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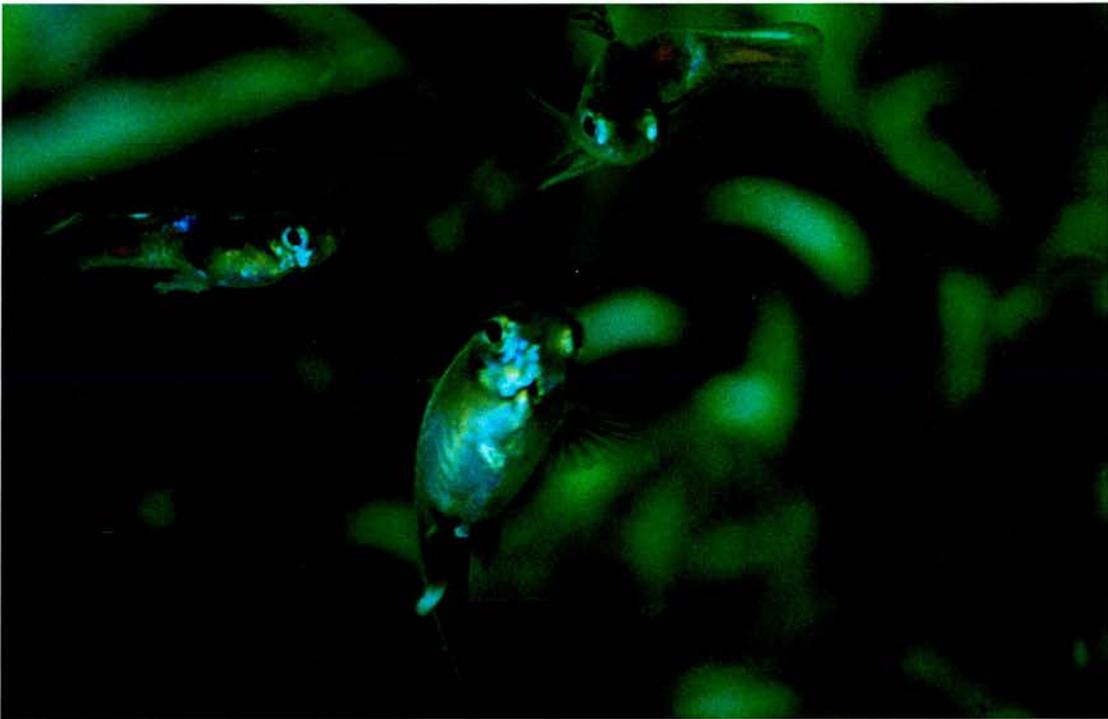
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Male mating strategies and sperm competition in the Trinidadian guppy

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Thesis submitted for the degree of doctor of philosophy,
University of St Andrews

September 2000



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Abstract

This thesis examines male mating strategies and sperm production characteristics in the Trinidadian guppy (*Poecilia reticulata*). In particular, it focuses on plasticity in these traits and explores the role of sperm competition in shaping them in natural and captive populations. Males can use sneaky mating (thrusts) as well as highly visual courtship displays (sigmoids) to achieve copulation. Data collected from wild populations revealed that males inhabiting low-predation environments, which are likely to be characterised by high levels of sperm competition, perform more sigmoids and have larger sperm reserves than their high-predation counterparts. The project also focused on the ontogeny of sexually-selected traits in males (courtship behaviour and body coloration) and revealed peaks in their expression which corresponded with maximum levels of sperm production in six-month-olds. Furthermore, sperm production (as estimated from stripped ejaculates) correlated positively with courtship behaviour in three- and six-month-old fish but not in the oldest (12-month) age class examined. These results, which are consistent with life-history theory, suggest that males maximise their reproductive investment early in life at the expense of reproductive vigour later on. Further experimental work showed that males reared in a male-biased sex ratio responded by increasing their rate of sneaky mating. However, contrary to prediction, their patterns of sperm production did not reflect this switch in mating strategy. Varying the fish density at rearing (where sex ratio was controlled) had no impact on the subsequent pattern of male mating behaviour. The project also examined sperm precedence patterns in guppies and revealed a bimodal distribution of paternity with either first or second male priority. It emerged that the best predictor of male reproductive success was the sigmoid display rate of the second male, suggesting that pre-copulatory female mate choice may facilitate sperm competition or sperm choice in the species. Finally, a range of potential female benefits through multiple mating was examined, and it was demonstrated that polyandry leads to increased brood sizes, shorter gestation periods and the production of offspring with better developed schooling abilities.

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Chapter one

General Introduction

(a) *Introduction*

Few species have been investigated as thoroughly as the Trinidadian guppy (*Poecilia reticulata*). The study of the behavioural ecology of guppies dates back to the middle of the 20th century, when Clark and Aronson (1951) examined their sexual behaviour and reproductive biology and Haskins and his co-workers (Haskins & Haskins 1950; Haskins *et al.* 1961) described geographic variations in these traits among natural populations. Subsequent work by Baerends *et al.* (1955) and Liley (1966) provided detailed observations of the guppy's courtship behaviour and laid the foundations for a number of ecological, behavioural and evolutionary studies (reviews by Houde 1997; Meffe & Snelson 1989). Arguably the most important research discipline to stem from these early studies was sexual selection, and chief among the practitioners in this field were John Endler and Anne Houde. Through their research, they each played a key part in revealing the role of sexual selection in generating the remarkable colour patterns observed in natural populations (Endler 1983; Endler & Houde 1995; Houde 1987; Houde & Endler 1990).

By the time Endler and Houde had published their initial findings (and consequently assured guppies a place in virtually every evolutionary textbook), the relatively new field of sperm competition (Parker 1970) was blossoming. This provided a new dimension to the 'classical' theory of sexual selection because it introduced a different arena (the female reproductive tract) in which male competition and female choice could potentially occur. Only 14 years after the concept was introduced, Smith's (1984) volume entitled *Sperm Competition and the Evolution of Animal Mating Systems* elegantly demonstrated the importance of sperm competition in the mating systems of a diverse range of animals. Among the taxonomic groups covered in this volume was the Poeciliidae, the family of live-bearing fish to which guppies belong (Constantz 1984). Despite this initial interest in sperm competition in poeciliids, few

subsequent studies have examined the role it plays in their mating systems (see Petersen & Warner 1998 p.455)

Whatever the underlying mechanisms of sperm competition, its outcome determines which males are successful at fertilizing eggs and which ones are not. It therefore has a profound effect on their reproductive biology and consequently raises a number of questions for evolutionary and reproductive biologists. For instance, how does sperm competition influence the evolution of male sexual behaviour and are males able to adjust their behaviour in response to cues indicating greater (or lesser) sperm competition risk? What are the behavioural, morphological and physiological traits that determine whether males are successful in sperm competition? And what (if any) benefits are accrued by females through multiple mating? These are the questions that form the basis of this thesis and I have addressed them by focusing on the mating strategies and reproductive biology of the Trinidadian guppy.

This introductory chapter provides a general background to the guppy's distribution and systematics (section *b*) and reviews the literature on its ecology, behaviour and reproductive biology (sections *c – e*). The final sections of the chapter review the relevant literature on sexual selection and sperm competition (sections *f – h*) and provide a basis for the experimental chapters that follow. The first three of these chapters focus on the male's courtship behaviour and sperm production characteristics and examine variation in these traits according to predation risk (chapter two), ontogeny (chapter three) and rearing demography (chapter four). Chapter five investigates the patterns of sperm precedence in guppies and identifies traits that may be important determinants of male reproductive success. The final experimental chapter examines sperm competition from the female perspective by looking at a range of potential benefits through multiple mating. Chapter seven concludes the thesis by exploring the wider implications of the research and presenting several possibilities for future work.

(b) *The Trinidadian guppy*

The guppy is perhaps the best known member of the Poeciliidae, a family of cyprinodontiformes endemic to tropical and subtropical latitudes of the New World (Parenti & Rauchenberger 1989; Rosen & Bailey 1963). The natural range of guppies

extends from north-eastern South America (Venezuela and Guyana) to the Caribbean islands of Trinidad and Tobago as well as several of the Windward and Leeward islands (Rosen & Bailey 1963). In addition, countless feral populations exist throughout the world wherever climatic conditions are favourable (Courtenay & Meffe 1989).

Despite their ubiquity, guppies have been studied most intensively in Trinidad (Magurran *et al.* 1995), which lies just 16 km off the Venezuelan coast. Until recently (between 10^3 and 10^4 years ago), Trinidad was part of mainland South America, a fact that is reflected by the island's unique natural history. One legacy of Trinidad's continental origins is the Northern Range mountain chain running along the island's northern flank (Fig. 1.1). Throughout these mountains, guppies are widely distributed and occur in all but the most marginal freshwater habitats (Meffe & Snelson 1989). Typical habitats include clear, fast-flowing mountain streams, turbid lowland rivers (Fig. 1.2) and, increasingly, the polluted drainage systems of several large towns. Although most guppies are found in freshwater habitats, they have good tolerance for salinity and several populations live in brackish waters (Houde 1997).

It is no coincidence that a number of evolutionary biologists have concentrated their efforts on Trinidad's guppy populations. The island's geography, and its recent separation from mainland South America, has resulted in the isolation and subsequent parallel evolution of numerous guppy populations. Moreover, the considerable morphological and behavioural variation that exists among guppy populations in Trinidad allows researchers to make replicated observations of populations in their natural environment. These populations are of particular interest to evolutionary biologists because much of the geographic variation that exists among them can be attributed solely to the selective influence of predation (Magurran *et al.* 1995).

(c) *Predation in Trinidadian rivers*

Two major river systems, the Caroni and the Oropuche, drain the southern slopes of Trinidad's Northern Range. The Caroni River flows westward into the Gulf of Paria, while the Oropuche River flows into the Atlantic Ocean to the east of Trinidad (see Fig. 1.1). The fish communities inhabiting these south-flowing rivers are typically South American in origin and include several cichlids (e.g. *Crenicichla alta* &

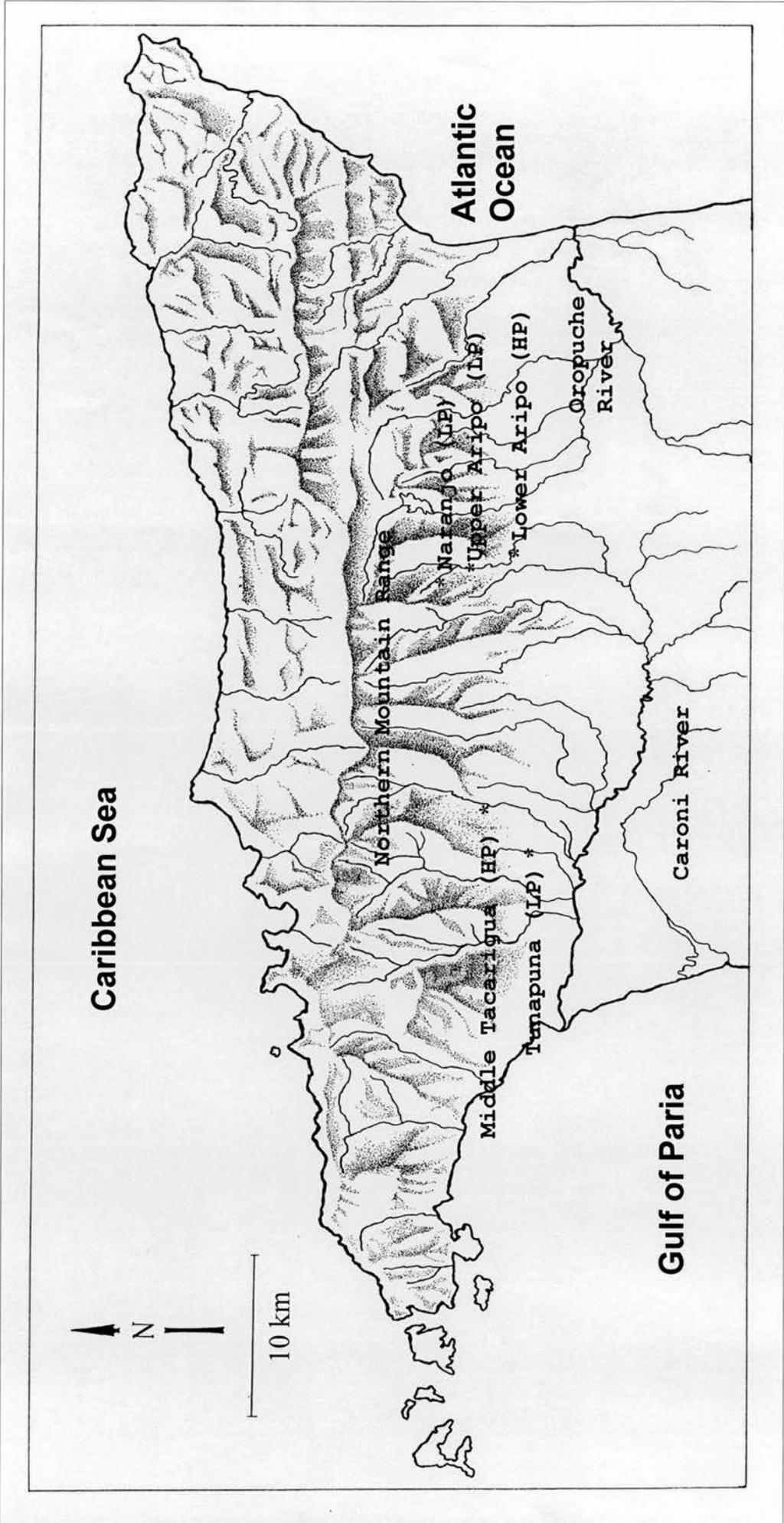


Figure 1.1 Map of northern Trinidad showing Northern Mountain Range, the two main rivers draining its southern slopes and stream locations where fish were collected (marked with an asterisk; see chapter two).

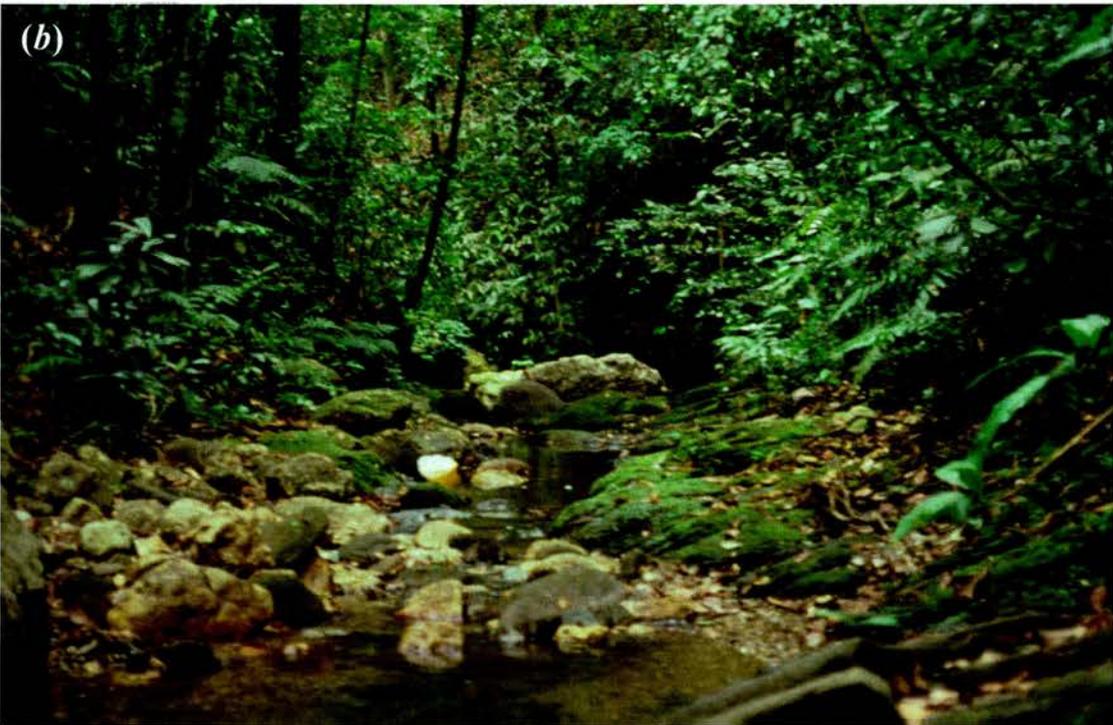
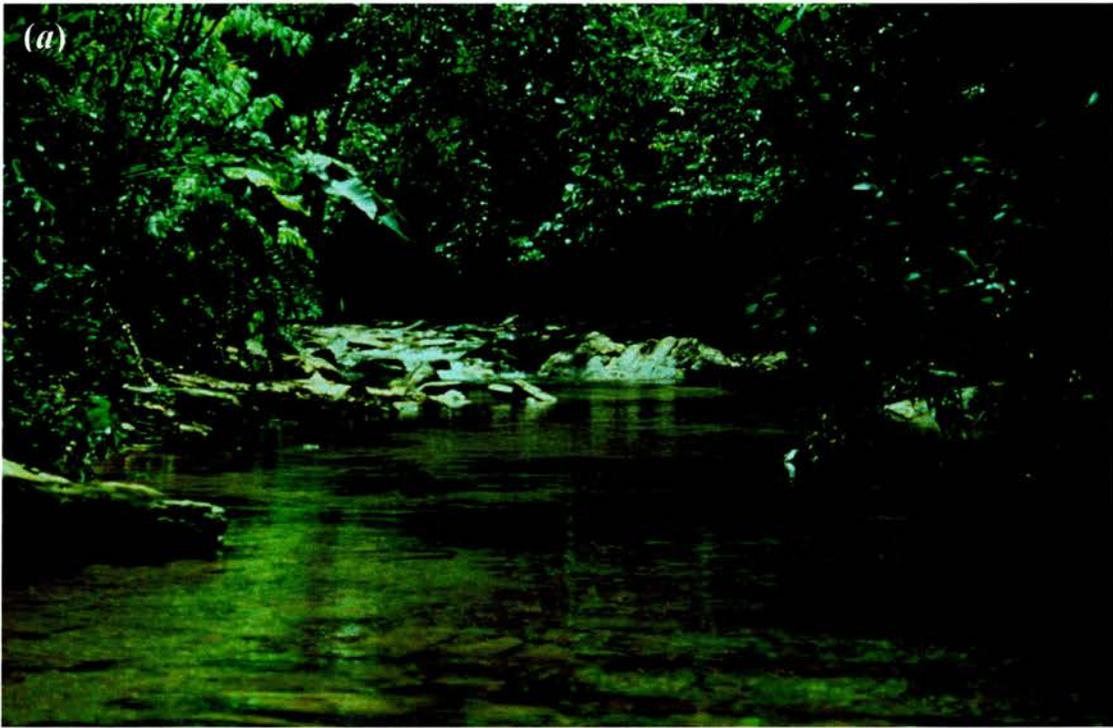


Figure 1.2 Typical (a) high- and (b) low-predation streams in Trinidad

Aequidens pulcher), characins (e.g. *Hoplias malabaricus* & *Astyanax bimaculatus*) and the cyprinodontid *Rivulus hartii* (Houde 1997). In contrast, the Northern Range's north-flowing rivers are home to a mainly Caribbean fauna that includes gobies (e.g. *Gobiomorus dormitor* & *Eleotris pisonis*), mullets (*Agonostomus monticola*) and freshwater prawns (*Macrobrachium crenulatum*).

A characteristic feature of rivers throughout the Northern Range is the presence of waterfalls that act as barriers to upstream fish migration. As a consequence, guppies inhabiting headwater streams often co-exist with a fairly impoverished fauna comprising relatively few predators. Notable among these is *Rivulus hartii*, which feeds mainly on immature size classes of guppies (Seghers 1973). Further downstream, below the barrier waterfalls, predation levels are usually far greater. Here, guppies occur sympatrically with several piscivorous predators that, in addition to *Rivulus*, include the cichlids *Crenicichla alta* and *Aequidens pulcher* and several characins such as *Astyanax bimaculatus* and *Hemibrycon dentatum* (Houde 1997).

Populations of Trinidadian guppies vary markedly in a host of morphological and behavioural traits, much of which can be attributed to differences in predation pressure. In particular, predation imposes costs on the expression of male secondary sexual characteristics such as coloration (Endler 1978; Endler 1980; Haskins *et al.* 1961), courtship behaviour (Liley & Seghers 1975) and ornament size (Endler & Houde 1995). Males inhabiting the low-risk streams above the waterfalls (which are often termed 'Rivulus sites') tend to be larger (Rodd & Reznick 1997) and more colourful (Endler 1978; Endler 1980; Endler 1983) than their downstream counterparts (i.e. in 'Crenicichla sites'). Haskins *et al.* (1961) were the first to note that these differences reflected the balance between sexual selection for conspicuousness and natural selection for crypsis. Further work by John Endler (Endler 1978; Endler 1980; Endler 1983) confirmed that male colour patterns were more conspicuous in low-risk populations than in high-risk ones.

Predation risk also accounts for population differences in life history tactics. Guppies that occur in high-predation streams tend to mature at an earlier age and a smaller size than those inhabiting low-predation environments. In addition, females in high-predation localities produce more, smaller offspring per brood and have higher

reproductive rates in relation to fish elsewhere (Reznick 1982; Reznick & Bryga 1996; Reznick *et al.* 1996; Reznick & Endler 1982). Most of these differences in life history tactics among natural populations reflect the shorter life expectancy of fish in high-predation environments. However, large size at maturity may also be favoured in low-risk sites because *Rivulus*, the principal predator in such localities, has a limited gape and can only take small size classes of guppies (Liley & Seghers 1975). Field experiments have confirmed that much of the variation in life history traits is attributable to natural selection by predators, and have thus provided some of the best evidence yet of evolutionary change in natural populations (Reznick *et al.* 1990; Reznick & Endler 1982).

Behavioural traits also vary in parallel with predation pressure. For example, guppies from high-predation localities form more cohesive schools and are more wary of predators than those from low-risk sites (reviewed by Magurran *et al.* 1995). In addition, male courtship behaviour is influenced by predation risk. In the presence of predators, males tend to switch from highly visual courtship displays (described below) to less conspicuous sneaky mating attempts (Dill *et al.* 1999; Endler 1987; Magurran & Seghers 1990). This switch in male courtship behaviour is also evident at high light intensities when males are presumably at greater risk from predators (Endler 1987; Reynolds *et al.* 1993). Female choosiness is also influenced by predation. For instance, females reduce their preference for brightly coloured males when predators (or models resembling them) are in view (Godin & Briggs 1996; Gong 1997). In comparative studies, Anne Houde and John Endler (Endler & Houde 1995; Houde & Endler 1990) confirmed that the strength of female preferences tended to be negatively associated with the risk of predation, which suggests that female preference patterns have evolved in response to natural selection by predators. All of these studies underlie the importance of predation in shaping male and female sexual behaviour.

Predator-mediated shifts in female sexual behaviour also influence the sexual behaviour of males. For example, Dill *et al.* (1999) have shown that males increase their rates of sneaky mating (described below) in response to female cues indicating greater predation risk rather than in direct response to the predator itself. Their experiment suggested that females at risk are less receptive to male courtship than

their low-risk counterparts. Such curtailment of female receptivity in the presence of predators is likely to influence the willingness of females to mate multiply, which is otherwise a common phenomenon in guppies (see below). One prediction, therefore, is that the extent of multiple mating by females (and ultimately the risk of sperm competition) will vary geographically in line with predation intensity (but see Kelly *et al.* 1999 for an alternative hypothesis – discussed in chapter seven). This prediction forms the basis of the first experimental chapter (two) of this thesis. Specifically, it is argued that male investment in spermatogenesis will be lower in populations experiencing elevated levels of predation. The experiment compares the mating behaviour and sperm production characteristics among males from five natural populations of Trinidadian guppies.

(d) *Mating behaviour*

Probably the most striking feature of guppy behaviour is the seemingly endless pursuit of females by males. Indeed, in some wild populations males devote the majority of their daily time budgets to courtship and mate searching (Endler 1987; Magurran & Seghers 1994a; Magurran & Seghers 1994b). The guppy's mating system is aptly described by Houde (1997) as promiscuous; females often mate with two or three males when they are receptive and males can potentially copulate several times per day. The description of male and female sexual behaviour given here is based on extensive behavioural observations made by Baerends *et al.* (1955) and Liley (1966).

Males use two distinct modes of insemination. The first requires female co-operation and involves an ordered behavioural sequence leading to copulation (Baerends *et al.* 1955; Liley 1966). The second, known as a gonopodial thrust, does not require co-operation and occurs when females are sexually unresponsive to males. The sequence of events leading to a solicited copulation often begins with the male following and watching a female (Liley 1966). Courting males perform a characteristic sigmoid display to stationary or slow-moving females (Baerends *et al.* 1955; Liley 1966). During the display the male orientates himself in front or to one side of a female and assumes a pronounced sigmoid posture while quivering his body with the caudal and dorsal fins either spread or closed (Fig. 1.3a). Following a sigmoid display, the male may 'jump' several centimetres away from the female, presumably to lead her away



Figure 1.3 (*a*) Male engaged in full sigmoid display whilst female watches; (*b*) contact during gonopodial thrust attempt.

from her current position (Baerends *et al.* 1955). If a female is receptive to a male's courtship attempts, she may 'glide' towards him in a manner quite unlike normal swimming (Liley 1966). When the female reaches the male, she arches her body by raising her head and tail slightly. At this point the male swings around the female and attempts to copulate. If the female co-operates, the two fish swim in tight circles ('wheeling') with the male on the outside and the female tilting her body so that her genital opening is exposed. During this wheeling stage, the male attempts to insert the tip of his gonopodium into the female's genital opening. If successful, the male performs a series of 'post-copulatory jerks' which last up to 3 minutes (Liley 1966). The function of these jerks is not clear (Constantz 1989) but they are a reliable indicator of successful sperm transfer (Liley 1966).

