and the molten gel was clear. The gel was then left to cool until approximately 40°C, whereby 5 µl (10 mg/ml) ethidium bromide (Sigma Chemical Co.) was added and the flask swirled to mix the contents thoroughly. Ethidium bromide acts as a fluorescent marker for

the DNA. The gel was then poured into a horizontal electrophoresis gel former (150 x 114 mm), and a 15-tooth comb added once the gel had been poured. The gel was then allowed to set for approximately 20 minutes at room temperature. If the gel was not for immediate use, it was stored at 5°C wrapped in cling-film for no longer than 48 hours.

Each well in the gel was loaded with the appropriate volume of ficol-bromophenol-blue loading buffer (x 6 concentration) (see Table 9.2) mixed with the DNA sample to be visualised. The gels were run using 0.5 x TBE running buffer, at 100V, for approximately 2 hours using a Bio-Rad power pack (model 1000/500). The gel tanks were custom-built (210 x 125 x 150 mm).

The bands on the gels were visualised using a UV transilluminator (UVP BioDoc-It System).

Table 9.1 Specifications of 10 x Trisborate (TBE) buffer.

Constituent	**Final concentration**	**Quantity added/ 1000 ml distilled water**	**Source**
Tris(hydroxymethyl)aminomethane	1.78 M	108 g	Fisons
Boric acid	1.78 M	55 g	Fisher Scientific
Ethylenediaminetetraacetic acid	4 mM	7.44 g	Sigma
pH adjusted to 8.3 using HCl			Fisher Scientific

Table 9.2 Constituents of 6 x ficol-bromophenol-blue loading buffer.

Constituent	Quantity added/ 1000 ml distilled water	Final concentration	Source
Glycerol	50 ml	50%	Fisher Scientific Ltd., Loughborough
Phenol blue	10 mg	0.001%	Sigma Chemical Co.
Ethylenediaminetetraacetic acid (EDTA)	25 ml	0.25 M	Sigmal Chemical Co.

9.2.6 ISSR primer selection

Four inter-ISSR primers were initially chosen (see Table 9.3). These primers were selected because they amplify common segments of DNA found in many plant and animal species. In addition, they have been used with some success in other invertebrate species (C. Ferris, personal communication). Several samples of DNA covering the five populations were picked arbitrarily to check the suitability of each primer for use in this study. Suitable primers show sufficient band resolution and observable band variation between samples. Primers AAG-1 and 855 were deemed to be unsuitable, as they did not show enough variation between samples, and in some cases produced too many bands to be able to distinguish between them. The two primers that were finally selected were AG-4 and CAC-1, as both showed adequate variation between samples (see Figures 9.3 and 9.4).

9.2.7 ISSR PCR (polymerase chain reaction) amplification

A reaction mixture was created for each primer (see Table 9.4), as much as was necessary for all of the DNA samples plus one negative control per gel, to which double-distilled water was added in place of DNA. An individual 0.5 ml microcentrifuge tube (Fisher Scientific UK Ltd., Loughborough) was used for each DNA sample for each

primer. Twenty-three microlitres of reaction mixture was put into each microcentrifuge tube along with 2 µl of DNA solution or, in the case of the negative controls, double-distilled water. The DNA, reaction mixture and its components were kept on ice during this process. The contents of each tube were vortexed for several seconds to ensure thorough homogenisation of the reaction mixture with the DNA. Two drops of mineral oil (Sigma Chemical Co.) was added to the surface of each mixture to prevent evaporation.

Table 9.3 ISSR primers used in the study (A: laboratory of Dr Mike Wilkinson, University of Reading; B: University of British Colombia, set nine).

Primer code	Primer sequence	Annealing temperature	Source
AAG-1	$CC(AAG)_5CC$	47 °C	A
AG-4	$CT(AG)_8GTG$	60°C	A
CAC-1	$GT(CAC)_5TG$	55°C	A
855	$(AC)_8YT$	58°C	B

To amplify the DNA samples a Perkin Elmer Gene Amp PCR system 9700 Thermal Cycler was used. The appropriate ISSR PCR cycle was activated, and the samples placed into the machine once the sample plate had reached a temperature of approximately 90°C. The PCR profile used for amplification of the DNA samples was: 1 cycle at 94°C for a duration of 4 minutes; 40 cycles with a duration of 20 seconds at 94°C, 30 seconds at x°C and 1 minute at 72°C respectively; 1 cycle at 72°C with a duration of 7 minutes followed finally by 1 cycle at 4°C for a duration of 30 minutes (x denotes the specific annealing temperature of the primer being used - see Table 9.3). The resulting PCR products were then stored at 5°C until required.

For visualisation of the ISSR bands, 5 µl of each of the PCR products was combined with 1.5 µl of loading buffer and run, adjacent with duplicate samples, on 1.6% agarose gels with a 1kb ladder on each gel.

Table 9.4 Constituents of reaction mixture for ISSR PCR amplification

Constituent	Concentration	Volume required per DNA sample (µl)	Source
NH_4 reaction buffer	x 10	2.5	New England Biolabs Inc., USA
dNTPs	2 mM	2.5	Ta Ka Ra Biomedicals, Japan
ISSR primer	15 mM	0.5	-
BIOTAQ DNA polymerase	5 u/µl	0.2	Bioline, UK
$MgCl_2$	50 mM	1.0	Bioline, UK
Double-distilled H_2O	-	16.3	-

9.2.8 Data analyses

The ISSR bands chosen to be included in the analysis were scored as presence/absence binary sequences. Since ISSR markers are interpreted as dominant diallelic markers, the presence of a band is determined by the dominant allele i.e. +/+ and +/− individuals have the (1) phenotype and −/− individuals have the (0) phenotype (Gupta

et al. 1994; Tsumura et al. 1996; Haig et al. 2003). The haplotype of each individual was determined and a pairwise genetic distance matrix between haplotypes was produced manually for each of the two primers used. These data were entered into MINSPNET, a computer program that calculates a Minimum Spanning Network from the genetic distances between haplotypes (Excoffier 1993).

In addition, the genetic distances between haplotypes were used to create neighbour-joining trees for both primers (Saitou & Nei 1987). These were constructed using the NEIGHBOR module from the Phylogeny Inference Package (PHYLIP V3.5c) (Felsenstein 1993). This program constructs trees by successive clustering of lineages, setting unconstrained branch lengths as the lineages join (Felsenstein 1993). The branch lengths are unconstrained because the trees produced do not assume an evolutionary clock and are unrooted.

Figure 9.1 Boulmer Haven, Northumberland: one of the chosen collection sites for *C. frigida*, showing a typical wrackbed where this species is found (photograph used with the kind permission of Les Bell).

