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Ciclo XXI

Sexual conflict in the guppy, *Poecilia reticulata*

Conflitto sessuale in *Poecilia reticulata*

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‘A fox, of a kind said to be peculiar to the island, and very rare in it, and which is an undescribed species, was sitting on the rocks. He was so intently absorbed in watching their manœuvres, that I was able, by quietly walking up behind, to knock him on the head with my geological hammer. This fox, more curious or more scientific, but less wise, than the generality of his brethren, is now mounted in the museum of the Zoological Society’

Charles Darwin
Voyage of the adventure and beagle
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List of papers

The thesis is based on the following papers (all unpublished manuscripts) which will be referred to by their roman numbers:

I Clelia Gasparini, Alessandro Devigili and Andrea Pilastro
Direct and indirect effects of sexual harassment in the guppy

II Clelia Gasparini, Ryan Dosselli and Andrea Pilastro
Quantitative genetics of pre- and post-copulatory sexually selected traits in the guppy

III Clelia Gasparini, Alfredo Peretti and Andrea Pilastro
Female presence affects sperm velocity in the guppy

IV Jonathan P. Evans, Clelia Gasparini, Indar W. Ramnarine and Andrea Pilastro
The influence of female population origin and male genital morphology on the success of forced copulations in Trinidadian guppies

V Clelia Gasparini, Andrea Pilastro and Jonathan P. Evans
Does size and shape of male genitalia influence female mate choice and sperm transfer in the guppy?

VI Jonathan P. Evans, Clelia Gasparini, Gregory I. Holwell, Indar W. Ramnarine, Trevor E. Pitcher and Andrea Pilastro
Intraspecific evidence for correlated patterns of male and female genital trait diversification

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Papers were written in co-authorship, reflecting the collaboration between authors. For papers IV and VI I performed experiments including behavioural trials and sperm extraction (IV) and female morphology analyses (VI) and I was involved in reviewing drafts in both papers. For papers I, II, III and V I had the main responsibility for experiments and for writing the papers.

ABSTRACT

Sexual conflict is a widespread phenomenon in nature and it occurs over a wide range of decisions involving female-male interactions. This conflict drives the evolution of sexually antagonistic traits. Under a sexual conflict scenario, a trait that is beneficial to one sex is detrimental to the other driving the evolution of counteradaptation in a sort of 'arm race'. In this process every evolutionary change in one sex is followed by a change in the opposite sex in order to minimize costs associated to the former. Mating frequency is one of the aspects of reproduction that could especially fuel sexual conflict and consequently sexually antagonistic coevolution because generally males have an optimal mating rate higher than females. My PhD thesis is aimed to study different aspects of sexual conflict using a small livebearing fish, *Poecilia reticulata*, as a model system. This species is particularly suitable for this purpose since sexual conflict is evident over mating rate, as revealed doubtless from the presence of high level of sexual harassment. I divided my researches into two main trends. First, I investigated costs and benefits of sexual conflict. Second, I focused on sexually antagonistic coevolution.

Costs and benefits of sexual conflict. Estimate costs and benefits of sexual conflict shed new light on the evolution of female resistance that is assumed to evolve to minimize the costs associated to superfluous matings. However, a recent hypothesis argues that female resistance evolves as a way to screen the more successful male and therefore operate via indirect benefits mechanism. Results from a study of the effects of sexual harassment on female's lifetime fecundity and on offspring's quality revealed that do not support this latter hypothesis. I demonstrated only costs of sexual harassment on offspring both in daughters and in sons. Moreover, study on heritability of males' traits influencing reproductive success both at pre- and postcopulatory level revealed scarce sire heritability that instead is a fundamental assumption of the hypothesis. The low heritability for males' traits may be explained by adaptive phenotypic plasticity that has been investigated for sperm velocity. This demonstrated an adjustment in sperm velocity by males according to mating opportunities.

Sexually antagonistic coevolution. My study of sexually antagonistic coevolution focuses on genitalia (male gonopodium and female oviduct). Gonopodial shape and dimension result important factors in determining the outcome of both consensual and coercive mating. Gonopodial length and shape resulted to be under sexual selection by female premating choice, with females mate more quickly with males possessing longer gonopodia with an enlarged shape. Gonopodium tip shape is revealed to be important in sperm transfer both in consensual and in forced matings. Another experiment demonstrated that females from low conflict population have minor control over sperm transfer in forced copulations by high conflict population males than relative counterparts. This result highlights the importance of male-female antagonistic coevolution in a sexual conflict scenario. An intraspecific study at population level corroborates this conclusion by providing evidence for a coevolutionary pattern between male and female concerning genitalia morphology. For this reason, genitalia might be the result of an evolutionary arm race between males and females in the struggle for control over mating rate and insemination success.

INTRODUCTION

Sexual conflict

Different sexes are defined by their gametes, females produce large nutritious eggs while males small motile sperm. The existence of anisogamy underlies the evolution of sex differences in physiology, behaviour and morphology. The reason why sexual reproduction evolved and it is maintained is so far poorly understood and under ongoing debate, but regardless how or why, sexual reproduction arose, once sexes exist also the circumstances for sexual conflict occur. Sexual reproduction causes the combination of genetic material from two parents to a shared progeny and therefore this operation has long been seen as an harmonious affair between mating partners fuel by the same purpose: leave the more descendants possible to the next generation. The real situation is actually far away from this view. Females and males, when come to reproduce, engage a potentially never ending battle: the battle between the sexes.

Coming back to gametes, it is worth to note that differences in the relative reproductive investment between sexes, result in different adaptations to maximize fitness (Parker 1979). Unitary cost in sperm production is smaller compared to that necessary to produce eggs (Trivers 1972), even if the traditional view of ‘cheap’ sperm has been demonstrated far to be universally true (Dewsbury 1982; Shapiro et al. 1994; Olsson et al. 1997). Eggs are costly to produce because a great investment in resources is required and consequently females are more carefully in the choice of mating partner with who share this costly investment. Males, on the other hand are less prudent on partner choice, because sperm reserves could be restore quickly and with less investment of energies. Typically, males are not limited in the number of sperm they can produce, and consequently in the number of potential offspring they can sire, while females experience physiological constrains in the actual number of gametes they can produce, and this number can not be increase with the number of sexual partners as for males (Bateman 1948). Therefore selection acts differently on males and females, leading to different ways to maximize life-time reproductive success. Males are selected to produce as many offspring as possible and females to produce high quality offspring. Sexual conflict enter the scene whenever there is any possibility to copulate with a different mating partner and therefore males and females interests will diverge if investment in that given reproductive event reduce its potentially future reproductive chances. Strict monogamy is the only situation in which the ideal harmonious cooperation between sexes could exist, but any other different situation ideally involves sexual conflict because lifetime reproductive interests of males and females do not coincide. Empirical evidences of the existence of sexual conflict in nature are ever increasing (see Arnqvist & Rowe 2005 for an exensive review). As defined originally by Parker (1979) ‘Sexual conflict is a conflict between the evolutionary interests of individuals of the two sexes’ and leads to evolution of traits beneficial for one sex despite detrimental for the other. Sexual conflict is therefore different from the other forms of sexual selection as not only it selects for traits beneficial to the bearer, but it selects for traits that advantage one sex (hereafter assumed to be male) but have harmful effect on the other sex (hereafter assumed to be female) fitness. However as this trait is detrimental to females, they are expected to evolve counteradaptations to decrease such costs in a sort of circle of adaptations and counteradaptations, process known as sexually antagonistic coevolution (see below).

Sexual conflict is not the same of sexual selection and may, or may not, derive from it (Parker 2006). There are adaptations shaped by sexual selection that has not consequences on the other sex and therefore sexual conflict does not occur.

Sexual conflict therefore acts because fitness of one sex is decreased by a trait expressed in the other sex, but nevertheless the mean fitness of each sex must to be equal to one due to the simple fact that in sexual reproducing species each has one father and one mother (Fisher 1930).

Intra and interlocus sexual conflict

There are two types of sexual conflict: intra- and interlocus conflict depending whether the target of selection is determined by alleles at one locus expressed in both sexes, or alleles at different interacting loci in males and females (Parker & Partridge 1998).

Intralocus conflict does not involve interaction between the sexes. It exists when traits expressed both in females and in males have different fitness optima, in other words for a given phenotypic trait selection favours different values when expressed in males or females. This can be best exemplified by an example on tail length in a bird species (see Arnqvist & Rowe 2005). Intralocus sexual conflict may occur because males are selected for a longer tail (used as an ornament, and therefore sexually selected by female choice) while females are selected for a shorter length (for example because more manoeuvrable). Alleles at the tail length locus are therefore under opposite selective forces and there is intralocus sexual conflict. If the sexes share the most part of genome (except to sexual chromosome) any alleles in the common genome that are under different selective pressures are likely to incur in this type of sexual conflict. The outcome of this scenario is that the resulting average phenotype is a sort of compromise between the evolutionary interests of the two sexes. However, given the widespread phenomenon of sexual dimorphism probably the constrain due to such a conflict is not absolute. Intralocus sexual conflict is potentially widespread (especially for traits under sexual selection) and evidence has been found in some studies (Vieira et al. 2000; Chippindale et al. 2001; MacKay 2002). However, its evolutionary importance is debated and the extent to which it limits the sex limited adaptive evolution remain poorly understood and need more empirical studies (e.g. Rice & Chippindale 2001; Bonduriansky & Rowe 2005). Due to the difficulties in detecting this type of evolutionary process, the relative interest of scientists in the intralocus conflict is less than that for the second type of sexual conflict, the interlocus sexual conflict.

Interlocus sexual conflict, different from the previous one, involves male-female interactions, and the most of the researches in sexual conflict topic focus on this. It occurs whenever interactions between the sexes may lead to different outcomes, which optimum is different in males and females (Rice & Holland 1997). Interlocus sexual conflict is the most studied and increasing body of evidence has been found to corroborate its importance as evolutionary force. This will be the main focus of my thesis. Virtually every interactions between the sexes may flow into interlocus sexual conflict, from mating rate to relative parental effort (Arnqvist & Rowe 2005). Mating rate is one of the aspects of reproduction that could especially fuel sexual conflict as generally males have an optimal mating rate higher than females (Rowe 1994; Choe & Crespi 1997; Arnqvist et al. 2000). In species with no-resources mating system, variance in male reproductive output is in theory high as males are not limited, at least as much as females, in gamete production. In females instead the optimum mating rate is the result of a delicate equilibrium between benefits and costs of matings that usually results in a lower ideal number of sexual partners compared to that of males. As a consequence of the selection on males to copulate with reluctant partners, traits associated to sexual coercion (such as sexual harassment and/or coercive mating strategies) are expected to evolve. Forced copulation occurs when males force females to copulate, while sexual harassment occurs when males repeatedly attempt to mate until the female either leaves or copulates with them (Clutton-Brock & Parker 1995). Both of these behaviours are often detrimental to the female fitness and have a number of different costs that have been investigates in several species. Increasing number of studies demonstrate that sexual harassment are costly to females in term of energy costs, lost of feeding time, increased predation risk or physical damaging (for an extensive review see Arnqvist & Rowe 2005).

In sexual conflict the optimal outcome differs between the two sexes, and therefore selection favours adaptations that lead their bearers to bias the outcome toward theirs own interests at the expenses of the partner fitness. To minimize the costs associated to a reduction in fitness, the ‘looser’ sex, in turn, is expected to evolve traits to counteract. Therefore, if we refer to an evolutionary novelty that increases male fitness, as an ‘adaptation’, we will predict, in a sexual conflict scenario, the evolution of ‘counteradaptation’ in females, in a sort of evolutionary arm race between the sexes, a process known as sexually antagonistic coevolution.

Sexually antagonistic coevolution

One way in which sexual conflict can drive evolutionary changes is expressed via sexually antagonistic coevolution (SAC) (Arnqvist & Rowe 2005). SAC is therefore the coevolution between males and females traits driven by sexual conflict, in a manner analogous to ‘red queen’ models of coevolution involving endless circle of changes through evolutionary time (Rice & Holland 1997). When adaptations that are detrimental to the other sex are followed by counteradaptations, sexually antagonistic coevolution is acting on the traits involved, that it is termed the ‘conflict trait’. For example, a new trait evolves in males that allow bearer to obtain more copulations (the ‘persistence’ trait). Persistent males are therefore able to increase the number of mate toward their optimum but this is detrimental to female fitness as female optimum is lower that male one. Females are expected to evolve some counteradaptations to compensate such costs (the ‘resistance’ trait). This counteradaptation of females that minimize (even balance) the costs of persistence traits, in turn, decreases the initial advantage gained by males that therefore are selected to exaggerate this trait or to evolve a different new trait and so on (see fig. 1).

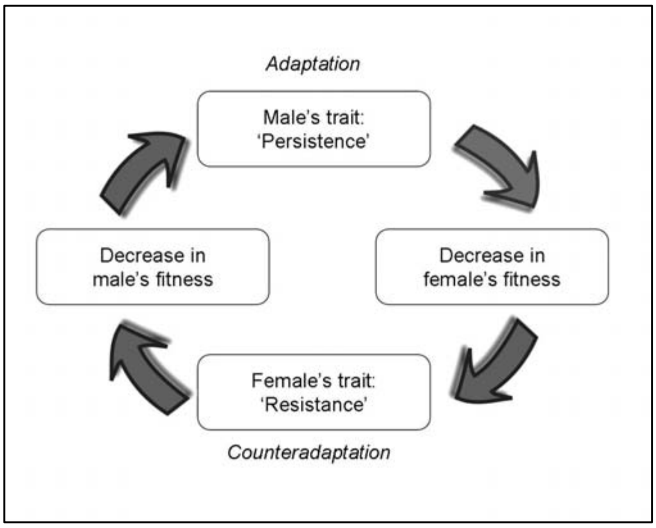


Fig. 1 Schematic representation of sexually antagonistic coevolution (SAC) between persistence and resistance traits.

One of the striking examples of sexually antagonistic coevolution comes from *Drosophila* species. During reproduction males transfer with ejaculate numerous substances such as 'acp proteins' produced by the male accessory glands. Acps have different functions that increase male reproductive success but are detrimental to female fitness; these proteins deeply influence physiology and behaviour of the females, inducing ovulation and reducing receptivity (Wolfner 1997). The great advantage for males has large implications for female fitness, as reduction in lifespan and consequently expected life-time reproductive success (Chapman et al. 1995). In this situation females are expected to evolve counteradaptations and therefore sexually antagonistic coevolution between toxic substance in males and female traits evolved to reduce the detrimental consequences they imply. An evidence for this coevolution has been demonstrated by experiments of Rice (1996), in which he demonstrate that if females are experimentally prevented to evolve, males seminal fluid results more detrimental to females, suggesting that seminal fluid becomes more toxic with females prevented to coevolve with males. As a consequence this indicates that females without the opportunity to coevolve suffered more the effect of male ejaculate fluid. An other evidence comes from the fact that females paired with males evolved in enforced monogamy life longer and had an increased overall reproductive fitness (Holland & Rice 1999), confirmed by the findings of Pitnick and colleagues (2001). *Drosophila* species remains one of the best studied system in sexual conflict and sexually antagonistic coevolution, however a lot of evidences come from comparative studies on different taxa, especially insects (e.g. Presgraves et al. 1999; Arnqvist & Rowe 2002b). The expected outcome of sexually antagonistic coevolution is a depression in the average female fitness, and therefore of population fitness. In the case of persistent and resistant arm race, assuming that resistance is costly to females, the result of SAC is a decline in female fitness and thereby in population fitness, as theoretical models predict (Holland & Rice 1998; Gavrilets et al. 2001). Sexual conflict over mating rate might therefore lead to new male traits that induce females to copulate over their optimum, with associated costs, like sexual coercion, that force females to copulate, but even evolution of traits that persuade females to copulate over optimum via seduction mechanism. In this latter case males evolve exaggerate elaborate traits that over stimulate females to mate, for example mediate by sensory bias as it occurs for the caudal fin (the sword) in *Xiphophorus* fish (Basolo 1990). In both situations if copulating beyond optimum is costly to females sexual antagonistic coevolution predicts evolution of resistance traits aimed to move the average mating rate towards females optimum.