In addition to solicited copulations, males can use the alternative method of gonopodial thrusting to achieve insemination (Farr 1980a; Liley 1966; Luyten & Liley 1991). A gonopodial thrust involves neither male display nor female co-operation. During a thrust attempt, the male approaches the female from behind, swings his gonopodium forward, and attempts to insert it into the female's genital opening (Fig. 1.3b). Gonopodial thrusting is described as an alternative mating tactic (Houde 1997) in the sense that it undermines female choice, but unlike alternative mating strategies used by other species (e.g. salmon, Gage 1995), sneaky mating in guppies is not a specialised behavioural phenotype. Instead, it is a facultative behaviour that males use opportunistically according to environmental (Luyten & Liley 1985; Luyten & Liley 1991), social (Farr 1976; Farr 1980b; Rodd & Sokolowski 1995) and ecological (Farr 1975) factors.

Although Houde (1997) briefly describes the ontogeny of male reproductive traits in her recent monograph, there has been no detailed description of the early ontogeny of male sexual behaviour. Indeed, Houde (1997) specifically advocates such an investigation in her chapter on *Reproductive Biology and Sexual Behavior* (p. 35). Furthermore, it is not clear how the development of male secondary sexual traits (notably courtship behaviour and coloration) is related to reproductive maturity in males (see Bisazza *et al.* 1996 for a similar investigation in the mosquitofish *Gambusia holbrooki*). The experiment described in chapter three addresses these

questions by examining the ontogeny of courtship, coloration and sperm production in four age classes of male guppies.

(e) *Reproductive biology*

Males possess a modified anal fin, or gonopodium, which serves as the intromittent organ. Unlike the gonopodia of some poeciliids, the guppy's organ is relatively short, bilaterally symmetrical (Constantz 1989; Rosen & Bailey 1963), and when erect forms a groove for sperm transport (Rosen & Bailey 1963). The gonopodium has a thin, hood-like caecum extending beyond its tip which, with the aid of an extensive nerve plexus, helps direct gonopodial thrusts towards the female's genital pore (Constantz 1989).

Like other poeciliids, male guppies have paired testes fused into a single tubular organ which is attached to the surface of the dorsal body wall (Billard 1986; Constantz 1989). Mature spermatozoa are formed after immature spermatogonial cells pass through a system of tubules and eventually join the sertoli cells of the central testes. After several generations, the spermatogonial cells undergo final meiotic division to become mature spermatozoa. These mature sperm form densely packed cysts known as spermatozuigmata (Fig. 1.4a & b). Based on detailed observations of cyst development, Billard (1969) calculated that the theoretical number of spermatozoa per spermatozuigmata was 32,768 (although his observations of mature spermatozuigmata revealed only 22,000 sperm per bundle). Spermatozuigmata are approximately 0.1-0.2 mm in diameter and distinctly double-concave shaped (personal observation). In common with most internally fertilizing sperm, the guppy's spermatozoa have elongated, lance-shaped nuclei and long flagellate tails (Jamieson 1991). Sperm motility is lengthy in the guppy, lasting up to two hours in some cases (Gardiner 1978).

Female guppies are livebearers with internal fertilization. Ova are matured within paired (but fused) ovaries in non-overlapping batches shortly before the birth of a litter (Constantz 1989). A few days after parturition, a mature batch of eggs is fertilized and gestation lasts between 25 and 35 days (Houde 1997). During gestation, a new batch of ova accumulates yolk and is subsequently fertilized upon production of the next brood. There is no maternal contribution to eggs after fertilization and young

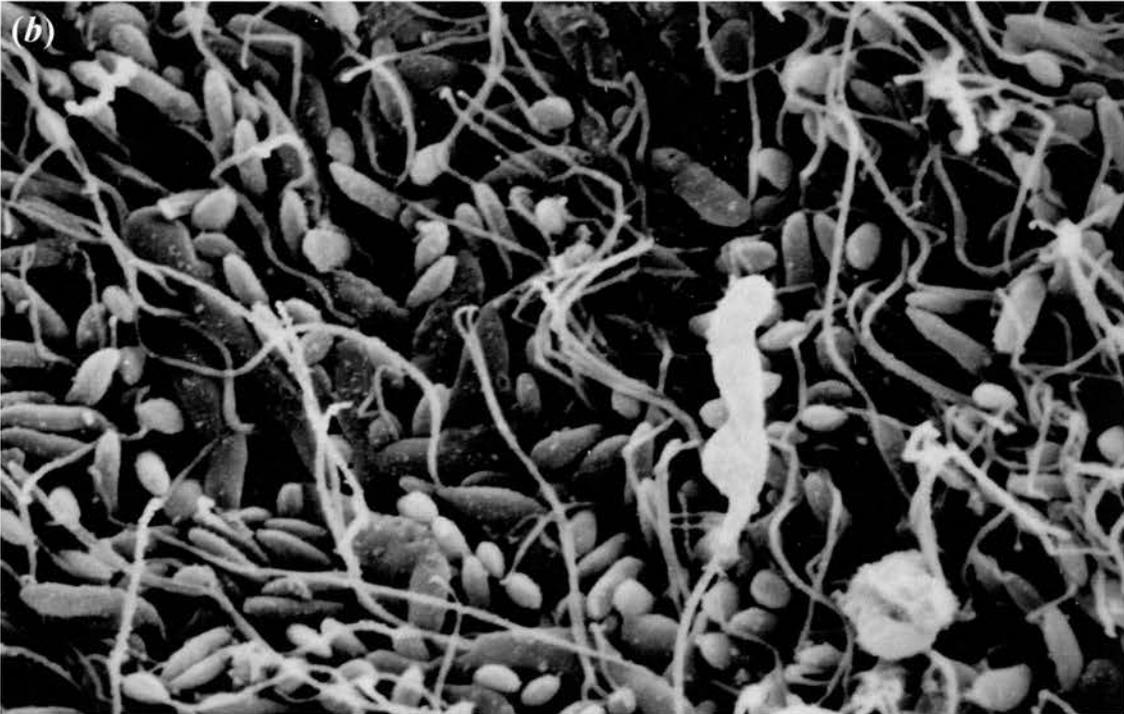
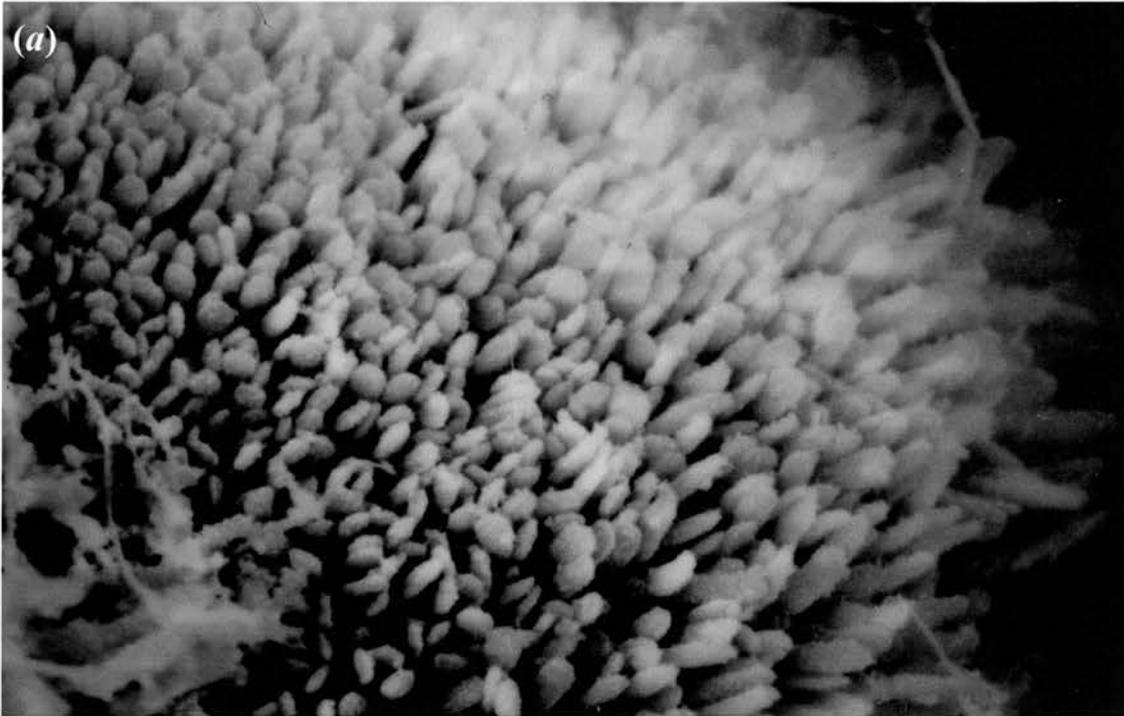


Figure 1.4 (a) Electron micrographs showing the surface of an intact sperm bundle (spermatozeugmata) and (b) individual spermatozoa with tails clearly visible. Note the presence of lance-shaped sperm heads in both micrographs. See text for details.

are born fully independent of their mother. During ovulation, females become increasingly receptive to male courtship (Liley 1966); at other times, females are sexually unresponsive. Female guppies store sperm for six to eight months (Constantz 1984) and can produce several successive broods without remating (Winge 1937). Sperm are stored within folds of the ovary (Constantz 1984) where they are nourished by extracellular ovarian sugars (Gardiner 1978).

(f) *Sexual selection*

Sexual selection was the mechanism proposed by Darwin (1871) to explain the evolution of exaggerated male traits such as fighting weapons, courtship displays, song and ornamentation. Such traits had hitherto appeared to contradict Darwin's (1859) theory of natural selection. Indeed, extravagant traits such as coloration in male guppies clearly increase their chances of succumbing to predation but nonetheless persist in nature (see Fig. 1.5*a* & *b*). Darwin's (1871) simple solution was to make a distinction between traits that enhance survival and those that enhance mating success. He proposed that selection on male traits, either through competition between males for access to females or via female mate choice, would inevitably lead to differential mating success among males. This is not to say that natural and sexual selection are mutually exclusive processes; in Andersson's (1994) review of sexual selection, several examples are provided in which male traits are shown to enhance both survival and mating success. Indeed, Endler (1978) has argued that in some guppy populations, male coloration may actually reduce male conspicuousness rather than enhance it. However, the fact that coloration in guppies occurs only in males strongly suggests that it has not evolved solely for crypsis. Instead, the colour patterns of males at a particular locality probably represent a trade-off between natural selection for crypsis and sexual selection for conspicuousness (Endler 1987; Haskins *et al.* 1961).

There is compelling evidence that conspicuous colour patterns and other phenotypic traits lead to differential mating success among male guppies. Several studies have demonstrated that the extent of male coloration is the most important factor affecting the mating preferences of females. Endler (1983), for example, was the first to collect quantitative data on the mating preferences of females. In his initial experiments, he confirmed that under low levels of predation, males very quickly evolved to be as



Figure 1.5 (a) Two males and (b) a female.

conspicuous as possible. When guppy populations were maintained for several generations in artificial streams, Endler (1980) found that males in coarse-gravel streams evolved smaller colour spots while those kept in fine-gravel streams evolved larger spots. To test his prediction that such rapid evolutionary change was driven by sexual selection, Endler (1983) used a dichotomous choice test to examine the mating preferences of females. Not only did his results confirm his prediction that females are attracted to brightly-coloured males, but they also demonstrated that the visual background (i.e. gravel size and colour) affects male mating success. Again, the most conspicuous males (i.e. those that had the most contrasting colours with the background gravel) achieved greater mating success than their less conspicuous counterparts.

Subsequent studies on mate choice in guppies have confirmed that females base their mating decisions on male colour patterns, and in particular on the area of carotenoid pigmentation. The carotenoid colours, which consist of red, orange and yellow, tend to reflect the nutritional health of males because they can only be obtained from the diet and cannot be synthesised (see Kodric-Brown 1989). Thus, female choice for brightly coloured males may be adaptive because it allows females to choose individuals with better foraging abilities (Houde 1997). Houde and Torio (1992) also found that the brightness of carotenoid spots was affected by parasite infection and that females consistently chose to mate with uninfected males (see also Barber *et al.* 2000; Skarstein & Folstad 1996). This is consistent with Hamilton and Zuk's (1982) hypothesis that female choice evolves because parasite-resistant genes are inherited by the offspring of parasite-resistant fathers. However, the inheritance of parasite resistance by (male and female) guppy offspring has not been demonstrated and it is also possible that females avoid mating with parasitized males to avoid being infected themselves (Kennedy *et al.* 1987; McMinn 1990).

Evidence to support the hypothesis that female guppies are attracted to orange pigmentation comes from numerous studies. For example, Kodric-Brown (1985) confirmed Endler's (1983) original suspicion that carotenoids were important for attracting females by conducting an exhaustive mate choice experiment in which she allowed females to choose between all possible combinations of eleven different colour patterns. In almost every case, females were attracted towards the males with

the highest levels of red and yellow pigment. Houde (1987) subsequently used three criteria to test for female colour preferences: the latency to mate, the frequency of female responses to non-displaying males, and the fraction of a male's courtship display that elicited a sexual response. In all three tests, females preferred males with higher than average orange coloration. Finally, Long and Houde (cited in Houde 1997) experimentally manipulated the visual conditions under which females chose males and found that females only discriminated between males when they were able to detect differences in orange pigment.

A number of experiments have examined female mating preferences for secondary sexual characteristics other than male colour patterns. For instance, Bischoff *et al.* (1985) found that domestic female guppies preferred large-tailed males to small-tailed ones. Sigmoid display rate (Bischoff *et al.* 1985; Kennedy *et al.* 1987; Kodric-Brown 1993; Nicoletto 1993), body length (Reynolds & Gross 1992) and spot asymmetry (Sheridan & Pomiankowski 1997) have also been shown to influence female mating preferences.

Although most researchers agree that female choice (i.e. intersexual selection) is the dominant mechanism of sexual selection in guppies, there is some evidence that male-male competition may also play a role. The guppy's mating system is clearly not one in which males engage in bloody combat: large males appear to have no obvious competitive advantage (Farr 1980b) and fighting weapons are absent. Nevertheless, male-male competition can operate in more subtle ways. Kodric-Brown (1993) assessed the effect of male-male interactions on mating success in guppies and found that the most dominant males (dominance assigned on the basis of nipping and chasing intensity) were also the most colourful and achieved the highest mating success. Intriguingly, Kodric-Brown (1993) also found that all matings performed by subordinate males were sneaky copulations, the success of which is not known. The association between dominance and colour patterns clearly demonstrates how both male-male competition and female choice can simultaneously play a role in sexual selection.

The studies cited above, and several others (see Houde 1997), have provided compelling evidence that female choice plays a central role in the guppy's mating

system. Subsequent work on sexual selection in the species has focused on establishing the function(s) that female choice serves. For instance, research has shown that female guppies sometimes reduce their preference for brighter males when predators (e.g. *Crenicichla alta*) are present, thus avoiding predation themselves (Godin & Briggs 1996). Females may also gain direct fertility benefits (Matthews *et al.* 1997) or avoid parasitic infection (Houde & Torio 1992; Kennedy *et al.* 1987; McMinn 1990) by mating with particular males. While proximate explanations such as these may be valid, much of the work on mate choice in guppies has been directed at explaining female choice in terms of indirect benefits (Breden & Hornaday 1994). Indirect benefits occur when female choice leads to fitness differences in offspring that result from traits inherited from males.

Broadly speaking, there are two types of 'indirect' model to explain the evolution and maintenance of female mate preferences and preferred male traits (Kodric-Brown 1990). Firstly, there is the adaptive – or 'good genes' – model, which postulates the evolution of secondary sexual traits (e.g. coloration, size, display frequency) that indicate a male's genetic quality (Andersson 1994). There are two essential components to this model: (1) a male's ornamentation must positively correlate with his overall genetic fitness; (2) high-quality males should pass on superior genes which in turn enhance the viability and/or fecundity of their male *and* female offspring. Secondly, there is the arbitrary – or 'Fisherian' – model of sexual selection which predicts a genetic correlation between an exaggerated male trait (say, coloration) and female preference for it (again, see Andersson 1994). The model is said to be arbitrary because female choice could potentially act to amplify any male trait for which there is an initial preference (assuming there is enough additive genetic variation for the trait in the population). The trait itself confers no fitness advantage to offspring and is only passed on to males, who in turn are more attractive to the next generation of females.

A number of studies have provided support for the adaptive model of sexual selection in guppies. Many of these studies have simply shown that desirable male traits, for example coloration and courtship intensity, are positively correlated with condition indices such as swimming performance and parasite resistance (Houde & Torio 1992; Kennedy *et al.* 1987; Nicoletto 1991). One study went further by showing that the

preferred male trait – in this case male size – was heritable and conferred fitness benefits to offspring. (Reynolds & Gross 1992). However, Brooks (2000) recently provided evidence of a genetic correlation between the attractiveness of male guppies and their male offspring which is clearly more consistent with the arbitrary model of sexual selection. Moreover, Brooks's (2000) study revealed a negative genetic correlation between male sexual attractiveness and survival, contradicting the predictions of the adaptive model of sexual selection (e.g. Møller & Alatalo 1999). Although it is tempting to view the adaptive and arbitrary models as alternative explanations for the evolution of female choice, the distinction between the two is not always clear-cut and in some cases both may operate simultaneously (see Nicoletto 1991 for further discussion).

(g) *Sperm competition*

In its original formulation, Darwin's (1871) theory of sexual selection made no allowance for female promiscuity and did not therefore account for the phenomenon of sperm competition. Even the most recent and thorough review of sexual selection (Andersson 1994) completely circumvents the issue of sperm competition. Yet sperm competition clearly plays a major role in the mating systems of many species (Birkhead & Møller 1998) and its incorporation into the theory of sexual selection is inevitable (e.g. see Møller 1998). In its simplest form, sperm competition, which was defined by Parker (1970) as the contest between the ejaculates from two or more males for the fertilization of a single female's ova, is the post-copulatory equivalent of pre-copulatory male competition. Competition among males for access to females was one of the two mechanisms underpinning Darwin's theory of sexual selection. The recent inclusion of cryptic female choice into sperm competition theory completes the scenario by incorporating sperm choice (the post-copulatory equivalent of female choice) into the theory of sexual selection (Eberhard 1996; 1998).

Sperm competition has been likened to a raffle, where the greater the number of sperm a male has competing for fertilization, the greater his chances of reproductive success (Parker *et al.* 1990). Assuming sperm competition operates on this principle, selection for high investment in spermatogenesis should be common among males in polygamous species. However, sperm – especially when packaged in bundles – are a costly resource (Dewsbury 1982) and males should optimise their investment

according to the magnitude of sperm competition they face (Parker 1982). In support of this prediction, comparative studies across a wide range of taxonomic groups have shown that male ejaculate expenditure is elevated in species experiencing high levels of sperm competition (for examples see Birkhead & Møller 1993; Gage 1994; Harcourt *et al.* 1981; Hosken 1997; Jennions & Passmore 1993; Møller 1988; Stockley *et al.* 1997; Svard & Wiklund 1989).

There is also evidence that in some insect species individual males can adjust ejaculate expenditure according sperm competition risk. For example, moths (Gage 1995) and crickets (Gage & Barnard 1996) adjust their rates of sperm production according to population density, thus adopting reproductive strategies to suit their social environment. Male guppies tend to increase their courtship display rate in the presence of competitors (Farr 1976; Farr & Herrnkind 1974) but it is not known whether this is reflected by their patterns of sperm production or by the size of their ejaculates. Recent work has confirmed that ejaculate expenditure is closely associated with male behaviour in guppies (Matthews *et al.* 1997) so it is possible that any change in male behaviour will be mirrored by changes in ejaculate characteristics. This question is addressed in chapter four in which the effect of rearing demography on sperm production (stripped ejaculate size and relative gonad size) and the mating behaviour of male guppies is investigated.

One of the simplest ways to investigate the outcome of sperm competition is to examine paternity patterns when two or more males mate consecutively with a female. Studies of this nature are common and in several cases (most notably among the insects) authors have reported last-male sperm precedence (reviewed by Simmons & Siva-Jothy 1998). Specifically, this term refers to the proportion of offspring sired by the last male to mate and is usually referred to as P_2 (Boorman & Parker 1976). If P_2 is significantly greater than 0.5, there is last male sperm precedence. In the past, attempts to document patterns of sperm precedence have been constrained by the methods available to assign paternity to offspring. For example, the irradiated male technique (Parker 1970) and those using genetic markers such as coloration (Houde 1988) or homozygous traits (Birkhead & Møller 1992) made it difficult to distinguish between more than two putative sires at a time. Since females often mate with more than two males during each reproductive cycle, the observation of high P_2 in the two-

male experiments may be overly simplistic (Zeh & Zeh 1994). With the advent of DNA fingerprinting techniques, such as microsatellite profiling (Blouin *et al.* 1996; Primmer *et al.* 1995; Queller *et al.* 1993), these problems have been largely overcome.

Microsatellites consist of tandem repeats of short nucleotide motifs that are flanked by known DNA sequences, or primer sites (Queller *et al.* 1993). DNA fingerprinting utilises the polymerase chain reaction (PCR) to amplify target microsatellite regions, the lengths of which are resolved on polyacrylamide gels (Sambrook *et al.* 1989). Because microsatellites tend to have variable numbers of repeats, they are potentially sensitive mendelian markers for determining kinship among individuals. Since the technique has the potential to unambiguously assign paternity to multiply-sired offspring, its use in the field of behavioural ecology is becoming increasingly widespread (e.g. Kellogg *et al.* 1995; Primmer *et al.* 1995; Zane *et al.* 1999).

Matthews (1998) recently found that in guppies, freshly inseminated sperm outperform stored sperm from a previous mating (i.e. sperm inseminated during the previous brood cycle). However, in subsequent broods the stored sperm from the first male took precedence over the more freshly inseminated sperm from the second male. Male guppies may therefore gain a short-term advantage in being the last to mate, but this initial advantage appears to be offset later on. To some extent these results parallel the findings from an earlier experiment (Hildemann & Wagner 1954) in which new sperm outperformed stored sperm. However, the switch from last-male sperm precedence to stored-sperm precedence was not apparent in Hildemann and Wagner's (1954) study. If sperm competition in the guppy follows the principle of a lottery, it is likely that recently inseminated sperm are initially more successful because many of the stored sperm are unavailable at the site of fertilization. The breakdown of this last-male advantage in later broods may result from there being few, if any new sperm left to fertilize subsequent batches of ova.

Patterns of sperm precedence in the guppy have not been documented following multiple copulation within a single reproductive cycle. In the above studies, Matthews (1998) and Hildemann and Wagner (1954) took post-partum females containing stored sperm and mated each of them with a new male. Both experiments were constrained to some extent by the methods available at the time for assigning paternity

to offspring. Hildemann and Wagner (1954) used genetic markers (coloration) while Matthews (1998) used random amplified polymorphic DNA markers (RAPDs). Neither method was sufficiently sensitive to assign parentage to offspring arising from two ejaculates competing within the same brood cycle and from the same genetic strain of males (i.e. from the same population in the case of Matthews 1998). Recently, however, a number of microsatellite markers have been developed for poeciliids, some of which are highly polymorphic and therefore appropriate for paternity studies (Taylor & Breden, unpublished data; Parker *et al.* 1998). With the advent of these new markers it is now possible to examine the outcome of sperm competition when females mate consecutively with two (or more) males *within* the same brood cycle. In chapter five, I describe an experiment in which two of these microsatellite DNA markers are used to investigate sperm precedence patterns when females mate consecutively with two males within a single brood cycle. Since female guppies regularly solicit copulations from more than one male during each receptive phase, observing the outcome of sperm competition between two fresh ejaculates is highly relevant.

(h) *The benefits of multiple mating*

Throughout its brief history, the study of sperm competition has essentially been an androcentric one. Sperm competition, it seemed, was simply male-male competition taking place in a different arena to that which Darwin originally envisaged. Females were believed to be monogamous and sperm competition existed only because males were inherently promiscuous. This male-orientated view of sperm competition may have had 19th century roots but it nonetheless remained intact for more than a century (Trivers 1972). Only in the last decade has the role of the female been questioned in relation to multiple mating. When it was discovered that it was often *females* that solicited copulations from more than one male (e.g. Kempenaers *et al.* 1992; Smith 1988), research began in earnest to uncover the function(s) of such behaviour.

