Mating behaviour and reproductive biology of the guppy, *Poecilia reticulata.*

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University of St Andrews.

September 1998

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Abstract

The aim of this dissertation was to investigate the alternative mating behaviour of the male Trinidadian guppy, *Poecilia reticulata*. Males of this species have two mating strategies. One involves female choice and co-operation, the other sneaky mating. Much has been written about female preferences and the guppy is now central to our understanding of sexual selection. However, our understanding of the role of sneaky mating is less clear. What are the implications of this behaviour for sperm competition? Does it have the potential to undermine female choice?

It is shown that male courtship behaviour is extremely sensitive to variation in environmental factors and represents an opportunistic response to changes in mating opportunities. Further, males that perform more gonopodial thrusts have higher sperm counts. A positive correlation also exists between male display rate and sperm numbers, providing a potential means for females to select males of higher fertility. Through combining information on sperm retention and embryo development, sneaky mating is shown to be a successful means of transferring sperm to 15% of non-receptive females. These ejaculates are likely to contain large numbers of sperm and have obvious implications for sperm competition. A histological investigation of the morphology of the ovary, in combination with the results of molecular analysis of sperm utilisation, suggests that the timing of matings may be of key importance. Recently inseminated sperm can successfully outcompete stored sperm in the competition to fertilise the next brood. However the precedence patterns in the two subsequent broods are reversed and the older stored sperm regain dominance. These findings have important evolutionary consequences in terms of population differentiation and reproductive isolation. Successful sneaky mating will increase gene flow and undermine female choice, and may provide the key to understanding the unexpected lack of speciation seen in the guppy.

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Contents

Appendices

Chapter 1

Introduction and study species

How successful are male alternative mating strategies? Is their importance limited to explaining multiple paternity, or do they have wider implications? This thesis will present data collected from the Trinidadian guppy, *Poecilia reticulata*, a species that has played a key role in furthering our understanding of female choice and sexual selection. Many important studies of the populations from the island's Northern Mountain Range have documented female preferences for a number of male traits. Despite this extensive research into which males females choose to mate with, far less attention has been paid to 'sneaky mating'. This dissertation therefore centres on the male's alternative mating tactic, also known as gonopodial thrusting, and investigates the role that it plays in the mating system of the guppy. It also addresses the potential that sneaky mating has for resulting in sperm competition and conflict between the sexes. For example, it has recently been suggested that alternative mating strategies may undermine female choice. Does it also have the potential to increase gene flow? Even more intriguingly can sneaky mating provide an answer to the paradoxical lack of speciation seen in Trinidad's guppy?

In recent years, the volume of research involving the guppy has increased dramatically and the guppy has now secured a place as one of the key organisms in studies of both ecology and ethology. A large body of literature exists detailing every aspect of the biology of the guppy, and only a brief overview of the main points will be provided here. Particular attention will be given to sexual behaviour and reproduction, although I will also introduce those aspects of the evolution and ecology of the guppy that have contributed to the guppy's current importance and are of importance to the dissertation. This chapter will also introduce the unique geography of Trinidad, concentrating on the rivers in the north of the island that have proved so informative to evolutionary and behavioural biologists.

Distribution and systematics

The guppy, *P. reticulata*, the best known member of the Poeciliidae, is a small teleost fish found widely throughout north-eastern South America and the adjacent islands of the Lesser Antilles, including Trinidad and Tobago (Rosen and Bailey 1963). The first individuals were collected by W. Peters in Venezuela in 1859, however the species' common name derived from R. J. Lechmere Guppy, a Victorian amateur naturalist. Full and comprehensive accounts of the systematics and zoogeography of the family Poeciliidae have been provided by Rosen and Bailey (1963) and Parenti and Rauchenberger (1989). Although some research has also been carried out in Venezuela (Winemiller *et al.* 1990), the most intensively studied populations are on the island of Trinidad, where the guppy is found widely through all but the extreme north east of the island (Kenny 1994). However, it is not known if this distribution is entirely natural, or has in part resulted from human interference and the use of guppies as a mosquito control (Kenny 1994). The majority of studies have involved populations from the north of the island, specifically the rivers of the Northern Mountain Range (figure 1.1; Magurran *et al.* 1995). These populations are of particular interest due to the significant variation in a range of morphological and behavioural traits and the degree to which this variation is attributable to a single ecological factor, namely predation (Endler 1995).

The rivers of Trinidad

The island of Trinidad lies 16km off the coast of Venezuela, and was until around 1000 years ago joined to the mainland by a land bridge (Kenny 1989). Guppies are found throughout the island, occurring in all but the most marginal freshwater environments (Boeseman 1960). In natural habitats they prefer still pools in small streams and the edges and side-branches of larger rivers (figure 1.2a and b). Only large females and small numbers of males are found in deeper faster moving water (Haskins *et al.* 1961; Mattingly and Butler 1994). The ubiquitous nature of their distribution is perhaps best highlighted by their occurrence in puddles of rainwater

Fig. 1.1 Map of northern Trinidad and the rivers draining the Northern Mountain Range.

Figure 1.1

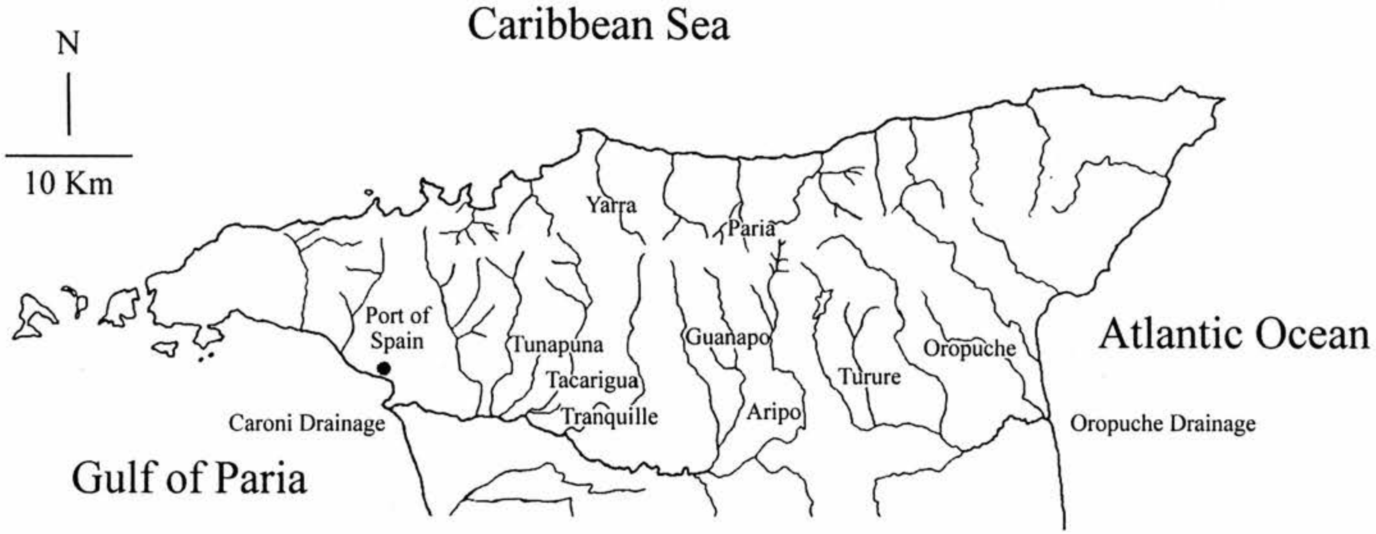

Caribbean Sea
N
10 Km
Yarra
Paria
Port of Spain
Tunapuna
Guanapo
Oropuche
Atlantic Ocean
Tacarigua
Turure
Tranquille
Aripo
Caroni Drainage
Oropuche Drainage
Gulf of Paria

Fig. 1.2a The Tunapuna river in Trinidad's Northern Mountain Range, provides an example of a typical low predation site. Importantly the Barrier waterfalls have prevented the upstream dispersal of major predators.

Fig. 1.2b In contrast to the Tunapuna, the Oropuche river, again in Trinidad's Northern Mountain Range, is typical of a high predation site. Downstream from the waterfalls, the guppies co-exist with important predators.

Figure 1.2


Photograph by Iain Matthews


Photograph by Iain Matthews

on the surface of Pitch Lake, a 36 hectare deposit of natural asphalt in southern Trinidad. However the populations subsequently referred to in this dissertation are all from the rivers of the island's Northern Range of mountains.

The rivers of Trinidad's Northern Range can be divided conveniently on the basis of the direction of their drainage. The rivers of the Oropuche drainage empty into the Atlantic Ocean, while the rivers of the Caroni drainage run west into the Gulf of Paria. It is thought that guppies colonised each of the drainages separately and the drainages have been shown to be genetically distinct (Carvalho *et al.* 1991; Shaw *et al.* 1991). Estimates of the length of the period of separation between drainages vary between 330,000 (based on mtDNA, Fajan and Breden 1992) and 500,000 years (based on allozymes, Carvalho *et al.* 1991). In addition to divisions based upon drainage, the diversity of fish fauna is the most common factor used to arrange populations.

Interest in the effects of predation regime on the biology of the guppy was first taken by Haskins *et al.* (1961). This early study was the first to establish a link between predation and colour pattern, a theme continued and expanded by several authors. The guppy is subjected to considerable predation both by larger fish and possibly birds and mammals (Seghers 1973). Haskins *et al.* (1961) identified five species of fish that preyed upon guppies in the Northern Range. Four of these, *Astyanax bimaculatus* (Characidae), *Aequidens pulcher* (Cichlidae), *Hoplias malabaricus* (Erythrinidae) and *Crenicichla alta* (Cichlidae) were termed 'serious predators', while the fifth, *Rivulus hartii* (Cyprinodontidae) was classified as 'less serious'. Several other species have since been identified as being important, though less severe, predators of the guppy (table 1.1). The most important point to be made concerning predation is that not all guppy populations are exposed to the same diversity of predators.

The slow flowing, wide, lowland rivers are home to a wide range of large piscivorous predators. The rivers of the Northern Range contain populations of *A.*

Palaemonidae	
	Macrobranchium crenulatum
Erythrinidae	
	Hoplias malabaricus
	Hoplerythrinus unitaeniatus
Characidae	
	Astyanax bimaculatus
	Hemibrycon taeniurus
Rivulidae	
	Rivulus hartii
Synbranchidae	
	Synbranchus marmoratus
Cichlidae	
	Crenicichla alta
	Aequidens pulcher
	Cichlasoma bimaculatum
Mugilidae	
	Agonostomus monticola
Gobiidae	
	Eleotris pisonsis
	Gobiomoris dormitor
	Dormitator maculatus

Table 1.1 Important piscivorous predators of *P. reticulata*. Data from Haskins *et al.* 1961; Seghers 1973; Liley and Seghers 1975; Endler 1978, 1983.

bimaculatus, *H. taeniurus*, *A. pulcher*, *H. malabaricus*, *C. alta*, *R. hartii* and freshwater prawns from the *Macrobrachium* genus. The importance of the first five of these species as predators has led to these rivers being referred to as 'high predation regimes'. However the positioning of natural barriers, such as waterfalls, has prevented the upstream migration of the majority of these species, and as a result they are absent from the headstreams (Haskins *et al.* 1961; Liley and Seghers 1975). The headstreams of the Northern Range are therefore free of the major predatory species. Only *R. hartii* and *Macrobrachium*, both of which are less important as predators, have successfully dispersed above these abrupt barriers (Haskins *et al.* 1961; Liley and Seghers 1975). The extent to which *Macrobrachium* preys upon guppies is still a topic of debate. Luyten and Liley (1985) suggest that it is of little impact, however Magurran and Seghers (1990a) and Endler (1978, 1980, 1983) believe that it may occasionally take guppies. While predation by *Rivulus hartii* has been confirmed, it is still only a relatively minor predator. Due to *Rivulus'* small size, it is only able to prey upon immature guppies (Seghers 1973; Liley and Seghers 1975; Seghers 1978; Mattingly and Butler 1994). The relatively lower level of predation threat in headstream waters has led to them being referred to as 'low predation regimes'.

This variation between populations in a single ecological factor has promoted considerable research into the effect of predation on a range of characteristics. Differences in predation pressure accounts for the majority of variation in life history traits between populations (Reznick and Endler 1982). For example, in high predation sites, guppies mature at a younger age (Reznick 1982), brood sizes are larger (Reznick 1989) and life span is shorter (Endler 1978). Colour patterns are also influenced by predation regime, in high predation sites males have a lower number of small less conspicuous colour spots (Endler 1978, 1980, 1983). Several behavioural traits are also affected. The effect of predation on courtship behaviour is dealt with in chapter 4, however female preferences (e.g. Houde and Endler 1990), schooling behaviour (Seghers 1974; Magurran and Seghers 1991; Seghers and Magurran 1995) and antipredator behaviour (e.g. Magurran and Seghers 1990b, 1994a, 1994b) are also influenced by the intensity of predation.

Courtship behaviour

The courtship behaviour of male guppies has been extensively recorded in a number of classical ethological studies (Clark and Aronson 1951; Baerends *et al.* 1955; Liley 1966). As male mating strategy is of particular importance to this thesis, I shall describe in some detail the main behaviours exhibited during mating. There are two distinct strategies that a male can adopt when attempting to transfer sperm. Males can display to a female and attempt to persuade her to cooperate in a mating. Alternatively males may attempt to transfer sperm without female co-operation, or possibly against the female's will, through sneaky mating.

The sigmoid display of the male is named after the characteristic 'S' – shape that the male adopts during courtship (figure 1.3a). The male follows the female, apparently attempting to gain a suitable position from which to display (Houde 1997). If the female stops or turns to face the male, the male will take up position in front of the female and begin to display. The male bends the body laterally into a sigmoid and extends his caudal and dorsal fins. This posture is maintained as the male 'quivers' and slowly circles the female (Liley 1966). Increase in the area of black colour spots is associated with changes in motivational state and in general males become much darker in colour whilst performing these sigmoids (Baerends *et al.* 1955). This sigmoid may proceed to copulation or the male may attempt to lead the female into an area with a lower density of males (Baerends *et al.* 1955).

However, as a result of the female reproductive cycle (see below), the majority of females (90%) are not receptive to male displays. Most sigmoids are therefore ignored, non-receptive females simply continue feeding or foraging, and are consequently brief. Several early papers, including Clark and Aronson (1951), considered females to be passive throughout courtship and mating. In contrast,

Fig. 1.3a Male guppy performing a sigmoid display. If sigmoids are successful, the female may co-operate in a full mating. Note the position of the male in front of the female and the characteristic posture of the male during the display.

Fig. 1.3b Male guppy attempting a sneaky mating. As mentioned in the text successful gonopodial thrusting relies on avoiding detection. The male approaches the female from behind and darts towards her, attempting to insert the tip of the gonopodium in the urogenital sinus.

Figure 1.3

Photograph by Iain Matthews

Photograph by Iain Matthews

receptive females turn to face the male and swim towards him in a characteristic smooth gliding movement (Liley 1966). Liley (1966) suggests that the smoothness of the approach acts to reassure the male that the female is not aggressive, and functions as an indication of her interest in mating. If the female does glide towards the male, then the male will continue performing lengthier, higher intensity sigmoids. After a period of circling, the female will also adopt a sigmoid posture and present her genital pore to the male. The male then swings his gonopodium forward and attempts to insert it into the urogenital sinus. If successful the pair circle rapidly, before parting and separating. After a successful copulation the male performs a series of sharp jerks, often compared to a 'bout of hiccups'. Although this prominent behaviour may continue for several minutes, its function has yet to be established. It is possible that the sharp jerks may be important in moving the next ejaculate in to the correct position in the gonopodium (Bowden 1969). Alternatively jerking may be a signal, to both males and females, of a successful insemination (Houde 1997).

In addition to performing a sigmoid display, males can also attempt to transfer sperm through sneaky mating. This second, less behaviourally complex strategy, also referred to as gonopodial thrusting, is attempted without male display or female co-operation. The male approaches the female from behind, possibly to avoid detection, and swings his gonopodium forward in an attempt to insert the tip into the female's genital pore (figure 1.3b). This behaviour has no signal function and there are no countersignals directed from the female to the male. As such it is not considered to be a display (Farr 1989).

Reproductive physiology

The sexes in *P. reticulata* are, as in all poeciliids, sexually dimorphic. In many members of the family this dimorphism may be only the expected differences in the genitalia (Farr 1989). However in the guppy the differences are far more pronounced and include both sexual dichromatism and sexual dimorphism in body size (figures 1.4a and b). The variation in body size between the sexes is primarily accounted for by the continued growth of females after reaching maturity (Seghers 1973). Females have a uniform olive brown colouration that is thought to be a defence against predation (Magurran *et al.* 1995). The only area of the female body carrying any colour is the dark spot around the genital pore (Constantz 1989), which may provide a target during gonopodial orientation (Peden 1972, 1973). Males display considerable intrasexual colour polymorphism (Haskins *et al.* 1961), the genetics and inheritance of which have been extensively studied (Winge 1922a, 1922b, 1927).

Both male and female guppies display a fascinating range of reproductive adaptations, many of which are unique to the poeciliids. Fertilization, as in all members of the family, is internal. The male anal fin is modified to form an intromittent organ. In immature males and adult females the anal fin normally has nine fin rays. In adult males, rays 3,4 and 5 are elongated and surrounded by muscles, nerves, blood vessels and connective tissue (Rosen and Tucker 1961). These fin rays form a short channel down which the sperm are transferred to the female (Clark and Aronson 1951). Spermatogenesis occurs within the single paired testis, where sperm are prepared in bundles and coated with a gelatinous matrix (Rosen and Bailey 1963). Individual sperm have a greatly enlarged nucleus, lance-shaped head and long flagellate tail (Jamieson 1996). Guppy sperm have plentiful supplies of stored glycogen (Gardiner 1978) and can remain motile for a relatively prolonged period (up to 2 hours, Billard 1986). It is not known how the encapsulated sperm bundles, spermatozeugmata, are transported to the gonopodium. The process underlying the subsequent transfer of the sperm to the female is also poorly understood.

The adaptations of the female reproductive tract are even more fascinating. As mentioned sperm are transferred to the female and fertilization occurs within the ovary. The guppy is ovoviviparous, the eggs therefore do not receive any further nourishment after fertilization (Thibault and Schultz 1978). Gestation takes around 28 days after which the female gives birth to a brood of between 2 – 18 juveniles (Wourms 1981; Reznick and Endler 1982; Reznick and Miles 1989). Females

Fig. 1.4a A female Trinidadian guppy. Despite their less colourful, cryptic appearance, females exhibit a suite of fascinating reproductive adaptations.

Fig. 1.4b A male guppy from the Tunapuna. Males possess highly polymorphic colour patterns. In addition the anal fin is modified into an intromittent organ, the gonopodium.

Figure 1.4

Photograph by Iain Matthews

Photograph by Iain Matthews

undergo regular cycles of egg production (Rosenthal 1952), and Turner (1937) distinguished three basic cycles, including the 'Lebistes' type with an interval of two days between parturition and ovulation. After parturition the females yolk the eggs of the next brood. This period between the birth of one brood and the fertilization of the next is marked by a distinct increase in the sexual receptivity of the female (Rosenthal 1952; Liley 1966; Crow and Liley 1979). Liley (1966) presented females to males for 15 minutes a day, starting on the day they gave birth. Copulations occurred during the observation periods of the first three days, after this copulations ceased completely and the number of sexual responses declined rapidly.

During this period of receptivity the female releases a chemical which causes an increase in male courtship behaviour. The chemical, which has not yet been identified but is thought to resemble oestrogen (Amouriq 1967), is often referred to as a pheromone (Crow and Liley 1979; Meyer and Liley 1982). However as is the case with other fish 'pheromones' (e.g. Schreckstoff, Smith 1997; Henderson *et al.* 1997), there is debate as to the correct term for these olfactory cues. As Henderson *et al.* (1997) point out the issue is primarily semantic, and centres on the definition of 'pheromone'. Williams (1992) proposed that the term be restricted to chemicals that were adaptive for the sender and that are produced, released and received by structures that have evolved for the purpose of communication. This excludes the majority of sex 'pheromones', which are produced, as seems likely to be the case in the guppy, as an incidental product of reproductive physiology. The act of parturition and the subsequent yolking of the next brood are both likely to be linked to the secretion of female gonadotrophins such as oestrogen. The release of the chemical has therefore not evolved as a signal to the males. However, Constantz (1989) and Farr (1989) both suggest that the chemical is a pheromone and that it functions to incite scramble competition amongst males. Males do indeed compete more for receptive females, however in itself this is insufficient to fulfil Williams' (1992) criteria. Irrespective of the terminology there is an increase in both female receptivity and male courtship activity that corresponds with the availability of yolked unfertilized ova (Kadow 1954).

Perhaps the most remarkable aspect of the female's reproductive biology is the ability to store viable sperm for prolonged periods. It has long been known that female guppies are able to produce several successive broods even in the absence of a male (Winge 1937). The prolonged survival of sperm in such an apparently hostile environment remains confusing. It has often been suggested that the ovary may provide nutrition for the stored sperm (Turner 1937). If this is the case in the guppy then ovarian sugars seem the most likely source of nourishment (Gardiner 1978). Further Hogarth and Sursham (1972) suggested that the high concentrations of oestradiol found in the ovary may reduce the rejection response to foreign cells. Sperm storage and the anatomy of the ovary are discussed in more detail in chapter 5.

Thesis and aims of this dissertation

The courtship behaviour of *P. reticulata* has been the subject of scientific investigation for several decades. However the previous studies have concentrated on female choice and male colouration. The sigmoids that males perform to receptive females have been described in depth. Sneaky mating meanwhile has been largely overlooked. This dissertation aims to address the role of sneaky mating in the reproductive biology of the guppy and to redress the balance between the two male mating strategies.

In order to further understand the factors influencing a male's choice of mating strategy, an initial set of experiments (Chapter 2) were carried out to establish the conditions under which males attempt sneaky mating. Predation intensity is known to affect the male's choice of mating strategy, but it is predicted that the receptivity of the female and the number of males competing for matings will also play a part. Behavioural observations of male courtship behaviour were undertaken to investigate the role of environmental and social factors in determining the relative proportions of sigmoids and thrusts. Chapter 3 again measured the behaviour of males, but in addition an ejaculate was also stripped from each male and the number of sperm recorded. Chapter 3 investigates the relationship between male courtship behaviour and ejaculate characteristics and tests Sheldon's (1994) phenotype-linked fertility insurance hypothesis. This hypothesis proposes that females that choose mates on the basis of key phenotypic characteristics gain information about their ability to father offspring. In addition the association between the number of thrusts a male attempts and his sperm count was also investigated.

How successful is sneaky mating at transferring sperm to non-receptive females? Laboratory studies determined the length of time that sperm remain in the ovary before being more permanently stored, and also the length of time taken by embryos to reach various developmental stages. Armed with this information, a field study (Chapter 4) was undertaken which allowed the determination of the number of unreceptive females that had recently received sperm as a result of gonopodial thrusting. If sneaky mating in the wild results in successful insemination then the potential exists for sperm competition and sexual conflict. What happens to sperm after they are inseminated? To address this issue, and with the aim of aiding our understanding of patterns of paternity and determining the fate of sperm received from sneaky matings, a microscopical and histological analysis of the ovary was undertaken (Chapter 5). The positioning of stored sperm and the access they have to the unfertilized ova will be one of the main factors that determine the outcome of sperm competition. Investigating the morphology of the female reproductive tract will aid in our understanding of the mechanisms of sperm competition and paternity determination in *P. reticulata*. In addition, it will help to determine the adaptive value of the behavioural strategies of males, which will only be fully understood following advances in the knowledge of anatomical and physiological mechanisms that come into play after copulation.

What are the patterns of sperm utilisation in the guppy? Female guppies store sperm in the ovary for several months (Winge 1937). Can sperm from more recent inseminations outcompete sperm already stored in the female reproductive tract? Chapter 6 reports the first study to undertake molecular analysis of paternity in the guppy. As a result of prolonged sperm storage, the ovary may still contain viable stored sperm at the time of remating. Does this lead to competition between the stored and the fresh, more recently inseminated sperm. An experiment was designed to investigate patterns of paternity following temporally separated inseminations from two different males. Finally the thesis, that sneaky mating is an important and thus far overlooked aspect of the reproductive behaviour of the guppy, is summarised in chapter 7. The results of the experimental chapters are brought together to suggest that sneaky mating may have implications in several aspects of the ecology of *P. reticulata*, and suggestions are made for potentially fruitful areas of future work.

Chapter 2

Male-male competition and female receptivity as determinants of male mating activity.

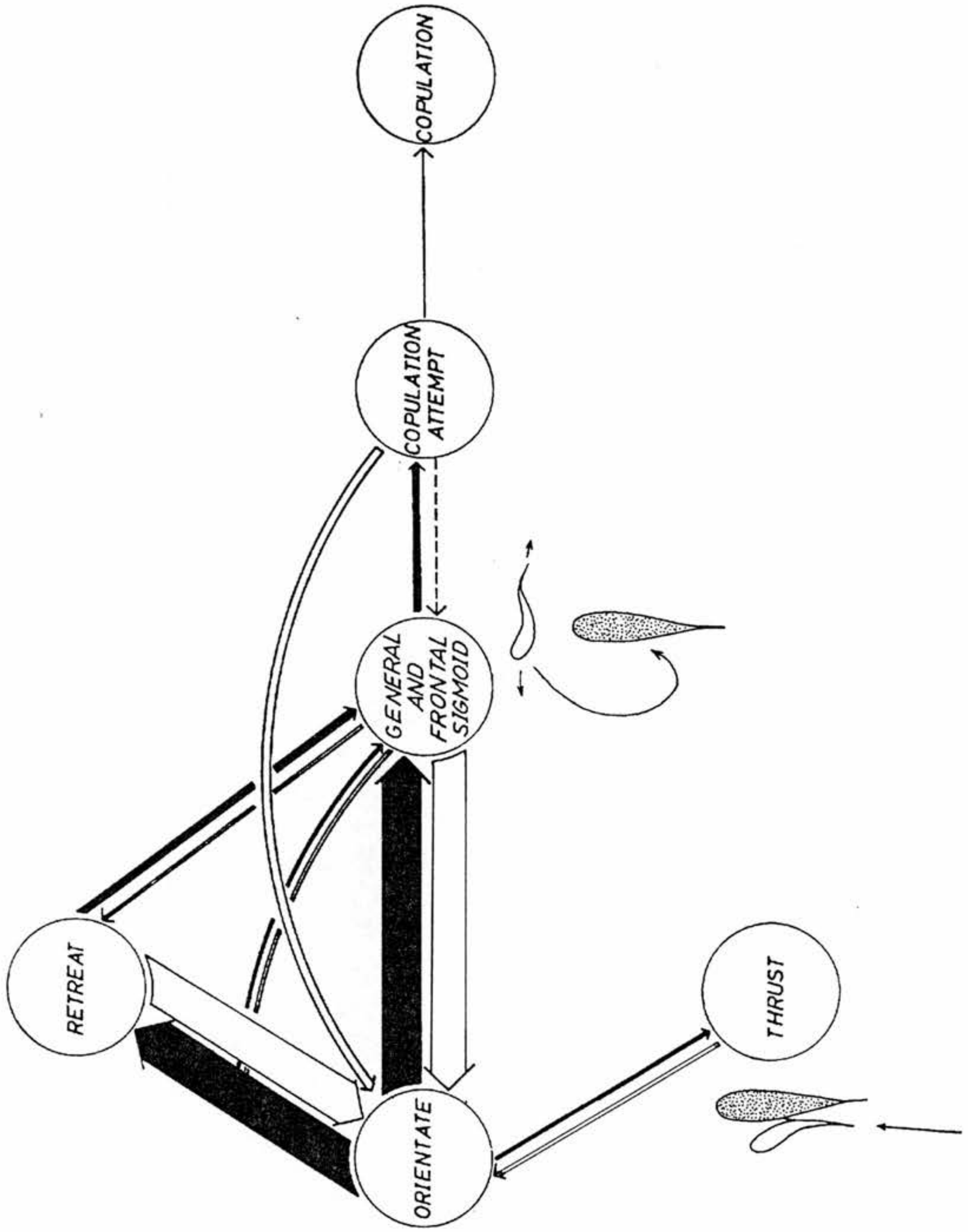
INTRODUCTION

In species in which males display alternative mating tactics (e.g. sticklebacks, gobies and guppies), mating strategy has been shown to be influenced by a number of factors. These variables include the social environment, internal state of the male, certain attributes of the female being courted and other environmental factors. Houde (1997) suggested that the best way to understand the behavioural decisions involved was as a series of trade-offs between current and future costs and benefits. The rate of encounter with females, success in gaining matings and the subsequent outcome of sperm competition are all determinants of the benefits of courtship. The costs of courtship and mating depend upon how the male's behaviour affects his chances of survival and his future reproductive success. In order to gain a more complete understanding of why males attempt sneaky mating at such high frequencies in the wild, the laboratory based experiment described in the chapter was carried out to tease apart the effects of three important variables.

The standard means of investigating these trade-offs is to examine how particular behaviours are affected by changes in environmental and social circumstances (Stearns 1992; Houde 1997). This still involves considerable labour, as a male's total courtship effort is the product of several individual behaviours (figure 2.1). In this study four behaviours; the time spent following the female, the number and duration of sigmoid displays and the number of sneaky mating attempts, were recorded as a measure of male courtship activity. Changes in male courtship were used as an indicator of the effect of three factors suspected of influencing male behaviour. All of these factors are found in wild populations and yet we have little understanding of how they combine to affect male sneaky mating rate. The factors investigated were

Figure 2.1 Sequence of the main behaviours involved in male courtship in *P. reticulata*. Modified from Liley (1966).

Figure 2.1


COPULATION
COPULATION ATTEMPT
GENERAL AND FRONTAL SIGMOID
RETREAT
ORIENTATE
THRUST

female receptivity, the predation pressure of the population from which fish were derived and the number of males competing for matings. By studying the factors that determine male mating strategy, it was hoped that further insights could be gained into the importance of sneaky mating in natural populations. In addition, this chapter aimed to establish which environmental factors could be experimentally manipulated to ensure that males attempted sneaky mating and did not perform sigmoids. The setting out of an experimental protocol that reliably leads to sneaky mating would prove valuable for subsequent studies of the success of the alternative mating strategy in this species.

Before detailing the experimental methods, I shall firstly review the literature covering the influence of environmental factors on male courtship behaviour.

Predation risk and courtship behaviour

Perhaps the most thoroughly investigated and best understood factor that affects the behaviour of guppies in the wild, and their laboratory bred descendants, is predation regime. In addition to affecting several other behaviours, including foraging behaviour (Magurran and Pitcher 1987; Magnhagen 1991; Sih 1994), it has been shown that several species, including guppies, are able to adjust their courtship behaviour in response to predation risk. Tuttle and Ryan (1982) and Ryan (1985) showed that male tree frogs (*Smilica smilica*) and Tungara frogs (*Physalaemus pustulosus*) reduce their calling frequency in the presence of a model predator. Sih *et al.* (1990) found that waterstriders (*Gerris remigis*) reduce both the frequency and duration of mating attempts in the presence of green sunfish (*Lepomis cyanellus*). As with foraging, the extent to which courtship is disturbed varies. So while fiddler crabs showed only a decrease in courtship activity when the perceived risk of predation is increased (Koga *et al.* 1998), Magnhagen (1990) found that black gobies (*Gobius niger*) exhibited a total cessation of spawning behaviour when under threat of predation by cod (*Gadus morhua*).

The most direct evidence of the effect of an increase in predation risk on courtship behaviour comes from studies of male behaviour in the presence of predators. Endler (1987) studied the courtship behaviour of a population of guppies living in predator free, artificial greenhouses. After 5 months a predator, *Crenicichla alta*, was introduced and the courtship behaviour was re-recorded. Endler (1987) found that the rate of sigmoid displays had decreased significantly, while the rate of gonopodial thrusts had significantly increased. However due to the mixed origins of the fish and the methods of the study he was unable to relate his findings to population variation in antipredator behaviour. Magurran and Seghers (1990a) addressed this issue with an investigation of the potential link between the tendency for a male to modify its courtship behaviour and the predation pressure in the male's natural population. Using wild-caught male guppies from above and below a barrier waterfall on the Aripo river, they recorded the number of sigmoids and thrusts firstly in the absence, then in the presence of two immature characins, *Astyanax bimaculatus*. Males from below the waterfall, the high predation site, showed a significant decrease in the frequency of sigmoid displays and a simultaneous increase in the number of thrusts. In contrast there was no change in the behaviour of the males from above the waterfall. The degree to which individuals alter their behaviour is therefore related to the degree of predation experienced by those fish in the wild.

The studies discussed so far have dealt with the effect of a predator on the behaviour of individuals within a population. In addition to these a number of studies have been carried out that have investigated the differences in courtship behaviour between populations with varying predation regimes. Farr (1976) found that males from populations with large predators of guppies, including *Crenicichla alta*, or from populations which were free from predators, had higher courtship rates than guppies from sites with *Rivulus hartii*. At first this may seem surprising as *R. hartii* is usually thought of as being less of a threat than the larger cichlids and characins (Haskins *et al.* 1961; Endler 1978). However *R. hartii* does still pose a threat to male guppies (Seghers 1973), and Farr (1975) suggested that the ambush hunting style of *R. hartii* may make displaying in locations rich in *R. hartii* especially dangerous. These findings were replicated by Magurran and Seghers (1990a) in a study of the courtship behaviour of males from sites experiencing either *C. alta* (Lower Aripo) or *R. hartii* (Upper Aripo) predation. Under laboratory conditions, when a predator was present, there was no significant difference in the display rates of the males from the two populations. However when the predator was removed guppies from the *C. alta* location were again found to have the higher display rate (Magurran and Seghers 1990a).

Other studies have produced conflicting results. Luyten and Liley (1985) also documented variation in male sexual behaviour between populations. Four rivers were examined; the Paria (low predation); the Naranjo (low predation); the Lower Aripo (high predation) and the Guayamare (high predation). Data were collected firstly for wild populations and later for laboratory bred offspring from each of the sites. Significant differences were found in both the number and the duration of sigmoid displays between high and low predation sites. However in contrast to the studies of Farr (1975) and Magurran and Seghers (1990a, 1994a), Luyten and Liley (1985) found that the highest rates of display were from males from low predation sites. Ballin's (1973; cited Luyten and Liley 1985) results provide a similar picture of high display rates in headstream (low predation) populations and lower rates in lowland (high predation) populations. While Crow's (1981; cited Luyten and Liley 1985) comparison of the Caparo (high predation) and the Naranjo and Paria (low predation) provided further evidence of higher display rates occurring under lower predation regimes. It is difficult to establish any reason for the differences in the findings between authors. Obviously it is problematic to compare the findings of different authors and their different study sites. However, while predation pressure is obviously an important factor, local variation in environmental conditions may also be involved in the determination of courtship strategy. For example, Luyten and Liley (1985) documented considerable variation between populations in water turbidity, while Crow (1981; cited Luyten and Liley 1985) found that increasing water velocity leads to a decrease in courtship activity. The large number of factors that influence male courtship behaviour makes it inevitable that there will be variation between studies. Further details of the differences in the exact range of microhabitats that were sampled by different authors may help to explain the variation in findings.

Ambient light levels and male mating strategy.

Guppies also modify their courtship behaviour in response to variation in light intensity, possibly as light level is correlated with predation risk. In Endler's (1987) experiment with guppies in artificial streams in the University of Utah's greenhouses, males performed more sigmoids during the winter study period (April) than the summer period (May – October). During both observation periods a single *Crenicichla alta* was also present in the streams. Endler (1987) suggested that the decrease in sigmoid rate might be due to the increase in ambient light levels. Similar patterns of lower display rates corresponding with bright conditions were recorded over the course of each day, with the number of sigmoids decreasing around midday. However light conditions did not affect the rate of sneaky mating attempts. It is possible that males may perceive the risk of predation as being increased at high light levels and therefore reduce the number of highly visible sigmoid displays. Indeed Endler (1987) also found that the pike cichlid was more likely to attack during periods of bright light (10.00 – 14.00 hours).

Endler's findings have since been developed and an experimental protocol established that allows the perceived risk of predation to be altered by varying light levels (Reynolds 1993; Reynolds *et al.* 1993). Studying laboratory bred fish descended from the Quaré river, it was found that when light levels were increased sigmoid rates, as predicted, decreased. Interestingly males responded differently depending on their body size. The reduction in sigmoid rate with increasing light levels was only observed in large males (Reynolds *et al.* 1993). Two possible explanations for the interaction between male size and light levels have been proposed. Reynolds *et al.* (1993) suggested that when predation risk is greatest, during periods of high light intensity, large males might be more vulnerable because size *per se* leads to greater conspicuousness. Alternatively, as females from the Quaré have been shown to prefer larger males (Reynolds *et al.* 1993), they may not need to court as intensively as their smaller rivals (Houde 1988b, Reynolds *et al.* 1993). Females may also alter their courtship behaviour during bright light conditions, becoming less interested in males during periods when they are most conspicuous to predators. Changes in the behaviour of the female will obviously feedback and affect the courtship behaviour of the male.

Ambient light levels within the river can also be affected by the turbidity of the water. Luyten and Liley (1985) found marked differences in the turbidity of their study sites. For all but a few hours, immediately following heavy rain, the Paria and the Naranjo remain clear. However the Guayamare is persistently turbid, with associated poor visibility, even after several days without rain. The high levels of gonopodial thrusting seen in the murky Guayamare may reflect the lower visibility of males to females. Under such conditions, displays may be less effective and sneaky mating may be a more appropriate behaviour (Luyten and Liley 1985).

Gonopodium length

Reynolds *et al.* (1993) found that males in the Quaré river exhibited a positive correlation between gonopodium length and frequency of gonopodial thrusting. However even small males with long gonopodia will also perform sigmoid displays. This finding is consistent with the hypothesis that gonopodium length is under selection for circumventing female choice. A similar result was obtained in a cross species analysis that compared the mating patterns of a range of poeciliids and related the findings to gonopodium length (Rosen and Tucker 1961). Those species with longer gonopodium were more likely to be the species that engaged in thrusting as a primary mating strategy rather than courtship.

Unlike the pattern generally assumed for poeciliids, smaller male guppies do not specialise in gonopodial thrusting. Four other species of poeciliid (*Poeciliopsis* *occidentalis*, *Gambusia affinis*, *Poecilia latipinna* and *Xiphophorus nigrensis*) are similar to guppies in that smaller males are less preferred by females. However, unlike guppies, small males in these species are subordinate. (Constantz 1975, Hughes 1985, Farr *et al.* 1986, Ryan and Causey 1989, Travis and Woodward 1989, Zimmerer and Kallman 1989, Reynolds *et al.* 1993). Gonopodial thrusting does therefore not fit into the typical 'best of a bad situation' tactic, where subordinate males attempt to gain matings through stealth (Dunbar 1982). One potential reason for the difference between *Poecilia reticulata* and these other poeciliids may be the lower levels of aggression in the guppy (see below). Smaller males may therefore be able to compensate for their relative unattractiveness by increasing the amount of courtship they perform without being attacked by rival males (Reynolds *et al.* 1993).

