

**INFLUENCE OF MALE MATING HISTORY ON FEMALE
MATE CHOICE IN THE TRINIDADIAN GUPPY
(*POECILIA RETICULATA*)**

by

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ABSTRACT

The phenotype-linked fertility hypothesis proposes that sexual selection should favour females who can accurately assess the fertilization potential of available males and preferentially avoid mating with males who may be sperm depleted so as to minimize the costs associated with reproduction. I investigated experimentally whether females of the Trinidadian guppy (*Poecilia reticulata*) are able to assess male functional fertility using a wide range of sensory cues and choose to avoid mating with males who may be sperm limited. On average, female guppies avoided males that were apparently previously mated or actually sperm depleted, while exhibiting a significant preference for the other non-sperm depleted male. Both visual and chemical cues from males appear to reveal their potential functional fertility and such cues were used by females to discriminate between sperm depleted and non-sperm depleted males. My novel results are important as they support the above stated hypothesis.

PREFACE

Of the two data chapters included in this thesis, Chapter 3 has been accepted for publication as a peer-reviewed article in the journal *Ethology*, but has not yet been published. My thesis supervisor, Dr. Jean-Guy Godin, and I collaboratively developed the research questions and experimental design for Chapter 3 and D. Chowdhury assisted with some of the behavioural observations and data collection. I performed the data analyses and wrote the first draft of the manuscript. Dr. Godin provided editorial suggestions and comments which improved the manuscript. I have received permission from both of my collaborators to include our collaborative manuscript as Chapter 3 in this thesis.

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CHAPTER 1

GENERAL INTRODUCTION

This thesis investigated whether females of a vertebrate species, the Trinidadian guppy (*Poecilia reticulata*), are able to assess male functional fertility using visual, chemical and socio-sexual cues and to use such cues in making mate-choice decisions. In the General Introduction, I first provide a general overview of why female should have evolved mating preferences for not only attractive males, but also for males who can provide enough sperm to ensure the successful fertilization of female ova. I then describe the study species used in all my experiments, and lastly I summarize the content of the remaining chapters of the thesis.

An overview of female mating preferences

There exists an extensive literature on the mechanisms underlying animal sexual behaviour and the role of sexual selection in shaping the evolution of specific traits associated with the survivorship and reproductive success of organisms. Specifically, sexual selection is the direct consequence of differences in the reproductive success of non-randomly breeding animals owing to both intra-sexual competition for available sexual partners and to inter-sexual selection (i.e. mate choice), which can result in the evolution of elaborated, conspicuous secondary sexual traits, including behaviour (Andersson 1994).

Non-random mating among males and females is ubiquitous in nature, especially in polygamous mating systems (Andersson 1994). In such mating systems, both sexes display many forms of direct, pre-copulatory competition over partners as they engage in courtship displays and contests. Moreover, as females commonly mate with more than one male during a given reproductive cycle (Simmons 2001), sperm competition is a

significant form of direct, post-copulatory competition that greatly affects both male and female reproductive success (Preston *et al.* 2001; Simmons 2001). Sperm competition is competition among sperm of different sires inside the reproductive system of multiply-mated females for the fertilization of their eggs. Such competition strongly enforces the evolution in males of optimal ejaculate size, sperm morphology and quality to maximize egg fertilization. On the other hand, females have evolved sophisticated reproductive structures and behaviours to counter male sexual harassment and deter unwanted sperm from fertilizing their eggs (Wedell *et al.* 2002).

Individual reproductive success is strongly driven by mate choice (Andersson 1994). In polygynous species, female rather than male mate choice is the strongest force behind sexual selection (Andersson 1994). As a direct consequence of female choosiness, a wide variety of secondary sexual characters has evolved in males, which function to enhance male overall attractiveness and reproductive success (Andersson 1994). Females can recognize the phenotypic traits that are reliable indicators of mate quality, and tend to mate preferentially with attractive partners (Andersson 1994; Gavrillets 2001). Owing to their sensory and cognitive systems, females can perceive and integrate visual, tactile, chemical and social cues that are correlated with the health, nutritional state, genotype and fertility of individual males, and tend to express their mating decisions accordingly (Andersson 1994; Iwasa and Pomiankowski 1999; Harris and Moore 2005). In comparison to males, females generally do not exhibit sexual ornamentation nor large variation in their reproductive success within populations (Andersson 1994), as their reproductive success strictly depends on indirect forms of competition for partners (Andersson 1994).

Mating preferences in females should evolve only if accrued fitness benefits exceed associated costs compared with random mating (Andersson 1994). Females may gain direct and indirect fitness benefits from being choosy when assessing potential mates. Direct fitness benefits include resources, protection and parental care received from their chosen mate, which further increase female fecundity, the likelihood of egg fertilization and the survivorship of offspring as well as their own. Indirect (genetic) benefits include increased survival and reproductive success of the female's offspring who inherit "good genes" from their healthy and sexually attractive father (Andersson 1994; Iwasa and Pomiankowski 1999; Pitcher and Evans 2001). By maximizing their fertilization potential and offspring survival, choosy females can minimize the high costs associated with reproduction.

Mating can be potentially very costly for females as it is associated with significant physiological constraints. More specifically, reproductive effort entails considerable energy expenditure, especially in females (Gavrilets et al. 2001), as the energetic investment into egg production is two to four orders of magnitude higher when compared to sperm production in general (Hayward and Gillooly 2011). Consequently, even if spermatogenesis is not limitless, males generally have a much higher reproductive potential than females, as they can produce high quantities of small gametes (sperm) at higher rates than females (Clutton-Brock and Vincent 1991). On the other hand, as offspring survival is usually dependent on zygote size, females are limited to producing very few large and energetically-expensive ova, further restricting their reproductive potential (Andersson 1994; Wedell *et al.* 2002; Hayward and Gillooly 2011). Moreover, females are frequently exposed to physical dangers while seeking sexual partners and

during copulation: they can potentially succumb to injury and predation, can contract diseases or get infected with toxic substances transferred along with the ejaculate (e.g. Magnhagen 1991; Gavrillets 2001). In order to minimize the costs associated with reproduction and to maximize their individual reproductive success, females should be selected to mate non-randomly with high quality, healthy males that can provide superior parental investment in offspring, “good genes” and compatible genotypes (e.g. Barbosa and Magurran 2006). High quality and good health in males is commonly honestly advertised by the expression of secondary sexual traits, as the maintenance of conspicuous phenotypes is costly and thus selected against by natural selection (Andersson 1994). As a result, choosy female will achieve higher reproductive success when mating with conspicuous males who not only manage to avoid predation and succeed in finding resourceful habitats, but also invest in parental care and maintain optimal health as a direct result of their superior genotypes (Kodric-Brown and Brown 1984).

Female preferences for certain phenotypic traits in males vary widely between populations and among individuals within the same population under different environmental conditions (e.g. Endler and Houde 1995; Houde 1997). Despite such variability in female preference, certain conspicuous phenotypes in males have been consistently selected for through female choice in taxonomically diverse polygynous vertebrates. Specifically, bright coloration and large body size in males are widely preferred by females, and have also been found to covary with different aspects of male quality. For example, females of many bird species preferentially reproduce with males displaying colourful plumage, which is commonly positively correlated with male

optimal nutritional state, growth and parental care (Iwasa and Pomiankowski 1999). Similarly, in the Trinidadian guppy, females show a strong sexual preference for males possessing bright red, orange and yellow body colour patterns (Houde 1997). Such carotenoid-based coloration in males is positively correlated with their swimming performance (Nicoletto 1991), overall health conditions and absence of parasites (Houde 1997), high courtship vigour (Houde 1997), and sperm quality and quantity per ejaculate (Pitcher and Evans 2001; Locatello *et al.* 2006; Pilastro *et al.* 2008).

As predicted by the phenotype-linked fertility hypothesis (Sheldon 1994), certain male phenotypes have been found to covary with male functional fertility (i.e. ability of sperm to successfully fertilize eggs), and can thus be important cues used by females to discriminate between available mates. Male functional fertility significantly influences female reproductive potential since a substantial amount of sperm is needed to guarantee the successful fertilization of female ova (Wedell *et al.* 2002; Härdling *et al.* 2008). As a result, sexual selection should favour females that recognize and mate preferentially with males displaying the traits associated with highly abundant and fertile sperm (Sheldon 1994).

Thesis overview

This thesis investigated whether females of the Trinidadian guppy are able to assess male functional fertility using a wide range of cues and to discriminate among available males as potential mates based on their prior mating history. The thesis comprises five chapters (including the current Chapter 1) and a Literature Cited section at the end. Chapter 2 describes methods common to the two data chapters (Chapters 3 and

4). In Chapter 3, I report and discuss the results of experiments that tested whether females are able to acquire information on male fertilization potential using socio-sexual cues associated with male mating history and if such information can ultimately influence female mate choice. I tested female preference by recording female association time in a dichotomous-choice apparatus, along with her sexual responsiveness in a full-interaction environment, towards either an apparently previously-mated male and a non-sperm depleted male. In Chapter 4, I tested whether females are able to assess male fertilization potential using chemical and visual cues originating from males in the absence of any direct information on male prior mating history. Lastly, Chapter 5 provides a summary of my main results and underlines their contribution to our understanding of the mechanisms driving female mate choice. I also suggest potential future areas of research that logically follow from my thesis results.

Study species

The Trinidadian guppy is an important model species for the study of sexual selection (Houde 1997) and is ideal to address the question raised in this thesis for the following reasons. This species is a sexually dimorphic, internally-fertilizing, ovoviparous freshwater fish native to Trinidad that exhibits a resource-free promiscuous mating system and mutual mate choice. There is no parental care of young. Sexual selection has driven the evolution of sexual dimorphism and of conspicuous secondary sexual traits in males (Houde 1997; Magurran 2005). Specifically, adult males are significantly smaller than females and are characterized by complex coloration patterns that include areas of carotenoid-based colours (red, orange, yellow), melanin

(black) colour and structural iridescent colours (white, blue, green) (Houde 1997). In comparison, females are relatively drab and olive-grey in coloration.

In natural populations, individuals live in mixed-sex shoals wherein females encounter multiple males simultaneously (Houde 1997; Jeswiet *et al.* 2011) and generally prefer to mate with the most colourful and larger males and with males engaging in high courtship display rates, depending on the population (Endler and Houde 1995; Houde 1997; Kodric-Brown and Nicoletto 2001). Females are only sexually receptive as virgins and for 2 - 3 days after giving birth (Houde 1997). They mate multiply with different males during each receptive period (Kelly *et al.* 1999; Neff *et al.* 2008) and are able to store viable sperm for several months (Liley 1966; Constantz 1984). Females use visual, chemical and social cues to assess and choose their mates (e.g. Houde 1997; Shohet and Watt 2004; Godin *et al.* 2005; Guevara-Fiore *et al.* 2009), and are therefore potentially able to recognize recently-mated and sperm-depleted males owing to the direct observation of male behaviour (Magurran 2005) and other secondary sexual traits in males (Houde 1997) such as courtship display rate and body coloration pattern (i.e. amount of orange colour), which covary positively with both the quantity and quality of their sperm and thus their functional fertility (Houde 1997; Matthews *et al.* 1997; Evans and Magurran 1999b; Pitcher and Evans 2001; Pilastro *et al.* 2008).

Males achieve copulations and potential fertilizations either by soliciting a female using courtship sigmoid displays or circumventing female choice through sneak gonopodial thrusting (Houde 1997; Pilastro and Bisazza 1999). They experience intense mating and sperm competition in nature (Kelly *et al.* 1999; Neff *et al.* 2008; Jeswiet *et al.* 2011). In the wild, males frequently attempt copulations, at rates between 0.1 to 1.2

attempts per minute on average depending on the population (Farr 1975; Luyten and Liley 1985; Magurran and Seghers 1994; Shaw *et al.* 1994; Godin 1995). The production of sperm ejaculates is rate limited in this species (Pilastro and Bisazza 1999). Moreover, up to 92% of available sperm can be transferred by a male to a consensual female, and males need up to 2-3 days post-copulation to replenish their sperm stores and to resume full fertility. Also, even though sneak copulations are usually less successful than consensual copulations, males may nonetheless be partially sperm depleted after engaging in a successful gonopodial thrust (Pilastro and Bisazza 1999). Sperm traits (quantity, quality and fertilization success) vary among males and, in some populations, covary positively with male phenotypic traits such as body coloration and courtship display rate (Matthews *et al.* 1997; Evans and Magurran 1999a,b; Pitcher and Evans 2001; Pilastro *et al.* 2002, 2008; Locatello *et al.* 2006).

CHAPTER 2

GENERAL METHODS

Study species and laboratory holding conditions

All experimental fish used in the experiments described in Chapters 3 and 4 of this thesis were lab-born and raised descendants of adults collected from the Upper Aripo River (Naranjo tributary), a low-predation population (Magurran 2005), in Trinidad, West Indies (10°41'70"N, 61°14'40"W). Since predation risk reduces the strength of female choosiness in guppies (Godin and Briggs 1996), females that are descendants of a population which has evolved under weak predation pressure are expected to express strong mating preferences (Endler and Houde 1995; Houde 1997).

The fish were held in stock aquaria filled with aged and filtered tap water maintained at 24-26°C and illuminated with overhead fluorescent (full-spectrum) lights on a 13 h L: 11 h D cycle in the Department of Biology at Carleton University. They were fed *ad libitum* twice daily with commercial flake food (Nutrafin™) and live brine shrimp nauplii (*Artemia franciscana*). Newborn fish were removed from the parental tanks and placed into separate smaller holding aquaria until their sex was recognizable visually. At the first visual sign of sexual maturation (i.e. body coloration and gonopodium formation), males were removed from the mixed-sex juvenile holding aquaria and placed in male-only aquaria. In doing so, females were raised in female-only aquaria so as to ensure their virginity at adulthood and maximal sexual responsiveness (Houde 1997). The female-only aquaria were placed between adjacent male-only aquaria to allow females and males to view each other and to familiarize themselves with the behaviour and appearance of the other sex as males commonly court females across the glass walls of adjacent aquaria.

Because guppies become familiar with other individuals after 12 days of association (Griffiths and Magurran 1997) and social familiarity can potentially affect female mate choice (Hughes *et al.* 1999; Kelley *et al.* 1999), the focal test female and the stimulus males used in any given mate choice trial were taken from different non-adjacent holding aquaria and were presumably unfamiliar with each other prior to testing. Female guppies use visual, chemical and social cues to assess and choose their mates (e.g. Houde 1997; Shohet and Watt 2004; Godin *et al.* 2005), and are therefore potentially able to recognize recently-mated males using such cues.