A commonly asked question is why a female should mate with several males during a single reproductive cycle when the sperm from a single, preferred male are sufficient to fertilize all of her eggs (Jennions & Petrie 2000; Keller & Reeve 1995; Yasui 1998). The question is not trivial; costs associated with multiple mating are listed in Keller and Reeve (1995) and include time and energy, loss of foraging opportunities,

increased vulnerability to predation and elevated risk of parasite transmission. In some cases, mating may even expose females to pathological substances in the male seminal plasma (Chapman *et al.* 1995). For some species, these costs are offset by direct benefits through fecundity-enhancing nutrient donations (Gwynne 1984) or the replenishment of sperm reserves (Fjerdingstad & Boomsma 1998). In theory, females should be able to obtain these direct benefits by mating repeatedly with a single male – assuming of course that this male is not severely sperm limited after a single copulation.

Explanations for polyandry, where females mate with more than one male within each reproductive cycle (Thornhill & Alcock 1983), often rely on genetic (i.e. indirect) benefits as these can only accrue from multiple copulations with different mating partners (e.g. Archer & Elgar 1999). For multiple mating to provide such benefits to females there must be a relationship between a male's genetic quality and the fertilization efficacy of his sperm. Assuming such a correlation exists, female-mediated sperm competition, as a result of multiple mating, should lead to more viable offspring. Madsen *et al.* (1992) provided support for such a mechanism by showing that multiple copulations by female adders (*Vipera berus*) increased offspring viability (measured as the proportion of live offspring at birth). In addition, they found that females tended to mate with a series of different males, and that sperm from 'better' males achieved the highest fertilization rates. Olsson *et al.* (1994) reported similar results when they showed that multiple mating enhanced the fitness of newborn sand lizards. However, neither study excluded the possibility that better (i.e. genetically superior) females attracted more mating partners (see commentary by Parker 1992). Furthermore, neither study was able to distinguish between repeated copulations with a single male and multiple copulations with several males. Recent work has addressed this issue and confirmed that in many cases, polyandrous females actively discriminate against previous mates, preferring instead new mating partners (Archer & Elgar 1999; Zeh *et al.* 1998). More importantly, Zeh (1997) confirmed that in the pseudoscorpion, repeated copulations with the same male did not increase offspring fitness, whereas females mated to several males experienced increased embryo survival and fewer brood failures.

In addition to selecting genes for enhanced offspring viability, females may have the potential to select genetically compatible gametes by mating with several males. Parker (1992) notes that it is unlikely that a female can reliably match the genotype of her suitor to her own in pre-copulatory mate choice (but see Drickamer & Lenington 1987 for an interesting exception in mice). Therefore, by accepting sperm from several males, females may exploit post-copulatory mechanisms (e.g. sperm competition, sperm selection and reallocation of maternal investment) to insure against costly genetic incompatibility (Zeh & Zeh 1997). For example, Price (1997) demonstrated conspecific sperm precedence, irrespective of mating order, in two species of *Drosophila*, suggesting that post-copulatory mechanisms may prevent hybridisation between closely related taxonomic groups. This possibility was also explored by Howard *et al.* (1998), who found that two closely related species of ground cricket (*Allonemobius* spp.) rarely produced hybrid offspring, even when pre-mating barriers to fertilization were controlled. Conspecific sperm precedence may therefore have the potential to severely limit gene flow between morphologically and anatomically similar species.

Precedence for genetically compatible sperm may occur within species as well as between closely related species. Several insect studies suggest that females mate with more than one male in order to reduce the risks associated with genetic incompatibility and inbreeding (reviewed in Jennions & Petrie 2000; Zeh & Zeh 1997). Zeh (1997) attributed poor reproductive success in pseudoscorpions to genetic incompatibility as no other hypotheses could account for her observation of reduced brood size in singly-mated females; certain males simply appeared to be more compatible with certain females. These results were corroborated in a recent study by Newcomer *et al.* (1999) in which they compared hatching success among female pseudoscorpions mated either twice to the same male (SM treatment) or twice to two different males (DM treatment). Their results indicate that DM females experienced a lower rate of spontaneous abortion than SM females and, as a consequence, the lifetime fecundity of polyandrous females was significantly enhanced. Tregenza and Wedell (1998) produced similar results when investigating the reproductive success of multiply-mated field crickets (*Gryllus bimaculatus*). In their experiment they assigned one, two or four males to each female but held the number of copulations constant among replicates. Their results showed that egg-hatching success increased with the

number of mates. A second experiment, in which they compared females mated twice (with a single male) with those mated four times (with a single male), revealed no such differences in hatching success (Tregenza & Wedell 1998). This clearly indicates that it is the number of males, rather than the number of copulations, that influences reproductive success in field crickets.

(i) *Why do female guppies mate multiply?*

There is evidence that female guppies discriminate against previous mating partners in favour of unfamiliar males (Farr 1977; Hughes *et al.* 1999). Such frequency-dependent mate choice behaviour by female guppies has been discussed only in relation to the maintenance of colour pattern polymorphism in wild populations (Hughes *et al.* 1999). To date, no studies have addressed the possible functional significance of these mating decisions by females. One interpretation of these studies (Farr 1977; see also Farr 1980b; Hughes *et al.* 1999) is that females show a preference for novel males in order to incite sperm competition or sperm choice. The mating behaviour of female guppies is certainly consistent with this view; polyandry has been documented in both laboratory (see Constantz 1984) and wild populations (Haskins *et al.* 1961; Kelly *et al.* 1999), even though it carries significant costs (listed in Houde 1997 and summarised in chapter six). The final experimental chapter of this thesis describes an experiment designed to investigate the possible benefits accrued by females through multiple mating. Since the guppy's mating system is completely resource free, few direct benefits can arise from polyandry. However, male guppies inseminate a large proportion of their available sperm during a single copulation (Pilastro & Bisazza 1999), so sperm limitation may be a problem for males (and ultimately for females). Females may therefore mate with several males to insure against sperm limitation (e.g. Wetton & Parkin 1991). Other than mating for more sperm, female guppies may obtain a range of indirect benefits through polyandry; these possibilities are also explored in chapter six.

(j) *Specific aims of project*

- To examine the patterns of sperm production and male courtship behaviour in natural guppy populations and to test the hypothesis that these traits co-vary with predation risk.

- To compare courtship behaviour, sperm production and colour patterns among four age classes of male.
- To test the hypothesis that male courtship and sperm production are phenotypically plastic traits that vary in accordance with rearing demography.
- To examine patterns of sperm precedence and uncover possible predictors of male reproductive success.
- To investigate the possible benefits (direct and indirect) accrued by females through multiple mating.

Chapter two

Geographic variation in sperm production and male courtship behaviour¹

ABSTRACT

In guppies, population comparisons have yielded important insights into adaptive differentiation. However, despite the current interest in sperm competition and spermatogenesis, geographic variation in these parameters has received little attention. Here, the hypothesis that sperm production co-varies with predation risk is tested by comparing five natural populations of guppies: two that inhabited dangerous *Crenicichla* localities, two from low-risk *Rivulus* sites and a ‘transplant’ population comprised of the descendants of guppies moved from one of the high-risk sites to a low-predation environment. As predicted, males from the three low-predation sites performed significantly more courtship displays and had larger sperm reserves than their high-predation counterparts. This result may imply higher rates of sperm competition in the low-risk sites.

INTRODUCTION

Sperm competition has been likened to a raffle (Parker 1990a) in which the probability of a male fathering offspring rises in proportion to the number of tickets (sperm) he enters (Parker 1970). It follows that males should invest more heavily in sperm production as the risk of sperm competition increases (Parker 1990b). Marked inter- and intra-specific variation in investment in spermatogenesis, predicted by sperm competition theory, has now been documented. For example, a comparative study of 89 fish species revealed a significant positive association between the intensity of sperm competition and sperm number per stripped ejaculate (Stockley *et al.* 1997). In some cases, males adjust their sperm production in line with the social

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environment in which they develop, or the mating strategy they adopt. For example, Gage (1995) found that when male moth larvae, *Plodia interpunctella*, develop at higher population densities, they grow bigger testes and produce ejaculates containing more sperm in anticipation of higher levels of sperm competition. Gage *et al.* (1995) also showed that precocious male salmon parr, *Salmo salar*, which mate by sneaking, have greater numbers of sperm, in relation to body size, than their anadromous male counterparts.

Although there is considerable interest in the relationship between male mating strategy and sperm production, geographic variation in these traits amongst populations of a single species has been largely ignored. Yet, as Foster & Endler (1999) point out, population comparisons yield important insights into adaptive differentiation. Natural populations often exhibit cascades of effects that stem from the correlation between behavioural, morphological and geographical traits. One of the best examples of this multiple-trait co-evolution is provided by guppy populations in Trinidad (Endler 1995). Haskins (1961) proposed that geographic variation in male colour patterns results from the interplay of (natural) selection by predators against conspicuousness and the (sexual) selection of colourful males by females (Endler 1978; Endler 1980; Endler 1983). It is now recognised that predators play a key role in generating the remarkable diversity of guppy populations in Trinidad (for reviews, see Endler 1995; Houde 1997; Magurran *et al.* 1995). Indeed, virtually every morphological, life-history or behavioural trait that has been examined is known to co-vary with risk, which in turn co-varies with biotic and abiotic factors such as forest cover and stream size (Endler 1995). Many of these traits exhibit complex interactions. For example, sexual selection and female receptivity is reduced in high-predation sites due to the combined effects of elevated predator avoidance behaviour, greater sexual harassment, more sneaky mating, increased food availability and higher water temperature (Endler 1995).

Experimental manipulations in the wild have confirmed that a shift in the predation regime results in evolution in guppy populations. For example, Endler (1980) conducted a transplant experiment that demonstrated heritable change in male colour patterns following a decrease in predation risk. In 1976 200 guppies were moved from a section of the Lower Aripo River in Trinidad to a previously guppy-free tributary

further upstream. Guppies in the Lower Aripo River co-occur with dangerous cichlid and characoid predators including *Crenicichla alta* and *Hoplias malabaricus* (Magurran & Seghers 1994; Reznick *et al.* 1990), whereas the tributary to which the transplanted fish were moved was originally home to just one species of fish, *Rivulus hartii* (Reznick *et al.* 1990). *Rivulus*, a member of the Cyprinodontidae, is at most a minor predator of guppies (Liley & Seghers 1975; Magurran *et al.* 1995). Within two years the male descendants of the transplanted guppies had become more conspicuous due to an increase in the size and number of their colour spots. Reznick & Endler (1982) and Reznick *et al.* (1990) demonstrated further evolutionary change in the transplanted population. In most respects, guppies introduced to the transplant site had evolved life-history (Reznick *et al.* 1990) and sexually-selected (Endler 1980) traits characteristic of low-predation populations. Artificial introductions in other Trinidadian rivers provide additional evidence of predator-mediated evolution in behaviour (Magurran 1998) and life history (Reznick *et al.* 1997).

Given the co-evolution of multiple traits in natural guppy populations, as highlighted by Endler (1995), it is likely that any geographic variation in sperm production will be complex and unpredictable. Nonetheless, since male courtship activity is often reduced in high-predation populations (Endler 1987; Magurran & Seghers 1990) and female receptivity is reduced in the presence of predators (Gong 1997) it seems likely that male investment in spermatogenesis will also be lower in these populations. The aim of this study is therefore to test the hypothesis that sperm production, as well as courtship intensity, is greater in males inhabiting low-predation populations than it is in males inhabiting high-predation ones. To achieve this, the sperm production and sexual behaviour of male guppies in Endler's transplanted population in the Aripo River, Trinidad, are contrasted with those of males in two high-predation (*Crenicichla*) populations (including the ancestral one), and two low-predation (*Rivulus*) populations. The inclusion of Endler's transplant population makes it possible to control for historical effects and excludes the possibility that any observed variation has arisen independently of predation regime. An increased level of sperm production by males in the transplant and *Rivulus* sites, relative to the *Crenicichla* ones, would be consistent with the hypothesis that sperm competition, like sexual selection, is more intense in low-risk localities.

METHODS

(a) *The study populations and their maintenance*

Male and female guppies were obtained from five populations: two high-predation populations (Lower Aripo River: grid reference PS 938 786; Middle Tacarigua River: PS 787 804), two low-predation populations in the same river systems (Naranjo River (Aripo): PS 936 809); Tunapuna (Tacarigua): PS 759 797) and Endler's Aripo Transplant (PS 932 799; see streams marked with asterisk in Fig. 1.1). Fish were returned to the laboratory in Trinidad where they were maintained in stock aquaria (~1:1 sex ratio) and used for the experiment within a month of collection.

Observations of male mating behaviour were made in a 45 x 23 x 20cm deep aquarium containing an airstone, filter and natural gravel, and maintained at 26°C. The fish were fed to satiation with commercially prepared flake food (Tetramin™) at least one hour before mating behaviour was recorded.

(b) *Mating behaviour*

Five males from each population were observed in the presence of five non-virgin females, all unfamiliar to the test males. The fish were allowed to settle for one hour before observations commenced. The number of sigmoid displays and gonopodial thrusts performed by each male was recorded over a 10-minute period. A sigmoid display is characterised by the male orientating itself in front of a female and vibrating its body in an S-shaped posture (Liley 1966). Sigmoids were only recorded if they were directed at a particular female. Females are receptive to males either as virgins or during the few days following parturition, and are unlikely to respond to male displays outside this period (Liley 1966). A gonopodial thrust is an alternative mating tactic and does not require female co-operation. During a thrust attempt a male approaches a female from behind, swings his gonopodium (intromittent organ) forward, and attempts to insert it into the female's genital opening (Liley 1966 and see chapter one). A male was judged to have successfully performed a thrust if his gonopodium made contact with a female's genital region. Differences in coloration made it possible to recognise each male individually.

(c) *Measuring sperm number*

Following behavioural observations, the males were isolated and humanely killed in a water bath containing a lethal dose of 0.4g l⁻¹ Benzocaine (ethyl p-amino benzoate). After removing excess water from each male, total body length (snout to tip of caudal fin) was measured and sperm were extracted. To collect sperm each male was placed on a Petri dish under a low-power dissection microscope. The gonopodium was swung forward and gentle pressure was applied to the side of the abdomen, just anterior to the base of the gonopodium. This action, which released sperm in the form of a number of spermatozeugmata (sperm bundles), was repeated in order to ensure all sperm bundles were removed. Following removal, the bundles were drawn up a Gilson™ pipette and added to 100µl of water. To break up the bundles and distribute sperm cells evenly, samples were repeatedly drawn up and expelled from the pipette. Sperm count was estimated by counting sperm cells on an 'improved Neubauer chamber' haemocytometer under x400 magnification. The distribution of sperm cells across the haemocytometer was checked visually for evenness before counts commenced. The number of sperm per stripped ejaculate was determined by multiplying the mean sperm count (from five counts) by the sample's dilution factor and initial volume. Sample sizes (number of male fish tested per population) were as follows: high-predation sites (Aripo: $n=24$; Tacarigua: $n = 20$); low-predation sites (Aripo: $n = 24$; Tunapuna: $n = 20$); Aripo Transplant site ($n = 21$).

RESULTS

Males from the high-predation sites performed significantly fewer sigmoid displays than those from the low-predation and transplant sites (Fig. 2.1a; ANOVA, $F_{4,102} = 10.82$, $P < 0.001$). This was confirmed using a post-hoc test for multiple comparisons (Tukey test, all values tested at $P = 0.05$). Thus, the courtship behaviour of the transplanted males resembled that of males from the low-predation sites more than it did the ancestral (high-predation) population. The rate at which males performed gonopodial thrusts also differed significantly among the populations (Fig. 2.1b; $F_{4,102} = 8.34$, $P < 0.001$).

To test the hypothesis that males from low-predation sites have higher sperm reserves than their high-predation counterparts, the sperm counts of males from the five populations were compared. Although sperm counts appeared to differ among males

from the different populations (Fig. 2.1c), there were also differences in male body size among the populations (Fig. 2.1d). Males from the high-predation sites were significantly smaller than those from the low-predation ones (ANOVA, $F_{4,102} = 42.9$, $P < 0.001$). To control for body size differences among the populations, an analysis of covariance (ANCOVA) was performed in which total length was the covariate. The difference in sperm number among the populations remained significant when controlling for body size; males from the three low-predation sites (two low-predation and transplant) had larger reserves of sperm than those from the high-predation sites (Fig. 2.1c; $F_{4,101} = 7.34$, $P < 0.0001$). This was confirmed with a post-hoc test for multiple comparisons (Tukey pairwise test, $P < 0.05$).

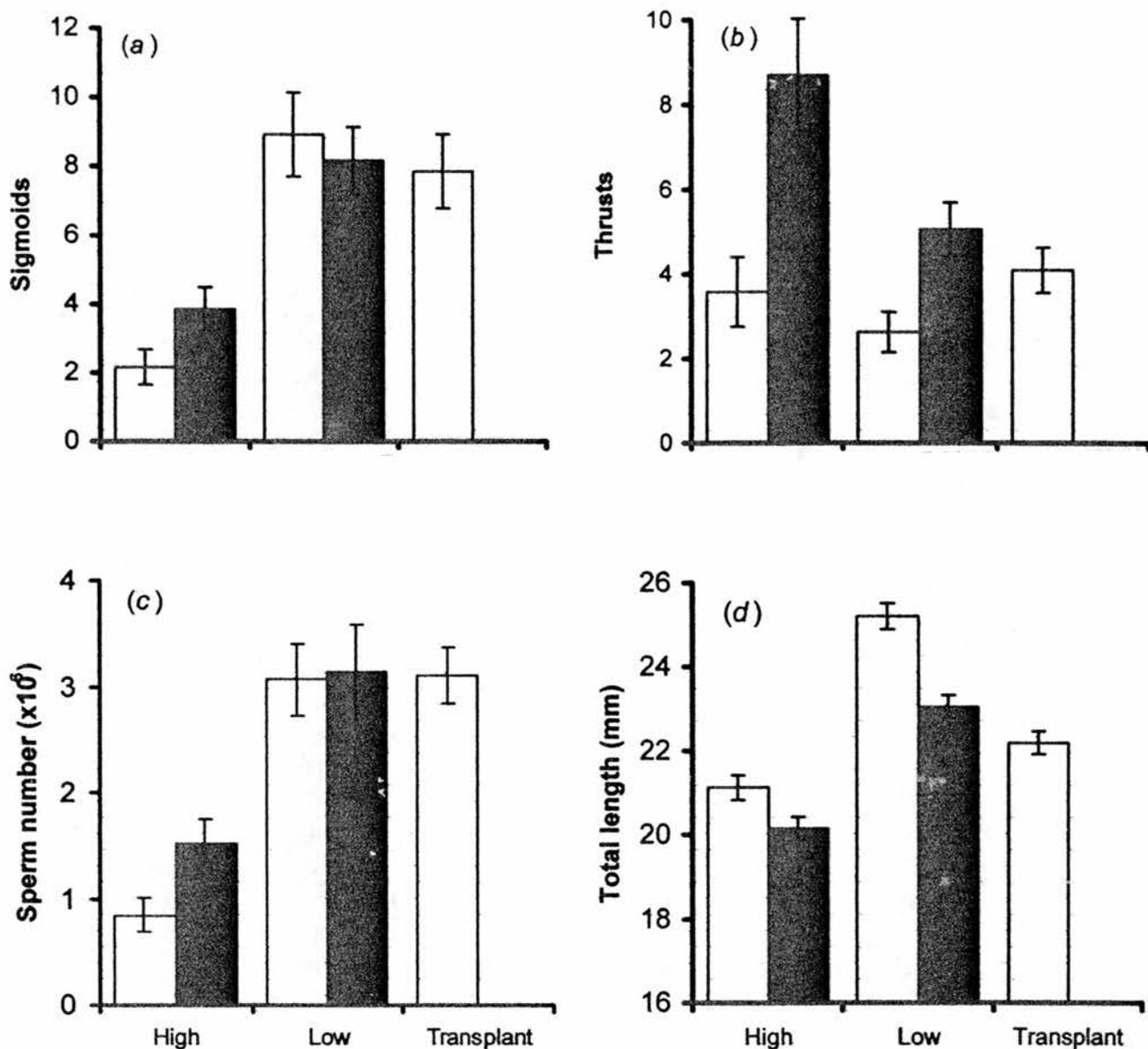


Figure 2.1 Sexual behaviour (a, b), sperm number (c) and body size (d) in male guppies from five localities in Trinidad. Figures are represented by the means \pm SE. Sample sizes: high predation $n = 24$ and 20 ; low predation $n = 24$ and 20 ; transplant $n = 21$. Shaded bars represent the Tunapuna (low predation) and Middle Tacarigua rivers (high predation) and open bars represent Lower Aripo (high predation), Naranjo (low predation) and Endler's (1980) Aripo transplant rivers (low predation).

DISCUSSION

Males inhabiting the three low-predation rivers had larger sperm reserves than their high-predation counterparts. One simple explanation for this is that males, which have determinate growth, are larger in *Rivulus* populations due to the combined effects of

water temperature (Liley & Seghers 1975) and selection on life-history traits (Reznick *et al.* 1997). Nevertheless, as demonstrated by the analysis in this study, the increased investment in sperm production by low-predation males cannot be accounted for by body size alone. Additionally, males in low-predation populations may have greater sperm reserves because they have fewer opportunities to mate. However, it should be noted that when male guppies are housed in standard conditions in the laboratory, with ample access to females, it is the individuals with high sperm reserves that exhibit consistently high courtship rates (Matthews *et al.* 1997). A further possibility is that fish inhabiting high-predation sites may channel their resources towards predator avoidance rather than reproduction (Havel & Dodson 1987). However, there is no evidence for this in wild guppy populations, and in studies carried out on captive descendants of wild guppies, no difference was found between the gonadosomatic indices of high- and low-predation males (Matthews *et al.* 1997).

A possible explanation for the observed difference in sperm reserves among the populations is that solicited copulations (as opposed to sneaky matings) following courtship displays require higher numbers of sperm than copulations following sneaky mating. Males inhabiting low-predation sites tend to rely more heavily on courtship displays to attract females than their high-predation counterparts (Farr 1975; Luyten & Liley 1985; Magurran & Seghers 1990) and this was confirmed by the present study. In a population of feral Brazilian guppies, Pilastro and Bisazza (1999) found that the number of sperm delivered after courtship was on average three times greater than when delivered after sneaky mating. Thus, the difference in sperm number detected in the present study of five Trinidadian populations may reflect the observed variation in mating strategies adopted by males in the different rivers. It would be interesting to compare the insemination efficiency (e.g. Pilastro & Bisazza 1999) of both mating strategies in natural populations to test these ideas further.

Males may also need larger sperm reserves in low-predation sites if the incidence of multiple mating in such populations is higher. As noted in the introduction, females in these *Rivulus* sites are more choosy during their receptive phase (Endler & Houde 1995; Houde 1997) and are unlikely to have their receptivity curtailed by the presence of predators. Thus, it is possible that the incidence of multiple mating by females is reduced in high-predation populations. However, such localities are characterised by

elevated levels of sneaky mating (see below) and this may mask any decrease in solicited copulations by females. Clearly, data on the relative incidence of multiple paternity in guppy populations in Trinidad are needed before such a direct link between predation and sperm competition risk can be claimed (see chapter seven for further discussion).

Although sperm competition may be reduced in high-predation populations it is unlikely to be absent. Guppy populations located in high-predation (*Crenicichla*) localities are less female biased than those in low-predation (*Rivulus*) ones (Rodd & Reznick 1997; Seghers 1973). This means that males in *Crenicichla* sites may have proportionally more competitors for each mating than their counterparts in *Rivulus* ones. The rate at which male guppies encounter one another could be increased further in risky localities as these fish have a higher schooling tendency (Magurran *et al.* 1992; Seghers 1974). Furthermore, for reasons including risk-sensitive courtship behaviour (Magurran & Seghers 1990), males in *Crenicichla* sites tend to rely more heavily on sneaky mating than those in *Rivulus* sites (Magurran & Seghers 1994). Although the high-predation males in the present study did not consistently exhibit this strategy, it remains possible that sneaky copulations undermine female choice more often in high-predation sites (see Kelly *et al.* 1999).

A number of factors may account for differences in male sexual behaviour between populations. These include short-term behavioural responses to predators (Endler 1987), differences in social environment (Rodd & Sokolowski 1995) and inherent genetic variation amongst populations (Luyten & Liley 1985). It has been shown that the presence of predators influences the degree to which males perform sigmoid displays and gonopodial thrusts (Endler 1987) although this can depend on the predation regime from which they originate (Magurran & Seghers 1990). Generally, males reduce their sigmoid display rate in favour of sneaky mating in the presence of predators (Dill *et al.* 1999; Endler 1987; Magurran & Seghers 1990). However, in the present study, males from the five localities were observed under standardised conditions and therefore short-term behavioural responses to predators cannot account for the observed differences in their behaviour. Despite this, however, the possibility that past experience, rather than inherent genetic variation, shaped the behaviour (and sperm production characteristics) of males in the study populations cannot be ruled

out. The degree to which males perform sigmoid displays and sneaky mating attempts is closely linked to the population demography they experienced as juveniles (see chapter four), as well as to heredity (Rodd & Sokolowski 1995). Indeed, the intensity of courtship by males in the Lower Aripo population has varied over time (Luyten & Liley 1985; Magurran & Seghers 1990; Magurran & Seghers 1994, this study). The finding that male display behaviour *and* sperm number co-varied in the transplant population, along with the fact that both traits resembled those seen in the two low risk sites, is consistent with the evolution of the transplant population. It will of course be necessary to raise fish from these populations under controlled conditions before any heritable changes can be claimed.