Parasite load

Evidence that a male's condition influences his courtship behaviour has been provided by studies of the effect of parasite load on courtship in the guppy. Kennedy *et al.* (1987) observed males infected with the common exoparasite *Gyrodactylus turnbulli*, and found a negative correlation between the number of parasites and display rate. Kennedy's most heavily parasitized male had twenty *G. turnbulli*, however this represents only a low level infection, and suggests that even mild parasitism may influence male behaviour. Houde (1997) suggests that the reduction in courtship may be an attempt to conserve energy supplies that will required to overcome the infection. This sensitivity of male courtship rate to male condition has been utilized by Schröder and Peters (1988) to provide a diagnostic test of environmental pollution. In addition male courtship behaviour is also sensitive to the inherited effects of irradiation (Speiser and Schröder 1980; Werner and Schröder 1980).

Female characteristics

Several characteristics of the female being courted have also been shown to affect male courtship activity. As female size increases so too do the benefits of mating, with larger females being potentially more fecund (Abrahams 1993). If females vary in their value as potential mates (Deutsch and Reynolds 1995) and males are limited in the number of females with which they can mate, they are predicted to exercise some degree of selectivity in their mate choice (Andersson 1994). Several studies in other fish species have documented male preferences for both receptive females (*Gambusia affinis,* Hughes 1985; *Poecilia latipinna,* Sumner *et al.* 1994) and larger females (*Cottus bairdi,* Downhower and Brown 1980; *Gasterosteus aculateus,* Rowland 1982; Sargent *et al.* 1986; Côté and Hunte 1989).

Receptive females produce a 'pheromone' that is detected by males (Liley 1972; Crow and Liley 1979; Meyer and Liley 1982). Evidence that an olfactory cue is produced is strengthened by the finding that males are not only attracted to the receptive female, but are also attracted to water in which the female has previously been held (Gandolfi 1969; Crow and Liley 1979). In low density populations males may rely on the detection of the pheromone to find receptive females, while in higher density populations the pheromone may allow the discrimination of receptive from unreceptive females. Liley (1966) presented males with either receptive females that had recently given birth or unreceptive females. Courtship display rates did not change, however courtship intensity (sigmoid length) was greater when the females were receptive. In addition gonopodial thrusting occurred at significantly higher levels when females were unreceptive. Liley (1966) suggested that males should always display at a high rate, initially performing low intensity displays to establish the receptivity of the female. If the female responds, then the male should increase the intensity of his displays, as females prefer displays of higher intensity (Farr 1977). If the female is unreceptive,a male should either change strategy and attempt gonopodial thrusting or search for another female. Display rate therefore remains high throughout, only display intensity and rate of gonopodial thrusting change. Farr

(1980b) also found higher levels of sneaky mating attempts when males were presented with unreceptive females.

In addition to female receptivity, male guppies have also been shown to respond to variation in female size. Abrahams (1993) found that males were more likely to leave a feeding station and begin courting if a larger female was presented then if the female was small. The interaction of receptivity and female size is once again unclear. Houde (1987) found that males preferred to court large unreceptive females rather than small receptive females. Houde (1997) suggests that the following of unreceptive females seems counterproductive, as these females are less likely to be interested in mating. Houde (1997) further proposes that this preference for large females will occasionally bring the male in to contact with a large receptive female. However, if males can detect female receptivity on the basis of the production of a pheromone, then this explanation is unsatisfactory. Instead it may be possible that the benefits of mating with a small receptive female are outweighed by the potential benefits of sneaky mating with a larger, more fecund unreceptive female. Sneaky mating may in certain conditions present larger benefits than full matings.

Aggression, dominance and male-male competition

While much work has been carried out on intersexual selection and female choice of male colour patterns, the possible role of male-male competition is much less clear. Both males and females are capable of aggression, although such behaviour is not thought to be directly related to competition for matings (Baerends *et al.* 1955; Liley 1966). In most cases, male guppies searching for females are reluctant to approach a female that is already being courted by another male (Houde 1997). As males move between females frequently, there are usually several unaccompanied females at any one time. Occasionally, a second male will persist in attempting to court a female that is already being attended by another male. In such circumstances the original male usually moves closer to the female and attempts to exclude the new male (Baerends *et al.* 1955). If the second male continues his courtship attempts then a chase may occur. During this both males follow the female attempting to gain position for a mating attempt. Eventually one male, most often the intruder, gives up and seeks another female (Houde 1997). Occasionally, both males may lose track of the female. Chases, in which two males pursue the same female, do not involve overt aggression between the males. Instead, each male appears to be trying to remain close to the female and to exclude the other male (Houde 1988b).

Whilst aggressive male behaviour is rarely observed in natural social groups in the field or in the laboratory (Farr 1975), aggression becomes more common when the sex ratio is artificially male biased (Gorlick 1976), when there are very few individuals in an aquarium (Bruce and White 1995), or when a defensible food resource is present (Magurran and Seghers 1991). Aggression is particularly common in groupings involving only two males even when there are two or more females (Houde 1997). In these cases, and in other laboratory studies, dominance is quickly established by one of the males (Gandolfi 1971; Gorlick 1976; Kodric-Brown 1992; Bruce and White 1995). Gandolfi (1971) and Gorlick (1976) concluded that male dominance in guppies is positively correlated with reproductive success, Farr (1975) however suggests that this seems unlikely to be the case in the wild. Instead, he argues that, if it were advantageous to be aggressive, one would expect observations of guppies in the field to reveal frequent agonistic interactions. In nature many other poeciliids are highly aggressive (e.g. *Poeciliopsis* spp. Moore and McKay, 1971; *Poeciliopsis occidentalis* Constantz, 1975; *Neoheterandria tridentiger* McPhail 1978; *Gambusia affinis* Martin 1975; *Mollienesia latipinna* Baird 1968), and it would seem that high levels of aggression would also be seen in guppies if it were an advantageous trait.

Male-male competition could operate in two ways in guppies that are distinct from the effects of female choice. First, males may compete indirectly (scramble competition), attempting to maximise their rate of encounter with co-operative females, but without directly interfering with other males. Each copulation that one male obtains is a gain for him, but a loss for other males because females only mate a limited number of times. Characters that increase a male's rate of obtaining copulations, for example patterns of search behaviour and display rate, would be favoured by sexual selection in this context. Second, males may attempt to increase their mating success by interfering directly with other males (interference competition). Male-male competition does not usually involve overt aggression in guppies (Farr 1975). More likely, males may be able to monopolise females at the expense of other males through more subtle interactions such as 'jockeying' for position, or by establishing dominance relationships with other males that affect the outcome of interactions.

The conclusions of these experiments (Farr 1980a; Houde 1988b; Kodric-Brown 1992, 1993) provide only preliminary information about the role of male-male competition in guppies. Most observations of natural social groupings suggest that dominance and aggression are not normally a very important factor, but more detailed observations and experiments are needed to determine the effects of subtle interactions between males on mating success. In combination with the influence of the other factors mentioned in this introduction, it is not surprising that male courtship behaviour in the guppy has proved difficult to study. This chapter aims to investigate the role of three of these variables in determining the when males abandon sigmoids and switch instead to sneaky mating. Examining the situations in which males attempt gonopodial thrusts will further our understanding of this behaviour in wild populations.

METHODS

Prior to the commencement of the experiment breeding populations of guppies from a number of sites in Trinidad were established in St Andrews. All guppies used in the study were either wild caught individuals or were recently descended from wild caught fish. Populations from two rivers were selected for the experiment. In one population, the Lower Tacarigua, guppies co-exist with a range of larger piscivorous fish, including the pike cichlid, *Crenicichla alta*, which are known to be important predators of guppies. In the second site, the Tunapuna, the presence of a barrier (most likely a waterfall) has prevented the upstream dispersal of these larger fish. Some lesser predators, such as *Rivulus hartii* and *Synbranchus* spp. are still present, however the predation pressure is considerably reduced. The choice of two populations that had been exposed to differing predation regimes allowed subsequent comparisons of the importance of environmental factors in determining male courtship strategy.

Sixty four males (32 from each population) were randomly selected and equally divided between eight tanks (60 x 30 x 35 cm deep aquaria, temperature was maintained between 24.5°C and 25.5°C and illumination was provided by an 18W bulb on a 12 hour light / dark cycle). Before the commencement of observations drawings were made of the colour patterns of each male. The extreme polymorphism of male colouration allowed males to be individually identified. In addition the weight and total length of all males was recorded at this stage. To provide receptive females, new born fish were collected from each population and raised in separate tanks. Whenever any signs appeared that an individual was male, it was removed and returned to an adult stock tank. Individuals that showed no development of pigmentation or gonopodium growth were removed and raised as virgin females. These females were maintained under the same lighting and temperature conditions as the mixed sex tanks. Experimental non-receptive females were obtained as required from mixed sex stock tanks. The weight and total length of experimental females was recorded on the day of their use.

A further two aquaria (60 x 30 x 35 cm deep) were set up as observation tanks. Houde (1997) suggests that gravel is necessary for the expression of normal swimming and foraging behaviour, and that upon the omission of gravel 'fish tend to press against the bottom of the tank and swim in agitated manner'. This resting, motionless on the bottom, is similar to the fright behaviour of guppies in the wild (Seghers 1973). However the inclusion of gravel makes it more difficult to catch and remove fish without harming them and possibly causing the release of alarm pheromones. In addition, in this study it was necessary to ensure that the tank was free of any cues left by receptive females that may have influenced male behaviour in subsequent observations. It was therefore decided to leave the tanks empty except for an air supply and a small patch of Java moss, *Vesicularia dubyana*, which was washed between trials. To reduce the risk of the female failing to behave normally around 10 sexually immature juvenile 'dithers' were also placed in each observation tank (Barlow 1967). Personal observations and the results of this study suggest that these steps were sufficient to prevent the female from showing the fright behaviour described by Houde (1997).

All fish were fed to satiation with Tetramin™ approximately one hour before behavioural observations were carried out. Each observation period lasted 10 minutes. During this time an event recorder was used to record the following four prominent behaviours: Time spent by focal male following the female; Length of the focal male's sigmoid displays; Number of sigmoids performed by the focal male; Number of sneaky mating attempts performed by the focal male.

These behaviours were recorded for each of the 32 males under four conditions: (a) when the focal male was placed individually with a receptive female; (b) when the focal male was placed individually with a non-receptive female; (c) when the focal male and a rival male were placed with a receptive female and (d) when the focal male and a rival male were placed with a non-receptive female, i.e.:

Receptive female - One male
Non-receptive female - One male
Receptive female - Two males
Non-receptive female - Two males

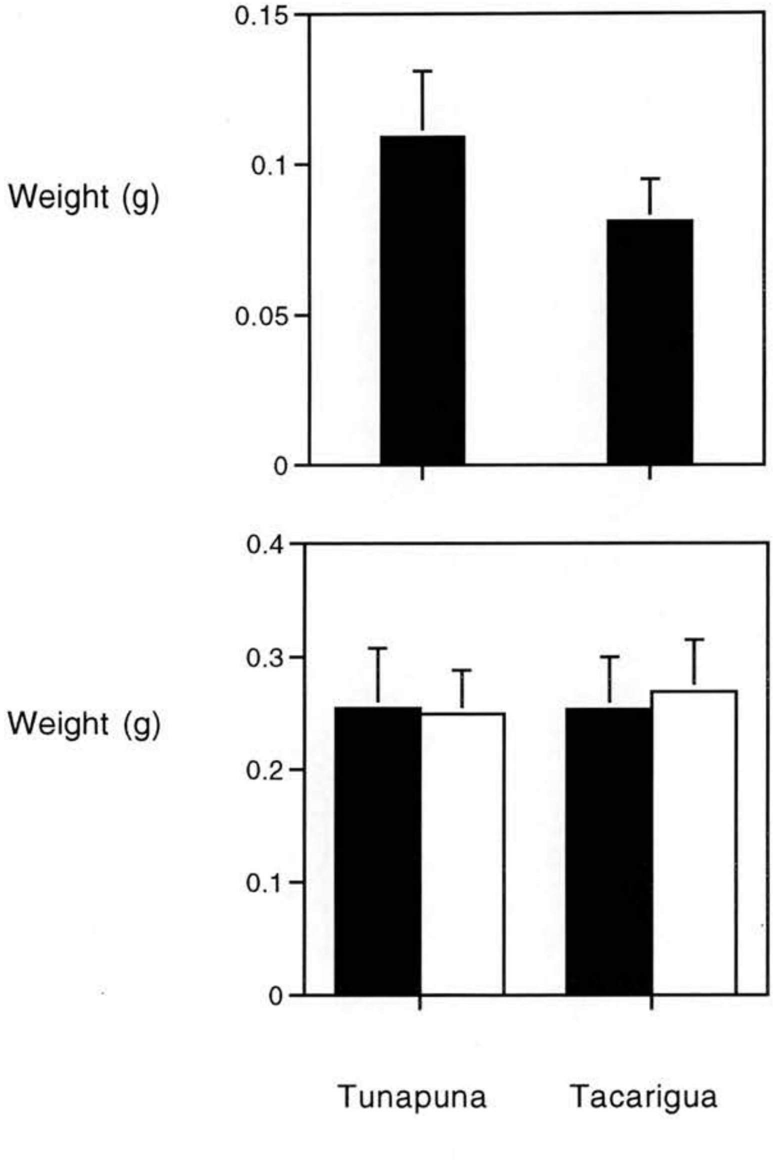
The female for each observation, plus the juveniles, were introduced in to the observation tank the evening before the observations. Males were introduced shortly before the observation period, which began when the male and female first interacted. After 10 minutes the males were removed. After a further 30 minutes a new male(s) were introduced. The order of presentation was randomised, however the same single female, receptive or non-receptive, was used on any one day. Each female was only used for one day, before being returned to a stock tank. The females used in this study were initially virgins, however data from Liley (1966) indicated that a virgin female's willingness to mate does not decrease until the third day after the first copulation. Receptive females could therefore be used for more than one observation, however to guarantee sexual responsiveness a new virgin female was used each day.

As mentioned in the introduction, males can determine female receptivity on the basis of the production of a pheromone. Males have been shown to increase their display rate in response to the presence of water that has housed a receptive female, even if the female herself is not present (Crow and Liley 1979; Meyer and Liley 1982). It was therefore decided that each evening the observation tank should be thoroughly cleaned and reassembled, before the introduction of the experimental female for the following day.

RESULTS

The mean weights of the males were not significantly different within either of the populations (Tunapuna $\bar{x} \pm$ S.D. = 0.109 g ± 0.022, $F_{3,28} = 0.888, p > 0.05$, Tacarigua 0.081 g ± 0.014, $F_{3,28} = 1.249, p > 0.05$). However Tacarigua males were on average significantly lighter than males from the Tunapuna ($t_{62} = 6.05, p < 0.0001$; figure 2.2). There was no significant difference in the wet weights of the four groups of females (Tunapuna: receptive ♀, $\bar{x} \pm$ S.D. = 0.254 g ± 0.054, non-receptive ♀, 0.249 g ± 0.039; Tacarigua: receptive ♀, 0.253 g ± 0.047, non-receptive ♀, 0.269 g ± 0.046, $F_{3,39} = 0.803, p > 0.05$; figure 2.1). As the behavioural data were non-normally distributed they were transformed, log (x + 1), prior to statistical analyses using a

Figure 2.2 Wet weight (g), with excess water removed, for n = 32 males from each population (above). Wet weight, again with excess water removed, for receptive (closed bars) and non-receptive (open bars) females (n = 14 for each group) (below). Data are given as means ± S.E.M.


0.15
0.1
0.05
0
Weight (g)
0.4
0.3
0.2
0.1
0
Weight (g)
Tunapuna
Tacarigua

three way ANOVA (Sokal and Rohlf 1995). All three of the primary factors investigated were found to have a significant effect on the amount of time that males spent following females (table 2.1, figure 2.3). Males spent significantly more time following the female when she was receptive ($F_{1,248} = 200.4$, $p < 0.0001$; table 2.1), or when the focal male was not having to compete against a second male for the female's attention ($F_{1,248} = 18.01$, $p < 0.0001$; table 2.1). Population also had a significant effect, males from the Tacarigua spending longer following the female than males from the Tunapuna ($F_{1,248} = 33.26$, $p < 0.0001$; table 2.1). As well as these three strongly significant findings, there were also significant differences in the way males from the two populations behaved towards females of different receptivity ($F_{1,248} = 7.61$, $p < 0.01$; table 2.1).

Table 2.1. Three-way ANOVA of male courtship behaviour, measured as time spent following the female. The factors are population, female receptivity and the number of males competing for matings.

Factor	df	Mean Square	F	*p*
Population	1	8101.24	33.26	< 0.0001
♀ Receptivity	1	48806.48	200.4	< 0.0001
Population x ♀ Receptivity	1	1852.39	7.61	0.006
♂ Number	1	4385.01	18.01	< 0.0001
Population x ♂ Number	1	252.99	1.04	0.309
♀ Receptivity x ♂ Number	1	616.68	2.53	0.113
Population x ♀ Receptivity x ♂ Number	1	1103.78	4.53	0.034
Residual	248	243.55		

As well as influencing the amount of time males spent chasing females, female receptivity also had a significant effect on the number of sigmoids that a male performed (table 2.2, figure 2.4). Overall males performed significantly more sigmoids towards receptive females ($F_{1,248} = 92.23$, $p < 0.0001$; table 2.2). The number of males competing for matings also had a significant effect. The number of

Figure 2.3 Mean time spent following either receptive females (closed bars) or non-receptive females (open bars) by a single male (above) and a focal male in the presence of a second rival male (below) from the Tunapuna and Tacarigua populations. n = 32 for each experimental group. Data are given as means ± S.E.M.

Figure 2.3

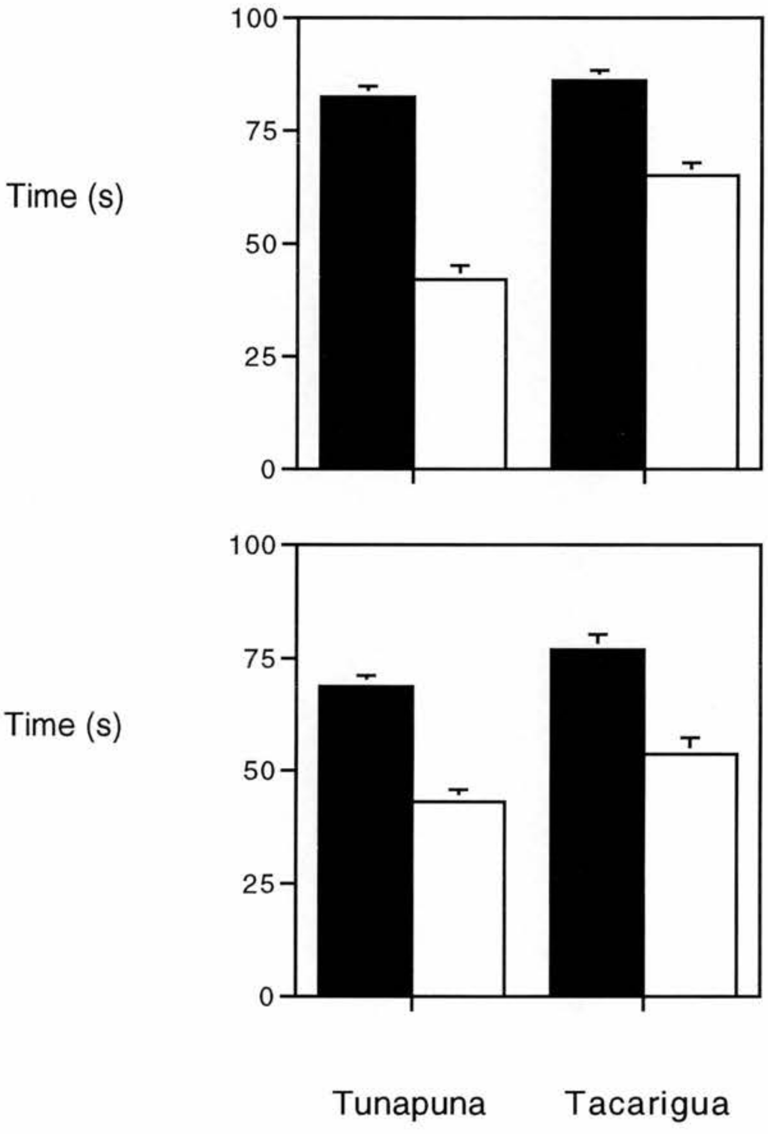
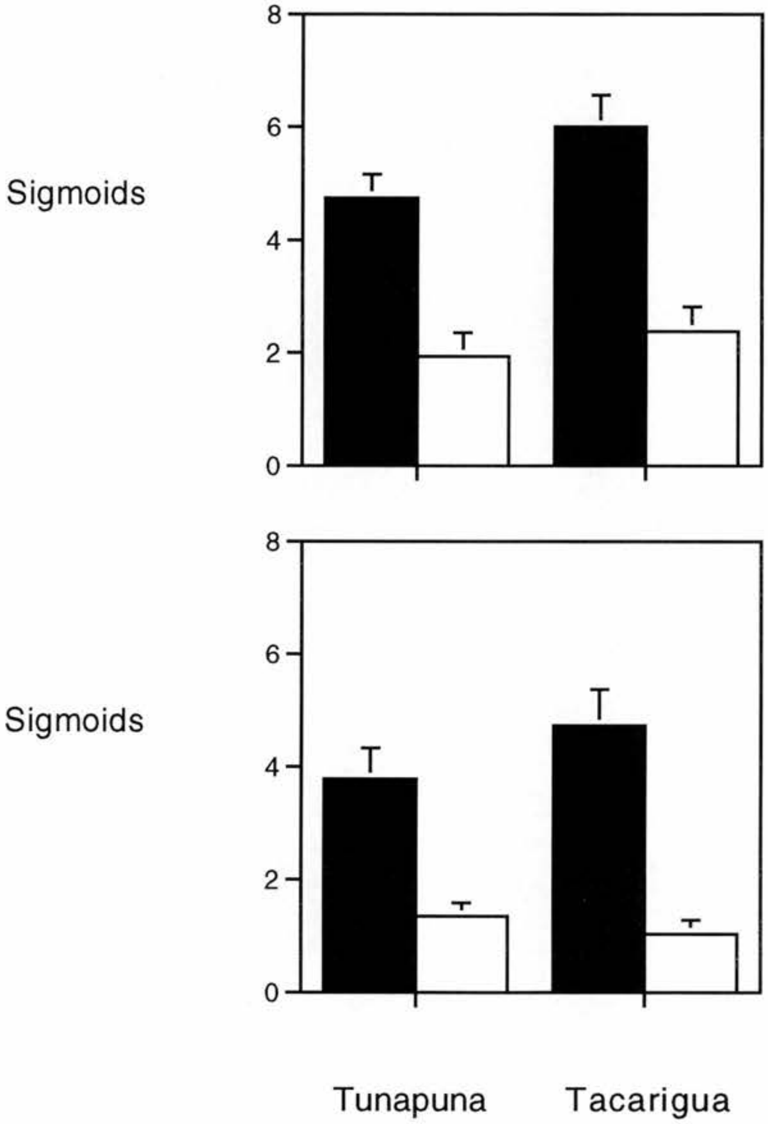


Figure 2.4 Number of sigmoids directed towards either receptive females (closed bars) or non-receptive females (open bars) by a single male (above) and a focal male in the presence of a second rival male (below) from the Tunapuna and Tacarigua populations. n = 32 for each experimental group. Data are given as means ± S.E.M.


Sigmoids
8
6
4
2
0
Sigmoids
8
6
4
2
0
Tunapuna
Tacarigua

sigmoids performed decreasing when a second male was introduced ($F_{1,248} = 10.25$, $p < 0.005$; table 2.2). Interestingly there was no significant difference between the populations in the number of sigmoids performed ($F_{1,248} = 3.13$, $p > 0.05$; table 2.2)

Table 2.2. Three-way ANOVA of male courtship behaviour, measured as number of sigmoids. The factors are population, female receptivity and the number of males competing for matings.

Factor	df.	Mean Square	F	p
Population	1	21.39	3.13	0.078
♀ Receptivity	1	631.27	92.23	< 0.0001
Population x ♀ Receptivity	1	17.02	2.49	0.116
♂ Number	1	70.14	10.25	0.002
Population x ♂ Number	1	4.52	0.66	0.417
♀ Receptivity x ♂ Number	1	0.39	0.06	0.811
Population x ♀ Receptivity x ♂ Number	1	0.77	0.11	0.738
Residual	248	6.85		

Not only did males perform more sigmoids when courting receptive females, these sigmoids were also more intense. Sigmoid length was significantly increased when courting a receptive female ($F_{1,248} = 283.1$, $p < 0.0001$; table 2.3, figure 2.5). While there was no difference between the populations in the number of male displays, the sigmoids performed by males from the Tunapuna were significantly longer ($F_{1,248} = 28.75$, $p < 0.0001$; table 2.3). Sigmoids also lasted longer when the sigmoiding male was not competing against a second male ($F_{1,248} = 82.84$, $p < 0.0001$; table 2.3). In addition there was a significant interaction between population and female receptivity, with males from the Tunapuna performing longer sigmoids to receptive females than males from the Tacarigua ($F_{1,248} = 17.19$, $p < 0.0001$; table 2.3).

Figure 2.5 Sigmoid length, a measure of sigmoid intensity, directed towards either receptive females (closed bars) or non-receptive females (open bars) by a single male (above) and a focal male in the presence of a second rival male (below) from the Tunapuna and Tacarigua populations. n = 32 for each experimental group. Data are given as means ± S.E.M.

Figure 2.5

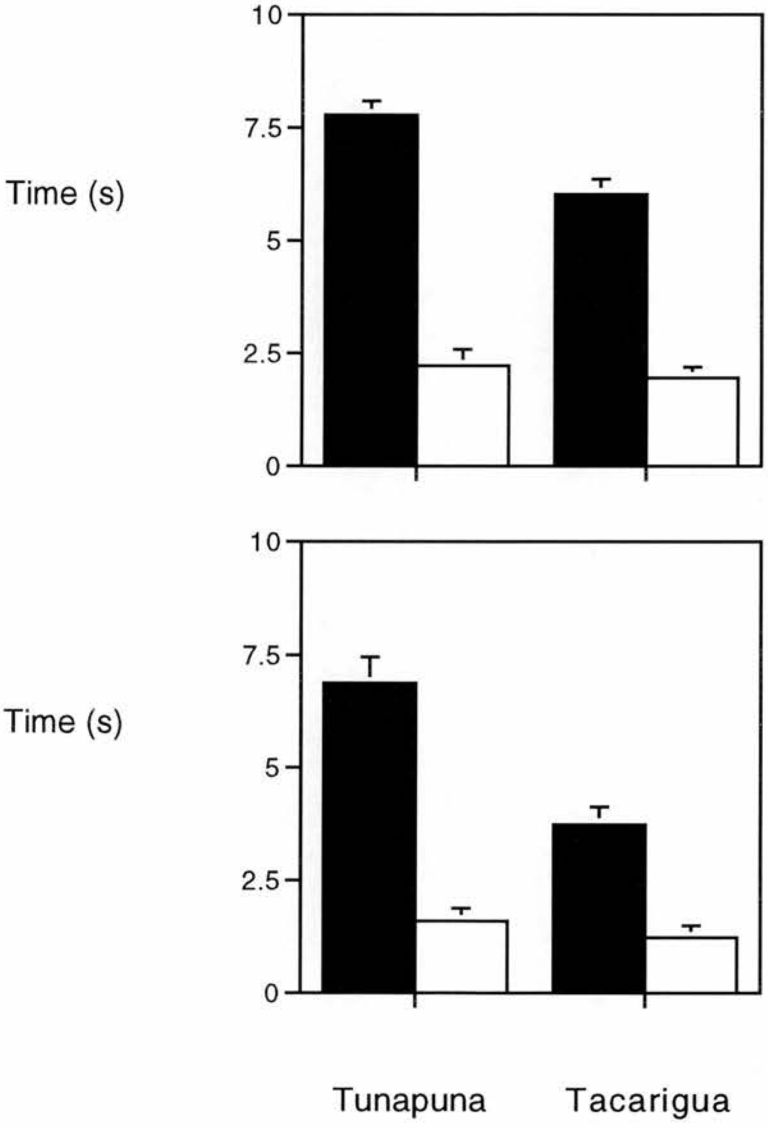


Table 2.3. Three-way ANOVA of male courtship behaviour, measured as mean sigmoid length. The factors are population, female receptivity and the number of males competing for matings.

Factor	df	Mean Square	F	P
Population	1	123.03	28.75	< 0.0001
♀ Receptivity	1	1211.26	283.1	< 0.0001
Population x ♀ Receptivity	1	73.54	17.19	< 0.0001
♂ Number	1	82.84	19.36	< 0.0001
Population x ♂ Number	1	8.71	2.04	0.155
♀ Receptivity x ♂ Number	1	13.66	3.19	0.075
Population x ♀ Receptivity x ♂ Number	1	6.64	1.55	0.214
Residual	248	4.28		

The number of sneaky matings attempted by a male was affected by several of the experimental factors. Surprisingly males from the Tunapuna never attempted sneaky mating with receptive females, and overall males from the Tacarigua performed significantly more gonopodial thrusts than males from the Tunapuna ($F_{1,248} = 149.9$, $p < 0.0001$; table 2.4, figure 2.6). Sneaky matings were also significantly more frequent when males were attempting to mate with a non-receptive female ($F_{1,248} = 66.91$, $p < 0.0001$; table 2.4). The introduction of a second male into the tank lead to an increase in the number of sneaky mating attempts ($F_{1,248} = 9.12$, $p < 0.005$; table 2.4). The interaction effect of population and receptivity revealed a significant difference in the way that males from the two populations responded to females of differing receptivity ($F_{1,248} = 4.82$, $p < 0.05$; table 2.4). Finally the interaction of all three experimental factors indicates that, while sneaky mating behaviour of males from the two populations is affected differently by changes in female receptivity, this behaviour is also sensitive to male – male competition ($F_{1,248} = 28.36$, $p < 0.0001$; table 2.4).

Figure 2.6 Number of thrusts directed towards either receptive females (closed bars) or non-receptive females (open bars) by a single male (above) and a focal male in the presence of a second rival male (below) from the Tunapuna and Tacarigua populations. n = 32 for each experimental group. Data are given as means ± S.E.M.

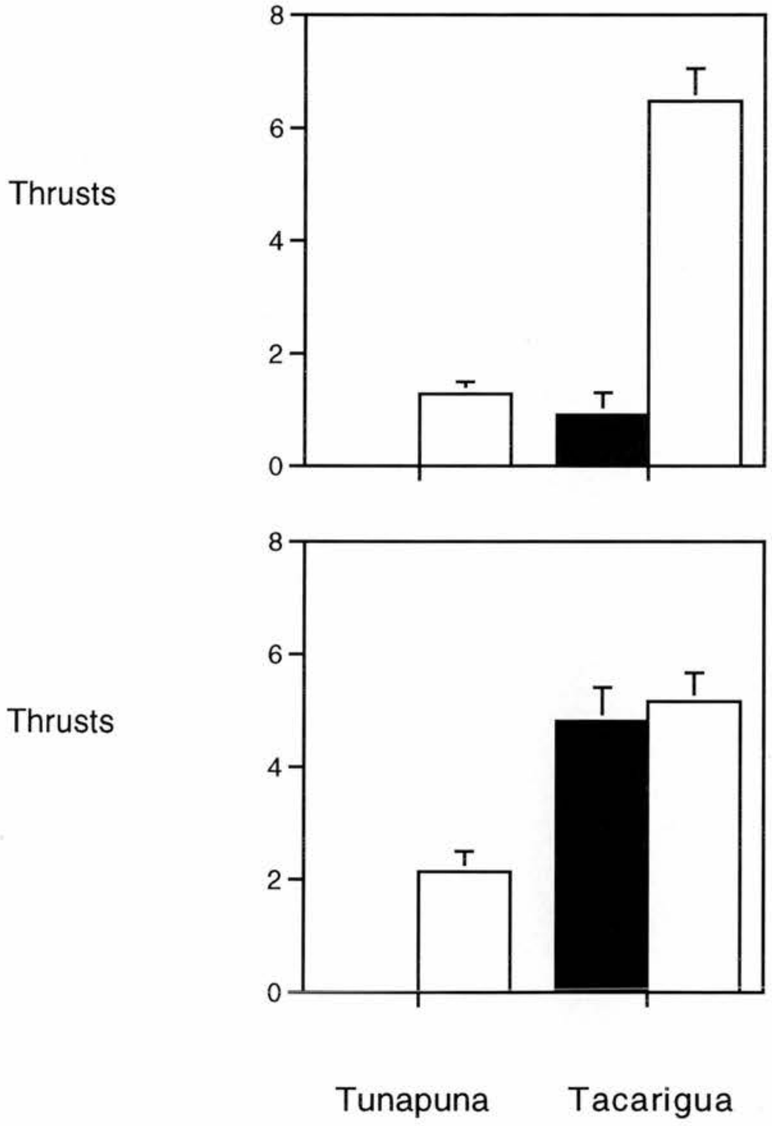

8
6
4
2
0
Thrusts
8
6
4
2
0
Thrusts
Tunapuna
Tacarigua

Table 2.4. Three-way ANOVA of male courtship behaviour, measured as number of gonopodial thrusts. The factors are population, female receptivity and the number of males competing for matings.

Factor	df	Mean Square	F	P
Population	1	777.02	149.9	< 0.0001
♀ Receptivity	1	346.89	66.91	< 0.0001
Population x ♀ Receptivity	1	25.00	4.82	0.029
♂ Number	1	47.27	9.12	0.003
Population x ♂ Number	1	12.25	2.36	0.126
♀ Receptivity x ♂ Number	1	76.56	14.77	0.0002
Population x ♀ Receptivity x ♂ Number	1	147.02	28.36	< 0.0001
Residual	248	5.18		

DISCUSSION

The results of this study indicate that all three of the factors investigated, individually and in combination, influence male courtship behaviour. Aside from the experimental variables, as many as possible of the factors mentioned in the introduction were standardized. Both the males and females used in the study were selected specifically to reduce any variation in a potentially confounding variable, body size. Further all observations were carried out under standard aquarium light levels which did not vary over the course of the study. While the possibility that the fish were infected with parasites was not investigated, there were no visible exoparasites and none of the guppies showed any obvious signs of ill health. Finally, to prevent males responding to any remaining pheromone that was left in the water, the tanks were cleaned between each trial.

As mentioned in the introduction, predation regime is one of the best studied selective forces influencing male courtship behaviour. The guppies of Trinidad have provided an ideal opportunity to study natural selection in the wild, and represent one of the finest examples of predator mediated variation in the literature. Predation pressure was first identified as an important selective force in Trinidadian guppies by Haskins *et al.* (1961). Seghers (1973) highlighted geographical variation in male behaviour and related this to the presence or absence of predators and recently Endler (1995) provided a definitive list of traits that have been found to covary adaptively with predation regime. The two populations used in this chapter were deliberately selected to allow a comparison between guppies from high (Tacarigua) and low (Tunapuna) predation sites.

Studies of male courtship behaviour of guppies from different Trinidadian populations have produced conflicting results. Several studies have reported higher display rates from sites where the guppy co-occurs with large predators (Farr 1975, 1976; Magurran and Seghers 1990a). However a second series of experiments produced exactly the opposite results, finding instead that male sigmoid rate was higher in low predation populations (Ballin 1973; cited Luyten and Liley 1985; Crow 1981; cited Luyten and Liley; Luyten and Liley 1985). Although population did not quite have a significant effect on the number of sigmoids a male performed ($F_{1,248} = 3.13$, $p = 0.078$), the results of this chapter provide further support for the view that sigmoids are more common in high predation sites. Farr (1975) suggested that predation by *R. hartii* may have been responsible for the observed low display rates in low predation sites. However several findings suggest that *Rivulus* is not an important predator of adult guppies. In a series of predation experiments, Liley and Seghers (1975) found that *Rivulus* were size selective predators, attacking only small males and juvenile guppies. Further, an analysis of the stomach contents of field collected *R. hartii* found only juveniles and immature males guppies (Liley and Seghers 1975). In combination, these studies provide persuasive evidence that *R. hartii* is unlikely to have had a major impact on the evolution of male courtship patterns in the guppy (Liley and Seghers 1975). It therefore seems unlikely that Farr's (1975) proposal, that the hunting styles of the various predators may account for differences in sigmoid rate, is correct. The suggestion of Magurran and Seghers'

(1990a), that the explanation may be a more complex combination of several ecological factors, is perhaps more plausible.

Whilst predation pressure did not affect sigmoids quantitatively, it did have a significant effect of the quality of the sigmoids. Males from the low predation Tunapuna, performed significantly longer sigmoids than their high predation Tacarigua counterparts. The intensity of male displays has received less attention than the number of sigmoids. The only previous study of the effect of predation regime on sigmoid length also found that sigmoids became shorter as predation risk increased (Farr 1975). This more subtle variation in courtship may be a further adaptive response to the higher risk of being attacked by a visually acute predator in the higher predation populations (Farr 1975; Endler 1995). Staying visible for longer periods may be more dangerous in high predation sites. Population also had a significant effect on the amount of gonopodial thrusting. The levels of sneaky mating found in this study were not quite as extreme as those reported in some other studies (Magurran and Seghers 1990a, 1994c). However all previous studies agree that males from high predation sites attempt sneaky mating more often than males from lower predation rivers (Farr 1975; Endler 1987; Luyten and Liley 1985, 1991; Magurran and Seghers 1990a), a finding further confirmed in this chapter. This correlation has now been established for males from several rivers in both the laboratory and field studies, and provides one of the best examples of heritable variation of behaviour in response to predation.

As well as influencing the relative proportions and intensities of the two mating strategies, the population from which the males were derived also had a significant effect on the amount of time males spent following females. This finding mirrors Magurran and Seghers' (1994c) observations of seven populations in the Caroni drainage. In the wild, female time budgets were found to be dominated by foraging, in contrast males spent much of their time chasing females (Magurran and Seghers 1994c). In both the previous study and this chapter, males from the higher predation population spent more time following females. This variation may reflect differences in the energetic requirements of males from the two sites. Males from high predation sites are typically smaller than upstream fish, and therefore have reduced energy needs (Liley and Seghers 1975). In addition upstream sites are more oligotrophic and have lower productivities. As a consequence of these sites being less profitable foraging areas, guppies must spend more of their time searching for food (Magurran and Seghers 1994c). An increase in time spent foraging, obviously leaves males with less time to chase females.

Although population had a strong effect, perhaps the most important factor in determining male courtship strategy was the receptivity of the female. The effects of variation in female receptivity have received far less attention than the effects of predation, however the issue has been addressed in both the guppy and other poeciliids. Perhaps one of the most thorough studies was carried out on a species closely related to the guppy, the sailfin molly, *Poecilia latipinna* (Farr and Travis 1986). Males of this species were found to dramatically increase their rate of sexual behaviour towards females immediately after they gave birth. Males also displayed higher rates of sexual behaviour towards virgin females than gravid females (Farr and Travis 1986; Sumner *et al.* 1994). Similarly the males in this chapter performed significantly more sigmoids to receptive than non-receptive females. The findings of Farr and Travis (1986) on *P. latipinna* and this chapter on *P. reticulata* differ from those of Liley (1966). This earlier study found that male display rate did not change with female receptivity. Instead males performed equal numbers of sigmoids to both receptive and non-receptive females, although there were differences in sigmoid length. Liley's (1966) suggestion that males should always perform sigmoids at a high rate is not supported by the findings of this chapter.