Measuring fish body length and male body coloration

At the end of each behavioural trial in the experiments described in Chapters 3 and 4, the focal female and all stimulus fish were dip-netted from the experimental apparatus, lightly anaesthetized with MS-222 and their left side photographed next to a ruler using a digital camera. Using *Image J* software (<http://imagej.nih.gov/ij/>), I measured the standard body length of each fish and quantified the body coloration pattern of each stimulus male as follows. I separately quantified the areas of black (melanin) and of red, orange and yellow (carotenoid, pteridine) pigmentation, hereafter referred to as orange coloration, on the left side of each male's body, excluding the fins. Black and orange coloration in males are sexually selected through female mate choice in the Trinidadian guppy (Endler and Houde 1995; Houde 1997). I then calculated the relative area of total body colour ($[\text{Black area} + \text{Orange area}] / \text{Total body area}$) for each male as their individual colour score. I analyzed all fish photographs to avoid inter-observer error.

CHAPTER 3

MALE MATING HISTORY INFLUENCES FEMALE MATE CHOICE IN THE TRINIDADIAN GUPPY

This chapter is based on a manuscript currently in press in *Ethology*.

ABSTRACT

Based on the phenotype-linked fertility hypothesis, sexual selection should favour females that can accurately assess the recent mating history of available sexual partners and preferentially avoid mating with recently-mated males (who may be sperm depleted) so as to minimize the risk of their eggs not being fertilized. This hypothesis has received to date only limited attention and empirical support. Therefore, in the current study, I investigated experimentally whether females of a vertebrate species, the Trinidadian guppy, are able to assess the recent mating history of males, and thus potentially their functional fertility, and choose to avoid mating with males that appear to have recently mated and who may be sperm limited. Individual virgin females were first given a dichotomous choice between a male that had not been recently observed to interact sexually with another female (i.e. not sperm-depleted) and another male that had been observed to interact sexually with a female (i.e. potentially sperm-depleted) as sexual partners. Paired males were matched for body length and coloration. Immediately following this test, the focal females were subjected to a free-swimming mate-choice test using the same paired stimulus males. As predicted, on average, female guppies avoided the apparently recently-mated (and potentially sperm-depleted) male and exhibited a significant preference for the other male not recently observed mating (and thus not likely sperm limited) during both tests. We do not yet fully understand the underlying mechanisms of this preference. Therefore, further research on the particular cues that females use to assess the recent mating history and fertility status of males is required.

INTRODUCTION

Gamete production in females is more energetically costly and limited than in males (Clutton-Brock 1991; Hayward and Gillooly 2011). As such, females generally have lower reproductive potential (Clutton-Brock and Parker 1992; Andersson 1994), and thus tend to be more choosy of their sexual partners (Andersson 1994, Andersson and Simmons 2006), than males (but see Edward and Chapman 2011 for male mate choice). However, sperm is not an unlimited, cost-free resource to produce (Dewsbury 1982; Wedell *et al.* 2002; Hayward and Gillooly 2011). In polygynous species, sperm depletion in males owing to recent successive matings is widespread taxonomically (e.g. Preston *et al.* 2001; Wedell *et al.* 2002; Pizzari *et al.* 2003; Olsson *et al.* 2004; Linklater *et al.* 2007; Rubolini 2007; Smith *et al.* 2009; Weir and Grant 2010). Because ejaculate size (= sperm numbers) is an important predictor of male fertilization success when males mate multiply and face sperm competition (Parker 1990, Simmons 2001) and because sperm numbers per ejaculate and the degree of sperm depletion/limitation vary widely among males within populations (e.g. Evans and Magurran 1999a, Doyle *et al.* 2011, Mautz *et al.* 2013; O’Dea *et al.* 2015), there should be selection on females to preferentially choose males with high functional fertility (= success of ejaculates in fertilizing eggs) as mates so as to insure maximal fertilization of their eggs by their preferred mate(s), all else being equal. In this context, the phenotype-linked fertility hypothesis (Sheldon 1994) predicts that ejaculate traits in males correlate with their phenotype, and that females

select for exaggerated or ornamented phenotypes to insure high fertilization success (Sheldon 1994).

In support of this hypothesis, it has been shown that male ornamentation and body size in birds are positively correlated with fertilization success (Sheldon 1994), and that male body coloration and courtship rate covary with sperm quantity, quality and fertilization efficiency in fishes (Matthews *et al.* 1997; Engen and Folstad 1999; Evans and Magurran 1999b; Pitcher and Evans 2001; Pilastro *et al.* 2002, 2008; Locatello *et al.* 2006; Weir and Grant 2010). Therefore, females could potentially assess male functional fertility indirectly using such sexually-selected secondary sexual traits. In addition, a male's mating history may provide females indirect cues of his functional fertility, as a recent or sequential mating in males leads to sperm depletion and decreased functional fertility and reproductive success in many vertebrate taxa (e.g. Nakatsuru and Kramer 1982, Preston *et al.* 2001, García-González 2004; Weir and Grant 2010). As males commonly require a certain refractory or 'time out' period after mating to replenish their sperm reservoirs (Dewsbury 1982, Clutton-Brock and Parker 1992, Preston *et al.* 2001), females will suffer a fitness cost (i.e. infertility) when mating with recently-mated, sperm-depleted males (Wedell *et al.* 2002, Härdling *et al.* 2008; Weir and Grant 2010; Loyau *et al.* 2014).

If males vary in their functional fertility owing to their mating history and limitations in their sperm production (Dewsbury 1982; Nakatsuru and Kramer 1982, Matthews *et al.* 1997; Preston *et al.* 2001; García-González 2004, Weir and Grant 2010), then sexual selection should favour females who can accurately assess differences in functional fertility among available males and avoid mating with those males that are

potentially sperm depleted so to minimize the risk of their eggs not being fertilized by preferred mates (Sheldon 1994; Wedell *et al.* 2002; Härdling *et al.* 2008), all else being equal. This particular prediction of the phenotype-linked fertility hypothesis (Sheldon 1994) has received to date only limited attention and empirical support in invertebrates (Harris and Moore 2005; Sato and Goshima 2007; Loyau *et al.* 2012; Mellan *et al.* 2014) and even less so in vertebrates (Nakatsuru and Kramer 1982). There is thus much scope for additional studies, especially with vertebrates.

Therefore, in the current study, I investigated experimentally whether females of a vertebrate species, the Trinidadian guppy, are able to assess the recent mating history of males, and thus potentially their functional fertility, and choose to avoid mating with males that appear to have recently mated and who might be sperm limited. I also tested whether females are consistent in their mating preferences, based on perceived male mating history, between two consecutive mate-choice tests that differed in available stimuli; namely, an initial dichotomous-choice test (wherein only male visual cues were available) and in a subsequent free-swimming, full-interaction test (visual, chemical and tactile/mechanical cues available). The guppy is an important model species for the study of sexual selection (Houde 1997) and is ideal to address these questions for reasons described in Chapter 2 above.

METHODS

Laboratory holding conditions

The general conditions under which the guppies were maintained in the laboratory prior to experimentation are described in Chapter 2.

Experimental apparatus

The experimental apparatus comprised a central glass test aquarium ($40 \times 20 \times 25$ cm; L \times W \times H), flanked at each end with a clear Plexiglas compartment ($15 \times 20 \times 22$ cm) (Figure 3.1). The test aquarium contained a focal test female and the end compartments contained the stimulus fish (see below). Stippled lines drawn on the back and front panes of the test aquarium demarcated a 10-cm wide preference zone at either end of the aquarium. The bottom of the test aquarium and end compartments was covered with aquarium gravel. The back wall of the test aquarium and three sides of the end compartments were covered externally with brown paper to reduce external disturbances. The central aquarium and end compartments were filled with aerated aged water (15 cm depth, 24-26°C). The entire apparatus was placed inside a blind and uniformly illuminated with overhead full-spectrum fluorescent lights.

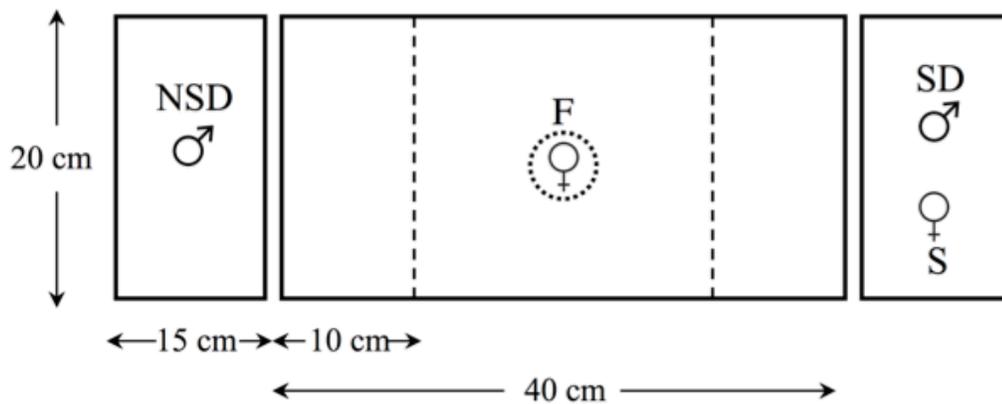


Figure 3.1. Schematic diagram (top view) of the experimental apparatus used in the dichotomous-choice test. The dotted circle in the centre of the test aquarium denotes the clear Plexiglas cylinder in which the focal female (F) was temporarily held. One end compartment contained the apparently sperm-depleted male (SD) and a stimulus female (S) with which he interacted sexually during the viewing period, and the other end compartment contained the non-sperm-depleted (NSD) male. The stippled lines at either end of the test aquarium demarcate the 10-cm wide preference zones.

The behaviour of the fish in the central test aquarium could be directly observed and video filmed through a small port in the blind and the sexual behaviour of the stimulus male that was paired with a stimulus female in one of the end compartments was filmed with an overhead video camera (see below).

General experimental protocol

Dichotomous-choice test

I quantified the mating preference of individual adult virgin females ($n = 58$; mean \pm SE standard body length = 20.9 ± 0.3 mm, range = 18.0 - 25.0 mm) using the well-established dichotomous mate-choice paradigm (e.g. Houde 1997; Wagner 1998; Jeswiet and Godin 2011) and the above apparatus (Figure 3.1). For any given trial, a focal female was given a choice between two stimulus males that differed in their apparent fertilization potential but otherwise matched for body length and coloration (see below for methodology). I used the time that a focal female spent in the preference zone near either stimulus male as a proxy of her mating preference. Such association time is a known reliable predictor of mating preference in both female (Dugatkin and Godin 1992) and male (Jeswiet and Godin 2011) guppies, as well as in other species (e.g. Witte 2006; Lehtonen and Lindström 2008; Walling *et al.* 2010). Although the two stimulus males in each trial were matched for body length and coloration, they possessed individually unique body coloration patterns (Houde 1997; see Appendix Figure 1), which allowed us

and presumably focal females to visually distinguish between them. Female guppies are able to recognize individual males using visual cues, including body coloration, and to subsequently remember their identity for at least 24 hours in making mate-choice decisions (e.g. Godin and Dugatkin 1996; Hughes *et al.* 1999; Pitcher *et al.* 2003; Pilastro *et al.* 2004; Godin *et al.* 2005). The formation of individual male recognition by female guppies can be very rapid, occurring in a matter of only a few minutes (Godin and Dugatkin 1996; Godin *et al.* 2005). In general, guppies have good associative learning abilities and memory based on colour cues (e.g. Reader *et al.* 2003; Bisazza *et al.* 2014).

Prior to the onset of a typical mate-choice trial, I first introduced a virgin female into the central test aquarium and a stimulus male in each of the two end compartments determined at random (Figure 3.1). The fish initially could not view each other because opaque partitions were placed between the end compartments and the central test aquarium. I chose one of the paired stimulus male (determined at random) to be the apparently sperm depleted (SD) male. I simulated potential sperm depletion in this male by placing a non-virgin, gravid (pregnant) stimulus female (similar in body length to the focal female) into his end compartment, thus allowing him to freely interact sexually with this female. Invariably, all SD males interacted sexually with the stimulus female presented. Following Jeswiet and Godin (2011) and Godin and Auld (2013), I used gravid stimulus females, who are generally unreceptive to male courtship and copulation attempts (Houde 1997), to present to SD males so as to ensure that the subsequent mating preference of focal females for either stimulus male based on their prior differential mating history (during the viewing period) would not be confounded by any sexual responses of the stimulus female toward the SD male. Male guppies commonly sexually

pursue, court and attempt to mate with previously-mated gravid females in both the wild and in the laboratory (Houde 1997; Guevara-Fiore *et al.* 2010; Jeswiet *et al.* 2011). They can inseminate unreceptive gravid females using forced copulations (i.e. coercive sneak gonopodial thrusting; Pilastro and Bisazza 1999). The other stimulus male (non-sperm depleted, NSD) did not have a female in his end compartment, and thus was not engaged in any sexual activity and his sperm store was intact.

Once in their respective compartments, the fish were allowed to acclimatize undisturbed for 30 min. After this period, the focal female was dip-netted and placed in a clear Plexiglas cylinder (7 cm diam.) in the centre of the test aquarium and allowed to view, but otherwise not sexually interact with, the paired stimulus males (with one of them interacting sexually with a stimulus female) in their respective end compartments for 15 min. During this viewing period, the apparently SD male and stimulus female were filmed using a digital video camera (CANON Vixia HFM-400) mounted 20 cm above their end compartment. The frequencies of sexual approaches, gonopore nips, courtship displays and copulation attempts (= gonopodial thrusts) (cf. Houde 1997; Godin and Auld 2013) exhibited by the SD male towards the stimulus female were later quantified from the video films. Because each of these sexual acts potentially contributes to male mating success in guppies (Houde 1997), they thus collectively represent a male's pre-copulatory mating effort (cf. Edward and Chapman 2011). At the end the viewing period, the opaque partitions were replaced, the stimulus female was removed from her end compartment and the focal female remained in the central cylinder until the onset of the mate-choice trial (see below).

Although attempted copulations by any stimulus SD male may have lead to successful transfer of sperm to the stimulus female during the viewing period, it was not clear from the analysis of our videos that this was the case because we did not observe unambiguous post-copulatory body ‘jerking’ behaviour, which signals successful sperm transfer in this species (Houde 1997). Nonetheless, because individual test females were simultaneously presented with a male (the SD male) that was observed sexually interacting with a stimulus female and another male (the NSD male) that was not observed near a female, it is reasonable to assume that the test females perceived the SD male as potentially sperm limited, as a result of his recent mating history with a stimulus female, compared to the paired NSD male.

Immediately (< 90 s) following the above viewing period, I started a typical mate-choice trial, which consisted of two consecutive 10-min mating preference tests, by removing the opaque partitions once again and gently raising the central cylinder, thus allowing the focal female to freely swim in the test aquarium and choose between the two stimulus males for an initial 10-min preference test (i.e. preference test 1) based on visual cues only. I observed the behaviour of the focal female from behind the blind, and recorded in real time (with stopwatches) the time the female spent in each preference zone and facing ($0 \pm 90^\circ$) the stimulus male in the end compartments. To control for any potential side bias, the end compartments were switched at the end of this first preference test, and the 10-min preference test was repeated (i.e. preference test 2). On average (\pm SE), only 78 ± 5 s elapsed between the end of the first 10-min preference test and the onset of the second one. A side bias was defined as a focal female spending more than 80% of her total association time in the same preference zone, even after the positions of

the end compartments containing the stimulus males had been switched (Schlupp and Ryan 1997; Dosen and Montgomerie 2004). A trial was discarded from further analysis if either the focal female exhibited a side bias or did not ‘visit’ both stimulus males at least once. Ten such trials were discarded. I used different focal and stimulus fish for each trial.