Chapter three

The ontogeny of courtship, colour and sperm production in males

ABSTRACT

In this chapter, courtship behaviour, colour morphology and sperm production are examined in four age classes of male guppies descended from a high-predation population in Trinidad. Specifically, these traits were measured in males aged 7-10 weeks, three months, six months and twelve months. The results revealed that six-month-old males performed higher numbers of sexual acts (both courtship displays and sneaky mating attempts), were more colourful and produced more sperm than males from the other age classes. Surprisingly, juvenile guppies displayed full sexual behaviour despite the fact that they were unable to produce sperm. A significant correlation between male courtship behaviour, sperm production and body size was detected in three- and six-month-old males but not in the other groups. These results suggest that male guppies maximise their mating activity and reproductive investment early in life at the expense of reproductive vigour later on. The data also suggest that information on male fertility status is only available to females from certain age classes of males.

INTRODUCTION

In Trinidad, male guppies engage in almost continuous mating activity and perform around one courtship act (sigmoid display or sneaky mating attempt) per minute (Magurran & Seghers 1994). This sexual ardour is undoubtedly rooted in the variance in fitness between the sexes. A female's reproductive success is ensured as long as she mates periodically to replenish her sperm stores, feeds efficiently and survives to produce successive broods. Indeed, a female's time budget in the wild is largely devoted to foraging, predator evasion and avoiding the persistent courtship of males (Magurran & Seghers 1994). Males, in contrast, may sire many offspring, but those that are neither preferred by females (Houde 1988) nor successful at sneaky mating

(Matthews & Magurran 2000) run the risk of fathering none at all. Furthermore, the bright colour patterns and flamboyant displays of males make them particularly vulnerable to predation (Endler 1978; Haskins *et al.* 1961). Thus, a male's fitness is constrained not only by his opportunities to mate but also by his risk of predation, particularly in those populations where guppies coexist with dangerous cichlid predators. Reznick *et al.* (1996) recorded up to 20% adult mortality per 12 days in such dangerous localities as opposed to 10% mortality over a similar period in less risky sites.

In the light of this mortality risk it is likely that there will be selection on male guppies to maximise their mating activity early in life and that this will be traded off against the reproductive vigour of older males (Charlesworth 1980). Work by Reznick and his colleagues (see for example Reznick & Bryga 1996; Reznick *et al.* 1997) reveals that wild males typically mature at approximately 50 days (high-predation sites) or 60 days (low-predation sites) after birth. These figures are based on the maturation of the male intromittent organ, the gonopodium. Males are considered sexually mature when the apical hood of the gonopodium extends beyond the tip of the anal fin (Houde 1997). Although relatively young males can successfully inseminate females when Reznick's maturity criterion is met (Houde 1997), it is not known whether adult patterns of sperm production can be achieved in such young fish. Furthermore, it is not clear whether courtship behaviour, morphology (including colour patterns) and spermatogenesis mature in synchrony in male guppies. The ontogeny of these three traits was therefore examined in four age classes of guppy descended from a high-predation population in Trinidad. It was also determined whether sperm production and courtship vigour is reduced in those older males that would, in the wild, almost certainly be dead.

METHODS

(a) *Origin of fish and maintenance*

The guppies used in this experiment were descendants of wild-caught fish from the lower Tacarigua River, Trinidad. The lower Tacarigua is a lowland site where guppies coexist with a variety of predators including the pike cichlid *Crenicichla alta*. To obtain males of known age and origin, sixty post-partum females were isolated and checked for broods daily. When produced, broods were isolated from their mother and

placed in 30 x 24 x 20 cm aquaria where they were fed a diet of Tetramin™ baby food, *Artemia* nauplii and subsequently commercially prepared flake food. Juvenile guppies were raised in several aquaria until required for behavioural trials. Each tank contained mixed-sex fish born within a two-week period, and was marked according to the age of fish it contained. On average, each tank accommodated approximately six separate broods comprising 40-50 fish. Temperature was maintained at $25 \pm 0.5^\circ\text{C}$ and illumination provided by an 18W bulb on a 12-h light/dark cycle.

(b) *Male mating behaviour*

At approximately five weeks of age males became morphologically distinguishable from females on the basis of anal fin development and emerging colour patterns. By 7-10 weeks, the gonopodium was distinctly rod-shaped and the apical hood was visible (see Houde 1997). At this stage of development (hereafter termed 'juveniles'), and at three months, six months and twelve months, a subset of males was placed in an observation tank for behavioural trials. Juveniles were only included in the mating trials if they had well formed gonopodia. In practice, it was relatively easy to check this by examining the males while they were swimming in the rearing tanks. Few males survived beyond twelve months and therefore the trials were halted at this point. Aeration was provided via airstones in a filter. Fish were fed to satiation with Tetramin™ flake food each morning of the trials. Male courtship behaviour was examined in juveniles ($n = 18$), three-month-olds ($n = 50$), six-month-olds ($n = 50$) and twelve-month-olds ($n = 20$).

In each trial, five males were placed in a 59 x 29 x 35 cm deep observation tank containing five fully mature non-virgin females, all unfamiliar to the test males. Individual males were recognised by their unique colour patterns. For each male, colour patterns were sketched to aid subsequent recognition of the fish. The observation tank contained gravel and a small clump of Java moss (*Vesicularia dubyana*). The fish were allowed to settle overnight before observations commenced. The number of sigmoid displays and gonopodial thrusts performed by each male was recorded over a fifteen-minute period. A sigmoid display is characterised by the male orientating itself in front of a female and vibrating its body in an S-shaped posture (Liley 1966). Sigmoids were only recorded if they were directed at a particular

female. A gonopodial thrust is an alternative mating tactic in which a male approaches a female from behind, swings his gonopodium forward, and attempts to insert it in her genital opening (Liley 1966). A male was judged to have successfully delivered a thrust if his gonopodium made contact with a female's genital region.

(c) Male morphology and colour patterns

Following behavioural observations males were isolated for two days (to replenish sperm reserves prior to stripping – see below) and anaesthetised in a water bath containing 0.4g l⁻¹ Benzocaine (ethyl p-amino benzoate). After removing excess water from the body surface, standard length and gonopodium length (base to apex) was recorded for each fish using a dissecting microscope fitted with a digital graticule. All measurements were accurate to within 0.1 mm. Following length measurements, anaesthetised males were photographed using a mounted camera (Nikon FM 35mm with a 100mm macro lens and two electronic flash guns) loaded with Kodak Ektachrome 64 slide film. For calibration, a section of ruler was included in each photograph. Slides were digitised and colours were quantified using a computerised image analysis software package (NIH image). The total surface area of each fish was measured in order to calculate the percentage of area covered by two main colour types: carotenoids (which include red, orange and yellow) and black spots / lines (melanin) (Endler 1978). Because relative area of coloration was used, the analysis eliminates the possible confounding effect of body size differences among the age classes.

(d) Measuring sperm number

Following photography, each anaesthetised male was placed on an inverted Petri dish under a low-power dissection microscope. The gonopodium was swung forward and gentle pressure applied to the side of the abdomen, just anterior to the base of the gonopodium. This action released a number of spermatozeugmata (sperm bundles). This procedure was repeated to ensure all sperm were removed. Following removal, sperm bundles were drawn up a Gilson™ pipette and added to 100µl of 0.9% saline solution. To break up sperm bundles and distribute individual spermatozoa evenly, samples were repeatedly drawn up and expelled from the pipette. Sperm count was estimated by counting sperm cells on an 'improved Neubauer chamber' haemocytometer under x400 magnification. The number of sperm in each stripped

sample was determined by multiplying the mean of five counts by the sample's dilution factor and initial volume. Sperm counts were expressed as the total number of spermatozoa per stripped ejaculate.

RESULTS

Six-month-old males performed more sigmoid displays (Fig. 3.1a; ANOVA, $F_{3,134} = 7.16$, $P < 0.0001$) and gonopodial thrusts (Fig. 3.1b; $F_{3,134} = 11.78$, $P < 0.0001$) than any of the other age groups. For both behaviours, six-month-olds were most active but the differences between them and twelve-month-olds were not significant (Tukey post-hoc test, $P > 0.05$). Sperm number showed a corresponding pattern to sexual behaviour: six-month-old males produced higher numbers of sperm, when controlling for differences in body size among the groups, than all other age classes (Fig. 3.1c; ANCOVA, $F_{3,128} = 12.09$, $P < 0.0001$). Again, the difference between sperm number among six- and twelve-month-olds was not significant. Of the eighteen juvenile males, only one produced sperm when stripped. Despite this, however, twelve of the eighteen juveniles performed sexual behaviour, albeit at a lower level than their adult counterparts (see Fig. 3.1a, b).

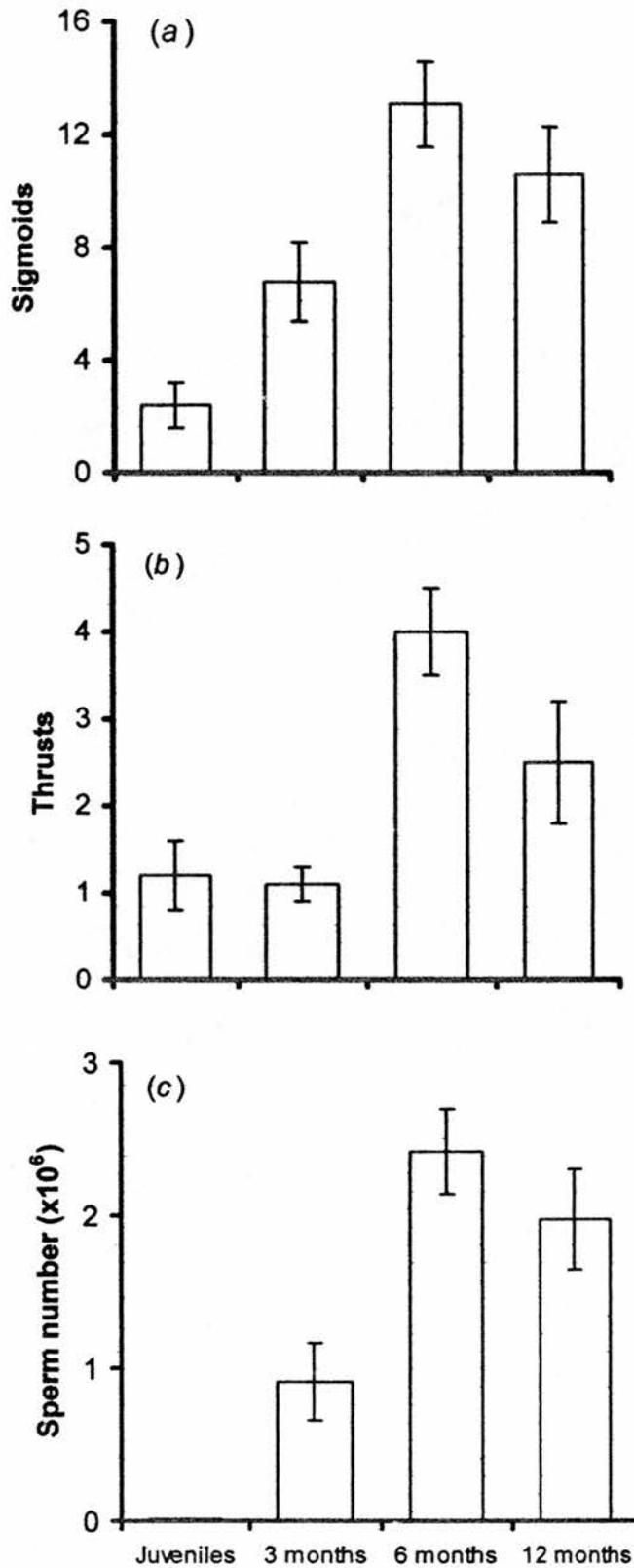


Figure 3.1 Mean (\pm SE) number of sigmoid displays (a) and gonopodial thrusts (b) over a fifteen-minute period and the number of sperm in a stripped ejaculate (c) in four age classes of male guppy.

It was only possible to obtain colour pattern data for juveniles ($n = 11$), three-month-olds ($n = 43$) and six-month-olds ($n = 43$). Unfortunately it was not possible to obtain colour data for twelve-month-old fish due to technical problems with the camera. Six-month-old guppies had a significantly greater percentage of their body covered with carotenoid (Fig. 3.2a; Kruskal-Wallis, $K_{2,95} = 16.46$, $P < 0.001$) and black (Fig. 3.2b; $K_{2,96} = 47.94$, $P < 0.001$) spots than younger males.

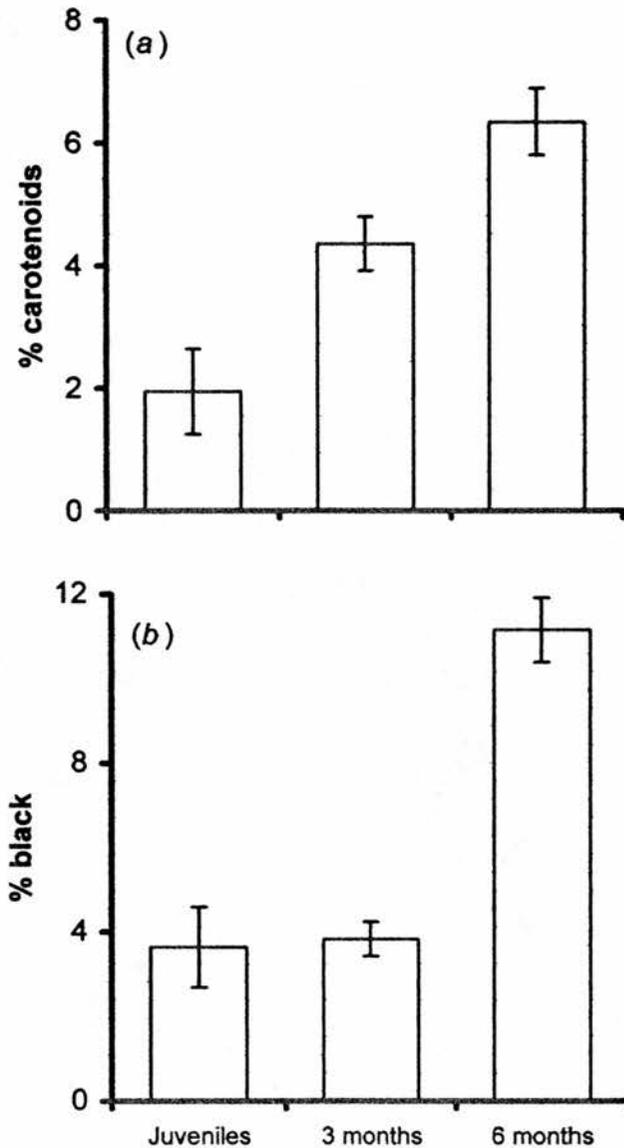


Figure 3.2 Percentage cover of red, yellow and orange carotenoids (a) and black melanin (b) in four age classes of male guppy. Figures are means \pm SE.

The results suggest that the gonopodium reaches its full length at the early stages of sexual development. Males in the juvenile and three-month-old groups had relatively larger gonopodia (when controlling for body size) than older males (Fig. 3.3; ANCOVA, $F_{3,128} = 3.68$, $P = 0.014$). Unlike an earlier study (Reynolds *et al.* 1993) no correlation existed between gonopodium length and thrust rate in any of the age groups (all P values > 0.50). Furthermore, the proportion of sexual acts devoted to gonopodial thrusting (number of thrusts / (sigmoids + thrusts)) did not differ among the age classes ($F_{3,107} = 0.52$, $P = 0.67$).

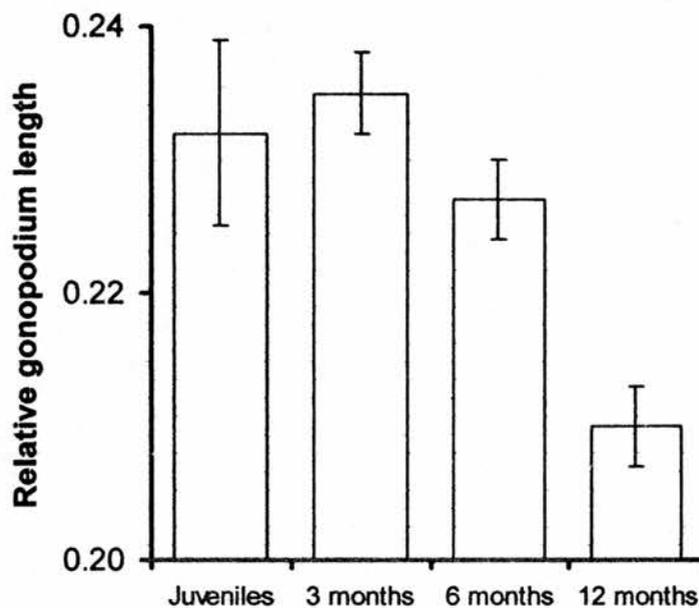


Figure 3.3 Relative gonopodium length (gonopodium length / standard length) among four age classes of male guppy. Figures are means \pm SE.

There was a highly significant correlation between sigmoid display rate and sperm number in three- ($r = 0.85$, $n=50$, $P < 0.0001$; Fig. 3.4a) and six-month-old fish ($r = 0.73$, $n=50$, $P < 0.0001$; Fig. 3.4a). However, no such correlation existed for twelve-month-olds ($P = 0.16$). The correlations between sigmoid display rate and sperm number remained significant in three- and six-month-olds after applying the Dunn-Šidák adjustment ($\alpha = 0.05$ corrected to $\alpha' = 0.009$) for multiple comparisons (Sokal & Rohlf 1995). The same pattern was evident for gonopodial thrusts; thrust rate

correlated with sperm number in three- ($r = 0.43$, $n=50$, $P = 0.002$) and six-month-olds ($r = 0.35$, $n = 50$, $P = 0.014$: NS after Dunn-Šidák correction) but not in twelve-month-olds ($P = 0.58$; see Fig. 3.4b). These data therefore support the findings of Matthews *et al.* (1997) who also described a significant correlation between male courtship behaviour and strippable sperm reserves. However, it is evident from the present study that the association between these variables is only apparent in certain age classes of males. Sperm number positively correlated with standard length in three- ($r = 0.44$, $P=0.001$) and six-month-olds ($r = 0.43$, $P = 0.002$) but not in twelve-month-olds ($r = 0.26$, $P = 0.26$).

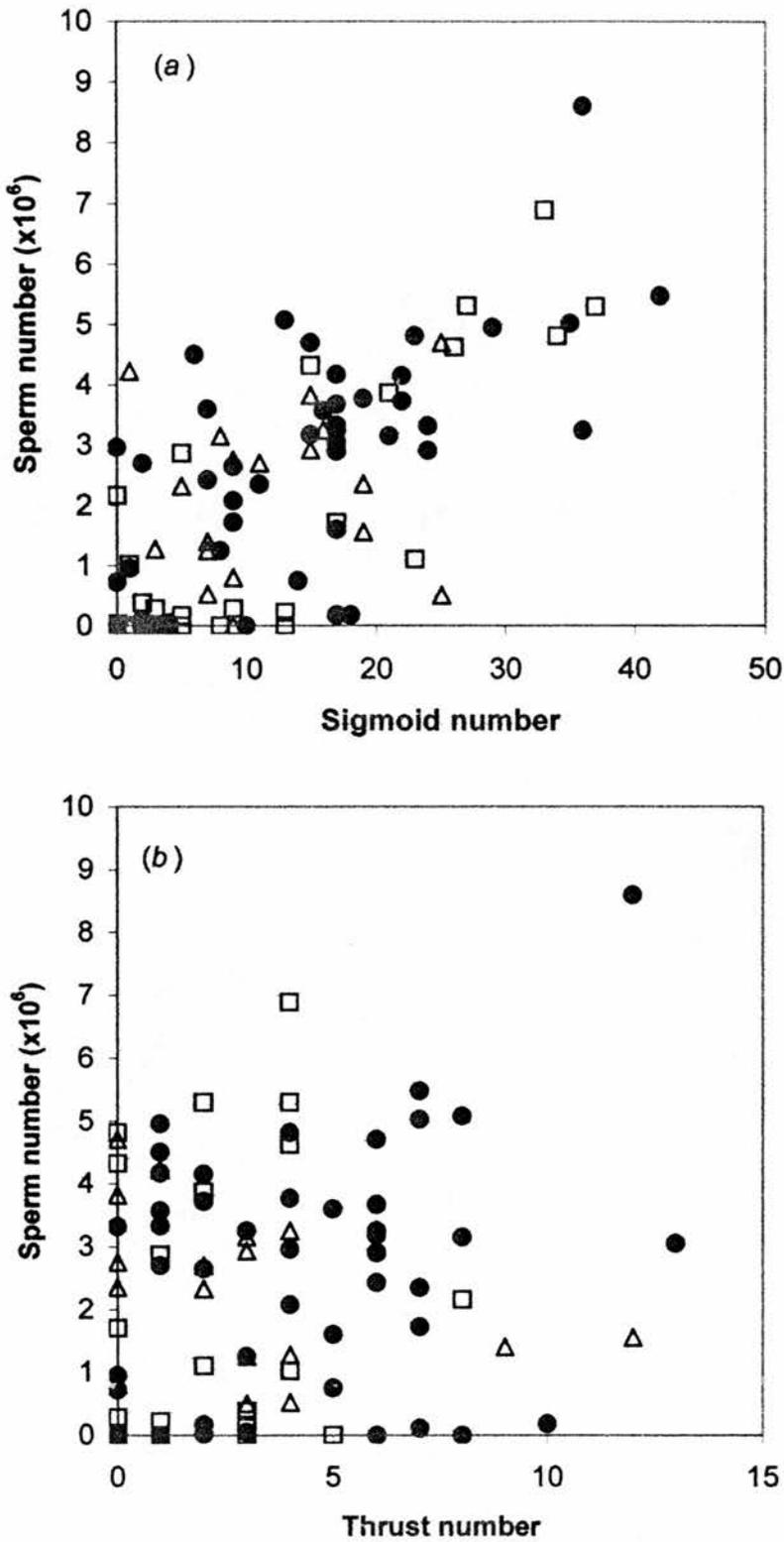


Figure 3.4 The relationship between sperm number per stripped ejaculate and (a) sigmoid and (b) thrust rate among three age classes of male guppy: (□) three months, (●) six months and (Δ) twelve months. See text for details.

DISCUSSION

The results presented here suggest that males are most sexually active at six months of age. The rate at which male guppies performed sigmoid displays, gonopodial thrusts and produced sperm was greatest in six-month-olds. The extent of carotenoid and melanistic coloration was also highest in six-month-old fish (although, as noted, it was not possible to collect colour data from twelve-month-old fish). Interestingly, there was a positive association between sperm production, sexual behaviour and body size in three- and six-month-old males but not in the oldest age class of males. This is consistent with the finding that males are most sexually active at six months and coincides with maximum levels of sperm production and coloration. Thus, although the decline in sexual behaviour and sperm number in the oldest age class of males was not statistically significant, the fact that *all* of these parameters showed a general decline in twelve-month-olds strongly suggests that males devote more energy to courtship early in life. Whether this decline was due to the poor health of males in the twelve-month group or differential resource allocation among the age groups remains unclear. However, males were included in the twelve-month trials only if they appeared to be in good condition (judged by their normal swimming activity).

A key prediction of life-history theory is that high rates of adult mortality should impose selection for early maturation and increased reproductive effort early in life (Charlesworth 1980). This prediction has been confirmed in a number of studies. Indeed, Reznick and his colleagues (Reznick & Bryga 1987; Reznick *et al.* 1990; Reznick & Endler 1982) have already provided evidence that the life-history patterns of guppies differ predictably among natural populations in relation to predation risk. For example, guppies from high-predation populations tend to be smaller, mature earlier and devote more resources to reproduction than their low-predation counterparts (Reznick *et al.* 1990; Reznick & Endler 1982). Given the low probability of extended survival following sexual maturity, particularly in high-predation localities such as the one from which the population used in this study descended (Reznick *et al.* 1996), it pays males to direct maximal resources towards reproduction early in life. It would be interesting to further examine the ontogeny of male courtship behaviour and associated reproductive traits in males from several low- and high-predation populations to test these predictions explicitly.

As described by Houde (1997), males begin to show sexual behaviour as the gonopodium differentiates, usually at about seven weeks. Specifically, male guppies are not considered sexually mature until the apical hood, a sensory protuberance on the gonopodium, extends beyond the tip of the gonopodium (Houde 1997). In the present study, the courtship behaviour of juvenile males was only examined if they had well-developed gonopodia ($n = 18$); males that were obviously incapable of inseminating females were not used for the study. Despite this, all but one of the males in the juvenile group did not produce sperm when manually stripped. Thus, the data suggest that male guppies begin to court females before they are physiologically capable of fertilising their eggs. Similar results were presented by Bisazza *et al.* (1996) when they studied male sexual behaviour in another poeciliid, the mosquitofish (*Gambusia holbrooki*). In that study they found that the sexual activity of immature male mosquitofish was as high as that of adults despite the fact that they were unable to transfer sperm. Because the reproductive success of male mosquitofish is strongly dependent on body size, which in turn is influenced by adult sex ratio, the authors suggested that male mosquitofish used sexual behaviour to predict their future reproductive success at a given size. Whether or not the precocious behaviour of young male guppies is also adaptive remains to be tested.