The method by which males can assess female receptivity in the sailfin molly has not yet been established. In the guppy however, females advertise their receptivity through the release of a pheromone from the reproductive tract. There is some confusion over the original function of this oestrogen like compound, and its classification as a pheromone is in doubt. Regardless of how males are able to assess females, there are obvious advantages to being able to distinguish receptive females. Receptive females are willing to cooperate in full matings, and presumably these matings are more likely to result in the successful transfer of sperm. Further as full matings involve a longer period of intromission it seems reasonable to propose that full matings may also allow the transfer of larger numbers of sperm and this may have implications for sperm competition (Parker 1990a).

As in Liley's (1966) study, the sigmoids that males performed to receptive females lasted longer than those performed to non-receptive females. There are two possible, but related, explanations. As mentioned above there are advantages to courting receptive females. It is therefore not surprising to find to find that males are willing to exhaust more energy performing longer sigmoids to more desirable females. Secondly, it is important to highlight the fact that females do not, as was once thought (Clark and Aronson 1951), play a passive role in courtship and mating. Liley (1966) provides detailed descriptions of the sexual responses of receptive females. These include a very characteristic, smooth 'gliding' manoeuvre, which it has been suggested signals the female's interest in the male. Although no full copulations were observed during this study, several females were seen responding to males. The increase in sigmoid length is most likely a response to some evidence of female interest in mating, and represents the beginning of an escalation in courtship, potentially leading up to mating.

When attempting to mate a non-receptive female, males show a dramatic switch in their courtship behaviour. As females do not mate outwith their period of receptivity, there is little point in performing energetically expensive sigmoids to non-receptive females. Godin (1995) pointed out that the decrease in sigmoid rate may be an indirect response to the reduction in female interest in the male. The increase in the number of thrusts may therefore be a response to the decrease in the chances of sigmoids eliciting a response from a non-receptive female (Godin and Briggs 1996). Early in trials with non-receptive females, males occasionally performed short 'exploratory' sigmoids, which are thought to test female responsiveness (Liley

1966). However when females were non-receptive there was a significant increase in the amount of gonopodial thrusting.

Male–male competition also had a significant effect on courtship behaviour. In the absence of competitors, males spent significantly longer following the female, performed more sigmoids and these sigmoids were longer. Whereas a single male was able to monopolise the female and direct all his attention to mating attempts, the introduction of a second male lead to intra-sexual competition. Direct male-male aggression is rare in field studies and even in laboratory experiments is only found under certain conditions (Kodric-Brown 1992, 1993; Bruce and White 1995). Aggression between males was observed, although not recorded, during this study. None of the encounters involved pronounced male aggression, and the most violent activity was occasional nipping at the gonopodium of the rival. However, although overt aggression is rare, males do appear to compete for position near the female, described by Houde (1988b, 1997) as 'jockeying for position'. Importantly, this may lead to variation in ability to successfully transfer sperm.

The decrease in time spent following the female was accounted for by an increase in the amount of time that males spent on other behaviours when unable to outcompete the rival male and gain position for mating. Sigmoid number and length were also reduced by the introduction of a second male. In contrast, Farr and Herrnkind (1974) observed various densities of male guppies to determine the effect of population density on male courtship behaviour. Single males were found to court at low levels, and the introduction of a second male lead to a significant increase in courtship activity. In this chapter the opposite trend was found. It is not known whether changes in courtship activity were due to the presence of the rival male or were mediated by changes in the behaviour of the female in response to increased male density.

In light of the differences between studies it is difficult to draw clear conclusions about male-male competition in guppies. These differences may be due to the fact that dominance relationships could be quickly established between two males, and prior interactions could have subtle effects on the ways males interact and on their mating success (Kodric-Brown 1992, 1993). Farr (1980a) demonstrated that, in his experimental pairings, the male performing the greater number of sigmoids obtained more matings. The mating success of this male was reduced if the other male performed a high rate of gonopodial thrusts, but not if the second male was more aggressive. These results suggest that being able to monopolise females is a key factor both in this and in earlier experiments. Interestingly there was a significant increase in the amount of sneaky mating that occurred when a second male was introduced. It has been shown, both theoretically and experimentally, that males increase the frequency with which they thrust when the female is distracted by a predator (Magurran and Novak 1991). A similar situation may occur when the female is being harassed by more than one male, and presumably she is less able to avoid the thrusts of the second male.

The importance of male-male interactions varies across different laboratory studies. Although Gorlick (1976), Farr (1980a) and Kodric-Brown (1993) reported the existence of dominance hierarchies, Houde (1988b) found little evidence of male-male competition. This chapter demonstrates that the number of males competing for the female does significantly affect male sexual behaviour, and suggests that male-male competition may play an important role in determining male choice of mating strategy. However, additional detailed observations are needed to better understand the role of interference competition between males in determining the distribution of matings between males.

In conclusion gonopodial thrusting is a commonly used mating tactic in the Trinidadian guppy. In contrast to some other fish species, in which alternative mating strategies involve separate developmental pathways and distinct morphologies, all male guppies perform sigmoid displays and attempt sneaky mating. Males can switch between strategies at will, and sneaky mating represents a facultative opportunistic behaviour. Male courtship is extremely sensitive to a number of environmental variables. Population and female receptivity appear to be particularly important, although male sexual behaviour appears to be sensitive to a range of more subtle and complex interactions between factors. Changes in these factors lead to an increase in the frequency with which sneaky mating is undertaken, and it was found that male guppies rapidly changed to gonopodial thrusting in several situations. The manipulation of experimental variables provides a potential mechanism for constraining the behaviour of males. A knowledge of the factors influencing male courtship may prove useful in studies wishing to ensure that males do not perform sigmoids. Sneaky mating in the guppy is therefore an important and integral part of male courtship behaviour and occurs at a frequency that highlights its potential role in the biology of the species.

Chapter 3

Male behaviour and sperm number : The phenotype-linked fertility insurance hypothesis.

INTRODUCTION

The benefits of mate choice.

The potential benefits that females might obtain, as a result of mate choice has become an area of increasing research in many species, including the guppy, *P. reticulata* (Houde 1997). Sexual selection theory has suggested two routes through which selection can influence the evolution of mate preferences. The two modes of selection, direct and indirect selection, are defined by the type of benefits that females stand to gain from a mating. Direct selection, can occur when the benefits or costs of a mating have a direct influence on the fitness of the female. For example the better a male is at providing food and guarding against predators the better his family's chances of survival (Davies 1992; Gwynne 1984a; Nisbett 1973). Direct benefits of mate choice may also include access to higher quality territory and the avoidance of infertile males (Gibson and Jewell 1982; Parker 1970a). In the guppy most of these direct benefits seem unlikely. Males do not control resources or oviposition sites nor do they provide any parental care. It is also unlikely, due to its small volume that the ejaculate acts as a nutritional contribution.

The provisioning of resources controlled by the male, time constraints and risks of mate choice associated with predation or parasites could also influence mate choice. In the guppy the costs of mating may involve a decrease in time available for foraging and an increase in the risk of predation (Magurran and Seghers 1994c). Examples of costs of mate choice that have been demonstrated in other species that may be of importance in guppies include an increase in the risk of parasite transmission (Rosenqvist and Johansson 1995) and the energetic costs of moving between males (Milinski and Bakker 1992). Several field studies have shown that the common guppy exoparasite, *Gyrodactylus turnbulli*, is prevalent in Trinidadian river systems. Males that are infected have less bright orange spots than their non-infected male siblings (Houde and Torio 1992). In dichotomous choice experiments females prefer the brighter, non-infected male. It is possible that the colour orange could be used by females as an indication of a male's recent history of parasite infection. Females that avoid less colourful males will therefore reduce their risk of becoming infected (Houde and Torio 1992).

In addition to studies of the direct benefits or costs incurred by the female, the guppy system offers one of the best opportunities for studying hypotheses concerning indirect selection. The question under investigation is how do female preferences evolve, if the preferences do not result in variation in female fecundity? A combination of several factors, including the ease with which they can be bred in captivity and female choice for heritable male colour patterns, has led to *Poecilia reticulata* becoming one of the key organisms in studies of indirect selection. Two main theories have been proposed. Mating preferences may arise, under indirect selection, firstly as a result of variation in heritable components of offspring survival and subsequent fitness ('good genes' models) or as a result of effects influencing the mating success of sons ('Fisherian' models). Much of the debate concerning indirect sexual selection has contrasted Fisher's (1930) model with the models associated with the good genes hypothesis, however no final resolution has yet been reached.

Fisher's (1930) model has been modelled mathematically by Lande (1981) and Kirkpatrick (1982). One of the predictions of Fisherian models is that a genetic co-variance will exist between male trait and female preference. Recent research has provided good evidence that such a co-evolution of trait and preference exists in the guppy. As mentioned earlier female mating preferences for male coloration vary geographically across populations in Trinidad (Houde 1988a). Houde and Endler (1990) showed that for seven of these populations the strength of a female's preference for orange spots was correlated with the amount of expression of that trait by males from her native population. These differences in preferences persisted after several generations in captivity, suggesting firstly that male trait and female preference have a genetic basis and secondly that they have diverged in parallel among populations. Unfortunately neither genetic variation in trait or preference, nor a genetic correlation between them provides conclusive support for Fisherian models. Such results are also predicted by other mechanisms. For example, habitat differences that favour transmission of different portions of the light spectra may also lead to the same outcome (Houde 1993).

An alternative to Fisher's runaway selection was proposed by Zahavi (1975). This second form of indirect benefit proposed that females prefer to mate with males that have demonstrated their superior genetic quality for survivorship. According to Zahavi (1975) females can ascertain this quality by assessing the magnitude of the handicap with which he is able to survive. Again the hypothesis relies on a genetic correlation, this time between the female preference and the male's good genes (i.e. some heritable component for increased viability). Evidence of females selecting males for their 'good genes' has been collected in many studies, the majority of which have involved birds. Possibly the finest of these is Petrie's (1994) study of the Peacock *Pavo cristatus*. Several previous studies had failed to account for females having access to more attractive males. These females may have increased the maternal contribution to the egg, resulting in an increase in viability, and confounding any paternal effects. Petrie (1994) controlled for maternal effects and instead of allowing females to chose males, mates were randomly assigned. In addition all offspring were raised under identical conditions. Male mating success has been shown to be correlated with the number of eye spots on the tail (Petrie *et al.* 1991), but in this later study (Petrie 1994) a related measurement, the mean area of the father's eye spots, was used as an index of male attractiveness. This value was found to be significantly correlated with offspring size at 84 days and survivorship at 24 months. In the guppy, similar evidence for the good genes hypothesis is provided from studies of the effect of the monogenean parasite *G. turnbulli*. Females prefer brighter, less parasitized, males, and as parasite resistance is heritable (Madhavi and Anderson 1985), females may be selecting mates to decrease the chances of their offspring being infected (Houde 1997).

A female preference for males that survive despite a costly handicap or display does not in itself provide conclusive support for the 'good genes' model of sexual selection. Most studies have failed to show that the condition dependence of the male trait involves additive genetic variation in traits affecting the survivorship of the offspring. The ultimate evidence needed is a demonstration of a genetic correlation between variation in the male character and some aspect of offspring fitness (Boake 1985). Reynolds and Gross' (1992) laboratory study using guppies produced evidence of such a relationship between a female preference for male body size. The results indicated that offspring of larger males did have significantly faster growth rates than young fathered by small males. In addition female offspring of larger males had a greater reproductive output. These results are consistent with the predictions of adaptive mate choice, however they have received criticism. The choice of body size as a male trait has caused some confusion. As well as being a trait preferred by females, this trait is also an important life history variable and correlations between size, growth rate and fecundity are not unexpected (Houde 1997). Reynolds and Gross' findings can therefore not be confirmed until it has been demonstrated conclusively that large body size does lead to increased offspring fitness. Further work is needed to investigate the importance of these factors in the evolution of adaptive mate choice and to estimate the relative importance of each of the hypotheses. At present however, 'good genes' models of indirect selection remain controversial. Strong directional selection resulting from mate choice will deplete genetic variation in those male traits preferred by females. Therefore in populations that are in equilibrium, fitness should not be heritable (Fisher 1958). The fitness of a female's offspring will be independent of parental genotype unless some other mechanism maintains additive genetic variance for fitness in the population. Several mechanisms have been proposed that might allow genetic variance (Charlesworth 1987; Stearns 1992), however it has been argued that the benefits that females stand to gain by selecting males on the basis of this variation is minimal (Williams 1992). Further, even though traits associated with fitness may be heritable, negative associations between life history traits means that overall fitness itself may not be heritable (Charlesworth 1987).

The phenotype-linked fertility insurance hypothesis.

In order to highlight that some of the research claiming to provide support for indirect benefits of mate choice may not be conclusive, Sheldon (1994) put forward an alternative hypothesis capable of explaining these findings. Several studies of birds had reported a correlation between a male trait and the male's success at obtaining extra-pair copulations or extra pair fertilizations. These studies had suggested that their results provided evidence that females are co-operating in these extrapair matings in order to gain, in one of the previously described ways, indirect benefits for their offspring (Kempenaers *et al.* 1992; Graves *et al.* 1993). Sheldon's (1994) alternative hypothesis instead suggested that females seek extra-pair copulations as an insurance against the functional infertility of their mates. The phenotype-linked fertility insurance hypothesis proposes that functional fertility covaries with male phenotype. If this were the case then females would obtain direct fertility benefits by mating with attractive males. The hypothesis predicts that sperm from an attractive male is either more viable or more abundant, and therefore more likely to fertilize a female's eggs than sperm from less attractive males. This means that females that choose mates on the basis of key phenotypic characteristics gain information about their viability as potential fathers (Sheldon 1994). As well as providing an equally good explanation of the results of existing studies, the hypothesis does not require the maintenance of genetic variation for fitness, it is therefore a more parsimonious explanation than those based on genetic benefits.

Several studies in a range of species have helped to elucidate the cues used by females in the selection of extra-pair males. Houtman (1992) showed that female zebra finches prefer males with redder bills and more complex songs than their partner. In the swallow, females are more likely to seek extra-pair matings from males with well developed tail feathers (Møller 1988a), while in the house sparrow females select on the basis of throat badge size (Møller 1988b, 1992a). These, and several other studies, have shown that a correlation exists between some aspect of male phenotype and male success at obtaining extra pair matings. It has also been demonstrated, through DNA fingerprinting, that male swallows with long tail feathers are less likely to be cuckolded (Møller 1994). However Sheldon (1994) suggests that the results of such studies can be explained by the phenotype-linked fertility insurance hypothesis. If variation exists between males in the likelihood of their sperm fertilizing a female's eggs, then there will be strong selection for females to select males that are producing large numbers of functionally fertile sperm. If functional fertility and male phenotype are correlated then the opportunity exists for females to select males with the largest trait and gain, in terms of functionally fertile sperm, direct benefits.

However empirical evidence for the phenotype-linked fertility insurance hypothesis is at present scarce. If measuring male fertility in wild populations is difficult (Sheldon 1994), then functional fertility is even more complex to determine. The production of a large number of sperm (i.e. high fertility) does not guarantee high functional fertility. High numbers of abnormal or damaged sperm or high mortality of sperm in the female reproductive tract may lead to a reduction in functional fertility (Austin 1975). The number of sperm that a male produces that actually survive and are capable of competing for fertilizations (his functional fertility) is for obvious reasons difficult to establish. In birds it has been shown that the proportion of fertilized eggs that a female produces is correlated with the number of sperm with which she was inseminated (Taneja and Gowe 1961). A similar situation, insufficient sperm leading to decreased brood size, has been documented in poeciliids (Vallowe 1953). In order to avoid this, it may pay females to select males with high functional fertility. Only a few studies have attempted to directly measure ejaculate characteristics and relate them to male phenotype.

Research by Mjelstad (1991) found that male capercaillie (*Tetrao urogallus*) with the highest display rates had the highest quality ejaculates. Further support was provided in a study by Nitchuk and Evans (1978) which showed that male sharp-tailed grouse, (*Tympanuchus phasinellus*), with central lek positions had higher testicular sperm counts. Both these papers found a correlation between a male trait and ejaculate characteristics. It is important to point out that in contrast to the studies providing support for the phenotype-linked fertility insurance hypothesis, studies of several species suggest that there is little evidence of a positive relationship between ejaculate features and phenotype. In the peafowl, *Pavo cristatus*, females preferentially mate with males with more elaborate trains, but Birkhead and Petrie (1995) failed to find any association between tail length and ejaculate quality. Møller (1994) found no relationship between tail length and fertility in barn swallows. In both the jungle fowl, *Gallus gallus spadiceus*, and the domestic fowl, *Gallus gallus domesticus*, females prefer males with large combs (Von Schantz *et al.*. 1995; Zuk *et al.* 1990). However in the domestic fowl testes size and comb size are negatively correlated (Von Schantz *et al.* 1995). Female sedge warblers, *Acrocephalus schoenobaenus*, preferentially pair with males with large element repertoires (Catchpole 1980; Catchpole *et al.* 1984), however neither sperm number in the seminal glomerula nor the proportion of morphologically abnormal sperm covaried with song repertoire size (Birkhead *et al.* 1997).

Recently Birkhead and Fletcher (1995) investigated male phenotype and ejaculate quality in the zebra finch. Two traits used by female zebra finches during mate choice, song rate (ten Cate 1984) and symmetry of chest band badges (Swaddle and Cuthill 1994) were studied. However it was found that these two variables were not correlated with sperm length, swimming velocity, number of sperm per ejaculate or percentage of live sperm (Birkhead and Fletcher 1995). It has been suggested that the use of captive birds may have influenced the findings of this study. Zebra finches have been the subject of intense captive breeding and artificial selection (Zann 1996), and this may have reduced the variance in male phenotypic traits and decreased the likelihood of a positive correlation (Birkhead and Fletcher 1995).

Further, Birkhead *et al.* (1997) suggested that while there is no convincing evidence for this hypothesis in any captive bird populations, it may be worth repeating the studies using wild populations (Birkhead and Fletcher 1995). In addition Roldan *et al.* (1998) have suggested that studies of birds have failed to provide support because females are assessing males on a number of other criteria such as ability as care givers. The idea that females chose males for direct benefits in fertility therefore remains highly controversial.

This chapter tests Sheldon's (1994) phenotype linked fertility insurance hypothesis by investigating the possibility that the male guppy's behaviour indicates ejaculate characteristics. Previous work (Reynolds and Gross 1992) has demonstrated that mate choice also enables female guppies to obtain heritable benefits ('good genes') for their offspring. It is not yet known whether males also signal their functional fertility, that is their viability as fathers, to female guppies. This chapter tests the hypothesis that a male guppy's behaviour reveals characteristics of his ejaculate (i.e. that phenotype is linked to fertility). The guppy seems an ideal species in which to test such hypotheses. Some of the most compelling evidence for female choice has been provided by studies of the guppy. Females are fertile for restricted periods (Liley 1966) during which they show preferences for males on the basis of a range of traits. Evidence exists to suggest that colour patterns (Houde 1987), morphology (Endler 1995) and courtship rate (Farr 1980b; Nicoletto 1993) are all implicated in female choice. It is not yet known if female choice selects for males of greater fertility. During this experiment I focused on the courtship behaviour of the male guppy, selecting the rate of mating attempts as the phenotypic variable.

METHODS

The study populations.

Male guppies used in this experiment were descendants of wild-caught stocks from the Upper Tunapuna and Lower Tacarigua. As described in earlier chapters, the Upper Tunapuna is a low predation river where guppies co-exist with *Rivulus hartii*, a minor predator, while the Lower Tacarigua is a dangerous locality for guppies as a variety of predators, including the pike cichlid, *Crenicichla alta*, are found there. The female guppies in this experiment were also descended from Trinidad populations.

Mating behaviour

Male mating behaviour was observed over a period of six weeks. The same five females, which were mixed-strain stock to exclude the possibility of biasing the behaviour of males from either population, were used throughout the trials. Observations were made in a 59 x 29 x 35 cm deep aquarium with water 27cm deep. The aquarium contained a small clump (6 cm high) of Java moss, *Vesicularia dubyana.* Temperature was maintained between 24.5°C and 25.5°C and illumination was provided by an 18W bulb on a 12 hour light/ dark cycle. The fish were fed to satiation with Tetramin ™ before behavioural observations were carried out.

In each trial, five males from one population were introduced to a tank containing the five females. The guppies were allowed to settle overnight before observations commenced. The number of sigmoid displays and sneaky matings performed by each male was recorded over a 15 minute period. This was repeated five times for each male. A male was judged to have successfully delivered a thrust to a female if his gonopodium made contact with a female's genital region. A total of eighty different males, forty from each population, were observed.

Ejaculate features

Each of the eighty males was then isolated overnight prior to analysis of his ejaculate features. The male was humanely killed by placing in ice cold water, and then placed on slightly damp laboratory tissue and any excess water removed from the body surface. This excess water was removed to prevent any unaccountable dilution of the ejaculate. The fish was transferred to a petri dish under a low power dissection microscope.

The gonopodium was swung forward and gentle pressure applied to the side of the abdomen, at the base of the gonopodium. This action released the ejaculate, in the form of a number of spermatozeugmata, which were then stroked down the gonopodium and onto the petri dish. It appears that some form of valve exists that prevents sperm being discharged when the gonopodium is not swung forward, however only after several frustrating attempts was the importance of moving the gonopodium discovered. Sperm cannot be released from a male whose gonopodium is held against the body. This stripping process was repeated three times for each fish to ensure that all available sperm had been removed. Occasionally some of the spermatozeugmata would become attached to the surface of the male and these were removed with forceps and added to the stripped ejaculate.

Once the ejaculate had been gathered, capillary tubes were used to measure its volume. This was probably the most awkward step of the protocol and may be subject to future modification. The main problem is the extremely small volumes that are involved. Ejaculates tended to be in the order of 1 - 2 μl, however the smallest recorded ejaculate volume was only 0.5 μl. Whilst it is possible to measure such volumes, there were additional problems due to the viscosity of the ejaculate. It was not always possible to achieve complete uptake of the sample, and there was always some ejaculate left on the petri dish. In an attempt to minimize any variables, I used the same petri dish and make of capillary tube throughout, and attempted to treat all samples in as similar a way as possible. Other methods of measuring ejaculate volume were investigated, including weighing the samples and measuring the area of the ejaculate between glass slides, however they all proved impractical and this method was selected as the most reliable.

After the volume had been measured, the ejaculate was expelled back onto the petri dish. Again there may have been a loss of sample, with some sperm remaining in the tube. However all samples were treated in this way, in an effort to standardize any error. A known volume of distilled water was then added and the spermatozeugmata were left to break down for 20 minutes. Other studies have used teleost ringer solution, however this appeared to cause problems with the salts crystallising and preventing counting. In order to aid the break down of sperm bundles and to distribute sperm evenly, the sample was repeatedly drawn up and expelled from a Gilson pipette.

Sperm were then counted twice for each male using an 'improved Neubauer chamber' haemocytometer. Number of sperm in each stripped ejaculate was determined by multiplying mean sperm count by the sample's dilution factor and initial ejaculate volume. Mean sperm lengths for each male were calculated as an average of 15 individual sperm lengths. The wet weight, wet weight minus gut, total and standard length and gonopodium length of each male was recorded. The testes were then removed and dried (24 hours at 60°C) along with the male's body to give dry weight and testes dry weight for each fish. Gonosomatic index was determined by calculating dry testes mass as a percentage of dry body mass (de Vlaming *et al.*. 1982).

RESULTS

Fish showed consistent courtship behaviour over the five 15 min observations (Kendall Coefficient for Tunapuna thrusts, W = 0.56, X^2 = 108.4, p < 0.001 and sigmoids, W = 0.726, X^2 = 141.57, p < 0.001; Tacarigua thrusts, W = 0.658, X^2 = 128.3, p < 0.001 and sigmoids, W = 0.776, X^2 = 151.3, p < 0.001). Given this consistency the median (out of the five) thrust and sigmoid frequencies was selected for each fish, and used in all subsequent statistical analyses.

Tacarigua males attempted significantly more sneaky matings per unit time than Tunapuna males (t_{78} = 2.0, p = 0.048; figure 3.1). The number of sigmoids performed did not significantly differ between the populations (t_{78} = 0.02, p = 0.98; figure 3.1). The number of gonopodial thrusts performed per unit time was positively correlated with the number of sperm per ejaculate in Tunapuna (r = 0.34, n = 40, $p <$ 0.05; figure 3.2) and Tacarigua (r = 0.59, n = 40, $p <$ 0.01; figure 3.2) males. Both populations also showed a highly significant positive correlation between sigmoid rate and numbers of sperm per ejaculate (Tunapuna r = 0.73, n = 40, $p <$ 0.01; Tacarigua r = 0.61, n = 40, $p <$ 0.01; figure 3.2). Number of thrusts and sigmoids correlated with mean sperm length in Tunapuna males (r = 0.35 and 0.40, n = 40, p < 0.05) but not in Tacarigua males (r = 0.29 and 0.26, n = 40, $p >$ 0.05). Interestingly sperm numbers per ejaculate correlated with mean sperm lengths in both Tunapuna (r = 0.49, n = 40, $p <$ 0.05; figure 3.3) and Tacarigua males (r = 0.52, n = 40, $p <$ 0.01; figure 3.3). There was no correlation between the GSI and either thrusts (r = 0.17, n = 40, $p >$ 0.05) or sigmoids (r = 0.003, n = 40, $p >$ 0.05) in Tunapuna males, but there were correlations between GSI and both thrusts and sigmoids in Tacarigua males (respective r values = 0.38 and 0.49, n = 40, $p <$ 0.05). Reynolds *et al.* (1993), found a significant correlation between gonopodium length and number of thrusts, however this was not found to be the case in either of the populations used in this study (Tunapuna r = 0.163, n = 40, $p >$ 0.05; Tacarigua r = 0.031, n = 40, $p >$ 0.05). Males from Tacarigua showed a positive correlation between number of sigmoids and number of thrusts (r = 0.416, n = 40, $p <$ 0.05), but no correlation was found for

Figure 3.1 Sigmoid rate (above) and thrust (below) per 15 min for n = 40 males from each population. Error bars indicate the 95% confidence interval.

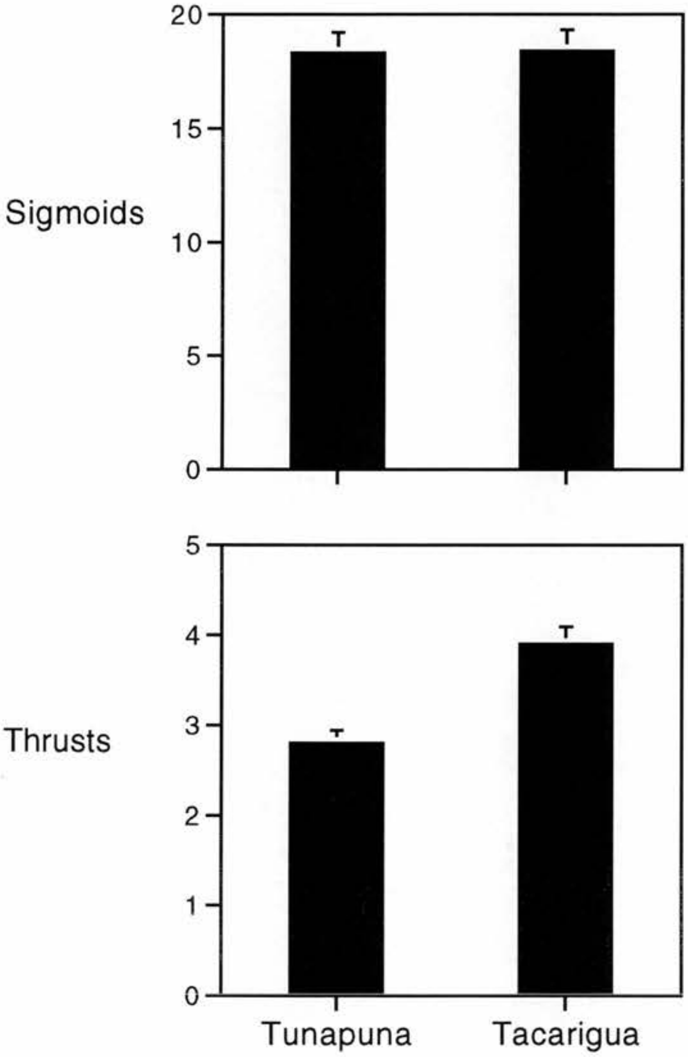

20
15
10
5
0
Sigmoids
5
4
3
2
1
0
Thrusts
Tunapuna
Tacarigua

Figure 3.2 The relationship between sperm numbers per ejaculate and sigmoid and thrust rate for Tacarigua (open circles) and Tunapuna (closed circles) males. Sperm numbers correlate with both sigmoids and thrusts for both populations. * = $p < 0.05$; ** = $p < 0.01$. Significance levels were calculated using a Bonferroni correction, see text for details.

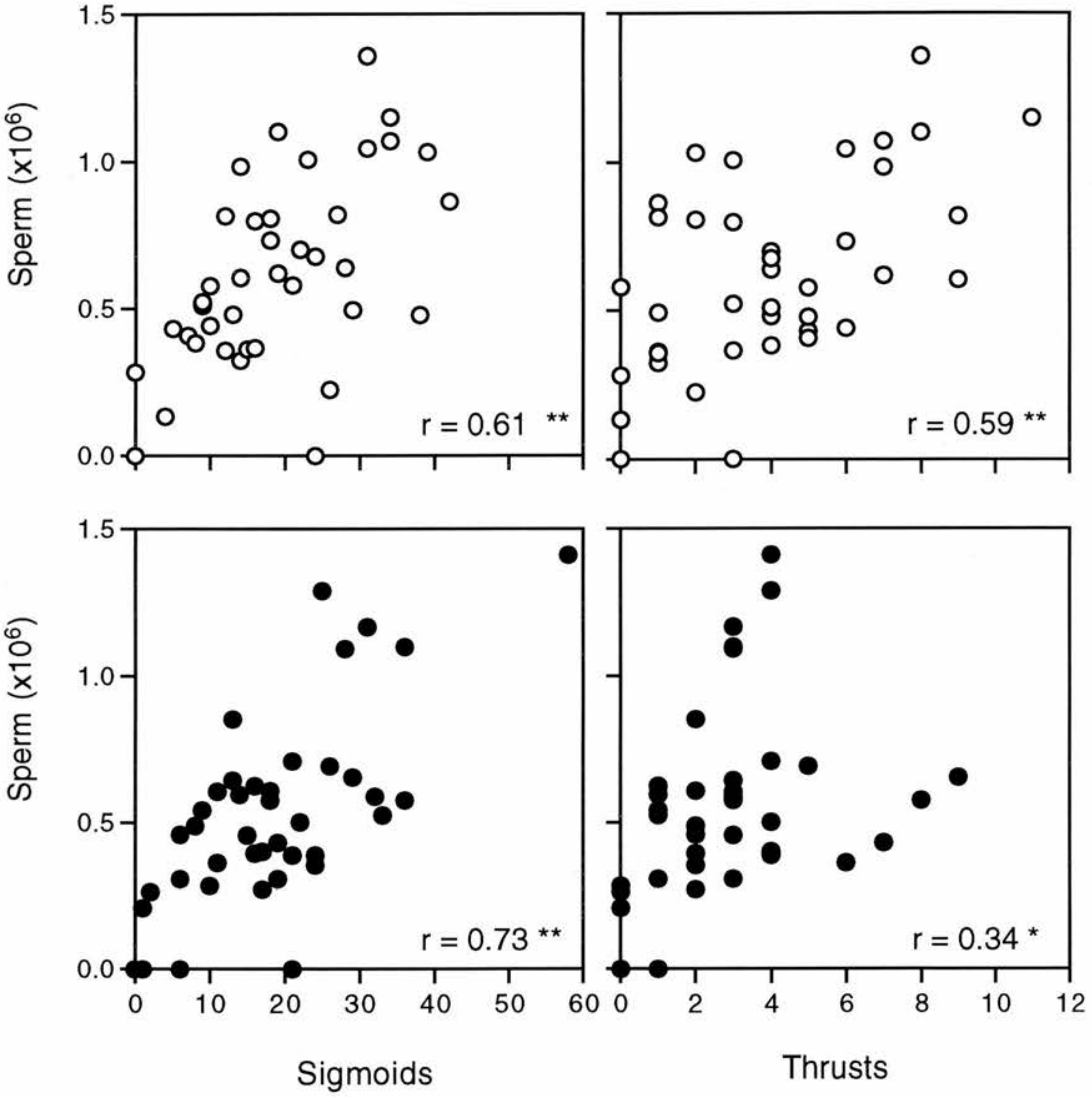
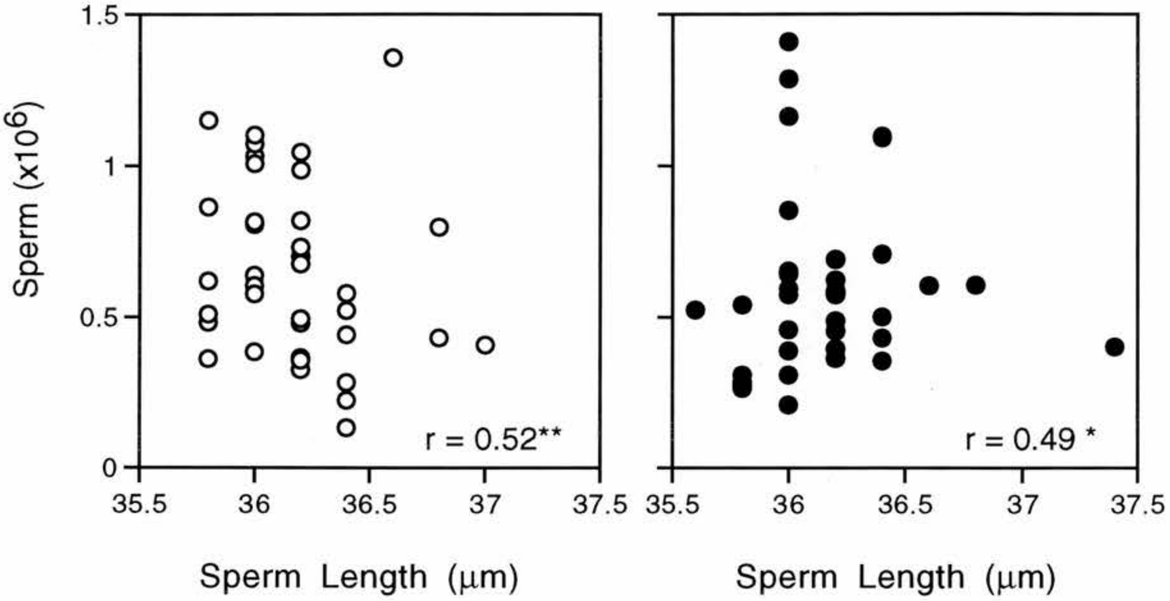
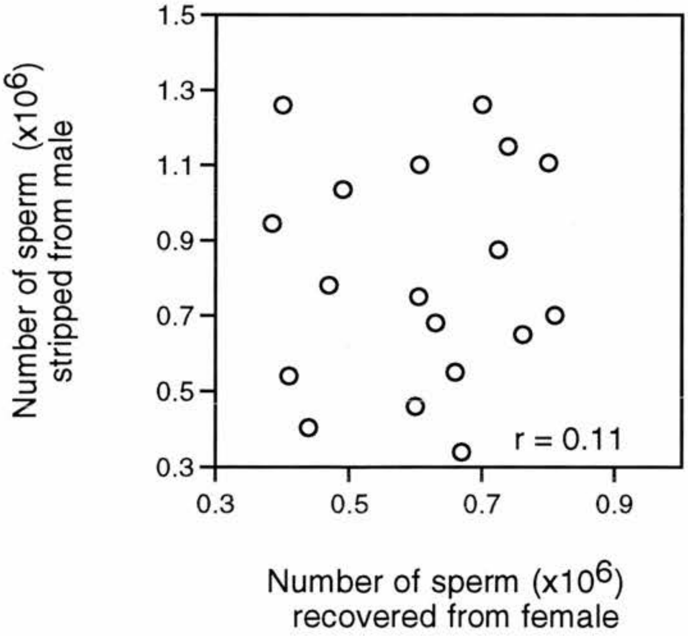

Sperm (x10^6)
1.5
1.0
0.5
0.0
r = 0.61 **
r = 0.59 **
r = 0.73 **
r = 0.34 *
0
10
20
30
40
50
60
0
2
4
6
8
10
12
Sigmoids
Thrusts

Figure 3.3 Sperm number per ejaculate against sperm lengths for males from Tacarigua (open circles) and Tunapuna (closed circles). * = $p < 0.05$; ** = $p < 0.01$. Significance levels were calculated using a Bonferroni correction, see text for details.

Figure 3.4 The relationship between sperm number per stripped ejaculate and sperm number recovered from females. No significant relationship was found ($r = 0.11$, $n = 18$, $p > 0.05$).

Figure 3.3
Figure 3.4





the Tunapuna males ($r = 0.312$, $n = 40$, $p > 0.05$). The significance levels of all correlations were calculated using a Bonferroni adjustment (0.05 and 0.01/13, (Sokal and Rohlf 1995) where 13 corresponds to the number of correlations performed per population).

Although Tacarigua males were significantly smaller (measured as dry-weight of fish) than Tunapuna males ($t_{77} = 2.7$, $p = 0.009$), the gonosomatic indices were virtually identical in both populations ($t_{77} = 0.01$, $p = 0.99$). Total ejaculate volumes, numbers of sperm per ejaculate, and sperm length did not differ significantly between the populations (t_{77} = 1.1, 0.86 and 0.04 respectively, $p > 0.05$).

DISCUSSION

The results of this study indicate that, for both populations, the number of sperm per stripped ejaculate is highly correlated with sigmoid rate (figure 3.2). This finding has possible implications for both male and female guppies. Firstly, it is obviously in the interest of a male not to waste energy chasing and displaying to females if he has little or no sperm ready for use. Bozynski (1994) has shown that males that have recently been artificially stripped of their ejaculate spend less time following females and perform fewer sigmoids than males whose sperm reserves are still intact. After spermatogenesis male guppies bundle the sperm into spermatozeugmata, each containing around 17,000 to 20,000 sperm (Billard 1969; Mann 1984). It has been estimated indirectly that a male can produce approximately 36 spermatozeugmata each day (Billard 1969). If spermatogenesis is constant then males may simply be more ardent in their mating attempts when they have greater reserves of sperm. It is possible that as the number of spermatozeugmata ready for use increases a male can sense some distension of the testes. Alternatively males may be capable of assessing the period since sperm were last released and therefore indirectly gauging the number of sperm available.

Irrespective of a male's ability to assess sperm supplies, the correlation between both sigmoid rate and thrusting rate and sperm number could be explained if males with large sperm stores are more ardent.