A focal female’s mating preference was taken as the time she spent near either stimulus male over 20 min (= two consecutive 10-min preference tests), the sum of which is referred to as her total association time. Additionally, a female was categorically classified as ‘preferring’ a particular stimulus male if she spent > 50% of her total association time near him (cf. Godin and Dugatkin 1995). These two metrics of female mating preference are complementary and are widely used in the sexual selection literature (e.g. Nakatsuru and Kramer 1982; Dugatkin and Godin 1992; Dugatkin 1996; Houde 1997; Wagner 1998; Witte 2006; Lehtonen and Lindström 2008; Walling *et al.* 2010). Even though the positions of the stimulus males were switched between the paired 10-min preference tests, the association times that focal females spent near either the SD male ($r_s = 0.53$, $p < 0.0001$) or NSD male ($r_s = 0.43$, $p = 0.0009$) were positively correlated (Spearman rank correlation), and 43 out of the 58 females categorically preferred the same male (greater than expected by chance; Binomial test, $p < 0.0003$), between the paired tests. Females were thus highly consistent in their mating preferences for a particular male over the course of the overall 20-min dichotomous-choice test period.

Full-interaction choice test

This test immediately followed (i.e. 221 ± 8 s later) the dichotomous-choice test for a sub-set of the focal females. The mating preference of 25 of the 58 focal females used in the dichotomous-choice test was ascertained again using a full-interaction choice test, in which individual focal females were presented with the same paired stimulus males that they experienced in the previous dichotomous-choice test. Shortly following the dichotomous-choice test and for any given trial, the focal female was temporarily placed in the central cylinder of the test aquarium and the two stimulus males were introduced into the same aquarium and allowed to swim freely whilst acclimatizing for 5 min. After this period, the cylinder was removed and the female was allowed to freely interact with the stimulus males for 20 min. I recorded the behaviour of the fish using a digital HD video camera (SONY Handycam HDR-PJ380), placed approximately 20 cm in front of the test aquarium and behind the observation blind to reduce external disturbances. In this choice test, the focal female had access to the full range of stimuli (visual, chemical, tactile, sound) originating from the two stimulus males. I changed the water in the test aquarium and end compartments with fresh aged water after every completed mate-choice trial.

From the video films and following Houde (1987, 1988), I recorded the number of courtship displays and copulation attempts (= gonopodial thrusts) directed by each stimulus male towards the focal female, the number of female sexual 'gliding' responses towards a displaying male (defined as the female orienting towards the displaying male, followed by an unambiguous 'gliding' movement towards him), and the number of female 'move towards' (similar to 'glides', but directed towards a non-displaying male). Female sexual interest and mating preference for either stimulus male was then expressed

in one of two ways (Houde 1988, 1997; Godin and Briggs 1996): (i) the proportion of courtship displays exhibited by a particular male that elicited a female sexual ('gliding') response, and (ii) the frequency of unsolicited 'move towards' directed by the female at a male that was not displaying at that time. Additionally, a female was categorically classified as 'preferring' a particular stimulus male if she directed > 50% of her 'glides' or 'move towards' at him (cf. Godin and Briggs 1996). These two behavioural measures of female mating preference predict male mating success in the guppy (Houde 1988). A trial was discarded from further analysis if the focal female did not exhibit any sexual interest (either did not glide or move towards) either of the stimulus males. Six such trials were discarded.

Measuring fish body length and male body coloration

When the testing of any focal female was completed, the focal female, the stimulus female (for the dichotomous-choice test only) and the paired stimulus males were lightly anaesthetized with MS-222, their left side digitally photographed, and their body length measured and body coloration (males only) quantified from the photographs following the methods described in Chapter 2. As I purposely aimed for each trial, paired stimulus males did not differ in either their standard body length (mean \pm SE, SD male = 16.89 ± 0.16 mm, NSD male = 16.92 ± 0.16 mm, $V_{57} = 751.5$, $p = 0.55$, Wilcoxon paired signed rank test) or colour score (SD male = 0.0386 ± 0.0026 , NSD male = 0.0391 ± 0.0025 , $V_{57} = 804$, $p = 0.86$).

Statistical analyses

All statistical analyses were carried out in the R statistical environment (R Development Core Team 2012) and all tests are two-tailed.

Dichotomous-choice test

I first tested the a priori prediction that focal females should prefer the non-sperm depleted (NSD) male over the apparently sperm depleted (SD) male by comparing their mating preference (association time) for either male using the paired t -test, as the data were normally distributed and homoscedastic following square-root transformation (Shapiro-Wilk test). Secondly, I tested whether the time focal females spent associating with the NSD male, and the number of females categorized as preferring the NSD male, differed from that expected by chance using the paired t -test and the Binomial test, respectively. Lastly, I used a linear model with Type I error to test for any effects of focal female body length, the body length and coloration score of the paired stimulus males, and the prior mating effort of the SD male directed towards the stimulus female during the viewing period on the mating preference of individual focal females. In the model, mating effort of the SD male was included both as the sum of all the sexual acts (sexual approaches, gonopore nips, courtships displays, copulation attempts) he directed towards the stimulus female and as the frequency of each of the component sexual acts separately. Female mating preference was expressed as the proportion of her total association time she spent near the NSD male. Because the body length of the focal female and the body length and coloration of either stimulus males did not significantly affect focal female mating preference (all $F_{1,47} < 2.57$, all $p > 0.116$), I therefore reduced the full model using the Akaike Information Criterion model selection procedure (Crawley 2007) to obtain the

qualitatively best explanatory and most parsimonious model. The final model included only the sexual acts of the SD males exhibited during the viewing period of the test as explanatory variables.

Full-interaction choice test

I first compared separately the frequencies of sexual acts (sexual approaches, gonopore nips, courtship displays, copulation attempts, and total acts) exhibited by each of the paired stimulus males and the female sexual responses directed towards each of these males using the Wilcoxon paired signed rank test, as these data were not normally distributed or homoscedastic (Shapiro-Wilk test). Second, I tested whether the number of females categorized as preferring the NSD male (based on their sexual responses towards either stimulus males) differed from that expected by chance using the Binomial test. Lastly, I tested for consistency of female mating preference for either of the paired stimulus males between the dichotomous-choice test and the full-interaction choice test using the *G*-test of independence (Sokal and Rohlf 1995).

RESULTS

Dichotomous-choice test

When provided only with prior visual information about male mating history, focal females subsequently spent significantly more time associating with the non-sperm-depleted (NSD) male than with the apparently sperm-depleted (SD) male ($t_{57} = -5.28$, $p < 0.0001$, Figure 3.2a), and significantly more so than expected by chance ($V_{57} = 1421$, $p <$

0.0001), as predicted. As such, 43 of the 58 focal females tested categorically preferred the NSD male, which is significantly greater than expected by chance (Binomial test, $p < 0.0003$). As revealed by my linear model, the total mating effort of the SD male directed towards the stimulus female during the viewing period of the test significantly affected the subsequent mating preference of focal females (linear model, $F_{1,53} = 13.77$, $p = 0.0005$; Figure 3.3a). Contributing most importantly to this observed negative relationship (Figure 3.3a) were the component acts of sexual approach (linear model, $F_{1,53} = 11.68$, $p = 0.001$) and courtship display ($F_{1,53} = 15.12$, $p = 0.0003$) of the SD male. More specifically and interestingly, the strength of focal female preference for the NSD male was inversely related to the prior rates of sexual approaches (Fig. 3.3b) and courtship displays (Figure 3.3c) exhibited by his rival, the SD male, toward another nearby (stimulus) female.

Full-interaction choice test

As expected, and consistent with the above results for the dichotomous-choice test, focal females preferred the NSD male over the paired SD male as evidenced by their directing significantly more sexual glides ($V_{24} = 36.5$, $p = 0.035$, Figure 3.2b) and marginally more ‘move towards’ ($V_{24} = 81.0$, $p = 0.083$, Figure 3.2c) at the NSD male than the SD male when free-swimming with them. As such, 18 out of the 25 focal females were categorized as preferring the NSD male in this test (Table 3.2), which is greater than expected by chance (Binomial test, $p = 0.029$).

Consistency in mate choice between tests

Because the paired stimulus males did not differ in their body length and coloration or in any component of their mating effort directed at the focal females (Table 3.1) and because of the lack of influence of the body length and coloration of the stimulus males on female mating preference (see results of my full linear model in the Statistical analyses section above), the observed strong female mating preference for the NSD male cannot be explained by any difference in the phenotypes of the males, as measured here. Although females showed a strong overall preference for the NSD males in both the dichotomous-choice and full-interaction choice tests, individual females were overall not consistent between these two choice tests in their preference for a particular male ($G_{\text{adj}} = 0.006$, $df = 1$, $p > 0.90$, Table 3.2).

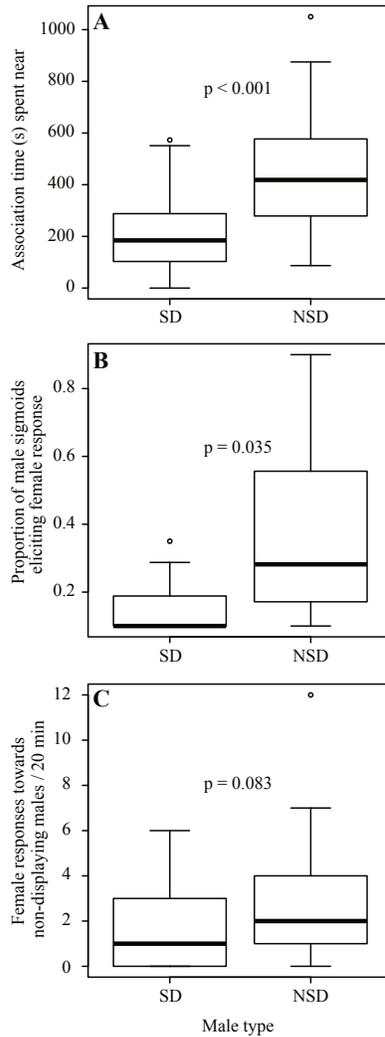


Figure 3.2. Female mating preference for either of the two stimulus males expressed as (a) association time spent near the apparently sperm depleted male or the apparently non-sperm-depleted male in the dichotomous-choice test, and as (b) the proportion of solicited sexual ‘glide’ responses exhibited towards a displaying stimulus male and (c) frequency of sexual responses (‘move towards’) towards non-displaying males in the full-interaction choice test. Box plots illustrate the median, 25th and 75th percentiles, inter-quartile range, and data points outside this range (open circles). The p-values shown were obtained using the Wilcoxon paired signed rank test.

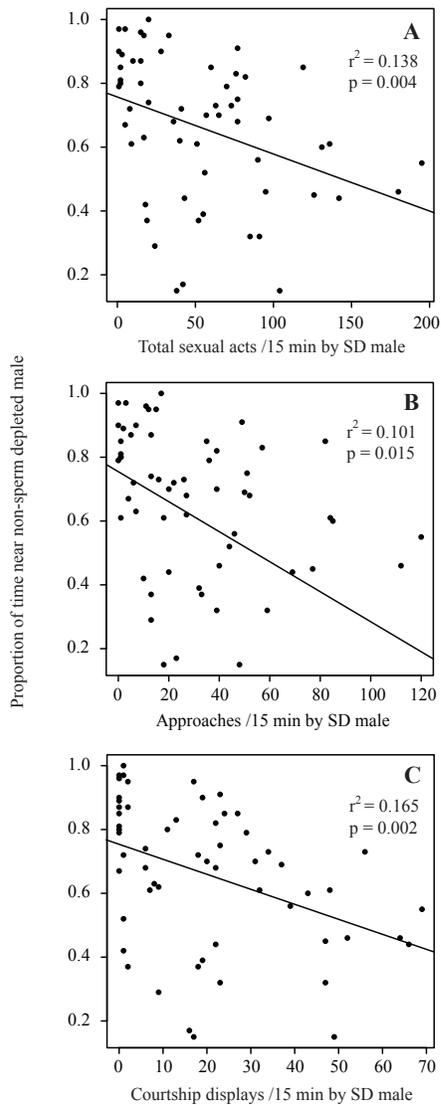


Figure 3.3. Female mating preference expressed as the proportion of total association time near the non-sperm depleted (NSD) male as a function of the frequencies of (a) total sexual acts, (b) sexual approaches, and (c) courtship displays exhibited by the other (apparently sperm depleted, SD) male towards the stimulus female during the 15-min viewing period of the dichotomous-choice trial. The lines of best fit, and associated statistics, shown were obtained using linear regression.

Table 3.1. The mean \pm SE standard body length, colour scores and frequencies of total and component sexual acts exhibited by the apparently sperm depleted (SD) male and the non-sperm-depleted (NSD) male towards a free-swimming female in the full-interaction choice test. Paired statistical comparisons were made using the Wilcoxon paired signed rank test (n = 25).

Phenotypic trait	SD male	NSD male	<i>V</i>	p
Body length (mm)	16.83 \pm 0.24	16.56 \pm 0.26	230.0	0.07
Body colour score	0.051 \pm 0.003	0.053 \pm 0.003	151.5	0.78
Courtship display	6.5 \pm 2.32	5.5 \pm 1.12	95.0	0.49
Gonopore nip	2.4 \pm 0.54	4.0 \pm 0.92	58.5	0.15
Gonopodial thrust	1.3 \pm 0.43	1.7 \pm 0.53	36.0	0.30
Total sexual acts	10.1 \pm 2.53	11.2 \pm 2.13	111.0	0.42

Table 3.2. Contingency table showing the number of females (out of a total of 25), which were tested in both the dichotomous-choice test and full-interaction-choice test, that categorically preferred either the apparently sperm depleted (SD) male or the non-sperm depleted (NSD) male in both choice tests. Data were analyzed using the *G*-test of independence with Williams correction. $G_{adj} = 0.006$, $df = 1$, $p > 0.90$.

		Dichotomous-choice test		
Full-interaction choice test		SD male	NSD male	Total
SD male		2	5	7
NSD male		6	12	18
Total		8	17	25

DISCUSSION

Because of their higher costs of reproduction and lower reproductive potential compared with males (Clutton-Brock 1991; Clutton-Brock and Parker 1992; Hayward and Gillooly 2011), there is strong selection on females to discriminate among potential sexual partners and to mate preferentially with males displaying secondary sexual traits that honestly advertise their overall quality, so as to maximize their reproductive success (Andersson 1994; Andersson and Simmons 2006). A component of male quality is functional fertility, that is, ejaculate size and the success of ejaculates in fertilizing eggs (Parker 1990, Sheldon 1994; Simmons 2001), which varies widely among males within populations (e.g. Evans and Magurran 1999a, Preston *et al.* 2001; Pizzari *et al.* 2003; Weir and Grant 2010; Doyle *et al.* 2011, O’Dea *et al.* 2015). By preferentially mating with males that have high functional fertility, females would maximize the fertilization of their eggs and thus their reproductive success (Sheldon 1994; Wedell *et al.* 2002; Härdling *et al.* 2008), all else being equal.