There was a significant association between sperm number and male sexual behaviour in three- and six-month-old males, but not in the oldest age class of males. These findings therefore concur with those of Matthews *et al.* (1997; but see Pilastro & Bisazza 1999) but in the present study it is apparent that the association between sperm number and behaviour exists only in certain age classes of males. Matthews *et al.* (1997) specifically set out to test Sheldon's (1994) fertility advertisement hypothesis and concluded that male guppies signalled their functional fertility to females when courting them. Although the present study was not designed to test this idea further, the data suggest that females may only gain this information from a subset of males. Additionally, standard length correlated with sperm number, confirming the results of a recent study by Pilastro and Bisazza (1999) on a population of feral Brazilian guppies. Reynolds *et al.* (1992) showed that female guppies were attracted to large males, and that male size had significant father-son heritability. Large-bodied males may therefore have a twofold advantage over smaller males: they are likely to

be more successful than their smaller counterparts during both pre-copulatory (female choice) and post-copulatory (sperm competition) sexual selection.

DECLARATION

In this study, Trevor Pitcher, of The University of Toronto, scanned and analysed the slides for the colour pattern data (see section *c*). Subsequent analyses of these data were performed in St Andrews.

Chapter four

The effect of rearing demography on patterns of adult male behaviour and sperm production¹

ABSTRACT

This experiment was designed to investigate the effect of rearing demography on the sexual behaviour and sperm production of adult males. The results indicate that male mating behaviour varies according to the rearing sex ratio: when reared in male-biased groups, males performed more forced copulations and fewer courtship displays but showed the opposite pattern of behaviour when reared in female-biased groups. The *a priori* prediction, based on sperm competition theory, that stripped sperm number would reflect social structure was not supported by the results. Instead, the overall level of sexual activity (gonopodial thrusts plus sigmoid displays) was a better predictor of sperm number among males in the sex ratio treatment. Rearing density, where sex ratio was controlled, did not significantly affect male mating behaviour or sperm traits. Males reared under the different sex ratios continued to show some of their characteristic behaviour patterns when placed in equal sex ratio tanks. It appears, therefore, that males adopt mating strategies to suit their social environment, and that these strategies remain fixed, for short periods at least, if the population structure changes.

INTRODUCTION

Darwin (1871) recognised that the evolution of male secondary sexual characteristics, such as weaponry, colourful plumage and flamboyant courtship displays, was the result of sexual selection. Subsequent research has revealed marked variation in the expression of these traits, even within populations of a single species. This variation arises because the intensity of sexual selection, and the cost to males of supporting the

¹ Data from this chapter have been published as Evans, J.P. & Magurran, A.E. (1999) Male mating behaviour and sperm production characteristics under varying sperm competition risk in guppies. *Animal Behaviour* **58**: 1001-1006

exaggerated characteristics, is not constant over space or time. For example, numerous studies have shown that food availability, predation and the physical environment can influence male morphology and behaviour (e.g. Weeks & Meffe 1996). In some cases this variation reflects heritable differences between individuals or populations (Lott 1984; Rodd & Sokolowski 1995). In other circumstances, an individual's genotype can produce a flexible response (a 'reaction norm') according to environmental conditions (Blanckenhorn 1998). Such variation can be attributed to phenotypic plasticity, which allows the expression of sexually selected traits to be tailored to short-term changes in local conditions (review by West-Eberhard 1989).

To date, the role of demographic structure in shaping male reproductive traits and behaviour has received relatively little attention. This is surprising because many species are comprised of demographically variable populations, and it is known that males may enhance their future reproductive success by responding optimally to the conditions they encounter as juveniles (Gage 1995). For example, males can respond to their early environment by adopting behavioural strategies that maximise their reproductive success as adults (Bisazza *et al.* 1996). Where levels of polyandry are high, males can improve their reproductive success by competing at the gametic level and investing relatively heavily in sperm production or other associated traits (Gage 1995; Harcourt *et al.* 1981; Oppliger *et al.* 1998).

The Trinidadian guppy provides one of the best known examples of geographic variation in any species. Field studies reveal considerable inter- and intra-population variation in the sexual behaviour of males (Endler & Houde 1995; Luyten & Liley 1985; Luyten & Liley 1991; Seghers 1973), much of which reflects the predation regime from which they originate (Endler 1995). Typically, those from low-predation populations perform higher levels of courtship display and lower levels of gonopodial thrusting than their high-predation counterparts (see preceding chapter and Luyten & Liley 1985). Although previous work has indicated that there is a genetic basis to this variation in behaviour (Reznick & Endler 1982), males may also adjust their courtship patterns in response to changes in social environment. For example, Rodd and her co-workers have attributed some of the variation in life-history traits (Rodd *et al.* 1997) and male sexual behaviour (Rodd & Sokolowski 1995) among guppy populations to their social environment (rearing demography).

As an indirect result of the differences in social structure among guppy populations, the magnitude of sperm competition faced by males is unlikely to be constant (see chapter two, but also Kelly *et al.* 1999). Theory predicts that ejaculate expenditure should co-vary with sperm competition risk, assuming of course that sperm compete numerically (Parker 1998). In support of this prediction, comparative studies across a wide range of taxa have shown that male ejaculate expenditure is higher in species experiencing high levels of sperm competition (Harcourt *et al.* 1981; Stockley *et al.* 1997). A number of studies have also shown that individual males have the ability to adjust ejaculate size according to the magnitude of sperm competition they face (Fuller 1998; Gage 1991; Gage 1995). It is not yet known whether male guppies are able to respond to the perceived risk of sperm competition by 'fine tuning' their sperm production and mating behaviour.

The aim of this study is to investigate the effect of variation in population structure on the sexual behaviour and sperm production of male guppies. Previous work indicated that early social environment influences male behaviour (Rodd & Sokolowski 1995), but did not disentangle the effects of density and sex ratio, or examine the consequences for sperm production. In this study, guppies were therefore reared from juveniles to adulthood under different sex ratios (where fish density was controlled) and different densities (where sex ratio was controlled) in order to investigate the effect that each parameter had on male sexual behaviour, sperm number and relative gonad size. It was anticipated that males reared in the male-biased treatment would rely more heavily on non-display mating tactics (i.e. sneaky mating) than their counterparts in the female-biased treatment. Furthermore, because the risk of sperm competition is likely to be affected by changes in the sex ratio, the prediction was also tested that males would respond to their social environment by adjusting their patterns of sperm production accordingly. A further aim of the experiment was to determine how male guppies respond to a change in population structure as adults. The sexual behaviour of males was therefore examined in two contexts. First, the behaviour of males was recorded in their rearing tank under whatever conditions they had been assigned as juveniles. Second, males from each of the experimental treatments were moved to a new tank, with an equal sex ratio and containing unfamiliar females. Male sexual behaviour was subsequently measured in the new context to see how it was

affected by short-term changes in population structure, and to determine whether past experience had any effect on male sexual behaviour in new surroundings.

METHODS

(a) *Origin of fish and maintenance*

The guppies used in this experiment were descendants of wild-caught fish from the lower Tacarigua River, Trinidad. The lower Tacarigua is characterised by clear, relatively fast-flowing water; guppies at this site occur sympatrically with several predators including the pike cichlid, *Crenicichla alta* (for further biological and physical information on this site refer to Magurran & Seghers 1994).

Juvenile guppies were reared under different sex ratios and densities in aquaria measuring 30 x 20 x 20 cm and filled to a depth of 19 cm. In the sex ratio treatment, fish were reared in low- (5 males:11 females) and high-male groups (11 males:5 females); in the density treatment, fish were reared at three densities: low (8), medium (16) and high (32). Sex ratios were maintained at unity in the density treatment and the medium group (8 males:8 females) doubled as the equal sex ratio treatment. Thus in the sex ratio treatment, equal fish densities were maintained across the treatments, while in the density treatment, sex ratios were maintained at unity. In addition, a separate male-only population ($n = 16$) was reared to examine male sexual behaviour and sperm production in the absence of females. Replicate tanks (at least two) were set up for each treatment to test for any differences between tanks. In all treatments, guppies were reared from juveniles (ca. 6-7 weeks old) and left in their allotted tanks until aged four months. Males were distinguishable from females at the juvenile stage on the basis of gonopodium (intromittent organ) development and emerging colour patterns (Houde 1997).

All fish were fed a diet of flake food throughout the rearing stage. To eliminate confounding dietary effects on male growth, all were fed on a per capita basis (enough food was given to feed each fish to satiation). To test this assertion the standard lengths and wet weights of males were measured after the behavioural trials were complete. All fish were reared under standard conditions; temperature was maintained at $25 \pm 0.5^\circ\text{C}$ and illumination was provided by an 18 W fluorescent bulb on a 12:12 h light:dark cycle. Tanks contained an air stone, gravel and a small clump of Java moss

(*Vesicularia dubyana*). Any offspring produced during the rearing stage of the experiment were removed and placed in stock aquaria.

(b) *Male mating behaviour*

Male mating behaviour was observed in two contexts. First, the behaviour of males was recorded under whatever treatment they had been assigned. Second, the behaviour of the same males was examined under standardised conditions (equal sex ratio) in a neutral, unfamiliar tank (hereafter referred to as 'standardised'). The standardised treatment tank contained eight non-virgin females, all unfamiliar to the eight test males. When moved to the standardised tank, males were allowed to settle for 24 h before behavioural observations commenced. Therefore, with the exception of the medium density tank (which also doubled as the equal sex ratio tank), all males were viewed under two contrasting conditions. Thus it was possible to examine how males responded to short-term changes in population demography.

Males use a characteristic sigmoid display to persuade receptive females to mate (see chapter 1, section *d*). In addition, they attempt forced copulations without prior display by means of a gonopodial thrust (Baerends *et al.* 1955; Liley 1966). The number of sigmoid displays and gonopodial thrusts performed by each male was recorded over a ten-minute period. The number of times each male actively chased a female was also counted. Chases were counted only if they were prolonged (> 5 s) and involved at least one other male. Male behaviour was recorded in both the rearing tank (home tank) and the unfamiliar one (standardised tank). On completion of the behavioural trials, all of the females were returned to the stock tanks.

(c) *Sperm number and gonadal investment*

Following the behavioural observations, males were placed in a water bath containing a lethal dose of anaesthetic (Benzocaine). After removing the excess water from each male, standard length (± 0.1 mm) and wet weight (± 1 mg) were recorded for each male and sperm were extracted (for methods, see preceding chapters). The total number of sperm in each stripped ejaculate was determined by counting cells on an improved Neubauer chamber haemocytometer. The samples were diluted in 100 μ l of 0.9% saline to bring the spermatozoa to counting concentration (ca.100 per chamber). Sperm number was determined by multiplying the mean sperm count (from five

counts) by the sample's dilution factor and initial volume. After sperm removal, testes were extracted from the body cavity and dried with the body (minus gut) for 24 h at 60 °C. The gonadosomatic index (GSI) was determined by calculating dry testis mass as a percentage of dried body mass (de Vlaming *et al.* 1982). All values were tested for normal distributions before statistical analyses.

RESULTS

There were no differences in the standard lengths and wet weights of fish reared at the different sex ratios (respective ANOVAs: $F_{3,55}=0.36$, $P = 0.78$; $F_{3,55}=1.02$, $P = 0.39$) or densities ($F_{3,55}=1.45$, $P = 0.25$; $F_{3,55}=0.19$, $P = 0.83$). Differential food intake had therefore not confounded the results. A one-way analysis of variance (ANOVA) was used to determine whether male behaviour differed between tanks within each treatment. No such differences were detected (all P values > 0.05). Given this consistency in male behaviour within each treatment, data were pooled for each group and parameters were expressed as means \pm SE.

(a) Sex ratio treatment

Male behaviour in home tanks

Males reared in tanks with a male-biased sex ratio performed significantly more thrusts than those reared in female-biased tanks ($F_{3,56}=6.48$, $P = 0.001$; Fig. 4.1a). Conversely, the same males performed significantly fewer sigmoid displays than their counterparts in the female-biased treatment ($F_{3,56}=9.62$, $P < 0.001$; Fig. 4.1b). In the male-only treatment, in which males displayed at each other in the absence of females, the sigmoid display rate resembled that of males reared in male-biased tanks (Fig. 4.1b). However, the male-only group performed lower rates of gonopodial thrusts than their counterparts in the male-biased tanks (Figs. 4.1a). Predictably, chases were more common among the males reared in male-biased tanks than in either of the other two ($F_{2,35}=8.14$, $P = 0.001$; Fig. 4.1c).

Male behaviour in standardised tank (1:1 sex ratio)

When moved to the unfamiliar (standardised) tank, males reared in male-biased tanks continued to thrust at higher rates than any of the other groups ($F_{3,56}=6.53$, $P = 0.001$; Fig. 4.1d). Thus, in this respect, they continued to show their characteristic behaviour patterns despite being exposed to a change in sex ratio. This suggests that past

experience affects male behaviour despite short-term changes in demography. However, the pattern of sigmoids shown by males reared under different sex ratios was not apparent when they were moved to the standardised tank; no difference in sigmoid display rate was detected among the male groups after being moved ($F_{3,56}=1.73$, NS). When the sexual behaviour of males in the standardised tank was compared to that of males in the home tanks, no significant differences were found (paired t tests: sigmoid rate: $t_{59}=1.6$, $P = 0.11$; thrust rate: $t_{59}=1.43$, $P = 0.16$). Again, this supports the assertion that male sexual behaviour is influenced by prior experience and not by short-term changes in population structure.

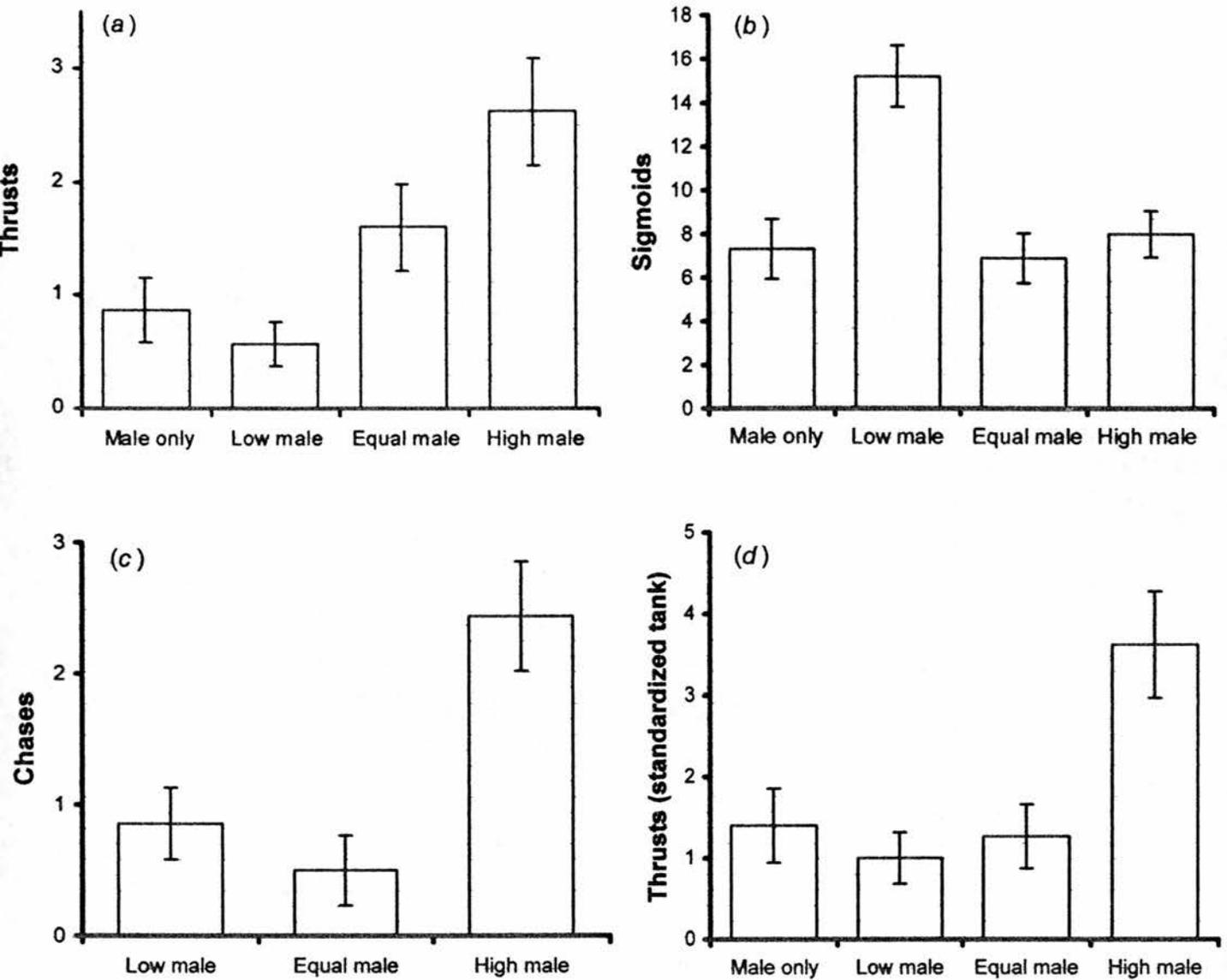


Figure 4.1 The sexual behaviour of male guppies reared under various sex ratios: the number of (a) thrusts, (b) sigmoid displays and (c) chases recorded over ten minutes in the home tank treatments (see text). (d) The number of thrusts over ten minutes in the standardized tank treatments. All values are means \pm SE. Sample sizes: male only $n = 15$; low male $n = 14$; equal male $n = 15$; high male $n = 16$.

Sperm number and gonadal investment

Sperm number differed significantly among the experimental groups ($F_{3,55}=7.47$, $P < 0.001$; Fig. 4.2). Males reared in the absence of females had the highest reserves of sperm. Using a post hoc test for multiple comparisons, it was evident that sperm number was lower in the equal sex ratio males than in any of the other groups (Tukey's pairwise test: $P < 0.05$). There was no significant difference in GSI among the four groups ($F_{3,51}=0.97$, $P = 0.415$).

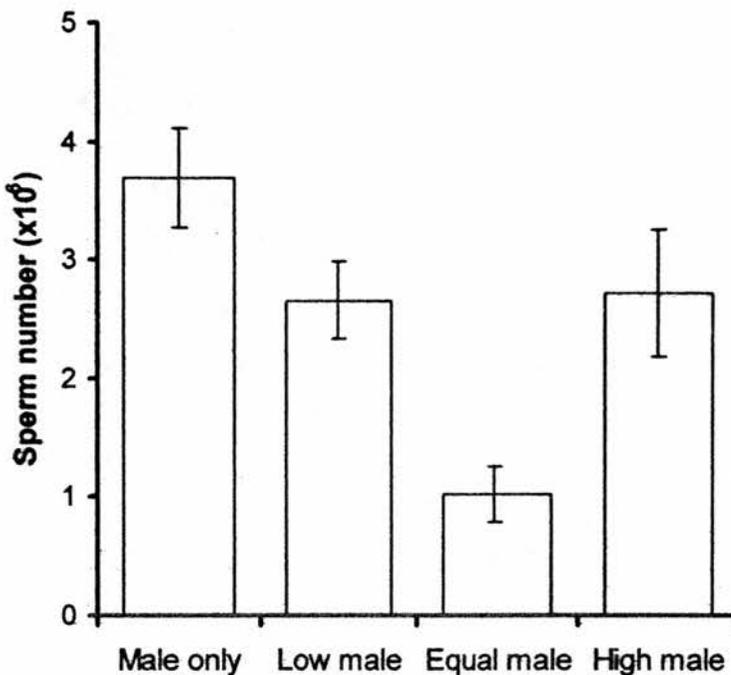


Figure 4.2 Sperm number per stripped ejaculate in male guppies reared under various sex ratios. Values are means \pm SE. See Fig. 4.1 for sample sizes.

(b) Density treatment

Fish density had no effect on male mating behaviour or any of the other traits measured (see Table 4.1 for descriptive statistics). Males from all three groups (high, medium and low density) showed similar sigmoid rates ($F_{2,39}=1.60$, $P = 0.207$), thrust rates ($F_{2,39}=0.460$, $P = 0.637$), sperm numbers ($F_{2,39}=2.80$, $P = 0.073$) and chases ($F_{2,39}=1.63$, $P = 0.212$). As in the sex ratio treatment, male behaviour did not differ between the standardised and the home tank treatments (paired t tests: NS). The

statistical power of the analyses for the density treatment was calculated according to Zar (1996, see Table 4.1).

Table 4.1. Sexual behaviour and sperm number of males reared at three densities (Low, Medium, High). Figures are mean values with SD in parentheses. See text for details.

	Low	Medium	High	<i>P</i> value	Statistical power
Sigmoids	11.09 (9.67)	6.87 (4.41)	10.75 (6.60)	0.207	0.70
Thrusts	1.09 (1.13)	1.60 (1.5)	1.375 (1.31)	0.637	0.94
Sperm*	2.52 (2.30)	1.02 (0.91)	1.60 (1.53)	0.073	0.40
Chases	0.45 (0.69)	0.50 (0.76)	0.12 (0.34)	0.212	0.90

* mean values are $\times 10^6$

DISCUSSION

This investigation indicates that the sexual behaviour of male guppies is phenotypically plastic and supports Rodd and Sokolowski's (1995) finding that males perform higher levels of gonopodial thrusting as competition between males for females intensifies (see also Jirotkul 1999a). However, their study, and another that produced similar results (Farr 1976), did not control for density, and could not disentangle the relative contribution of each factor in shaping male sexual behaviour patterns. In the present study it is evident that it is sex ratio, rather than density, that influences male sexual behaviour (but see Jirotkul 1999b). This result is perhaps unsurprising; gonopodial thrusting (i.e. sneaky mating) is presumably more profitable when the operational sex ratio (OSR = proportion of receptive females in the population; Emlen & Oring 1977) is low. The probability that a male will achieve successful copulation through courtship display lessens as the proportion of receptive females in the population decreases. By rearing fish at different densities, but maintaining sex ratios at unity, the OSR was unlikely to differ among the three density treatments. Clearly this argument depends on there being no relationship between rearing density and female receptivity (e.g. see Gage 1995). In guppies, there is no evidence that females alter their mating preferences or copulation rates in response to changes in population density (Jirotkul 1999b). Female receptivity is influenced by predation risk (review by Houde 1997) but this does not vary predictably with population density (A. E. Magurran & I. Ramnarine, unpublished data). It is therefore unlikely that females use density as a cue to alter their mating patterns and preferences.

The second aim of this experiment was to determine whether male behaviour patterns were maintained during short-term changes in adult population structure. The results show that males continue to show their characteristic patterns of sneaky mating when moved to new surroundings, irrespective of a change in sex ratio. These data again support the findings of Rodd and Sokolowski (1995) who also noted that the rearing environment influences behaviour, even when males are observed in standard conditions. In Rodd and Sokolowski's experiments, a male encountered a single, unfamiliar female in a novel tank. Taken together, these studies underline the importance of early experience in shaping behaviour. The sex ratios of natural populations of guppies vary considerably, both across and within predation regimes (Haskins *et al.* 1961; Rodd & Reznick 1997; Seghers 1973) as well as at the same locality from one year to another. For example, the overall ratio of males to females in the Upper Tunapuna River in Trinidad fell from 0.8:1 in 1991 to 0.47:1 in 1996 (B.H. Seghers & A.E. Magurran, unpublished data). Given that it takes at least 7 weeks for a male guppy to mature (Reznick *et al.* 1997), and that adult mortality rates are high (Reznick *et al.* 1996), the sex ratio that a male experiences as a juvenile is strongly predictive of the conditions he must face as an adult. Phenotypic plasticity in male mating behaviour is clearly adaptive.

Although this investigation confirms that male guppies respond to their early social environment by modifying their sexual behaviour, no effect of population structure on gonadal investment was detected in either of the treatments (sex ratio or density). Sperm number varied among the four sex ratio groups (the male-only group and three different sex ratios) but the pattern did not correspond with initial predictions. Because sperm competition often intensifies when large numbers of males compete for access to a limited number of females (Gage 1991; Gage 1995; Oppliger *et al.* 1998), it was anticipated that sperm competition would be more prevalent in male-biased groups. Assuming sperm compete numerically in guppies, males facing high levels of sperm competition should invest relatively heavily in sperm production. One possible reason why this was not apparent in the current study is that sperm numbers were estimated by artificially stripping males and not by collecting natural ejaculates. The data therefore provided a good estimate of the numbers of sperm available for mating, but not of the actual number delivered per copulation. Although the number

of stripped sperm appears to be a good predictor of ejaculate size in guppies (Pilastro & Bisazza 1999), it is not known whether males are able to adjust the size of their ejaculates in response to variation in sperm competition risk. It would be interesting to examine male ejaculation strategies under varying sperm competition risk and intensity to test these ideas further (see discussion in chapter seven, section c). Even if male guppies cannot adjust ejaculate size (e.g. due to constraints at mating), they may respond to increased sperm competition risk by adjusting copulation rate or postcopulatory mate guarding behaviour (e.g. Parker 1998).