Secondly, since the behaviour of a male conveys information on his sperm availability, females may be able to assess a male's ejaculate features. The data therefore provide support for Sheldon's (1994) phenotype-linked fertility insurance hypothesis that female preferences arise because the morphological and behavioural features that females find attractive covary with ejaculate features. It has long been accepted that female guppies exert choice and base their mating preferences upon individually variable male coloration and morphology (Endler 1983; Endler 1995; Houde 1987; Kodric-Brown 1985), but there is also experimental evidence that female guppies choose males on the basis of their behaviour (Farr 1980b). Nicoletto (1993) found that females responded more readily to males that had higher display rates. Given the strong correlation between sigmoid frequency and sperm number, females that select males on the basis of courtship rate will be choosing males with higher sperm counts. This means that in addition to gaining indirect benefits, such as those proposed by the 'good genes' model of sexual selection (Reynolds and Gross 1992), female guppies can obtain direct benefits as a consequence of mate choice. Further work on the relationship between male coloration and behaviour, ejaculate characteristics and female choice is needed in order to distinguish between these two possibilities.

Even if females are not using display rate to select males of high fertility, the relationship between thrusts and sperm number will still benefit males that successfully inseminate unreceptive females. As previously mentioned, females solicit matings for only a few days during each reproductive cycle. At other times they face continual sexual harassment from males. The amount of paternity achieved by sneaky mating is still unknown (Magurran 1996), but as shown in chapter four wild females are being successfully inseminated whilst unreceptive (i.e. through sneaky mating). This suggests that it could occasionally undermine female choice.

This hypothesis is further supported by our results, which indicate that the males that engage most actively in sneaky mating have highest sperm counts. During their period of receptivity, it is known that females may choose to mate with several males (Houde 1987). Furthermore the high frequencies of sneaky mating attempts observed in the wild (Magurran and Seghers 1994a) will introduce sperm from yet more males into the female reproductive tract. Sperm competition will be an inevitable consequence of these behaviours.

Models of sperm competition are discussed in more detail in chapter six, but briefly if sperm competition follows the rules of a lottery (Parker 1990a), the greater the number of sperm that a male has competing for fertilizations then the greater his chance of success. Further models suggest that competition for fertilizations may take the form of a race. Under such conditions males would be selected to produce longer sperm, which will be able to swim faster and therefore reach the egg before slower, shorter sperm (Briskie and Montgomerie 1992; Gage 1994; Parker 1990b). Our results found an interesting correlation within each population between sperm length and the number of sperm per ejaculate (figure 3.3), indicating that male guppies are using both strategies to maximize reproductive success. This result is contradictory to Parker's theory (Parker 1982) that a trade-off exists between sperm size and sperm number, and deserves further investigation.

Observations in the wild indicate that risky sites, such as the Tacarigua, are characterized by high levels of courtship activity, particularly sneaky matings (Magurran and Seghers 1994a). This relationship between predation regime and male courtship behaviour was also evident in this study (figure 3.1). Interestingly, the relationship between sneaky mating frequency and sperm number is also strongest in fish descended from the high predation population (Tacarigua) (figure 3.2). I would predict therefore that sneaky mating will account for proportionally more fertilizations in high predation localities than in low predation ones. There may also be a higher incidence of multiple paternity in such localities. Once suitably polymorphic genetic markers, such as microsatellites, have been developed, these questions also deserve attention.

If males vary in their ability to fertilize, perhaps because of differences in the their ability to produce sperm, female choice of the most fertile males should reduce the risk of producing unfertile eggs (Williams 1992). In the frog, *Uperoleia laevigata*, females move around the areas where males chorus and apparently listen to several males before approaching a selected individual. The male then climbs onto the female's back and she carries him to the nearest pond where mating occurs. Females have been shown to prefer the calls of heavy males over light males (Robertson 1986), and were further found to prefer synthetic calls corresponding to those of males that were 70% of their own body weight (Robertson 1990). When pairs of mating frogs were sampled males tended to weigh around 70% of the female to which he was paired. Hatching success of the subsequent spawn has been shown to be highest if the male's weight is between 65-80% of female mass (Robertson 1990). If a female chooses a male that is too heavy, she may be unable to swim and risk drowning. Light males (i.e. less than 65% of female body weight) may be easier to carry during mating, but may have insufficient sperm to fertilize an entire clutch. In this example females gain an obvious benefit, increased fertilization success, from mating with males of the correct phenotype.

In the fruitfly, *Drosophila melanogaster*, it has been demonstrated that multiple mating by a male can lead to a reduction in his fertility. Despite this temporary sterility, caused by a depletion of accessory gland secretions, males will continue to court and attempt to mate. However when virgin females were given a choice between a virgin male and a recently mated male, virgin males were chosen significantly more often (Markow *et al.* 1978). The lack of successful mating by non-virgin males could not be explained by a reduction in courtship behaviour. No evidence was found to suggest that virgin males initiated courtship faster or spent longer courting than mated males. Whilst no phenotypic trait was found that could account for this selection it is possible that an olfactory cue or a variation in the organization of courtship elements is involved. Again the potential advantage resulting from discrimination against low fertility males was highlighted. Halliday (1978) has also reported a similar finding in the smooth newt, *Triturus vulgaris*. In this species, the rate of male display during early phases of courtship is highly correlated with the number of spermatophores later deposited. During mating a male normally releases two or three spermatophores (Halliday 1976). There then follows a period of post-copulatory sperm depletion from which a male may require several hours and up to a day to recover (Halliday 1976). Courtship in newts is intrinsically unreliable as a means of sperm transfer. Males release spermatophores on to the ground and then try to coax females to walk behind them over the spermatophores. Even in laboratory situations designed to maximize sperm transfer, females only collected 43% of the spermatophores (Halliday and Houston 1978). However the chances of sperm transfer being successful increase with the number of spermatophores released. As a result it may not pay a male to court if he has only one spermatophore ready to deposit. By depositing the available single spermatophore he will increase the delay before the production of two or three sperm bundles. The amount of energy a male invests in display is therefore related to his chances of successful sperm transfer. Display rate is therefore an indicator of the subsequent number of sperm to be released, again a phenotypic trait correlated with fertility that could be used by females. However no evidence exists that females assess display rate during mate choice in this species (Halliday 1978).

A final example of females selecting against males with reduced fertility was provided in a study of fertilization success in the lemon tetra, *Hyphessobrycon pulchripinnis* (Nakatsuru and Kramer 1982). Traditionally, although this is presently being reassessed, sperm have been thought of as being relatively cheap to produce. As a result males being sperm limited was thought to be rare. Laboratory studies that have investigated this problem have found that males do run out of sperm after repeated matings. However such studies have been criticized for staging matings at frequencies far in excess of those found in nature. In the lemon tetra males defend territories containing spawning sites. Females, when ready to spawn, enter a territory and approach the male. After a brief courtship, male and female approach a spawning site and release sperm and eggs respectively. On average females spawn 23 times, releasing a total of 160 eggs, every fourth day during the breeding season. Individual males were allowed to establish territories, a female was then introduced and allowed to spawn. When 10 spawning events had occurred the mating was halted and the eggs removed. The mating was then continued for a further 10 spawnings after which these eggs were also collected. All eggs were incubated to determine the number that had been successfully fertilized. Males achieved significantly higher fertilization rates in the first set of ten spawnings compared to the second. When female choice tests were carried out it was found that females spent more time near unspawned males. No details were provided by Nakatsuru and Kramer (1982) concerning the possible phenotypic traits that females might use to assess male fertility, but their study does highlight two important points. First, even under natural conditions males may run out of sperm and this may affect a female's ability to successfully fertilize all her eggs. Secondly, females can select against males that have less sperm and therefore a lower fertilization success. As a group these studies combine to demonstrate the fertility benefits that can be gained through mate choice. If sperm are limited, differences in fertility among potential mates will favour mate choice for males of higher functional fertility.

At present there is no evidence to suggest that male guppies are limited in their ability to produce sperm. However I would expect that under certain conditions, primarily during periods of food shortage, the production of sperm may decrease or exceptionally cease. It would be interesting to know if there is any seasonal change in testis size in the guppy. It has been shown that several environmental factors, including light levels, temperature and rainfall can influence testes size in birds (Murton and Westwood 1977). However, in contrast to the guppy, none of the species studied bred throughout the year. In addition to seasonal patterns, diurnal variation in sperm production has also been reported. In the house sparrow, for example, sperm production peaks several hours before dawn with copulation soon after dawn (Møller 1987). Again it is possible that similar patterns may be found in the guppy, with sperm production peaking during periods of high light intensity, in preparation for matings during the lower risk darker periods. However as males attempt sneaky mating with such frequency, it seems likely that sperm production will be continuous over the shorter time scale, with possible variation over the longer scale caused by lack of food.

Sperm depletion in males has been demonstrated in a number of species (see Dewsbury 1982; Birkhead and Møller 1992). Unfortunately no information is available on any of the poeciliids. However if we compare Billard's (1969) data, that males produce around 750,000 sperm a day, with the average number of sperm per ejaculate recorded in this study, then males would only have enough sperm to inseminate one female a day. However it should be noted that the values in this study are numbers of sperm per stripped ejaculate. An attempt was made to establish a correlation between the number of sperm in a stripped sample and the number of sperm a male inseminated in a natural ejaculate. Males were isolated for 48 hours to ensure that no recent matings had depleted their sperm stores. A virgin female was then introduced, and the male allowed a single mating. The female was then removed and an attempt was made to recover as many sperm as possible from her reproductive tract. These sperm were then counted as described previously. The male was then allowed a further 48 hours to recover, after which time a stripped sperm sample was taken and the number of sperm counted. Unfortunately there was no correlation between the number of sperm recovered from the female and the number of sperm later stripped from the same male ($r = 0.11$, $n = 18$, $p > 0.05$; figure 3.4). It is difficult to believe that all sperm were recovered from the female, however even allowing for a 10% failure in recovery, there is still no clear relationship. Only 18 pairs of fish were sampled and it is possible that a larger sample may provide a clearer picture. If an association could be established between the number of sperm in natural and stripped ejaculates, it would allow us to increase our confidence in the application of these results to the situation observed in the wild. It would also allow more detailed investigation of issues of sperm availability. How this problem can be addressed is difficult to assess. The use of freeze dried females and false cloacae have proved most informative in studies of birds, but seem unlikely to succeed in the more complex courtship of the guppy. Perhaps the ejaculate can be caught before it leaves the male, possibly by cannulating the gonopodium. Alternatively some form of receptacle could be introduced into the opening of the ovary, which could later be extracted and the ejaculate recovered.

As mentioned laboratory studies have shown that in *Xiphophorus* an insufficient supply of sperm leads to a decrease in brood size (Vallowe 1953). No similar results exist in the guppy. Female guppies can produce up to eight broods following only a short period of exposure to a male (Hildemann and Wagner 1954). This would seem to suggest that during this short period, females can obtain sufficient supplies of sperm to maximise their reproductive output for a considerable time. However it would be worth establishing if there is a significant difference in the number of offspring produced by females that receive matings over 24 hours and are then isolated until brood production ceases, and females that are reinseminated after each brood. If females that receive regular sperm are able to produce more offspring, or perhaps offspring that are in some way fitter, then there may be a reduction in female fitness through not being regularly inseminated. Sperm limitation would seem more likely if females benefited from receiving sperm on a monthly basis rather than only needing two or three successful matings a year. On the other hand, if female guppies in the wild prove to have either copious stores of sperm or plentiful opportunities to obtain sperm (i.e. are not sperm limited), then they should have no problem in obtaining sufficient sperm to fertilize their offspring. Females would therefore have little to gain in terms of reproductive success, by selecting males with the greatest sperm reserves. However no work has been carried out, in the wild, to establish if sperm is in fact this abundant. If sperm is not limited, the relationship between sperm reserves and male behaviour will be male-driven rather than female-driven, and females might be expected to weight reliable male signals such as coloration more highly when choosing a mate.

Even if females stand to gain from choosing males of higher fertility Birkhead and Fletcher (1995) have argued that consistent differences between males in ejaculate features are required if females are to choose between males on the basis of differences in their ability to fertilize eggs. In this study each male was only sampled once and therefore no information was collected on the consistency of ejaculate features within males. However this does not preclude it from providing evidence for Sheldon's (1994) hypothesis. Because sperm were stripped from males soon after the behavioural observations, we can be confident of the relationship between sperm number and courtship rate. I would predict that any subsequent change in ejaculate characteristics would be accompanied by corresponding changes in behaviour, so that at any time the display rate of the male reveals his current sperm count. However it would be worth investigating whether male guppies show consistent patterns of sperm production during their adulthood. Studies following individual males over longer time periods, perhaps the six months following first sperm production, would provide valuable information on the variance of male ejaculate characteristics over time. Further work on the relationship between qualitative features of the ejaculate and display rate and between ejaculate characteristics and other phenotypic traits may also provide further interesting results.

It is confusing that some studies support Sheldon's (1994) hypothesis whilst others refute it. The possible reasons for this difference are not clear. Perhaps in some species, there is a relationship between phenotype and ejaculate features, while this association is absent in others. It would appear more probable that the correlation exists between some phenotypic characters and ejaculate features and not others. Perhaps the species that have so far been cited as failing to provide evidence for the phenotype-linked fertility insurance hypothesis would provide support if different characters were studied. Would Birkhead and Petrie (1995) have found a correlation between some other aspect of the peacock's tail and ejaculate characteristics? Is there also a correlation between male colouration and sperm number in guppies? Alternatively it is possible that the conditions in which the study animals are maintained may influence the findings (T.R. Birkhead pers. comm.). The collection of evidence to support Sheldon's (1994) hypothesis and to distinguish between the 'good genes' model and the phenotype-linked fertility insurance hypothesis therefore remains a topic for further work.

Chapter 4

How successful is sneaky mating at transferring sperm?

INTRODUCTION

As discussed in chapter 1 female guppies are ovoviviparous and fertilization is internal, with sperm transfer achieved via the male's modified anal fin, the gonopodium (Constantz 1989). Females mate promiscuously and as they can store sperm for up to six months, a single insemination may result in the production of several broods (Winge 1937; Kadow 1954). Male guppies have two distinct mating strategies. To briefly reiterate, males may firstly court a female, perform a sigmoid display, and try to persuade her to copulate (Liley 1966; Luyten & Liley 1985). If the female accepts the male's advances, she will cooperate in the mating and swim in a tight circle, exposing the genital pore (Liley 1966). If the male's displays are unsuccessful, either because the female is not receptive to courtship, or she does not wish to mate, then the male may attempt to transfer sperm through sneaky mating (Liley 1966). The male approaches the female from behind and attempts to insert the gonopodium into the female's genital pore. Importantly, this second mating strategy is undertaken without female cooperation.

Females are responsive to male displays and will cooperate in full matings only when virgins or for approximately 36 hours following the birth of a brood. During the remainder of the 24-28 day brood cycle females are unreceptive and do not cooperate in any matings (Liley 1966; Luyten & Liley 1985). A male's only chance of inseminating such females is through sneaky mating, and importantly female sperm storage means that such inseminations can compete for fertilizations. Thrusts can occur at extremely high frequencies and in some natural populations females receive up to one sneaky mating attempt per minute (Magurran & Seghers 1994a).

The success of alternative mating strategies in other fish.

The occurrence of alternative male mating strategies has been documented in a range of both internally and externally fertilizing fish species, however few data are available on the relative success rates of each strategy. Males of the bluegill sunfish, *Lepomis macrochirus*, adopt one of three mating strategies (Gross 1979, 1982; Dominey 1980). Large 'resident' males build and defend nests to which they attract females. Streaker males interrupt and join mating pairs, similarly female mimics also join spawning pairs and release their sperm. In a study of mating success in bluegill sunfish (Phillipp and Gross 1994), it was found that in both laboratory and wild populations, males adopting one of the alternative mating strategies were able to fertilize eggs. In the laboratory, sneakers fathered 32.9% of the available eggs, while in four wild populations sneakers successfully fertilized between 0% and 58.7% of the eggs (Phillipp and Gross 1994). The tactics are exclusive alternatives and not simply age dependent differences (Gross and Charnov 1980), and the success of each strategy is thought to be negatively frequency dependent (Gross 1991). An experiment involving crossing of individuals from the F_1 generation suggests that the alternative male tactics in this species are not due to genetic differences (Dominey 1984).

The relative success of 'parasitic' and 'nesting' males has also been investigated in three species of salmonid. In *Salvelinus malma miyabei* an average of 7% of eggs were fertilized by 'parasitic' males (Maekawa and Onozato 1986; Taborsky 1994). Foote *et al.* (1997) determined the relative spawning success of jack sockeye salmon, *Oncorhynchus nerka*. Spawning success was found to be highly variable (3-93%) but was not significantly different from that of large males. In the Atlantic salmon, *Salmo salar*, it was found that 10.8% of the eggs collected had been fertilized by 'parasitic males' (Jordan and Youngson 1992). Moran *et al.* (1996) tested the success of parr in the presence of an adult male and female spawning pair. Parr were able to fertilize between 24.7 and 89.3 per cent of the total eggs. While Hutchings and Myers's (1988) experiments, in which a range of densities of parr spawned in artificial redds with females, showed that parr were capable of fertilizing up to 25% of the available eggs. Thomaz *et al.* (1997) used polymorphic minisatellite DNA markers to analyse paternity in a series of mating experiments where the number and body size of parr were manipulated. The fraction of eggs fertilized by mature parr ranged from 26 to 40%, with individual parr fertilizing up to 26% of the eggs. A strong positive correlation was found between parr size and reproductive success.(Thomaz *et al.* 1997). Despite small size, parr weigh only 0.15% of the average body mass of anadromous males, and occupying a disfavoured mating role, parr achieve an unexpectedly high number of fertilizations. Gage *et al.* (1995) measured a range of reproductive traits for both male strategies. Anadromous males have absolutely larger testes and produce greater numbers of sperm than parr males. However, parr invest relatively more heavily into total spermatogenesis, and have a larger gonosomatic index (testes mass as a % of body mass) than anadromous males. Relative to body size, it was found that the stripped ejaculates of parr were of greater volume and also contained greater numbers of longer lived sperm.

The success of alternative mating tactics has also been studied in the African cichlid *Pelvicachromis pulcher* (Martin & Taborsky 1997). Males of this species may either reproduce monogamously (pair males), polygynously (harem males), or be tolerated in a harem territory (satellite males). The reproductive success of harem males was found to be 3.3 times higher than that of pair males and 7 times higher than that of the average satellite male. However dominant satellite males were as successful as monogamous pair males. The males of the previously mentioned fish specialise as either sneaks or courting males. However in guppies, as in sticklebacks (Goldschmidt *et. al.* 1992), males adopt both strategies and can switch between them at will. Sneaky mating therefore represents a facultative, opportunistic behaviour rather than a specialised behavioural phenotype (Houde 1997). In the stickleback, following the identification of a probe allowing paternity assessment (Rico *et al* 1991a), an initial study Rico *et al.* (1991b) examined DNA from the guardian male and 10 randomly selected fry from 10 nests. Earlier behavioural studies suggested that males shed sperm into the nests of other males (van den Assem 1967) and occasionally steal eggs from other nests (Stacy and Owings 1978). However all the offspring analyzed by Rico *et al.* (1991b) were fathered by the guarding male. In a second, more extensive study of the resident male and 10 randomly selected fry from a further 17 nests, successful fertilization by sneaker males was reported (Rico *et al* 1992). In one nest five of the fry had been fathered by a sneaker male, in two nests the guardian male had stolen eggs from another male's nest and a final nest one egg had been fertilized by a sneaker and three of the eggs had been stolen. In the fourspine stickleback, *Apeltes quadracus*, males use female mimicry to sneak over to nest-building males and rarely attempt to enter the nest until after the female has spawned and left. Males have been shown to release spawn in the nests of rival males, but further research including DNA fingerprinting of offspring to determine paternity in nests fertilized by both the owner and a sneaker has yet to be carried out (Willmott & Foster 1995).

Sneaky mating in *Poecilia reticulata.*

The earliest observation of mating in a poeciliid was presented by Ryder (1885). Reporting on mating in *Gambusia*, he observed that the male's 'prolonged anal fin was thrust into the external opening of the oviduct'. Despite this seemingly reliable account of a full mating, early observations of gonopodial thrusts are scarce, the few that do exist are anecdotal and often inaccurate. Schmidt (1920) suggested that contact between male and female guppies was not necessary for sperm transfer. Having observed the male approach the female and swing his gonopodium forward, he proposed that the sperm were fired through the water, adhered to the female's abdomen and later entered the female reproductive tract. This idea was accepted by Winge (1927, 1937) and was presented in the literature as late as 1951 (Whitney 1951). However Breder and Coates (1935) did suggest that there was 'momentary contact' between the sexes during sneaky mating, and this view was echoed by Purser (1941) and Haskins and Haskins (1949).

Thus, until the early 1950's it was wrongly believed that the vast majority of sneaky mating attempts, regardless of whether contact was made, resulted in insemination (Liley 1966; Houde 1997). However this view was challenged by three major studies carried out in that decade which suggested that thrusting very rarely leads to successful insemination. Clark and Aronson (1951) observed more than 1000 thrusts, none of which were reported to have successfully transferred sperm. Kadow (1954) found two of the twelve 'copulation thrusts' that she observed to have resulted in the transfer of 'small numbers of sperm', Baerends *et al.* (1955) obtained similar results. In 197 cases of 'attempted copulation not followed by jerking' (i.e. sneaky mating), no sperm were found in oviduct samples from females. However in '30 cases of more complete copulation attempts which were all followed by post-copulatory jerking' all but 3 gave indications that insemination had occurred. As a result of these, and his own studies, Liley (1966) reported that 'a considerable body of evidence exists that insemination occurs almost entirely during recognisable acts of copulation which are preceded by sigmoid display', and that 'most of the fleeting contacts involved in Thrust do not result in insemination'.

Previous studies of *P. reticulata* have therefore presented two contrasting views of male sexual behaviour. Several authors have reported the tendency of males to attempt sneaky matings (Liley 1966; Farr 1976; Luyten and Liley 1985; Magurran and Seghers 1994a). However, as was discussed in the previous section, these gonopodial thrusts are thought to have only a marginal success rate (Clark & Aronson 1951; Kadow 1954; Baerends *et al.* 1955; Liley 1966). The importance of sneaky mating in the guppy, or indeed any internally fertilizing fish, has received little attention and even in recent studies it has generally assumed to be largely unsuccessful (Endler 1987; Kodric-Brown 1993; Godin 1995; Houde 1997). Despite such reports, the expenditure of energy on, and persistence of, gonopodial thrusting suggests some reproductive success. In view of the high incidence of this behaviour in the wild, this experiment investigated whether sneaky mating is indeed an effective method of transferring sperm to unreceptive females, and if so, what proportion of females are receiving unsolicited sperm.

METHODS

Two hundred and fifty females, from several rivers in the Caroni drainage system in Trinidad, were collected during a four week period. Each female was humanely killed, using a terminal dose of anaesthetic, and examined for both the presence of sperm and the developmental stage of her embryos within 12 hours of capture from the wild.

Checking for sperm in the female reproductive tract.

Micropipettes were used to sample the female's ovarian fluid for the presence of sperm. These were machine pulled from glass capillary tubes (Clark Electromedical Instruments) and the end removed with a diamond-tipped pen. A drop of distilled water was taken up, and the capillary tube introduced into the opening of the female's genital pore. The fluid was then expelled into, and taken up from, the ovarian cavity. This was repeated three times for each female. These samples were examined, using a high power microscope, for the presence of sperm (Kadow 1954; Liley 1966).

Prior to the fieldwork, a laboratory study was carried out to determine how long, after a mating, sperm remained recoverable in the oviduct. Full matings of sixteen virgin females and sixteen receptive, but non-virgin females were observed. Immediately after mating the male was removed and the female isolated for subsequent analysis. The oviducts of two virgin and two non-virgin females were checked for the presence of sperm on each of the next eight days. Each female was only sampled once. Sperm were most numerous in the samples taken from females 24 hours after mating, and became increasingly less common, until after eight days none of the four females yielded recoverable sperm. Our own observations support N.R. Liley's finding (personal communication) that sperm can be recovered from a female guppy's oviduct for up to seven days following insemination.

We further confirmed that stored sperm were not recovered by the procedure used in this study. Twenty eight virgin females were mated, and then kept in isolation until the birth of a brood confirmed insemination had been successful. Each female was then subjected to the above protocol. None of the females yielded recoverable sperm, despite producing subsequent broods. A single insemination has been shown to be sufficient to produce several broods (Winge 1937). We also confirmed that the protocol did not recover sperm from females that had been mated but not yet produced broods. A further seven virgin females were mated, isolated and examined after 14 days. Again no sperm was recovered from any of these females, and all went on to produce offspring within the next 22 days.

Determining the receptivity of the female.

In order to establish the means by which sperm had been inseminated, it was necessary to establish the sexual receptivity of the female at the time of insemination. Following the data provided by Haynes (1995; appendix 4.1), I therefore established, in the laboratory, a system for categorizing the developmental stage of a female's embryos as a guide to her sexual receptivity, and related this to the presence of recoverable sperm in her oviduct. Twenty-one virgin females were mated and then isolated. Three females were dissected on each of the next seven days, and the developmental stage of the embryos recorded. As guppies show no evidence of superfetation (Turner 1937), individuals within a batch of embryos are all of a similar stage of development. These laboratory experiments showed that a week after a mating has taken place, embryos developing in females at 25°C (the typical temperature of Trinidadian rivers) have not advanced beyond the end of stage 2, that is the primitive streak stage in Haynes' (1995) classification of poeciliid embryonic development (appendix 4.1).

The ovary was dissected out of each of the 250 experimental fish and the females were assigned to one of four categories according to the developmental stage of the embryos. A female with ova that had not yet been fertilized was classed as being in group one (figure 4.1a), while females with embryos from blastodisc to primitive streak embryo were placed in group two (figure 4.1b). Females with more advanced embryos, from optic cup embryo to late-eyed embryo, were categorised as being in group three (figure 4.2a), and females with fully developed embryos, from very late eyed embryo onwards, were assigned to group four (figure 4.2b).

RESULTS

Fifteen percent of the 117 females with embryos in stages one and two (figure 4.3) had recoverable sperm. Given the mating system of the guppy (Houde 1987) we cannot exclude the possibility that these sperm were from accepted matings preceded by sigmoid displays. The remaining 133 females contained mature embryos. We detected sperm in the oviducts of 16% of females with stage 3 embryos and 13% of females with stage 4 embryos. As at least a week, and in many cases much longer, had elapsed since the females were last receptive to male courtship, we can conclude that these sperm must have been the product of sneaky matings. In total, 15% of the 250 females had received sperm from sneaky matings.

DISCUSSION

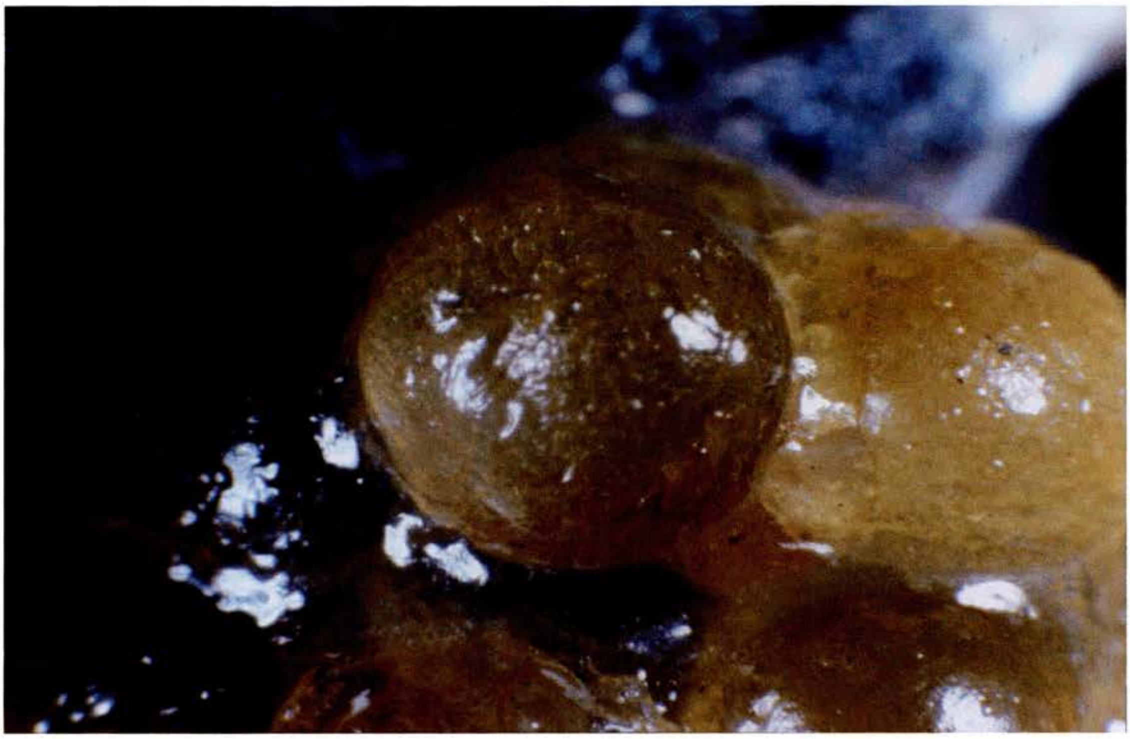
This study showed 15% of the females with embryos in stages 3 and 4 to have recoverable sperm. Due to the seven day refractory period prior to the movement of sperm from the oviduct to the storage sites, sperm from any one insemination only remain recoverable for up to one week. I have demonstrated that the protocol used in this study does not recover sperm from long-term storage sites. Further, our laboratory studies have shown that females in these two later stages had been unreceptive for longer than seven days. It can thus reliably be assumed that any recoverable sperm came from a successful sneaky mating. We must therefore revise

Figure 4.1a Ovary of a stage one female (centre of photograph) containing several small unyolked and unfertilized oocytes. The female is either a virgin or more probably has recently given birth. The oocytes are unfertilized and the female is receptive to male displays.

Figure 4.1b Section of the ovary of a stage two female guppy. The oocytes have been yolked and fertilized. The yolk is translucent golden yellow in colour and the beginnings of the developing embryo can be seen as a white region on the left of the central oocyte. This brood has been recently fertilized and the female's reproductive may still contain sperm from accepted matings.

Figure 4.1

Photograph by Iain Matthews

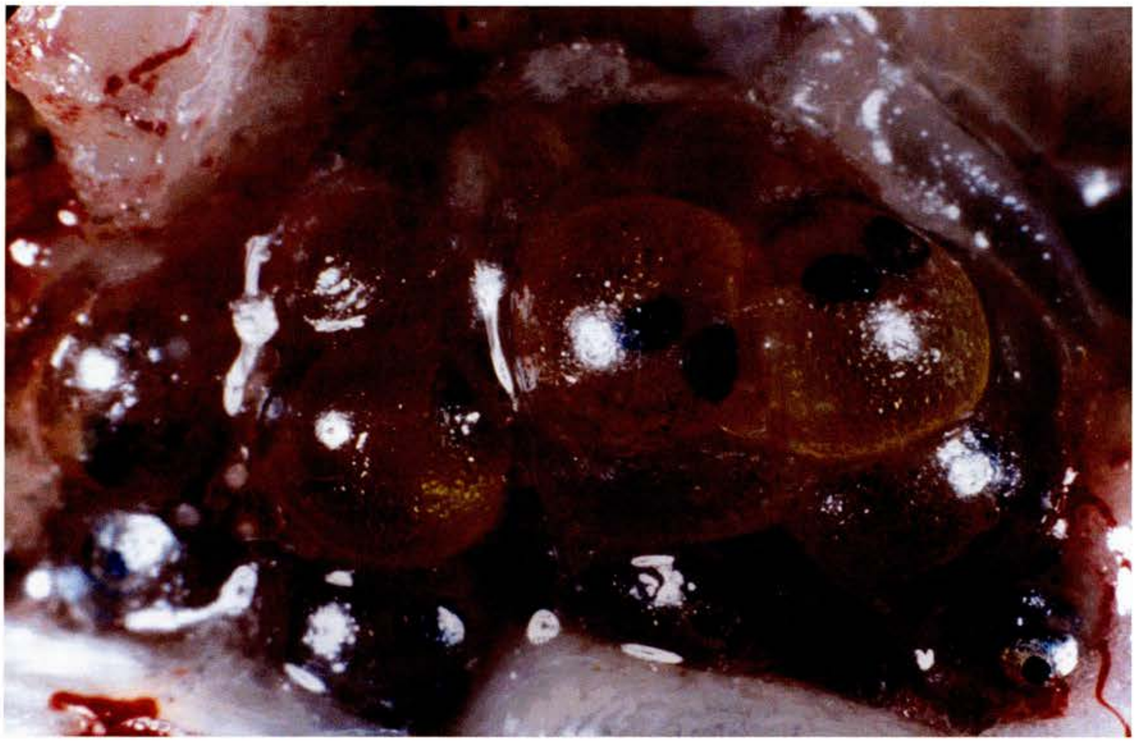


Photograph by Iain Matthews

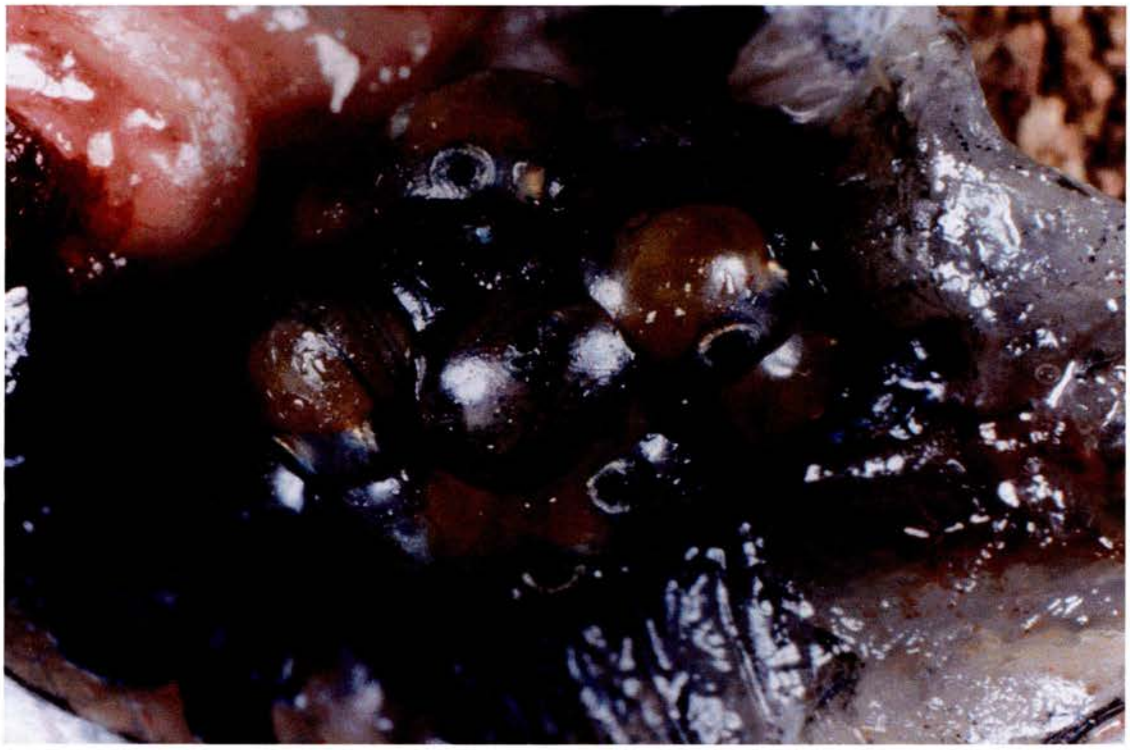
Figure 4.2a Clearly visible embryos in the ovary of a stage three female. The eyes have become pigmented and the initiation of dorsal pigmentation can be seen on the back of the head. The yolk has turned a more opaque darker shade of yellow. These embryos have been developing for at least one week, and any sperm that are recoverable from the reproductive tract of such females must have been inseminated through gonopodial thrusting.

Figure 4.2b The ovary of a stage four female guppy. The embryos have become more heavily pigmented and the volume of yolk has reduced considerably. Pigmentation is nearing completion and the eyes are fully formed. Any sperm recovered from females containing such advanced embryos must have been received from successful sneaky matings.

Figure 4.2



Photograph by Iain Matthews



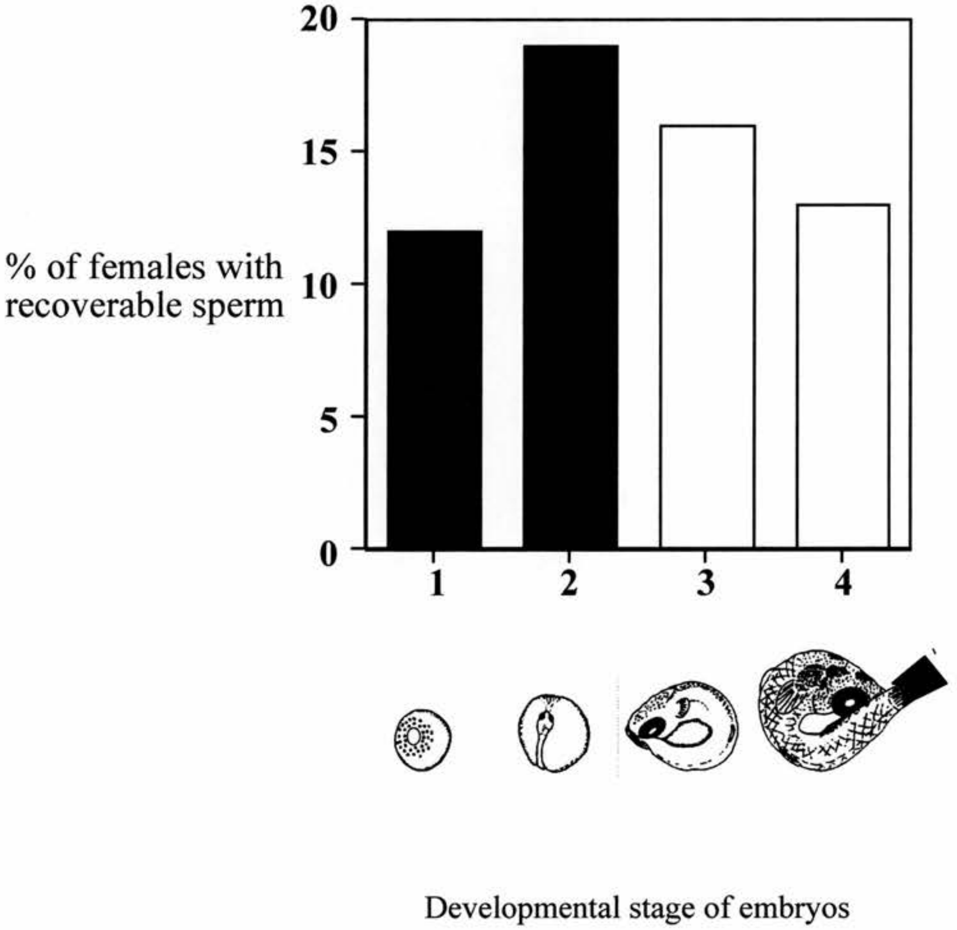
Photograph by Iain Matthews

Figure 4.3	Percentage of females with recoverable sperm in relation to the developmental stage of the embryos. Females were collected from natural populations in the Caroni drainage in Trinidad and their oviducts checked for the presence of sperm (Kadow 1954; Liley 1966). Developmental stages were a reassignment of, and are redrawn from, those of Haynes (1995). Values in parentheses are the number of females in each stage.