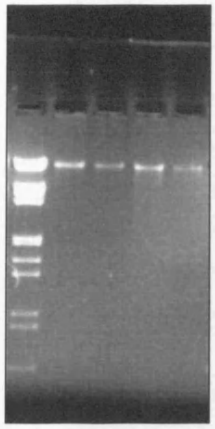


Figure 9.2 Gel showing the presence of DNA from four samples with a 1 kb ladder running adjacent on the left.

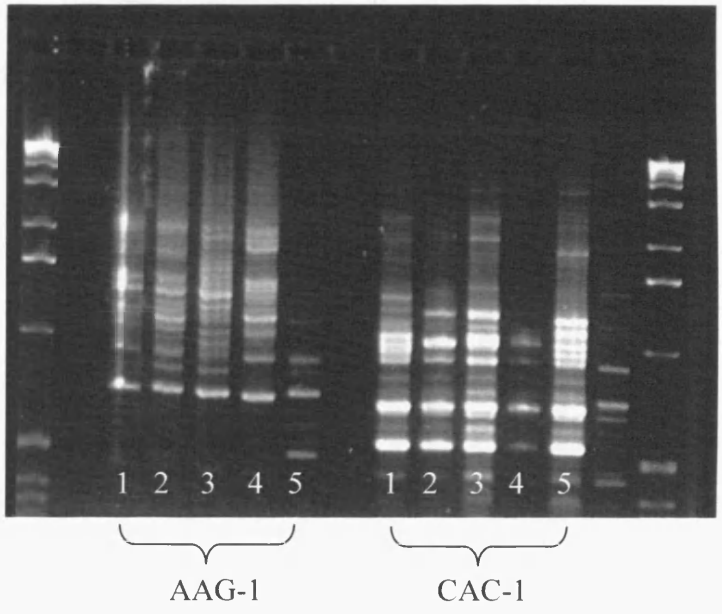


Figure 9.3 Gel showing 5 samples amplified with primers AAG-1 and CAC-1. 1 kb ladders were run on either side of the gel.

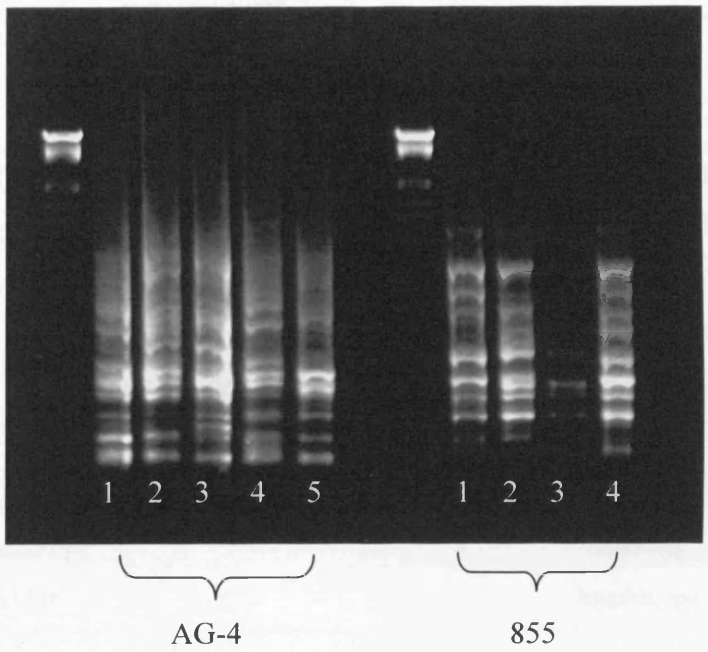


Figure 9.4 Gel showing 5 samples amplified with primer AG-4 and four with 855. 1 kb ladders were run on either side of the gel.

9.3 Results

The DNA from a total of 41 individuals from the 5 populations was examined using primers AG-4 and CAC-1. This included 10 individuals from St Mary's Island, 8 from Belhaven Bay, 10 from Barns Ness, 10 from Longhoughton and 3 from Boulmer. From these 41 individuals, 28 different haplotypes were detected (labelled A to BB) using primer AG-4 (see Table 9.5). These haplotypes differed at 7 polymorphic sites. The genetic distances between the haplotypes are presented in Figure 9.5. Primer CAC-1 detected 35 haplotypes (labelled A to II) differing at 8 polymorphic sites (see Table 9.6). The genetic distances between these haplotypes are shown in Figure 9.6. The bands produced by both primers appeared to be highly reliable and reproducible since the duplicate samples yielded the same haplotypes

There were a number of individuals that shared the same haplotype, though these were rarely from the same population (see Table 9.7). When the haplotype data from both primers were combined, each individual was found to be completely unique.

Table 9.7 Haplotypes shared by 2 or more individuals and the populations from which they come, for primers AG-4 and CAC-1 (SMI = St Mary's Island; BB = Belhaven Bay; BN = Barns Ness; LO = Longhoughton; BO = Boulmer).

AG-4			CAC-1		
Haplotype	**N° individuals sharing haplotype**	**Population/s**	**Haplotype**	**N° individuals sharing haplotype**	**Population/s**
A	3	SMI, BN, LO	B	2	SMI, BB
C	4	SMI, LO, BO	G	2	SMI, BO
D	2	SMI, BN	Q	2	BB, BN
G	2	SMI	R	2	BN, LO
J	3	BB	S	2	BN, BO
L	3	BB, BN, LO	DD	2	LO
M	2	BB, BO	-	-	-
O	2	BN, LO	-	-	-

The genetic distances between the haplotypes were used to create neighbour-joining trees for each primer to detect any patterns of genetic variation within and between populations (see Figures 9.7 and 9.8). The relationships between haplotypes appeared to be scattered throughout the populations with no apparent patterns with both primers. This was particularly the case with primer CAC-1. The neighbour-joining tree created using primer AG-4 also showed a scattered pattern of haplotypes, though there was some minor aggregation of haplotypes in the population from Longhoughton (haplotypes R, Q, S and T).

The genetic distances were also used to generate minimum spanning networks (MSN). Though MSNs were created to represent the relationships between the haplotypes produced by both primers, the large number of connections between haplotypes and the absence of any major high-frequency haplotypes resulted in MSNs of such complexity that they were uninformative. The haplotype connections and their additional alternative links generated by MINSPNET are given in Figures 9.9 and 9.10, but the MSNs have not been presented.

Table 9.5 Binary sequences for each haplotype produced using primer AG-4.

Haplotype	Haplotype binary sequence						
A	1	1	1	1	1	0	1
B	1	0	1	1	0	0	0
C	1	1	1	1	1	1	0
D	0	1	1	1	1	1	1
E	0	1	1	1	0	0	0
F	1	1	1	0	1	0	0
G	1	1	1	0	1	0	1
H	0	1	0	0	1	0	0
I	0	0	0	1	0	1	1
J	1	1	0	1	1	1	1
K	0	1	1	1	0	0	1
L	1	1	1	1	1	1	1
M	1	1	1	1	0	0	1
N	0	1	0	1	0	0	0
O	0	1	1	1	0	1	1
P	0	1	1	1	1	1	0
Q	0	1	0	0	1	1	0
R	0	1	0	0	1	0	1
S	0	1	0	0	1	1	1
T	0	1	1	0	1	1	1

Table 9.5 continued. Binary sequences for each haplotype produced using primer AG-4.