Superfluous matings are costly to females in terms of increased predation risk, energetic expenditure and decrease of benefits associated to female choice. Moreover, mating per se can be costly due to harmful adaptations in males as the well-known traumatic insemination in bedbugs (Stutt & Siva-Jothy 2001; Morrow & Arnqvist 2003) and spiny genitalia as in *Callosobruchus maculatus* (Crudgington & Siva-Jothy 2000).

Resistance traits documented include morphology, physiology and behaviour. For example, in the different genera of bedbugs in which hypodermic insemination occurs several studies demonstrated associated costs, in term of high stress for the immune system that leads higher female mortality (Usinger 1966; Newberry 1989) and reduce longevity (Stutt & Siva-Jothy 2001). Different female counterstrategies have been reported. Females often engage pre mating struggle to escape harassing males (Usinger 1966), there are some physiologically adaptations to male ejaculate demonstrated by interspecific studies (Ryckman & Ueshima 1964; Davis 1965; Newberry 1989) and females possess a sort of ‘secondary reproductive system’ (the paragenital system Carayon 1966) that reduces direct costs of traumatic insemination (Reinhardt et al. 2003; Morrow & Arnqvist 2003).

There is a large amount of experimental data suggesting that direct costs of superfluous mating are enough to account for the evolution of female resistance (for an extensive review see Arnqvist & Rowe 2005), but recent theoretical works advanced the idea of the evolution of resistance as a way to females to screen for superior males, favoured by indirect selection for genetic benefits (Eberhard 1996; Pizzari & Snook 2003; Cordero & Eberhard 2003). In this hypothesis, also known as ‘selective resistance’ (Eberhard 2002), direct costs suffered by females may be compensated by indirect benefit that females obtain by mating with the most persistent male. In other words female resistance is a way to test males (resistance as screening, (resistance as screening, see Blanckenhorn et al. 2000) aimed to filter out individuals that are unable to overcome such resistance (Berry & Shine 1980; Eberhard 2002). If conflict traits involved in acquire more mates are heritable from sires to sons, females resistance will benefit from producing sons with the same ability of sire, via a mechanism similar to the ‘sexy sons’ mechanism, proposed for the evolution of elaborate sexual secondary traits under sexual selection (Fisher 1930). Those males, more efficient in overcome female resistance, are either those that can transmit ‘good genes’ because persistent males are likely to be more vigorous, and therefore of superior quality. However, indirect genetic benefits are often considered a weak force compared to direct selection (Kirkpatrick 1996; Kirkpatrick & Barton 1997; Cameron et al. 2003). Both empirical and theoretical models do not support this idea suggesting the low probability that direct costs of resistance can be counterbalanced by any indirect effects (Cameron et al. 2003) but more empirical works are needed to clarify to relative costs and benefits of females resistance.

AIMS OF THE THESIS

My PhD thesis is aimed to study different aspects of sexual conflict using *Poecilia reticulata* as a model system. In particular, I grouped the six papers into two major research trends:

A) Costs and benefits of sexual conflict

In this part I analyzed the relative contributions of direct costs of sexual harassment on female life-time reproductive success and the effect on offspring fitness (**Paper I**). Theoretical models suggesting the selective resistance hypothesis, assume a heritable mechanism for male traits from sires to sons. Demonstrating the existence (or non-existence) of heritability of male characteristics could have a strong impact in clarify the possibility of this hypothesis. Therefore, I tested the heritability in male traits associated to sexual conflict as a measure of potential benefits that could be acquired by indirect selection (**Paper II**). Heritability of sexually selected traits influencing male reproductive fitness are often considerably low, due to the erosion of genetic variance in consequence to strong directional selection or even due to the fact that traits influencing fitness are costly to be maintained and show high phenotypic plasticity in response to different cues. I studied if sperm swimming velocity, thought to be an important factor in determine fertilization outcome, shows this phenotypic plasticity according to mating opportunities (**Paper III**)

B) Sexually antagonistic coevolution

I focused my antagonistic coevolution study on genital evolution. First of all I investigated the role of genital size and morphology in forced (**Paper IV**) and cooperative matings (**Paper V**). Moreover I used a comparative intraspecific approach to study evolution of male genitalia according to different sexual conflict pressure and potential coevolution between male and female genitalia (**Paper VI**).

STUDY SPECIES

Poecilia reticulata

The study species of my thesis is the guppy (*Poecilia reticulata*), a live-bearing freshwater fish with a promiscuous non resource-based mating system.

Guppies show elevated degree of sexual dimorphism and polymorphism in the colour pattern of males. Females show a coloration resembling the gravel present on the bottom of freshwater strain they inhabit, while males, generally smaller than females, exhibit a bright colour pattern formed by a series of coloured spots peculiar in the position and relative body area covered. The colour pattern is inherited from paternal lineage and there are evidence of Y-linkage (Houde 1992). Colour pattern results from the combinations of spots grouped together in three big categories based on the main origin of colour: orange and yellow spots ('orange') formed by carotenoids and pterins; melanistic black ('black'); structural colour including blue, green, violet and white ('iridescent').

Fertilization is internal and the male uses his modified anal fin (the gonopodium) to transfer sperm to the female. The **gonopodium** is formed by the union of three rays of the anal fin thickened and elongated together to form an elaborate rode-like structure that forms a channel allowing the passage of sperm bundles, packages of sperm termed spermatozeugmata (Houde 1997). The distal region of the gonopodium is equipped with a pair of 'hooks' and a fleshy structure, the 'hood', which appears to have a sensory function (Clark & Aronson 1951). Even if the role of the hooks it is not yet clearly demonstrated, recent works revealed that the experimental removal of the paired hooks at the gonopodium distal tip impairs sperm transfer (Cheng 2004). Sperm bundles contained about 27000 sperm cells (Evans et al. 2004b) and previous work reported that the number of sperm per bundle does not correlate with either the body size or the extent of body coloration (Evans et al. 2003c). Females actively choose mating partners and base their mating decision on a number of morphological and behavioural traits; in particular colourful males (with particular attention to relative area of orange) with high rates of courtship are preferred (Houde 1997). Courtship behaviour in the guppy is a complex behaviour, called '**sigmoid display**', that consists in the vibration of the male body in front of the female in a particular S-shaped posture, during which the body is arched and the fins are extended (Liley 1966). Females are sexually receptive both when they are virgin and two or three days after parturition, thus, the operational sex ratio is typically biased towards males (Magurran 2005). The female discrimination together with the brevity of receptivity period favour the evolution of male traits intended to overcome the female mating resistance. To force reluctant females to mate outside periods of sexual receptivity, males have the potential to undermine precopulatory female choice by using sneaky matings, a form of forced copulation called '**gonopodial thrusts**' (Liley 1966). This alternative mating strategy has, on average, a low insemination success (Matthews & Magurran 2000; Russell et al. 2006) although sometimes large ejaculates are delivered during sneaky matings (Pilastro & Bisazza 1999; Evans et al. 2003b). Individual males employ both mating tactics, courtship and gonopodial thrusting, interchangeably. The degree to which either tactic is used depends on female receptivity (Liley 1966) and on several ecological factors, such as population demography (Rodd & Sokolowski 1995; Rodd et al. 1997), light intensity (Endler 1987; Reynolds et al.

1993) and predation intensity (Endler 1987; Magurran & Seghers 1990; Magurran & Nowak 1991; Godin 1995). In this species the level of sexual harassment, the incessant behaviour of mating attempts by males, is particularly high: females receive, on average, one coercive mating attempt per minute along the entire lifetime (Magurran & Seghers 1994a). Pilastro and colleagues (2004) showed that the number of sperm inseminated during solicited copulations is influenced by the female perception of male attractiveness, independently from any direct manipulation of the focal males themselves. The mechanism by which this control is achieved is through copulation duration, the longer is the copulation the more sperm are transferred (Pilastro et al. 2007). When mating occur, even if coercitive, males performed a number of postcopulatory jerks that has been demonstrated to correlate to the number of sperm transferred (Pilastro et al. 2007). Females carry developing embryos until necessary (ranges from 20-30 days) and they give birth to live young that could be numerous (twenty or more per litter). In nature females copulates with more than one male over the same reproductive cycle and as a consequence, offspring are often sired by multiple males, brood are typically sired by two-three males with a proportion of multiply sired brood about 95% (Becher & Magurran 2004; Hain & Neff 2007). Promiscuity and the ability for females to store sperm for months after copulation lead to a high level of sperm competition faced by males of this species. Of particular interest is the peculiar pattern of geographical distribution of natural population in Trinidad. Within the same river inhabited by guppies is usually possible to recognize two different types of localities, characterized by different predation regime. Generally in the lower parts of the river guppies coexist with several predator species and are therefore called 'high predation' localities (also known as '*Chrenicicla*' localities, due to the most dangerous predator, the pike ciclid, *Crenicichla alta*. In the upper parts few predators occur, of which the most important is the killfish, *Rivulus hartii* that predate mainly on juveniles; these sites are known as 'low predation' localities. Transition between low and high predation localities is due to natural barrier, like waterfall that prevent movement of predators between localities and maintaining differences between population even closely geographical related. The variation in predator assemblages results in marked differences among population involving differentiation in colour pattern, behaviour and life history traits. Guppies from high predation sites exhibit a less conspicuous coloration, mature quickly, tend to produce many small offspring and more often than their counterparts from low predation localities.

Sexual conflict in the guppy

Sexual conflict is likely to dominate mating system of the guppies for several reasons (Magurran 2005). For example, females are able to store sperm for several months and fertilise consecutive batches of eggs following a single copulation (Constantz 1989). Although females can gain substantial direct and indirect benefits from mating polyandrously (Evans & Magurran 2000; Ojanguren et al. 2005), optimal mating rates for males are still likely to be far higher than those for females. Indeed, males perform up to one mating attempt per minute in natural populations (Magurran & Seghers 1994b), with potentially important impacts on female fitness (Ojanguren & Magurran 2007; and see **paper I**). It has been demonstrated that sexual harassment led to a reduction of 25% in foraging. Moreover, superfluous matings may make females more vulnerable to predators (Pocklington & Dill 1995) and may undermine female choice, potentially reducing any genetic benefits associated with mate choice (Reynolds & Gross 1992; Nicoletto 1995). As Magurran and Seghers (1994b) have previously noted, some (if not all) of these costs are likely to be elevated in high-predation populations where females are subject to higher levels of forced matings than their low-predation counterparts.

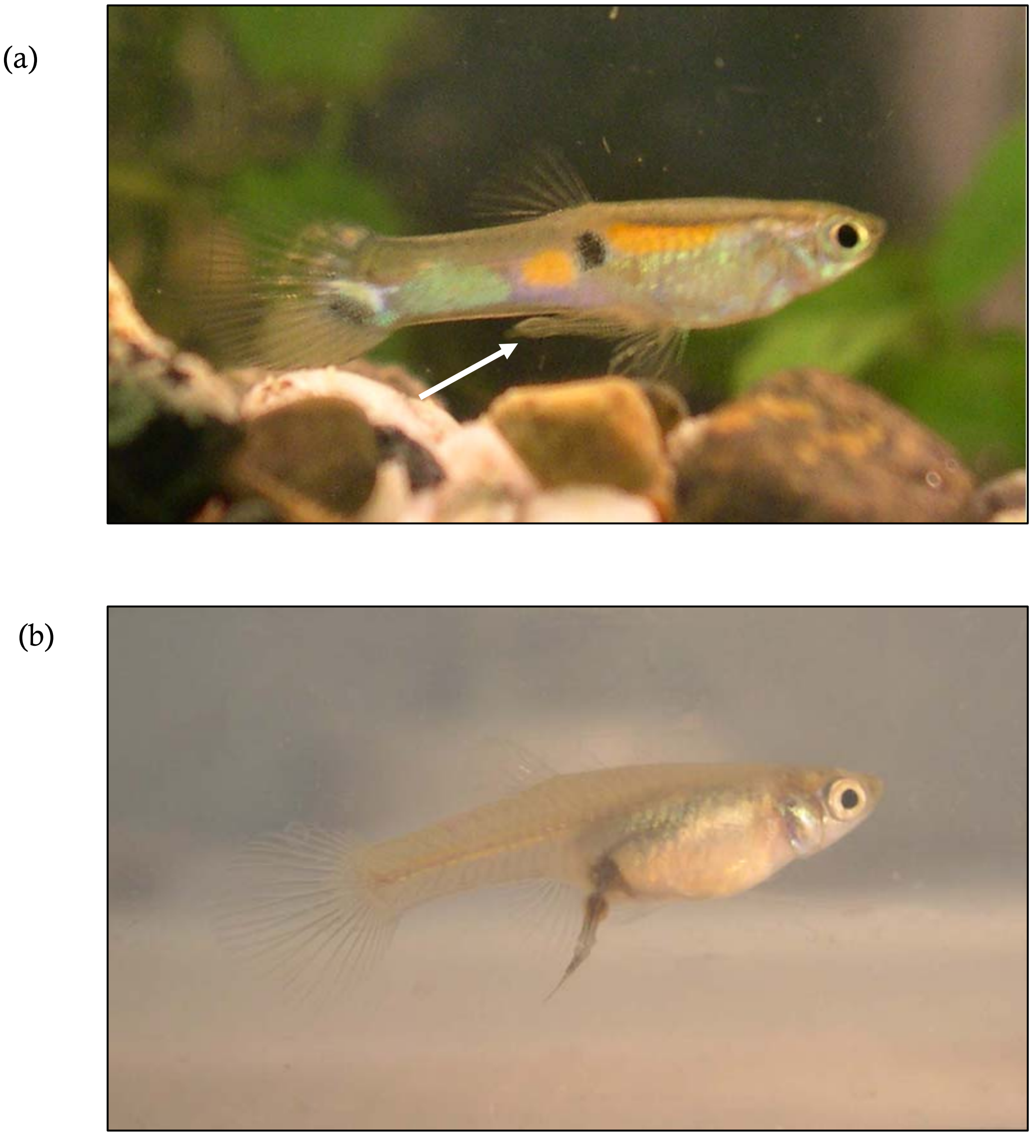


Fig. 1 (a) A male showing the bright colour pattern typical of this species, white arrow indicates the male's intromittent organ; (b) A female during parturition