Stage 1 - (75) -	Immature ovum to mature ovum.
Stage 2 - (42) -	Blastodisc embryo to primitive streak embryo.
Stage 3 - (81) -	Optic cup embryo to late eyed embryo.
Stage 4 - (52) -	Very late eyed embryo to mature embryo.

It is not possible to assign sperm recovered from females in stage 1 + 2 (black bars) to either full or sneaky matings. However any sperm found in females in stage 3 + 4 (white bars) can only have been inseminated as a result of successful sneaky mating. An example of an embryo from each stage is shown beneath the appropriate column.

Figure 4.3


20
15
10
5
0
% of females with recoverable sperm
1
2
3
4
Developmental stage of embryos

the traditional concepts that females are only rarely inseminated through sneaky matings.

The insemination assay used in this study was not able to establish if all the sperm recovered were from one insemination, or indeed from one male. It is possible that each female had received more than one successful sneaky mating during the last week, and that the sperm recovered were from a number of inseminations possibly from a range of males. The assay only reveals that at least one male has successfully transferred sperm without female co-operation during the last seven days. Furthermore there may have been females from stages 3 and 4, that did not yield recoverable sperm, but that had received successful sneaky mating. Any sperm inseminated during the period from one week after the end of the female's period of receptivity until the birth of the next brood can only have come from sneaky mating. However if the sperm were inseminated during this period, but more than eight days before sampling then they would not have been picked up in this experiment. 15% may therefore be an underestimate of the number of females successfully inseminated as a result of sneaky mating, and as it is not possible to distinguish between inseminations it is almost certainly an underestimate of the number of successful sneaky matings.

Direct observations of sneaky mating in the laboratory such as those mentioned in the introduction, have lead researchers to believe that successful insemination by this means is infrequent. However even with a conservative estimate of success rates as low as 1 insemination per 1000 attempts, it is still predicted that with sneaky mating occurring in high predation sites at a rate of one attempt per minute, this may result in a male guppy achieving over 250 extra matings a year. Due to the ability of the female to store sperm for prolonged periods, any successful insemination may compete for a number of successive broods. That males in some populations expend such large amounts of their time sneaking has always suggested that there is likely to be a direct benefit from sneaky mating. Until now this benefit has been assumed to be marginal. Whilst this study does not present any data on the subsequent use of sperm from sneaky mating, the potential of this strategy for gaining additional paternity for a male is clear. Paternity analysis and DNA fingerprinting studies now need to be undertaken in order to determine the amount of paternity a male can gain by sneaky mating.

One surprising finding of this experiment is the lower than expected percentage of receptive females (stage 1 females) that yielded recoverable sperm. The period during which females have unfertilized ova corresponds with the release of a 'pheromone' from the female reproductive tract. This chemical cue leads to an increase in male sexual activity. Furthermore, females in stage one, immediately following parturition, are receptive to male sigmoids. It was therefore predicted that a large proportion of these females would have received recent inseminations. It was unexpected to find that only 12% of stage one female have been recently mated. Brillard and Bakst (1990) have recently shown, using artificial inseminations in laying turkeys, that the sperm storage tubules are much more receptive to sperm at this stage of the breeding cycle. During the period when eggs are being fertilized, the uptake of sperm by the sperm storage tubules is both faster, and greater, than in non-laying females. Similar results have also been obtained using natural copulations and Bengalese finches (Birkhead and Møller 1992). If a similar situation occurs in receptive female guppies, then the expected inseminations may be stored more rapidly than in females in other stages. If the sperm in stage one females is stored more rapidly, then this would explain the lower than expected number of females that appear to have been inseminated.

Recent experimental evidence in the sand lizard (*Lacerta agilis*) has suggested the intriguing possibility of post copulatory sexual selection (Olsson 1996 *et al*). However, studies of other species have raised questions concerning the generality of the findings (Simmons *et al* 1996; Stockley 1997). These studies have dealt with a female's ability to discriminate against sperm from related males and so avoid the costs of inbreeding. As opinion for even this form of sperm selection is divided, it seems fair to assume, at this stage, that female guppies can not discriminate against sperm on the basis of how they were inseminated (i.e. matings preceded by sigmoids against gonopodial thrusts). In addition, studies based on heritable colour patterns have shown that the majority of females, both in the wild and in aquaria, are multiply sired. Most broods were found to be sired by two males, a few by three or more (Luyten & Liley 1991; Houde 1997). It is not known whether the degree of multiple paternity is in some way determined by the female, or is simply a reflection of the abundance, in the oviduct, of sperm from different males. If females cannot actively select sperm, then any sperm that a male introduces into a female's reproductive tract will compete for fertilizations. If paternity is a function of the number of sperm inseminated (Parker 1990a) then the more sperm a male inseminates the higher his chance of gaining paternity. Earlier work has shown that male guppies that engage most actively in sneaky mating have the highest numbers of sperm ready to ejaculate (Matthews *et al.* 1997). If sperm competition in guppies follows this lottery model, then it is predicted that sperm successfully transferred through sneaky mating will father a significant number of offspring.

If successful insemination is converted into paternity there are two areas of key interest that may be influenced. The frequency of sneaky mating attempts is highest in populations subject to severe predation risk (Luyten & Liley 1985; Endler 1987) and males in these populations are drabber in appearance than those from less dangerous habitats (Endler 1980). Selection by predators is the conventional explanation for the relationship between coloration and risk but, if female choice is compromised in dangerous localities, then an additional reason for population variation in male colour patterns emerges (Houde & Hankes 1997). Female guppies have been shown to select males on the basis of a range of traits, including morphology (Endler & Houde 1995), courtship rate (Farr 1980b; Nicoletto 1993) and colour patterns (Houde 1987). It is possible that sneaky mating may undermine these female preferences. Female guppies from another Trinidadian river, the Yarra, prefer males that have a greater amount of orange body colouration (Houde & Hankes 1997). Theory predicts that the degree of preference for orange colouration should have evolved to match the extent to which this colour is expressed in natural populations. However in the Yarra, the males are less orange than predicted on the basis of results from female choice experiments. This difference in the extent of the female preference for a trait and the degree to which males express that trait may have arisen a result of successful sneaky mating.

A further example of the way in which the sneaky mating may affect Trinidadian guppies can be illustrated by looking at the differentiation of populations. Natural populations of guppies evolve rapidly in response to changes in predation pressure. For example, male colour patterns become more flamboyant (Endler 1980) and antipredator behaviour diminishes (Magurran *et al.* 1992; Shaw *et al.* 1992) following a relaxation of predation regime. The dramatic response to natural selection in these fish was recently highlighted in a paper by Reznick *et al.* (1997) which assessed evolutionary changes in two life-history traits: age at maturity and size at maturity. Reznick et al. (1997) concluded that the observed rate of evolution in wild guppies was on a par with artificial selection experiments and seven orders of magnitude faster than in the fossil record. This capacity for rapid evolution, combined with variation in sexually selected traits and a genetic correlation between female preferences and male colour patterns (Houde 1994) should facilitate rapid speciation (Lande 1981). There is, however, no reproductive isolation between populations that have been separated for at least a million generations (Magurran *et al.* 1996). Females do not actively discriminate against males from genetically divergent populations (Endler & Houde 1995; Magurran *et al.* 1996). This situation contrasts dramatically with that seen in some other groups of fish, for example, Lake Malawi cichlids some of which are thought to have speciated within the last few hundred years (Owen *et al.* 1990). Male mating behaviour and the success of sneaky matings could help explain why, despite marked population differentiation, there is no speciation in Trinidadian guppies.

In conclusion some of the most compelling evidence for female choice has been provided by studies of the Trinidadian guppy, *Poecilia reticulata*. Females show preferences for males on the basis of colour patterns, morphology and courtship rate.

However, male guppies also attempt inseminations without female cooperation. Until now it has been accepted, primarily on the basis of laboratory studies, that such sneaky matings are rarely successful. Our investigation of female guppies in Trinidad tests the hypothesis that sneaky matings are in fact an important means of sperm transfer in natural populations. We found that 15% of nonreceptive females had recoverable sperm that must have come from successful sneaky matings. These results provide the first evidence that sneaky mating is indeed a successful method of sperm transfer in wild populations of the guppy.

Chapter 5

The morphology of the female reproductive tract

INTRODUCTION

All of the poeciliids studied so far are internal fertilizers, and all but one of the 150 species (*Tomeurus gracilis* has internal fertilization but external embryo development) so far studied are viviparous (Rosen & Bailey 1963). Despite this there is still considerable variation within the family for a range of reproductive traits, for example the number of offspring in each brood ranges from single figures to over 300 (Krumholz 1948). Once internal fertilization had evolved, a number of modifications in the reproductive system were possible, for example changes in ova size, ova number, size of embryos, amount of nutritional supplement from mother to embryos, degree of placental development, degree of superfetation (the simultaneous development of multiple broods within a single ovary), brood interval, duration of reproductive season and length of reproductive life (Thibault and Schultz 1978). Turner (1937, 1940, 1947) carried out several studies of adaptations for internal fertilization and subsequent embryo development in poeciliids, but concluded that there was no relationship between the evolution of viviparity in this family and the ecological conditions of the environments they inhabited.

One generalisation that holds true across the family is that female poeciliids are able, to a greater or lesser degree, to store viable sperm for subsequent fertilizations. This ability of females to store sperm is a common feature of the reproductive physiology of not only poeciliids, but of many species across many phyla. In order to understand the mechanisms that underlie the competition between sperm in the female reproductive tract, it is necessary to know in more detail the basic morphology of the storage sites in the ovary. The occurrence of sperm competition can be established through the observation of within brood multiple paternity. However, the mechanism for the observed patterns of paternity can only be established after analysis of the environment (the female reproductive tract) in which this competition occurs. However the exact morphology of the seminal receptacle or the ovarian cavity has not yet been investigated. It was therefore decided to attempt to establish if the ovarian cavity is merely a linear tubule or if side pockets or branches run from the main cavity. In order to provide a fuller understanding of the possible adaptations that exist for sperm storage, and to highlight the range of means by which female retain viable sperm, I shall firstly review the literature on sperm storage in other species. I shall then outline the methods used in this study and provide details of the distribution of sperm in the ovary of *P. reticulata*. Finally the potential implications of sperm storage and distribution for sperm competition in the guppy are discussed. I shall continue by reviewing the available information on sperm storage firstly in invertebrates and subsequently in vertebrates.

Sperm storage in invertebrates

Sperm competition has yet to be extensively studied in the majority of invertebrates. Data has been collected on arachnids (Austad 1984; Thomas and Zeh 1984; Watson 1991a, b; Zeh and Zeh 1994) and molluscs (Cigliano 1995; Vianey-Liaud 1995; Todd *et al.* 1997). However the most comprehensive studies have centred on insects and it is on one particular member of this phlyum that I shall concentrate.

Insects

The volume of literature that has been published on the mechanisms of sperm storage in insects is too large to be covered in this thesis. I will therefore concentrate on one of the best studied groups, *Drosophila*, although sperm storage has been documented in several other orders.

Female *Drosophila* have three sperm storage organs: the dorsal, paired spermathecae and the ventral seminal recepticle (Fowler 1973). Each of these spermathecae consists of a hemi-spherical cup, the inside walls of which are formed of a pigmented cuticular layer. This is surrounded by a single layer of epithelial cells and a second covering of endocrine cells. The glandular cells secrete directly into the lumen of the spermatheca following insemination. It has been suggested that this substance acts as a nutrient for stored sperm (Gromko *et al.* 1984). The spermathecae are connected by ducts to the anterior, dorsal end of the uterus. In addition to the spermathecae, females also have a separate storage site, the seminal receptacle. This long tightly coiled tube lies against the anterior end of the uterus, is composed of cuboidal epithelial cells and opens into the uterus. The seminal receptacle is surrounded by a layer of muscle and nerve fibres run between the coils (Fowler 1973).

Whilst the anatomy of the female reproductive tract has been extensively investigated, the movements of sperm in the uterus are still unclear. Sperm are ejaculated into the anterior portion of the uterus, immediately surrounded by the ducts to the storage organs. However *in vitro* observations of sperm have shown that directional propulsion is rare. It appears that sperm from several *Drosophila* species may have such disproportionately long tails that they are no longer able to swim (Lefevre and Jonsson 1962). Observations of sperm in the female genital chamber reveal that sperm appear to be moving in channels, possibly micro-tubules in the seminal fluid (Fowler 1973; Gromko *et al.* 1984). The muscular nature of the genital chamber may also contribute to sperm movement (Fowler 1973). Males of *Drosophila subobscura* produce distinct classes of short and long sperm. The importance of variation in tail length is not yet clear. In an average ejaculate of around 20,000 sperm, 66% were short and 34% long. Thirty hours after mating, the receptacle and spermathecae were found to contain a total of around 900 sperm (Bressac and Hauschteck-Jungen 1996). Interestingly it appears that females preferentially selected to store long sperm. After females had been allowed to lay eggs for seven days, only one third of the initial amount of sperm remained in the storage organs. During this time the percentage of short sperm increased suggesting that long sperm were preferentially used for fertilization (Bressac and Hauschteck-Jungen 1996).

Sperm storage in Vertebrates

With few exceptions, some degree of sperm storage within the female reproductive tract occurs in most vertebrate orders with internal fertilisation (Smith 1984). However, as already detailed with invertebrates, the manner in which sperm storage operates and the nature of the adaptations and counter adaptations are extremely diverse.

Fish

As the majority of fish species are external fertilizers, sperm storage in the phylum is restricted to relatively few species. However internal fertilization and some degree of subsequent sperm storage has been recorded in several families of fresh water teleost (table 5.1).

In addition, internal fertilization is universal amongst chondrichthyan fishes (Dodd 1983) and also occurs in sea water teleosts (e.g. Embiotocidae and Cottidae). Internal fertilization can be followed by either the release of fertilized eggs (e.g. *Trachycoristes*, von Ihering 1937), larvae (*Sebastodes paucispinus*, Moser 1967), juveniles (Poeciliidae, Turner 1947) or even sexually mature offspring (*Cymatogaster aggregata*, Wiebe 1968). Despite the diversity in modes of gestation in internally fertilizing fish very little is known about *in vivo* sperm storage. Instead the majority of research has concentrated on the artificial preservation and storage of stripped sperm samples from commercially important species.

Amphibians

The amphibians are split into three orders; of these the anurans (frogs and toads) are mostly external fertilizers, while the majority of the apodans (caecilians) and urodeles (newts and salamanders) are internal fertilizers. The morphology of the storage sites in newts and salamanders has been studied extensively. In general, male urodeles

- Clupeiformes
    - Pantodontidae

- Cypriniformes
    - Characidae
    - Cyprinidae
    - Auchenipteridae

- Beloniformes
    - Hemiramphidae

- Cyprinidontiformes
    - Adrianichthyidae
    - Goodeidae
    - Jenynsiidae
    - Anablepidae
    - Poeciliidae
    - Tomeuridae
    - Horaichthyidae

- Phallostethiformes
    - Phallostethidae
    - Neostethidae

- Perciformes
    - Embiotocidae
    - Comephoridae

Table 5.1 Families of freshwater teleost in which internal fertilization has been recorded.

deposit spermatophores with are then taken up into the female cloaca (Haliday 1976). From here sperm are transferred, possibly by cilia (Pool and Hoage 1973) or along a chemical gradient (Boisseau and Joly 1975), to the adjacent spermatheca. Two basic types of spermathecae have been distinguished. The two morphological types are characterized by differences in the way that the tubules join the cloaca. For example in *Necturus beyeri* the numerous tubules are short and open directly onto the roof of the cloaca. In contrast, the tubules of *Eurycea quadridigitatus* have relatively few tubules which open into a common duct which then opens onto the cloaca (Boisseau and Joly 1975). While the gross anatomy of urodele spermathecae shows marked interspecific variation, the microscopic structure is comparatively uniform. The tubules have an inner lining of epithelial cells and an outer lining of myoepithelial cells. The epithelial cells possess secretory vacuoles containing glycoproteins. These molecules are released into the lumen and bathe the stored sperm (Sever and Bart 1996). It has been suggested that the inner lining cells provide nutrition for the sperm during storage, while the myoepithelial cells allow contraction of the tubules and the expulsion of sperm prior to fertilization (Dent 1970). If this is the case then the ability of the female to produce glycoproteins may be the factor that limits the duration of sperm storage (Sever and Bart 1996).

In *Notophthalamus viridescens* the sperm are found in close contact with the epithelial cells (Dent 1970), while in *Diemyctylus pyrrhogaster* the sperm were found buried into the epithelium (Tsutsui 1931; cited Halliday and Verrell 1984). Once stored, sperm can remain viable for considerable periods. In many urodeles there is an interval of up to 8 months between mating and oviposition. In *Salmandra salamandra* sperm are known to remain viable for at least a year and possibly upto two years (Boisseau and Joly 1975). Such prolonged storage has been proposed as further evidence that the epithelial cells provide nourishment for the sperm (Halliday and Verrell 1984). However it has also been reported that in *N. viridescens* (Dent 1970; Sever *et al.* 1996) and *Plethodon cinereus* (Sever 1997), the spermathecal epithelial cells ingest sperm by phagocytosis and that long term storage may allow less viable sperm to be selected out (Halliday and Verrell 1984).

Reptiles

Ectothermy in reptiles, and fish and amphibians, may account, in part at least, for their protracted duration of sperm storage. One consequence of ectothermy and a low metabolic rate is that it can result in very long reproductive cycles. Because the interval between mating and egg-laying or birth can be protracted, the optimum time for copulation may not correspond with the optimal birth season. Under these conditions selection may favour the uncoupling of copulation, fertilisation and egg-laying or birth. This can be achieved by storing sperm, and females can then ovulate and fertilize their eggs at a time that results in young being born when their chances of survival are greatest. Consequently it is not too surprising to find that the longest sperm storage duration for vertebrates come from the reptiles. Sperm storage has been studied in several groups including crocodilians (Davenport 1995), turtles (Gist and Jones 1989; Gist and Fischer 1993), lizards (Conner and Crews 1980; Ruth Shantha Kumari *et al.* 1990; Whittier *et al.* 1994; Srinivas *et al.* 1995) and snakes (Aldridge 1992). In contrast to other phyla, the adaptations for sperm storage are limited, and across taxa sperm receptacles are most common in the vaginal and infundibular regions (Gist and Jones 1989). Tubular pockets exist in both the infundibulum and the posterior region of the vagina, and it is in these regions that sperm are found with the acrosomal portion of the sperm perpendicular to the epithelium (Whittier *et al.* 1994).

Birds

In birds, the primary sperm storage site, the area containing the sperm storage tubules (SSTs), is low down in the reproductive tract at the utero-vaginal junction. Shugart (1988) and Schuppin *et al.* (1984) both described the storage sites in the utero-vaginal junction as sperm storage 'glands', however unlike the amphibian spermathecae there is no evidence that they have any secretory ability (Bakst 1987). Mero and Ogasawara (1970) first applied the now universal term sperm storage tubules. These tubules have been found in a number of species and may prove to be ubiquitous among birds (Birkhead 1988; Shugart 1988; Birkhead and Møller 1992; Birkhead and Petrie 1995).

The sperm storage tubules are situated within folds of the lumen of the oviduct at the utero-vaginal junction. In all species examined so far the sperm storage tubules have a similar anatomical structure; they are branched or unbranched tubules in the lamina propria of the mucosal folds, lined by tall columnar cells with basal nuclei and apical microvilli. The sperm storage tubules have a well developed blood supply but unlike the spermathecae of many insects they have no contractile elements or nerve fibres (Gilbert 1979). Avian sperm storage sites share the same microscopic structure but show considerable interspecific variation in their number, length and degree of branching. Following copulation sperm are deposited within the vagina and travel up the oviduct. Sperm begin to enter the storage tubules in the utero-vaginal junction about one hour after mating (Bakst 1981) and, in the turkey, sperm may continue to enter the sperm storage tubules from the vagina for 48 hours following insemination (Brillard and Bakst 1990). Only viable sperm successfully enter the sperm storage tubules (Bakst 1981), although it is not known precisely how non-viable sperm are excluded. In the turkey, only around 10% of sperm transferred during insemination are retained in the female tract (Brillard and Bakst 1990). The rest are subsequently eliminated through the cloaca (Howarth 1971).

The mechanism by which sperm survive after storage is unclear (Zavaleta and Ogasawara 1987). Stored sperm are found at the distal end of the tubule, with their heads oriented towards the blind end of the tube. In some species the sperm are stored as head to head agglutinations (Van Krey *et al.* 1981). Van Krey *et al.* (1981) suggested that, in some avian species, this process may play an important role in sperm storage and release. He suggested that as sperm in the storage tubules age, their ability to agglutinate decreases. Some sperm may then break away from the bundle and be released in to the oviduct. In contrast to some other species, there is no evidence that sperm associate with, and receive nutrients from, the epithelium of the storage site. The mechanism underlying the subsequent release of stored sperm is therefore unclear. Some authors have suggested that the mechanical process of ovulation or oviposition triggers the release of sperm from sperm storage tubules (Bobr *et al.* 1964). Alternatively it has been proposed that as the bundles break down, sperm leave the storage tubules more or less continuously, so that there is a constant supply of sperm at available to fertilize eggs (Brillard and Bakst 1990; Wishart 1987).

Mammals.

The duration of sperm storage in most mammals is relatively short compared with other phyla. As fertilization generally occurs within 24 hours of mating, sperm only survive for a short period in the female tract (Gomendio and Roldan 1993). Generally, female mammals lack the specialised sperm storage organs that allow prolonged storage as described for insects and birds. However, some species are capable of sperm storage for extended periods.

The ability of monotremes to store sperm is still controversial. It has been suggested that storage may allow delayed use of sperm from matings before hibernation in both the echidna (Geiser and Seymour 1989) and the platypus (Griffith 1978). In marsupials extended periods of sperm storage (2 – 3 weeks) have been reported for three families. In both the Dasyuridae (Selwood & McCallum 1987) and the Didelphidae (Bedford *et al.* 1984) sperm are stored in specialised crypts in the isthmus. While in the Peramelidae (Bedford *et al.* 1984; Selwood & McCallum 1987) sperm storage occurs in the vaginal caeca.

Several studies of eutherian mammals have provided evidence of extended sperm storage. In the European hare (Martinet and Raynaud 1975), stored sperm remain fertile throughout the 40 day gestation period. While in the domestic dog, motile sperm have been found in the uterine glands for up to 11 days after insemination (Doak *et al.* 1967). In general, mammals appear to store sperm for relatively short periods, however some species of bat store sperm for nearly 200 days (Fenton 1984;

Hosken 1997). The forces leading to extended sperm storage in this group are unknown. It has been suggested that prolonged sperm storage allows females to mate before hibernation and delay fertilization, thus temporally separating mating and birth (Fenton 1984). However sperm storage also occurs in tropical non-hibernating species suggesting that this is unlikely to have been a selective force favouring storage.

Aims of the study

The female guppy's ability to store viable sperm has potential implications for several key aspects of the biology of *P. reticulata*, including the determination of paternity, the evolution of male and female mating behaviour and, in some circumstances, the maintenance of genetic diversity. Whilst sperm storage has been studied in several species drawn from diverse groups, accurate information on the internal morphology, both on a gross and more detailed microscopical level, is lacking for internally fertilizing fish. This information is vital if we are to begin to understand the interactions that occur between ejaculates within the female reproductive tract. The adaptive value of the behavioural strategies of males will only be fully understood following advances in knowledge of the anatomical and physiological mechanisms that come into play after copulation. As has been shown there is undoubtedly great potential for sperm competition in the guppy. Females mate promiscuously whilst receptive (Houde 1987) and in addition receive frequent sneaky mating attempts (Magurran & Seghers 1994a). The outcome of this contest will be decided by many factors, not least the positioning of the sperm within the ovary. For example, the size and shape of the storage organs may help to explain the patterns of paternity, either through sperm mixing as in rats (Dewsbury 1984) and field crickets (Parker *et al.*. 1990) or sperm precedence as in spiders (Austad 1984) or certain butterflies (Gwynne 1984b). This study therefore investigated the anatomy of the ovary of *P. reticulata*, with the aim of aiding the understanding of patterns of paternity and the determining the fate of sperm received from sneaky matings. In addition to investigating the more obvious, larger scale features of the ovary, finer scale microscopical features were also analysed.

METHODS

The procedures detailed below were followed in the course of this experiment.

Inks and corrosion casting

In an attempt to investigate the gross internal morphology of the ovary and to establish the position of potential sperm storage areas, several attempts were made to introduce coloured dyes or inks into the ovary. The aim was to flood the lumen of the ovary with a clearly identifiable medium, which would allow subsequent observation of the areas to which sperm have access. Two easily identifiable colours of commercially available India inks (blue and black) and a 5% solution of methylene blue were selected. Females were humanely killed and the micropipettes designed for recovering sperm from females (Chapter 4) were used to introduce the dye into the female reproductive tract. Once again these were machine pulled from glass capillary tubes (Clark Electromedical Instruments) and the end removed with a diamond-tipped pen. A drop of dye was taken up, and the capillary tube introduced into the opening of the female's genital pore. The fluid was then expelled into the ovarian cavity. The female was left for 30 minutes, and after this time an incision was made along the ventral surface, allowing the ovary to be visualised.

Subsequently it was decided to produce corrosion casts of the lumen of the ovary. Corrosion casting involves the introduction of an acrylic resin into the cavity or vascular system to be studied, this is then allowed to set and the surrounding tissue removed (Lametscwandtner *et al.* 1990; Aharinejad and Lametscwandtner 1992). In order to reduce the viscosity of the ovarian fluid and allow the resin to enter the ovary more easily, the ovary was first thoroughly flushed out with a physiological ringer solution (NaCl, 207mM; KCl, 5.4mM; $CaCl_2$ 1.3mM; $MgSO_4$, 0.41mM; $NaHCO_3$, pH 7.6). The acrylic casting material (Mercox CL-2B, Ladd Research Industries Inc., Vermont, USA) consists of two components, a resin and a catalyst. Once the two components are mixed they have a relatively short working time before they solidify. Therefore the catalyst was only added to the resin after the ovary had been fully prepared. 1ml of resin was combined with 24mg of catalyst and the two components mixed. This was then taken up in to the pipette and, ensuring that no air bubbles were present, the casting material was expelled into the ovary.

Scanning electron microscopy

6 males and 6 females were randomly selected from mixed sex stock tanks, humanely killed and their testes and ovaries respectively removed. Males were also sampled to provide an indication of the appearance of sperm under SEM, although information was also gained on the structure of the spermatozeugmata. Tissues were fixed in 2.5% gluteraldehyde phosphate buffer (9ml insect buffer (0.408g anahydrous KH_2PO_4 + anahydrous Na_2HPO_4 in 500ml distilled water), 1ml 25% gluteraldehyde, 0.06g sucrose) prior to being dehydrated using the protocol in appendix 5.1. Three of the dehydrated ovaries and three of the testes were cut into sections to allow the visualisation of their interiors. All samples were lowered into specimen holders and placed in the chamber of a Samdri 780 critical point dryer (Tousimis Research Corporation, Rockville, Maryland, USA). The chamber of the critical point dryer was then flooded with liquid CO_2, cooled and maintained at 0°C. The chamber was flushed and purged with fresh cold CO_2, so that all traces of ethanol were removed. The chamber and samples were then heated to the critical temperature, while the volume of the chamber was held constant. This causes a rise in pressure within the chamber and the liquid CO_2 enters the gaseous phase. Finally the gaseous CO_2 is exhausted from the chamber and the samples slowly returned to room temperature and pressure. Samples were fixed to microscope mounting cores, sputter coated with a fine covering of gold and placed in the electron chamber for viewing.

Haematoxylin and eosin staining

Four females were taken from mixed population stock tanks. As the tanks contain males and females, it was assumed that all females would have stored sperm. Females were humanely killed in iced water and an incision made along the ventral surface. This allowed the ovary to be visualised and the entire area of the body containing the ovary and urogenital sinus was then removed. These were fixed whole in Bouins solution (5ml glacial acetic acid, 25ml 40% formaldehyde solution, 75ml saturated picric acid aqueous solution) for twenty four hours then rinsed with, and stored in, 70% ethanol at 4°C until processed further.

Preparation for wax sectioning

The whole sections underwent dehydration through a series of alcohols to xylene as detailed in appendix 5.2 in preparation for sectioning. Each ovary was then embedded in paraffin wax (BDH, Glasgow, solidification point 51-53°C) and maintained at 55-57°C for at least 2.5 hours to allow the wax to penetrate the tissues before being allowed to cool and harden overnight.

Sectioning and mounting

The embedded samples were sectioned to 7μm using a microtome, then mounted upon glass slides. Strips of wax containing between three and five sections were floated on the surface of a gelatinised waterbath at 43°C (distilled water, 1g gelatine and a pinch of potassium dichromate) to flatten out before being coaxed onto slides and left to dry at 40°C overnight.

Rehydration

The sections were dewaxed and rehydrated through xylene and ethanol solutions to deionised water following the protocol in appendix 5.3.

Histological staining

The sections were stained with the nuclear stain haematoxylin. Cytoplasm was then counter-stained with the anionic xanthene dye, eosin, before undergoing a further dehydration to xylene as detailed in appendix 5.4.

Photography

Using 64T slide film (Eastman Kodak Company, New York) photographs were taken of the areas containing sperm within the ovary (Dialux 20 microscope, E. Leitz Instruments Ltd., Wetzlar, Germany; Wild MPS 45 photoautomat and Wild MPS 51S SPOT shutterpiece, Wild Heerbrugg Ltd., Heerbrugg, Switzerland).

RESULTS

Unfortunately, while the introduction of dyes into the ovary did provide some indication of the complexity of the lumen, it was not possible to clearly envisage the exact form of the numerous offshoots from the main cavity. A main tubule running along the dorsal side of the ovary was clear in all females into which dye was successfully introduced. However, the presence of other substances in the side branches of this tubule seems to have prevented any further diffusion of the coloured inks or dyes. A subsequent investigation, in which the females were left for longer before dissection found no evidence of the dyes having advanced.

The use of the casting medium proved to be even less successful and failed to provide any information on the morphology of the ovary. It was hoped that the use of resin-catalyst corrosion casting would provide detailed information on the presence of side branches or pockets of the lumen. The small diameter particles should in theory have entered even the narrowest of tubules and eventually solidified. Once the surrounding tissue had been removed it was hoped that the volume of these casts could be measured and analysed using scanning electron microscopy. On several occasions it appeared that the Mercox had been successfully introduced into the lumen. However upon dissection it was consistently found that the resin-catalyst mix had entered the body cavity. Despite attempts at flushing out the viscous ovarian fluid from the lumen, the introduction of the Mercox seems to have increased the pressure within the ovary, leading to the bursting of the lumen wall. This then allowed the catalyst to spill out in to the body cavity and resulted in the casts failing to fully flood the areas of interest.

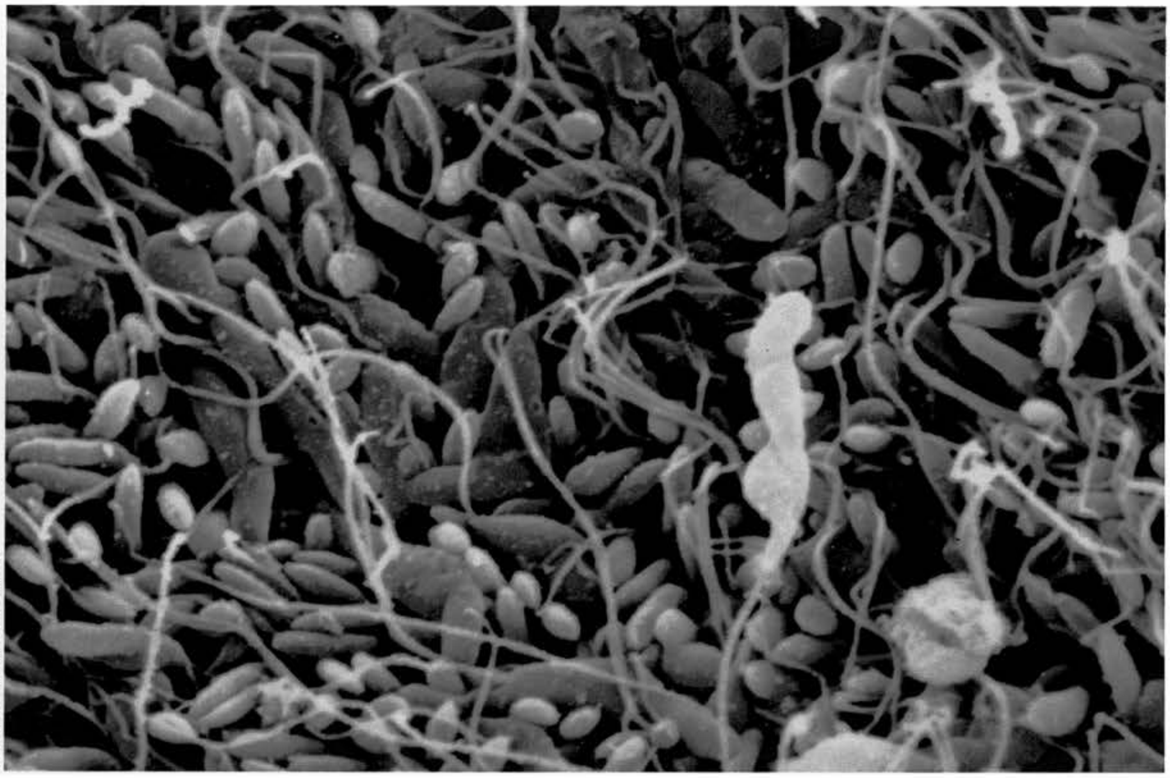
While the SEM samples provided useful information on the appearance of sperm in the testes and the structure and packaging of spermatozeugmata (figure 5.1), no sperm could be found in any of the six ovaries. As these females were all sexually mature and had been in recent contact with males it seems safe to assume that sperm were present. Further the analysis of the six males' testes provided an indication of the form in which sperm would appear following preparation for scanning electron microscopy. Knowing the form in which the sperm would be found, it seems unlikely that they would have been overlooked. It must therefore be the case that although sperm were present, they were not made apparent by this SEM based approach. It is possible that alternative approaches, such as confocal laser microscopy, may prove more successful.

Analysis of the histological sections with a light microscope provided information of the anatomy of the ovary. The ovaries of the four females were found to contain yolked but unfertilised oocytes that, following the methods of chapter 4, would have lead to the females being classified as belonging to stage 1. The ovary in *P. reticulata*, as in all poeciliids, consists of two paired ovaries fused into a single, large organ. The ovary is heavily vascularized and dorsally suspended occupying a major portion of the body cavity (Turner 1937; Rosen and Bailey 1963; see figures 4.1 - 4.4 in previous chapter for photographs of ovaries containing various developmental stages of embryo). The ovary consists of relatively few tissue types (Constantz 1989), and comprises primarily of an external sac, formed by peritoneal folds (Wourms

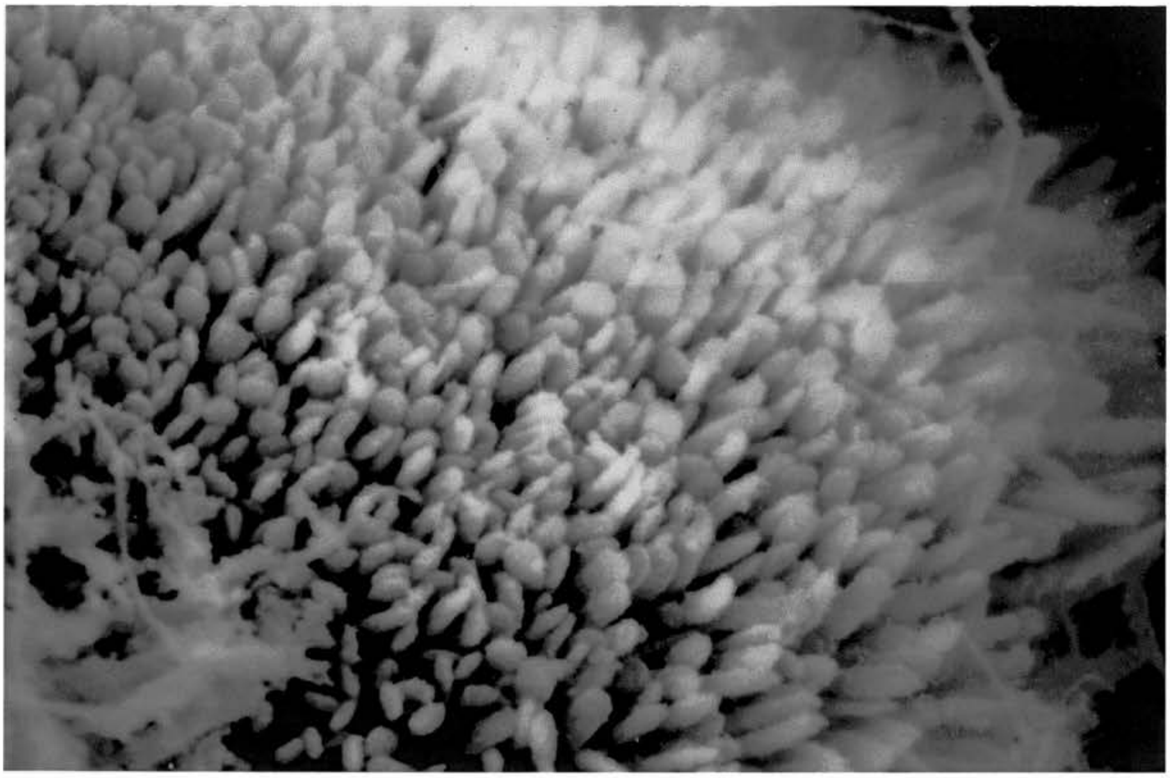
Fig. 5.1a Scanning electron micrograph of sperm in the testis of a Trinidadian guppy.

Fig. 5.1b Scanning electron micrograph of the surface of a spermatozeugmata. Each bundle contains around 16,000 sperm, which are packed in tail-tail agglutinations prior to ejaculation.

Figure 5.1



Photograph by Iain Matthews



Photograph by Iain Matthews

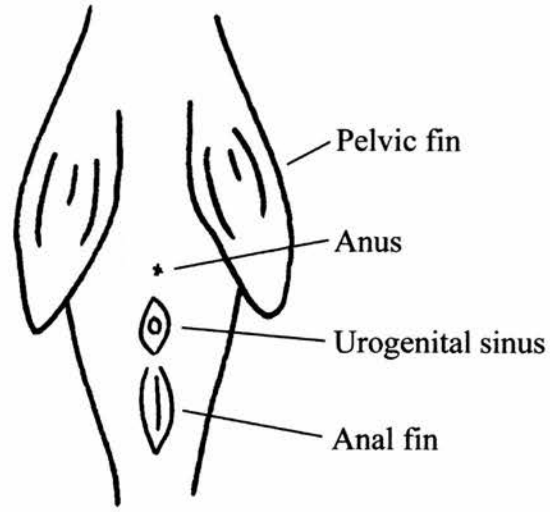
1981), and an inner lumen. Within the ovary the lumen occupies mainly the postero-dorsal region and connects to the urogenital sinus via a short duct, which exits just posterior to the anus (figure 5.2a and b). It is in to this opening (the urogenital sinus) that the tip of the gonopodium is thrust and sperm released during copulation (Kadow 1954). It has been suggested that the hooks on the gonopodium may damage the lining of the urogenital sinus (Constantz 1984). However, no evidence was found of any tearing or bleeding in the urogenital sinus of any of the females in this study. The duct from the urogenital sinus proceeds anteriorly to form the lumen of the ovary. The complete ovary has a shape resembling a tear-drop broadening towards the anterior end (figure 5.3). In the posterior portion of the ovary the oocytes are lateral and ventral to the lumen, but more anteriorly, where the ovary is wider and the oocytes more densely packed, they restrict the lumen. In this area of the ovary, projections of the lumen were found extending amongst the oocytes. These localised expansions of the lumen epithelium run down to, and make contact with the oocytes, and are referred to as delles (Winge 1922a). The distribution of these delles varied between females and does not appear to follow any set pattern, however in all cases they appear to terminate in close proximity to the oocytes.