Here, I showed that mating preferences in female Trinidadian guppies can be influenced by the female’s observation of the recent mating history of available males, such that they significantly preferred males that were not observed to mate recently (and thus not likely to be sperm depleted) over males that were recently observed sexually interacting with another female and consequently more likely to be sperm depleted. This mating preference, observed in both my mate-choice tests, cannot be explained by any differences in measured phenotypic traits between the paired stimulus males, as they did not differ in their body length and colour score in either mate-choice tests, nor in their

mating effort in the full-interaction choice test. Moreover, any behavioural interactions between the paired stimulus males is unlikely to explain the observed female preference for the non-sperm depleted male in the latter test, as there is no clear evidence for female mate choice based on male aggression or sexual interference in the Trinidadian guppy (Houde 1997). This result strongly suggests that female guppies avoid recently mated and potentially sperm depleted males as mates, as predicted by the phenotype-linked fertility hypothesis (Sheldon 1994). Alternatively, this avoidance may be owing to focal females having perceived the SD male as being sexually unattractive because of the non responsiveness of the gravid stimulus female towards him during the viewing period. But this is unlikely because the SD male exhibited sexually-attractive courtship displays, whereas the NSD male did not, during the viewing period. My findings are relatively novel for vertebrates; female sexual discrimination among males based on their recent mating history and potential functional fertility has been reported previously for only one other vertebrate species, the lemon tetra *Hypessobrycon pulchripinnis* (Nakatsuru and Kramer 1982), and a few invertebrates (Harris and Moore 2005; Sato and Goshima 2007; Loyau *et al.* 2012; Mellan *et al.* 2014) to my knowledge.

According to the phenotype-linked fertility hypothesis (Sheldon 1994), male functional fertility may be reliably indicated by costly sexually-selected traits, such as body ornamentation and courtship rate, and that females could potentially assess male functional fertility indirectly using such sexual traits when making their mating decisions. In addition, a male's mating history may provide females indirect cues of his functional fertility, as a recent or sequential mating in males leads to sperm depletion and decreased functional fertility and reproductive success in many vertebrate taxa (e.g. Nakatsuru and

Kramer 1982, Preston *et al.* 2001, García-González 2004; Weir and Grant 2010).

Although female guppies in the current study overall preferred the non-sperm depleted males and thus avoided the sperm depleted males, the magnitude of female avoidance of apparently sperm depleted males in the dichotomous-choice test was negatively correlated with the males' prior rates of total sexual acts, comprising most importantly of sexual approaches and courtship displays, directed towards another nearby (stimulus) female during the observation period of this test. This relationship suggests that focal female guppies used observed differences in the apparent recent mating history of the paired stimulus males, as revealed by their prior sexual interactions (namely, courtship displays and sexual approaches) with other females, in making their decision to avoid the male that was recently observed to sexually interact with another female in favour of the other male that was observed not to have recently interacted with any other female. This is a plausible mechanism as a male's courtship effort is a known predictor of his functional fertility and the degree of depletion of his sperm stores in fishes, including the guppy (Nakatsuru and Kramer 1982; Houde 1988; Matthews *et al.* 1997; Engen and Folstad 1999; Evans and Magurran 1999b; Pitcher and Evans 2001; Weir and Grant 2010). In addition to public visual cues, chemical cues can serve as indicators of a male's recent mating history and thus potential sperm depletion in invertebrates (Harris and Moore 2005; Sato and Goshima 2007; Loyau *et al.* 2012), but this remains to be determined in the guppy.

Different sensory cues were available to focal female guppies to discriminate between the paired stimulus males in our dichotomous-choice and full-interaction tests, with only visual cues from the paired stimulus males (and stimulus female) in the

dichotomous test and the full range of natural cues (visual, chemical, tactile/mechanical) in the full-interaction test being available. Although female guppies exhibited a strong preference for the non-sperm depleted males overall in both the dichotomous-choice and full-interaction choice tests, not all individual females were consistent between the two choice tests in their preference for a particular male (14 were consistent, 11 were not). Because female Trinidadian guppies are usually consistent over time in the expression of their individual mating preferences when their sensory environment remains similar (e.g. Godin and Dugatkin 1995; Brooks 1996; Godin *et al.* 2005), the observed inconsistency in individual female mating preferences between our two choice tests here requires explanation. One plausible explanation is that such inconsistency in mating preference may be attributable to inter-individual variation among focal females in their use of available cues, which differed between the two tests, to assess and distinguish between the stimulus males. Second, females may have varied in their ability to perceive and remember (from the previous dichotomous-choice test) the SD male as being potentially sperm depleted because of the likely more difficult cognitive task of distinguishing between two paired stimulus males of similar phenotype (Table 3.1) in the more sensorially complex environment of the full-interaction test. Lastly, repeatedly testing individual focal females three times (in two consecutive 10-min preference tests in the dichotomous-choice test, followed shortly thereafter by a 20-min full-interaction test) may have adversely affected their behavioural consistency. The latter is not a likely explanation because the number of sequential repeated tests does not affect repeatability (consistency) estimates of behaviour, and repeatability estimates are higher for behaviours measured close in time (as is the case for my current study), as revealed by a

recent meta-analysis of the literature by Bell *et al.* (2009).

It is now well recognized that an animal's social environment and available social information can importantly affect its behavioural decisions (Westneat *et al.* 2000; Danchin *et al.* 2004). In the guppy, females can use social cues in their immediate environment to copy the observed mate choice of another nearby conspecific female (e.g. Dugatkin and Godin 1992; Godin *et al.* 2005). Interestingly, in previous mate-choice copying studies (e.g. Dugatkin and Godin 1992; Godin *et al.* 2005), female guppies were attracted to and more likely to prefer a male that they had previously seen near another (model) female than otherwise, whereas in the current study females avoided males that were previously observed near and sexually interacting with another female. The difference in the sexual responses of female guppies between our current study and previous mate-choice copying studies is likely attributable to differences in the nature of the social information originating from the stimulus male that is near the model stimulus female (cf. Westneat *et al.* 2000). In the current study, one of the paired stimulus males was allowed to fully and physically interact sexually with a stimulus female (including copulation attempts) during the viewing period, whereas in previous mate-choice copying studies with the guppy (e.g. Dugatkin and Godin 1992; Godin *et al.* 2005) and in other such studies with fish (e.g. Schlupp and Ryan 1997) the stimulus male and the nearby model stimulus female were separated by a clear partition and thus not allowed to physically interact (no sexual pursuit, gonopore nips or copulation attempts possible). So it is plausible that the social information arising from the (physical) sexual interactions between a stimulus male and a stimulus female in the current study indicated a likely sperm-depleted male to focal females and led to female avoidance (as expected from the

phenotype-linked fertility hypothesis, Sheldon 1994), which is not the case in mate-choice copying studies in fishes (e.g. Dugatkin and Godin 1992; Schlupp and Ryan 1997; Godin *et al.* 2005).

To date, investigations on the mechanisms underlying female assessment of male mating history, and related functional fertility, in the context of mate choice are relatively few (Nakatsuru and Kramer 1982; Harris and Moore 2005; Sato and Goshima 2007; Weir and Grant 2010; Loyau *et al.* 2012; Mellan *et al.* 2014). My findings presented here contribute importantly to this limited body of knowledge and are novel for the guppy. Following up on this first study with the guppy (current Chapter 3), I investigated female choice for males that have not recently mated (and thus have their sperm reserves intact) versus sperm-depleted males (that have naturally exhausted their sperm reserves through previous copulations), in the absence of prior visual social information regarding male mating history available to focal females. My follow-up study (Chapter 4) was designed to address whether female discrimination between sperm-depleted and sperm-intact males could be owing to changes in male behaviour and/or emission of chemical cues, which could indicate reduced male functional fertility, as a result of recent copulations with other females. It would also be important to demonstrate in future any reproductive cost to mating with sperm-depleted males in female guppies.

CHAPTER 4

FEMALE GUPPIES USE OLFACTORY AND SOCIO-SEXUAL CUES TO ASSESS MALE FUNCTIONAL FERTILITY WHEN CHOOSING MATES

ABSTRACT

Recently, a growing body of research has been paying close attention to the possibility that choosy animals may use multiple cues, rather than rely only on one, to assess male quality and choose a mating partner. As a logical follow-up to my findings reported in Chapter 3, I was interested in testing the ability of females of the Trinidadian guppy to use multiple cues to obtain information about male functional fertility when no direct information on their recent mating history is available and, if so, to use such information to avoid sperm depleted males as potential mates, as predicted by the phenotype-linked fertility hypothesis. Here, I provide empirical evidence that, when only male chemical cues were available, female guppies avoided males that were sperm depleted owing to recent copulations with another female, and they exhibited much stronger sexual responses and interest towards non-sperm depleted males in a more sensorially complex environment with multiple male cues available. Such mating preferences can be explained by differences in potential functional fertility of available males because they did not differ in their body length, weight and coloration and because no preference was expressed by the females towards any particular male when males were matched for their phenotypic traits (body length, weight and colour) and did not differ in their prior mating history.

INTRODUCTION

Even if males can be choosy (Edward and Chapman 2011), mate choice has evolved to be particularly strong in females because of the high costs and risks associated with reproduction. In fact, females usually have a lower reproductive potential than males (Clutton-Brock & Parker 1992; Andersson 1994) because the production of female gametes is energetically more costly than the production of male gametes (Clutton-Brock 1991; Hayward & Gillooly 2011). As a result, females should be selected to be able to use a wide range of cues, rather than relying only on one sensory modality, to assess the quality of available males in order to mate with the most suitable partner. However, there is still limited evidence for the use of multiple cues by females in mate choice (Candolin 2003). Male fertilization potential (i.e. functional fertility) is an important aspect of male quality as a significant amount of sperm is needed to ensure fertilization of female ova (Wedell *et al.* 2002; Härdling *et al.* 2008). By avoiding sperm depleted males and by preferentially mating with males that have high functional fertility, females would significantly decrease the costs associated with reproduction while maximizing the fertilization of their eggs and thus their reproductive success (Sheldon 1994; Wedell *et al.* 2002; Härdling *et al.* 2008), all else being equal.

The findings reported in Chapter 3 suggest that female guppies are able to indirectly assess male functional fertility based on information obtained from observing the recent mating history of available males with other females. These findings raise the question about the particular sensory cues that female guppies use to assess male functional fertility, which presently are not well known. In nature, female guppies may

not always be able to observe sexual interactions between males and other females, and consequently assess their functional fertility based on such social information, due to the fission-fusion nature of their societies wherein males move frequently between mixed-sex shoals in search of sexually receptive females (Croft *et al.* 2006). Consequently, the use of other (non-visual) cues by females that might indicate male functional fertility would be expected. Therefore, I was interested in testing the ability of female Trinidadian guppies to use multiple cues in assessing the functional fertility of males and in avoiding sperm depleted males as mates when no direct information on the recent mating history of males is available to them, as a follow-up study to my findings reported in Chapter 3.

Potential socio-sexual cues that may indicate male quality include visual, chemical, tactile and mechanical (e.g. sounds) cues (Candolin 2003). Males of numerous invertebrate and vertebrate species are not only characterized by visually conspicuous secondary sexual traits, such as bright coloration and elaborate ornaments, but also engage in vigorous sexual behaviours (e.g. courtships displays) and intra-sexual competition (e.g. aggression, sexual interference behaviour) (Andersson 1994; Houde 1997). In general, females tend to prefer males that exhibit high courting display rates (Andersson 1994; Houde 1997), as these males are usually superior in their quality and can provide direct benefits such as resources and parental care (Knapp and Kovach 1991).

In promiscuous and resource-free mating systems wherein males do not provide any direct benefits to females (Andersson 1994), female preference for large, colourful and high-courting males has been hypothesized to increase female fitness and the survival and reproductive success of their young, as these males are able to provide indirect

genetic benefits to females (Pilastro *et al.* 2002). For example, males who engage in vigorous behaviours are more likely to transmit “good genes”, sexually attractive features and resistance against diseases to their offspring (e.g. Andersson 1994; Pellitteri-Rosa *et al.* 2011).

Moreover, female preference for males displaying conspicuous secondary sexual traits and high courtship rates might have been selected for if these sexual traits in males covary with their fertilization potential, thereby potentially conferring direct fertility benefits to females (Sheldon 1994; Pilastro *et al.* 2002). Evidence supporting this phenotype-linked fertility hypothesis (Sheldon 1994) has been obtained mainly by testing if the evolution of female preference for certain secondary sexual traits in males, such as colour and body size, could have occurred owing to a positive correlation between such traits and the size and quality of male ejaculates (e.g. Pilastro *et al.* 2002; Pitcher *et al.* 2009). In comparison, relatively little research effort has been dedicated to characterizing the relationship between conspicuous sexual behaviours, such as courtship displays, and male functional fertility. Nonetheless, given that courtship behaviour covaries positively with ejaculate quality and male functional fertility (Evans and Magurran 1999, Pilastro and Bisazza 1999; Charge *et al.* 2010) and male copulation and resultant sperm depletion may lead to changes in male sexual behaviour (e.g. reduction in courtship rate, Matthews *et al.* 1997) in some species, female mate choice could potentially be influenced by visual cues obtained by females from directly observing the courtship displays of individual males, which might honestly advertise their functional fertility.

In addition to visual cues, olfactory cues have also been shown to play an important role in providing information about male quality and in female mate choice in

taxonomically diverse terrestrial and aquatic species (e.g. Blows and Allan 1998; Landry *et al.* 2001; Candolin 2003; Shohet and Watt 2004; Plenderleith *et al.* 2005). Sex pheromones are chemical compounds that allow animals to recognize mating partners of the opposite sex, and are very diverse in their chemical structure across different taxonomic groups (reviewed in Gomez-Diaz and Benton 2013). Females and males of many insects, nematodes and mammals have been found to strongly rely on these chemical signals when searching for and assessing mating partners. For example, sex pheromones play a central role when preventing cross-species and strain mating, especially in morphologically similar organisms who inhabit overlapping niches, and can advertise sexual maturity and aging. In addition, as a pheromone's profile can change depending on an individual's mating status, individuals might be able to assess the recent mating history and fertilization potential of available partners using chemical cues. Investigations of such potential use of sexual pheromones have been largely limited to insects and other invertebrates (reviewed in Harris and Moore 2005; Sato & Goshima 2007; Gomez-Diaz and Benton 2013). In fishes, sexual pheromones and other chemicals produced by individuals mediate a wide range of behaviours, including mating, shoaling, conspecific recognition and predator recognition, in others (e.g. Shohet and Watt 2004; Plenderleith *et al.* 2005; Ward and Currie 2013). Moreover, fish are able to use chemical cues to assess the phenotypic traits of conspecifics, such as body length, and mediate the size-assortativeness of shoals (Ward and Currie 2013).