Haplotype	Haplotype binary sequence						
U	0	1	0	1	1	0	0
V	1	1	1	1	0	0	0
W	1	1	0	1	0	1	0
X	1	0	0	1	0	0	1
Y	0	0	1	1	1	1	1
Z	1	1	0	1	0	1	1
AA	1	1	0	1	1	1	0
BB	0	1	0	1	0	0	1

Table 9.6 Binary sequences for each haplotype produced using primer CAC-1.

Haplotypes	Haplotype binary sequence							
A	0	1	1	0	0	0	1	1
B	0	1	1	0	1	1	0	1
C	1	0	1	1	1	1	1	0
D	1	0	1	1	1	0	1	0
E	0	1	1	1	0	1	1	1
F	1	1	1	1	1	1	1	0
G	1	1	1	1	0	1	0	0
H	0	0	1	0	0	1	0	0
I	1	0	1	1	1	0	1	1
J	1	0	1	1	1	0	0	0
K	0	1	1	0	1	0	0	0
L	0	1	1	0	1	1	1	1
M	1	1	1	1	1	0	0	1
N	0	1	1	1	1	0	0	1
O	1	0	0	0	1	1	0	0
P	1	1	1	0	1	1	0	1
Q	1	1	1	1	1	1	0	1
R	0	1	1	1	1	0	1	1
S	1	0	1	1	1	1	0	1
T	1	0	0	1	1	1	0	1
U	0	1	1	1	1	1	0	1
V	0	0	1	0	1	0	0	0
W	1	1	0	1	1	0	0	1
X	1	0	1	0	1	1	1	0
Y	1	0	1	0	1	0	0	0
Z	1	1	1	1	1	0	1	0
AA	0	0	1	1	1	1	1	0
BB	0	1	1	1	1	1	0	0
CC	1	1	1	1	1	1	0	0
DD	1	1	0	1	1	0	1	0
EE	1	0	1	1	1	0	0	1
FF	0	0	1	0	1	1	1	0
GG	1	1	0	0	1	0	1	0
HH	1	1	1	1	0	1	0	1
II	1	1	1	1	0	1	1	1

Figure 9.5 Lower diagonal matrix showing the genetic distances between haplotypes produced by primer AG-4.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	BB
A	0																											
B	3	0																										
C	2	3	0																									
D	2	5	2	0																								
E	3	2	3	3	0																							
F	2	3	2	4	3	0																						
G	1	4	3	3	4	1	0																					
H	4	5	4	4	3	2	3	0																				
I	5	4	5	3	4	7	6	5	0																			
J	2	5	2	2	5	4	3	4	3	0																		
K	2	3	4	2	1	4	3	4	3	4	0																	
L	1	4	1	1	4	3	2	5	4	1	3	0																
M	1	2	3	3	2	3	2	5	4	3	1	2	0															
N	4	3	4	4	1	4	5	2	3	4	1	5	3	0														
O	3	4	3	1	2	5	4	5	2	3	1	2	2	3	0													
P	3	4	1	1	2	2	4	3	4	3	3	2	4	3	2	0												
Q	5	6	3	3	4	2	4	1	4	3	5	4	6	3	4	2	0											
R	3	6	5	3	4	4	3	1	4	3	3	4	4	3	4	4	2	0										
S	4	7	4	2	5	4	3	2	3	2	4	3	5	4	3	3	1	1	0									
T	3	6	3	1	4	5	2	3	4	3	3	2	4	5	2	2	2	2	1	0								
U	3	5	3	3	2	2	4	1	3	3	3	4	4	1	4	2	2	2	3	4	0							
V	2	1	2	4	1	1	3	4	5	4	2	3	1	2	3	3	4	5	6	5	3	0						
W	4	3	2	4	3	3	5	4	3	2	4	3	3	2	3	3	3	5	4	5	3	2	0					
X	3	2	5	5	4	4	4	5	2	3	3	4	2	3	4	6	6	4	5	6	4	3	3	0				
Y	3	4	3	1	4	4	4	5	2	3	3	2	4	5	2	2	4	4	3	2	4	5	5	4	0			
Z	3	4	3	3	4	4	4	5	2	1	3	2	2	3	2	4	4	4	3	4	4	3	1	2	4	0		
AA	3	4	1	3	4	4	4	3	4	1	5	2	4	3	4	2	2	4	3	4	2	3	1	4	4	2	0	
BB	3	4	5	3	2	2	4	3	2	3	1	4	2	1	2	4	4	2	3	4	2	3	3	2	4	2	4	0

Figure 9.6 Lower diagonal matrix showing the genetic distances between haplotypes produced by primer CAC-1 (matrix is continued over page).

A	0																					
B	3	0																				
C	6	5	0																			
D	6	6	1	0																		
E	5	3	4	5	0																	
F	2	4	1	2	3	0																
G	5	4	3	4	3	2	0															
H	5	3	4	5	4	5	3	0														
I	4	5	2	1	4	4	5	6	0													
J	4	5	2	1	6	3	3	4	2	0												
K	6	5	5	4	5	4	4	3	5	3	0											
L	4	2	4	5	2	3	5	4	4	6	3	0										
M	2	1	4	3	4	3	3	6	2	2	3	4	0									
N	4	3	5	4	3	4	4	5	3	3	2	3	1	0								
O	3	2	3	4	7	4	4	3	5	3	4	5	5	6	0							
P	4	4	4	5	4	3	3	4	4	4	3	2	2	3	3	0						
Q	5	1	3	4	3	2	2	5	3	3	4	3	1	2	4	1	0					
R	2	2	4	3	2	3	4	6	2	4	3	2	2	1	7	4	3	0				
S	6	3	2	3	4	3	3	4	2	2	5	4	2	3	3	2	1	4	0			
T	7	4	3	4	5	4	4	5	3	3	6	5	3	4	2	3	2	5	1	0		
U	4	1	4	5	2	3	3	4	4	4	3	2	2	1	5	2	1	2	2	3	0	
V	4	3	4	3	6	5	5	2	4	2	1	4	4	3	3	4	5	4	4	5	4	0
W	5	4	5	4	5	4	4	7	3	3	4	5	1	2	4	3	2	3	3	2	3	5	0

Figure 9.6 continued. Lower diagonal matrix showing the genetic distances between haplotypes produced by primer CAC-1