Oocyte distribution within the ovary is correlated with oocyte maturity. In sections that were histologically prepared, as well as in the ovaries of females analyzed in chapter 4, the smallest unyolked oocytes occurred centrally in the ovary, in some cases lying amongst the developing embryos. Sperm were clearly visible in several areas of the lumen and while no quantitative measurements were taken, abundances appeared highest in the delles themselves (figure 5.4). Not all delles were found to contain sperm. Sperm were also found, but in lower densities, in association with the dorsal epithelium of the lumen. However no sperm were found in the anterior blind-ending tubule formed by the lumen.

Fig.5.2a Diagrammatic representation of the ventral surface of a female guppy, showing the external genitalia. Modified from (Constantz 1989).

Fig 5.2b Diagrammatic representation of the relative positions of the digestive, urinary and reproductive tracts. Modified from Constantz (1989).

Figure 5.2


Pelvic fin
Anus
Urogenital sinus
Anal fin

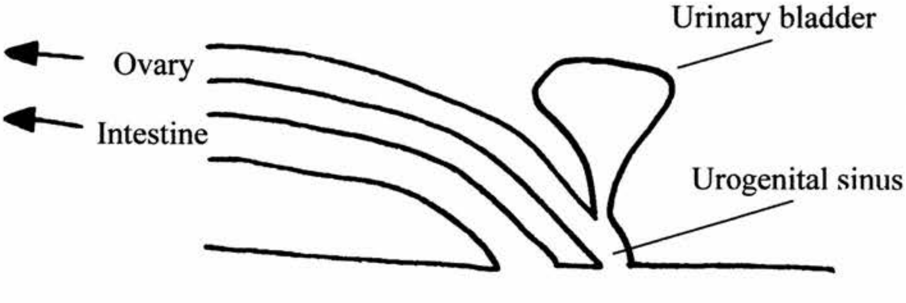

Urinary bladder
Ovary
Intestine
Urogenital sinus

Fig 5.3 Longitudinal section of the guppy ovary, illustrating the positions of the main features mentioned in the text.

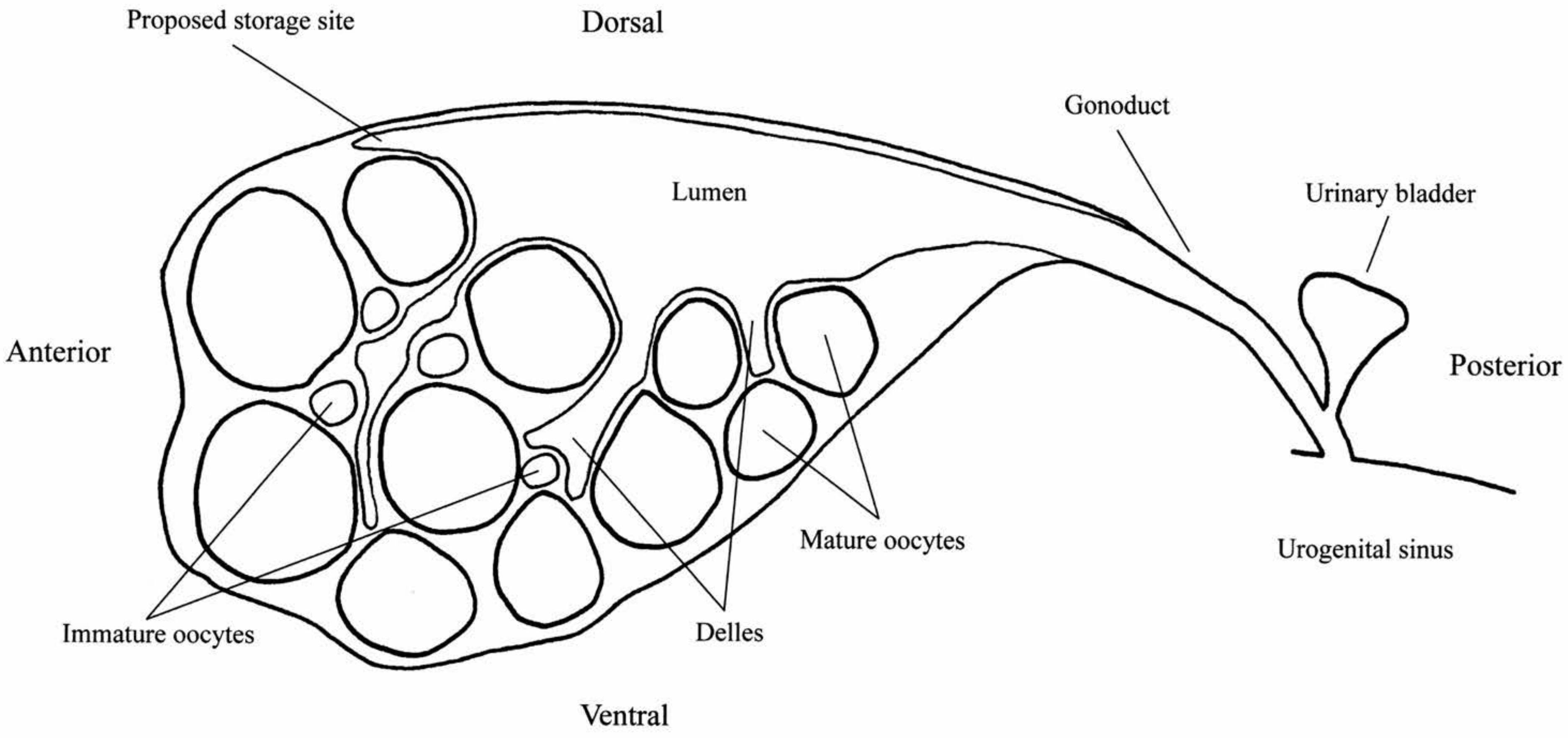
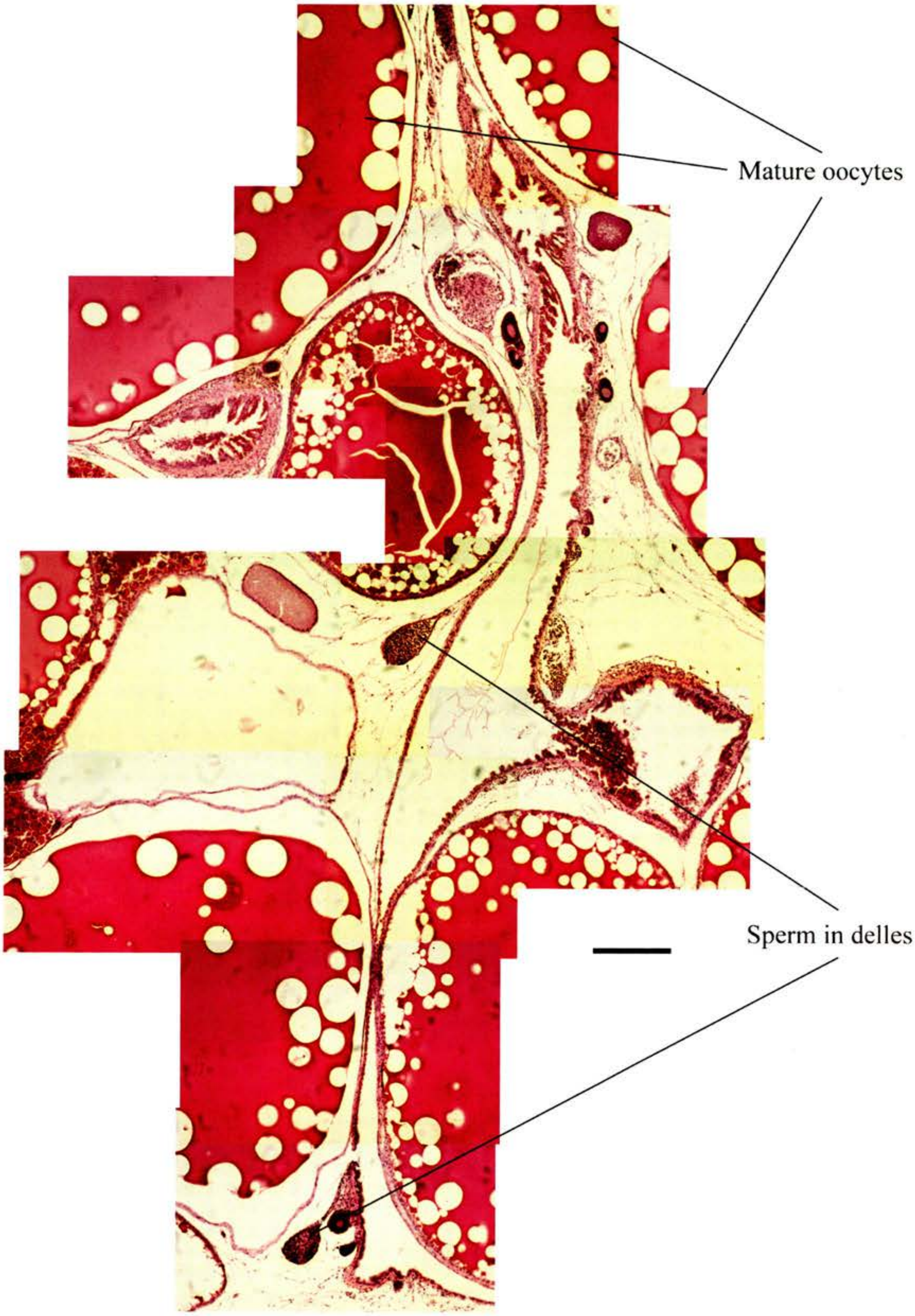

Proposed storage site
Dorsal
Gonoduct
Lumen
Urinary bladder
Anterior
Posterior
Mature oocytes
Urogenital sinus
Immature oocytes
Delles
Ventral

Figure 5.3

Fig 5.4 Reconstructed montage of a section of the ovary highlighting the position of oocytes and sperm. Scale bar = 200µm.


Mature oocytes
Sperm in delles

DISCUSSION

Several studies have investigated reproductive adaptations for viviparity in poeciliids (Turner 1940, 1947; Thibault and Schultz 1978; Wourms 1981). This chapter represents the first concerted attempt to investigate the adaptations allowing long term storage of viable spermatozoa in the guppy. Particular attention was paid to the structure of the ovary and the presence of any side branches of the lumen, as these areas may have implications for sperm competition and the order of sperm utilisation.

Despite there being only a few tissue types (Constantz 1989), the ovary of *P. reticulata* is an extremely complex organ that serves several functions. Not only is it the site of oogenesis, but also it is the site of fertilisation. In addition, it provides the necessary requirements for internal embryo development. In many poeciliids, only gas exchange and protection are provided by the ovary, but in other species, including the guppy, the ovary also provides the nutritional requirements of the embryo (Thibault and Schultz, 1978; Wourms 1981).

The mode of viviparity seen in the guppy can only occur in conjunction with special modifications for fertilisation and sperm storage (Thibault and Schultz 1978). As the oocyte does not enter the lumen before fertilisation, there must be some alternative way for the sperm to reach the unfertilized eggs (Turner 1947). In the guppy, this problem is apparently solved by the contact between extensions of the lumen and oocytes. The intimacy of the association between the oocyte and the extension of the lumen provides a means by which the sperm can be transported to the oocytes. The delle typically terminates directly onto the follicle and during fertilization it has been reported that the terminal end of the delle and the adjacent area of the follicle break down (Nagahama 1983). The follicle (glandular granulosa) surrounding the eggs in *P. reticulata* is continuous and does not have a micropyle, the opening through which the sperm pass before reaching the zona pelucida (Laale 1980). The breakdown of the two regions that separate the sperm from the egg may therefore be an adaptation to overcome the absence of a micropyle. Indeed in viviparous species in which the egg does have a micropyle the formation of delles does not occur (Nagahama 1983). These sites will undoubtedly be important in determining the outcome of sperm competition. As this region is as close as the sperm can get to the eggs until the breakdown of the final wall before the egg, then the ejaculate that can gain numerical predominance in these sites will presumably have a statistical advantage when it comes to gaining successful fertilizations.

The development of the tubules and oocytes has not been described. However, it appears that the association develops early on in the reproductive cycle, as tubules were found adjoining even immature oocytes. Presumably the extensions of the lumen regrow and become associated with the new oocytes prior to the fertilization of each brood. Unfortunately, nothing is known about temporal changes in tubule occurrence or distribution. Further, it is not known at what age the delles first form. In turkeys the sperm storage tubules develop early and are present at 30 weeks of age, well before the oviduct and ovaries have matured (Bakst 1987). Fulmars have also been found to possess SSTs before their ovaries have fully developed (Hatch 1983). It would be interesting to know at what stage the lumen of the ovary in juvenile females develops a system of delles.

In chickens not all sperm storage tubules receive sperm, regardless of the number of inseminations (Bakst 1989). These findings have lead to the speculation that, in birds, females might have the ability to control which sperm storage tubules are available for use and may therefore be able to influence the outcome of sperm competition (Birkhead and Møller 1992). In this chapter, sperm were found in tubules of all females studied. Sperm do not appear to discriminate between tubules and were found in delles associated with unfertilized oocytes of varying age. The mechanism by which sperm enter the delles is not known. In birds, the epithelium of the utero-vaginal junction is covered in microvilli, and the cells at the entrance of the sperm storage tubules are ciliated. These two in combination probably play an important role in drawing sperm into the sperm storage tubules. No evidence was found of any similar structures in the guppy, and it is not possible to conclude from this chapter, whether females have the ability to influence the distribution of sperm between delles. Further work is needed to investigate this possibility. Given the structural complexity of the guppy ovary, if females were able to control sperm movement the implications for sperm competition would be profound.

Kadow (1954) and Jalabert and Billard (1969) both identified diverticulae in the antero-dorsal portion of the ovary. They reported the existence of an association between the spermatozoa and the plasma membrane of the lumenal epithelial cells in this region, which suggests that this area may be a site of long term storage. Unfortunately there was no evidence to support these earlier studies from the females examined in this chapter. An anterior blind-ending tubule formed by the lumen was present in the region referred to by these papers, but there was no evidence of any sperm in this area. Lack of time prevented the sectioning of further females, although this may provide information to support the notion of sperm storage in a more general seminal receptacle.

Despite not finding any general sperm storage site, the reproductive biology of female guppies suggests that such an area must exist. We know that at least 6 broods can be fertilized from a single insemination (Carvalho *et al.* 1996). Yet, if sperm were stored solely in the tubules, the eggs of several broods would have to be present in the ovary when fertilization occurred. However, guppies show no evidence of superfetation (two or more broods at different stages of development within a single female (Thibault and Schultz 1978; Wourms 1981)). Also as is documented in the following chapter, whilst sperm from a recent mating fertilize the majority of the next brood, paternity of the subsequent broods is dominated by the stored sperm. Again, if the tubules were the sole sperm storage sites, this change in patterns of paternity could be accounted for only if sperm from re-inseminations were selective against tubules associated with the least mature oocytes. This does not appear to be the case. Chapter four showed that sperm can be manually removed from the ovary for up to seven days following insemination. After this time the procedure used did not recover sperm.

As mentioned, nothing is known about how the sperm enter the tubules and in addition nothing is known about the viability of sperm in the delles. If sperm viability outside of the specialised area of sperm storage is short, then even though sperm entering the tubules early may gain dominance of the key site allowing access to the eggs, these sperm may no longer be viable by the time the sperm are released onto the ova. Curiously and in contrast to this findings of this chapter, Jalabert and Billard (1969) do not mention finding any sperm in the tubules. They conclude that the only site of sperm storage is in the alveolated diverticulae of the antero-dorsal portion of the ovary, a finding with which this chapter disagrees. In some cases, sperm were also found associated with the epithelium of the main portion of the ovarian lumen, particularly with the dorsal epithelium. However, due to low sperm density and irregular sperm orientation in these structures, it seems unlikely that this area is an important site of sperm storage.

The most tenable model at this time is that a more general sperm storage receptacle exists in addition to the tubules. At copulation, a portion of the sperm move to the tubules and a portion move to a receptacle in the antero-dorsal portion of the ovary. Sperm in the tubules fertilize oocytes as they mature. After parturition, another copulation again sends sperm to both regions of the ovary. Those going to the tubules gain access before the oocytes mature, and as the oocytes mature, these sperm fertilize them before sperm from the previous insemination can gain access to the oocytes. Those sperm from the re-insemination which enter the sperm storage receptacle are stored in a position that gives them a competitive edge over already existing sperm, and thus gives them precedence if a new insemination does not occur. As the sperm from the most recent insemination are used up, sperm from previous inseminations can again increase their success in fertilizing eggs. An examination of how sperm from different inseminations are distributed in the antero-dorsal ovarian storage site would give some insight into this problem.

A virgin female will obviously have no sperm in her reproductive tract. In a situation where such a female receives a single mating, some of the sperm inseminated will enter the tubules and become positioned at the distal ends of the delles. The remaining sperm will either perish or will be stored in the epithelial lining of the lumen or in the antero-dorsal storage organ (Jalabert and Billard 1969). When the oocytes are fully yolked, the end of the delles break down allowing the sperm access to the eggs. If after the brood are born, the female fails to remate then there will be no sperm in the tubules to fertilize the next batch of oocytes. The release of sperm from storage will allow sperm from the previous ejaculate to move through the ovary and into the key area of interest, the delles.

Alternatively, if the female remates after parturition then as before, a portion of the sperm will move into the tubules and the remainder will be stored. At the time of fertilization the sperm in the tubules from the new ejaculate will reach the unfertilized eggs before rival stored sperm from previous matings. The newly received sperm will have gained precedence as a result of numerical superiority at the site of fertilization. If sperm from storage are released only as a last resort following failure to remate (i.e. they are released shortly before fertilization), then any sperm that are inseminated after the delles have been formed will be able to gain an advantage. The sperm of the first male to mate after the formation of the tubules will have the chance to dominate close to the site of fertilization. If delles are formed while the female is still non-receptive then sneaky mating may provide a means by which a male can ensure that his ejaculate has a numerical advantage in the subsequent competition between new and stored sperm.

The anatomy of the ovary, and in particular the oocyte's lack of a micropyle, has resulted in the necessity for an adaptation to bring sperm and egg into contact. This problem is overcome by the formation of extensions of the lumen (delles). These tubules are the key sites in which the competition for fertilizations will occur. The outcome of sperm competition will be determined in part by the proportions of sperm from different ejaculates in the delles. This in turn will be decided by the timing of delle formation, the timing of new inseminations and the release of stored sperm. These factors require investigation to increase our understanding of how the reproductive physiology of the influences the success of male behavioural strategies such as sneaky mating.

Chapter 6

Multiple paternity and sperm precedence patterns in the guppy

INTRODUCTION

Sperm competition and the mechanisms underlying patterns of sperm precedence have yet to be fully investigated in any taxa (Birkhead and Hunter 1990), but the information available for fish, especially internal fertilizers, is particularly scarce. As is typical throughout the phylum, little is known about patterns of paternity in the Poeciliidae. During their period of receptivity, it has been shown that female guppies choose to mate with several males (Houde 1987). High frequencies of sneaky mating attempts (Magurran & Seghers 1994a) will introduce sperm from yet more males into the female reproductive tract. Sperm competition will be an inevitable consequence of these behaviours. Indeed multiple paternity has been recorded for several members of the family, confirming the occurrence of sperm competition (Constantz 1984). In conjunction with the analysis of the anatomy of the ovary (chapter 5), it was decided to undertake an experiment to investigate patterns of paternity following temporally separated inseminations from two different males. Although female guppies store sperm in the ovary for several months (Winge 1937), they become sexually receptive on an approximately monthly basis. As a result of prolonged sperm storage, the ovary may still contain viable stored sperm at the time of remating. This in turn will lead to competition between the stored and the fresh, more recently inseminated sperm. The outcome of the competition between sperm that has been stored from previous matings and sperm that has been recently inseminated will determine the paternity of the subsequent brood. It is the paternity of such broods that is investigated in this chapter, the first attempt to use molecular techniques to determine paternity in the guppy.

Before further detailing the experimental plan, I shall briefly outline techniques used for the investigation of sperm precedence and provide a review of the literature available on multiple matings.

Techniques for investigating sperm precedence

In species with internal fertilization, or close physical or behavioural links between mother and offspring, maternity is often evident. However due to promiscuous mating patterns (Ridley 1988, 1990; Ginsburg and Huck 1989; Møller and Birkhead 1989) and the ability of some females to store sperm (Parker 1984; Birkhead and Møller 1992) the establishment of paternity is often more complex. Several solutions to determine which male successfully fathers offspring have been developed and utilized in a number of studies. As with the anatomy of the reproductive tract (chapter 5) far more is known concerning patterns of paternity in insects (Gwynne 1984b) and birds (Birkhead and Møller 1992) than other phyla. The first prerequisite in any study of sperm precedence is a way of identifying the offspring of different males. Traditionally, and until recently, the most common of these was the use of genetic markers, such as body or plumage colour (Cheng *et al.* 1983; Sims *et al.* 1987; Birkhead *et al.* 1988a, 1989) or body size (Compton *et al.* 1978). All studies using morphological markers follow the same basic plan; one of the genes for the selected characteristic is recessive, the other dominant and only known homozygous individuals are used. Therefore when a recessive female mates with a homozygous dominant male, the offspring will carry the dominant genes. Conversely when a recessive female mates with a recessive male all the offspring will be of a recessive phenotype. Then, when two males of different genotype are mated to the same homozygous female the offspring of each male can easily be distinguished. This technique has proved extremely useful in laboratory studies, but for obvious reasons its use in the field in limited. Studies involving genetic markers have been undertaken using male colouration in *P. reticulata*. In Haskins *et al.'s* initial experiment (1961) competitive breeding tests were undertaken, in which males from two different colour pattern strains competed for access to a female. As with the studies of birds, analysis of the colour patterns of offspring and their putative fathers allowed subsequent determination of paternity. Interestingly Haskins *et al.* (1961) were not attempting to investigate sperm competition, but instead to establish whether more colourful males achieved a greater mating success. However the technique was quickly adopted by others and has been used in several subsequent studies (Farr 1980a, b; Bischoff *et al.* 1985; Houde 1988b; Reynolds and Gross 1992; Kodric-Brown 1993; Brooks 1996; cited Houde 1997).

A second approach used commonly in studies of sperm precedence in insects involves the use of sterile males (Parker 1970a). One of a pair of males is irradiated and sterilised. This male's sperm can still fertilize eggs but due to the high levels of lethal mutations the zygote never develops. Reciprocal, sequential matings with sterile and normal males are carried out, with the proportion of developing eggs indicating the proportion of zygotes fathered by viable sperm. Several authors have utilised this technique (Gwynne 1984a; Ridley 1988; Jennions and Passmore 1993) with last male sperm precedence reported as the most frequent outcome. This technique has not been attempted in the guppy, however no evidence exists to suggest that this approach would not provide useful data.

In addition several molecular techniques, allowing the assignment of paternity, have also been developed. Many paternity studies have utilized protein electrophoresis or autosomal DNA markers. Protein samples (in fish usually eye, liver and muscle) are collected, homogenized and run on starch gels. Subsequent histochemical staining singles out the products of a particular gene, producing banding patterns from which paternity is established. This technique has been used to provide data on multiple paternity in a diverse range of species including mice (Birdsall and Nash 1973); prairie dogs (Hoogland and Foltz 1982); indigo buntings (Westneat 1987); house wrens (Price *et al.* 1989); field sparrows (Petter *et al.* 1990); lobsters (Nelson and Hedgecock 1977); salamanders (Tilley and Hausman 1976) and *Drosophila* (Milkman and Zeitler 1974).

Recently an alternative means of assessing paternity has been developed. In 1985 Jeffreys *et al.* (1985) discovered the first minisatellite sequences in humans. The likelihood of individuals sharing the same set of these DNA sequences was established as being so remote that, when samples were appropriately analyzed, these minisatellites provide each person with a unique banding pattern. Multilocus fingerprinting has led to a huge boom in paternity studies and since 1987 this technique has almost entirely replaced the use of phenotypic characteristics and other molecular techniques in paternity analysis. As a result of the technique's ability to reliably determine paternity, a large body of literature now exists based on this new method.

Using DNA fingerprinting, paternity studies have now been undertaken in many species, for example hydrozoans (Levitan and Grosberg 1993); Subalpine ant (Evans 1993); Harlequin beetle-riding pseudoscorpion (Zeh and Zeh 1994; Zeh *et al.* 1997); bees (Blanchetot 1992); dragonfly (Hadrys *et al.* 1993); bushcricket (Achmann *et al.* 1992); Red-eyed treefrog (D'Orgeix and Turner 1995); Sand lizard (Gullberg *et al.* 1997); Larger mouse-eared bat (Petri *et al.* 1997); Common shrew (Tegelstrom *et al.* 1991); California mouse (Ribble 1991); Pika (Peacock and Smith 1997); Grey seal (Amos *et al.* 1993); Hooded seal (McRae and Kovacs 1994); Barbary macaque (von Segesser 1994) and Toque macaque (Keane *et al.* 1997). However birds remain the most frequently studied group, for example, Indigo bunting (Payne and Payne 1989; Westneat 1990); Snow goose (Quinn *et al.* 1987); Red-winged blackbird (Gibbs *et al.* 1990); Dunnock (Burke *et al.* 1989); Purple martin (Morton *et al.* 1990); Zebra finch (Birkhead *et al.* 1990); Blue tit (Kempenaers *et al.* 1992); Pied flycatcher (Ellegren *et al.* 1995); Short-tailed shearwater (Austin and Parkin 1996). Through these and numerous other studies, researchers have been able to investigate patterns of paternity and the mechanisms underlying sperm competition

As well as differences in the means of determining paternity, studies of sperm precedence in birds can be divided into three sections based on the situation under which the mating occurred and the means of insemination (Birkhead and Møller

1992). Firstly, the earliest studies were those involving mate replacement. A male is allowed to mate with a female, and subsequently removed and replaced with a second male (Sims *et al.* 1987; Birkhead *et al.* 1988a). This is also the approach used in this study. Secondly, several authors have done away with normal matings and opted for the greater control gained through the use of artificial insemination. This technique has several advantages; the number and timing of inseminations and the number of sperm per insemination can all be controlled, a situation impossible under normal conditions. This approach has been widely used in commercially important species such as chickens and turkeys (Compton *et al.* 1978) but some other species have also been studied (Cheng *et al.* 1983). However the use of artificial insemination has been criticised (Birkhead and Møller 1992) as being unrealistic and unable to provide insights in to frequently seen behaviours such as extra pair copulations. However a third category of studies, notably those of Tim Birkhead and his co-workers, have specifically addressed the outcome of extra pair copulations. Field studies have revealed that extra pair copulations occur in many species, including the zebra finch (Birkhead *et al.* 1988b). If a female is mated repeatedly with her partner, but allowed to receive one final mating from a second male (equivalent to an extra pair copulation) then up to 84% of offspring will be fathered by the last male (Birkhead *et al.* 1988a). In addition, DNA fingerprinting has shown that these matings do father offspring in the wild (Birkhead *et al.* 1990).

Patterns of paternity

The introduction of molecular techniques has revolutionised the detection of sperm competition and evidence now exists for virtually all animal taxa. However no general rule has emerged concerning patterns of paternity. Excluding broadcast spawning species, in which several males release sperm simultaneously, there is normally a time delay between inseminations. Many studies have investigated the proportion of offspring fertilised by males in relation to their order of mating. Boorman and Parker (1976) established the now standard protocol of referring to the proportion of offspring sired by the second male as the P2 value. P2 values range widely across taxa from very low (i.e. the first male to mate fathers the majority of the offspring) in spiders (Austad 1984) and the adder *Vipera berus* (Hoggren 1995), through situations where paternity is shared, as in rats (Dewsbury 1984) and field crickets (Parker *et al.* 1990), to very high P2 values found in some birds (Birkhead and Møller 1992), locusts (Parker and Smith 1975) and certain butterflies (Gwynne 1984b).

Far less information is available on patterns of paternity in fish. In some species it would appear that one male is guaranteed exclusive paternity. For example, sperm competition is not thought to occur in the pipefishes and seahorses (Syngnathidae). Females pass their eggs into a male's brood pouch, where they are fertilized and subsequently develop. Fertilization is internal, but occurs within the male thus removing the scope for sperm competition and multiple paternity - although males do occasionally receive eggs from more than one female producing broods of multiple maternity (Jones and Avise 1997). In *Oreochromis aureus*, matings occur between a female and a single male and the eggs are fertilized and develop in the female's mouth (Chao *et al.* 1987). However males of most fish species appear to face higher intensities of sperm competition (Breder and Rosen 1966; Stockley *et al.* 1996, 1997).

Whilst little data exists on patterns of paternity in internally fertilizing fish species, there are references to females producing multiple paternity broods in several species. Paternity analyses indicate that sperm from more than one male compete for fertilizations in the shiner perch, *Cymatogaster aggregata*, (Darling *et al.* 1980) and the sculpin, *Alcichthys alcicornis*, (Munehara *et al.* 1990). The most comprehensive data on multiple paternity within broods comes from the Poeciliidae (Constantz 1984). Multiple inseminations leading to mixed paternity broods have so far been demonstrated for nine members of the Poeciliidae (table 6.1). Estimates of the proportion of females carrying broods of multiple paternity range from 23% up to 85%. However there is speculation that these results are artificially low and that in

Species	Frequency of multiple insemination	Analysis	References
Xiphophorus variatus	42 % of females produced mixed paternity broods	Offspring colour patterns	Borowsky and Khouri 1976
Poeciliopsis monacha	6 of 26 females (23%) inseminated by at least two different males	Enzyme protein phenotypes of mother and offspring	Leslie and Vrijenhoek 1977
Neoheterandria tridentiger	2 of 6 (33%) broods were fathered by two males	Analysis of enzyme phenotype of mother offspring combinations.	Hall 1980; cited Constantz 1984
Gambusia affinis	At least 56% of females had received sperm from at least two males		Chesser *et al* 1984
	Between 50-80% of females had received sperm from more than one male.	Electrophoretic survey of enzyme loci polymorphism	Greene and Brown 1991
	80% of females produced multiply sired broods	Electrophoresis of mother and offspring enzyme proteins	Robbins *et al* 1987
Poecilia latipinna	12 of 23 females were carrying multiple sired broods	Electrophoretic survey of enzyme loci polymorphism	Travis *et al* 1990
	From 9 - 85% of females, depending on population, produced broods with multiple paternity	Electrophoresis of polymorphic allozyme loci	Trexler *et al* 1997

Poecilia reticulata	The majority of females produced broods containing offspring fathered by two or three males	Offspring colour patterns	Haskins *et al* 1961
	Two or more males had inseminated 64% of females	Autoradiographic analysis of radiolabelled sperm samples	Luyten and Liley 1991
	No data presented	Offspring colour patterns	Winge 1937 Rosenthal 1952 Hildemann and Wagner 1954 Gandolfi 1971 Schmidt 1919; cited Constantz 1984 Houde 1988b, 1997
Poeciliopsis prolifica	No data presented	Analysis of inheritance of mutant phenotype	Schultz 1963
Limia perugiae	11 of 21 females produced broods fathered by two males.	DNA fingerprinting	Schartl *et al* 1993
Xiphophorus maculatus	48% of females fertilized by two males, 24% by three males and 4% by four males.	Observed colour patterns of offspring's tails	Borowsky and Kallman 1976
	No data presented	Offspring colour patterns	Gordon 1947 Vallowe 1953 Kallman 1965, 1970

Table 6.1 Members of the family Poeciliidae for which within brood multiple paternity has been reported.

natural populations the rate of multiply inseminated females may approach 100% (Chesser *et al.* 1984).

The development of increasingly sensitive genetic markers has resulted in observations of multiple paternity being extended to diverse species previously thought to be monogamous (e.g. Yezerniac *et al.* 1995) or polygynous (e.g. Westneat 1993; Weatherhead and Boag 1995). In conclusion multiple inseminations, leading to multiple paternity, are now seen as routine in the majority of species, including members of the Poeciliidae.

Adaptive significance of multiple mating

Courtship and mating incur costs to both males and females. These costs include the time and energy devoted to courtship and copulation (Daly 1978; Magurran and Seghers 1994a); the risk of disease transmission (Sheldon 1993); the negative effects of some seminal fluids transferred by males (Chapman *et al.* 1993, 1994) and the increased risk of predation during mating (Magnhagen 1991; Magurran and Seghers 1990a). Despite these potential costs females of many species mate with several males. In addition to the high levels of sneaky mating, female guppies are known to accept matings promiscuously during their fertile periods. Several hypotheses have been put forward to explain why females mate multiply in spite of the apparent costs. Firstly, mating may allow the female to ensure she has full sperm stores (Thornhill 1976; Lewis and Austad 1994). In poeciliids females with depleted sperm stores exhibit degeneration and absorption of unfertilized eggs (McKay 1971, Tavolga 1949). Consequently insufficient supplies of sperm lead to a decrease in brood size (Vallowe 1953), whereas reinseminated females exhibit a return to typical numbers of offspring per brood (Rosenthal 1952). It has therefore been suggested that multiple mating acts as a mechanism allowing females with inadequate sperm supplies to refill their stores (Boorman and Parker 1976). Whether females in the wild ever have to reduce their brood size due to insufficient supplies of sperm has yet to be investigated.

Interestingly, in female *Poecilia latipinna* a relationship has been shown between the number of males a female mates with and subsequent brood size (Travis *et al.* 1990; Trexler *et al.* 1997). While in the guppy, females that produced broods sired by both of the two experimental males produced larger broods than those that had only received sperm from one male (Hughes *et al.* MS). Unfortunately no information was presented on how many matings each male obtained, but it is possible that the increase in brood size may reflect an increase in sperm availability. As mentioned earlier, females in some Trinidadian populations receive up to one mating attempt a minute (Magurran and Seghers 1994a). Whilst not all of these attempts will successfully result in sperm transfer, it seems unlikely that females will ever be sperm limited. In birds the number of sperm necessary to fertilize the entire clutch is low and can often be obtained from only one mating (Taneja and Gowe 1961; Lake and Ravie 1987; Wishart 1987; Bakst 1989). In guppies it is not known how many sperm are required to fertilize each brood. If the fertilization of a brood requires more than one insemination then females may multiple mate to acquire sufficient sperm. However it has been shown that one mating provides a female guppy with enough sperm to fertilize several successive broods (Purser 1938). More precisely Billard (1969) calculated that male guppies produce approximately 750,000 sperm per 24 hours. Based on this estimate 750,000 sperm are sufficient for the fertilization of several subsequent broods. This therefore seems an unlikely explanation for female promiscuity in the guppy. Good data exists on the average number of sperm in stripped ejaculates (Chapter 3; J. Evans *pers. comm.*) and it would be worthwhile carrying out artificial inseminations with varying sperm numbers to determine if sperm shortage is one factor contributing to multiple mating.

Secondly, in many insect species the male gives the female nuptial gifts during courtship and copulation. Gifts may take the form of food obtained by the male and passed to the female, secretions such as products from the salivary or integumental glands and nutrients passed to the female with the sperm, including high protein content spermatophores (Wedell and Arak 1989) and nutrients in the seminal fluid (Gwynne 1994a). Studies have shown that these nutrients are incorporated into both the body and the eggs of females (Boggs and Gilbert 1979; Gwynne 1984a; Pitnick *et al.* 1991). The volume of the ejaculate in the guppy is, in contrast to most insects, relatively small. It therefore seems unlikely that male guppies transfer sufficient nutrients during mating to provide any benefit to females. However it would be worth carrying out the necessary experiments to establish if the ejaculate in guppies does act as a nutritional gift.

Among monogamous bird species courtship feeding is widespread (e.g. Brown 1967). In some species a direct link exists between courtship feeding and pair copulations (e.g. red-billed gulls, Tasker and Mills 1981). While in two polygynous bird species, orange-rumped honeyguides and purple-throated Carib hummingbirds, evidence exists that males trade food for copulations. In both cases males defend areas of food, bees' nests and nectar bearing flowers respectively, and only allow females to forage in their area in return for copulations. No evidence of courtship feeding exists for the guppy or any other poeciliid. In addition male poeciliids do not hold territories or defend areas of resources against females. Another possible material benefit that females may gain from multiple mating is increased paternal care. For example, in the dunnock, females often mate with, and receive help from two males (Davies 1992). DNA fingerprinting has shown that if both males successfully mated with the female, then both males were likely to father offspring (Burke *et al.* 1989) The second male provides parental care only if he has mated with the female (Davies *et al.* 1991). Again this seems an unlikely explanation for promiscuous mating in the guppy. In poeciliids the females provide all the nutrients required by the developing ova, and after parturition neither sex engages in parental care.

Kempenaers *et al.* (1992) suggested that multiple mating and extra-pair paternity in the blue tit resulted from the female's preference for high quality males. Several other authors have also hypothesised that as a result of multiple mating, females may increase their probability of mating with a high quality male (Birkhead and Møller 1992, 1993; Møller 1992b). The theory suggested that females would benefit from engaging in extra-pair copulations with males that are of better quality than their partner (Walker 1980). Two reasons, both concerning gains in genetic quality, have been put forward. Females may either obtain genes allowing the production of 'sexy sons' (Fisher 1930; Weatherhead and Robertson 1979) or they may obtain genes for increased health and resistance to parasites and pathogens (Zahavi 1975, 1977; Hamilton and Zuk 1982). The literature concerning potential genetic benefits and mate choice is too large to be covered here and outside the scope of this dissertation (see Andersson 1994 for an excellent review). Briefly in the guppy for example, orange carotenoid spots have been shown to increase the attractiveness of males in some populations (Kodric-Brown 1985). The brightness of these spots increases with the carotenoid content of the diet and may reflect the male's foraging ability (Kodric-Brown 1989). In addition, Nicoletto (1991) found that carotenoid spots are a reliable guide to a male's swimming performance. Endler (1980, 1983) suggested that the female preference for bright spots has evolved through an indicator process based on heritable genetic variation, reflected in the foraging success of males. In conclusion female guppies exhibit preferences for certain males, and these preferences may reflect traits that improve the survival of offspring. Females mate promiscuously during their fertile period, and it would be interesting to know if females reject matings from males of a lower quality than those from which they have already received sperm. It is therefore possible that females are choosing to mate with several males in order to gain 'good genes' for their offspring.