The Trinidadian guppy is a suitable model species for investigating whether adult females can assess male functional fertility using chemical cues. This species has evolved a strong sense of smell and shows a wide range of predictable behavioural responses to

water-borne chemical cues, such as predator avoidance, detection and recognition of conspecifics, kin recognition and changes in gestation period (Nordell 1998; Brown and Godin 1999; Shoehet and Watt 2004, Evans *et al.* 2007). Moreover, mate choice in male guppies is influenced by olfactory cues, as males are able to assess female reproductive value based on their production of sexual pheromones that vary over their gestation period (Houde 1997), and they show stronger sexual interest in and attempt to copulate more frequently with virgin females than with previously-mated females (Guevara-Fiore and Watt 2009).

In the current study (Chapter 4), I tested the hypothesis that, in the absence of any direct information on the prior mating history of males, female Trinidadian guppies use both visual and olfactory cues from individual males to assess their functional fertility and avoid mating with sperm depleted males in preference of males with intact sperm stores. I tested for the mating preference of individual focal females by presenting them with paired stimulus males that differed in their functional fertility (with one male being sperm depleted/limited and the other possessing a full sperm store) in each of three consecutive sensory cue treatments: Treatment 1 (male olfactory cues only), Treatment 2 (male visual cues only) and Treatment 3 (multiple sensory cues available).

METHODS

Laboratory holding conditions

The general conditions under which the guppies were maintained in the laboratory prior to experimentation are described in Chapter 2.

To ensure that stimulus males used in the current study had previous courting and mating experience, they were exposed to free-swimming females and allowed to mate with them in their respective holding aquaria up to seven days before the onset of their use in experimental trials. At the end of this period of continuous sexual interactions with females, the males would likely have been fully or partially sperm depleted. On the other hand, males would have full sperm stores if 7 days had elapsed since they had last mated with any female, because male guppies require up to 2-3 continuous days of not mating to replenish their sperm stores (Pilastro and Bisazza 1999).

Experimental apparatus

The experimental apparatus comprised a central glass test aquarium (40 × 20 × 25 cm; L × W × H), flanked at each end with a clear Plexiglas compartment (15 × 20 × 22 cm) (Figure 4.1). The test aquarium contained a focal test female and the end compartments contained one stimulus male each (see below). Stippled lines drawn on the back and front panes of the test aquarium demarcated a 10-cm wide preference zone at either end of the aquarium. The bottom of the test aquarium and end compartments was covered with aquarium gravel. The back wall of the test aquarium and three sides of the end compartments were covered externally with brown paper to reduce external disturbances. The central aquarium and end compartments (see details below) were filled with aerated aged water (15 cm depth, 24-26°C). The entire apparatus was placed inside a blind and uniformly illuminated with overhead full-spectrum fluorescent lights. The behaviour of the fish in the central test aquarium could be directly observed and video filmed through a small port in the blind and the sexual behaviour of both stimulus males

was filmed with overhead video cameras placed either overhead (for Treatments 1 and 2, see below) or directly in front of the central aquaria (Treatment 3).

A variable-flow peristaltic pump (model #3385, VWR International, West Chester, PA, USA) was located near each of the end compartments in Treatment 1 only. The two pumps could be simultaneously activated to slowly pump stimulus water from each of the stimulus male end compartments into the respective ends of the central test aquarium (Figure 4.1a). Prior to experimentation, the pumps were calibrated (with food colouring added to the water of the end compartments) to deliver water at similar rates (0.586 and 0.625 ml/min, respectively), so as to create a gradient of chemical cues from either end of the central aquarium towards its centre.

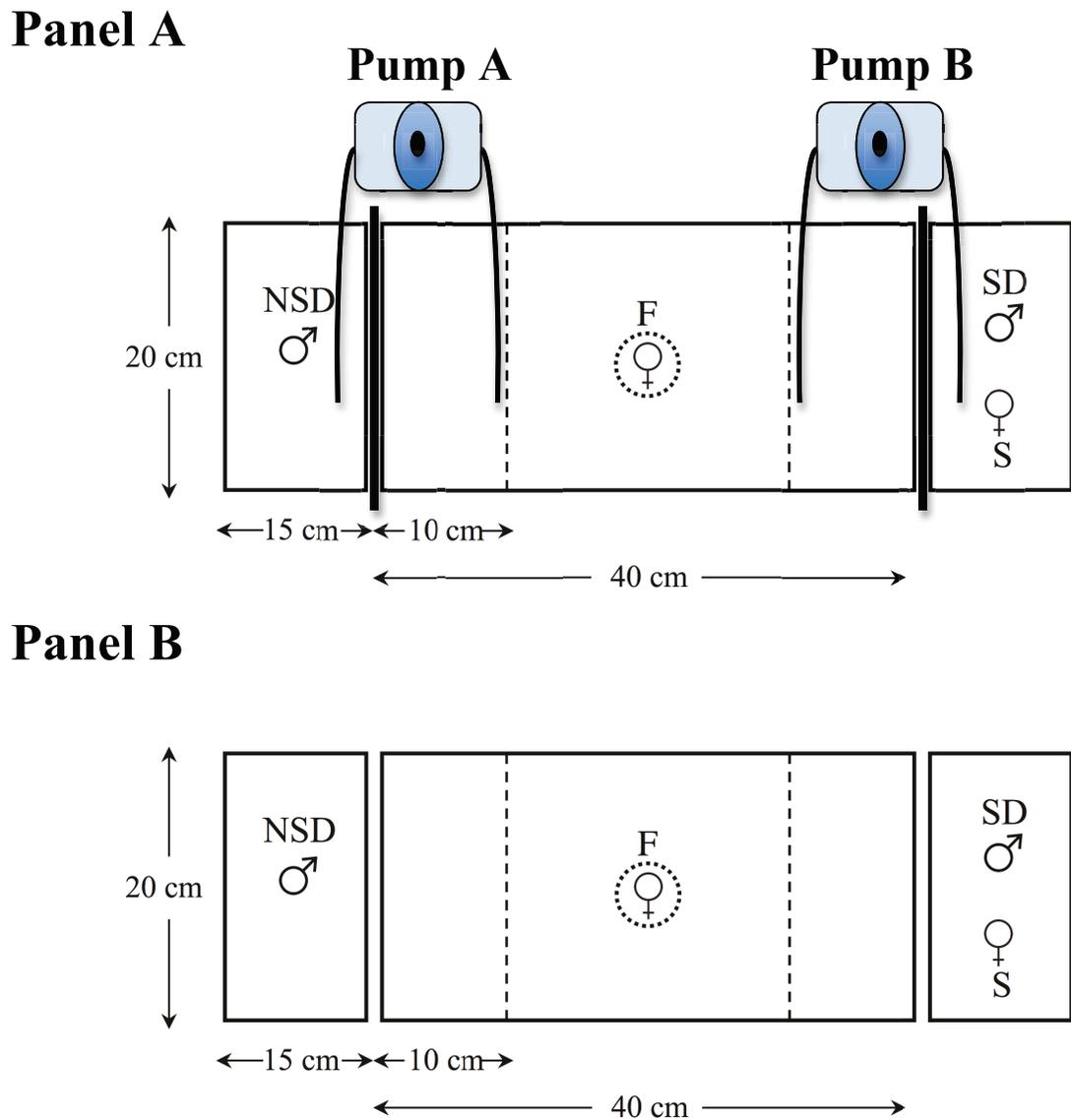


Figure 4.1. Schematic diagram (top view) of the experimental apparatus used in the dichotomous-choice test of Treatment 1 (chemical cues only, panel A) and Treatment 2: (visual cues only, panel B) of the experimental trials. One end compartment contained a sperm-depleted (SD) male and the other end compartment contained a non-sperm-depleted (NSD) male. Two peristaltic pumps were used to slowly deliver water containing male chemical cues from each of the end compartments into the central test aquarium in Treatment 1. The thick dark lines separating the central aquarium from both end compartments in panel A denote opaque Plexiglas partitions, which were kept in

place during the entire duration of Treatment 1 trials to prevent the focal female from directly observing the stimulus males. The opaque partitions and the pumps were removed for Treatment 2 to allow the focal female to view the stimulus males, but not receive olfactory cues from them. The dotted circle in the centre of the test aquarium denotes a clear Plexiglas cylinder in which the focal female (F) was temporarily held. The stippled lines at either end of the test aquarium demarcate the 10-cm wide mating preference zones. For Treatment 3 (full interaction), only the central aquarium was used and it contained the female and both stimulus males. The apparatus was similarly set up for the control trials, although one compartment contained the right-side (R) male and the other contained the left-side (L) male.

General experimental protocol

Experimental trials

In the Experimental trials, focal test females were individually presented with paired stimulus males that differed in their functional fertility (i.e. sperm stores) and allowed to choose between them as potential mates. The two stimulus males, which had been prevented from mating with females for at least 7 days, were dip-netted from their holding aquarium and were matched by eye for both body length and overall body coloration (which was later confirmed using digital photographs of the males). One of the paired males (determined at random), deemed the sperm depleted (SD) male, was then placed in a small breeding aquarium with a sexually receptive virgin female and allowed to interact with her for one day (including 13 h of light and 11 h darkness). With this manipulation, the male was expected to have copulated with the female and become naturally sperm depleted during this period. To confirm that this male had successfully copulated and exhausted some or all of his sperm reservoir, the virgin female was subsequently isolated in a separate holding aquarium until she give birth (which normally occurs within four weeks; Houde 1997) or was visibly gravid (pregnant). In cases where the female did not give birth and showed no outward sign of pregnancy after 8 weeks, the females were euthanized (with overdose of MS-222) and dissected to check for the presence of fertilized eggs or developing embryos (see Appendix Table 1). The other stimulus male, deemed the non-sperm depleted (NSD) male, was placed in another similar breeding aquarium with a different virgin female for the same amount of time, but the two fish were separated by a clear, perforated mesh partition that divided the aquarium in halves and were thus prevented from physically interacting and mating.

However, this male was expected to be sexually active after being exposed to the presence of a female. The water in these small breeding aquaria was completely replaced with clean, aged city water at the end of each trial.

Individual females ($n = 20$) were each tested sequentially with the same pair of stimulus males ($n = 20$ pairs) in three different sensory cue treatments. I used a different pair of stimulus males for each focal test female. Each treatment presented the females with different sensory cues originating from the paired males, as follows.

Treatment 1: Chemical cues only

This treatment tested whether females could distinguish between the sperm depleted (SD) stimulus male and the non-sperm depleted (NSD) male based solely on olfactory cues that might be associated with prior mating in males (and thus their potential functional fertility).

I quantified the mating preference of individual adult virgin females ($n = 20$; mean \pm SE standard body length = 20.8 ± 0.31 mm, range = 19.0 - 24.0 mm) using the well-established dichotomous mate-choice paradigm (e.g. Houde 1997; Wagner 1998; Jeswiet and Godin 2011) and the above apparatus (Figure 4.1a). For any given trial, a focal female was given a choice between two stimulus males that differed in their fertilization potential but otherwise matched for body length and coloration. I used the time that a focal female spent in the preference zone near either stimulus male as a proxy of her mating preference. Such association time is a known reliable predictor of mating preference in both female (Dugatkin and Godin 1992) and male (Jeswiet and Godin

2011) guppies, as well as in other species (e.g. Witte 2006; Lehtonen and Lindström 2008; Walling *et al.* 2010).

Prior to the onset of a typical mate-choice trial, I first introduced a virgin female into the central test aquarium and a stimulus male in each of the two end compartments determined at random (Figure 4.1a), which were partially filled with aged city water, and partially with the water originating from the respective male breeding aquaria. The fish could not view each other at any time because opaque Plexiglas partitions were placed between the end compartments and the central test aquarium throughout the entire duration of the behavioural trial. Once in their respective compartments, the fish were allowed to acclimatize undisturbed for 30 min. After this period, the focal female was dip-netted and placed in a clear Plexiglas cylinder (7 cm diam.) in the centre of the test aquarium for 15 minutes. Stimulus water from each end compartment was then pumped simultaneously at a similar rate into the respective preference zones of the central aquarium for 1 min before releasing the female from the cylinder and during the entire 20-min behavioural trial to expose the female to chemical cues originating from each of the stimulus males. After the first minute of pump activation, the focal female was released from the central cylinder and allowed to freely swim in the central aquarium for 20 min. During this period, I quantified her mating preference as the association time she spent in each of the two preference zones. At the end of a trial, the test female was gently dip-netted from the central test aquarium and temporarily held in an opaque container until her use in the next treatment (Treatment 2). A trial was discarded from further analysis if either the focal female did not ‘visit’ both preference zones at least once or did

not resume swimming normally after being released from the cylinder. Four such trials were discarded.

Treatment 2: Visual cues only

This treatment tested whether females could distinguish between the SD stimulus male and the NSD male based solely on visual cues that might indicate prior mating in males (and thus their potential functional fertility). Although the two stimulus males in each trial were matched for body length and coloration, they possessed individually unique body coloration patterns (Houde 1997; see Appendix Figure 1), which allowed me (and presumably focal females) to visually distinguish between them in this treatment (as well as in Treatment 3 and the Control trials below).

For this treatment, the central test aquarium was replaced with an identical aquarium containing fresh, aerated and aged city water (24-26 °C) so as to eliminate any water-borne residual chemical cues from the previous Treatment 1 trial. The focal female was then poured from her temporary holding container into the central aquarium. As no carry-over chemical cues were present in the central test aquarium, female preference for either one of the (same) paired stimulus males was expected to be based solely on visual cues originating from male behaviour.

Before the onset of a typical trial, the fish were acclimatized to the apparatus for 45 min to avoid any disturbance in behaviour caused by the above aquarium replacement procedure. At the end of this period, the female was reintroduced in the central cylinder , and allowed to observe the males for 15 min after the opaque partitions between the end compartments and central test aquarium were removed (Figure 4.1b). At the end the

viewing period, I started a 20-min mate choice trial, which consisted of two consecutive 10-min mating preference tests, by gently raising the central cylinder and allowing the focal female to freely swim in the test aquarium and choose between the two stimulus males based on visual cues only. In the initial 10-min preference test (i.e. preference test 1), I observed the behaviour of the focal female from behind the blind, and recorded in real time (with stopwatches) the time the female spent in each preference zone and facing ($0 \pm 90^\circ$) the stimulus male in the end compartments. To control for any potential side bias, the end compartments were switched at the end of this first preference test, and the same test was repeated for 10 min (i.e. preference test 2). No more than 90 seconds elapsed between the paired preference tests. A trial was discarded from further analysis if either the focal female exhibited a side bias or did not ‘visit’ both stimulus males at least once. A side bias was defined as a focal female spending more than 80% of her association time in one of the two preference zones (Schlupp and Ryan 1997; Dosen and Montgomerie 2004). Five such trials were discarded.