Sperm numbers (per stripped sample) were lower in males reared under an equal sex ratio than in those reared in the high- or low-male treatments, or even the male-only treatment. This result is at first perplexing; it was predicted that males reared in male-biased groups would have greater reserves of sperm because they faced higher levels of sperm competition. However, as noted above, the total number of sperm in a stripped sample may not correspond with that of a natural ejaculate. Instead, it is possible that the number of stripped sperm corresponds with the motivational state of males in each treatment. There is good evidence that the number of stripped sperm correlates with male sexual behaviour in guppies (Matthews *et al.* 1997). The present study suggests that males perform high levels of courtship in the low-male treatment and high levels of sneaky mating in the high-male treatment. Overall, the level of sexual behaviour was lower in males reared in the equal sex ratio treatment. When this difference was analysed it was evident that the sum of sexual behaviour (sigmoid displays + gonopodial thrusts) differed significantly between the treatments ($F_{3,56}=7.68$, $P < 0.001$); males in the equal sex ratio treatment were significantly less active in both respects than their high- and low-male counterparts (Tukey's pairwise test: $P < 0.05$). This pattern of sexual behaviour among the three treatments corresponds with that of stripped sperm numbers (see Fig. 4.2). One plausible conclusion, therefore, is that males respond to differences in sex ratio by adjusting their level of sexual activity, as well as the degree to which they use courtship displays and sneaky mating attempts. This, in turn, is reflected by their strippable sperm reserves.

Chapter five

Sperm precedence patterns and predictors of paternity

ABSTRACT

Despite its widespread occurrence in animals, sperm competition has been studied in a limited range of taxa. Among the most neglected groups in this respect are internally fertilizing fish in which virtually nothing is known about the dynamics of sperm utilisation. In this study we examined the outcome of sperm competition when virgin female guppies mated with two males. Behavioural cues were used to ensure that each male mated once and that sperm were successfully inseminated at copulation. Two polymorphic microsatellite loci were used to estimate the proportion of offspring sired by the second male to mate (P_2). The results revealed a bimodal distribution with either first or (more often) second male priority; in no instance was paternity shared equally among male pairs. The observed P_2 distribution differed from that expected under the 'fair raffle' model of sperm competition. Random sperm mixing is therefore unlikely to account for the observed variance in P_2 in this study. A further aim of the study was to identify factors that influence male reproductive success. Interestingly, stripped sperm number did not predict male reproductive success. Instead, the best predictors of paternity were time to remating and the difference in courtship display rate between first and second males. Since females are attracted to high-displaying males, our results suggest that female choice may facilitate sperm competition and / or sperm choice in guppies.

INTRODUCTION

It is now recognised that sperm competition (Parker 1970) is ubiquitous in the animal kingdom, occurring in taxa as diverse as birds, insects, amphibians, mammals and fish (Birkhead & Møller 1998; Smith 1984). Indeed, even species previously thought to be strictly monogamous have been shown, with the aid of paternity markers, to mate promiscuously (e.g. Birkhead & Møller 1992). Theory predicts that a male's

fertilization success will increase proportionately with the number of his sperm relative to that of other males (Parker 1990; Parker *et al.* 1990). In support of this prediction, comparative studies across a wide range of taxa have shown that male ejaculate expenditure is higher in species experiencing high levels of sperm competition (Harcourt *et al.* 1981; Stockley *et al.* 1997). A number of studies have also shown that individual males have the ability to adjust ejaculate size according to the magnitude of sperm competition they face (Fuller 1998; Gage 1991; Gage 1995).

One of the primary objectives of researchers working on sperm competition (in internally fertilizing species) is to establish patterns of paternity when two males mate consecutively with a female (e.g. Birkhead & Møller 1998). Usually, paternity patterns are described in terms of second (or last) male sperm precedence (P_2); that is, the proportion of offspring sired by the second male to mate (Boorman & Parker 1976). Patterns of paternity are often highly variable both within and between species (Lewis & Austad 1990) and it is this variability that allows researchers to make inferences about the mechanisms that underlie them (Birkhead 1998; Birkhead & Hunter 1990). However, uncovering such mechanisms (e.g. sperm displacement; sperm mixing; passive sperm loss; female sperm choice) is notoriously difficult and only a few studies have provided convincing evidence of their roles in sperm competition (e.g. Birkhead & Biggins 1998; Colegrave *et al.* 1995; Price *et al.* 1999; Simmons & Siva-Jothy 1998).

The majority of studies of sperm precedence have been confined to insects (Gwynne 1984; Simmons & Siva-Jothy 1998) and birds (Birkhead & Møller 1992); very little is known about patterns of paternity in other groups of internal fertilizers. This is especially true of fish in which the outcome of sperm competition in internal fertilizers has never been studied in detail (Petersen & Warner 1998). In the poeciliids, for example, sperm competition is known to be intense (Constantz 1984; Hildemann & Wagner 1954; Zane *et al.* 1999) but is nonetheless poorly understood. Among these, the guppy has been the subject of intense study, especially relating to the evolution of secondary sexual characters through female choice (Houde 1997). Guppies have a promiscuous mating system in which female choice plays an important role (Houde 1987; Kodric-Brown 1985). As virgins, and during their brief receptive phase (ca. monthly), female guppies solicit copulations from several males

(Houde 1997) and subsequently produce mixed-paternity broods (Hildemann & Wagner 1954; Kelly *et al.* 1999). Female guppies store sperm for several months within the folds of the ovary (Constantz 1984) where they are nourished by extracellular ovarian sugars (Gardiner 1978).

In order to attract females, male guppies use a highly conspicuous courtship posture, known as a sigmoid display (Baerends *et al.* 1955; Liley 1966). This display serves to show off the male's coloration and may signal viability (e.g. Nicoletto 1993) or fertility (Matthews *et al.* 1997). In addition, males employ the sneaky mating tactic, termed gonopodial thrust, to circumvent female choice. Gonopodial thrusts are forced copulations, not preceded by courtship display, in which the male attempts to thrust his gonopodium (the intromittent organ) into the female's genital pore (Baerends *et al.* 1955; Liley 1966). While both strategies are used interchangeably (and continually) by individual males, the degree to which either tactic is employed can depend on several factors including social environment, (Rodd & Sokolowski 1995 and see previous chapter), predation pressure (Dill *et al.* 1999; Endler 1987; Magurran & Seghers 1990) and female receptivity (Liley 1966).

Some of the earliest work on sperm competition was performed using poeciliids as a model system. Indeed, long before the term sperm competition was coined, Winge (1937) noted that multiply-mated female guppies gave birth to successive broods over several months. Later work examined the pattern of sperm precedence when post-partum females subsequently mated with a second male. This work uncovered a strong last male advantage and suggested that fresh sperm were at a competitive advantage over previously stored sperm (Hildemann & Wagner 1954). Matthews (1998) used DNA markers to assign paternity to offspring from twice-mated females and provided further evidence that fresh sperm outperform stored sperm. However, no study has examined the outcome of sperm competition when two ejaculates are inseminated within the same brood cycle. Furthermore, although the stripped sperm number correlates with several male traits including courtship intensity (Matthews *et al.* 1997) and body size (Pilastro & Bisazza 1999), it is not known whether these accurately predict male reproductive success. The aims of the present study are therefore to examine sperm precedence patterns when receptive virgin females are sequentially paired with two randomly chosen males, and to relate these patterns to

several male traits with the aim of uncovering possible predictors of male reproductive success.

METHODS

(a) *The study population and its maintenance*

The guppies used in this experiment were descendants of wild-caught fish from the lower Tacarigua River in Trinidad. This site is characterised by high levels of predation on guppies, notably by the pike cichlid *Crenicichla alta* (Magurran & Seghers 1994). Virgin females were reared for this experiment in single sex groups as soon as they became morphologically distinguishable from males (ca. 5-6 weeks, see Houde 1997). The male guppies used in this study were maintained in mixed-sex aquaria (~1:1 sex ratio) until used in the experiment. On the morning of the mating trials all fish were fed to satiation with commercially prepared flake food (TetraMin™).

(b) *Mating trials*

Mating trials took place between 09:00 and 11:00 in an aquarium (60 x 30 x 38 cm) containing gravel, a small clump of Java moss (*Vesicularia dubyana*) and an air filter. Tank temperature was maintained at $25 \pm 0.5^\circ\text{C}$ and light was provided by an 18 W fluorescent bulb on a 12:12h light / dark cycle. Virgin female guppies, aged six months and approximately matched for size, were used for the mating trials. In each trial a female was sequentially paired with two randomly chosen males taken from stock aquaria. Copulation success was judged according to Liley's (1966) detailed description of guppy mating behaviour. Copulations were considered successful only if they were followed by post-copulatory jerks (PCJs) by the male. Such jerking movements by male guppies are highly conspicuous and invariably signal sperm transfer (Liley 1966). Post-copulatory jerks usually last several minutes and are followed by a refractory period that can last up to one hour (Houde 1997). Copulations not followed by PCJs do not result in successful sperm transfer (Liley 1966) and males immediately resume courtship after such attempts (personal observation).

The protocol for the mating trials was simple: each female was allowed to settle in the mating arena overnight and on the following morning a male (Male 1) was carefully

placed into the tank. As soon as the pair successfully copulated, Male 1 was removed and placed in a small holding tank (30 x 20 x 22 cm) for five days to replenish his sperm reserves prior to measuring his behaviour and sperm number (see below). At this point the time taken for Male 1 to successfully copulate with the female was noted. Exactly one hour after Male 1 had copulated, a second male (Male 2) was placed in the tank and was observed until he successfully inseminated the female. The time taken for the second male to mate was recorded. After copulation, Male 2 was removed and placed with Male 1 in the holding tank for 5 days. Each male's colour pattern was then sketched to aid subsequent recognition of the fish. If Male 2 did not successfully copulate with the female within 30 minutes he was replaced by a third male. If this third male was successful, the time taken for him to mate was recorded. In practice, only three of the successful mating trials (where females were double mated) involved a third male. If the third male was unsuccessful, the trial was terminated and the female and (three) males were returned to a stock tank. The stock tank containing females and males from failed trials was clearly labelled and fish within it played no further part in subsequent mating trials. The one-hour gap between matings was necessary to allow the female to settle and resume 'normal' swimming and foraging activities.

A total of 55 mating trials were performed yielding 30 successful double matings. In each of these successful trials the female was only inseminated once by each male and the interval between matings never exceeded two hours. Following double matings, females were isolated in plastic bottles (4l volume), each containing a small clump of Java moss and an airstone, until the production of their first brood. Twenty-one of the thirty double-mated females gave birth to broods, of which nineteen comprised three or more offspring (mean brood size = 7.9 ± 0.72 SE). Only broods containing three or more offspring were used for the paternity analysis. In practice only one brood comprised three individuals; all other broods contained at least five juveniles.

(c) *Estimating P_2*

Second-male sperm precedence (P_2) was estimated using two polymorphic microsatellite DNA markers specifically designed for poeciliids. The first, isolated and characterised by J. S. Taylor and F. Breden, (unpublished; Genbank accession number AF164205) had a core (TAA)_n repeat, with primer sequences 5'-GTG ACC

GAA CGA AAG GAT A-3' and 5'-CCC CAA AGG AAC ACT GTA-3'. The second locus (Pooc-G49) was isolated by Parker *et al.* (1998) (accession number AF026459) and had a (GT)₆, GC, (GT)₄, GC, (GT)₇ repeat with primer sequences 5'-CAT AGA TTC TGC AGG CAG TG-3' and 5'-CTC AGT GAC TAT AAG GCC AAC-3'.

After giving birth, females were anaesthetised in a water bath containing 0.4g l⁻¹ Benzocaine (ethyl p-amino benzoate). When fully subdued, each female's standard length was recorded and a small clipping (approx. 25 µg) was taken from her caudal fin. Fin-clipped females were revived in conditioned fresh water (Stress Coat®, Aquarium Pharmaceuticals Inc.) and placed in stock aquaria. Juveniles were too small to survive the fin-clipping procedure and were therefore humanely killed in a water bath containing a lethal dose of Benzocaine. Caudal fin tissue was taken from the two putative sires after observing their behaviour and stripping their sperm (see below).

Genomic DNA was extracted from tissue samples taken from each family (two putative sires, mother and offspring) using a rapid one-tube extraction method (Estoup *et al.* 1996). Polymerase chain reaction (PCR) amplifications were performed on a PTC-100 thermal cycler (MJ Research Inc.). Reaction volumes of 10 µl for the first (TAA)_n locus consisted of 1.6 µl of DNA template, 0.07µl of each dNTP (each dNTP = 10 mM), 2.0 mM MgCl₂, 5 pmol of each primer pair and 0.4 units of Taq polymerase (Promega). Thermal cycles consisted of 1 min at 95 °C followed by 27 cycles of 52°C for 30 s, 72°C for 30 s and 95°C for 10 s. A terminal extension step of 5 min at 72°C completed the program. PCR conditions for Pooc-G49 were identical except for an annealing temperature of 63.2 °C and an Mg Cl₂ concentration of 1.5 mM. PCR products were resolved on 6% polyacrylamide gels (Sambrook *et al.* 1989) and paternity was assigned according to allele sharing between putative sires, mother and offspring (Fig. 5.1).

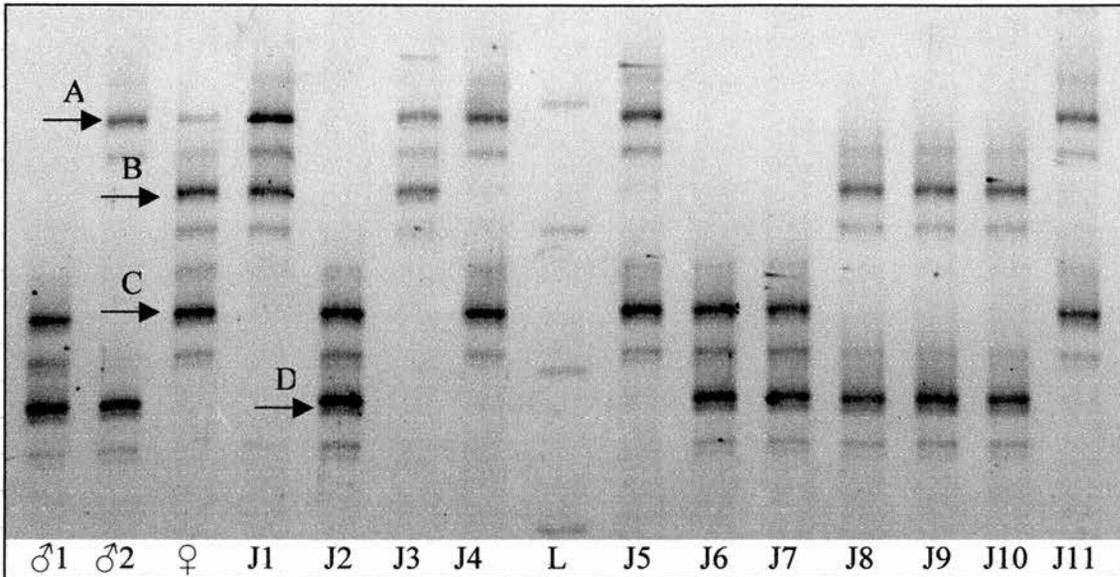


Figure 5.1 An example DNA fingerprint prepared using the $(TAA)_n$ locus showing two putative sires (σ^1 & σ^2), mother (♀) and offspring (J1-J11). A 10 base pair ladder (L) was used as a reference to size each allele. Four alleles (A-D) are shown and paternity was assigned to each offspring on the basis of band sharing between putative sire and offspring. Note that σ^1 & σ^2 share the D allele making paternity assignment for some offspring ambiguous. For example, from this gel paternity was resolved for offspring 1,3,4,5 & 11 (assigned to σ^2 on the basis of band sharing at the 'A' allele). Paternity assignment for the remaining offspring (2,6-10) was achieved using the second microsatellite marker PooC-G49 (not shown here).

A total of 146 offspring were genotyped of which 134 (91.8%) could be unambiguously assigned to one of the putative sires. The results of the paternity analysis were 'blind' checked by an impartial observer not involved in the study but experienced in gel scoring techniques. Offspring that could not be assigned to one of the two males ($n = 12$) using both microsatellite loci were not included in the subsequent paternity analysis. Four of the 12 offspring for which paternity could not be assigned constituted an entire brood and therefore a total of 18 families were used in the final analysis.

(d) Male mating behaviour

Five days after the mating trials, pairs of males from each trial were moved into an observation tank (45 x 30 x 30 cm) containing two unfamiliar, non-virgin females already acclimatised to the tank (ca. 24h). The observation tank contained gravel, moss and an air filter. Male courtship behaviour (sigmoid display and gonopodial thrust rate) towards the two females was recorded over a 10-min period as soon as the males settled into the tank and commenced courtship. Sigmoid displays were recorded when the focal male moved in front (or to one side) of the female, arched his body in a pronounced S-shape, and quivered (Baerends *et al.* 1955; Liley 1966). Gonopodial thrusts were recorded when the focal male attempted to make (or succeeded in making) physical contact with a female's genital region without her co-operation.

(e) Sperm counts

Following behavioural observations, male pairs were humanely killed in a water bath containing a lethal dose of Benzocaine. After removing excess water from each male, standard length (± 0.1 mm) was determined and sperm were extracted. To strip sperm each male was placed on an inverted petri dish under low-power magnification. The gonopodium was swung forward and gentle pressure was applied to the abdomen, just anterior to the base of the gonopodium (where the testes are located). This procedure released sperm in the form of spermatozeugmata (sperm bundles) and was repeated to ensure that all sperm bundles were extracted. Following removal, sperm bundles were drawn up a Gilson™ pipette and added to 100 μ l of water. To break down sperm bundles and distribute sperm cells evenly, samples were repeatedly drawn up and expelled from the pipette. Sperm count was estimated by counting sperm cells on an 'improved Neubauer chamber' haemocytometer under x400 magnification. The distribution of sperm cells across the haemocytometer was checked visually for evenness before counts commenced. The number of sperm was determined by multiplying the mean sperm count (from five counts) by the sample's dilution factor and initial volume. Sperm counts were expressed as the total number of spermatozoa per stripped ejaculate.

(f) Analysis

Second male paternity (P_2) was estimated by determining the proportion of offspring sired by the second male to mate. Observed values of P_2 were compared with

theoretical expected values of sperm precedence based on a model assuming random sperm mixing (Parker *et al.* 1990). Assuming a fair 'raffle', in which individual sperm from both males have an equal chance of fertilizing ova and are not used preferentially by the female, the probability of second male paternity will be

$$P_2 = S_2 / (S_1 + S_2),$$

where S_1 and S_2 represent the number of sperm ejaculated by the first and second male respectively. Recent work suggests that the number of stripped sperm is a good predictor of ejaculate size in guppies (Pilastro & Bisazza 1999) and therefore values of S_1 and S_2 were estimated by comparing mean (stripped) sperm counts from first and second males. This generated a range of expected P_2 values for each of the 18 families tested. From these data the mean and standard deviation were used to generate a normal curve (see line in Fig. 5.2) which was compared with the observed P_2 distribution (bars in Fig. 5.2) using a Kolmogorov-Smirnov goodness-of-fit test (see Cook *et al.* 1997).

Generalised linear regression was used to determine whether the independent variables measured for each male (sperm number, sigmoid rate, thrust rate, body size and time to remating) significantly affected P_2 . Because P_2 data are proportional, they were analysed using logistic modelling with a logit link function (Genstat 5 Committee 1993). Four of the independent variables represented differences in the explanatory variables between first and second males (i.e. Male 2 trait minus Male 1 trait). The fifth independent variable to be fitted into the model was time to remating (= time from introduction to copulation for second – or third – males). Male 2 success and failure (success = number of offspring sired by Male 2 within each brood; failure = number sired by Male 1) were entered as the binary response variable.

RESULTS

(a) Pattern of P_2

The proportion of offspring sired by the second male ranged from 0% and 100% (mean \pm SD: 0.64 ± 0.45) and clearly showed a bimodal distribution (Fig. 5.2). The number of stripped sperm did not differ significantly among first and second males ($t_{34} = 0.53$, $p = 0.60$) and these data provided the basis for the expected P_2 distribution

under the fair raffle model of sperm competition. The expected and observed distribution of P_2 differed significantly (Kolmogorov-Smirnov goodness-of-fit test: $D_{0.5} = 0.56$, $n = 18$, $p < 0.01$; Fig. 5.2), suggesting non-random mixing of sperm. There was either first or (more often) last male priority and in no instance was paternity shared equally by both males.

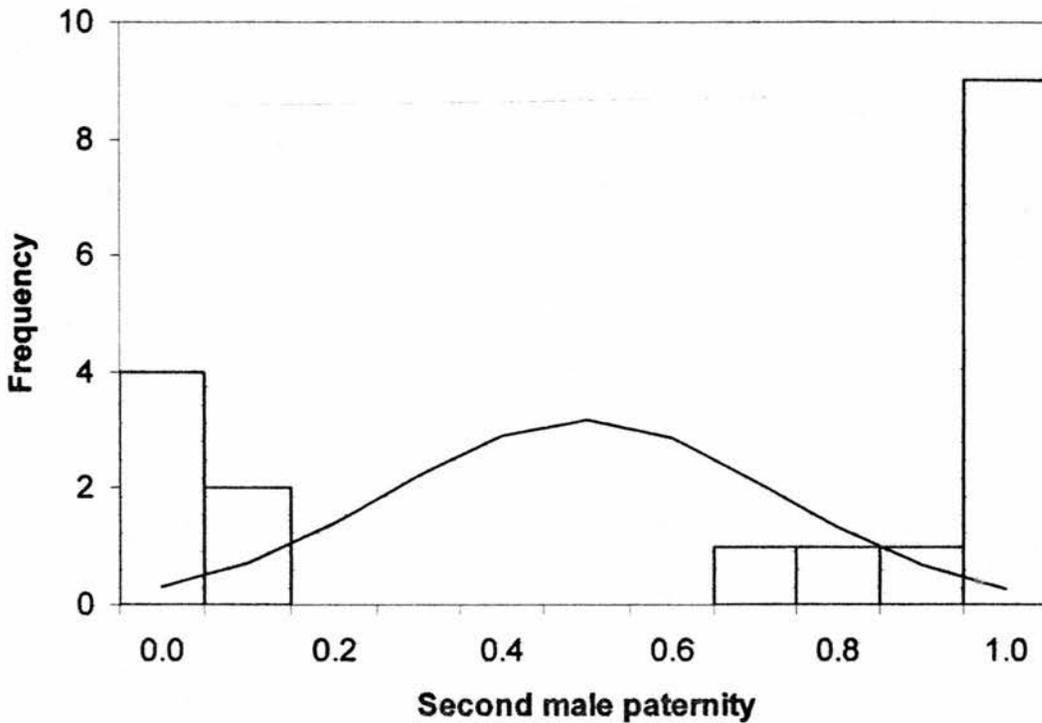


Figure 5.2 Expected P_2 distribution (line) if sperm mix randomly compared with observed data (bars). The observed distribution is bimodal and shows either first or last male sperm precedence. For further details see text.

(b) *Predictors of fertilization success*

Table 5.1 shows the results from the logistical multiple regression and reveals that time to remating and relative differences in sigmoid rate among competing males accounted for more than 63% of the deviance in the model. Relative male size showed a sequence effect when entered into the model and was non-significant if relative difference in sigmoid rate among male pairs was entered first (Table 5.1). This arises because sigmoid rate and body size were correlated in this data set; relative differences in sigmoid rate and the time to remating were always significant

irrespective of input order. Figure 5.3 shows the fitted curves for sigmoid rate and remating interval and reveals that males who perform relatively more sigmoids obtain greater parentage (Fig. 5.3 *a*) with P_2 declining rapidly when the time to remating exceeds 17 minutes (Fig. 5.3 *b*).

Table 5.1 Results from the generalised logistic linear modelling of P_2 against five independent variables. The significance of terms is inferred from changes in the deviance of the model due to each variable, which is distributed as χ^2 . Non-significant (NS) probabilities occur where $P > 0.05$.

Source of deviance	df	Deviance (% of total)	Probability
Sperm number	1	0.126 (0.04%)	NS
Thrusts	1	0.791 (0.05%)	NS
Sigmoid rate	1	21.442 (14.68%)	<0.001
Body size	1	1.032 (0.7%)	NS
Time to remating	1	70.595 (48.33%)	<0.001
Residual	10	52.059 (36.2%)	-

In subsequent analyses, individual males within each pair were classified as being successful (sired > 50% of the brood) or unsuccessful (< 50%). Successful males mated, on average, 7.1 (± 1.4 SE) minutes after being introduced to the female. Unsuccessful males, however, took an average of 14.6 (± 3.5) minutes to copulate (two-tailed t-test comparing mean time to mating for successful and unsuccessful males: $t_{32} = 1.98$, $P = 0.062$). Interestingly, stripped sperm number was not a good predictor of fertilization success (see Table 5.1). Successful males had similar sperm counts (stripped ejaculate size = $3.65 \times 10^6 \pm 0.45$ SE) to unsuccessful males ($3.36 \times 10^6 \pm 0.58$; $t_{34} = 0.4$, $P = 0.69$). Furthermore, successful males were not larger than their unsuccessful counterparts ($t_{34} = 0.36$, $P = 0.72$), nor did they have higher rates of gonopodial thrusting ($t_{32} = 0.28$, $P = 0.78$).