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	BB	CC	DD	EE	FF	GG	HH	II
X	5	4	1	2	5	2	4	3	3	3	4	3	5	6	2	3	4	5	3	4	5	3	6	0											
Y	5	4	3	2	7	4	4	3	3	1	2	5	3	4	2	3	4	5	3	4	5	1	4	2	0										
Z	4	5	2	1	4	1	3	6	2	2	3	4	2	3	5	4	3	2	4	5	4	4	3	3	3	0									
AA	5	4	1	2	3	2	4	3	3	3	4	3	5	4	4	5	4	3	3	4	3	3	6	2	4	3	0								
BB	5	2	3	4	3	2	2	3	5	3	2	3	3	2	4	3	2	3	3	4	1	3	4	4	4	3	2	0							
CC	6	3	2	3	4	1	1	4	4	2	3	4	2	3	3	2	1	4	2	3	2	4	3	3	3	2	3	1	0						
DD	5	6	3	2	5	2	4	7	3	3	4	5	3	4	4	5	4	3	5	4	5	5	2	4	4	1	4	4	3	0					
EE	5	4	3	2	5	4	4	5	1	1	4	5	1	2	4	3	2	3	1	2	3	3	2	4	2	3	4	4	3	4	0				
FF	4	3	2	3	4	3	5	2	4	4	3	1	6	5	3	4	5	4	4	5	4	2	7	1	3	4	1	3	4	5	5	0			
GG	4	5	4	3	6	3	5	6	4	4	3	4	4	5	3	4	5	4	6	5	6	4	3	3	3	2	5	5	4	1	5	4	0		
HH	4	3	4	5	2	3	1	4	4	4	5	4	2	3	5	2	1	4	2	3	2	6	3	5	5	4	5	3	2	5	3	6	4	0	
II	3	4	3	4	1	2	2	5	3	5	6	3	3	4	6	3	2	3	3	4	3	7	4	4	6	3	4	4	3	4	4	5	5	1	0

Figure 9.7 Unrooted neighbour-joining tree of haplotypes produced by primer AG-4. (Population key: St Mary's Island, Belhaven Bay, Barns Ness, Longhoughton, Boulmer)

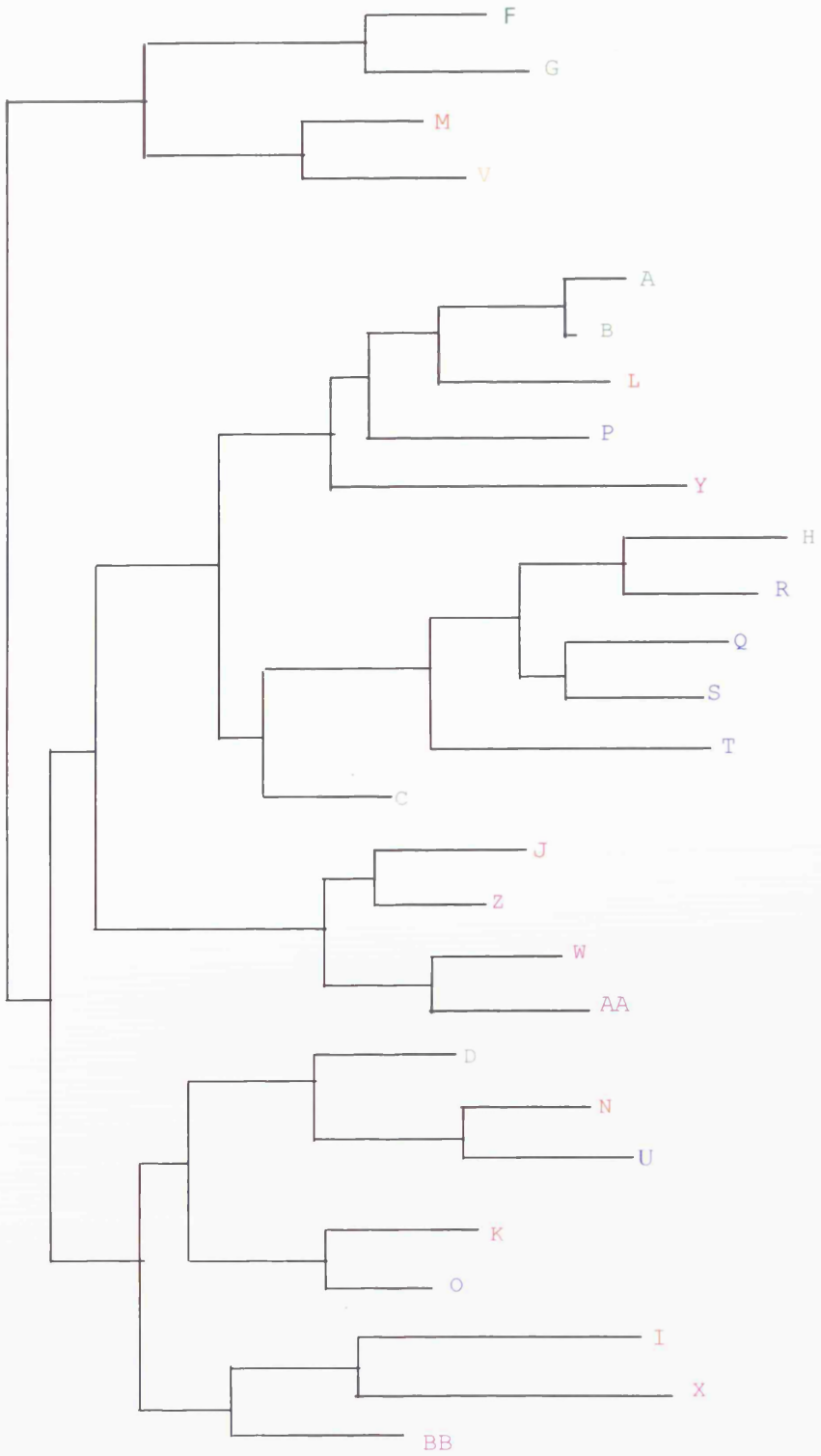


Figure 9.8 Unrooted neighbour-joining tree of haplotypes produced by primer CAC-1. (Population key: St Mary's Island, Belhaven Bay, Barns Ness, Longhoughton, Boulmer)