PART A:
COSTS AND BENEFITS OF SEXUAL CONFLICT

Sexual conflict over mating rate is particularly evident in the guppy mating system due to the brevity of receptive period and the strong discrimination over preferred phenotype by females (Houde 1997; Magurran 2005). Males can force reluctant females to mate using the tactic termed gonopodial thrust resulting in an elevated level of sexual harassment in this species (see *study species*). It has been demonstrated that it is unlike for females to be sperm depleted, given that one copulation is sufficient to fertilize the entire batch of eggs produced by a single female (Pilastro et al. 2008). If females require only one, or few, mating it is clear that male and female interests over mating rate strongly differ. Mating and sexual harassment are costly for females in term of increased risk of predation (Pocklington & Dill 1995), decreased foraging ability (Magurran & Seghers 1994a), and other potential costs such as increasing risk of wounds and parasite transmission during copulation. Costs of sexual harassment in guppy, as well as in other poeciliid species (for examples see Pilastro et al. 2003; Plath et al. 2007), are often believed to have important consequences on female fitness, but only few empirical studies addressed this topic. Within the poeciliid family three studies investigated the effect of sexual harassment on female fitness with contrasting results (Smith & Sargent 2006; Head & Brooks 2006; Ojanguren & Magurran 2007). It is also important to note that these studies focused on a single or few reproductive cycles and therefore are not exhaustive in the understanding of the effects on female, because they do not consider life-time fitness. Therefore I performed a study aimed to estimate the overall effects of sexual harassment on females (in term of life-time fecundity) and the effects on offspring (**paper I**). Evaluating potential costs and/or benefits on offspring due to the sexual harassment suffered by females could shed new light on the evolution of female resistance. If female resistance mainly evolves to minimize costs due to sexual harassment I will not expect to find any benefits on offspring. Nevertheless, some theoretical works (Eberhard 1996; Cordero & Eberhard 2003; Pizzari & Snook 2004) suggest that direct costs of sexual conflict might be overweighted by indirect benefits gained via selection of the most persistent males. Therefore results from exploring effects on offspring fitness could have at least two possible outcomes. If resistance evolves via indirect benefits, I will expect that mothers that underwent a high level of sexual harassment gain some benefits for their offspring in terms of enhanced overall fitness for sons and daughters. In fact, persistent males are supposed to be also the fittest and therefore are likely to produce offspring with enhanced fitness. Females may gain also benefits in terms of elevated reproductive success of sons, as sons of persistent males will inherit persistence traits that allow them to overcome female resistance. This latter hypothesis requires that those male traits are inherited from sires to sons. Therefore, evaluating the heritability of male traits provides an estimation of the potentiality for mechanisms based on indirect benefits to occur. I estimated the heritability of several male traits that are likely to be involved in reproductive success (**paper II**). Among these traits I considered those involved in mate acquisition, such as ornamentation and sexual behaviour (i.e. gonopodial thrusts and courtship displays), and in fertilization success, such as some ejaculate traits (i.e. sperm swimming velocity and quantity). Heritability estimates were evaluated using a full-sibs/half-sibs breeding design and results were compared to estimates for traits, such as body coloration, previously demonstrated to be Y-linked (Houde 1992). However, heritability of fitness traits is complicated by several factors as maternal non genetic components, erosion of additive genetic variance due to strong directional selection and phenotypic plasticity. Phenotypic plasticity is one possible adaptation to heterogeneous environments (Via et al. 1995). Sperm swimming velocity has a great impact on male fitness than previously believed (Snook 2005), with evidences that elevated sperm velocity is associated with a higher fertilization success both in presence (Froman et al. 1999; Levitan 2000; Kupriyanova & Havenhand 2002) and in absence (Birkhead et al. 1999; Gage et al. 2004; Denk et al. 2005) of sperm competition. Although experimental estimates of the cost of producing faster sperm are still lacking, producing sperm with enhanced swimming performances is likely to be costly. For example, higher sperm speed is associated with higher sperm ATP content (Froman & Feltmann 1998; Lahnsteiner et al. 1998; Burness et al. 2004; Locatello et al. 2007). Therefore some degree of phenotypic plasticity in sperm velocity is likely to evolve to save energy that could be allocated in other activities and in future reproductive events. I investigated if male guppies are able to adjust sperm velocity in response to mating opportunities (**paper III**).

Results of these papers and relevant conclusions are briefly reported below, for details see the complete papers from page 37.

Paper I: Direct and indirect effects of sexual harassment in the guppy

Experimental design consists of 62 females randomly assigned to one of the two opposite treatments. The two treatments differed for the level of sexual harassment experienced by females: ‘low level’ (hereafter LC) and ‘high level’ (hereafter HC). Treatments were started with females at their first parturition. In HC treatment three males were placed simultaneously in the female’s tank and allowed to interact freely with the resident focal female for 8 consecutive days during her unreceptive period (from 8th to 15th day after the parturition); in LC treatment three males, under the same conditions, were allowed to interact with the female but only in her receptive period (24 hours following the parturition). I recorded the total number of offspring produced per female, and the exact interbrood interval. This was done until each experimental female died to obtain a precise estimation of life-time reproductive success. Moreover, I measured female survival and growth that resulted not different between treatments. On average, lifetime fecundity did not differ between treatments but there is evidence of a different timing of reproductive allocation (interaction treatment*brood number= 6.30, P=0.012, fig. 1). In particular, HC females showed a relatively greater fecundity early in life, but their reproductive output declined afterwards. In contrast, LC females, which initially produced smaller broods, continuously increased their brood size. The offspring production was therefore not equally distributed between the two treatments. In nature sexual harassment increases the risk of being predate (Pocklington & Dill 1995) and therefore females suffered a high level of sexual harassment are likely to perceive a greater risk of mortality respect to their low level counterparts. Therefore, HC females perceived a high risk of mortality, mediate by a more intense level of sexual harassment, and this could explain the asymmetry in offspring life-time allocation. Another possible explanation is that the observed temporal shift in offspring production was not a consequence of female life history ‘decision’ but of a pre- or postcopulatory manipulation by harassing males due, for example, to seduction traits or other factors such as harmful behaviours or eventually seminal fluid component that might stimulate embryogenesis over female optimum.

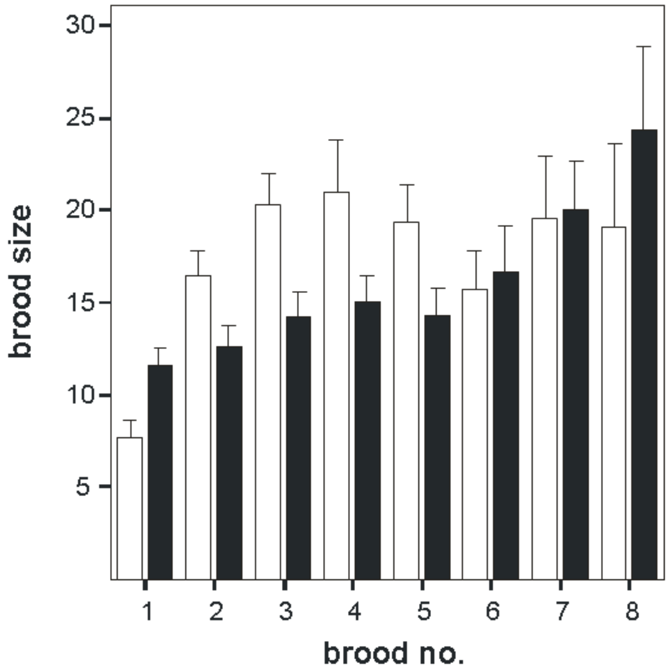


Fig. 1 Mean (± SE) number of offspring produced per each brood by females assigned to HC treatment (open bars) and LC treatment (filled bars) numbers indicate sample size (number of females) for each group

I measured offspring quality at birth and after sexual maturity. Measurements in newborns revealed no differences between treatments in sex ratio, body size at birth and antipredator performance (linked to early fitness). Instead, measurement of body size after sexual maturity (4-months old) provides evidence of a negative effects of sexual harassment endured by mothers, in terms of smaller body size, and therefore lower fecundity (Reznick & Endler 1982; Farr 1989), for daughter of HC females (fig. 2). This cost may be a consequence of female condition causes by male harassment (costs mediate by female conditions) or by the diminished opportunity of exert sexual choice in a conflicting context (costs mediate by low quality sire genes). The cost, in this latter case, is attributable to a minor strength of sexual selection in presence of sexual conflict that decrease benefits associated to female choice.

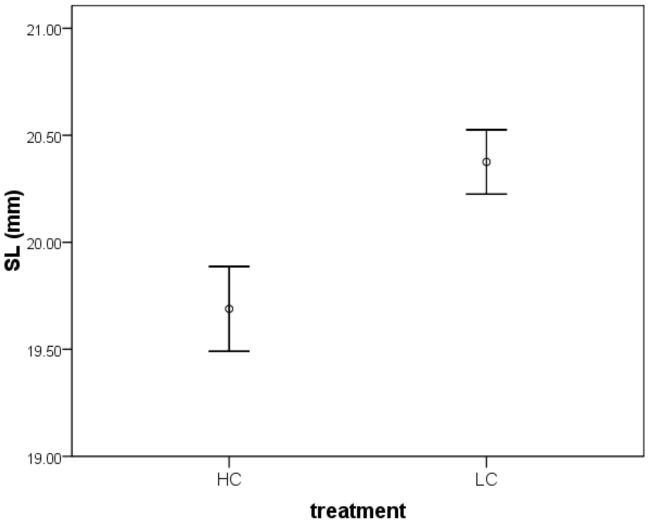


Fig. 2 Mean (± SE) of standard length (SL) in daughters of females belong to opposite treatments

I measured traits that are likely to be associated to reproductive success in sons. I considered males sexual behaviour, attractiveness, development of ornaments and measure of sperm quality and quantity. Our measurements on reproduction success in sons revealed that LC males had a longer gonopodium, were preferred by females and showed superior ability in achieving sneaky copulations. As discuss for result on daughters' size, negative effects revealed in sons could be the consequence of costs mediate by female condition or low quality genes (see above). Female worst conditions due to the presence of sexual harassment could affect egg production or embryos development and therefore compromise the general quality of offspring expressed on traits associated to reproductive success. In mosquitofish males with longer gonopodium are preferred by females in the precopulatory choice, but has been demonstrated to be costly, suggesting that only superior males could endure this cost (Langerhans et al. 2005). In addition, **paper V** showed that also in the guppy a longer gonopodium is preferred by females and therefore this finding could be explained by greater opportunities for LC females to choose preferred males that, in turn, possess a longer gonopodium.

In conclusion, we found several female fitness costs associated with male sexual harassment, all of which were visible in the offspring upon sexual maturity. Sexual harassment reduced the expected reproductive success of the progeny of both sexes, i.e. body size of daughters and sexual attractiveness and success in gonopodial thrusts of the sons.These findings are particularly interesting for our understanding of the evolution of female's resistance. Our results allow us to conclude that there is no support for the idea that female resistance evolves through indirect benefit achieved by screening the more persistent males. At least in the guppy, the magnitude of costs of imposed by sexual conflict over mating rate and the evolution of coercive mating tactic in the male does not seem to be compensated by any indirect benefits.

Paper II: Quantitative genetics of pre- and post-copulatory sexually selected traits in the guppy

I used a quantitative genetic approach to measure sire heritability of male traits that are likely to be associated to reproductive success, as behaviour and sperm traits (quality and quantity). I compared the additive genetic variance of these traits to which are known to be under directional selection through female preference, as body coloration (Bischoff et al. 1985; Kodric-Brown 1985; Houde & Endler 1990). If female resistance evolves and it is maintained through acquisition of indirect benefits for sons, I will expect a substantial sire additive genetic variance for the male traits associated to reproductive success. Experimental design consisted of a half-sibs/full-sibs breeding design. Each male (sire, N=33) was used to inseminate several females (dams, N=66) and data derived from the males offspring (N=238) were analyzed. Measurements on sexual behaviour were performed in four different repetitions; sperm quality, in term of sperm velocity, was measured using a Ceros Sperm Tracker from which three different measures of sperm velocity were obtained (VAP, VSL and VCL). These three measures provide an estimate of progressive velocity and have been demonstrated to be a good predictor of fertilization success in different taxa (e.g. Birkhead et al. 1999; Levitan 2000; Gage et al. 2004) and in the guppy (C. Boschetto, C. Gasparini and A. Pilastro, in prep.). The number of sperm collected from each male was counted with an ‘improved Neubauer’ haematocytometer. Coloration and body size were analysed from digital photographs using ‘Image tool’ analysis software. For all the traits considered I reported the value of heritability (h^2) and coefficient of additive genetic variance (CVA, 'evolvability' sensu Houle 1992). In particular CV_A is a standardized measure that provides a better estimation of heritability and allows the comparison among different traits and indicates the possibility for a given character to respond to selective pressure. Results for some male traits considered are shown in table 1.

trait	Mean	SD	df	F	*P*	CV_A	h^2
% orange	13.34	4.11	32	4.377	<0.001	46.08	1.921
Courtship displays	6.70	3.76	32	1.264	0.252	21.85	0.151
Gonopodial thrusts	14.12	8.98	32	1.125	0.369	26.44	0.170
Sperm velocity (VAP)	104.24	17.99	32	1.396	0.178	10.08	0.342
Sperm number (x10^6)	13.52	8.43	32	3.321	<0.001	66.37	1.151

Table 1 Coefficients of additive genetic variance (CV_A) and corresponding heritabilities (h^2)

Our results confirm previous quantitative genetic studies on male ornaments in guppies (size of colour spots), which nearly invariably show heritability estimates greater than 1 for colour components, suggesting Y-linked inheritance (Houde 1992; Brooks 2000; Brooks & Endler 2001a). The size of orange spots is subject, in this population, to directional precopulatory (Evans et al. 2004a) and postcopulatory sexual selection (Evans et al. 2003c; Pilastro et al. 2004). Despite very high heritability of orange spots, which should allow directional selection to rapidly erode additive genetic variance, I found substantial sire additive genetic variance for the area of the spots of orange and iridescent. This result indirectly support the importance of other non directional selection pressures, such as negative frequency-dependent survival (Olendorf et al. 2006), complex female preference functions (Brooks & Endler 2001b) and female preference for unfamiliar males (Hughes et al. 1999).

Reproductive behaviour, gonopodial thrusts and courtship displays, did not show significant sire heritability, even if our analysis revealed significant repeatability within male. The results agree with previous studied in which it has been demonstrated that behavioural pattern show low heritability compared to physiological and morphological traits (Mousseau & Roff 1987; Moore 1990; Lynch 1994). Behavioural patterns have often been reported to have low additive genetic variance. Explanation for the low heritabilities could rest on genetic architecture at the bases of complex behaviours that could reflect genetic non additive effects of dominance or epistasis. Evolution of behaviours is also strongly subjected to environmental influences. When individuals evolved in a unpredictable environment it is unlikely that a single phenotype will confer the highest fitness in all the situations, and therefore phenotypic plasticity is one possible adaptive solution to heterogeneous environments (Via et al. 1995). In the guppy the use and the relative success of the two alternative tactics are strongly dependent on a number of biotic (female receptivity, presence of rival competitors, operational sex ratio) and abiotic (light, visibility, water flow) variables (reviewed in Houde 1997; Magurran 2005). Indeed, as discussed for ornamentations, there are time and space fluctuations in the success of a male that could make a male more successful in one situation than in an other, and in consequence the outcome of displays or gonopodial thrusts could be different.