The behaviour of each of the stimulus males was filmed during the 15 min viewing period and the two 10-min preference tests using two overhead HD digital video cameras (CANON Vixia HFM-400 and SONY Handycam HDR-PJ380) mounted 20 cm above each of the end compartment, to determine if the behaviour of the stimulus males (whilst in their respective end compartments) had a significant effect on female mating preference. Each overhead camera was positioned to film half of the end compartment it was assigned to, and half of the adjacent preference zone of the central compartment. Using video playback, the frequency of courtship displays and the time spent ‘nosing’ the side of the end compartment facing ($0 \pm 90^\circ$) the female was recorded for each of the

stimulus males during the 15-min viewing period. In addition, for the two 10-min preference tests, the frequency of courtship displays and ‘nosing’ time were recorded for each stimulus male when the female entered the preference zone near him and was directly facing ($0 \pm 90^\circ$) him and observing his behaviour.

Treatment 3: Full interaction (multiple sensory cues)

This treatment tested whether females could distinguish between the SD stimulus male and the NSD male when freely interacting with them and thus in the presence of the natural range of sensory cues originating from the males that might indicate their prior mating history and potentially their functional fertility.

This treatment immediately followed Treatment 2. Individual focal females were presented with the same paired stimulus males that they experienced in the previous Treatment 1 and Treatment 2 trials. Prior to the onset of a typical trial, the focal female was temporarily placed in the central cylinder of the test aquarium and the two stimulus males were then dip-netted from their end compartments and introduced into the central test aquarium and allowed to swim freely for 3 min. Following this acclimatization period, the cylinder was removed and the female allowed to freely interact with the stimulus males for 20 min. During this period, I recorded the behaviour of the fish using a digital HD video camera (SONY Handycam HDR-PJ380), placed approximately 20 cm in front of the test aquarium and behind the observation blind to reduce external disturbances. In Treatment 3, the focal females had access to the full natural range of stimuli (visual, chemical, sound, tactile) originating from the two stimulus males. I changed the water in the test aquarium after every completed mate-choice trial.

From the video films and following Houde (1987, 1988), I recorded the number of courtship displays, copulation attempts (= gonopodial thrusts) and gonopore nips directed by each stimulus male towards the focal female, the number of female sexual 'gliding' responses towards a displaying male (defined as the female orienting towards the displaying male, followed by an unambiguous 'gliding' movement towards him), the number of female 'move towards' (similar to 'glides', but directed towards a non-displaying male). Female sexual interest and mating preference for either stimulus male was then expressed in one of three ways (Houde 1988, 1997; Godin and Briggs 1996): (i) female total sexual effort, as the sum of the frequency of her 'glides' and 'move towards', (ii) the proportion of courtship displays exhibited by a particular male that elicited a female sexual ('gliding') response, and (iii) the frequency of unsolicited 'move towards' directed by the female at a male that was not displaying at that time. Additionally, a female was categorically classified as 'preferring' a particular stimulus male if she directed > 50% of her total sexual effort, 'glides' or 'move towards' at him (cf. Godin and Briggs 1996). These three behavioural measures of female mating preference predict male mating success in the guppy (Houde 1988).

A trial was discarded from further analysis if the focal female did not exhibit any sexual interest in (did not 'glide' or did not 'move towards') either of the stimulus males. One such trial was discarded.

Control trials

To control for any potential intrinsic differences in the behaviour and (or) pheromone production of the paired stimulus males, I carried out Control trials with

different focal females ($n = 20$; mean \pm SE standard body length = 21.7 ± 0.32 mm, range = 20.0 - 24.0 mm) and stimulus males, which were matched for both body length and coloration. In the Control trials, the paired stimulus males were manipulated in the same manner as described above for the Experimental trials, except that both males were maintained unmated (i.e. had fully intact sperm stores) as they had previously being placed separately in a breeding aquarium with a virgin female for one day but separated from the female by a clear, perforated partition. The Control trials followed exactly the same experimental protocol (i.e. Treatments 1, 2 and 3) as described above for the Experimental trials. In the Control trials, female mating preference for either stimulus male was predicted to be random, as no significant differences in sexual behaviour and chemical cues released between the paired stimulus males was expected and the paired males were similar in body length and overall body coloration. Three and two trials were discarded for Treatments 1 and 2 trials, respectively, because the focal female had either not visited both male preference zones or exhibited a side bias.

Measuring fish body length and male body coloration

When the testing of any focal female was completed, the focal female and the paired stimulus males were lightly anaesthetized with MS-222, their left side digitally photographed, and their body length measured and body coloration (males only) quantified from the photographs following the methods described in Chapter 2.

For the Experimental trials, the paired stimulus males did not differ either in their standard body length (mean \pm SE, SD male = 17.33 ± 0.19 mm, NSD male = 17.30 ± 0.21 mm, $t_{19} = 0.21$, $p = 0.834$, paired t -test), body weight (SD male = 108.4 ± 4.02 mg,

NSD male = 111.3 ± 5.57 mg, $t_{19} = -0.679$, $p = 0.505$, paired t -test), or colour score (SD male = 0.058 ± 0.0047 , NSD male = 0.053 ± 0.0036 , $t_{19} = 1.19$, $p = 0.249$, paired t -test). For the Control trials, paired stimulus males similarly did not differ in either colour score (SD male = 0.058 ± 0.0051 , NSD male = 0.056 ± 0.0045 , $t_{19} = 0.341$, $p = 0.737$, paired t -test) or weight (SD male = 97.4 ± 2.70 mg, NSD male = 100.6 ± 3.56 mg, $t_{19} = -1.13$, $p = 0.271$, paired t -test), but differed slightly in their standard body length (SD male = 16.60 ± 0.187 mm, NSD male = 16.95 ± 0.25 mm, $V_{19} = 47.5$, $p = 0.033$, Wilcoxon paired signed rank test).

Statistical analyses

All statistical analyses were carried out in the R statistical environment (R Development Core Team 2012) and all tests are two-tailed.

Experimental trials

Treatment 1: Chemical cues only

I first tested the a priori prediction that focal females should prefer the non-sperm depleted (NSD) male over the sperm depleted (SD) male by comparing their mating preference (proportion of association time) for either male using the paired t -test. Secondly, I tested whether the time focal females spent associating with the NSD male, and the number of females categorized as preferring the NSD male, differed from that expected by chance using the paired t -test and the Binomial test, respectively. Lastly, I used a linear model to test for any effects on female preference of focal female body

length, of the side of the end compartment where the NSD male was placed, and differences in body weight and coloration score between the paired stimulus males.

Treatment 2: Visual cues only

I tested the a priori prediction that focal females should prefer the non-sperm depleted (NSD) male over the sperm depleted (SD) male by comparing their mating preference (total association time) for either male using the paired *t*-test. Secondly, I tested whether the number of females categorized as preferring the NSD male (based on their association time spent near this male) differed from that expected by chance using the Binomial test.

Treatment 3: Full interaction (multiple sensory cues)

I first compared separately the frequencies of sexual acts (courtship displays, gonopore nips, gonopodial thrusts, and total acts) exhibited by each of the paired stimulus males and female sexual responses directed towards each males using the Wilcoxon paired signed rank test, as these data were not normally distributed. Second, I tested whether focal females expressed stronger and more frequent sexual responses towards either one of the stimulus males using the Wilcoxon paired signed rank test, as these data were also not normally distributed. I then tested whether the number of females categorized as preferring the NSD male (based on their total mating effort towards either stimulus males) differed from that expected by chance using the Binomial test.

Testing for consistency of female mate choice between treatments

Lastly, I tested for consistency of female mating preference for either of the paired stimulus males between all possible paired combinations of the three treatments using the Binomial test (Sokal and Rohlf 1995).

Control trials

The a priori expectation of no choice between paired stimulus males (that were similar in phenotype and did not differ in their recent prior mating history) was tested using the same analysis as described for the Experimental trials. No linear models were generated as no significant differences in female preference for either stimulus males was observed in Treatments 1 and 2 of the Control trials.

RESULTS

Confirmation of sperm depletion in the SD males

To create sperm depleted (SD) males to be used as stimulus males in the Experimental trials, I had placed each of 20 males with a virgin female in individual breeding aquaria for one day to allow them to mate. Of the 20 virgin females that were so exposed to a putative SD male, 6 subsequently produced live offspring following the Experimental trials. Of the remaining 14 females that were dissected, 2 had developing embryos in their ovaries (Appendix Table 1). There is therefore direct evidence that 8 of the 20 stimulus males categorized as SD males had successfully copulated with, and transferred sperm, to a female and were to some (unknown) degree sperm depleted when

used in the Experimental trials. The remaining 12 males might also have copulated and transferred sperm to a female, but no successful fertilization of eggs resulted and thus no direct evidence of sperm depletion is available for these males (see Discussion for rationale).

Experimental trials

Treatment 1: Chemical cues only

When provided with chemical cues introduced from the end compartment of both a previously-mated (SD) male and a non-sperm depleted (NSD) male, focal females subsequently spent significantly more time in the preference zone associated with the NSD male than that of the SD male (paired $t_{19} = -2.70$, $p = 0.0141$, Figure 4.2a), and significantly more so than expected by chance ($t_{19} = 2.60$, $p = 0.0178$), as predicted. As such, 17 of the 20 focal females tested categorically preferred the NSD male, which is significantly greater than expected by chance (Binomial test, $p = 0.001$). As revealed by a linear model, none of the non-olfactory explanatory variables included in the model significantly explained the observed mate choice of the focal females (Table 4.1).

Treatment 2: Visual cues only

The paired stimulus males did not differ significantly in their sexual behaviours either during the 15-min viewing period preceding the mate-choice test (sigmoid displays $V_{19} = 1.5$, $p = 0.59$, Figure 4.3a; nosing $t_{19} = -0.306$, $p = 0.76$, Figure 4.3b) or during the 20-min mate-choice test (sigmoid displays $V_{19} = 19$, $p = 0.069$, Figure 4.3c; nosing $t_{19} = 0.071$, $p = 0.944$, Figure 4.3d). When provided only with visual cues originating from

males exhibiting similar sexual behaviours, focal females on average did not spend significantly more time associating with the NSD male than with SD male ($t_{19} = -1.09$, $p = 0.285$, Figure 4.2b), even if 14 of the 20 focal females categorically chose the NSD male over the SD one (greater than expected by chance; Binomial test, $p = 0.037$).

Treatment 3: Full interaction (multiple sensory cues)

As expected, and consistently with the above results for the dichotomous-choice test of Treatment 1, focal females preferred the NSD male over the paired SD male as evidenced by their directing a greater overall mating effort (calculated as the sum of the frequency of ‘glides’ and ‘move towards’, $V_{19} = 41$, $p = 0.0175$, Figure 4.2c) and more frequent ‘move towards’ responses ($V_{19} = 2.5$, $p = 0.0027$, Figure 4.2e) towards the NSD male than the SD male when allowed to freely interact with the males. Moreover, focal females exhibited proportionally more sexual ‘glides’ towards the NSD male (mean \pm SE = 0.146 ± 0.044) than towards the SD male (0.075 ± 0.040), but this difference is not significant ($V_{19} = 31.5$, $p = 0.345$, Figure 4.2d). Based on their overall mating effort (Figure 4.2c), 15 out of the 20 focal females were categorized as preferring the NSD male, which is greater than expected by chance (Binomial test, $p = 0.015$). Mirroring this female choice, NSD males exhibited a greater total mating effort (calculated as the sum of their courtship displays, copulation attempts and gonopore nips) towards the focal females than the SD males did ($V_{19} = 49.5$, $p = 0.0399$, Table 4.2).

Consistency in female mate choice between treatments

Because the paired stimulus males did not differ in their body length, weight and coloration (see supporting data in Methods above), the observed strong female mating preference for the NSD male cannot be explained by any difference in the phenotypes of the males (Table 4.1), as measured here. Females not only showed a strong overall preference for the NSD males in both the dichotomous-choice test of Treatment 1 (chemical cues only; Figure 4.2a) and the full-interaction choice test of Treatment 3 (Figure 4.2c-e), they were also consistent in their preference for the NSD male between these two treatments (Binomial test, $p = 0.037$, Table 4.3). Females were also consistent in their preference for the NSD male between the dichotomous-choice test of Treatments 1 and 2 (Binomial test, $p = 0.015$, Table 4.3), and between the dichotomous-choice test of Treatment 2 and the full-interaction choice test of Treatment 3 (Binomial test, $p = 0.005$, Table 4.3). On the other hand, females were marginally not consistent in their preference for the NSD male across all three treatments (Binomial test, $p = 0.074$, Table 4.3).

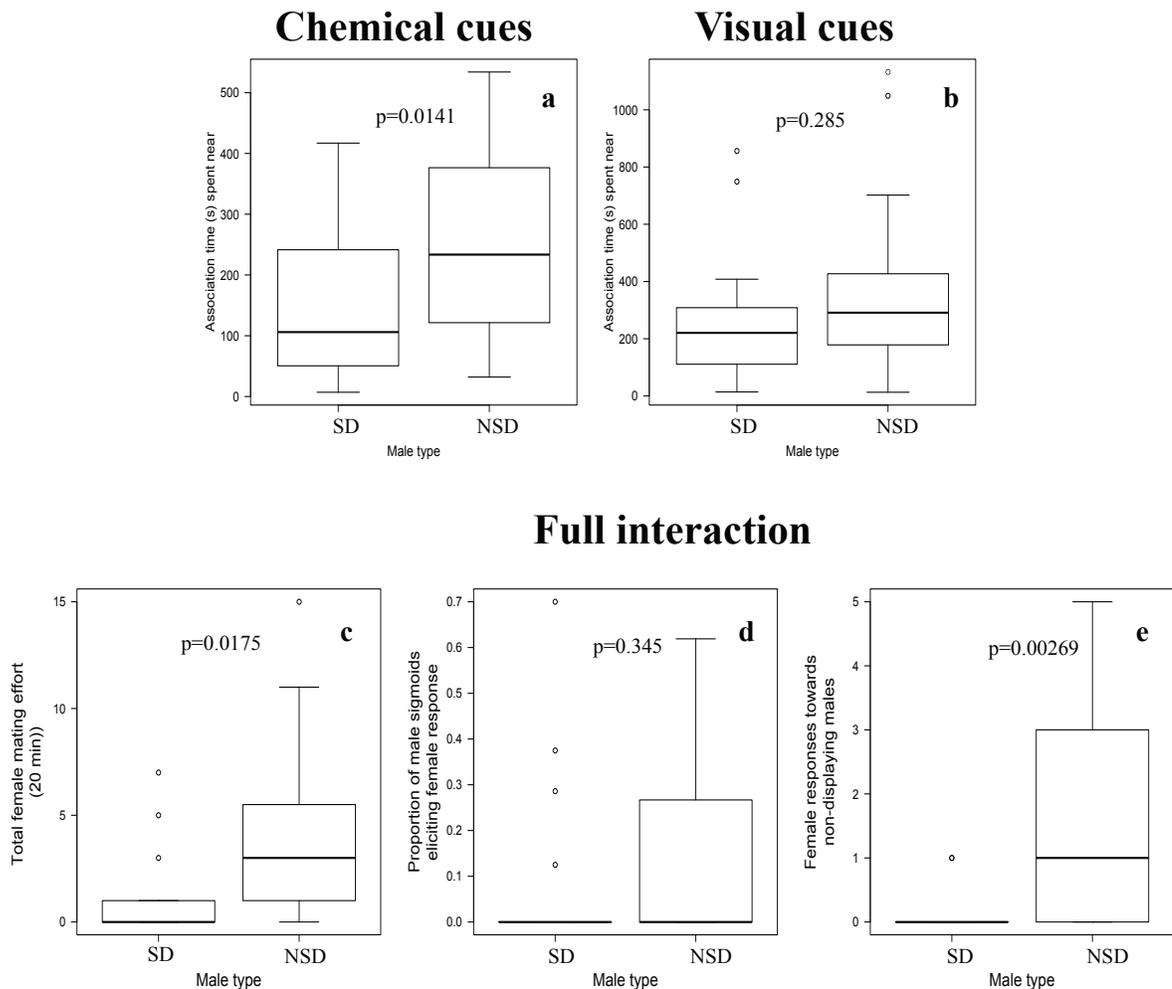


Figure 4.2. Female mating preference for either of the two stimulus males during the dichotomous-choice test of (a) Treatment 1 (chemical cues only) and (b) Treatment 2 (visual cues only) of the experimental trials, expressed as female association time spent near the paired SD male and NSD male. Female mating preference in Treatment 3 (full interaction test) of the experimental trials was expressed as (c) female total mating effort calculated as the sum of sexual ‘glide’ and ‘move towards’ responses exhibited towards either of the males, (d) the proportion of solicited sexual ‘glide’ responses exhibited towards a displaying stimulus male, and (e) frequency of sexual responses (‘move towards’) towards non-displaying males. Box plots illustrate the median, 25th and 75th percentiles, inter-quartile range, and data points outside this range (open circles). The p-values shown were obtained using either the paired *t*-test when the data was normally distributed and homoscedastic (panels a and b) or the Wilcoxon paired signed rank test when such conditions were not satisfied (panels c and e).