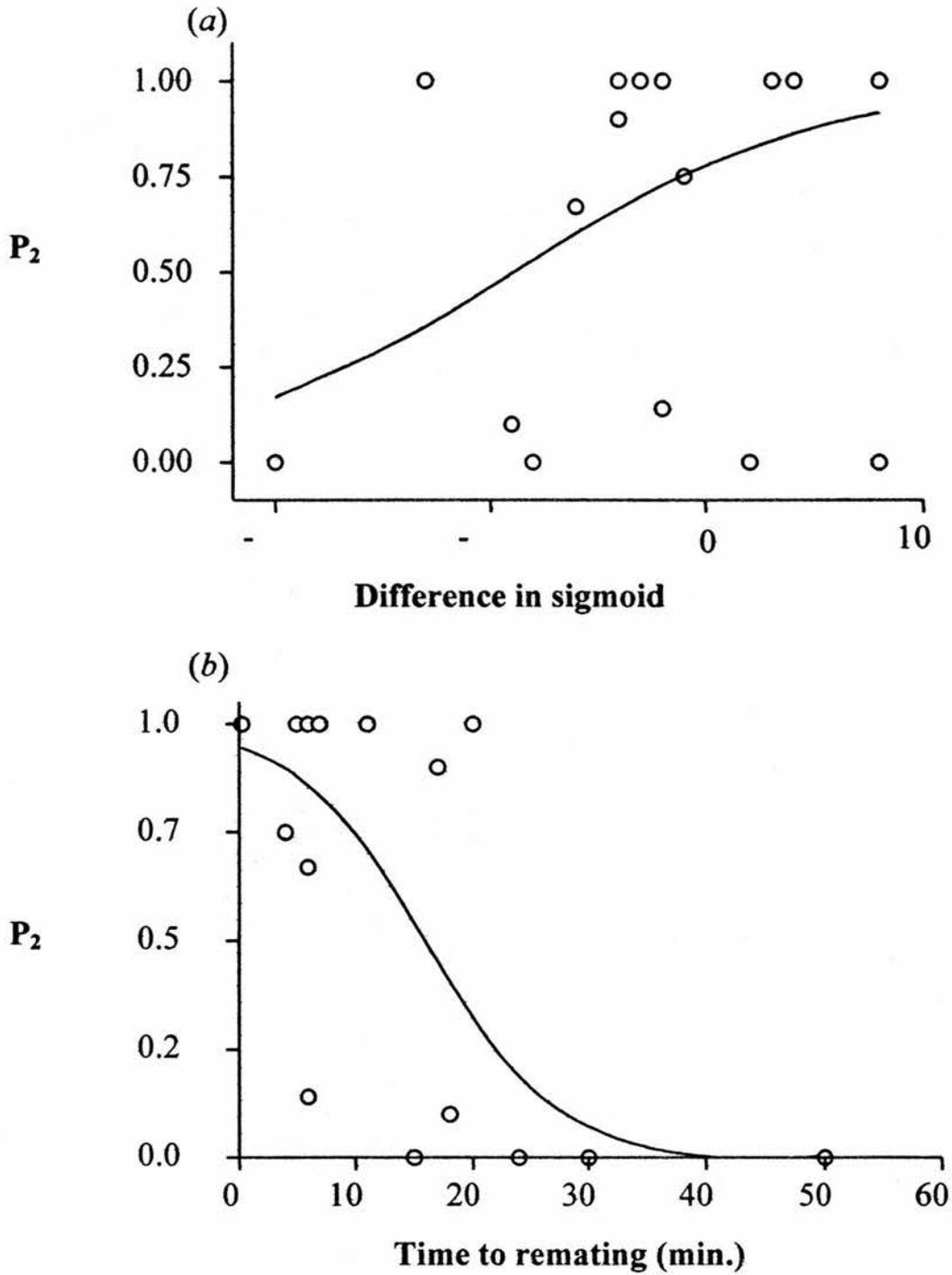


Figure 5.3 Proportion of offspring sired by the second male (P_2) as a function of (a) differences in display rate among 1st and 2nd males, and (b) time to remating. Curves are the fitted lines from the generalised linear model.

DISCUSSION

In summary, paternity patterns in the guppy showed a bimodal distribution with either first or (more often) last male priority. The observed P_2 distribution clearly differed from that predicted by the fair raffle model of sperm competition (Parker *et al.* 1990) and the best predictors of P_2 were the insemination interval and the intensity of second males' courtship displays. Clearly, both factors are likely to be linked: males with high courtship rates are likely to attract females more quickly than their less vigorous counterparts. However, it is not clear why such males should sire proportionally more offspring than males without such attributes.

The insemination interval may be important because sperm inseminated beyond a certain time are unavailable for fertilization. However, virgin female guppies are receptive to male courtship for at least three days (Houde 1997; Liley 1966) and it is unlikely that sperm inseminated within just two hours of an initial copulation are disadvantaged to any great extent. More likely, it is the willingness of females to mate with certain males that truly affects male reproductive success. Females show a clear preference for high-displaying males (e.g. Nicoletto 1993). Thus, attractive males may copulate more quickly than their unsuccessful counterparts and their subsequent success will be irrespective of the (short) insemination interval. This suggests two possible – but admittedly broad – mechanisms responsible for the success of such high-quality males: either females themselves influence the outcome of sperm competition via complex interactions at the level of the genome (Zeh & Zeh 1997) or gamete (Eberhard 1996); or high quality males (i.e. those with high display rates) produce superior sperm with better fertilizing ability (Yasui 1997). Distinguishing between these processes is extremely difficult, especially since they may not be mutually exclusive (Birkhead 1998; Eberhard 1998).

In order to make inferences about the mechanisms of sperm competition from P_2 data it is necessary to have a detailed understanding of the processes of sperm transfer and storage (Simmons & Siva-Jothy 1998). Fortunately, there is some – albeit limited – information on these processes in poeciliids. Clark and Aronson (1951) noted that during the seven-day period following insemination, sperm become increasingly undetectable in the female's reproductive system. They suggested that this reduction in sperm number resulted from the gradual accumulation of sperm in the folds of the

ovary (i.e. sperm stores). However, no such reduction in sperm was apparent within a few hours of mating (Clark & Aronson 1951). Since male guppies cannot displace previously deposited sperm (Constantz 1984), ejaculates arising from two males mating in quick succession (as in the present study) are likely to occur simultaneously in the female reproductive tract. This further supports the idea that the relationship between the insemination interval and the observed pattern of sperm precedence is unlikely to be causative.

The data presented here suggest that the pattern of P_2 differs from Parker *et al.*'s (1990) fair raffle model of sperm competition. This assertion rests critically on the assumption that ejaculate size did not differ among male pairs. Although there was no statistical difference between the number of stripped sperm in the first and second males, actual ejaculate size may have differed among the two groups. However, there are two reasons for suspecting that this was not an important factor in the present study. First, male guppies were sequentially paired with a female and therefore had no way of 'knowing' that they were the first or second to mate. Second, if males did increase ejaculate size in response to female cues indicating greater sperm competition risk, the study should have uncovered a stronger last-male effect with a mean (significantly) above 0.5 (Parker *et al.* 1990). Instead, the results showed that certain males did better than others irrespective of mating order. Furthermore, even when males were classified as being successful or unsuccessful, there was no statistical difference in stripped ejaculate size among either group.

The skew in the distribution of paternity may have been a consequence of the experimental design in which females were able to choose whether or not to copulate with either of the two males. In the case of first males, all females invariably copulated with them in a matter of minutes. Indeed, virgin females often mate indiscriminately when first encountering a male (Houde 1997). However, females were more reluctant to mate with subsequent males (aptly demonstrated by the difficulty in achieving a high number of successful double matings) and the skewed pattern of paternity towards second males may have been a consequence of females only choosing to re-mate with superior males. This form of 'trading up' is common in birds (e.g. Møller 1992) and may account for the widespread occurrence of last male sperm precedence reported in several avian studies (Birkhead & Møller 1992). Clearly

it would be instructive to perform a series of artificial inseminations involving the sperm from two males to determine whether last male priority exists in the absence of female choice. Furthermore, on development of such a technique, it would also be useful to determine whether 'high-quality' males (judged on the basis of their morphology and courtship behaviour) achieve disproportionately high reproductive success when the sperm from two males are inseminated simultaneously.

Irrespective of the mechanisms underlying male reproductive success, it is clear that females are not passive participants in the process. Females evidently solicit copulations from more than one male and in so doing provide the arena for sperm competition (or sperm choice) in the first place. The fact that males with varying phenotypes experience differential reproductive success is likely to have far-reaching evolutionary implications for both males and females. For females, sperm competition or sperm choice may be a way to bias paternity towards the most suitable males. From the male perspective, those that signal the vigour, quality or size of their ejaculates (via cues used during mate choice) will have a twofold advantage over others because they will be more attractive to females and will be more successful during sperm competition. There is already good evidence that in guppies, male phenotype does indeed signal ejaculate characteristics (Matthews *et al.* 1997). This may be especially important for females since their choice of mating partners is often compromised by sneaky mating. On the other hand, sneaky mating may be a means by which males can undermine both pre- and post-copulatory female choice, depending on the timing of such attempts (Hildemann & Wagner 1954; Matthews 1998). An important challenge for future research is to determine whether males or females are ahead in this evolutionary battle of the sexes, and to what extent.

Chapter six

Female benefits of multiple mating¹

ABSTRACT

The rewards of promiscuity for males are undisputed. But why should a female mate promiscuously, particularly when her partners offer no resources other than sperm and increase her chances of succumbing to predation or disease? This question has been hotly debated but at present remains largely unresolved. One possibility is that females exploit post-copulatory mechanisms, such as sperm competition, to increase both the quality and quantity of their offspring. In this experiment the guppy, a species with a resource-free mating system, is used to test the hypothesis that females gain benefits from multiple mating. Virgin female guppies were assigned to one of two experimental treatments: single, where each female mated repeatedly with the same male on four successive days, and multiple, where each female mated with a different male on each of the four days. The results indicate that multiply-mated females secure substantive advantages: they have shorter gestation times, larger broods and produce offspring with better developed schooling abilities and escape responses than their singly-mated counterparts.

INTRODUCTION

In most species with typical sex roles, male reproductive success is directly proportional to the number of inseminations secured. The widespread occurrence of multiple mating by males is therefore easily explicable. Polyandry, where females mate with two or more males over a single reproductive cycle (Thornhill & Alcock 1983), is less easy to explain, especially when the sperm from a single insemination are often sufficient to fertilize an entire clutch of eggs. Moreover, mating can involve a number of indirect costs for females such as decreased foraging efficiency (Magurran & Seghers 1994a), increased predation risk (Magurran & Nowak 1991)

¹ Data from this chapter have been published as Evans, J. P. & Magurran, A. E. (2000) Multiple benefits of multiple mating in guppies. *Proceedings of the National Academy of Sciences USA* **76**: 10074-10076

and elevated risk of parasite transmission (e.g. Rosenqvist & Johansson 1995). In some cases mating can expose females to pathological substances in the male seminal fluid (Chapman *et al.* 1995). Understanding the adaptive (i.e. functional) significance of polyandry has become a major goal of many recent studies (Fjerdingstad & Boomsma 1998; Hoodland 1998; Tregenza & Wedell 1998; Watson 1998).

In many species, the costs to females associated with polyandry are offset by substantive direct benefits, such as nutrient donations which enhance female fecundity (Andersson 1994). However, in the absence of such direct, material benefits it is widely assumed that females mate multiply to secure genetic benefits for their offspring (Jennions & Petrie 2000). Unlike nongenetic benefits, which may accrue from repeated copulations with the same male, genetic benefits through polyandry can only arise from multiple copulations with different males (e.g. Archer & Elgar 1999). One way in which females can benefit through polyandry is by trading previous mates for potentially superior ones. Most examples of 'trading up' are confined to birds which sometimes perform extra-pair copulations to obtain genetic benefits for their offspring while simultaneously obtaining paternal support from their original (non-preferred) mate (see Birkhead & Møller 1992). However, another way in which polyandry may benefit females is by insuring against male infertility, assuming of course that males become sperm limited after a single copulation. For example, females may mate with more than one male to replenish dwindling sperm stores (Fjerdingstad & Boomsma 1998) or to insure against male infertility (Sheldon 1994).

The recently documented association between secondary sexual characteristics and sperm number in male guppies (Matthews *et al.* 1997) suggests that females may indeed choose to mate with 'sperm-rich' males. Guppies have a non-resource based mating system in which female choice for brightly coloured males plays a central role (Houde 1997). Female guppies are receptive to male courtship as virgins or shortly after (c. 24h) the production of a brood. During this brief receptive phase, they typically solicit copulations from several males (Kelly *et al.* 1999); outside this period females are subject to frequent sneaky mating attempts (Magurran & Seghers 1994b). Gestation usually lasts between 25 and 35 days (Houde 1997) and, as in other poeciliids, offspring are born live and fully independent from their mother. Evidence from work on the related sailfin molly (*Poecilia latipinna*) suggests multiple mating

may be advantageous because it leads to increased brood sizes (Travis *et al.* 1990). Here, the prediction is tested that multiply-mated female guppies will also benefit from higher fecundity. Since fecundity can be increased by reducing the length of the brood cycle (Lewontin 1965) and/or by producing more offspring, both variables are measured. Furthermore, as newborn guppies are vulnerable to predation (Magurran 1990) and cannibalism (Meffe & Snelson 1989), predator evasion skills, namely schooling behaviour and the escape response (Fuiman & Magurran 1994), were quantified to evaluate the performance of newborn offspring born to singly- and multiply-mated females.

METHODS

(a) *Mating trials*

All fish used in this experiment were descendants of wild-caught fish from the lower Tacarigua River, Trinidad. The lower Tacarigua is a high-predation locality where guppies occur sympatrically with several predators including the pike cichlid, *Crenicichla alta*. Mating trials took place in aquaria (30 x 20 x 20 cm) containing gravel, an air filter and a small clump of Java moss. Temperature was maintained at 25 ± 0.5 °C and illumination provided by an 18 W fluorescent bulb on a 12:12 h light:dark cycle.

Virgin females, aged between six and nine months and approximately matched for size, were randomly assigned to one of two experimental treatments, hereafter termed single and multiple. For both treatments, females were placed singly in the mating tanks and allowed to settle overnight. The males used in both treatments were taken from mixed sex aquaria (~ 1:1 sex ratio) and, as with the females, were roughly matched for size. The experimental design ensured that females in both groups were given equal opportunities to mate. Females allocated to the single treatment were allowed to mate repeatedly with a single, randomly chosen male over the course of four days. Those allocated to the multiple treatment were presented with a different male on four successive days. The males in the single treatment were added and removed from the tanks in the same way as those in the multiple treatment in order that females in both treatments would be exposed to the same level of disturbance. Each consecutive mating trial lasted four hours and during this time the number of

successful copulations (i.e. those followed by male post-copulatory jerking: Liley 1966) was recorded.

Tanks were assigned randomly for each treatment and none of the females had visual access to the other tanks. In total, 76 mating trials were performed (38 for each treatment) and 42 broods were produced (21 for each treatment). Following the mating trials, females were isolated until giving birth. After measuring schooling behaviour (see below), the multiply-sired broods were humanely killed by ice immersion and preserved (along with the putative sires and mothers) in 70% ethanol prior to DNA extraction.

(b) *DNA fingerprinting*

To determine whether multiply-mated females produced multiply-sired broods, a subsample ($n = 10$) of broods from the multiple treatment were genotyped. Genomic DNA was extracted from tissue samples taken from each of the ten families (putative sires, mothers and offspring) using a rapid one-tube extraction method (Estoup *et al.* 1996). The polymerase chain reaction (PCR) was used to amplify a polymorphic microsatellite locus with seven alleles for the Lower Tacarigua population (Genbank accession number AF164205; J.S Taylor & F. Breden, personal communication). PCR products were resolved on 6% polyacrylamide gels (Sambrook *et al.* 1989) and paternity was assigned to individual juveniles according to allele sharing between the four putative sires, mother and offspring. Using this method it was possible to unambiguously assign paternity to 48% of the offspring (total number of offspring assayed was 101). Offspring that could not be assigned to one of the four males were excluded from the paternity analyses and therefore the estimate of mean number of sires per brood is likely to be a conservative one.

(c) *Measuring juvenile schooling behaviour*

Newborn guppies were tested the day after birth; all were fed with TetraMin™ baby fish food for livebearers prior to observations. Pairs of fish were randomly selected from a brood and gently placed in a circular cream-coloured arena (diameter 44 cm, water 2.5 cm deep and 25.5°C). The guppies were given 2 minutes to settle and explore the arena; schooling behaviour was measured for the subsequent 5 minutes

using a standard method (Magurran & Seghers 1990). The length of time that each pair spent schooling was recorded; the criteria were that the fish should be no more than 3 cm (approximately 3.5 body lengths) apart and swimming and turning in synchrony. In practice it was straightforward to record schooling times as the fish tended to either swim synchronously in close proximity or be widely separated in the arena. The procedure was repeated until all pairs within a brood had been accounted for. Mean schooling times (% time spent schooling) were then calculated for each of the 20 broods (with >1 offspring) per treatment. Data were arcsine transformed.

(d) Juvenile escape response behaviour

As noted by Birkhead *et al.* (1998), the time taken to capture an animal represents a measure, by proxy, of the likelihood that it will escape a predator in the wild. This parameter was therefore used as a measure of juvenile 'quality', employing a similar method to the one used by Birkhead *et al.* (1998). Using a small net, each individual newborn was captured from the schooling arena in a standardised way. The mean time to capture was calculated for broods from $n = 19$ singly-mated and $n = 20$ multiply-mated females. The one-day-old guppies showed well-developed escape responses and were adept at evading capture. Mean capture times per brood were calculated and correlated with mean schooling times per brood (arcsine transformed proportions).

RESULTS

The results from this study clearly indicate that female guppies gain more from multiple copulations with different partners than from repeated copulations with the same partner. Copulation frequency did not significantly differ between the two treatments (mean copulations per 30 min: single = 1.50 ± 0.38 s.e., multiple = 1.46 ± 0.42 ; $t_{39} = 0.20$, $P = 0.84$) and after confirming that there was no difference in female body size among the treatments ($t_{39} = 0.35$, $P = 0.73$), it was evident that multiply-mated females produced larger broods than their singly-mated counterparts (ANCOVA with body length as a covariate: $F_{1,39} = 10.10$, $P = 0.003$; Fig. 6.1a). Furthermore, gestation (the sum of time from insemination to fertilization plus development time from fertilization to birth) was significantly shorter in multiply-mated females than in singly-mated ones ($t_{40} = 2.57$, $P = 0.014$). On average, multiply-mated females produced broods 8.76 days sooner than singly-mated females (Fig.

6.1*b*). The results from the analysis of microsatellites confirmed that, on average, each brood in the multiple treatment was sired by $1.62 (\pm 0.45 \text{ s.e.})$ males.

The progeny of multiply-mated females spent a significantly higher proportion of their time schooling, when tested in pairs, than those born to singly-mated females ($t_{33} = 2.76, P = 0.009$; Fig. 6.2*a*). Furthermore, multiply sired offspring had longer capture times than singly sired fish ($t_{36} = 2.0, P = 0.05$; Fig. 6.2*b*), although this was not significant following a correction for ($k = 4$) multiple comparisons using the Dunn-Šidák method (Sokal & Rohlf 1995). All other tests (above) remained significant after applying this correction factor to the significant levels. Finally, capture times were significantly correlated with schooling times among juveniles from both treatments ($r = 0.327, n = 35, P = 0.04$).

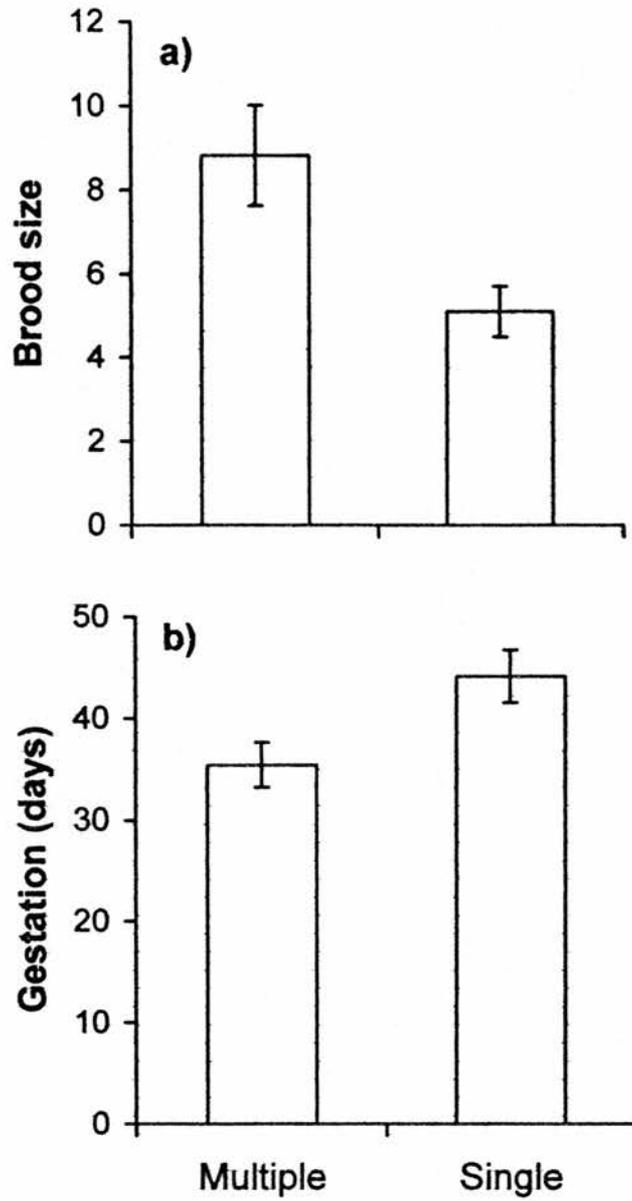


Figure 6.1 Mean \pm SE brood size (a) and gestation (b) in multiply- and singly-mated female guppies.

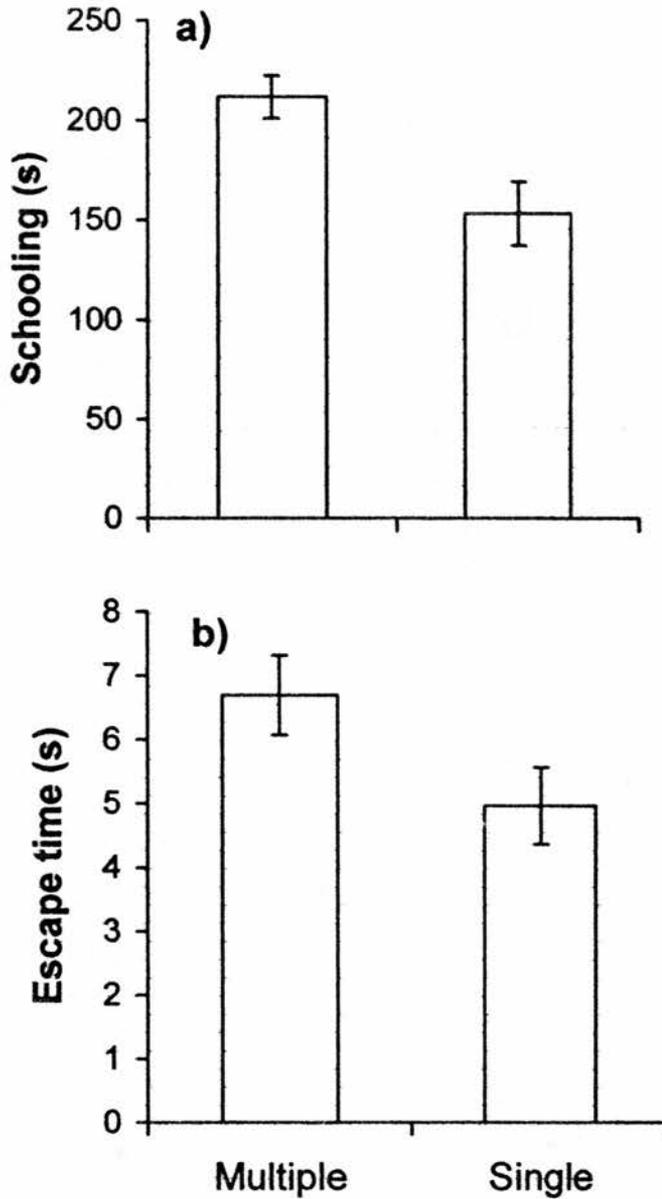


Figure 6.2 Mean \pm SE schooling duration (a) and escape times (b) by broods of singly- and multiply-mated female guppies.