Results for sperm velocity traits suggest that the evolvability of these traits is very low. There may be different explanations for the lack of evolvability of sperm velocity observed in the guppy. One of the most plausible explanations is that the intense directional selection may have eroded the genetic variance underlying sperm traits associated with sperm competition success, such as sperm velocity. Despite the very low coefficients of additive genetic variance I found for sperm velocity, that one for sperm number was high and comparable to value obtained for orange coloration. The high sire heritability for sperm number it is a not unexpected result. It has been suggested that traits related to sperm production (testes size, ejaculate volume and sperm number) may have relatively higher levels of evolvability than sperm morphology and performance. In several species sperm competitiveness may be influenced by the number of sperm a male is able to inseminate besides differences in sperm quality (Snook 2005). One may therefore argue that female guppies primarily select sires with the highest sperm production and not with highest sperm quality. Anyway, in this species it seems improbably that sperm production (in term of sperm number) influences fertilization success. In fact, it has been shown that female guppies actively limit, during cooperative copulations with unattractive males, the number of sperm transferred (Pilastro et al. 2004), by reducing the duration of the copulation (Pilastro et al. 2007). Moreover, the number of sperm transferred during forced copulations is much lower (about 50×103 sperm, Pilastro & Bisazza 1999) compared to the daily sperm production (750 x10^3 sperm, Billard 1986) and therefore it is improbable for males to be sperm depleted. Although the number of sperm transferred could be important in a sperm competition scenario (Boschetto C., Gasparini C. and Pilastro A. in prep.), the sperm production it is unlikely to limit male fertilization success. This suggests that sperm production is not under a strong selective pressure and that genetic variance is not eroded by strong directional selection.

In conclusion due to the low heritability in traits associated to mating acquisition and fertilization success, our study do not support the ‘selective resistance’ hypothesis that suggests that the evolution of female resistance is driven by acquisition of indirect benefits of producing more successful sons.

Paper III: Female presence affects sperm velocity in the guppy

If sperm production is costly, males are expected to strategically adjust their allocation to sperm production in response to mating opportunities, in order to minimise such costs. Indeed, adjustment in sperm number has been reported in several taxa, but only few study investigated whether sperm quality can show adaptive phenotypic plasticity. On the basis of the low unitary cost of producing sperm, it has long been assumed that producing sperm is cheap and males are rarely, if ever, sperm limited. In contrast to this view, there is now ample evidence that sperm production is costly (Dewsbury 1982; Nakatsuru & Kramer 1982; Shapiro et al. 1994; Olsson et al. 1997) and that sperm production is strategically adjusted according to mating opportunities and hence expected sperm utilization (e.g. Shapiro et al. 1994; Aspbury & Gabor 2004; Scharer et al. 2004; Pyter et al. 2005; Bjork et al. 2007). Recently, it has been recognized that not only numbers are involved in determining fertilization success, but also that sperm quality, such as viability and velocity, has a great impact on male fitness and has been found to influence fertilization success in several animal taxa (Snook 2005). In particular elevated sperm velocity is associated with a higher fertilization success both in presence (Froman et al. 1999; Levitan 2000; Kupriyanova & Havenhand 2002) and in absence (Birkhead et al. 1999; Gage et al. 2004; Denk et al. 2005) of sperm competition. Although experimental estimates of the cost of producing faster sperm are still lacking, producing sperm with enhanced swimming performances is likely to be costly. For example, higher sperm velocity is associated with higher sperm ATP content (Froman & Feltmann 1998; Lahnsteiner et al. 1998; Burness et al. 2004; Locatello et al. 2007). While there is evidence that sperm velocity and viability are adjusted to the perceived level of sperm competition (Kilgallon & Simmons 2005; Rudolfsen et al. 2006; Simmons et al. 2007), whether or not males adjust their sperm quality in response to mating opportunities has never been investigated so far. In the guppy it has been demonstrated that males that were maintained subject to visual and possibly chemical contact with a female exhibited larger amount of strippable sperm when compared to isolated males (Bozynski & Liley 2003). This result suggests that male guppies adjust their sperm production to mating opportunities as a way to save energy when no mates are available. Experimental males were individually isolated for 3 days in small transparent tank (1l), right next to an identical container which either contained three virgin females selected from dedicate tanks or was empty. After treatment period males were stripped and sperm were collected for subsequent analysis with Ceros Sperm Tracker. Our analysis revealed that males produced significantly faster sperm in the presence of females than in isolation ($F_{1,45}$=6.784; P=0.012, see fig 3).

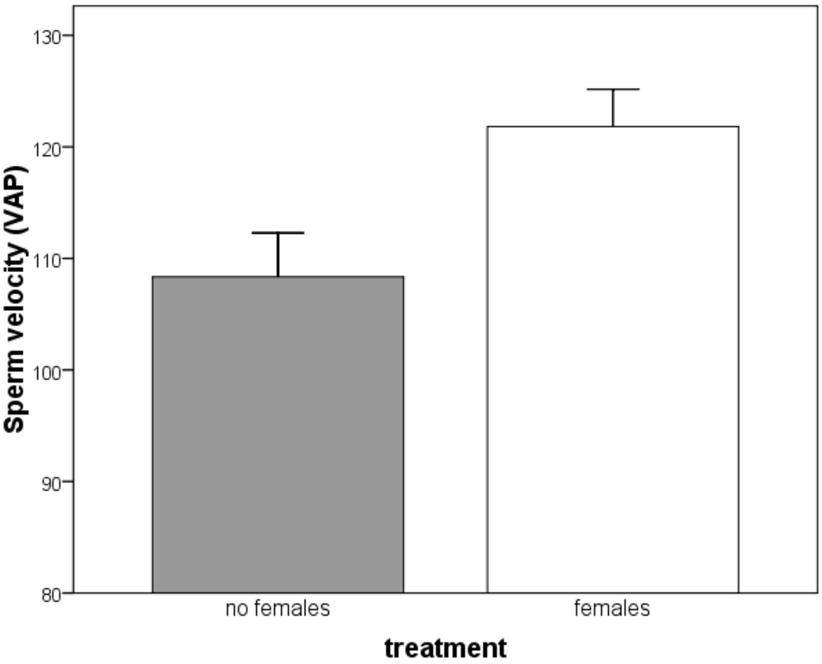


Fig. 3 Sperm swimming speed (mean ± SE) for males in the two treatments.

These results, associated with those obtained by Bozynski and Liley (2003) suggest that male guppies reduce the number and the quality of their strippable sperm when isolated from females. Our results demonstrated that the capability of adjust sperm quality have evolved also in response to the presence of potential mates. This study demonstrated the phenotype plasticity in sperm velocity that may explain the low heritability found for sperm velocity in this species (**Paper II**).

Conclusions

The study on costs and benefits of sexual harassment does not support the hypothesis of evolution of female resistance as a way to select the more successful male. Females that suffered higher level of sexual harassment produce more offspring early in life, but this production declines later on life respect to their counterparts which produce brood size more linearly. This indicate that the formers allocate more resources to early reproduction, probably affecting late-life resources available for reproduction. Different life-time strategies in females according to different levels of sexual conflict are therefore demonstrated. In addition, results indicate that a more intense sexual harassment results in a diminished daughter size, that reflect a lower expected fecundity. Males offspring from females that were subjected to more sexual harassment were less successful in achieving forced matings, were more attractive and moreover possess a longer gonopodium. These latter results may reflect lower opportunities to exert female choice in a conflict situation. Therefore, given the absence of benefits and the presence of costs in offspring as a consequence of male sexual harassment, these data do not support the hypothesis that female resistance evolves via indirect benefits mechanisms.

Moreover male traits associated to reproductive success both at pre and postcopulatory level do not show the elevated sire heritability that would be expected if this hypothesis effectively operate in the guppy mating system. Sperm velocity, a trait likely associated to fertilization success, has been found to possess low heritability and this could reflect an adaptive phenotypic plasticity. This idea is corroborated by the findings of **paper III**. Males can adjust sperm velocity according to mating opportunities, suggesting an evolutionary adaptive plasticity that, in turn, is not consistent with high heritability.

PART B:
SEXUALLY ANTAGONISTIC COEVOLUTION

The great diversification and evolution of genital morphology observed in internal-fertilizing species has long been debated among biologists. Genital morphology has been demonstrated to be the most rapidly evolving morphological trait (Eberhard 1985). Male genitalia are much more complex structures than necessary to the simple aim of transferring sperm. Evidences that sexual selection is responsible for such diversity are now widespread (Eberhard, 1985, Hosken & Stockley, 2004, Sirot, 2003), coming from studies linking variation in male genital morphology to mating success (Bertin & Fairbairn, 2005, Langerhans et al., 2005, Brooks & Caithness, 1995, Preziosi & Fairbairn, 1996) and to relative fertilization success (Arnqvist & Danielsson, 1999, Danielsson & Askenmo, 1999, House & Simmons, 2005, Wenninger & Averill, 2006). Evidences for sexual selection on genitalia come from comparative studies revealing evolutionary divergence in male genital shape among populations or strains that differ in the intensity of sexual selection acting on male genital traits (e.g. Arnqvist, 1998, Ramm, 2007). More recently, sexual conflict is believed to be involved in fuelling such diversification, and sexually antagonistic coevolution (SAC) is considered the main responsible for the pattern of divergence found in male and female genitalia. If possessing a certain genital morphology confers to males some advantage in reproduction although detrimental to female fitness, females are expected to evolve counteradaptations aimed to minimize the associated costs to the former, as predicted by SAC. Although, in general, male genitalia show higher degree of diversification than female genitalia (Eberhard 1985), some interesting works provided evidence for interspecific coevolution pattern in genitalia (e.g. Cordoba-Aguilar 2005; Ronn et al. 2007). For example, using a comparative approach in 16 waterfowl species Brennan and colleagues (2007) reported a correlation between female and male genital morphology. Comparative studies among different species showing correlated evolution are not anyway conclusive, as males and females belonging to the same species might show similar evolution in response to a common environment rather than a consequence of sexually antagonistic coevolution. Interspecific studies therefore need to be carefully interpreted before making conclusive explanations for correlated pattern. Only few studies reported intraspecific correlated pattern genitalia (e.g. Arnqvist & Rowe 1995) and none tested for their predicted covariance among populations exhibiting divergent genital traits.

In poeciliid family sexual conflict is likely to be pervasive and the high level of sexual conflict over mating decisions typically results in sexual coercion traits. Within this family the length of the intromittent organ (called gonopodium) varies predictably according to mating system, with species dominate by sexual coercion showing the highest relative length of the gonopodium (up to 70% of male's body length, Rosen & Tucker 1961). Therefore, gonopodium length has always been considered as one of the most important antagonistic traits subjected to strong selection as it favours males in achieving forced copulations. However, Langerhans and coworkers (2005) reported a premating preference for longer gonopodium in *Gambusia,* a species showing both courtship and gonopodial thrusts suggesting the possible role of sexual selection in shaping genital morphology. The scenario is further complicated by the fact that it has been hypothesized that different selective pressures might act on different aspects of male genital morphology (Werner & Simmons 2008).

In the guppy it has been demonstrated that gonopodium length differs among populations according to the level of predation pressure, in turn associated with the use of forced mating and therefore with the level of sexual conflict. Males in high conflict populations have a longer gonopodium, if compared to their low conflict counterparts (Kelly et al. 2000; **Paper VI**). Guppy genitalia are therefore likely to be a conflict trait, but the role of gonopodium morphology in the insemination success has scarcely been investigated. My aims were to investigate whether, and to which extent, gonopodial shape influences male reproductive success (namely mating acquisition and insemination success) both in coercive (**Paper IV**) and consensual (**Paper V**) matings in order to shed light on the type of selective pressure involved in genital evolution. Then, I studied, using an intraspecific approach, whether male genitalia differentiation among populations is associated with differentiation in female genital tract, to test the occurrence of sexually antagonistic coevolution (**Paper VI**). Main results and conclusions are briefly reported below, for details see the complete papers from page 69.

Paper IV: The influence of female population origin and male genital morphology on the success of forced copulations in Trinidadian guppies

Sexually antagonistic coevolution is difficult to detect as correlative pattern between the sexes could result from different evolutionary processes, and therefore distinguish among them is a challenging intend. Verbal models that aim to distinguish SAC from other forms of sexual selection have been proposed (Holland & Rice, 1998, Parker & Partridge, 1998). One basic prediction arising from these models is that where reproductive traits that are shaped by sexual conflict diverge among populations, females will be more resistant to males from their own population, with whom they have coevolved, than those from alien populations against whom they lack adaptations to resist superfluous matings (Holland & Rice 1998). In this paper I determined whether males originating from a focal high-predation (HP) population exhibited higher insemination success during forced matings when paired with unreceptive females from a population characterised by relatively low levels (LP) of predation (and therefore relatively low levels of sexual harassment). To do this, I compared the insemination success of 102 males paired with females from the same population (HP, N=36), with females from a different population characterized by the same predation pressure (HPc, N=33) and with females from a different population, but within the same river of male population, characterized by the opposite level of predation (LP, N=33). I recorded sexual behaviour, in particular the number of gonopodial thrusts that result in genital contact (therefore more successful in sperm transfer), and I retrieved the sperm from female oviduct in order to estimate the actual number of sperm transferred during these coercive matings. Moreover, I estimated male genital length and shape using tps software that provides dimensionless measurements of shape; I found that the proportion of gonopodial thrust attempts that resulted in genital contact tended to be higher in the trials involving low-predation females. The analysis revealed that females from the low-predation population had higher numbers of recoverable sperm in their oviducts ($F_{2,\ 47}$=3.58, P=0.036, Fig. 4). Morphometric analyses revealed a significant effect of male genital morphology on insemination success, estimated as the presence (success) or absence (failure) of recoverable sperm from the female's reproductive tract. I found that variance in the shape of both the gonopodium distal tip (overall shape of distal tip) and its genital hooks predicted the likelihood of sperm transfer during coercive mating attempts. The results obtained in this study showed that male genital shape influences the outcome of forced matings, indicating that it is a potential target of antagonistic selection.