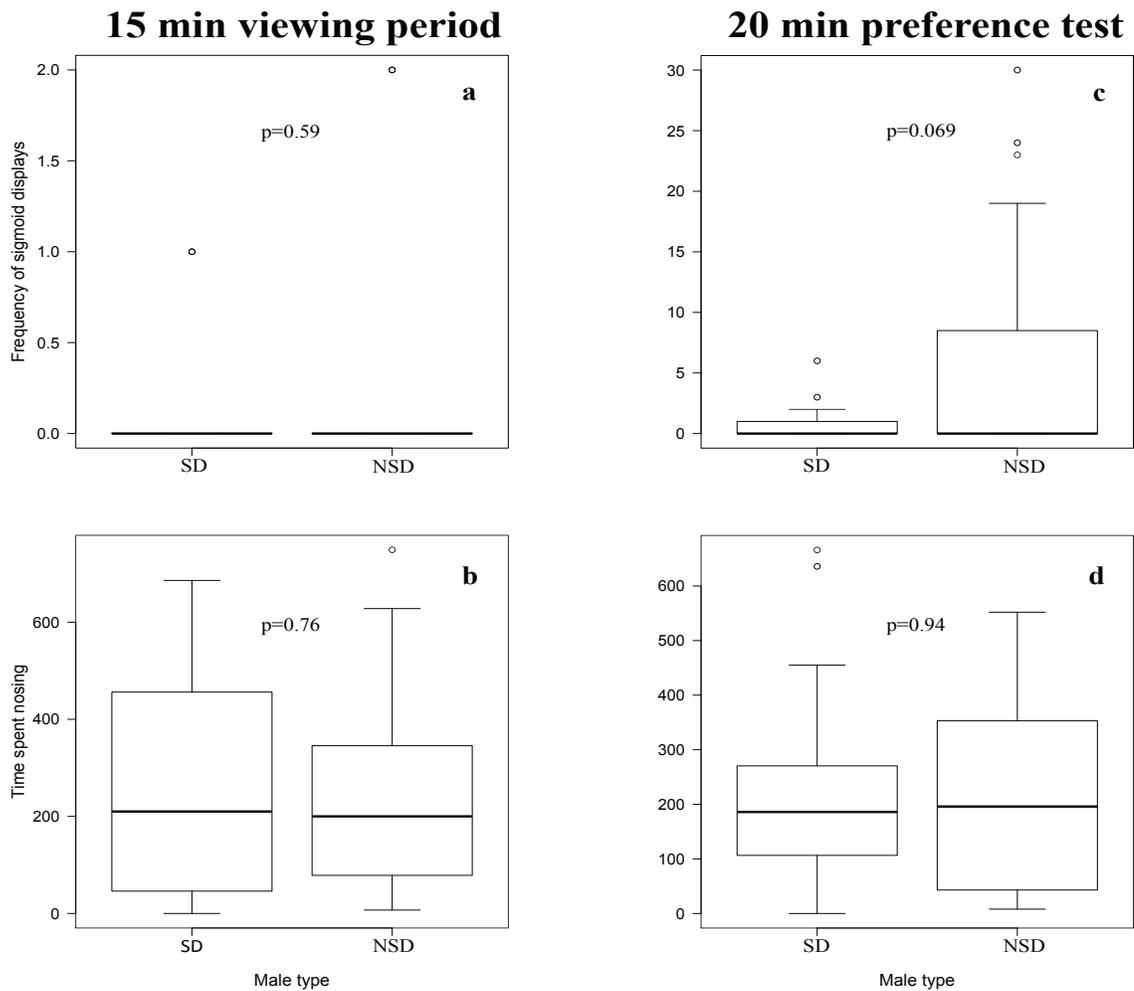


Figure 4.3. Frequency of sigmoid courtship displays and total time spent ‘nosing’ against the side of the end compartment facing the female exhibited by the paired the SD and NSD stimulus male during the 15-min viewing period (panels a and b) and the 20-min preference test (panels c and d) of the dichotomous-choice test in Treatment 2 (visual cues only) of the experimental trials. Box plots illustrate the median, 25th and 75th percentiles, inter-quartile range, and data points outside this range (open circles). The p-values shown were obtained using the Wilcoxon paired signed rank test

Table 4.1. Statistical results for the explanatory variables obtained from a linear model for Treatment 1 of the Experimental trials. $r^2_{\text{adj}} = -0.147$, overall $F_{6,13} = 0.593$, $p = 0.73$. The dependent variable in the model was the association time that the focal females spent in the preference zone associated with the NSD male. Side NSD = the particular side (left or right) of the dichotomous-choice apparatus that the end compartment containing the NSD male was located in each trial.

Explanatory variable	Model estimate	V	p
Side NSD	-0.213	2.400	0.146
Colour score SD	1.550	0.198	0.664
Colour score NSD	-3.280	0.218	0.649
Body weight SD	-0.00308	0.347	0.566
Body weight NSD	0.00022	0.181	0.677
Female body length	0.00279	0.219	0.648

Table 4.2. Mean \pm SE frequencies of sexual behaviours (numbers per 20 min) directed by the paired SD and NSD males towards the focal female during the full-interaction choice test (Treatment 3) of the Experimental trials. The statistical results shown were obtained using the Wilcoxon paired signed rank test.

Sexual behaviour	SD male	NSD male	<i>V</i>	p
Courtship display	2.15 \pm 0.95	7.10 \pm 1.99	40	0.088
Copulation attempt	1.75 \pm 0.05	2.75 \pm 0.60	52	0.025
Gonopore nip	1.05 \pm 0.37	2.25 \pm 0.52	25.5	0.052
Total sexual acts	4.95 \pm 1.63	12.10 \pm 2.41	49.5	0.039

Table 4.3. Contingency table showing the number of focal females (out of a total of 20), which were tested in the dichotomous-choice test of Treatments 1 and 2 and in the full-interaction choice test (Treatment 3) of the Experimental trials, that consistently preferred the non-sperm depleted (NSD) male between different paired combinations of the three treatments. Observed frequencies were compared against frequencies expected by chance using the Binomial test.

Treatment comparison	Consistent females	p
Treatments 1 vs. 2	15	0.015
Treatments 1 vs. 3	14	0.037
Treatments 2 vs. 3	16	0.005
Treatments 1 vs. 2 vs. 3	13	0.074

Control trials

The focal females tested in the control trials did not express any significant preference for either the right male or the left male in any of the treatments. On average, females did not differ significantly in the time they spent associating with either stimulus males in the dichotomous-choice test of Treatment 1 (chemical cues only; paired $t_{19} = -0.220$, $p = 0.828$, Figure 4.4a) and Treatment 2 (visual cues only; $t_{19} = 0.865$, $p = 0.398$, Figure 4.4b). Similarly, they did not differ significantly in their total mating effort ($V_{19} = 111.5$, $p = 0.822$, Figure 4.4c), sexual ‘glide’ responses towards a displaying male ($V_{19} = 64$, $p = 0.842$, Figure 4.4d), or their sexual ‘move towards’ a non-displaying male ($V_{19} = 71$, $p = 0.541$, Figure 4.4e) for either of the paired stimulus males.

The sexual behaviours that the paired stimulus males directed towards the focal females were similar in both Treatment 2 (visual cues only) and Treatment 3 (full interaction). For Treatment 2, the paired males did not differ significantly in the frequencies of their sigmoid displays ($V_{19} = 0$, $p = 0$, Figure 4.5a) or ‘nosing’ behaviour ($V_{19} = 94$, $p = 0.070$, Figure 4.5b) during the 15-min viewing period (when the males were still in their end compartments). Similarly, during the 20-min mate-choice test (when the males were freely swimming with the focal female) of this treatment, the paired males did not differ significantly in the frequencies of their sigmoid courtship displays ($V_{19} = 75.5$, $p = 0.157$, Figure 4.5c) or ‘nosing’ behaviour ($t_{19} = -0.319$, $p = 0.699$, Figure 4.5d). In the full-interaction choice test (Treatment 3), the sexual behaviours of the paired stimulus males also did not differ significantly (Table 4.4).

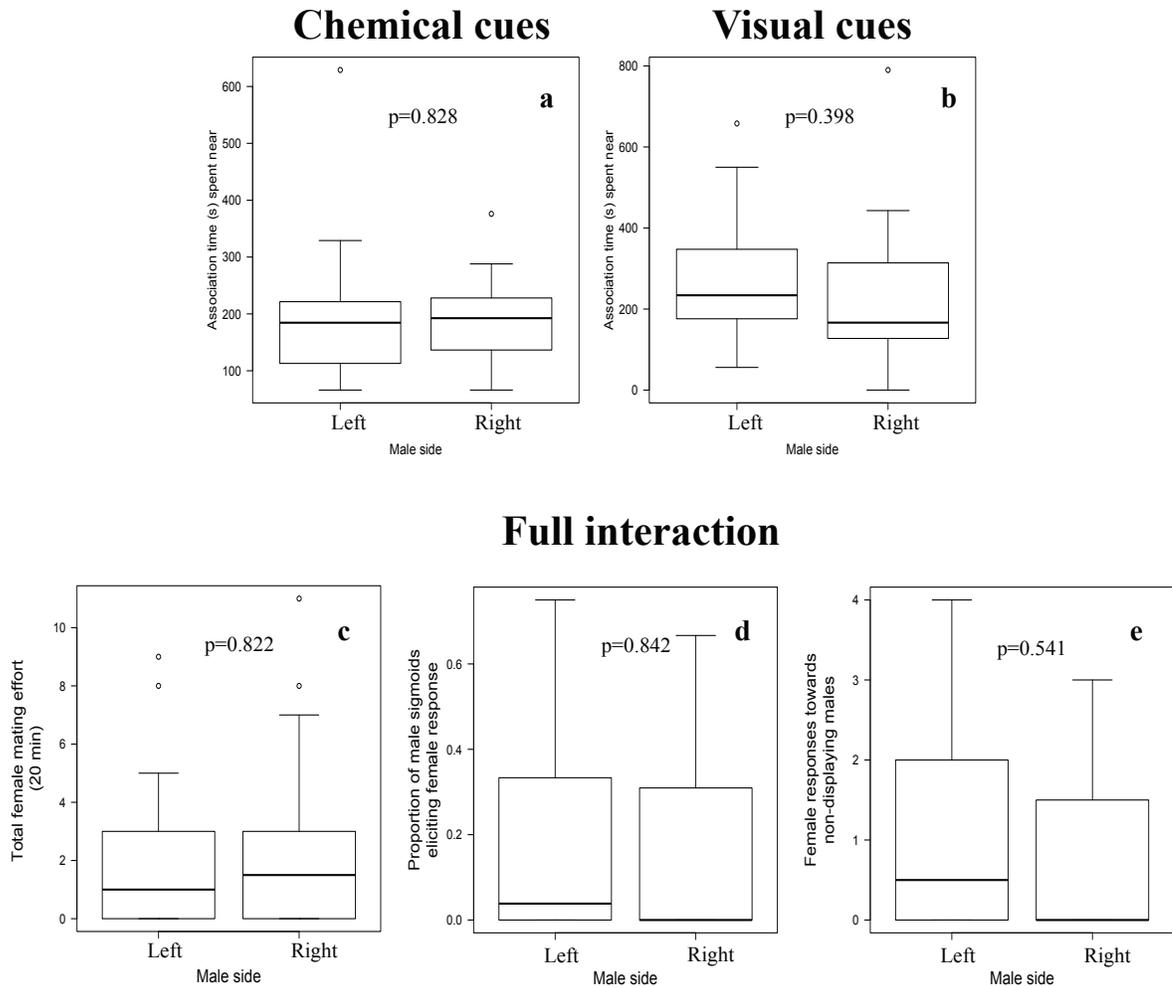


Figure 4.4. Female mating preference for either of the two stimulus males during the dichotomous-choice test of (a) Treatment 1 (chemical cues only) and (b) Treatment 2 (visual cues only) of the Control trials, expressed as female association time spent near the paired stimulus male (Left male, Right male). Female mating preference in Treatment 3 (full interaction test) was expressed as (c) female total mating effort calculated as the sum of sexual ‘glide’ and ‘move towards’ responses exhibited towards either of the males, (d) the proportion of solicited sexual ‘glide’ responses exhibited towards a displaying stimulus male, and (e) frequency of sexual responses (‘move towards’) towards non-displaying males. Box plots illustrate the median, 25th and 75th percentiles, inter-quartile range, and data points outside this range (open circles). The p-values shown were obtained using either the paired *t*-test when the data was normally distributed and homoscedastic (panels a and b) or the Wilcoxon paired signed rank test when such conditions were not satisfied (panels c-e).