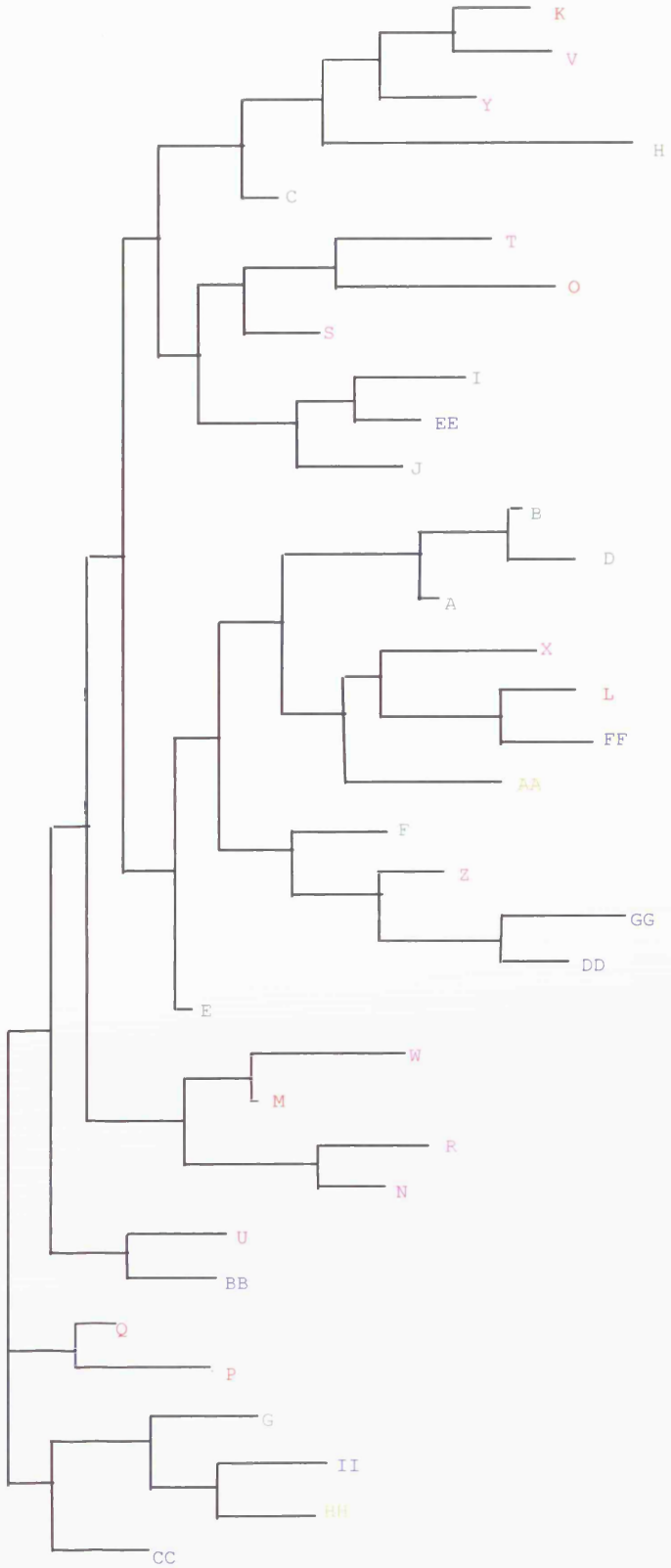


Figure 9.9 Relationships between haplotypes produced by MINSPNET for primer AG-4, with connection lengths. Alternative links are also given with connection lengths in parentheses.

Haplotype 1	Haplotype 2	Connection length
A	G	1.00000
G	F	1.00000
A	L	1.00000
L	C	1.00000
L	J	1.00000
L	D	1.00000
A	M	1.00000
M	K	1.00000
K	E	1.00000
K	N	1.00000
D	O	1.00000
C	P	1.00000
D	T	1.00000
T	S	1.00000
S	Q	1.00000
Q	H	1.00000
S	R	1.00000
N	U	1.00000
F	V	1.00000
V	B	1.00000
D	Y	1.00000
J	Z	1.00000
Z	W	1.00000
C	AA	1.00000
K	BB	1.00000
O	I	2.00000
M	X	2.00000

Haplotype	Alternative link/s (connection length/s)
D	P (1.00000)
E	N (1.00000) V (1.00000)
H	R (1.00000) U (1.00000)
I	X (2.00000) Y (2.00000) Z (2.00000) BB (2.00000)
J	AA (1.00000)
K	O (1.00000)
M	V (1.00000)
N	BB (1.00000)
W	AA (1.00000)
X	B (2.00000) Z (2.00000) BB (2.00000)

Figure 9.10 Relationships between haplotypes produced by MINSPNET for primer CAC-1 with connection lengths. Alternative links are also given with connection lengths in parentheses.

Haplotype 1	Haplotype 2	Connection length
A	F	2.00000
F	C	1.00000
C	D	1.00000
D	I	1.00000
D	J	1.00000
C	X	1.00000
J	Y	1.00000
Y	U	1.00000
V	K	1.00000
F	Z	1.00000
C	AA	1.00000
F	CC	1.00000
CC	Q	1.00000
Q	P	1.00000
Q	S	1.00000
S	T	1.00000
Q	T	1.00000
U	N	1.00000
N	R	1.00000
CC	G	1.00000
Q	B	1.00000
CC	BB	1.00000
Q	M	1.00000
M	W	1.00000
Z	DD	1.00000
I	EE	1.00000
X	FF	1.00000
FF	L	1.00000
DD	GG	1.00000
Q	HH	1.00000
HH	II	1.00000
II	E	1.00000
II	O	2.00000
V	H	2.00000

Haplotype	Alternative link/s (connection length/s)
B	M (1.00000) U (1.00000)
D	Z (1.00000)
F	D (2.00000)
G	HH (1.00000)
H	FF (2.00000)
J	EE (1.00000)
M	N (1.00000) EE (1.00000)
O	B (2.00000) T (2.00000) Y (2.00000)
S	EE (1.00000)
U	BB (1.00000)
AA	FF (1.00000)

9.4 Discussion

The results of this study present strong evidence for the effectiveness of ISSR markers in detecting genetic variation between individual *C. frigida* flies, and therefore their potential as a robust tool in the characterisation of parentage in this species and other invertebrate taxa. The high number of haplotypes generated by the two primers chosen for use in this study make them ideal in differentiating between individuals, though less effective in the study of inter-population genetic variation and gene flow (however, several of the samples came from the same coastline, so perhaps a greater degree of diversity would be seen between samples from more widely separated populations). Though a small number of individuals shared the same haplotype when generated with the same primer, when the haplotypes produced by both primers were combined, each individual was found to be unique. The ability of ISSR markers to discriminate between individuals rather than between populations was further demonstrated by the two neighbour-joining trees constructed from the genetic distances between haplotypes. These revealed complex relationships between haplotypes with little population-associated patterns being revealed. However, this study's primary aim was to establish the suitability of ISSR markers for future use in studies where the paternity of individuals from a wide range of possible known fathers needs to be determined, rather than genetic variation between populations.

Though several primers were checked for suitability before AG-4 and CAC-1 were picked, the number of bands produced by these two primers was still, on occasion, too numerous, which made band-scoring difficult. The technique would certainly benefit from the construction of primers specifically for use with coelopids, which would give clearer band resolution and possibly identify individual-specific ISSR loci, such as those seen in a study on parentage and relatedness in the comb-crested jacana, *Irediparra gallinacea*, which used ISSR markers (Haig et al. 2003).