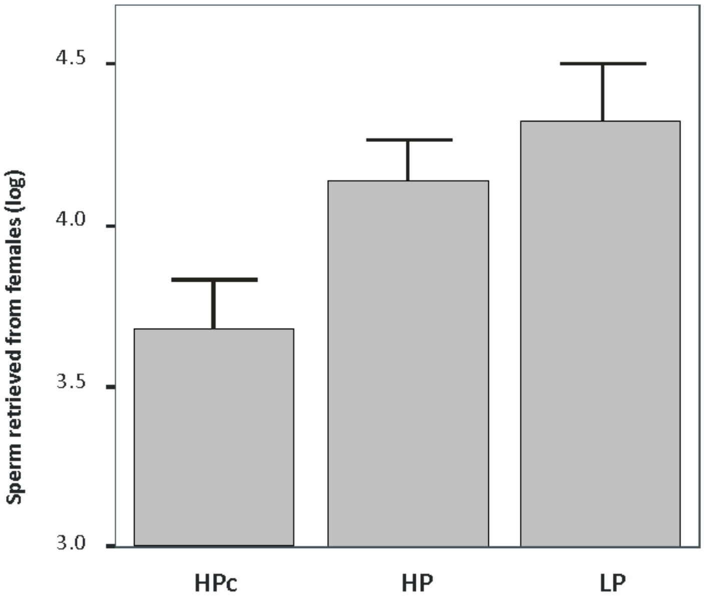


Fig. 4 Mean (± SE) number of sperm extracted from female oviducts in the three populations.

Paper V: Does size and shape of male genitalia influence female mate choice and sperm transfer in the guppy?

In this study I investigated the role of gonopodium morphology in male-female consensual interactions in a series of experiments involving only cooperative matings. In particular, I examined both female precopulatory preference and the number of sperm transferred in relation to gonopodium length and shape. Mating trials were conducted using virgin, therefore receptive, females paired to two consecutive males. In total I obtained 41 trials resulted in successful successive matings between the female and each of the two males (total number of males=82). I considered copulation latency as a measure of female preference and number of postcopulatory jerks as a measure of sperm transfer (Pilastro et al. 2007). Traits considered were: male sexual behaviour, male ornaments (body coloration) and characteristics of the gonopodium. In particular, I measured gonopodium linear length and shape, using tps software, as for **paper IV**. Copulation latency, measured as time taken for females to copulate following the introduction of the male into the tank, did not differ between the first and the second males. Copulation latency was influenced by male courtship rate (F=51.606, P<0.001), gonopodium linear length (F=6.286, P=0.015) and gonopodium shape, as indicated by a relative warps (F=5.619, P=0.021). Female mated more quickly with males that performed more displays, with a longer gonopodium and also larger in shape. Results concerning sperm transfer success did not reveal any significant predictor, nevertheless shape of the distal tip of gonopodium was slightly non significant associated with sperm transfer (F=3.117, P=0.081), indicating that males possess a shorter hook in the gonopodium distal tip transferred more sperm.

Paper VI: Intraspecific evidence for correlated patterns of male and female genital trait diversification

In this paper I estimated intraspecific divergence in male and female genital traits and these patterns were related to differences in the intensity of predation among populations. Guppies that inhabit lowland river populations typically endure high levels of predation from a variety of fish predators, while those inhabiting upstream sites are usually exposed to far lower levels of predation (Houde 1997). Across these ecological gradients, males use both forced mating attempts and courtship to acquire copulations, but the extent to which they employ either tactic is influenced by the level of predation. Under elevated predation risk, males tend to switch from highly visual courtship displays to less conspicuous forced matings (Godin 1995), possibly because forced matings are more profitable when females are preoccupied with predator evasion (Evans et al. 2002). Consequently, females from high-predation localities typically endure higher levels of sexual harassment (and associated costs) than their low-predation counterparts (Magurran & Seghers 1994b). About 20 male and 20 female guppies from 10 populations in Trinidad were collected. Within each river system, guppies were sampled from one upstream (low-predation site) and one downstream site (high-predation site). I examined variation in the shape of the whole gonopodium and the gonopodium distal tip using geometric morphometric analyses as for **papers IV** and **V**. In addition, I estimated linear measures for gonopodium length and width. Females were kept individually isolate until they produce brood, and after 3-4 days humanely sacrificed for subsequent analysis, in order to have females at approximately the same gestational stage to minimise variation in reproductive tract anatomy among females. I focused on linear measures of the female's oviduct, extending from the urogenital sinus to the posterior end of the egg sac. Analyses revealed differences in the size and shape of the gonopodium across predation gradients. In accord with previous work (Kelly et al. 2000) I found that males captured from high-predation populations had relatively longer gonopodia than their low-predation counterparts, when controlling for variation in body size among males ($F_{1,211}$=6.96, P=0.009). Furthermore, geometric morphometric analyses revealed highly significant variation in gonopodial shape among populations, both in the whole gonopodia and in the gonopodial tip. On average, males from high-predation populations exhibited gonopodia with less elongated distal tips and reduced gonopodial claw angles compared to their low-predation counterparts. Analyses from female oviduct revealed a significant association with predation regimes across rivers ($F_{1,178}$=8.26, P=0.005), with females from high-predation sites possess a higher relative oviduct width. Given the highly significant divergence among populations in both male and female genital traits, I looked for evidence of coevolutionary patterns of selection in these traits. There were two main results to emphasize. First, in populations in which males have lower hook angle females tend to have relatively wider oviducts (*r*=-0.769, P=0.009, see fig. 5). Second, on average, males from the high-predation populations had thinner gonopodia in relation to the depth of the females' oviduct than their low predation counterparts ($F_{1,8}$=8.54, P=0.043). Results suggest that males inhabiting high-predation populations exhibit adaptations that reflect their higher levels of forced mating activity and that counter-selection on female traits may serve to reduce the success of forced matings in these populations. Some not mutually explanations might be advanced. In high-predation sites, where the prevalence of forced matings is relatively high, wider oviducts may reduce the male ability to effectively clasp onto the female's internal reproductive tract during forced matings. Such an adaptation may therefore enable greater control by females over the duration of copulations, which has recently been shown to covary with the number of sperm transferred during matings (Pilastro et al. 2007). Furthermore, increased oviduct width may be an adaptation that reduces the damage inflicted by gonopodial hooks during forced matings.

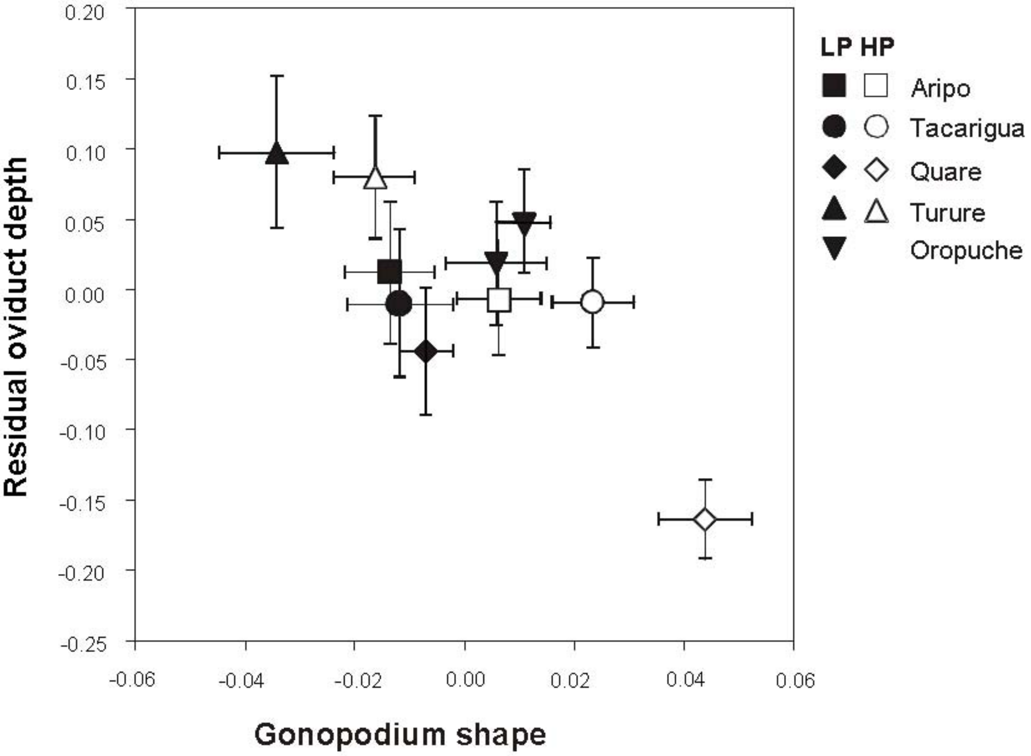


Fig. 5 The relative width of the females' oviduct in relation to the shape of gonopodium tip in the 10 populations sampled. Plotted values are population means with standard error for male (x error bars) and female (y error bars) traits.

Conclusions:

Gonopodial shape and dimension result important factors in determining the outcome of both consensual and coercive mating. Gonopodial length and shape resulted to be under sexual selection by female premating choice, in fact females mate more quickly with males possessing longer gonopodia and also with an enlarged shape. Moreover, there is a tendency (even marginally not significant) for male possess a shorter hook, in the gonopodium tip, to transfer more sperm. Gonopodium tip is also involved in the probability of sperm transfer during forced mating. Again, tip morphology vary among populations according predation pressure that in turn is associated with an increasing tendency for males to use coercive matings and therefore with sexual conflict. Taken together, these findings suggest that male genitalia evolution is shaped by different evolutionary forces that favour different components of male gonopodium shape in response to the different sexual tactics (consensual or forced). I can speculate that these different selective pressures may generate a trade-off between genitalia morphology and that may explain, at least in part, the variability found in the wild. Sexual conflict seems therefore to play a determinant role in genitalia evolution couple together with sexual selection. The study at population level revealed a coevolutionary pattern between male and female genitalia controlling for difference in ecology that comparative interspecific study can not completely rule out. Therefore, given also the fact that females that did not have the possibility to coevolve with males in high conflict context have little control over sperm transfer in forced copulations, evidences for sexually antagonistic coevolution between genitalia traits in the guppy are provided. For this reason, genitalia might be the result of an evolutionary arms race between males and females in the struggle for control over mating rate and fertilization success.

GENERAL CONCLUSIONS

Sexual conflict is likely to be pervasive in all but sexually monogamous animals. Triggered by sexual differences in the reproductive investment, sexual conflict exists because male and female evolutionary interests mostly do not coincide (Parker 1979). As showed in pioneer research of Bateman on *Drosophila*, the number of ideal sexual partners differs between the sexes, with the number of offspring increasing proportionally with the number of sexual partners for males but not for females (Bateman 1948). Evolutionary forces therefore act on opposite directions in the two sexes to maximize reproductive fitness and, if this occurs at other sex's expenses, it is predicted to generate coevolutionary patterns of divergence in male (persistence) and female (resistance) traits (Arnqvist & Rowe 2005). Sexual conflict over mating rate is likely to dominate all the guppy mating system and to have caused the evolution of forced mating and sexual harassment as well as in other animal species (Clutton-Brock & Parker 1995). The principal findings of my thesis are the follows.

First (**paper I**), sexual harassment did not affect overall female lifetime fecundity of females, but caused a different timing of reproductive allocation. Females that suffered more intense sexual harassment produced larger brood size at early parturitions, but brood size declined after few reproductive cycles, while females that were experimentally subjected to a lower level of sexual harassment continued to increase brood size linearly during successive reproductive cycles. This suggests that the formers allocated more resources to early reproduction, probably affecting late-life resources available for reproduction. Furthermore results revealed that male sexual harassment is associated with several female fitness costs visible in the offspring upon sexual maturity. Sexual harassment reduces the expected reproductive success of the progeny of both sexes, i.e. body size of daughters and sexual attractiveness and success in gonopodial thrusts of the sons. Taken together, these findings are particularly interesting for understanding the evolution of female resistance. It has been recently proposed that female resistance evolves as a way to screen among the available mates in order to select the best males that are able to overcome female resistance and, in turn, sire more offspring. This 'selective resistance' hypothesis, as argued by some theoretical models (Eberhard 2002; Pizzari & Snook 2003; Cordero & Eberhard 2003), is therefore not supported by these findings. At least in the guppy, the magnitude of costs of imposed by sexual conflict over mating rate and the evolution of coercive mating tactic in males does not seem to be compensated by any indirect benefits. This hypothesis is based on the assumption that male traits that are likely to be associated with reproductive success are inherited from sires to sons. Results (**paper II**) revealed that most of the traits involved in male reproductive success are not likely to be inherited by sons (but see discussion on the paper for heritability of sperm number). Therefore, the study of sire heritability in guppy does not provide evidences for the assumption on which selective resistance hypothesis is based. Phenotypic plasticity may explain the low heritabilities found for fitness-related traits. According to this, I found that male guppies adjust sperm velocity in relation to mating opportunities (**paper III**).

Taken together the results from these papers support the hypothesis of the evolution of female resistance as a consequence of direct costs associated to superfluous matings, while is not consistent with the evolution of female resistance via indirect benefits. In conclusion, sexual conflict over mating rate is likely to have deep evolutionary consequences in shaping the evolution of this species. *Poecilia reticulata* is therefore an ideal model to test sexually antagonistic selection on traits that are likely to determine the outcome of male-female interaction over mating rate.

In the second part of my thesis I focused on genitalia as a target of sexually antagonistic coevolution. In fact, in the poeciliid family, comparative studies suggest that gonopodium length is related to the level of sexual conflict, with males of species with only coercive mating possessing a longer gonopodium (Rosen & Tucker 1961). Also in guppy, long gonopodium is usually associated with coercive matings (Reynolds et al. 1993). Anyway, it has been showed that genitalia are also involved in premating female choice indicating a role of sexual selection in their evolution (Langerhans et al. 2005). The role of genitalia in the outcome of male-female interaction in the guppy has been scarcely investigated and aims of my thesis were to detect whether and at which extent male genitalia influence the outcome in coercive (**paper IV**) and consensual (**paper V**) matings. Results indicate that males with a longer gonopodium are preferred by females and that gonopodium tip shape is related to the success in sperm transfer in both situation. The gonopodium tip is the portion of male's genitalia that physically interacts with the female's genital pore during copulations (Rosen & Gordon 1953; Clark et al. 1954). My findings showed that especially this region (in particular the hook morphology) is important in sperm transfer (see also Cheng 2004). Taken together, these findings indicate that different process are involved in the evolution of male genitalia and probably in maintaining polymorphism on this trait. The importance of male-female coevolution in the outcome of sexual conflict is highlighted by findings of **paper IV.** The results are best explained by sexually antagonistic coevolution, as female are less resistant to mating attempts performed by males with which they have not coevolved. This coevolution pattern is furthermore corroborated by the findings of **paper VI**. This intraspecific study, that examined the natural range among different populations subjected to different level of sexual conflict, provides evidence for coevolutionary pattern between male and female genitalia. Differences among populations and the relative coevolution between male and female genitalia, indicate the evolutionary importance of sexual conflict and sexually antagonistic coevolution. In particular, this situation has the potential to fuel differences among populations that could lead the evolution of reproductive isolation and therefore promote speciation.