It has also been suggested that females may mate more than once as an insurance against any one male being sterile (Walker 1980; Gibson and Jewel 1982). Drummond (1984) expanded this and suggested that, in Lepidoptera, females should mate with several males for four reasons. (1) The partner may be incapable of transferring sperm during mating. (2) The partner may transfer insufficient sperm to fertilize all the female's eggs, or (3) the male may transfer sufficient sperm, but it may deteriorate before the female has fertilized all her eggs. (4) The male's sperm may simply be incapable of fertilizing eggs. Alcock *et al.* (1978) suggested that as intense selection exists for males to produce sperm, male sterility will be rare and therefore unlikely to account for female promiscuity. However, male infertility can occur for a number of non-genetic reasons. In human studies about one in 25 males is infertile, and the primary cause is low sperm counts. Human male sterility can be permanent or temporary and may be the result of one or more factors (Smith 1984). Poultry also suffer reduced sperm counts caused by factors such as food quality and parasite infections (Romanoff 1960).

Birkhead *et al.* (1988b, 1989) showed that even in males capable of producing adequate numbers of viable spermatozoa, some behaviourally successful copulations fail to result in sperm transfer. They suggested this may be due to either the male's failure to ejaculate during mating, or because despite ejaculation taking place, insemination does not occur. In birds both types of failure probably occur if the cloacae are incorrectly juxtaposed during mounting. A similar situation could arise in guppies. The positioning of the gonopodium is likely to be crucial to successful sperm transfer, and incorrect alignment of the male's gonopodium and the gonoduct is likely to result in sperm either being released into the water or not being ejaculated far enough into the oviduct. It is not known whether this kind of failure to inseminate a female occurs in nature. However, the occasional lack of sperm transfer during copulation may be one reason why females in many species, including poeciliids, copulate several times during the receptive period.

Birkhead and Møller (1992) pointed out that having a sterile partner will be particularly costly in lekking species in which females typically copulate once or a very few times per clutch. In such species selection will have operated to ensure that both males and females are able to recognise unsuccessful copulations. Among lekking birds selection will also favour female choice for males capable of producing an ejaculate with sufficient sperm to fertilize their entire clutch (Avery 1984). In male sharp tailed grouse, the amount of testicular sperm, but not the size of the testes, differs in relation to a male's position on the lek. Central males (which are likely to have a higher copulation success) had larger amounts of testicular sperm than peripheral males (Nitchuk and Evans 1978). This study is one of the few providing support for Sheldon's (1994) phenotype-linked fertility insurance hypothesis. According to this hypothesis females can observe a phenotypic characteristic of a male and from this information determine key features of his ejaculate. A high correlation between display rate and sperm number has been demonstrated in the guppy (Matthews *et al.* 1997), these data are consistent with, and provide evidence for the phenotype-linked fertility insurance hypothesis. If, as was suggested in Chapter 3, female guppies can tell how large an ejaculate a male has ready for use, then female guppies may only need to multiple mate if the attempted mating from a selected male does not successfully transfer sperm. However the numbers of sperm per ejaculate produced by different males of the same species can vary consistently and dramatically (Romanoff 1960; Gibson and Jewel 1982; data on stripped ejaculates in guppies in Chapter 3). If a female was to mate with a male that transferred only a small number of sperm it may, as mentioned, adversely affect brood size.

Several recent papers have suggested that if there is a correlation between sperm quality and offspring quality (e.g. Evans and Geffen 1998) then females should mate with more than one male in order to promote sperm competition. Field studies of the adder, *Vipera berus*, (Madsen *et al.* 1992) and the sand lizard, *Lacerta agilis* (Olsson 1992; Olsson *et al.* 1994a, b) have shown that multiply sired broods have higher embryonic survival, fewer deformities and, in the sand lizard, the offspring are heavier and have higher first year survival. Madsen *et al.* (1992) proposed that females may promote competition between sperm of different quality from different males, and that the offspring traits they recorded were due to the relationship between sperm quality and offspring viability. Parker (1992) cast doubt on this interpretation pointing out that the sperm's haploid genome is thought to be unlikely to determine sperm performance. However Nayernia *et al.* (1996) demonstrated that, in mammals, morphological and functional aspects of sperm develop primarily under the control of haploid genome. The relationship described by Madsen *et al.* (1992) could exist if the male's genome influences both the number of detrimental alleles carried by the sperm and sperm's performance. As a result the genome of a high performance sperm would result in the production of a zygote of increased viability. This hypothesis is still in its early stages and at present has only been investigated in the adder and sand lizard. However it may prove to be another contributory factor leading to multiple matings.

As well as gaining benefits in terms of genetic quality, it has been proposed that multiple mating may result in an increase in the genetic diversity of the offspring. Williams (1975) suggested that in an unpredictable environment increased genetic diversity of offspring increases the likelihood of some survival and reproduction. This hypothesis has been adapted to focus on the ephemeral nature of the environment inhabited by many poeciliids (Meffe and Snelson 1989a, b). Constantz (1984) suggested that selection might favour female poeciliids that produce heterogeneous offspring in a fluctuating environment. In other words, carrying sperm from several males may allow a female to produce a brood in which the offspring are of varied phenotype, thus increasing the chances of some young surviving in shallow, seasonally variable habitats. An alternative genetic diversity theory in poeciliids was proposed by Chesser *et al.* (1984). They put forward the idea that females mating with more than one male leads to a larger effective population size than would be predicted if females were strictly monogamous. They suggested that if five or six females were then to become isolated in a new pool, the number of effective founders would be increased if each female carried sperm from several different males. However Chesser *et al.'s* (1984) proposal has been criticized as being framed at the individual level and yet having tones of group selectionism. Constantz (1984) took care to rephrase the proposal at the individual level, highlighting the possibility that females in isolated pools could reduce the amount of inbreeding amongst the second generation by producing broods of mixed paternity.

Two factors suggest that an increase in genetic diversity is an unlikely explanation. Williams (1975) pointed out that as a result of meiosis and recombination, multiple mating does not produce considerably greater genetic diversity than mating singly. Secondly any advantage that is gained may be outweighed through increased competition between paternally unrelated individuals in the brood (Parker 1984). Genetic diversity is therefore not generally regarded as a plausible explanation for multiple mating (Birkhead and Parker 1997).

It has been reported that in many species infanticide by males results in increased reproductive success by eliminating the offspring of competitors and decreasing the delay before the mother becomes receptive again. This theory was first proposed by Hrdy (1979) to explain multiple mating in primates, but evidence has since been provided in a range of mammals and some birds (e.g. Veiga 1990). This seems an unlikely explanation for multiple mating in the guppy. As a result of laboratory observations it is clear females practise that infanticide, however I have never seen males kill or eat offspring. More importantly female guppies become receptive on a regular monthly cycle (Liley 1966), the period of receptivity coinciding with parturition. As females are receptive as they give birth, males have nothing to gain by killing existing juveniles.

A final explanation, and one that may be of particular relevance to the Poeciliidae was put forward by Halliday and Arnold (1987). They suggested that females might benefit from multiple mating because eggs that are fertilized by old stored sperm are more likely to suffer from genetic abnormalities than eggs fertilized by newly inseminated sperm. Experimental evidence providing support for this hypothesis comes primarily from studies of birds (Birkhead 1988). In the ring dove hatching success decreases as sperm age increases (Riddle and Behre 1921). Similar patterns have also been recorded in the turkey, chicken, mallard and Japanese quail (Birkhead and Møller 1992). However the relationship between hatching success and time for which sperm have been stored has not been apparent in two more recent studies (Birkhead *et al.* 1989; Birkhead and Møller 1992). Unfortunately this hypothesis has not been studied in any of the poeciliids. Certainly after an initial single insemination the number of offspring does decrease over consecutive broods (Rosenthal 1952). However this reduction may be due to a decrease in sperm numbers, rather than any decrease in viability or increase in genetic defects caused by storage.

In conclusion, where females gain a direct benefit from multiple mating, such as nutrients (either in a spermatophore (Butlin *et al.* 1987; Wedell and Arak 1989) or as a gift of prey (Thornhill 1976, 1980)) or an increase in parental care it is clear that there may be a benefit to the female. However in other cases where the benefits are indirect, such as an increase in genetic quality or diversity, the benefits gained by females are still unclear and remain to be investigated.

It has been already been shown that female guppies mate promiscuously during their brief period of receptivity (Houde 1987). Successful gonopodial thrusting while the female is unreceptive will introduce sperm from yet more males into the ovary and increase the competition for fertilizations. It has been shown, using colour patterns, that females produce broods of multiple paternity (table 6.1), however this experiment uses DNA fingerprinting to investigate both the patterns of paternity within broods and the changes in these patterns over consecutive broods.

METHODS

Animal material

All fish used in this experiment were bred in the laboratory. These fish were descendants of wild caught individuals from rivers in Trinidad's Northern Mountain Range. The water from these mountains drains to the east in the Caroni drainage and the west in the Oropuche drainage. Two populations were used. One (Lower Tacarigua) from the Caroni Drainage and the other (Oropuche) from the Oropuche Drainage. A preliminary study suggested that the arbitrary primers being used would not allow unambiguous assignment of paternity between males within a population. It was therefore necessary to conduct matings between two populations and separate the offspring on the basis of from which population the father was derived.

35 females were taken from each population and their weight and total length recorded. An attempt was made to select 'middle-aged' females that were of a reasonable size, as both small females and geriatric females tend to produce low brood sizes precluding paternity analysis. Females were from single population stock tanks and were isolated until the birth of the first brood. This brood was taken to indicate that the female was capable of producing young, and had been receiving successful insemination attempts from males of her own population. This brood can only have been fertilized using stored sperm from the female's native population and the offspring were not kept for analysis. As soon as possible following birth of her first brood the female was placed with two males from the second population. These two males were left with the female for 48h (the period of female receptivity to male courtship (Liley 1966)), and then removed and preserved in ethanol. The female was then isolated again to await the birth of subsequent broods. These broods were collected and immediately preserved in ethanol. At the end of the breeding period those females who had given birth to broods which were to be analysed were also preserved, while those that had not produced broods were returned to the stock tanks. DNA fingerprinting was then carried out to establish the paternity of these offspring.

RAPD analyses and extraction of DNA

The RAPD (Random Amplified Polymorphic DNA) fingerprinting technique is a PCR (polymerase chain reaction) based protocol which uses a single arbitrary short oligonucleotide as a primer to scan a genome for small inverted repeats and amplifies the intervening DNA segments (Williams *et al.* 1990). These fragments serve as dominant genetic markers, and intra-specific polymorphisms can be used to fingerprint anonymous genomes.

Groups of a female, the two males with which she had mated and offspring from each of her subsequent broods were prepared for RAPD analyses. Whole genomic DNA was extracted from all samples using a DNA isolation kit (Puregene, Gentra Systems, Inc.). In the case of adults 20mg of caudal muscle was used, while for juveniles the entire fish was used. Each sample was placed in 600µl of chilled Cell Lysis Solution. This was then heated at 65°C for 20 minutes and the tissue homogenised using 40 strokes of a microfuge tube pestle. The lysate was incubated at 65°C for 60 minutes. After this period 3µl of RNAase was added, the tube inverted 25 times and the sample incubated at 37°C for a further 60 minutes. The sample was then removed from the oven and allowed to cool to room temperature (<21°C). When sufficiently cooled, 200µl of Protein Precipitation Solution was added to the lysate. The sample was then centrifuged at 13,000 rpm for 3 minutes, leaving the precipitated proteins on the bottom of the tube. The supernatant was decanted in a clean 1.5ml eppendorf containing 600µl 100% isopropanol. The tube was then inverted 50 times revealing the DNA as a clump of white threads. This sample was centrifuged at 13,000 rpm for 1 minute, leaving a tight pellet of DNA and allowing the supernatant to be poured off. To wash the DNA 600µl of 70% ethanol was added to the tube, which was then inverted 30 times. The sample was centrifuged for 1 minute and the ethanol carefully poured off. After the DNA had been allowed to dry for 30 minutes, 100µl of DNA Hydration Solution was added. The DNA was left to rehydrate overnight at room temperature, prior to storage at 5°C.

For each PCR reaction I used 0.25µl of a 2 unit/µl solution of DyNAzyme DNA polymerase (Flowgen), 1.25µl of 2mM dNTP mix and 2.5 µl of DyNAzyme reaction buffer. To this was added either 0.2 µl of 25 pmol/µl or 1.0µl of 5 pmol/µl of a single, random sequence, ten nucleotide oligomer (Operon Technologies Inc., Alameda, California, USA). Each of the amplifications was made up to 22.5 µl with sterilised distilled water, to which was added 2.5µl of 4 ng/µl of sample DNA. Finally each well was topped off with a drop of mineral oil.

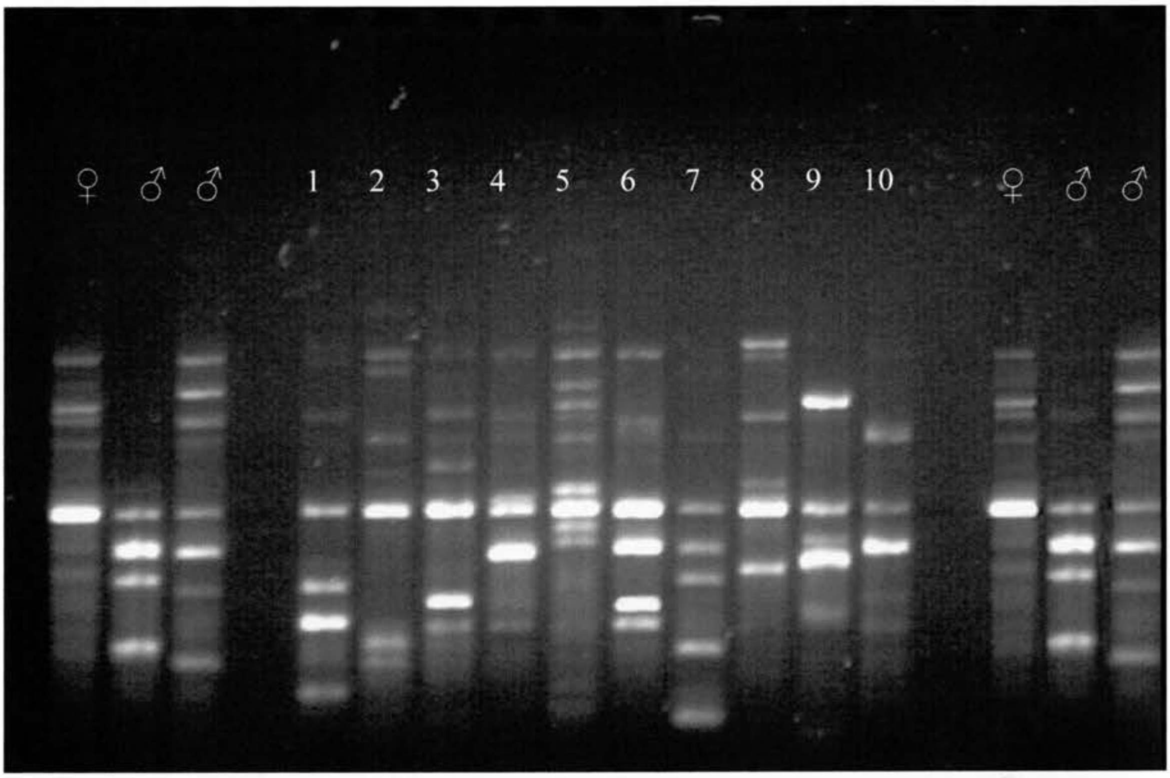
The 96 well plate was then placed in a PTC-100 thermal cycler (M.J. Research, Inc.). The first cycle consisted of 3.5 minutes at 94°C, 45 seconds at 40°C and 1.5 minutes at 72°C. Amplification was allowed to continue for 44 additional cycles, each comprising 30 seconds at 94°C, 45 seconds at 40°C and 1.5 minutes at 72°C. The PCR products were electrophoretically analysed, adding 5µl of loading buffer to each sample and transferring this mixture to wells in a 1.4% agarose gel stained with ethidium bromide (figure 6.1). Gels were run in 0.5X TBE at 90V for 5 h, and then viewed and recorded under UV light, using an E.A.S.Y. Store camera and computer system (Herolab GmbH, Wiesloch, Germany).

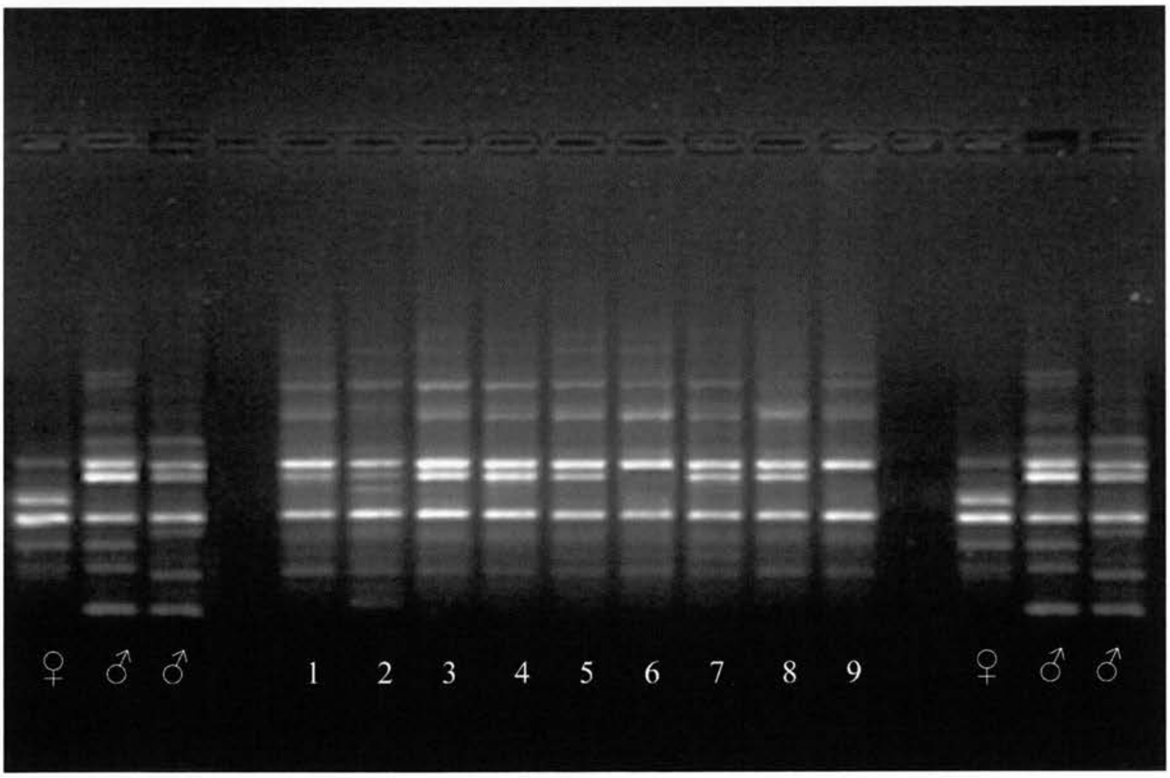
As RAPD fingerprinting is PCR based and involves the use of short primer sequences and low annealing temperatures, one of the technique's principal limitations arises from its sensitivity to the reaction conditions. Slight changes in these conditions may result in the production of extraneous 'artefact' bands, and thereby affect the reproducibility of amplification products (Williams *et al.* 1990; Arnold *et al.* 1991; Carlson *et al.* 1991; Klein-Lankhorst *et al.* 1991). The technique is sensitive to (a) the shape of the temperature profile (i.e. the range and duration of temperatures to which the sample is exposed), (b) the type of polymerase used and (c) Mg^{2+} concentration of the PCR mix. Only strictly standardized reaction conditions will ensure reproducible PCR products. Therefore the shape of the temperature profile (see preceding section), the concentration of Mg^{2+} in the PCR reaction mixture and the brand of polymerase (DyNAzyme) were optimised and standardized. In order to further ensure the reproducibility of amplification the concentration of the DNA (4ng/ul) was also standardized and the same thermal cycler was used throughout the study

60 primers were screened to ascertain their suitability for paternity analysis in this study. None of the primers were able to separate unambiguously individuals within a population. However several were able to distinguish between populations. All primers were commercially prepared ten nucleotide oligomers (Operon Technologies Inc., Alameda, California, USA). Five primers which amplified suitably polymorphic bands were selected (OPA10; OPB12; OPB14; OPB17 and OPH05).

Paternity was established from gels on the basis of the presence of diagnostic bands that allowed the assignment of parents to offspring, or *vice-versa*. This approach involved searching for marker bands present in offspring that could be used to

Figure 6.1 Sample gels showing the banding patterns produced for two families of offspring, their mother and the two potential fathers from the second population. Males and females were run in duplicate down either side to ensure that bands were running straight down the gels and to ease scoring.


♀
♂
♂
1
2
3
4
5
6
7
8
9
10
♀
♂
♂


♀
♂
♂
1
2
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4
5
6
7
8
9
♀
♂
♂

exclude one, or more of the potential fathers (exclusion), or searching for bands that were unique to a single male, and then assigning all offspring with those bands to that parent (inclusion). Even if a band was found that was unique to one male, it is possible that the stored sperm may also have carried this band. In order to ensure that offspring were allocated to the correct parent, a further 10 males from each population were run with each primer. Any bands that were shared between the two groups were discounted from further analysis. This ensured that only bands that were genuinely population specific were used to assign paternity. No attempt was made to assign offspring separately between the two males, both of which were from the same population, that had provided the recent ejaculates.

RESULTS

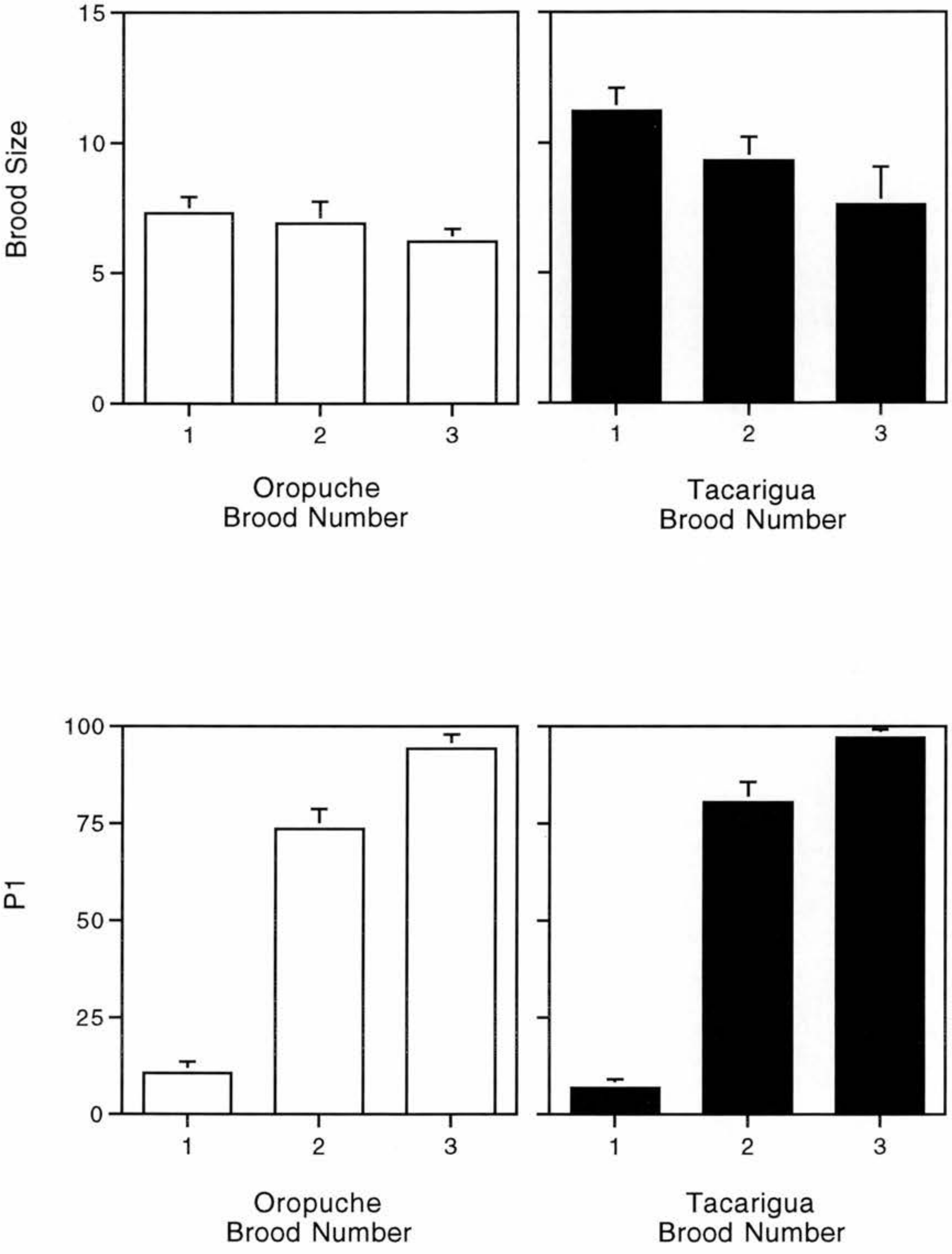
Of the 35 females from each population that were initially isolated, 26 of the Tacarigua females and 19 of the Oropuche females produced first broods. The number of females producing offspring decreased with each subsequent brood. 12 Tacarigua females and 8 of the Oropuche females produced second broods, while only 7 Tacarigua and 5 Oropuche females gave birth to third broods.

In both populations the number of offspring per brood decreased over the three consecutive broods (figure 6.2a). In neither of the populations was the decrease in brood size over time significant (Oropuche : $F_{44} = 2.38$, $p > 0.1$; Tacarigua : $F_{31} = 0.36$, $p > 0.5$). First broods produced by Oropuche females were significantly smaller than the first broods of Tacarigua females (7.3 ± 0.64, 11.1 ± 0.89; $t_{43} = 3.27$, $p < 0.005$). Broods 2 and 3 from Oropuche females were also smaller than their Tacarigua counterparts, but there was no significant difference (Brood 2 : 6.8 ± 0.85, 9.3 ± 0.9; $t_{18} = 1.82$, $p > 0.05$; Brood 3 : 6.2 ± 0.49, 7.6 ± 1.46; $t_{10} = 0.76$, $p > 0.1$). At the commencement of the experiment there was no significant difference in either female weight (Oropuche : 0.31g ± 0.008; Tacarigua : 0.33 ± 0.008; $t_{43} = 1.88$, $p > 0.05$) or total length (Oropuche : 3.1cm ± 0.04; Tacarigua : 3.1 ± 0.04; $t_{43} = 0.34$, p

Figure 6.2a The mean number of offspring per brood produced by females from Oropuche (open bars) and Tacarigua (closed bars). Data are given as means ± S.E.M.

Figure 6.2b Variation in the percentage of paternity gained by stored sperm (P1) over the course of three consecutive broods produced by females from the Oropuche (open bars) and Tacarigua (closed bars). Data are given as means ± S.E.M.

Figure 6.2


Brood Size
15
10
5
0
1
2
3
Oropuche
Brood Number
1
2
3
Tacarigua
Brood Number
P1
100
75
50
25
0
1
2
3
Oropuche
Brood Number
1
2
3
Tacarigua
Brood Number

>0.1) between the populations. Perhaps not surprisingly weight and length were highly correlated for both Oropuche ($r = 0.64$, $n = 19$, $p < 0.003$) and Tacarigua females ($r = 0.81$, $n = 26$, $p < 0.0001$).

In all cases, the most recently inseminated sperm fertilized the majority of offspring in the first brood (i.e. high P2 values for brood 1). In 27 of the 45 first broods new sperm fertilized 100% of the offspring (10 broods from Oropuche females and 17 broods from Tacarigua females). Stored sperm fertilized only a small number of offspring in first broods (Oropuche females + Tacarigua males : 10.6% ± 3.0; Tacarigua females + Oropuche males : 6.86% ± 2.2 (figure 6.2b). All other young could be unambiguously assigned to one of the males that had provided new sperm.

In the second broods that were analysed, the situation was drastically reversed and in all cases the majority of offspring were fertilized by stored sperm (Oropuche females + Tacarigua males : 73.6% ± 5.0; Tacarigua females + Oropuche males : 80.4% ± 5.2). In 1 of 8 broods from Oropuche females and 4 of 12 broods from Tacarigua females, stored sperm gained exclusive paternity. In the few females that produced third broods the amount of paternity gained by stored sperm continued to increase (Oropuche females + Tacarigua males : 94.1 ± 3.6; Tacarigua females + Oropuche males : 97.0% ± 2.1). The proportion of broods that were exclusively fathered by stored sperm also increased, 3 of 5 broods from Oropuche females and 5 of 7 broods from Tacarigua females.

There was no significant difference in the amount of paternity gained by stored sperm between the two populations for any of the three broods (brood 1: $t_{43} = 1.021$, $p > 0.1$; brood 2 : $t_{18} = 0.897$, $p > 0.1$; brood 3 : $t_{10} = 0.722$, $p > 0.1$). However ANOVA revealed that, in both populations there was a significant increase in the degree of paternity gained by stored sperm over the three consecutive broods (Oropuche female + Tacarigua male : $F_{31} = 121.77$, $p < 0.0001$; Tacarigua female + Oropuche male : $F_{44} = 216.49$, $p < 0.0001$). When both populations are combined stored sperm gains only 8.4% ± 1.8 of paternity in the first brood, rising sharply to 77.7% ± 3.7 in brood two and continuing to rise to 95.8 ±1.9 in the third brood.

There was no significant correlation between length (Oropuche $r = -0.15$, $n = 19$, $p > 0.5$; Tacarigua $r = -0.02$, $n = 26$, $p > 0.1$) or weight (Oropuche $r = 0.12$, $n = 19$, $p > 0.5$; Tacarigua $r = -0.09$, $n = 26$, $p > 0.5$) of the female and the degree of paternity achieved by stored sperm in the first brood. Intriguingly however, for females from Oropuche, there was a significant correlation between the size of the brood and the amount of paternity gained by stored sperm ($r = -0.7$, $n = 19$, $p < 0.001$ (figure 6.3). Females from Tacarigua also showed a negative correlation between brood size and paternity awarded to stored sperm ($r = -0.38$, $n = 26$, $p = 0.057$), however the correlation was not quite significant. This negative correlation was continued for both populations over the next two broods, although the relationship was only significant ($r = -0.6$, $n = 12$, $p < 0.05$) for the second brood from Tacarigua females.

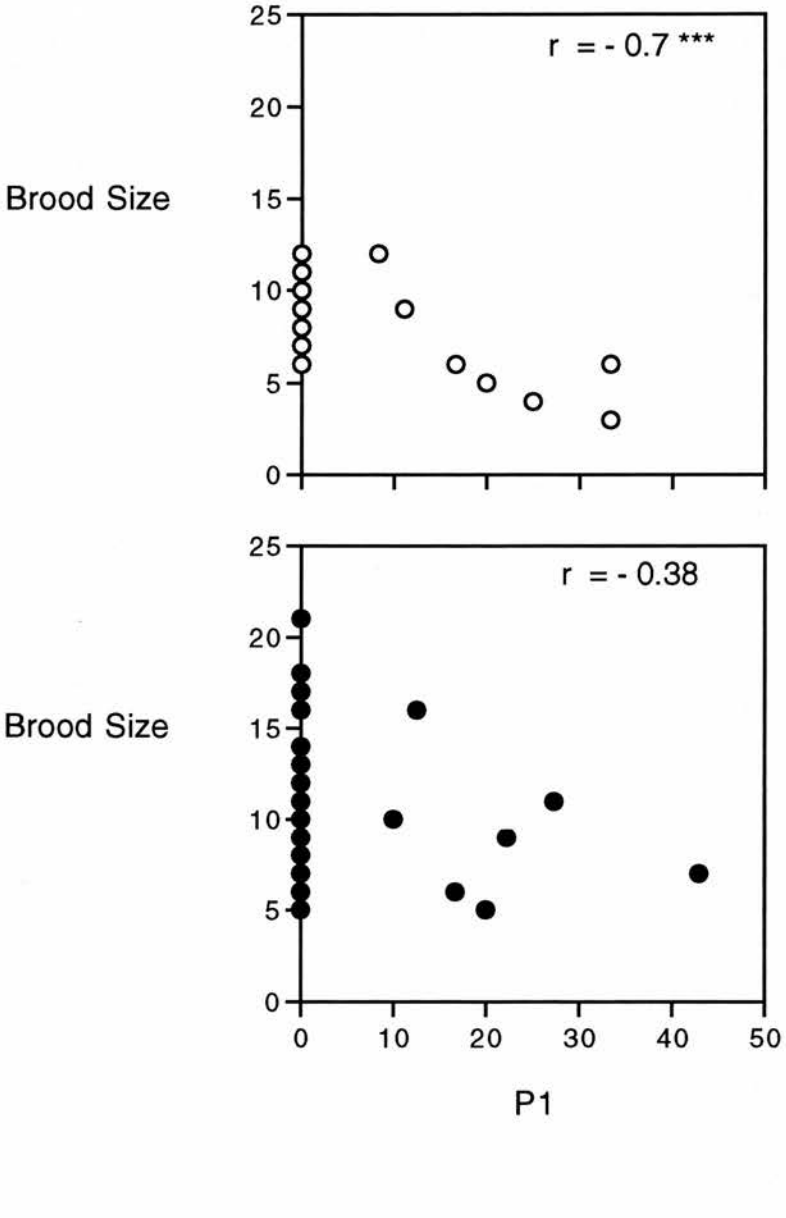
DISCUSSION

The results provide further support for the findings (table 6.1) that multiple mating and the production of broods of mixed paternity is common in poeciliids. Females of both populations produced mixed paternity broods and paternity was shared in 36 of the 77 broods. In the remaining cases either newly inseminated or stored sperm gained exclusive paternity. In first broods all incidences of exclusive paternity were attributable to new sperm (i.e. $P2 = 1.0$). In second and third broods the reverse was true with all cases of exclusive paternity accounted for by stored sperm.

In all females the most recently inseminated ejaculate is rewarded with the majority, if not the entirety, of the paternity of the next brood. New sperm fertilized more than 50% of the first brood offspring in all of the 45 females in this study. This suggests that in some way the new sperm are gaining an advantage over sperm from previous inseminations. There are several possible explanations for these findings. Before

Figure 6.3 The relationship between number of offspring and the amount of paternity gained by stored sperm in the first brood for females from the Oropuche (open symbols) and Tacarigua (closed symbols). The correlation is only significant for females from the Oropuche *** = $p < 0.01$.

Figure 6.3


r = - 0.7 ***
Brood Size
25
20
15
10
5
0
r = - 0.38
Brood Size
25
20
15
10
5
0
0
10
20
30
40
50
P1

discussing the results I will briefly outline the main theories concerning sperm competition and sperm precedence.

Mechanisms of sperm precedence

Direct observation of fertilizations and sperm competition is impossible, therefore the primary approach taken in understanding patterns of paternity has been the construction of mathematical models. The assumptions of these models can then be tested against the observed patterns of paternity. Following this protocol many hypotheses have been put forward to explain patterns of paternity, most have been based on the two best studied groups, insects and birds, however most of these mechanisms may be generalized to include internally fertilizing fish.

Several mechanisms have so far been proposed. The first and simplest of these are models of sperm mixing without displacement, also known as 'raffle' or 'lottery' models. Two separate lottery based models have been put forward. The first of these, the fair raffle, assumes that all the sperm from each of the males has an equal chance of entering the fertilization set (those sperm able to fertilize). The probability of paternity for the second male is therefore the total number of sperm inseminated by the second male divided by the total number of sperm from both males (Parker *et al.* 1990). A more biologically realistic model may be the loaded raffle in which not all sperm have an equal chance of achieving paternity (Parker et al. 1997). This situation may occur if, for example, sperm from an earlier ejaculate achieve greater numerical predominance around the site of fertilization than sperm from the second male. A similar result may also be obtained if at the time of the first mating no mature oocytes are available for fertilization. By the time of the second insemination, many of the first male's sperm will have died resulting in a reduced contribution to the fertilization set. The degree to which the raffle is loaded, or the unfairness of the raffle, may depend on several factors. If random sperm mortality occurs in the female reproductive tract, the length of time between matings may influence the proportion of sperm from each male that survive to enter the fertilization set. Similarly, the inter-mating interval may influence the patterns of paternity if a competitive race occurs between sperm to reach the unfertilized eggs. Schwagameyer and Foltz (1990) found that P2 declined with the interval between matings in 13 lined ground squirrels. In ground squirrels, as in most mammals, sperm are ejaculated into the uterus and have to 'race' to the site of fertilization, the Fallopian tubes. The sperm of the first male will have a head start in this race, and will make up a higher proportion of the sperm available to fertilize the eggs (Parker *et al.* 1990).

In light of the success of these earlier models, Lessells and Birkhead (1990) also constructed a series of mathematical models in an attempt to clarify the mechanism responsible for the level of sperm precedence found by Compton *et al.* (1978) in domestic fowl. One of these models tested the effect of 'passive sperm loss', and is in many ways similar to Parker's (1990a) loaded raffle. The model suggested that last male precedence might be due to earlier inseminations decreasing in number as a result of greater mortality. Sperm do leak from the storage tubules (Wishart 1987), however the rate of loss is not sufficiently high to explain Compton *et al.'s* (1978) P2 value of 0.77. However passive loss of sperm from the first ejaculate was sufficient to explain patterns of paternity found in two experiments on domesticated zebra finches (Birkhead *et al.* 1995; Colegrave *et al.* 1995). In the first experiment, a female copulated several times with a first male, followed by a similar number of matings from a second male. Despite both males receiving the same number of matings, the second male fathered the majority of the offspring. In the second experiment, designed to test the efficiency of extra-pair copulations, the first male was allowed nine matings while the second male only mated once. Again the second male fertilized over half of all eggs. The results were found to be extremely similar to those predicted by the passive loss model.

A second hypothesis, for which support is now declining, is sperm stratification (Birkhead and Møller 1992). The majority of evidence for this mechanism comes predictably from poultry. Compton *et al.* (1978) proposed that the last male sperm precedence seen commonly in birds arises as a result of sperm stratification. Using 'dwarfing' as a genetic marker they showed that when females were artificially inseminated, left for four hours and then inseminated again with sperm from a second male, 77% of the offspring were fathered by the second ejaculate, regardless of male genotype.

Three pieces of evidence had led to the suggestion of stratification as a possible mechanism: high levels of last male paternity, the finding of blind ending sperm storage tubules and the breakdown of sperm precedence if matings occur within 4h of each other. When two inseminations occurred less than four hours apart the sperm mixed before entering the storage tubules and no last male mating effect occurred. However when the matings were separated by more than four hours it was proposed that the first ejaculate entered the sperm storage tubules in advance of the second insemination resulting in sperm stratification. It has since become clear that several of Compton *et al.'s* (1978) results are inconsistent with the stratification model. An important prediction of this 'first in – last out' model is that as sperm from the second insemination are depleted, sperm from the first ejaculate should start to be released from the tubules. This should be reflected in an increase in the paternity achieved by the first male. In Compton *et al.*'s (1978) study the proportion of offspring fathered by each male stayed constant over time. Similar results were also obtained in a study of sperm precedence in the zebra finch (Birkhead *et al.* 1988a). Only two studies, both in the turkey, provide support for the uncovering of the first ejaculate and subsequent increase in P1 (Christensen 1981; Christensen and Bagley 1989).