As stated earlier, it is clear that ISSR markers would be an invaluable tool in laboratory-based studies involving flies of known genotype, however the possibility of using ISSR-based methods for parentage analysis and the determination of genealogical relationships in natural populations of *C. frigida*, also needs to be evaluated. Since females of this species mate with many different males (see Chapter 5) and populations can be of a very high density, assigning paternity using methods such as exclusion, categorical

allocation (Meagher & Thompson 1986) and fractional allocation (Devlin et al. 1988; Sancristobal & Chevalet 1997; Marshall et al. 1998; Gerber et al. 2000) is impossible. These methods require a finite set of individuals and a comprehensive assessment of all genetically possible parents (Jones & Ardren 2003), which is not feasible in high-density natural populations of *C. frigida*. However, these approaches may be of use when investigating paternity and relationships in small, low-density populations of other coelopid species.

A possible alternative that may uncover interesting results is the use of parental reconstruction (Jones & Avise 1997). This technique uses the multilocus genotypes of parents and offspring to reconstruct the genotypes of unknown parents providing gametes to a progeny array for which one parent is known (Jones 2001; Jones & Ardren 2003). This can be achieved using an algorithm applied by a computer program called GERUD (Jones 2001). The reconstructed genotypes are then compared either (1) to a set of candidate parents or (2) to each other, which allows the detection of multiple mating. The second type of comparison can be carried out even if there are no genetic samples from one of the sexes (Jones & Avise 1997; Jones et al. 1998). This method of paternity analysis could potentially reveal patterns of post-copulatory sperm use in wild populations of *C. frigida* if fertilised females were collected from the wild and allowed to oviposit in the laboratory. This would ensure that any offspring could be collected in a family group and both the progeny and the mother's genotypes could be determined using ISSR markers, allowing successful parental reconstruction. However, as with other similar techniques, this method may have one possible drawback - it is computationally demanding and may perhaps become too time-consuming for progeny arrays with many fathers (Jones & Ardren 2003). If this proves to be the case, the molecular data gathered could still be useful in assessing some mating system features, such as the frequency of multiple paternity (Ritland 2002).

This study highlights the great potential for molecular markers, particularly ISSRs, to be used in paternity and kinship analyses of *C. frigida*, generating countless exciting possibilities for investigations into the field of post-copulatory sperm use in this species. The future use of molecular techniques in the study of the mating system of *C. frigida* may help to answer many questions concerning sperm competition and cryptic female choice, among others, which are currently unresolved.

Chapter 10. General discussion

Until now, studies involving seaweed flies have primarily focussed on pre-copulatory mechanisms of sexual selection, in particular those associated with sexual conflict (e.g. Crean & Gilburn 1998; Dunn et al. 1999; Shuker 2002). The results of the experiments carried out as part of this thesis have built on these studies and have provided valuable information regarding the effect of the αβ inversion system on the behavioural components of the *Coelopa frigida* mating system. Numerous earlier studies have demonstrated the marked effect of male size on the willingness of males to mount females (e.g. Butlin et al. 1982, Gilburn & Day 1994a,b,c, Day et al. 1996, Dunn 2001) and the relationship between male size and mating success (e.g. Dunn et al. 1999). The αβ inversion system is known to be an important factor in determining male size (Butlin 1983), though very little work has previously been carried out on the effect of αβ inversion karyotype on mating interactions (but see Shuker 1998). Surprisingly perhaps, karyotype has no direct influence on male willingness to mate or other behaviours such as copulation duration or struggle duration, though an indirect effect, through its impact on size, may be possible. Interestingly, male size and karyotype seem to have a much greater influence on female rejection behaviour, for example, females use the abdomen curling rejection response more often, and the shaking and kicking response less often, with larger αα males than with smaller αβ or ββ males. There appears to be no clear genetic effect of female karyotype on female rejection behaviour, which leaves the question as to why αα females are mated with more often, unanswered. There is certainly scope for further investigation into this topic; for example, the energetic intensity of individual rejection responses could be looked at in greater detail. In addition, it may be possible that the order in which females exhibit rejection behaviours may be of some importance.

Though the mating system of coelopids has been previously recognised as being based on convenience polyandry, this thesis is the first to attempt to determine the extent to which female re-mating frequency might occur in wild populations of *C. frigida*. The discovery that female *C. frigida* do not avoid wrack beds when not ovipositing as a means of preventing superfluous copulations, generates a wide variety of possible questions regarding the potential benefits that females might gain from

staying near wrack beds. Even more fascinating is the finding that females may mate over a thousand times in their lifetime. This level of promiscuity is astounding, and far exceeds that of any other invertebrate studied to date, indicating that there is great potential for sexual conflict, through post-copulatory mechanisms of sexual selection, to work in *C. frigida*. Furthermore, this study highlights the need for more data to be gathered on female re-mating frequencies in wild invertebrate populations, as there is currently very little available.

As discussed above, there is huge potential for post-copulatory sexual selection in *C. frigida* due to the exceptionally high re-mating frequency exhibited as a consequence of their convenience polyandry-based mating system. Though studies involving pre-copulatory sexual selection in *C. frigida* are numerous (e.g. Butlin et al. 1982b; Gilburn et al. 1992; Gilburn & Day 1994a, b, c; Crean & Gilburn 1998; Crean et al. 2000; Dunn et al. 1999; this thesis), few studies have yet determined the extent to which post-copulatory sexual selection may play a role in the mating system of this species (but see Day & Gilburn 1997). This thesis presents evidence for cryptic female mate choice after copulation with a single male - female *C. frigida* may have the ability to select sperm from within the ejaculate of a single heterokaryotypic male to produce fitter heterokaryotypic offspring. Though the various methods used to demonstrate cryptic female choice are currently under much discussion and this field of research remains controversial, results of the type presented here can only be a positive addition to the debate. With new ideas and methods being developed at this time in the pursuit of evidence for cryptic female choice, seaweed fly researchers have an important role to play, since it is clear that post-copulatory mechanisms of sexual selection are at work in seaweed flies. In addition to the possibility of cryptic female choice functioning in *C. frigida*, the experiments carried out for this thesis also show that the time interval between copulations and the order of polyandrous copulations with males of different chromosomal karyotypes, interact in their effects on the proportion of offspring fathered by the second male to mate (P_2). Also, the consistent finding of last male sperm precedence when there is an interval of 24 hours between copulations, regardless of the mating order, implies that sperm loss probably occurs from the female within 24 hours of mating. It would be interesting to find out whether this is the case, and by which mechanisms this may occur. Do females actively eject sperm or is sperm lost through

sperm death and degradation? Surely with females mating many hundreds of times in high-density populations, all of the sperm transferred by males cannot possibly be stored in the females' spermathecae. Once copulation has taken place in seaweed flies, virtually nothing is known about the movement, storage or utilisation of ejaculates. In order to fully understand the mechanisms of post-copulatory sexual selection and conflict, experiments need to be constructed to rectify this gap in the current knowledge. The quantity of sperm stored within the female spermathecae could be measured following single and multiple matings to investigate sperm storage processes, like those carried out in *Tribolium castaneum* (Lewis & Jutkiewicz 1998). If sperm from different males could be recognised as such within a mixture of ejaculates from different males, there are a number of possible experiments that could be done. The spermathecae of *C. frigida* females have been dissected in an attempt to investigate sperm storage (J. Blyth, unpublished results), but until sperm markers have been developed it is extremely difficult to determine the fate of sperm from different males' ejaculates.