PAPERS

PAPER I
Direct and indirect effects of sexual harassment in the guppy

Clelia Gasparini, Alessandro Devigili & Andrea Pilastro

Abstract

The fitness consequences of sexual conflict are a source of ongoing debate in evolutionary biology. In this work we investigated the relative importance of direct and indirect effects of sexual conflict in a small tropical fish, *Poecilia reticulata*, in which sexual conflict occurs over mating rates and females endure a high rate of sexual harassment. We studied if there are benefits associated with sexual conflict that could balance direct costs suffer by females, as suggested by theoretical works, by measuring female fecundity and various aspects of offspring performance and viability and traits associated to reproductive success. We experimentally manipulated the level of sexual harassment and we found that sexual conflict leads to a differential life-time allocation in offspring number. An additional cost for females that underwent a more intense level of sexual harassment is revealed by measurements on offspring. After maturity we found difference between treatments in daughter body size, and therefore in the expected reproductive output, smaller for daughters of females that endured more sexual harassment. Moreover, we found that sons of females subjected to low level of sexual harassment were preferred, in female mate choice tests, over their counterparts, were more able in achieving forced copulations and possessed a longer gonopodium. We therefore demonstrated an additional cost of sexual harassment on female fitness. The lack of benefits in offspring suggest that, in this species, it is unlikely the evolution of female resistance via indirect benefits.

Introduction

Sexual conflict is widespread in nature and it occurs over a wide range of decisions involving female-male interactions driving the evolution of sexually antagonistic traits (Rice 1996; Rice & Holland 1997; Chapman et al. 2003; Arnqvist & Rowe 2005). Mating frequency is one of the aspects of reproduction that typically fuels sexual conflict because generally males have an optimal mating rate which is higher than that of females (Rowe 1994; Choe & Crespi 1997; Arnqvist et al. 2000). The costs of mating at the equilibrium mating rate may be substantial and females are expected to evolve resistance traits to reduce these costs. The genetic models of sexual conflict over mating rate predict a rapid evolution of male persistence traits in response to female resistance, possibly resulting in sexually antagonistic coevolution (Holland & Rice 1999; Gavrilets et al. 2001).

Various costs of mating have been identified, such as, for example, increased disease transmission (Daly 1978), energy costs (Magurran & Seghers 1994a; Watson 1998; Schlupp et al. 2001; Pilastro et al. 2003), predation risk (Arnqvist 1989; Fairbairn 1993; Rowe 1994; Pocklington & Dill 1995), physical injuries (Stutt & Siva-Jothy 2001; Blanckenhorn et al. 2002), and reduced fecundity (Arnqvist 1989; Byrne & Roberts 1999; Byrne & Roberts 2000; Stutt & Siva-Jothy 2001; Maklakov & Lubin 2004; Maklakov et al. 2005). Despite the costs associated with multiple matings, females typically mate with multiple males in nature (Jennions & Petrie 2000; Arnqvist & Nilsson 2000; Hosken & Stockley 2003). Indeed, it has been shown that, at least in insects, polyandry is associated to direct benefits (Arnqvist & Nilsson 2000). However, these benefits tend to decrease as the mating rate increases above the female remating optimum, in which case matings tend to be deleterious to females. Thus, even when female optimal rate is above one, sexual conflict is likely to eventually dominate resource-free mating systems in which males are not limited as much as females in gamete production (see Bjork & Pitnick 2006 for an exception). Thus, although the relationship between mating rate and female fitness depends on the interaction between costs and benefits associated with multiple mating (Arnqvist 2004), sexual conflict over mating rate is expected to arise (Arnqvist & Nilsson 2000) and high mating rate usually results in lower female fitness (Arnqvist 1989; Chapman & Partridge 1996; Holland & Rice 1999; Byrne & Roberts 1999; Byrne & Roberts 2000; Stutt & Siva-Jothy 2001; Martin & Hosken 2004; Maklakov & Lubin 2004).

In contrast with this view some authors argued that female resistance could instead represent a subtle form of female choice (Pizzari & Snook 2003; Cordero & Eberhard 2003). Under this view, the costs imposed by males could be more than compensated by indirect benefits of selecting the most persistent males. The rationale is similar to that of the ‘sexy sons’ process (Fisher 1930; Pomiankowski et al. 1991), as resisting females will be inseminated by the most persistent males, and will in turn produce sons with enhanced mating success.

Despite the appealing of these verbal models, theoretical works suggest that it is unlikely that direct costs associated with male sexual coercion could be counterbalanced by indirect ‘persistent sons’ effects (Kirkpatrick & Barton 1997; Cameron et al. 2003). The first reason is that it is unlikely that the strength of any indirect benefit, such as increased offspring viability or increased mating success of sons, will be greater than that of direct costs to females of antagonistic adaptations, as usually direct benefits (or costs in the case of antagonistic traits) are larger than indirect benefits (Møller & Jennions 2001). Second, quantitative genetic models of sexual selection suggest that the variance in female resistance is expected to be greater than the additive genetic covariance between female resistance and male persistence. Therefore, indirect effects will be scaled by a factor that is less than the factor scaling the direct effects (Cameron et al. 2003). Although these conclusions have been reached for female preference and positive female fitness (Kirkpatrick 1996; Kirkpatrick & Barton 1997), it is important to note that they will equally apply whether the direct effects of the preference on female fitness are positive (as in direct benefits models) or negative (as in sexual conflict).

However, a male’s persistence may also be associated with his quality/condition, and it may result in greater offspring viability. Although the effect of sexy sons indirect benefits has not effect on the evolution of female preference (or resistance) at equilibrium (Cameron et al. 2003), indirect benefits via a traditional ‘good genes’ mechanism can (e.g. Kirkpatrick 1985; 1996). Thus, according to the type of indirect benefits, ‘sexy sons’ or ‘good genes’, these might alter the stability properties of equilibria if the indirect effects are strong enough (Gavrilets et al. 2001). Furthermore, the relationship between female mating rate and female fitness depends on the form of the functions linking the costs and the benefits associated with multiple mating and their interaction (Arnqvist & Nilsson 2000; Arnqvist 2004; Maklakov et al. 2005) and may therefore vary in different species and different conditions (e.g. Chapman & Partridge 1996; Maklakov et al. 2005). The most striking example of sexual conflict over mating rate is probably represented by poeciliids, a fish family with internal fertilization. In these fishes, females are sexually receptive only 2-3 days per month. However, any insemination can result in paternity as sperm are stored in the ovary for months after copulation (Constantz 1989). Thus, males constantly attempt to obtain forced copulations (male poeciliids adopt a coercive mating tactic, gonopodial thrusting, through which males can forcibly inseminate the female, Pilastro & Bisazza 1999), making up to one gonopodial thrust attempt per min (Magurran & Seghers 1994b; Bisazza & Pilastro 1997; Schlupp et al. 2001; Pilastro et al. 2003; Plath et al. 2007). Furthermore, in more than a half of the species, males never overtly court the female and all matings are achieved through gonopodial thrusting (Farr 1989; Bisazza 1993).

With specific reference to those poeciliids in which females are never (overtly) sexually receptive, it has been suggested that females resist to male mating attempts as a way to select the best mate (Bisazza et al. 2000). Whereas the continuous male sexual harassment results in a significant reduction of female foraging rate (Magurran & Seghers 1994a; Schlupp et al. 2001; Pilastro et al. 2003; Plath et al. 2007), studies aimed at testing the effect of male sexual harassment on female fecundity lead to contrasting results, and while one study found evidence of fecundity costs associated with male sexual harassment (Ojanguren & Magurran 2007) other studies did not (Smith & Sargent 2006; Head & Brooks 2006; Smith 2007). These results suggest that fecundity costs of the conflict over mating rate may not necessarily be very large in poeciliids. However, the three studies above have measured the effect of a relatively brief exposition to male sexual harassment on short-term female fecundity, whereas females can live for more than one year, produce one brood per month on average and are exposed to sexual harassment throughout their life (Magurran 2005). Furthermore, the effect of the level of sexual harassment experienced by the mother on the reproductive success of the offspring has never been studied. Thus, the possibility that female resistance results in male offspring with higher reproductive success cannot be ruled out. On the other hand, if female choice is associated to indirect benefits (Evans et al. 2004b), male sexual coercion could represent, in addition to the other costs mentioned before, a cost in term of decreasing opportunities to exert precopulatory choice, thus diminishing genetic benefits related to female choice.

In order to understand the consequences of the conflict over mating rate on female fitness, we measured female longevity and life-time female fecundity in two groups of guppy females exposed, throughout their life, to different levels of male sexual harassment. Furthermore, we measured offspring body size at birth. Finally, we raised to maturity one brood (the forth one) of each female of the two groups and measured body size of male and female offspring, and, for male offspring only, the size of the sexual secondary characters, gonopodium length, ejaculate quality traits, sexual attractiveness and success in coercive matings. We tested the following prediction: 1) male sexual harassment results in a reduce female survival and lifetime fecundity; 2) females exposed to high levels of sexual harassment will produce male offspring with enhanced success in coercive mating tactic and, possibly, with reduced success in courtship tactic.

Materials and Methods

The study species

We focus here on the guppy *Poecilia reticulata*, a poeciliid species with a polyandrous, nonresource-based mating system (Houde 1997). During precopulatory mate choice, male courtship rate and the size of orange coloration influences female mating decisions (Endler & Houde 1995; Houde 1997). Females obtain indirect benefits, but not direct fecundity benefits (Pilastro et al. 2008), from mating with attractive males (Reynolds & Gross 1992). Furthermore, females obtain both direct and indirect benefits by multiple mating (Evans & Magurran 2000; Ojanguren et al. 2005). During their sexual receptivity phase, which lasts usually for 2-3 days after parturition, females mate with more than one male, typically 2-3 (Evans & Magurran 2000; Pitcher et al. 2003). For the rest of the breeding cycle, which lasts on average 25-35 days, females usually refuse to copulate (Liley 1966). As a consequence, the operational sex ratio is typically biased towards males (Magurran 2005).

Males, however, have the potential to undermine precopulatory female choice using forced 'gonopodial thrusts' (Liley 1966). Individual males employ both mating tactics, courtship and gonopodial thrusting, interchangeably. The degree to which either tactic is used depends on female receptivity (Liley 1966) and on several ecological factors, such as population demography (Rodd & Sokolowski 1995; Rodd et al. 1997), light intensity (Endler 1987; Reynolds et al. 1993) and predation intensity (Endler 1987; Magurran & Seghers 1990; Magurran & Nowak 1991; Godin 1995). The sperm delivered during copulation are stored for months in the ovary (Constantz 1989) and a female can produce broods six or more months after last copulation (Winge 1937). Thus, forced copulations, during which males can deliver as many sperm as during a cooperative copulation (Pilastro & Bisazza 1999) are potentially capable to result in fertilisation. As a result, the conflict over mating rate in this species is particularly high and females receive, on average, one coercive mating attempt per minute (Magurran & Seghers 1994a). Male sexual harassment imposes several costs to females. First, sexual harassment increases the risk of predation: a pair is more conspicuous to predators and it has been shown that in a mix-sex pair females are preferentially attacked (Pocklington & Dill 1995). Second, sexual harassment causes a reduction (up to 25 %) of female foraging efficiency, which in turn are likely to reduce female fecundity (Magurran & Seghers 1994a). Direct measures of female fecundity (growth rate and survival) in relation to male sexual harassment, however, reached contrasting results. Ojanguren and Magurran (2007) found that female guppies harassed by several males produce smaller broods than females who encounter the same number of males, but sequentially, suggesting a fecundity cost of male sexual harassment. In contrast, another studies did not find evidence that elevated rate of sexual coercion decreases female fecundity and offspring survival (Head & Brooks 2006). Similarly, sexual coercion did not affect directional selection on male sexual ornaments and behaviour (Head et al. 2008).

The study populations and its maintenance

We used guppies descendent of wild-caught fish from the lower part of Tacarigua River, Trinidad. Fish were maintained in the temperature regime of 26 ± 1° and under a 12 h light/dark cycle. All fish were fed a mixed diet of brine shrimp nauplii and commercially prepared flake food. Males and females were maintained in stock aquaria with roughly 1:1 sex ratio. To obtain virgin females, babies were isolated from adults and reared to maturity in dedicate tanks; once recognizable as females, usually within 2-3 weeks, virgins were kept separately from males until they were used for experiments.

Experimental design

Experimental design consists of 62 females randomly assigned to one of the two opposite treatments. The two treatments differed for the level of sexual harassment experienced by females: 'low level' (hereafter LC) and 'high level' (hereafter HC).

Treatments were started with females at their first parturition. In order to obtain females at the same stage of reproductive cycle, and with the same sexual experience, we artificially inseminated virgin females (4 to 6 month-old) with equal number of sperm bundles (packages of sperm) collected from 7 males showing similar colour pattern. We chose to inseminate only few bundles to minimize the risk of storaging of surplus sperm. Artificial insemination (AI) were performed following a standard protocol used in our laboratory (see Pilastro et al. 2008). Briefly, each male was stripped after been anaesthetized in a solution of MS-222, and sperm bundles were then collected. Sperm bundles were immediately injected into female reproductive trait (4 bundles per female) using a Drummond micropipette. After insemination females spent early gestation time in individual containers, and once they could be visually recognized as pregnant (about 15 days after the AI) they were placed individually in the dedicate sector of the experimental tank (43x52x36cm) equipped with filter, plastic plants, and gravel substratum, and maintained at the same condition of light and temperature of stock populations. Each tank (a total of 31 tanks were used) was divided by an opaque partition into two identical sectors (a total of 62 sectors) each one containing a focal female, assigned to opposite treatment. Partitions allowed free water flow between sectors but prevented visual interactions between neighbouring fish.

In HC treatment three males were placed simultaneously in the female's tank and allowed to interact freely with the resident focal female for 8 consecutive days during her unreceptive period (from 8th to 15th day after the parturition); in LC treatment three males, under the same conditions, were allowed to interact with the female but, differently from HC treatment, only in her receptive period (24 hours following the parturition). Therefore, treatments were identical except for the choice of timing and the duration of the period in which males could interact with females. In order to minimize the difference in the level of food competition between the two treatments, three virgin females (visually selected of similar size of experimental males) were placed with LC female from day 8 to day 15. Males were chosen randomly among 200 males reared in tanks with sex ratio 1:1 and isolated from females the day before. Males were replaced in their original tanks after the experiment. Treatments started after the first parturition and were performed at every reproductive cycle throughout female lifetime (i.e. until each female died).

Behavioural observations

For each experimental female observations of males' sexual behaviour were made in three different time blocks: during the first, second and forth treatments. Observations took place an hour after feeding time. We recorded frequency of sigmoid displays, gonopodial thrusts and the number of copulations (either forced and cooperative) underwent by each female (for description of behaviours see Houde 1997; Head & Brooks 2006). Because observations were recorded over variable periods (30 or 45 minutes per female), all behaviours are expressed per minute.

Life-time fecundity

Experimental tanks were checked for fry at least twice a day in order to recorder the exact number of offspring produced per female, and the exact interbrood interval (hence gestation time). This was done until each experimental female died.

Females' growth rate

Female standard length (SL) was measured twice: one week following the first and after the forth parturition (i.e. after 3 treatment cycles; 91.35 days ± 1.23 after the first measure). We measured female size without anaesthesia, to avoid unnecessary stress that could have falsify subsequent measurements. Indeed, a female was gently placed in a small transparent box with a millimetre rule on background (to calibrate measurements); once female was in focusing a photo was taken with a digital camera. Female weight was measured at the same moment of reproductive cycle for all females (4 days after parturition) to avoid differences in weight due to different embryos development. We measured weight by calibrating a small box fill of water and subsequently weighed the box with the female. The average of three measures to the nearest 0.01 g was used into analysis.