DISCUSSION

Females in the multiple treatment clearly benefited from increased fecundity: they produced larger broods more quickly than their singly-mated counterparts. If reduced female fecundity was attributable to genetic incompatibility or male sterility, a higher proportion of females in the single treatment would have failed to produce offspring. Instead, the proportion of females producing broods was identical (21 broods produced from 38 trials) among the two experimental treatments. An alternative

explanation for the reduction in brood size in the singly-mated group is that females were sperm limited and would have benefited directly by increasing their sperm stores. Until recently it was assumed that sperm limitation was unlikely to be a factor in promoting multiple mating by female guppies. This is because information on male fertility (reflected by their strippable sperm reserves) is available to females via courtship display (Matthews *et al.* 1997) and body size (Pilastro & Bisazza 1999). Thus, on the basis of clear phenotypic cues used in mate choice, females can presumably choose to mate with highly fertile males. However, it is not clear whether such 'high quality' males actually deliver larger ejaculates at mating (see also chapter five on sperm precedence); such males may actually invest less sperm than each female requires because they partition their ejaculates more sparingly among females (Pitnick & Markow 1994). Indeed, when male guppies do mate successfully, ejaculate size can vary greatly with some males inseminating up to 92% of their available sperm during a single insemination (Pilastro & Bisazza 1999). Thus, males that mate repeatedly with a single female may allocate progressively smaller ejaculates over time. Furthermore, as indicated in chapter three, information on male fertility status is clearly not 'accurate' throughout the reproductive lives of male guppies and thus such fertility cues may be unreliable. The difference in brood size among the two treatments may therefore have been a result of sperm limitation in singly-mated females rather than any fecundity enhancing effect of multiple mating (e.g. see Travis *et al.* 1990).

Sperm limitation cannot account for the other fecundity effect detected in this study: gestation was significantly shorter in multiply-mated females than in singly-mated ones. Lewontin (1965) has noted that even a relatively small reduction in gestation length (in his example egg-laying time) equates to a large increase in fecundity. Thus, although the conclusions from this study are restricted to the first reproductive cycle of female guppies, the combined effects of brood size and gestation may predict significant gains to the lifetime reproductive success of multiply-mated females. Clearly, a comprehensive study of the lifetime reproductive success of multiply-mated females is needed before this assertion can be validated. However, given the high mortality rates of adult fish, particularly in high predation populations such as the one from which the fish in this study originated (Reznick *et al.* 1996), there are clear advantages to females in maximising fecundity in their early reproductive life.

Anti-predator skills were better developed in the multiply-sired broods. These progeny spent a significantly higher proportion of their time schooling, when tested in pairs, than those born to singly-mated females. Furthermore, multiply-sired offspring took longer to capture than singly-sired fish. Juvenile fish benefit from the dilution and confusion effects of schooling from the moment they begin to associate with one another (Fuiman & Magurran 1994). Enhanced evasive skills are equally important in reducing the capture success of predators (Fuiman & Magurran 1994). The significant association between capture and schooling times indicates that broods with the highest schooling tendency are also better at evading capture and thus more likely to survive this vulnerable stage. In line with previous work (Griffiths & Magurran 1999) there is no evidence that greater relatedness enhances the schooling behaviour of juvenile guppies. If it did, higher schooling tendencies in the progeny of singly-sired broods would have been predicted rather than the opposite pattern found here.

Although it is unknown whether the above benefits to juveniles are transmitted genetically or are maternally induced (Gil *et al.* 1999), a plausible explanation for them is that genetically superior males produce more, or better quality sperm (Yasui 1997). Thus, 'fit' males, which can afford to invest heavily in sperm production and associated traits, will be at an advantage in sperm competition (see discussion by Parker 1992). Previous work confirms that such a mechanism is possible in guppies. As noted above, sperm production rate is positively correlated with courtship intensity (Matthews *et al.* 1997) and body size (Pilastro & Bisazza 1999), which in turn are cues used by females during mate choice (Houde 1997). These phenotypic traits are both condition-dependent and heritable (Reynolds & Gross 1992) so the association between them and ejaculate size will favour viable males in sperm competition. As a consequence, multiple mating may yield more viable offspring. Whether these benefits extend to sneaky mating, to which females are frequently exposed (Magurran & Nowak 1991), remains to be tested.

A question that arises from the results described here is why, if multiple mating is costly, do females continue to mate with 'poor quality' males? There are two possible answers. First, a female may copulate indiscriminately to increase the likelihood of finding a genetically compatible mate (Parker 1992). However, as noted above, this

idea is not consistent with the finding that the proportion of females producing broods did not differ among the single and multiple treatments. Moreover, since female guppies are provided with multiple cues during mate choice, several of which correlate with female mating preferences and offspring characteristics (Kodric-Brown 1993; Reynolds & Gross 1992), it seems improbable that the selection of genetically compatible partners is relegated to chance. Second, females may mate with more than one male in order to 'upgrade' previous matings (Jennions & Petrie 2000). This hypothesis is consistent with the present finding that gestation is shorter in multiply-mated females. The above experimental design was such that females were given no choice of partner during the mating trials; females were sequentially paired with randomly chosen males (multiple treatment) or a single male (single treatment). On this basis, females assigned to the single-male treatment may have delayed the process of fertilization in case a more 'suitable' male was encountered. This may also explain the very long gestation times of some of the singly-mated females in contrast to the normal interbrood interval of 25-35 days (Houde 1997). Female control of fertilisation is clearly an intriguing topic for further study.

Recent work (Kelly *et al.* 1999) indicates that multiple paternity is widespread in natural populations of guppies in Trinidad but until now it has been unclear why females should seek multiple partners. This study has uncovered some of the possible benefits of this behaviour suggesting that promiscuity is evidently rewarding for females as well as for males.

Chapter seven

Summary and future directions

In addition to the work presented in this thesis, recently published studies (Matthews & Magurran 2000; Pilastro & Bisazza 1999; Kelly *et al.* 2000; 1999) on guppies and other poeciliids (Zane *et al.* 1999) have highlighted the importance of sperm competition in shaping the behaviour and reproductive biology of both males and females. Research into sperm competition in the Poeciliidae is in its infancy and there are numerous possible directions for further work. The aim of this final chapter is to address a number of questions that have been raised in previous chapters, and to present suggestions for future research in areas of particular interest.

(a) Is sperm competition risk mediated by predation?

The results from chapter two revealed that male courtship behaviour and sperm production co-vary with predation risk. Males from the three low-predation sites performed more courtship displays and produced more sperm than their high-predation counterparts. It was argued that sperm competition risk would also co-vary with predation intensity because female receptivity (and thus the propensity of females to re-mate) is curtailed in high-predation populations. However, to provide support for this argument it is necessary to demonstrate a direct link between predation intensity and female multiple mating. This approach was recently adopted by Kelly *et al.* (1999) when they examined the incidence of multiple paternity among ten natural populations of guppies. Kelly *et al.* offered an alternative hypothesis to the one tested in chapter two and argued that predator-mediated shifts in male mating behaviour would lead to elevated, rather than decreased, sperm competition risk in high-predation localities. Their argument is based on the observation that under a high level of predation, males tend to use sneaky mating rather than highly visual courtship displays to achieve copulation (Dill *et al.* 1999; Endler 1987; Magurran & Seghers 1990). Indeed, the results from chapter two were largely consistent with this observation; Tunapuna (low-predation) males performed significantly fewer gonopodial thrusts than Middle Tacarigua (high-predation) males.

Kelly *et al.* (1999) argue that on account of sneaky mating, female choice will be undermined more frequently in high-predation populations and this will result in elevated levels of concurrent multiple paternity in such localities. The data presented by Kelly *et al.* (1999) were consistent with this hypothesis. They used two microsatellite DNA markers and detected elevated levels of concurrent multiple paternity in high-predation populations. However, their analysis was incomplete because it did not control for differences in loci polymorphism among the populations under investigation (Westneat *et al.* 1987). For example, in Kelly *et al.*'s study the resolving power of their most powerful microsatellite locus (Taylor & Breden, Genbank accession number AF164205) was considerably lower in the low-predation populations (mean allele frequency = 7.0 ± 4.1 SD) than in the high-predation ones (mean allele frequency = 11.4 ± 4.4 SD). If the analysis does not control for such differences in resolving power, the ability to detect multiple paternity (i.e. the ability to detect more than three paternal alleles among offspring within each brood) will be greater in the high-predation populations.

In a subsequent re-analysis of Kelly *et al.*'s data, in which loci polymorphism was controlled, Neff and Pitcher (submitted manuscript) found no difference in the occurrence of multiple paternity among predation regimes. However, they also noted that the original analysis performed by Kelly *et al.* (1999) had low statistical power ($\approx 10\%$). They suggested that at least four or five loci (each with a similar resolving power to the ones used) would be necessary to detect differences among the populations with any degree of certainty.

There are now at least four polymorphic microsatellite loci available for guppies, two of which were used to estimate the sperm precedence patterns described in chapter five. It should therefore be possible to determine the actual number of sires contributing towards broods in high- and low-predation populations (see Dewoody *et al.* 2000 for statistical aspects). (Note that Kelly *et al.* provided an estimate of the frequency of broods that were multiply mated in each population, rather than the number of sires contributing *per* brood.) However, even when such an investigation is carried out, the issue of whether predation influences female multiple mating may

prove difficult to resolve. As Kelly *et al.* (1999) note, the propensity of males to adopt the sneaky mating strategy is influenced by predation risk, which may in turn mediate sexual selection through sperm competition. However, as argued in chapter two, variation among populations in female mating behaviour is also likely to influence sperm competition risk. Both hypotheses, which are summarised below (see Fig. 7.1a & b), are likely to be overly simplistic because neither considers the combined mating strategies of both males and females. Since female receptivity (i.e. willingness to mate) is curtailed in the presence of predators, any increase in mating activity due to sneaky mating may be masked by a corresponding decrease in solicited copulations. In reality, the level of sperm competition at any given locality is likely to be influenced by male and female behaviour. The more complex scenario depicted in Figure 7.1c accounts for all possible interactions between predation, male sexual behaviour and female receptivity patterns. It recognises that predation risk affects the sexual behaviour of males and females (Godin & Briggs 1996; Magurran & Seghers 1990), and also that male behaviour is indirectly affected by predation risk through changes in female receptivity (Dill *et al.* 1999). While it is clear that predation has the capacity to indirectly influence sperm competition risk, local levels of sperm competition will depend on the delicate balance between all of these parameters.

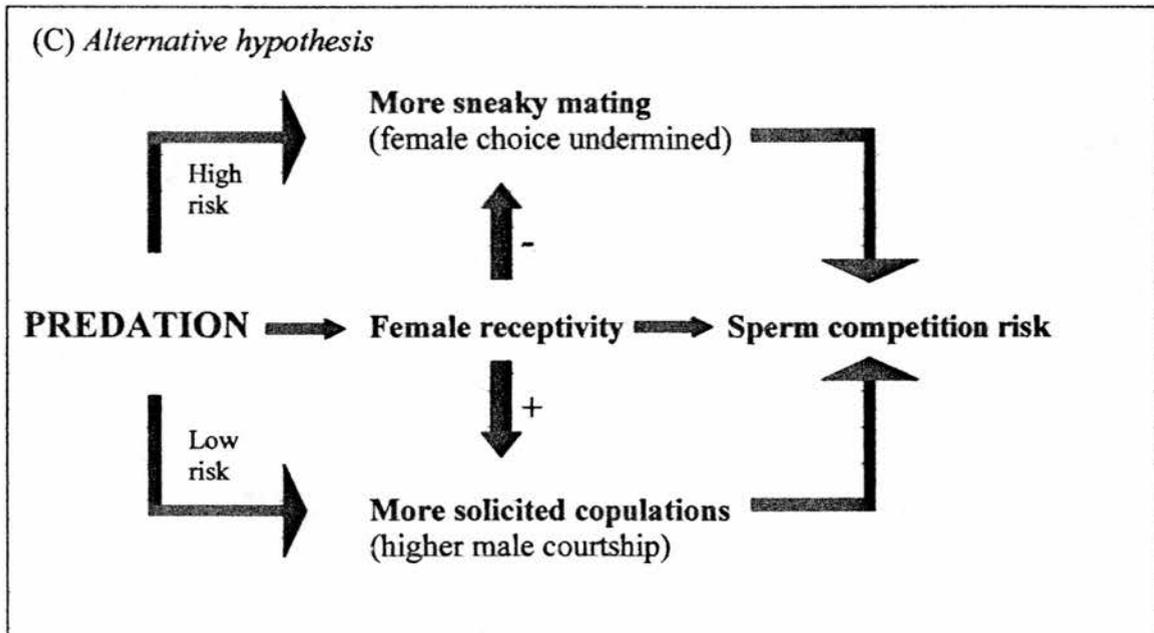
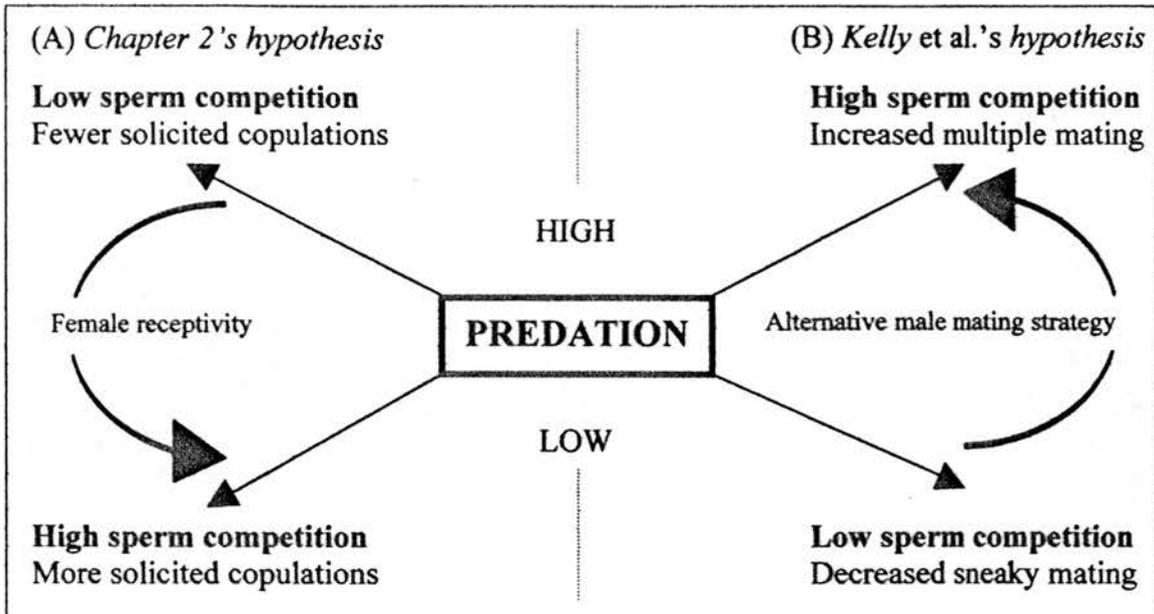


Figure 7.1 The hypotheses presented in chapter two (A) and by Kelly *et al.* (B). Note that hypothesis A is based on predator-mediated changes in female behaviour while hypothesis B considers only variation in male mating behaviour. An alternative scenario is presented (C) in which sperm competition risk reflects the balance between male and female behaviour and their interaction.

(b) How successful is sneaky mating?

Throughout this thesis I refer to 'sneaky mating', whether in relation to geographic variation in mating behaviour, ontogeny, rearing demography, possible predictors of paternity, or even as a potential benefit to females. Yet we have little idea how successful the strategy is in terms of its potential to fertilize eggs (Houde 1997). Recent evidence suggests that in the wild, 15 percent of females contain stored sperm that could only have arisen from sneaky mating (Matthews & Magurran 2000). Moreover, when gonopodial thrusts do lead to sperm transfer, insemination efficiency is often extremely high (Pilastro & Bisazza 1999). However, no study has yet been undertaken to determine whether such mating attempts actually lead to fertilization – a prerequisite for the strategy to be truly successful. Again, the reason for this is simple: measuring the success of the strategy depends upon the ability to assign paternity to offspring, which in the past has been limited. Now that there are several DNA fingerprinting markers available for the poeciliids it should be fairly straightforward to determine whether sneaky mating leads to successful fertilization. An experiment, in which mid-cycle (i.e. non-receptive) females are paired for a short period with males of known genotype (genetic screening is a simple procedure), would suffice. Any offspring sired by these males could only result from forced copulations since mid-cycle females are completely unreceptive to male courtship.

Such an experiment would enable researchers to begin to address several questions that have hitherto remained unanswered. For example, to what extent are males able to undermine female choice and therefore impede the process of population differentiation and ultimately speciation (Magurran 1998)? Can predator-mediated shifts in male behaviour affect sperm competition risk in natural populations (see above)? And to what extent can females control the outcome of sperm competition? If sneaky mating is a successful strategy, it is presumably because it undermines both pre-copulatory (mate choice) and post-copulatory (cryptic) female choice.

(c) Testing models of sperm competition on the guppy

The data presented in chapter four suggest that male guppies respond to their early rearing environment (i.e. the sex ratio) by modifying their sexual behaviour. However, contrary to the initial predictions, they did not invest more heavily in sperm production when the perceived risk of sperm competition was high (i.e. when the sex

ratio was male biased). On the contrary, the number of stripped sperm was virtually identical in the low and high male treatments, and lowest where sex ratios were maintained at unity. As mentioned in chapter four's discussion, the number of stripped sperm may not be a good estimate of ejaculate size in guppies (but see Pilastro & Bisazza 1999), especially if males are able to adjust the size of their ejaculates in response to varying levels of sperm competition. However, this problem may be overcome if natural ejaculates are collected in place of stripped ejaculates. A recent study by Pilastro and Bisazza (1999) has confirmed that it is possible to extract freshly inseminated sperm from female guppies using a fine-tipped glass micropipette. This new technique makes it feasible to conduct experiments in which sperm competition theory is tested explicitly.

It would be possible to test Parker *et al.*'s 'risk' (1997) and 'intensity' (1996) models of sperm competition on the guppy. These models distinguish between sperm competition 'risk', where competition is rare and at most involves two ejaculates, and sperm competition 'intensity', where two or more ejaculates compete for fertilization. The predicted patterns of ejaculate expenditure depend upon the number of males competing for fertilization. In competition 'risk' situations (Parker *et al.* 1997), males benefit from producing increasingly large ejaculates as the probability of competition increases. When the risk of sperm competition is zero, only the minimum number of sperm required to ensure fertilization should be expended, thus assuring paternity with minimal investment. Surprisingly, however, in competition 'intensity' situations (Parker *et al.* 1996), males benefit from actually *decreasing* their expenditure on sperm as the number of competing males increases above two. This is explained by the fact that the expected gain from each additional unit of ejaculate expenditure decreases proportionally as the number of competitors increases. Just like the lottery principle on which these models are based, the potential benefit from buying extra tickets (i.e. sperm) decreases as the number of subscribers to the raffle increases.

It would be simple to manipulate the perceived level of sperm competition in guppies in order to test the above predictions. Indeed, a similar investigation is currently underway in Italy using another species of poeciliid, the eastern mosquitofish *Gambusia holbrooki* (A. Pilastro, M. Pierotti & J. P. Evans). The preliminary results

from these trials are promising and suggest that the poeciliids may provide an ideal model system in which to investigate the dynamics of sperm competition.

(d) *The use of artificial insemination in guppies*

In chapter five, an experiment is reported in which two microsatellite markers were used to estimate the pattern of sperm precedence in guppies. The study produced intriguing results. Firstly, the observed pattern of sperm precedence differed from a theoretical one generated by a model based on random sperm mixing. This suggests that differential sperm usage among females or differential sperm competitive ability among male pairs led to the bimodal distribution of paternity revealed by the study. Secondly, the best predictors of P_2 were the insemination interval (= time elapsed between the two matings) and the sigmoid display rate of the second male. As noted in the discussion (chapter five), the association between insemination interval and male reproductive success does not imply causation. Instead, male courtship displays may indicate some other trait (e.g. sperm competitive ability), which in turn biases paternity towards the second male. On the other hand, males with high display rates are likely to attract females more quickly and in so doing benefit from their sperm reaching the site of fertilization before their rival's. One way to distinguish between these possibilities is to perform a series of artificial inseminations on anaesthetised females. As mentioned above, a technique to extract ejaculates from recently mated females has been devised, so the reverse technique of artificial insemination should also be possible. An experiment in which sperm were inseminated into females at various time intervals would determine whether 'high-quality' males still achieve disproportionately high fertilization success in the absence of pre-copulatory female choice, and also whether the insemination interval affects the outcome of sperm competition. A second experiment in which the sperm from two males are simultaneously inseminated into virgin females would also be useful because it would eliminate the possible confounding effects of sperm age, sperm number and mating order.

(e) *Multiple mating revisited*

The experiment described in chapter six adds to the increasing volume of research documenting female benefits of polyandry. In line with previous studies, it provides an insight into the possible adaptive significance of polyandry, but at the same time

raises a number of interesting questions. Among these is whether sperm limitation influences female mating decisions. Specifically, it was argued that female guppies remate in order to maintain egg fertility over their brief (ca. 48h) receptive period. The recent finding that males inseminate a large proportion of their available sperm during a single copulation (Pilastro & Bisazza 1999) suggests that males (and ultimately females) may become sperm limited over the course of time. Females that were paired with four different males presumably received a continued supply of fresh sperm on each successive day of the mating trials. However, those assigned to the single-male group may have become increasingly sperm limited over the course of the trials. This effect is likely to be especially marked if virgin females do not have eggs immediately available for fertilization when first encountering a male. There is evidence that in guppies this is the case; the period between insemination and brood production – which in chapter six is referred to as ‘gestation’ – is usually more prolonged in virgin than in non-virgin females (Liley 1966). This suggests that despite being sexually receptive, virgin females may contain eggs at various stages of maturity. If they receive a plentiful supply of sperm over the course of several days, they are likely to have mature eggs available at the exact time of insemination. Their single-mated counterparts, however, will have to rely upon a stored and presumably dwindling sperm supply from an initial large ejaculate. This prediction could be tested using a fairly simple – but admittedly invasive – experiment in which egg maturity is examined in virgin and recently mated females over the course of several days.

Two questions arise from the above considerations: do non-virgin females also ‘benefit’ from increased fertility through multiple mating? and is brood size directly related to the number of sperm inseminated? Again, using artificial insemination to mimic natural copulations (while controlling sperm number and other unwanted variables) may help answer these questions. To address the first, it would be necessary to take post-partum females and (artificially) inseminate them either with sperm from a single male or sperm collected from several males. (Post-partum females are sexually receptive for at least two days after producing a brood (Liley 1966) and would therefore have eggs ready for fertilization.) In either case the number of sperm inseminated should be equal. Any increase in female fecundity in the multiple-male group could then be attributed to male number and not sperm number. The second question could be addressed by inseminating virgin females with varying numbers of

sperm and comparing the brood sizes among pairs of sibling females, one of each pair inseminated with a small sperm 'packet' and the other with a large one. Each female within a pair would receive sperm from the same male, thus minimising the possible confounding effect of genetic incompatibility among males and females.

Chapter six also raises a number of mechanistic questions relating to female benefits. For example, do females invest differentially in offspring according to arbitrary cues used during mate choice (Gil *et al.* 1999, and see review by Møller, 1998) or do genetically superior males produce inherently better or more sperm (see review by Jennions & Petrie 2000)? The former possibility suggests females may be able to obtain information from males during pre-copulatory mate choice, thus negating the need to mate with several males. However, where potential mates are encountered sequentially (as in guppies), multiple mating may be a means of 'trading' previous poor quality mates for superior ones. Furthermore, where the genetic compatibility of prospective mates cannot be assessed during pre-copulatory mate choice, polyandry extends the scope for selection (and thus for differential investment) at the genomic level. Alternatively, if males with good 'viability genes' produce better quality sperm, paternity bias towards these males will yield more viable offspring. As noted in chapter six, several condition-dependent traits correlate positively with stripped ejaculate size in male guppies; viable males may therefore perform well in sperm competition. However, differential female investment and genetic benefits are not necessarily mutually exclusive mechanisms and females may apportion more resources towards embryos from genetically superior males.

A further question raised relates to the control of fertilization by females. To what extent are females able to manipulate the processes of sperm storage and fertilization? In chapter six, an explanation was proposed to account for the difference in gestation periods among the two experimental groups. It was argued that singly-mated females delayed the process of fertilization until they encountered superior males. Thus, the difference in 'gestation' periods detected in the study may in fact have been due to a difference in the interval between insemination and fertilization and not the development time. To test this hypothesis it would be a simple matter to dissect females at various intervals from insemination to fertilization to examine the stages of embryo development.

(f) *Concluding remarks*

For over 50 years the guppy's mating system has been used as a model for the study of sexual selection. The insights provided throughout this period have been illuminating and far-reaching. While studies on mate choice continue apace, the blossoming field of sperm competition offers exciting and hitherto unexplored avenues of research into the guppy's mating system. The aim of this thesis has been to highlight the importance of sperm competition in this mating system, and specifically to consider the role it plays in shaping male behaviour and its effect on sperm production. When the work leading to this thesis began in 1997, no other research into sperm competition in the poeciliids was being conducted. Today, at least three other groups are active in this area. Their contributions to this growing field of research are much needed and eagerly anticipated.

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