Another factor which has been highlighted by the findings of this thesis - that the pattern of paternity biases varies with the time interval between matings in *C. frigida* - is that population density may have a major impact on levels of post-copulatory sexual selection in seaweed flies. This, in turn, suggests that population density may have a significant effect on the evolution of the *C. frigida* mating system, as this will determine the frequency with which females mate and therefore the opportunity for cryptic female choice and sperm competition to operate. Population density effects need to be examined in more detail to determine the exact nature of such interactions.

The preliminary investigation into the use of ISSRs as a technique to investigate genetic variation in *C. frigida*, with a view to using this technique in future studies of post-copulatory sexual selection through paternity analysis, was a resounding success. It seems that research into mating interactions and the evolution of mating systems in seaweed flies can be enhanced greatly by the introduction of molecular techniques into this predominantly behavioural field. Though behavioural experiments will always be of the utmost importance, it is reassuring to know that particular molecular techniques can be utilised successfully when needed. This may also create a more comprehensive link

between purely behavioural studies and the considerable number of genetic studies carried out using seaweed flies.

In addition to new molecular techniques, other approaches have been introduced by this thesis - namely, the comparative method. Though the comparative method has been around for a number of years, it has not been applied to the mating system of seaweed flies. Now that the number of seaweed fly species studied has been increased through the research described in Chapter 6, the possibility of such approaches revealing important information about the evolution of mating behaviour in seaweed flies through antagonistic co-evolution may be achieved. The comparative study carried out here yielded a number of exciting, though perplexing results. Hopefully this will instigate further investigation into the possibility of demonstrating sexually antagonistic co-evolution in seaweed flies, which may play a crucial role in the evolution of their mating systems.

This thesis has endeavoured to broaden the scope of seaweed fly research in addition to increasing our knowledge of sexual conflict and post-copulatory mechanisms of sexual selection in seaweed flies, and it has achieved these aims. Future researchers can build on the knowledge gained here to utilise new and innovative molecular techniques, greatly expanding the possibilities for experiments in the field of post-copulatory sexual selection in coelopids. Furthermore, this thesis has applied approaches such as the comparative method to an ever-expanding dataset of mating interactions in coelopids, helping to recognize the potential that such a comprehensive dataset has for the study of mating system evolution.

The future of coelopid research looks like an exciting prospect and I eagerly anticipate further developments.

Acknowledgements

Firstly I would like to thank my supervisor, Dr. André Gilburn, for his help throughout my PhD and for proofreading this thesis. I would also like to thank Judy Jackson for showing me the ropes, and Helen Pearson and Jean Liggins for their help with the flies. Many thanks also to Dr. David McAlpine from the Australian Museum for chauffeuring me around the beaches north of Sydney, for letting me use his amazing collection of coelopid specimens and for his patience and kindness. I would also like to thank Jack van Berkel and the staff of the Edward Percival field station in Kaikoura for making me feel very welcome during my stay in New Zealand.

Many people from the Biology department at Leicester gave me advice and help during my time there – many thanks to you all. I would especially like to thank Frank Clark, Ezio Rosato and Paul Hart for their suggestions and encouragement. Also thanks to Richard Gornall, for the time we spent deciphering independent contrasts, and Colin Ferris, for his help with the molecular study. Particular thanks must go to John MacMullan (and his Ozzie Osbourne CDs) for successfully helping me through the unfamiliar territory of the 'molecular lab'.

I would also like to thank Prof. Mike Ritchie at the University of St Andrews for giving me my first taste of research and introducing me to the world of flies!

Outside the lab, many people made my stay in Leicester great fun. Huge thanks to the Portignol Network (you know who you are!) for the countless nights of entertainment in the Marquis and for keeping me in the loop while writing up. Thanks to Sandra for good times, lots of laughs and for being a great housemate. Many thanks also to Fiona, who gave me lots of encouragement and a sympathetic ear when it was needed, and Veryan for her advice and friendship.

Finally, I would like to thank my family for their constant support and encouragement, and George for his patience, understanding and advice, especially through times of stress while writing up.

This work was supported by an NERC studentship.

Appendix 7.1 Behavioural and size data used to generate independent contrasts for each species.

Species	Male wing length (log)	CV male wing length	Female wing length (log)	Mean wing length (log)	Size dimorphism	Struggle duration reject (log)	Struggle duration accept (log)	Proportion mounted	Proportion copulated	Proportion of males rejected	Proportion of females rejected	Strength of sexual selection
A. blanchae	0.395	0.165	0.416	0.405	0.953	1.409	1.373	0.476	0.567	0.100	0.333	0.618
C. littoralis	0.826	0.100	0.628	0.738	1.578	1.940	2.155	0.296	0.333	0.250	0.417	0.166
C. sydneyensis	0.806	0.113	0.754	0.781	1.127	2.011	2.040	0.435	0.500	0.243	0.257	0.266
G. keyzeri	0.693	0.147	0.607	0.652	1.221	2.178	2.449	0.571	0.510	0.333	0.167	0.506
T. canus	0.364	0.178	0.509	0.443	0.717	1.273	1.321	0.484	0.610	0.068	0.322	0.081
C. ursina	0.739	0.110	0.696	0.718	1.106	0.951	1.053	0.720	0.421	0.522	0.057	0.029
C. nebularum	0.792	0.124	0.720	0.758	1.184	1.346	1.230	0.870	0.421	0.390	0.052	0.023
C. pilipes	0.712	0.088	0.703	0.708	1.022	0.981	1.202	0.547	0.628	0.327	0.050	0.024
G. musgravei	0.748	0.090	0.754	0.751	0.987	0.930	0.921	0.900	0.333	0.566	0.101	0.107
C. vanduzeei	0.849	0.716	0.781	0.816	1.170	1.303	1.542	0.310	0.595	0.243	0.160	0.097
G. nitida	0.649	0.073	0.628	0.639	1.051	0.602	1.120	0.944	0.824	0.059	0.118	0.308
C. frigida	0.794	0.128	0.749	0.772	1.112	0.974	1.566	0.695	0.461	0.411	0.031	0.012
D. australis	0.629	0.068	0.600	0.615	1.069	0.000	0.426	0.233	0.730	0.000	0.340	-0.281

Appendix 7.2 Independent contrasts generated by the CONTRAST module of PHYLIP for each node on the Coelopidae phylogeny.