Offspring measurement at birth

We measured body size (length and abdomen width) for newborns collected in nine different birth, from brood number 2 to brood number 10, for a total of 231 broods (HC: n=113; LC: n=118). We took photos the day after the birth with a digital camera. We measured all the offspring for each brood up to 25 offspring per brood (i.e. for broods larger than 25, we randomly selected 25 offspring for body size measurement) using Image Tool (http://ddsdx.uthsca.edu/dig/download.html).

We tested offspring performance in offspring collected at the birth number two, following methods described in (Evans et al. 2004b). Specifically, we analysed anti-predator behaviour in newborns (24 hours after the birth) in order to estimate two different traits: schooling time and escape response. These two measured are likely to influence the ability of newborn fish to evade predators and will therefore affect the probability of juvenile survival in the wild (Magurran 2005). Briefly, the schooling test enabled us to estimate the percentage of time spent in schooling behaviour. We tested a pair of individuals at time in a circular arena of 35 cm diameter. Juveniles behaviour was recorded with a video camera (Sony Handycam CCD-TR718E) positioned above the schooling arena. Schooling time was calculated as percentage of time siblings spent swimming in synchrony within two body lengths of each other over a 10-min period. Moreover, we measured escape response as the time (sec) an observer required catching the pair with a hand net.

Offspring measurement at sexual maturity

To study if sexual harassment affects late offspring fitness, we collected brood at birth number 4 (i.e. after four treatments). Hereafter the sons/daughters of HC or LC females were referred to as the HC or LC sons/daughter respectively. The babies were reared in small plastic tanks (1l) in groups of maximum 6 individuals, labelled according to the treatment and the mother identity. All the babies grew under the same temperature, light and food regimes in a recirculating water system, in which water, shared among tanks, was constantly renovated throughout a electronic system and kept clean and filtered by mechanical filter and UV lamp.

We measured body size (SL) on daughters and sons at 4 months of age, when fish are fully sexual mature, and we estimated sex ratio. After this latter measure males were used for further experiments.

We raised at maturity a total of 196 sons (HC: 99, from 13 females; LC: 97, from 17 females). Dichotomous choice trials were performed to test whether female guppies showed a precopulatory preference between HC and LC males. We used sexually receptive (virgin) females previously reared in single sex tanks. Males were isolated three days before the experiment. The experiment followed the methods described in (Evans et al. 2004a). The experimental tank was divided in three parts: one main central chamber and two smaller chambers at the sides. The central chamber (30x20x28cm filled with water up to 20cm) contained one virgin female. Males from each group, LC and HC, were randomly assigned in a compartment on either side of the central chamber (each measuring 8x20x28cm), and the relative position between HC and LC males was reciprocated during following tests. The central chamber was divided in five sections of equal length using an indelible pen. Opaque dividers, placed in front of the two smaller chambers, prevented visual access by the female into the two side chambers during an initial acclimatisation period (20 min). Following this period the opaque dividers were raised, allowing the female to observe males. Female's position were register every 5 sec over a 30 min period, in order to obtain a score of female's preference.

Sexual behaviour trials were conducted to investigate whether differences between HC and LC males in sexual behaviour existed. Each male were observed individually with a female the day after the dichotomous choice test. One unreceptive female from stock was allowed to acclimatize during the morning. In the afternoon test male was put the tank and the sexual behaviour was observed for 20 min. The number of sigmoid displays, sneaky matings (gonopodial thrusts), and swings (movement of the gonopodium) were recorded over that period. Gonopodial thrusts were divided into three different categories, according to the distance reach by male's gonopodium respect to gonopore. In particular, we considered more successful gonopodial thrusts in which there was a contact between the gonopodium tip and female genital opening ('genital contact'), clearly evident by a sudden jerk of the female (Pilastro et al. 1997). After each observation the male was gently transferred back to his original tank.

Males were photographed using a digital camera (Nikon CoolPix 4300) and stripped to obtain sperm samples. Photos were analysed using image analysis software (Image Tool, http://ddsdx.uthsca.edu/dig/download.html). For each male we estimated standard (SL) and total (TL) length, gonopodium length, total area of the body and relative area covered by coloured spots (orange, black and iridescent spots).

Total number of sperm per ejaculate was estimated by stripping males following procedures described above for AI. We obtained sperm from 194 out of 196 males. Free sperm solution were obtained by vortex all sperm bundles for 1 minute and the total number of sperm was counted with an 'improved Neubauer' haematocytometer (see Pilastro et al. 2007).

When all the previous experiments were completed we performed sperm velocity analysis on a subset of the available fish (n=44). Using the same methods previously described, males were stripped and single sperm bundles were taken with a micropipette (3μl) for analysis. Each bundle was individually placed on a slide, previously sprayed with silicon oil. To ensure the depth needed from sperm to swim, we used a layer of Parafilm at the edge of the slide. Once a bundle was set, a drop of activating solution (250 mM of KCL and 4 mg/ml bovine serum albumin) was added, and gently covered with a cover glass. Sperm was analysed using a Ceros Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA, version 12.3). Sperm velocity were measured on mobile sperm (mean: 59.54 ± 4.93), average of two measures from two different bundles was used. These measures include average path velocity (VAP); straight line velocity (VSL) and curvilinear velocity (VCL). The threshold values defining static cells were predetermined at 25 μm/s for VAP and 20 μm/s for VSL. These three measures provide an estimate of progressive velocity and have been shown to correlate with fertilization rates in various vertebrate species and has been suggested to have important role on fertilization also in guppy (Skinner & Watt 2007).

Statistical analysis

Statistical analyses were performed using SPSS 15.0. We checked the distributions of our data for normality and for homogeneity of variance and used appropriate transformations where necessary. When we compared multiple data collected from the same female (such as brood size in successive broods; phenotypic differences between offspring, etc.), we used Linear Mixed Models, in which the identity of the mother was entered as random factor, to control for non independence of the data.

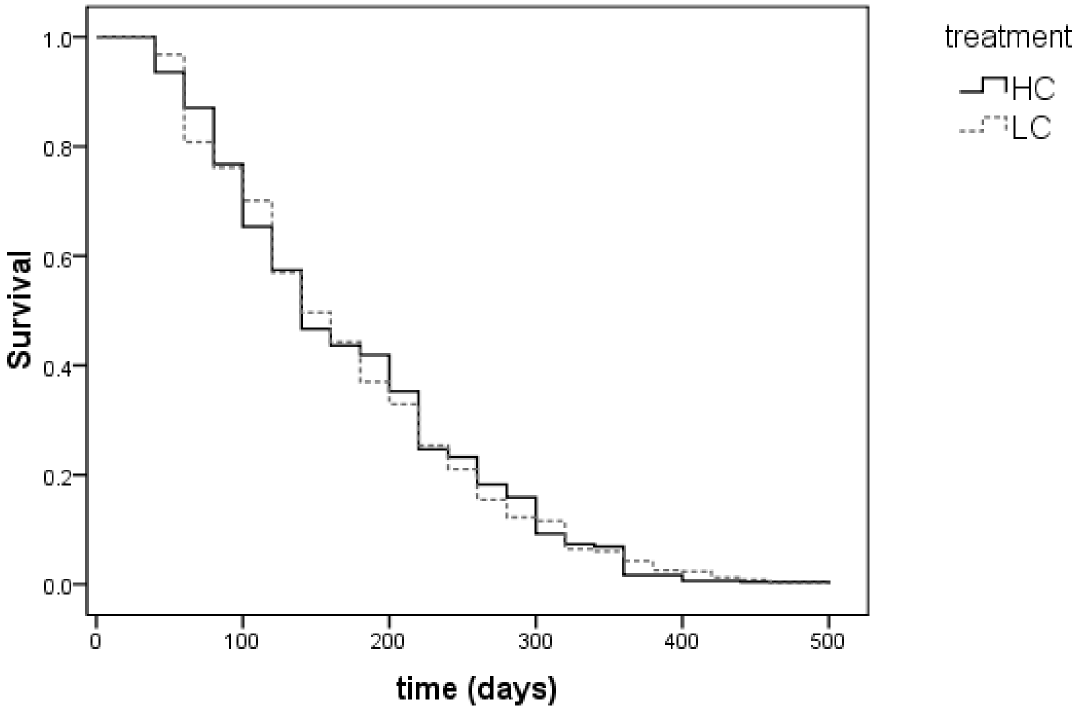
Results

Effect of treatment on the intensity of male sexual harassment

As expected females in HC treatment suffered more sexual harassment respect to their counterparts in LC treatment. This is due, first, because experimental design was intended to left HC males to interact with females for a longer period than LC males (i.e. 8 days vs. 24 hours). In addition, we found HC females received significantly more courtship displays and gonopodial thrusts (see table 1).

	HC (min^{-1})	LC (min^{-1})	HC (total)	LC (total)	*df*	*F*	*P*
Gonopodial thrusts	1.11 ± 0.08	0.62 ± 0.05	106.29 ± 7.66	7.47 ± 0.59	1,58	165.250	<0.001
Courtship displays	0.51 ± 0.03	0.20 ± 0.02	19.56 ± 2.92	10.94 ± 2.13	1,58	5.699	0.020
Copulations	0.005 ± 0.00	0.007 ± 0.00	0.52 ± 0.14	0.65 ± 0.14	1,58	0.421	0.519

Table 1. Mean (± SE) frequency of male mating behaviour and copulations in the two experimental groups expressed per minute and for the total time of interaction in the two groups. Statistics are referred to the total time of interaction (8 days vs 1 days).



Effect of treatment on female growth, survival and lifetime fecundity

Female growth and survival

Females were randomly assigned to treatments and there was no difference in their initial standard length ($F_{1,61}$=0.978, P=0.327) and weight ($F_{1,61}$=1.687, P=0.199). As expected, females' standard length increased significantly between measurements (repeated measure ANOVA: $F_{1,34}$=187.52, P<0.001), but did not differ between treatments ($F_{1,34}$=2.293, P=0.139). The same pattern was observed for females' body mass that increased between the two measurements ($F_{1,37}$=139.32, P<0.001) but not between treatments ($F_{1,37}$=3.104, P=0.09). Similarly, daily growth rate did not differ between treatments (SL: $F_{1,37}$=0.762, P=0.388; body mass: $F_{1,37}$=3.088, P=0.087).

Female longevity did not differ between groups (lifespan LC: 211.1 days ± 120.8 SD, range=43-489; HC: 208.2 days ± 114.0 SD, range=32-483; Mann-Whitney U test, U=480, z=0.01, P=0.99), and mortality was distributed equally between groups along the experiment (Mantel Cox test, Chi square = 0.015, P=0.90; Fig. 1).

Female lifetime fecundity

On average, lifetime fecundity did not differ significantly between treatments (mean number of offspring, HC: 85.06 ± 14.25, range=0-301, LC: 72.16 ± 11.78, range=0-296; $t_{1,60}$=0.698, P=0.488). These results were substantially unchanged when the females that died before producing their first brood were excluded (P=0.458). Mean brood size did not differ significantly between treatments but there was a significant interaction between treatment and brood number, suggesting a different timing of reproductive allocation (treatment, Wald chi square = 2.08, P=0.149; brood number, Wald chi square = 10.93, P<0.001; interaction

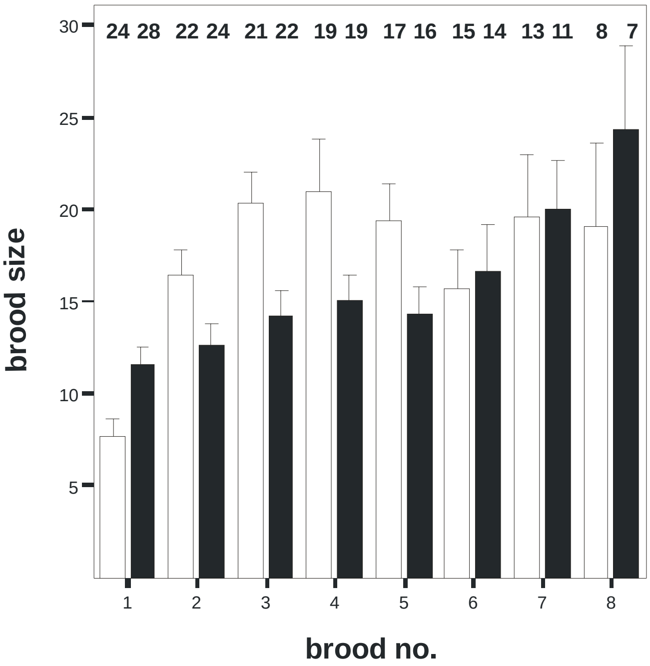


Fig. 2 Mean (± SE) number of offspring produced per each brood by females assigned to HC treatment (open bars) and LC treatment (filled bars). Numbers above the bars indicate sample size (number of females producing a brood).

treatment*brood number = 6.30, *P*=0.012, cubic smoothing spline; linear mixed model, female ID = random factor). In particular, HC females showed a relatively greater fecundity early in life, but their reproductive output remained stable, or declined, afterwards. In contrast, LC females, which initially produced smaller broods, continuously increased their brood size until brood 8 (Fig. 2). After brood no. 8, the number of alive females dropped to 9 (HC: n=4, LC: n=5, Fig. 1) and brood size declined in both groups, with the exception of a single HC female whose last brood before dying was very large (31 offspring).

In contrast, interbrood interval did not differ significantly between groups (HC: 31.97 days ± 0.39 SE; LC: 32.4 days ± 0.33 SE; treatment $F_{1,122.908}$=0.008, P=0.927; brood no. F=42.146, P<0.001; interaction treatment*brood no. F=0.028, P=0.868 linear mixed model, female ID = random factor).

Effect of treatment on offspring characteristics

Offspring size and schooling behaviour at birth

Mean body size of the offspring at birth did not differ between treatments after controlling for brood size (newborn body length, treatment: Wald chi square=2.265, P=0.139; brood size: Wald chi square=4.142, P=0.043; newborn abdomen width: treatment: Wald chi square=0.933, P=0.339, brood size, Wald chi square=5.505, P=0.020; linear mixed model, female ID = random factors; *n* of newborns measured = 3665, *n* of broods measured = 231, *n* of females = 50).

Treatment did not influence newborns' anti-predator behaviour, neither expressed as time spent in schooling (treatment, $F_{1,42}$=0.01, P=0.92, gestation length, $F_{1,42}$=0.01, P=0.95) nor as capture time (treatment, $F_{1,42}$=0.12, P=0.73, gestation length, $F_{1,42}$=6.08, P=0.018; interaction treatment * gestation time = NS).

Brood sex ratio

In total we sexed 389 offspring from 29 brood collected after parturition number four. Sex ratio did not differ between treatments (mean sex ratio, LC: 0.493 ± 0.167, n=17; HC: 0.490 ± 0.143, n=12; treatment, deviance ratio = 1.37, P=0.25, logistic regression).