In addition there is no empirical evidence for stratification of sperm within the storage tubules. Van Krey *et al.* (1981) attempted to investigate stratification by inseminating female domestic fowl firstly with [^{3}H] thymidine labelled sperm, waiting four hours, then inseminating again with unlabelled sperm. Unfortunately autoradiographic analysis revealed both labelled and unlabelled sperm in storage tubules and it was impossible to conclusively determine whether sperm were stratified. A second attempt at the same experiment was attempted, replacing tritiated thymidine with a fluorescent dye (Hoescht 33342). This approach also failed as, once stored, labelled sperm released their dye and stained both unlabelled sperm and storage tubules, thereby preventing any attempts at discrimination. The information available to date is therefore seen as providing relatively little support for the stratification hypothesis (Birkhead and Møller 1992), and the general consensus is that this mechanism should now been rejected as a model of sperm competition in birds (Birkhead and Parker 1997).

The second main hypothesis for high P2 values, and the one most commonly cited in studies of insects, is sperm displacement (Parker *et al.* 1990). In several insects evidence suggests that the last male to mate removes some of the sperm of rival males before his own mating attempt (Lefevre and Jonsson 1962; Siva-Jothy and Tsubaki 1989). This sperm displacement can be either direct, as in the damselfly (Waage 1979) or indirect, as in the yellow dungfly (Parker 1990b). Adaptations for direct, or mechanical, displacement have been studied in a number of species including the beetle, *Tenebrio molitor*, (Gage 1992), the bushcricket, *Metaplastes ornatus*, (Helversen and Helversen 1991) damselflies (Waage 1979), longicorn beetles, *Psacothea hilaris* (Yokoi 1990) and rove beetles, *Aleochara curtala* (Gack and Peschke 1994), and is usually associated with adaptations of the intromittent organ, such as hooks or spines. These modifications have been shown to 'scour' the female genital tract and remove any rival sperm (Wagge 1979; Gage 1992). In some odonate species the second male can instead of removing it, push rival sperm to sites where it is has a lower probability of successful fertilization (Waage 1984; Siva-Jothy 1987). A further form of sperm displacement has also been demonstrated in the dunnock, where before mating the male pecks at the cloaca of the female and in response she ejects a droplet of sperm from her cloaca (Davies 1992).

Indirect sperm displacement occurs when the sperm from a second mating pushes out sperm already inseminated by another male (Parker *et al.* 1990), and has been identified as the mechanism of last male precedence in several insects including, the sperm–eating tree-cricket, *Truljalia hibinonis*, (Ono *et al.* 1989) and *Spodoptera* *litura* (Etman and Hooper 1979). Parker *et al.* (1990) proposed the division of sperm displacement in to two separate mathematical models: sperm displacement with instant mixing and sperm displacement with mixing after displacement. To highlight the differences in these two models Birkhead and Parker (1997) put forward the analogy of a large (water) tank with two pipes, one incoming and the other outgoing. The sperm inside this tank represent the sperm that are able to fertilize the eggs, the fertilization set. In the model with mixing after displacement, sperm flow in to the tank and before mixing begin pushing out the old sperm. The proportion of sperm from the last male therefore rises linearly. The rate of increase equalling the volume of sperm entering per unit time divided by the volume of the tank. If however the sperm being inseminated mix immediately with the sperm already in the fertilization set, then the presently mating male will displace some of his own sperm. At first the sperm being lost will mainly be rival sperm, but as the male continues to increase the proportion of his sperm in the tank, the proportion of his own sperm being displaced will also increase (self-displacement). Eventually, as the mating continues, the proportion of self-displaced sperm will increase until finally the majority of sperm being lost are newly inseminated sperm. The proportion of new sperm in the fertilization set therefore increases during the mating, but at an exponentially decreasing rate (Parker and Simmons 1991).

The predictions of the indirect displacement hypothesis have been most rigorously tested in the yellow dungfly (*Scatophaga stercoraria*). Despite the fact that virtually all females contain sufficient sperm to fertilize their eggs, females remate before oviposition, with the male mating and guarding the female immediately prior to egg laying fathering over 80% of the current batch of eggs (Parker 1970b). Several factors suggest that the high P2 value in the dung fly can be explained with a model of sperm displacement with instantaneous mixing. The P2 value has been shown to increase with copula duration (Parker 1970b), and optimality models have shown that males mate long enough to obtain to the observed 80% of paternity despite the fact that longer matings would yield higher fertilization success (Parker 1970b).

Further the rise in P2 with copula duration is not linear, but follows the law of diminishing returns suggesting that self-displacement occurs as the mating continues.

In addition to these three main mechanisms, two others of note have also been proposed. One of the explanations discussed in Compton *et al.'s* (1978) study was that the second ejaculate may in some way physiologically depress the activity of sperm already present in the reproductive tract. It was shown that sperm from a single mating continue to fertilize the hen's eggs for around 18 days. If two males mate with the female, sperm from the first male only remain active for 15 days (Birkhead 1988). In addition when an artificial insemination is carried out with more than four hours separating the introductions of sperm, the sperm from the second insemination have a longer fertilizing capacity (Birkhead and Møller 1992), suggesting that some depression of the first ejaculate is occurring. This idea has received little further attention.

Secondly, in an extension of the idea that rival ejaculates might physiologically inhibit each other, Silberglied *et al.* (1984) suggested that, in Lepidoptera, some sperm might be designed not to fertilize eggs, but to destroy, disable or block the passage of rival sperm. The presence of several sperm types in mammals has also been proposed as being an adaptation for sperm competition (Baker and Bellis 1988, Bellis *et al.* 1990). In particular they discussed the possibility of kamikaze sperm forming plugs and preventing subsequent inseminations from reaching the site of fertilization. The mechanisms underlying physiological incapacitation of rival sperm have been investigated primarily in insects. The most likely explanation appears to be that some agent in the seminal fluid is able to recognize and disable stored sperm. For example, if some molecule, secreted by the female, binds to sperm during storage, this may allow the differential destruction of sperm, and allow the current insemination to achieve precedence. Such a function has been proposed for accessory gland products in the seminal fluid of *Drosophila melanogaster* (Clark *et al.* 1995). However the kamikaze sperm hypothesis has attracted criticism (Harcourt 1991) and remains to be investigated in the majority of taxa.

Patterns of paternity in *Poecilia reticulata*

Before discussing the relevance of these models to the findings of this study, it is important to mention other factors that may have influenced the results. The first of these concerns the experimental conditions. To allow the successful completion of the fingerprinting section of this study, it was necessary to mate females with males from a different population. This was an unfortunate added variable, but only in this way was it possible to obtain offspring that produced sufficiently variable banding patterns to allow unambiguous paternity analysis using RAPDs. It is possible that these cross-population breeding attempts may have influenced the outcome of the study. Females are known to select males on the basis of a range of geographically varying traits (see Chapter 1), and it is possible that the males from the second population may have been in some way disadvantaged as a result of female preferences.

However, Magurran *et al.* (1996) carried out visual choice tests with females from Oropuche and Tacarigua. A male from each population was isolated in a transparent plastic 1l bottle. The bottles were introduced into the female's aquarium, one at either end, and the frequency of approaches and duration of interaction between female and male recorded. Females did not discriminate in favour of the male from their own population. Further in a similar experimental situation in which the three fish could freely interact (Magurran *et al.* 1996), the female approached each male approximately equally. However, despite females spending time with Oropuche males, nearly all copulations were with Tacarigua males. In this study females were isolated with two males from the second population, thus eliminating competition between males from different populations. If females were refusing to mate with these males, or *vice versa*, then we would expect to find that females produced broods devoid of offspring fertilized by new sperm. However newly inseminated sperm gained paternity in all of the first broods that were analysed, confirming that all 45 females were successfully inseminated by males from the second population.

This result supports the findings of other authors that females do not reject matings from males on the basis of population.

While we know that all females mated with experimental males, it is more difficult to account for inter-population differences in the characteristics of the sperm. It is theoretically possible that sperm from males of one of the populations may be, for example, faster swimmers, longer lived, more able to fertilise ova or in some other way more vigorous. If this were the case then these sperm could have an advantage in fertilising eggs and gaining paternity. In other words sperm from different populations may have differential fertilizing capacities (Lanier *et al.* 1979). While no information was collected on the ejaculate features of Oropuche males, the data in chapter 3 showed no significant difference in sperm length between males from the Tacarigua and the Tunapuna. If sperm from one of the two populations in this study were consistently better at fertilizing, then we would expect this variation to be reflected in the amount of paternity achieved by those sperm. The amount of paternity gained by newly inseminated sperm was not significantly different between populations. This result was consistent over each of the three broods, and it therefore seems reasonable to assume that the sperm from both populations were equally efficient at obtaining fertilisations.

Of the mechanisms of sperm precedence outlined previously, at least one can be rejected as being an unlikely candidate in the guppy. As mentioned the males of some species show morphological adaptations of the intromission organ (Waage, 1979; McVey and Smittle, 1984). These hooks and spines may allow the removal of sperm deposited by the previous male, thus giving the new male's sperm a greater chance of paternity. Poeciliid gonopodia are structurally complex and highly variable among species in the size and shape of their bony projections. It is possible that these gonopodial projections could be adaptations for sperm removal. However several factors suggest that direct sperm removal by rival males seems extremely unlikely in *P. reticulata*. In contrast to insects, male guppies are unable to physically manipulate females. Male insects are able to clasp the female's body allowing positive genital coupling and the displacement of previously deposited sperm (Waage 1979). The absence of clasping limbs and genitalic locking mechanisms precludes male manipulation of female sperm stores in the guppy (Constantz, 1989). Furthermore as was shown earlier, sperm are stored deep within the ovary and the small size of the gonopodium and the shallowness of penetration during copulation would prevent direct sperm removal. Direct sperm removal takes considerable time (Waage 1979), and the brief copulation duration observed in the guppy would probably be insufficient to allow the displacement of rival sperm. However there is some evidence that these skeletal elements may serve to reduce remating by tearing or irritating the female genital tissue. Copulation, especially forced copulations such as sneaky mating, may result in vaginal bleeding, oviducal blood clots and leave the female subsequently unreceptive to copulation. This minimises sperm replacement in two ways. Firstly an injured female may be sexually unreceptive until after fertilisation has occurred. Secondly a blood clot may serve as a copulatory plug (*sensu* Ross and Crews, 1977; Murie and McLean, 1980). In extreme cases mating may lead to internal bleeding or even death (Houde 1997).

The rejection of mechanical sperm removal does not preclude the occurrence of indirect sperm displacement. However given the volume of both the ovary and the proposed sperm storage sites in relation to the volume of an average ejaculate, it seems highly unlikely that a male could influence the position of rival sperm with his own ejaculate. In addition indirect sperm displacement, or sperm flushing, is again predicted to take time and, as mentioned, copulation in the guppy is extremely brief.

The stratification of sperm in the storage organ and the tubules, although possible, also seems unlikely. One of the predictions of the model is that the level of last male precedence decreases, as sperm from earlier inseminations are uncovered. Until now only two studies (Christensen 1981; Christensen and Bagley 1989) have provided empirical evidence to support this prediction. In this study, there is an increase in the amount of precedence gained by stored sperm over successive broods. However, as will be explained, this may arise through an alternative and simpler mechanism.

Further work, probably using artificial inseminations, would be needed to provide more information, before this hypothesis can be rejected. Information on the distribution of sperm from separate ejaculates within the ovary would also be most enlightening.

The occurrence of non-fertilizing sperm in the ejaculates of poeciliids has not been investigated. However it is possible that such sperm exist. At present it is therefore impossible to comment on the likelihood of such a mechanism occurring in the *P. reticulata.* A closer analysis of the characteristics of the sperm within guppy ejaculates may provide useful information, and allow insights into the possibility of this mechanism being involved in sperm precedence in this species.

The simplest mathematical explanation of the paternity patterns in this study concerns the proportions of the two sperm types present in the female reproductive tract at the time of fertilisation, Parker's 'fair raffle' (1990a). Unfortunately no information was collected concerning either the number of sperm held by females in storage, or the number of sperm in the recent matings. Before the experiment all females had been in mixed sex stock tanks and it seems likely that at the commencement of the experiment they had full stores of sperm. If we assume the female ovary can contain more than the number of sperm contained in one ejaculate then it seems unlikely that the high P2 values seen in first broods can have been obtained on the basis of numerical predominance alone. However it is likely that not all sperm are in a position from which they are able to fertilize eggs, such sperm would therefore not be included in the fertilization set, and if this is the case the loaded raffle model would be more appropriate. This situation may arise in the ovary of the guppy if not all the sperm in the storage organ are released at the time of fertilization, or if, in some other way, the newly inseminated sperm gain numerical predominance at the site of fertilization. On the basis of the morphological data from the previous chapter it appears possible that this may occur in the guppy.

If sperm competition in the guppy does conform to Parker's (1990a) loaded raffle model then, from our paternity results, we can make predictions about the relative abundances of new and stored sperm that were present when each of the three broods was fertilised. The paternity results for brood one suggest that newly inseminated sperm heavily outnumbered stored sperm. In second broods the amount of paternity gained by stored sperm rose sharply. This suggests that a reversal occurred, with stored sperm outnumbering new sperm, although in 16 out of 20 females new sperm were evidently still present in the oviduct. Again it is possible to imagine a way that these results could arise through, and be explained by, the loaded raffle model. In this experiment the females were kept in isolation and not allowed to remate. Therefore the proportion of each sperm type in the fertilization set will have been altered. It is possible that the increased success of stored sperm is due to an increase in the number of sperm from the female's own population, possibly as more were released from the storage sites. Alternatively, the number of 'new' (although now one month old) sperm may have fallen. Numbers of new sperm will be diminished during fertilisation and this decrease may be considerable if large numbers of sperm are required to fertilize each brood. Assuming paternity reflects sperm abundances, the mix of paternity between new and stored sperm will reflect the proportions of sperm from each population. By the third brood the paternity results indicate that stored sperm made up the majority, and new sperm the minority, of sperm in the oviduct. In 8 of the 12 broods stored sperms fathered all offspring. Again this is likely to be due to stored sperm being released and the reserves of new sperm being depleted during the two previous fertilisations.

There are other possible explanations for the patterns of paternity found in this study. Sperm viability may decrease over time, such that even though stored sperm remain capable of fertilization, they can be outcompeted by fresher, newly inseminated sperm. For example, in this experiment the new sperm may have been able to swim faster than the stored sperm. This may have lead to both a larger number of new sperm reaching the eggs and the new sperm reaching the site of fertilization sooner. After one month in the ovary, the difference in performance between the two sets of sperm may have been reduced, such that stored sperm were no longer competitively equal. Irrespective of the mechanism responsible for the last male precedence it is apparent from the results that a male that successfully inseminates a females in the 24 hours following parturition can, if no further matings occur, expect to fertilize the majority of the next brood. This may have important implications for paternity in natural populations.

One final aspect of interest requiring further investigation is the surprising speed with which stored sperm regained precedence. Previous evidence suggests female guppies are able to produce several successive broods even in the absence of a male (Winge 1937). It is not known how many sperm are used to fertilise each brood. Perhaps females can control the number of sperm that are used, alternatively brood size may be constrained by sperm availability. It is possible that females can assess the amount of sperm they have stored and that the females in this study had such plentiful supplies of stored sperm that they utilized the majority of the most recent insemination to fertilize the first brood, thus explaining the resurgence of stored sperm. Alternatively it is possible that the female may be able to discriminate between the sperm of males from her own population and that of males from other populations. A considerable, and currently expanding, body of literature has recently documented the occurrence of such preferences for conspecific sperm within the female reproductive tract.

Price (1997) raised virgin males and females of three closely related species, *Drosophila simulans*, *D. sechelia* and *D. mauritiana*. On the fourth day after eclosion a virgin female was mated with the first male and if that was successful two days later the female was mated with the second male. All interspecific crosses produced offspring, although the male offspring were sterile. Intraspecific matings in all three species result in a second male sperm precedence. However the result of the interspecific matings provided a number of surprising results. When a *D. mauritiana* male was mated with a *D. mauritiana* female it produced an average of 81 ± 14.9 offspring. When a *D. mauritiana* male was then mated with a *D. simulans* female it produced 104 ± 12.5 offspring. This suggests surprisingly that heterospecific sperm are better at achieving fertilizations. However *D. mauritiana* males suffer a severe reproductive disadvantage when competing against *D. simulans* males for access to *D. simulans* female's eggs. When the heterospecific male mates last he fathers on average 20 ± 11.6 offspring, while when the heterospecific mating occurs first the male only fathers an average of 1.1 ± 0.6 offspring. (c.f. 104 ± 12.5 after a single heterospecific mating). When a female *D. mauritiana* mates first with a *D. simulans* male and then a D. mauritiana male the results are reversed and the *D. mauritiana* male fathers on average 93% of the offspring. Matings with the heterospecific male mating second were not possible due to the discriminatory behaviour of *D. mauritiana* females. This could therefore just be a simple second male sperm precedence, but does indicate that *D. mauritiana* sperm are not universally poor competitors. Price (1997) suggests that something happens inside the female so that sperm acquire a disadvantage inside the reproductive tract of heterospecific females. Similar findings have also been reported in the alpine grasshopper *Podisma pedestris* (Hewitt *et al.* 1989); flour beetles, *Tribolium castaneum* and *Tribolium freemani* (Robinson *et al.*. 1994; Wade *et al.* 1994); Louisiana irises, *Iris brevicaulis*, *I. fulva* and *I. hexagona* (Emms *et al.* 1996) and the beetle, *Callosobruchus maculatus* (Wilson *et al.* 1997). The mechanism by which the sperm of certain male phenotypes do better in the reproductive tracts of females of certain genotypes is as yet unclear.

Although the above studies deal with sperm preferences between species, the geography of Trinidad and the distribution of the two populations used in this chapter provide an analogous situation involving populations. The Tacarigua and Oropuche rivers run from the Northern Mountain Range into two separate drainages. These drainages are thought to have been separated for at least 330,000 (based on mtDNA, Fajan and Breden 1992) and possibly for longer (based on allozymes, Carvalho *et al.* 1991). In light of the genetic differences between the two populations used in this study, it seems plausible that such female preferences for homo-population sperm may eventually provide an explanation for the speed with which stored sperm regain dominance.

In conclusion more must be known about basic aspects of the reproductive biology of the guppy before conclusions can be drawn as to the mechanism(s) underlying sperm competition and sperm precedence. For example in order to fully evaluate the passive loss model in the zebra finch, information was required on the relative numbers of sperm from each male within the female reproductive tract. In order to determine even these seemingly simple values it was necessary to establish the number of sperm inseminated by each male, the timing of the inseminations and the rate of loss of sperm from the female tract. In zebra finches all of this data has now been collected. Unfortunately even basic information such as this is unavailable for any of the poeciliids. It is clear from this study that sperm competition occurs in the guppy and that sperm precedence patterns and their underlying mechanisms deserve further study.

Chapter 7

Discussion and further work

This concluding chapter will provide a summary of the results of this thesis, bringing together the findings of the experimental work presented in this dissertation to highlight the potential importance of gonopodial thrusting in the Trinidadian guppy. Finally consideration will be given to some future areas of research that may provide further interesting insights into the role of sneaky mating and sperm competition in the guppy.

Implications and importance of sneaky mating in *P. reticulata*

This section will discuss the role of the male guppy's alternative mating strategy, sneaky mating, in the context of increasing gene flow and preventing reproductive isolation. In recent years the Trinidadian guppy has proved one of the most revealing species in studies of population differentiation and the evolution of biological diversity. The geography of Trinidad provides a 'natural laboratory' in which to study the effect of variation in an important selection factor, namely the presence or absence of predators (Haskins *et al.* 1961). In some areas, especially the lower reaches of rivers, guppies occur with a wide range of fish predators, including important guppy predators, such as *Crenicichla alta.* However restriction in the distribution of these larger predators, caused by barrier waterfalls, provides a sharp discontinuity in predation regime. Upstream, above any waterfalls, the guppy co-occurs with a reduced fauna of minor predators, such as *Rivulus hartii.* The importance and influence of predation has been highlighted in a recent review by Endler (1995) that listed 47 traits that covary adaptively with predation regime. However Trinidad's guppy populations provide more than mere correlational evidence for the role of natural selection in shaping these populations. The rivers of the Northern Mountain Range provide evidence of a direct causal link between predation risk and evolution. In effect, the island's guppy populations can demonstrate 'evolution in action'.

The best known of these experiments providing direct evidence of heritable changes was performed by John Endler in 1976. Endler's experiment (1980) involved moving guppies from the high predation, Lower Aripo, and transplanting them upstream into a low predation area. This freedom from predation pressure produced remarkable results and over the next 2 years, or 4-6 generations, male colour patterns underwent a period of rapid evolution. Variation was found in the number of spots, the total area covered by spots and the colour diversity of the spots (Endler 1980). Subsequent studies have shown that male colour patterns are not the only traits capable of such accelerated evolution.

During the allozyme based survey of genetic diversity undertaken by Shaw *et al.* (1991) an interesting anomaly was uncovered. The guppies sampled from the Turure river, part of the Oropuche drainage, were genetically more closely related to fish from the Caroni drainage on the other side of Trinidad (Shaw *et al.* 1992). Further investigation revealed that in 1957 Caryl Haskins had performed his own unpublished transplant experiment. The rediscovery of this transplanted population provided an opportunity to investigate how behaviour had evolved over a longer time scale. Again the fish had been moved from a high predation to a low predation site, and over the 34 years, around 100 generations, since the transplant occurred the schooling behaviour had, as expected, become significantly reduced.

The most dramatic evidence of accelerated evolution comes from a recent study of changes in two life-history traits, age and size at maturity (Reznick *et al.* 1997). Using data from an additional transplant (Reznick and Bryga 1987), supplemented by data from existing transplant populations (Reznick *et al.* 1990), they calculated the rate at which populations were evolving. The rate was estimated to be between 5.3 - 45 Darwins (Reznick *et al.* 1997). The observed rate of evolution is therefore on a par with that found in artificial selection experiments, and over seven orders of magnitude greater than the rate found in the fossil record. In combination these experiments provide conclusive evidence of the evolution of traits in response to relaxation in selection pressure. The most remarkable feature however is the speed with which the traits evolve, variation is noticeable not only over the more normal extended time period, but within the lives of the scientists carrying out the experiments.

In addition to these studies documenting rapid evolution, there are several aspects of the biology of female guppies that may also promote population differentiation and move the guppy further towards speciation. Several studies have demonstrated that female guppies exert strong preferences for certain male traits (Houde 1987, 1988a, 1997; Houde and Endler 1990). For example females from the Paria prefer males with areas of red colour spots (Houde 1987; Houde and Torio 1992). Importantly Houde (1994) was recently able to progress further and establish the existence of a genetic correlation between the male trait and the female preference. Such a relationship between a sexually selected trait and the preference for that trait was one of the factors that Lande (1981) recognised as enabling rapid speciation. A further facet of female behaviour that may promote population differentiation are female preferences for other females as school mates. It has been found that female Trinidadian guppies prefer the company of familiar school partners (Griffiths and Magurran 1997a, b). It seems likely that guppy schools are not transitory social groupings, but instead that they represent more permanent structures that remain stable over time. Not only do females form regular groups but these schools tend not to disperse, and females are in general less mobile than males (Magurran and Seghers 1994c; Magurran 1998). These female preferences, both for mates and schooling partners will facilitate the evolution of pre-mating isolation, which in turn may lead to speciation (Butlin and Tregenza 1998; Coyne and Orr 1998).

There is one final aspect of the female guppy's biology that may under certain circumstances have profound genetic implications; the ability of females to store sperm for prolonged periods and produce several broods from only a single mating

(Purser 1937; Winge 1937). If a female is moved to a new location, perhaps by a hurricane or flood or through human activity for mosquito control or even as part of a transplant experiment, she has the potential to found a new population. While there is no evidence of this having occurred in the wild, a case of the successful colonisation of a new environment by a single female has been documented. Professor Kenny, the retired head of the Zoology Department of the University of the West Indies, took a single female guppy and moved her into an isolated pond. When the population genetics of the descendants was investigated a decade later, it was found there had been a dramatic reduction in heterozygosity and also that significant genetic divergence had occurred (Carvalho *et al.* 1996). Founder effects have also been implicated in speciation, although their role is controversial (Barton 1989). However it is clear that females have the potential to cause population differentiation in a number of different ways.

In light of the speed and extent of the evolution reported in Trinidadian guppy populations, it would seem intuitive that *P. reticulata* is undergoing rapid speciation. This does not appear to be the case. As part of their genetic survey Shaw *et al.* (1991, 1992) found that some of the populations are extremely ancient. Genetic markers estimate the Caroni and Oropuche drainages to have been separated for around 500,000 years, approximately 1-2 million generations. Paradoxically there has still not been any reproductive isolation between the drainages (Endler and Houde 1995; Magurran *et al.* 1996). It appears that the Trinidadian guppy system has all the elements required for speciation; a range if traits have been shown to evolve rapidly under natural selection mediated through predation, in addition females exert preferences for a diversity of male traits that vary geographically. This should lead to population differentiation, reproductive isolation and speciation. Several studies provide undeniable evidence that this is not occurring. Therefore while it was traditionally thought that the system was in a state of incipient speciation, this opinion is now being revised. What factors can account for this lack of speciation?

Endler (1995) proposed three possible explanations. Firstly as he correctly points out, the potential for speciation may be reduced because guppies live in relatively ephemeral habitats. Guppies can be found in a range of pools and ditches which are subjected to natural seasonal perturbations. In addition the boundaries between populations may also be transitory. It is therefore possible that these populations do not persist for sufficient periods to become reproductively isolated. However, the rapidity with which guppies evolve in response to variation in predation pressure suggests that the response is much faster than the longevity of these waterfalls. The ephemeral nature of geological features seems unlikely to be the sole reason for the lack of speciation.

A second possible explanation centres on the finding that as predation pressure increases, so female preferences become weaker (Houde and Endler 1990; Shaw *et al*. 1994; Endler and Houde 1995). Waterfalls allow more downstream than upstream gene flow. Downstream sites will also be receiving more inward migration from the upstream tributaries (Endler 1995). These effects will prevent or decrease downstream divergence and reproductive isolation from upstream populations. However there is some concern over the generality of the reduction of female choosiness in high predation sites. As Magurran (1998) points out caution is needed before concluding that the findings of Houde and Endler (1995) apply to low risk populations in other drainages.

Finally, Endler (1995) points out that the geographical scale of gene flow relative to that of selection determines how strongly populations can differentiate. The scale of gene flow in Trinidadian rivers has been estimated at around 0.75 km of stream length (Endler 1977; Endler 1989). The zones of single selection regimes (particular levels of predation, food availability, etc.) are small, consisting of less 100 'gene flow units'. This means that every guppy population is not very far away (in gene flow units) from other populations that experience different selection pressures. Thus while selection for some traits is strong, the small spatial scale of selection relative to gene flow may undermine differentiation. It seems that gene flow must hold the key to understanding the lack of speciation of Trinidad's guppy populations, and it is here that the sexual behaviour of the male may be especially important.

To explain why we return to the male's two different mating strategies. As has been pointed out throughout this dissertation males can either perform sigmoid displays or attempt gonopodial thrusts. In chapter two it was shown that male courtship behaviour is extremely sensitive to a range of environmental factors. Male guppies respond to variation in these factors and switch between strategies as the circumstances dictate. Predation regime and the number of males competing for matings both had a significant effect on male strategy. However perhaps the most important factor determining the relative numbers of sigmoids and thrusts was the receptivity of the female. There were significant, but more subtle interactions between the effects, which also lead to an increase in the frequency of sneaky mating. Importantly males adopt sneaky mating in a wide number of situations.

Chapter three revealed a strong relationship between sperm number and male courtship behaviour. Both the number of sigmoids and the number of thrusts were significantly correlated with the number of sperm that a male had ready to ejaculate. As described earlier this means that females could use male display rate as a way of selecting males with more sperm. Even if females are not using sigmoid rate to select males of high fertility, the relationship between thrusts and sperm number will still benefit males that successfully inseminate unreceptive females. Whilst sneaky mating has been shown to occur at extra-ordinary levels in the wild (Magurran and Seghers 1990a, 1994c), nothing was known concerning how successful thrusts were at transferring sperm to non-receptive females. This issue was addressed directly in chapter four. By combining information on the duration of recoverable sperm after insemination and the rates at which embryos develop it was possible to identify females that had been successfully sneaky mated. A significant proportion (15%) of the females sampled from several sites in Trinidad were found to be carrying highly developed embryos and also to have recoverable sperm. The combination of mature embryos and recoverable sperm is indicative of successful gonopodial thrusting. This is a conservative estimate but confirms for the first time that sneaky mating successfully transfers sperm to females in wild Trinidadian populations. Not only are females receiving unsolicited inseminations, but importantly as was shown in chapter 3, males that attempt sneaky mating more often have higher sperm counts. In combination these results suggest that, not only are females being successfully sneaky mated, but potentially these ejaculates may contain large numbers of sperm. The number of sperm inseminated by sneaky mating may have important implications for its subsequent success during sperm competition (Parker 1990a).

Demonstrating that these sperm gain paternity is a further challenge, but in order to begin to understand how sperm interact and compete within the female reproductive tract, it is necessary to know more about the morphology of the ovary. Therefore a series of histological sections of the ovary were examined. Chapter five provides details of the results and puts forward initial information on a potential mechanism which may make sneaky mating even more likely to undermine female choice. During the period of receptivity, females accept matings from preferred males. This sperm is then used to fertilize the next brood (Winge 1937). However the morphology of the female reproductive tract, in particular the positioning of sperm relative to unfertilised oocytes, suggests that sperm inseminated whilst the female is non-receptive may be able gain numerical dominance at the site of fertilization. This will also have important implications for the outcome of sperm competition (Parker 1990a).

The occurrence of sperm competition in the guppy has been inferred from observations of multiple paternity within broods (Winge 1937; Hildemann and Wagner 1954; Haskins *et al.* 1961; Houde 1988b). Chapter six details the first attempt to utilise molecular techniques to investigate this phenomenon in more detail. In addition it investigates changes in the patterns of sperm utilisation over consecutive broods. Stored sperm fertilized the majority of offspring of second and third broods. However in the first brood after insemination with new sperm, this more recent ejaculate gained the majority, and in most cases the entirety, of the available fertilizations. This confirms that stored sperm can be outcompeted by more recently inseminated sperm. Therefore, if a female fails to mate in the brief period following parturition, sperm received from successful sneaky mating will be able to dominate the paternity of the subsequent brood. Even if a female does accept a mating whilst receptive, the correlation between sneaky mating and sperm number suggests that a large number of sperm may be available to compete against those from the accepted mating.

In conclusion the ardour with which males pursue sneaky matings and the time and energy that they invest in thrusting imply that, in evolutionary terms, this is an important strategy. This dissertation provides the first evidence that sneaky mating does result in the successful transfer of sperm to non-receptive females. Further these inseminations may contain large numbers of sperm. As a result of the timing of inseminations and the positioning of ejaculates within the ovary, and in light of the evidence of competition between stored and recently inseminated sperm, it is clear that sneaky mating has a central role in the biology of the Trinidadian guppy. The success of sneaky mating may well offer the solution to the paradox surrounding the lack of speciation. The aim of this dissertation was to test the hypothesis that sneaky mating and sperm competition undermine female choice in the guppy. The results of this dissertation; the sensitivity of male courtship to environmental factors, the correlation of sperm number and frequency of sneaky mating attempts, the success of gonopodial thrusting and the information on the morphology of the ovary and patterns of sperm use all point to the fact that sneaky mating will act to undermine female choice and play an important part in the maintenance of high levels of gene flow. This may explain the lack of reproductive isolation and speciation seen in populations of Trinidadian guppy.

Further Work

There are still several aspects of the reproductive biology and physiology of the guppy that require further investigation, and whilst this dissertation provides data on a number of important areas, it serves only as a preliminary study. I hope the following suggestions may stimulate further work on this topic.

The most obvious question arising from this thesis concerns the fate of sperm from sneaky mating once they have entered the ovary. Chapter 3 revealed that 15% of unreceptive females in the wild have recoverable sperm. These sperm can only have been inseminated as a result of successful sneaky mating. In addition, chapter six successfully used molecular techniques to demonstrate the occurrence of competition between stored and recently inseminated ejaculates. Unfortunately, due to the low polymorphism of the RAPD fingerprinting technique, within populations paternity analysis was not possible. Therefore, the experiment required that the paternity analysis was based on offspring raised from cross drainage mating experiments. The results support the observations of a lack of reproductive isolation between populations, but as mentioned previously do not account for any population differences in sperm characteristics. The development of more highly polymorphic and sensitive microsatellite markers is vital and is being undertaken in St Andrews at present. Hopefully the successful design of these primers will allow unambiguous paternity assignment within populations. A combination of careful experimental planning and the use of microsatellites will also allow the first direct assessment of the paternity rewards of sneaky mating. A further testable hypothesis arising from the results of chapter three is the prediction that, due to the correlation between thrusts and sperm number, sneaky mating will be more successful in high predation populations. It is also predicted that this relationship may lead to an elevated frequency of multiple paternity in these sites.

The results of chapter three demonstrate a correlation between the numbers of sigmoids and thrusts a male performs and the amount of available sperm. However all data in the chapter were derived from stripped ejaculates. Data on true ejaculates released under natural conditions are extremely rare and numbers of stripped sperm are widely used as an alternative measure (Stockley *et al.* 1996). It is difficult to envisage a way in which this problem can be overcome. Attempts to establish a relationship between the number of sperm inseminated by a male and the number of sperm subsequently available for stripping failed to produce a significant correlation. However, some progress has been made on the collection of natural ejaculates in salmonids. Recent work has centred on attempting to attach a 'condom' over the male gonopore and catch the sperm as they are shed (N.R. Liley, pers. comm.). It is possible that this technology could be modified for use in poeciliids. Personal observations suggest that even after sperm have been artificially removed, males still have sperm available in their testes. However, until a means has been established to demonstrate an association between stripped and natural ejaculate size, the results of the correlation should be treated with caution.

Chapter five provided information on the anatomy of the ovary and adaptations for sperm storage. Further investigations should address the development of the tubules in relation to the timing of fertilizations. When are the delles formed? When are sperm first able to enter the tubules? Can sperm from sneaky matings take up position in the delles before the female becomes receptive? The number of sperm recoverable from the ovary decreases in the seven days following insemination. What process accounts for this decline in abundance? How do sperm enter storage? As a result of sperm storage, females can fertilize broods without males being present, but what factors initiate the release of stored sperm? In addition, it would be interesting to know if there is any evidence for the storage of different ejaculates in different areas of the ovary. The use of labelling techniques allowing the individual identification of competing ejaculates should be investigated as a possible means of addressing this issue. As it appears that the timing of matings will have profound implications for the outcome of sperm competition and the success of sneaky mating, further molecular work is also required. Hopefully microsatellite development will prove successful. Even if it fails, the RAPD protocol in chapter 6 could still be modified and used to investigate the success of inseminations at various times during the female reproductive cycle.

Only through further sustained investigations, integrating behaviour and physiology, of both the male and female guppy can we hope to increase our understanding of courtship and reproduction in this species. Trinidad's unique environmental conditions and the biology of the guppy look set to continue to provide illuminating insights into several important areas of behavioural and evolutionary ecology.

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Appendices

Appendix 4.1

Protocol for assessing embryonic development in the guppy (modified from Haynes 1995). For the purposes of this dissertation females with embryos in stages 1 – 3 were classified as group A, embryos in stages 4 – 5 made up group B, females with more advanced embryos in stages 7 – 9 were classed as group C and embryos in stages 10 – 11 placed a female in group D.

Stage One : Immature ovum
Ovum small, opaque white in colour, distributed throughout the ovary, and often packed within yolking ova or early developing embryos.

Stage Two : Early yolked ovum
Ovum in the process of yolking, opaque yellow orange in colour, but not full sized. Oil droplets, if present, not evenly distributed.

Stage Three : Mature ovum
Ovum fully yolked, translucent golden yellow in colour. Oil droplets evenly distributed over yolk surface.

Stage Four : Blastodisc embryo.
Ovum recently fertilized, embryo appears as a small white cap on the yolk surface. Oil droplets under blastodisc at animal pole.

Stage Five : Primitive streak embryo.
Blastodisc spread to form embryonic shield which nearly covers one half of yolk. Primitive streak appears as thin white line in the centre of the shield

.

Stage Six : Optic cup embryo.
Optic cup and otic vesicles present. Little or no eye pigmentation.

Stage Seven : Early eyed embryo.
Eyes pigmented but not complete. Head greatly enlarged compared to trunk, caudal and pectoral fin buds present. Dorsal pigmentation initiated.

Stage Eight : Middle eyed embryo.

Eyes complete but not yet full sized. Head and trunk proportionate, some dorsal and possibly lateral pigmentation. Dorsal and anal fin buds present. Caudal fin rays forming. Operculae present but inconspicuous.

Stage Nine : Late eyed embryo.

Eyes enlarged but not full sized. Moderate dorsal pigmentation. Pigment present along the lateral line. Pectoral fin rays present.

Stage Ten : Very late eyed embryo.

Eyes full sized. Operculae conspicuous and may be pigmented. Dorsal and anal fin rays present. Embryo much more elongate.

Stage Eleven : Mature embryo

Yolk sac mostly absorbed and pectoral fins elongate. Scales present. Embryo resembles small adult.

Appendix 5.1

Dehydration	
Solution	**Time**
50% ethanol	10 minutes
70% ethanol	10 minutes
96% ethanol	10 minutes
Absolute ethanol I	10 minutes
Absolute ethanol II	10 minutes

Appendix 5.1: Dehydration procedure prior to scanning electron microscopy.

Appendix 5.2

Dehydration and cutting	
Solution	**Time**
80% ethanol	3 hours
90% ethanol	1 hour
95% ethanol	Overnight
Absolute ethanol I	1 hour
Absolute ethanol II	30 minutes
50% ethanol / 50% xylene	5 minutes
Xylene	5 minutes

Appendix 5.2: Dehydration and cutting procedure.

Appendix 5.3

Rehydration	
Solution	**Time**
Xylene I	20 minutes
Xylene II	20 minutes
Absolute ethanol I	60 seconds
Absolute ethanol II	30 seconds
95% ethanol I	15 seconds
95% ethanol II	15 seconds
90% ethanol	15 seconds
80% ethanol	15 seconds
Deionised water	15 seconds

Appendix 5.3: Rehydration procedure.

Appendix 5.4

Histological staining and final dehydration	
Solution	**Time**
Haematoxylin	5 minutes
Tap water I	5 minutes
Tap water II	5 minutes
Tap water III	5 minutes
Distilled water	5 minutes
1% eosin solution	15 seconds
70% ethanol	15 seconds
80% ethanol	15 seconds
90% ethanol	15 seconds
95% ethanol	15 seconds
95% ethanol	15 seconds
Absolute ethanol I	30 seconds
Absolute ethanol II	60 seconds
Xylene I	2 minutes 30 seconds
Xylene II	2 minutes 30 seconds

Appendix 5.4 Histological staining and dehydration procedure.