Node	Male wing length (log)	CV male wing length	Female wing length (log)	Mean wing length (log)	Size dimorphism	Struggle duration reject (log)	Struggle duration accept (log)	Proportion mounted	Proportion copulated	Proportion of males rejected	Proportion of females rejected	Strength of sexual selection
1	0.02186	-0.00921	-0.06543	-0.02635	0.16666	0.09643	0.03691	-0.00621	-0.03805	0.02285	0.00846	0.37925
2	-0.01399	0.00921	0.08920	0.03024	-0.31839	0.57793	-0.08145	0.10536	0.12997	-0.00518	-0.12006	0.07050
3	0.10004	0.02022	0.04869	0.07742	0.15163	0.85668	0.89360	0.07613	-0.15933	0.13310	0.00273	0.26582
4	0.06971	0.01222	0.08893	0.07923	-0.04505	0.23212	-0.14095	-0.08202	-0.44392	0.38357	-0.01181	-0.14196
5	-0.00281	0.03491	-0.04486	-0.02272	0.10784	0.75440	0.76342	-0.30444	-0.06335	0.00521	0.03098	0.15970
6	0.01416	-0.00708	-0.00106	0.00748	0.04525	-0.16390	-0.09048	-0.22301	-0.01565	-0.06911	0.07502	-0.11967
7	-0.00127	-0.00273	-0.02051	-0.01024	0.05085	0.26297	-0.23755	0.20342	-0.03096	-0.01651	0.01450	0.00727
8	-0.02897	-0.00830	-0.02039	-0.02496	-0.02254	-0.11175	-0.18408	-0.05799	-0.01170	0.07340	0.00800	0.00590
9	0.03563	-0.02151	0.02862	0.03235	0.01830	0.10556	0.13250	-0.24918	0.08588	-0.10212	0.05110	0.03409
10	-0.02333	-0.00034	-0.02444	-0.02355	0.00632	-0.00451	0.04010	-0.00861	0.01695	-0.04049	0.04076	0.03266
11	-0.01117	-0.00327	0.00251	-0.00478	-0.03608	-0.04505	-0.06351	-0.01572	0.03335	0.00752	-0.03360	-0.02424
12	-0.08925	0.01835	-0.05762	-0.07306	-0.06961	0.05936	-0.00572	-0.02949	0.00950	-0.05643	0.04924	0.06436

Appendix 8.1 The karyotype of the female and the second male, the time interval between matings, the number of female offspring produced and the P_2 value for each of the 42 families.

Female Karyotype	2nd Male Karyotype	Time Interval (h)	Number of Offspring	P_2 value
αα	αα	0	66	0
αα	αα	0	64	0.6719
αα	αα	0	55	0.2182
αα	αα	0	60	0
αα	αα	0	13	0
αα	αα	24	24	0.0417
αα	αα	24	25	0.56
αα	αα	24	26	0.7308
αα	αα	24	22	0.8182
αα	αα	24	59	1
αα	αα	24	48	0.7708
αα	αα	24	14	0.9286
αα	ββ	0	32	0.875
αα	ββ	0	34	0.8824
αα	ββ	0	57	0.9474
αα	ββ	0	75	0.9067
αα	ββ	0	38	1
αα	ββ	24	57	0.6667
αα	ββ	24	52	1
αα	ββ	24	43	1
αα	ββ	24	28	0.9643
αα	ββ	24	34	0.3235
αα	ββ	24	73	0
αα	ββ	24	79	0.5316
αα	ββ	24	46	1
ββ	αα	0	13	0.8462
ββ	αα	0	30	0.7
ββ	αα	0	87	0.4483
ββ	αα	0	69	1
ββ	αα	0	47	0.766
ββ	αα	24	69	0
ββ	αα	24	68	0.8382
ββ	αα	24	29	1
ββ	αα	24	20	0.9
ββ	ββ	0	36	0.4444
ββ	ββ	0	16	0
ββ	ββ	0	21	0
ββ	ββ	0	71	0.831
ββ	ββ	0	65	0
ββ	ββ	0	72	1
ββ	ββ	24	56	0.9107
ββ	ββ	24	16	0.4375

Appendix 9.1 The step-by-step method for DNA purification and isolation of genomic DNA from *C. frigida* (taken from the DNeasy® Tissue Handbook). Suppliers of the specific equipment and chemicals used are given in parentheses.

1. Place insect tissue in a 1.5ml microcentrifuge tube (Fisher Scientific UK Ltd., Loughborough).
2. Freeze with liquid nitrogen (BOC Gases, Leicester) and grind up tissue with a disposable microtube pestle (Fisher Scientific UK Ltd., Loughborough).
3. Add 180µl Buffer ATL (Qiagen).
4. Add 20µl proteinase K (Bioline, UK), mix by vortexing (Whirlimixer®, FSA Laboratory Supplies, UK), and incubate at 55°C in a water bath (model SB1, Grant Instruments Ltd., Cambridge) until the tissue is completely lysed (approximately 2 hours). Vortex occasionally during incubation to disperse the sample.
5. Add 4 µl of RNase A (Qiagen) (100mg/ml), mix by vortexing, and incubate for 2 minutes at room temperature (15-20°C).
6. Vortex for 15 seconds. Add 200µl Buffer AL (Qiagen) to the sample, mix thoroughly by vortexing, and incubate at 70°C in a water bath for 10 minutes.
7. Add 200µl ethanol (96-100%) (Sigma, UK) to the sample, and mix thoroughly by vortexing.
8. Pipette the mixture into the Dneasy Mini Spin Column (Qiagen) placed in a 2ml collection tube (Qiagen). Centrifuge (Micro Centaur MSE/SANYO centrifuge) at 8000rpm for 1 minute. Discard flow-through and collection tube.
9. Place Dneasy Mini Spin Column in a new 2ml collection tube, add 500µl Buffer AW1 (Qiagen), and centrifuge for 1 minute at 8000rpm. Discard flow-through and collection tube.
10. Place the Dneasy Mini Spin Column in a 2ml collection tube, add 500µl Buffer AW2 (Qiagen) and centrifuge for 3 minutes at 14,000rpm to dry the Dneasy membrane. Discard flow-through and collection tube.
11. Place the Dneasy Mini Spin Column in a clean 1.5ml microcentrifuge tube and pipette 100µl Buffer AE directly onto the Dneasy membrane. Incubate at room temperature for 1 minute, and then centrifuge for 1 minute at 8000rpm to elute.
12. Repeat elution once as described in step 11.

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