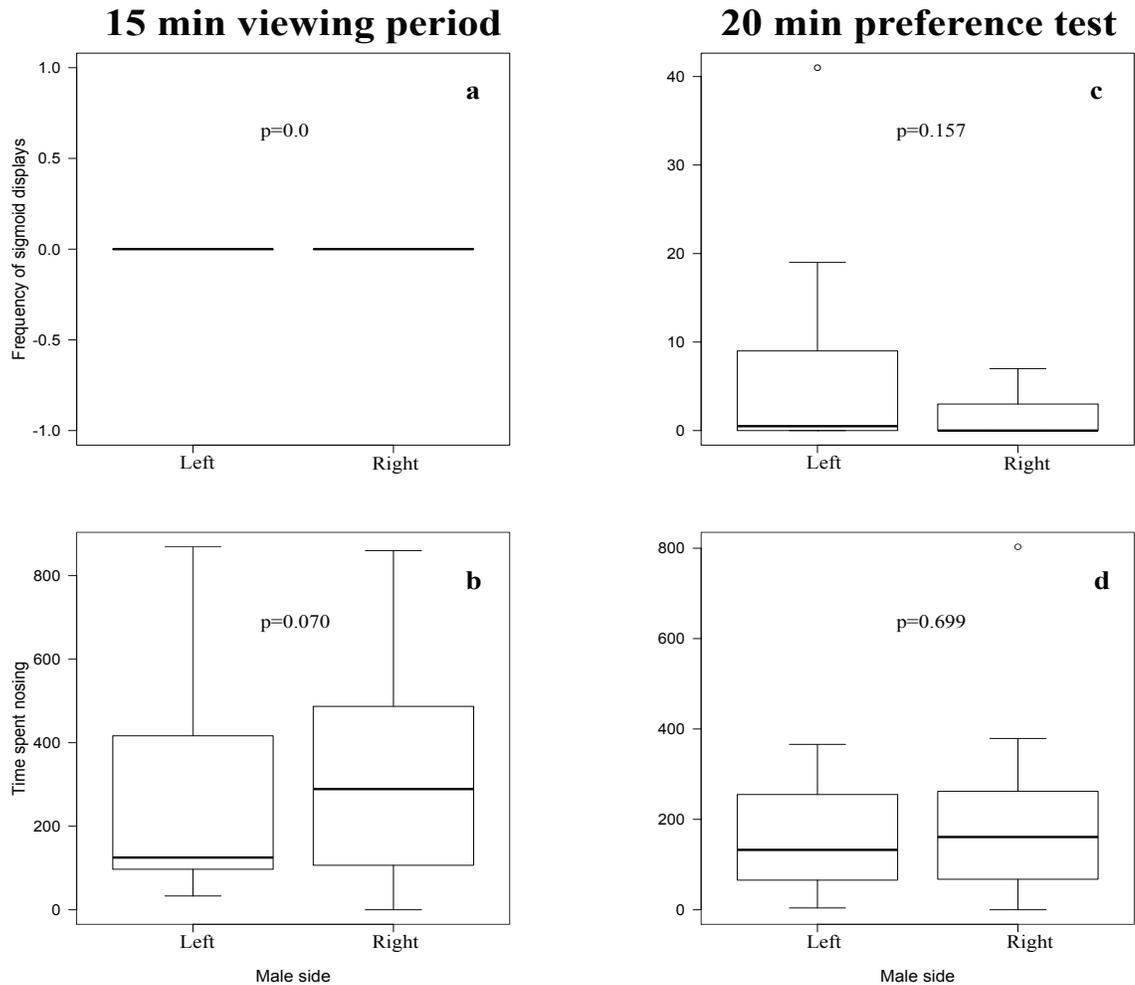


Figure 4.5. Frequency of sigmoid courtship displays and total time spent ‘nosing’ against the side of the end compartment facing the female exhibited by the paired the SD and NSD stimulus male during the 15-min viewing period (panels a and b) and the 20-min preference test (panels c and d) of the dichotomous-choice test in Treatment 2 (visual cues only) of the control trials. Box plots illustrate the median, 25th and 75th percentiles, inter-quartile range, and data points outside this range (open circles). The p-values shown were obtained using the Wilcoxon paired signed rank test.

Table 4.4. Mean \pm SE frequencies of sexual behaviours (numbers per 20 min) directed by the paired stimulus males towards the focal female during the full-interaction choice test (Treatment 3) of the Control trials. For any given pair of stimulus males, the male that had previously been in the right end compartment of the dichotomous-choice apparatus (at the onset of tests in Treatments 1 and 2) was denoted the Right male and the male that had been in the left end compartment was denoted the Left male for Treatment 3.

Sexual behaviour	Left male	Right male	V	p
Sigmoid courtship display	5.10 \pm 1.36	3.80 \pm 1.15	92.5	0.462
Copulation attempt	2.90 \pm 0.71	2.30 \pm 0.60	96	0.366
Gonopore nip	2.25 \pm 0.49	1.45 \pm 0.37	58.5	0.132
Total sexual acts	10.25 \pm 1.89	7.55 \pm 1.48	131	0.350

DISCUSSION

Animals may use multiple sensory cues, rather than rely only on one, to assess the quality of available mates and to choose their preferred mate (Candolin 2003). In this chapter, I have provided for the first time empirical evidence that female guppies rely on a combination of at least olfactory and visual cues originating from available males to assess their functional fertility and to avoid sperm depleted males in favour of males that have fully intact sperm stores, when no other information about the recent mating history of the males was available. These results are novel, not only for the guppy but also for vertebrates in general.

Specifically, when only male chemical cues were available, females showed a significant tendency to avoid males that had presumably depleted their sperm reservoirs owing to recent copulations with another female. Moreover, females displayed much stronger sexual responses and interest towards the non-sperm depleted males when the female and stimulus males were physically interacting with each other in the more sensorially complex environment of Treatment 3. Such a mating preference for non-sperm depleted males over sperm depleted ones, observed in both Treatments 1 and 3, cannot be explained by any differences in measured phenotypic traits between the paired stimulus males, as they did not differ in their body length, weight and colour score. Moreover, any behavioural interactions between the paired stimulus males in Treatment 3 is unlikely to explain the observed female preference for the non-sperm depleted male, as there is no clear evidence for female mate choice based on male aggression or sexual interference in the Trinidadian guppy (Houde 1997). In comparison, females exhibited no significant preference for either stimulus males when they had only visual cues

originating from the males (Treatment 2), presumably because the paired males were similar in key phenotypic traits (namely, body length, body weight, body coloration and sexual behaviour) that are known to reflect male quality in the guppy (Houde 1997; Matthews *et al.* 1997; Evans and Magurran 1999b; Pitcher and Evans 2001; Pilastro *et al.* 2002, 2008; Locatello *et al.* 2006). As revealed by the Control trials here, when both paired males had fully intact sperm stores and were similar in measured phenotypic traits (body size, weight and coloration and sexual behaviour), females did not exhibit a clear mating preference for either male. Collectively, these results strongly suggest that female guppies can use male chemical cues only, or chemical cues in combination with other cues (most likely visual cues), to distinguish between males that have different recent mating history and to preferentially avoid males that have recently mated with another female and who likely have depleted sperm stores, as predicted by the phenotype-linked fertility hypothesis (Sheldon 1994). These findings are complementary to and corroborate those presented in Chapter 3.

The fact that I was able to collect direct evidence of successful copulation and egg fertilization for only 8 out of 20 females does not necessarily mean that only 8 of the SD males were actually sperm depleted and that the other 12 males were not sperm depleted to some degree. Even if it is possible that these latter 12 males did indeed copulate with and transferred sperm into the female that they were interacting with in the breeding aquaria, their sperm might have been unsuccessful in fertilizing any eggs, any fertilized eggs/early zygote died, or the female selectively did not use inseminated sperm from the male to fertilize her eggs. In the Trinidadian guppy, the success of inseminated sperm in fertilizing eggs within a female's reproductive tract varies widely among males and local

conditions (Pilastro & Bisazza 1999; Pilastro *et al.* 2002), females can exert post-copulatory cryptic choice and reject sperm from particular males (Pilastro *et al.* 2004), and embryos can die within the ovaries before parturition and their tissues potentially reabsorbed (Constantz 1989). These latter phenomena could lead to under-estimation of successful copulation, sperm transfer and sperm depletion in male guppies, as might be the case in my study.

In the current study, focal females were significantly consistent across treatments (different sensory contexts) in their preference for the non-sperm depleted (NSD) male. Previous studies have demonstrated that female guppies are generally consistent in their mate choice when their sensory environment remains unchanged (e.g. Godin and Dugatkin 1995; Brooks 1996; Godin *et al.* 2005). On average, females appeared to be consistent in their mating preference between pairs of treatments in the current study, suggesting that they are able to adjust their use of cues depending on their availability in their environment. On the other hand, only a marginally significant trend in the consistency of female preference for the NSD male was observed across all three treatments. Given that sequential repeated tests on individual animals does not generally affect the repeatability (consistency) estimates of their behaviour (Bell *et al.* 2009), a plausible explanation for the absence of a significant consistency between Treatments 1, 2 and 3 here is that females varied in their assessment of male quality depending on the available male cues, which differed between the three treatments. It is known that different female guppies can assess the same males as potential mates differently (Brooks and Endler 2001).

To my knowledge, there is only limited empirical evidence explaining the mechanisms and the signals used by females when assessing male mating history, and related functional fertility, in the context of mate choice (Nakatsuru and Kramer 1982; Harris and Moore 2005; Sato and Goshima 2007; Weir and Grant 2010; Loyau *et al.* 2012; Mellan *et al.* 2014). My findings presented here therefore contribute importantly to advancing this (limited) body of knowledge and are novel for the Trinidadian guppy.

CHAPTER 5

GENERAL DISCUSSION

Overview of salient results

As predicted by the phenotype-linked fertility hypothesis (Sheldon 1994), sexual selection should favour females that recognize and mate preferentially with males that possess abundant and fertile sperm, while avoiding recently-mated males (who would be sperm depleted to varying degree) so as to minimize the risk of their eggs not being fertilized. As the production of female ova is more energetically costly and limited than gamete production in males (Clutton-Brock 1991; Hayward and Gillooly 2011), and as such females generally have lower reproductive potential (Clutton-Brock and Parker 1992; Andersson 1994), females should be selected to preferentially choose males with high functional fertility as mates so as to ensure the maximal fertilization of their eggs, all else being equal. This particular prediction of the phenotype-linked fertility hypothesis (Sheldon 1994) has received to date only limited attention and empirical support in invertebrates (Harris and Moore 2005; Sato and Goshima 2007; Loyau *et al.* 2012; Mellan *et al.* 2014) and less so in vertebrates (Nakatsuru and Kramer 1982). The main objective of my thesis was therefore to test if male mating history and fertilization potential influence female mate choice in the Trinidadian guppy. Female guppies use visual, chemical and social cues to assess and choose their mates (e.g. Houde 1997; Shohet and Watt 2004; Godin *et al.* 2005), and are therefore potentially able to recognize recently-mated males using such cues.

In Chapter 3, I provided empirical evidence that the mating preference of females is influenced by (visual) information on the recent mating history of available males that they acquired by observing the behaviour of males who interacted with another female and of other males who were observed in the absence of another female. Specifically,

females significantly preferred males that were not observed to mate recently (and thus not likely to be sperm depleted) over males that were recently observed sexually interacting with another female and consequently more likely to be sperm depleted. Although female guppies overall preferred the non-sperm depleted males, the magnitude of female avoidance of apparently sperm depleted males was negatively correlated with the male's prior sexual interest towards another nearby female. This relationship suggests that focal females closely observe male mating behaviour and use their courtship effort as a proxy to assess their fertilization potential. In fact, courtship display rate in males covaries positively with ejaculate quality and functional fertility in the guppy and other species (e.g. Evans and Magurran 1999, Pilastro and Bisazza 1999; Charge *et al.* 2010). As investigations on the mechanisms underlying female assessment of male mating history, and related functional fertility, in the context of mate choice are relatively few (Nakatsuru and Kramer 1982; Harris and Moore 2005; Sato and Goshima 2007; Weir and Grant 2010; Loyau *et al.* 2012; Mellan *et al.* 2014) and as a follow-up to my initial results reported in Chapter 3, I then investigated which types of cues influence female preference for non-sperm depleted males in the absence of prior visual social information regarding male mating history.

In Chapter 4, I tested the ability of females of the Trinidadian guppy to use multiple cues to assess male functional fertility when no direct observation of male interactions with other females is possible. Females appeared to be able to use a combination of olfactory cues and visual cues originating from the behaviour of males to assess their functional fertility. Specifically, when only chemical cues were available, females showed a significant tendency to avoid males that had previously depleted their

sperm reservoirs owing to prior copulation with another female, and exhibited much stronger sexual responses and interest towards the non-sperm depleted males when allowed to physically interact with each other (all natural sensory cues available). Females were generally consistent in their choice of mate between the sensory-cue treatments.

Collectively, my results support the phenotype-linked fertility hypothesis, are novel not only for the guppy but for vertebrates in general, and contribute further to our understanding of the sensory cues used by females to assess male quality, specifically their mating history and fertilization potential, when making mate-choice decisions.

Future research

The results presented in Chapters 3 and 4 further contribute to scientific knowledge on female mate choice and on the mechanisms driving female preference for males that have not recently mated and have high functional fertility. By being able to assess male functional fertility using chemical and visual cues originating from males that had recently interacted sexually and mated with other females or not, females can avoid mating with males that have exhausted their sperm reservoir and thereby minimize the risks and costs that are generally associated with reproduction.

Future research should attempt to quantify the potential costs to females of mating with sperm depleted males (and thus indirectly the benefits of avoiding to mate with such males). Additionally, more research effort is needed to expand and deepen our understanding of the role of sexual pheromones in mate choice and on the origin and chemical structure of these compounds. The system of hormonal pheromones in fish is in

fact very complex and only limited information is available on the chemical compounds used by some species (e.g. goby, cyprinids and salmonids) in regulating reproductive behaviours (Stacey 2003).

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APPENDICES



Appendix Figure 1. Examples of the individually unique body coloration pattern of three pairs of stimulus male guppies.

Appendix Table 1. Summary of the reproductive outcome of stimulus female guppies (n = 20) that were allowed to sexually interact with a male (deemed the SD male) for 24 hours preceding each behavioural trial in the experiment described in Chapter 4.

Stimulus female #	Eggs fertilized	Observation
5	NO	No embryos. Only yolked eggs (10) in ovary
7	NO	No embryos. Only small eggs (6) in ovary
8	YES	Newly-emerged fry collected
9	NO	No embryos. Only small eggs (8) in ovary
10	NO	No embryos. Only yolked eggs (11) in ovary
12	NO	No embryos. Only yolked eggs (2) in ovary
14	YES	Newly-emerged fry collected
15	YES	Newly-emerged fry collected
16	YES	Newly-emerged fry collected
20	NO	No embryos. Only yolked eggs (5) in ovary
22	YES	Newly-emerged fry collected
23	YES	1 early-stage embryo found in ovary
24	NO	No embryos. Only yolked eggs (7) in ovary
25	NO	No embryos. Only yolked eggs (8) in ovary
26	YES	1 early-stage embryo found in ovary
27	NO	No embryos. Only yolked eggs (10) in ovary
28	NO	No embryos. Only yolked eggs (9) in ovary
29	YES	Newly-emerged fry collected
31	NO	No embryos. Only yolked eggs (8) in ovary
32	NO	No embryos. Only yolked eggs (5) in ovary
Total = 8 YES/12 NO		