Body size at sexual maturity

Survival rate of the offspring to the age of four months did not differ between treatments (HC=0.92 ± 0.10 SE; LC=0.91 ± 0.14 SE; deviance ratio = 0.10, d.f. 1,28, P=0.75, logistic regression with correction for overdispersion, phi=0.1298). Body size (SL) was measured at the age of four months in 312 offspring (157 daughters and 155 sons) which were obtained from the fourth brood of 25 females (LC: n=14; HC: n=11). As expected, a significant difference in body size between the sexes was found; body size was further influenced by treatment, brood size and tank sex ratio (fish were grown in tanks containing 6 fish each and the no. of males per tank varied between 0 and 6). In particular, the level of sexual harassment experienced by the mother negatively affected body size at maturity. This effect, however, was particularly evident in daughters' body size, as suggested by the significant interaction between sex and treatment. Furthermore, female body size was positively influenced by a male-biased sex ratio (Table 2, Fig. 3).

Predictor	*F*	*P*	*b* ± *SE*
Treatment	7.096	0.016	-0.294 ± 0.251 [a]
Sex	71.727	<0.001	3.330 ± 0.364 [b]
Sex ratio	22.222	<0.001	2.733 ± 0.669
Brood size	10.571	0.003	0.101 ± 0.031
Treatment * sex	4.911	0.027	
Sex ratio * sex	16.683	<0.001	

[a] LC as factor's reference level

[a] Male as factor's reference level

Table 2. Effect of experimental treatment on body size at maturity (four months) of sons and daughters (linear mixed model, dependent variable: body size (SL); predictors: treatment, sex, sex ratio in the tank and brood size; random factor: female identity). Only significant interactions were included in the final model. While female identity explained a significant proportion of the variance in the offspring body size (0.169 ± 0.088), variance component due to the tank was estimated to be = 0 and was therefore excluded from the final model.

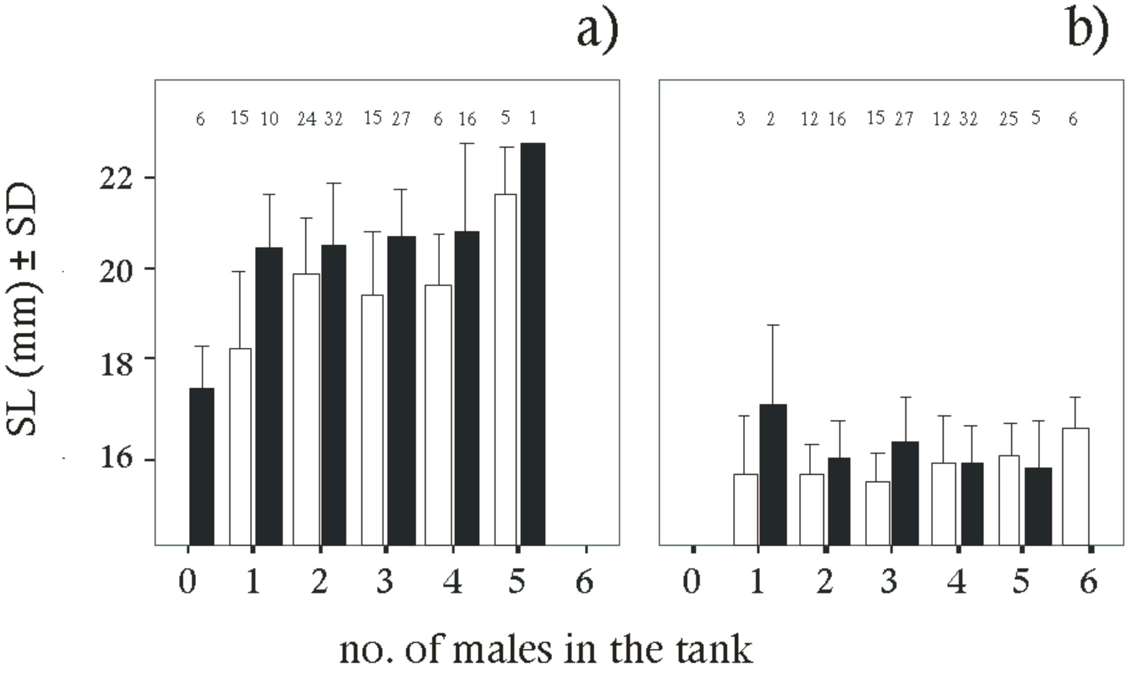


Fig. 3. Offspring body size (SL) at age 4 months in relation to treatment (LC=filled bars; HC=open bars), sex (a=females, b=males) and the sex ratio (no. of males) in the tank where the fish were raised to maturity in groups of six. Numbers above the bars represent the number of offspring.

Sons' ornaments and gonopodium length

Results of a Multivariate Linear Mixed model suggest that the size of the spots of the three colour components (orange, melanistic and iridescent) did no vary significantly according to treatment (Wald chi square = 1.21, P = 0.304, female ID = random factor, HC: n_{sons}=99, n_{broods}=13 LC: n_{sons}=97, n_{broods}=17). None of the three colour categories, analysed separately, revealed any significant effect of the treatment (all P>0.090). In contrast, gonopodium length differed significantly between treatments (HC: 3.58 ± 0.03 SE, LC: 3.65 ± 0.02 SE), after controlling for body size, with male offspring from the LC treatment have relatively longer gonopodium (treatment: F=4.882, P=0.029, body size: F=17.31, P<0.001; HC: n_{sons}=98, n_{broods}=13 LC: n_{sons}=97, n_{broods}=17, see fig. 4). Gonopodium length was not affected by the sex ratio in the rearing tank (P>0.55)

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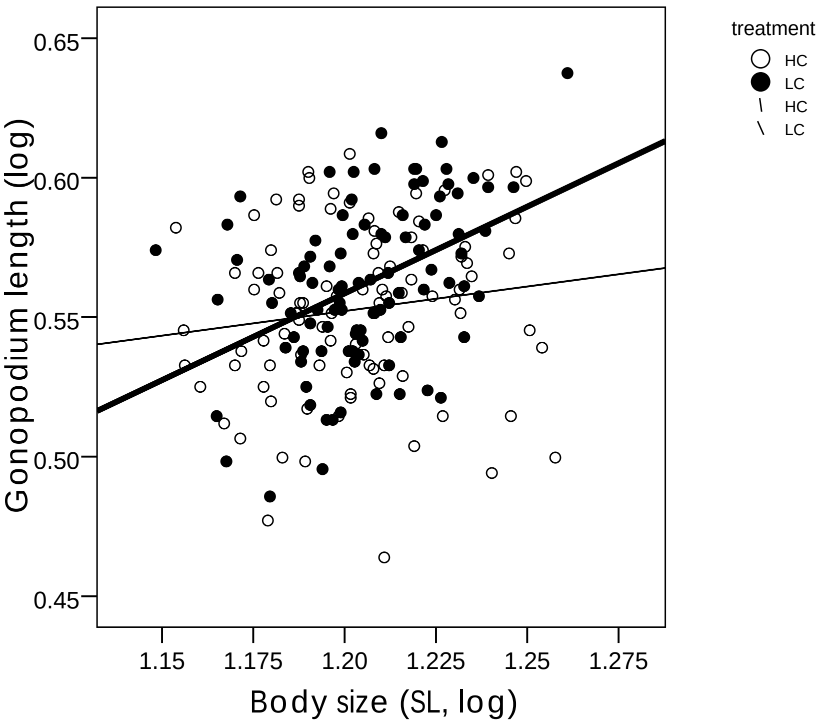

Fig. 4 Relation between gonopodium length and body size

Sons' sexual behaviour

The proportion of successful gonopodial thrusts was positively correlated with female size (Wald chi square=11.89, P=0.001, b=0.073, SE= 0.021) and negatively correlated with male size (Wald chi square=3.41, P=0.065, b=-0.150, SE= 0.082), and resulted to be higher in the sons of mothers that experienced low sexual harassment (HC: 0.138 ± 0.017 SE, n_{sons}=78, n_{broods}=13; LC: 0.157 ± 0.015 SE, n_{sons}=89, n_{broods}=17; Wald chi square=4.86, P=0.028, b=0.28, SE=0.13). The estimated effects (*b*) of the fixed factor remained substantially unchanged when mother identity was kept into account as a random factor (treatment: b=0.27, SE=0.16). In contrast, the experimental treatment did not affect the number of sigmoid displays, the total number of gonopodial thrusts and the number of gonopodial swings (all P>0.30).

Sons' attractiveness

Dichotomous mate choice tests using virgin females revealed that sons of LC mothers were significantly more attractive than their HC counterparts (% time spent near HC: 47.19 ± 2.12, near LC: 52.81 ± 2.12) and the difference remained significant also after statistically controlling for differences in body size and size of colour spots (see Table 3).

Full model

	b	Se	df	*F*	*P*
Treatment[a]	19.67	9.04	1,174	4.739	0.031
Body size (SL)	4.82	5.72	1,174	0.711	0.400
Orange	1.08	1.20	1,174	0.802	0.372
Melanistic	3.92	4.27	1,174	0.843	0.360
Iridescent	-2.21	2.12	1,174	1.089	0.298

Reduced model

	b	Se	df	*F*	*P*
Treatment[a]	18.94	8.76	1,178	4.672	0.032

[a] HC group is taken as reference level.

Table 3. Effect of treatment on sons' attractiveness (mixed model with family and choice trial as random factors, and treatment as fixed factor; male body size and area of three components of male colours were entered as covariates).

Sons' ejaculate characteristics

Sons from the HC group had on average larger sperm stores (HC: 6.88×10^6 sperm ± 0.49 SE, n_{sons}=98, n_{broods}=13; LC: 5.87×10^6 sperm ± 0.46 SE, n_{sons}=96, n_{broods}=17), but the difference was not significant once the identity of the mother was kept into account (treatment: F=2.51, P=0.125; mother ID = random factor, P<0.001, linear mixed model). Including body size (SL) and sex ratio in the rearing tank as covariates did not change the results (treatment: F=2.87, P=0.104), but revealed a significant positive effect of sex ratio (no. of males) on sperm reserves (b=3.36 ±1.34 SE, F=6.25, P=0.014; SL, F=0.01, P=0.92). Similarly, sperm velocity (VCL) did not differ between groups (HC: 61.01 ± 2.98 SE, n_{sons}=21, n_{broods}=10; LC: 63.54 ± 4.28 SE, n_{sons}=23, n_{broods}=13; F=0.37, P=0.548; linear mixed model, mother ID = random factor, P=0.98).

Discussion

In this study we provided an estimate of the effects of male sexual harassment on female fecundity and offspring fitness at birth and at maturity. Our experimental design was aimed at avoiding the confounding effect of differences in the number of potential mates by keeping the number of males constant between treatments. Our behavioural observations suggest that the number of mates per females per brood cycle was not significantly different between treatments. We did not perform any paternity analysis and therefore we cannot rule out that the mean number of actual sires per brood did not differ between treatments. However, an experiment in which the opportunities for sexual harassment (and coercive matings) were experimentally manipulated revealed no differences in the variance of male reproductive success (Head et al. 2008). In contrast, males directed towards HC females significantly more sexual attempts than in their LC counterparts, as the frequency of male coercive mating attempts per minute did not differ between treatments, but males stayed with the females for 1 day in the LC group vs. 8 days in the HC group.

Our results suggest that, at least in the conditions in which the experiment was carried out, sexual harassment did not affect overall female lifetime fecundity. However, we found that female reproductive investment (as expressed by brood size) varied, between treatments, across subsequent brood cycles. In particular, after a reduced brood size observed in the first brood of HC females respect to LC females (see Ojanguren & Magurran 2007 for a similar result), brood size subsequently increased more rapidly in the HC group. After brood number four, however, brood size in the HC females remained approximately stable. In contrast, brood size of the females from the LC continued to increase linearly during the experiment until brood eight, suggesting that HC females allocated more resources to early reproduction, probably affecting late-life resources available for reproduction. Why then this different timing of reproductive allocation between treatments? We can envisage two alternative, although not mutually exclusive, mechanisms to explain this result. Firstly, high levels of male harassment may be associated with increased predation risk (e.g. Pocklington & Dill 1995) or female extrinsic mortality due, for example, to sexually transmitted diseases. Extrinsic mortality risk is expected to select for early reproduction (Reznick & Endler 1982) and, in those cases in which predation risk varies across a female's life, it may be advantageous to increase her reproductive effort early in life when the perceived mortality risk is high. Indeed, female guppies from this population reduce their gestation time in response to a dummy predator in this population (Evans et al. 2007), suggesting that the pattern of reproductive investment is influenced by predation risk. We did not find, however, evidence of a significant change in gestation time in our experiment. Thus, a second possibility is that the increased reproductive effort early in life may observed in HC females may be the result of the antagonistic manipulation of females by males, either at the pre- or post- copulatory level. For example, in several poeciliid species, forced copulations result in wounds in female genital opening and vaginal bleeding (Constantz 1984), which may reduce her willingness to copulate or induce the females to invest more in the current reproduction (Arnqvist & Rowe 2005). These traits may be selected in males because they may facilitate sperm transfer or because they induce female to increase their current reproduction. As in this species males and females do not form stable groups (Magurran 2005), and sperm competition is strong, males have little interest in female future reproduction other than the next brood. Male guppies may also produce seminal fluid component that might stimulate embryogenesis over female optimum, as it has been observed in *Drosophila* (e.g. Chapman et al. 1995). Nothing is known about the composition of seminal fluids in poeciliids, and there is certainly avenue for future research in this field.

Whatever the explanation of the differential reproductive allocation observed in our experiment, we did not find differences between treatments in offspring body size and anti-predator performances at birth, and no effects on secondary sex ratio (number of males and females at sexual maturity). Similarly, we did not find any significant effect of sexual harassment on female mortality rate and growth. This was somehow surprising, as previous studies on the guppy and other poeciliids (Magurran & Seghers 1994a; Schlupp et al. 2001; Pilastro et al. 2003; Plath et al. 2007) demonstrated a significant reduction of female foraging rate (more than 25%) in the presence of harassing males. However, these studies may have overestimate the effect of sexual harassment on female foraging rate as often employed hungry females and fed males, whereas in our experiment both males and female had to forage, possibly reducing the effect of sexual harassment on female foraging rate. Finally, fish were fed ad libitum, and this may have masked costs sexual harassment that become visible only in more natural conditions. Finally, clearly the effect of sexual harassment on predation, which may be substantial in nature, was not included in the present study. Collectively, these results suggest that the increased early reproductive effort observed in the HC females was not associated with a decline of female survival or female overall fecundity.

Although the offspring did not differ in quality between treatments at birth, we found significant differences in several fitness-related traits in male and female offspring at maturity. In particular, we found that the females that experience a high level of sexual harassment produced daughters with smaller body size than their low-harassment counterparts. Female fecundity is positively correlated with body size (Farr 1989) and, although we did not directly measure female offspring fecundity, it is therefore likely that the sexual harassment experienced by the mother has an impact on daughters' reproductive success. This result indicates that even if sexual harassment has not effect on lifetime fecundity of the mother, still it may significantly affect her fitness by reducing the reproductive success of her daughters. We can only speculate on the mechanisms linking the level of sexual harassment experienced by the mother with the growth rate of female offspring. One explanation may be that males that are particularly successful in obtaining coercive matings passed onto the progeny genes that, expressed in the daughters, result in a reduced growth rate. Negative correlation between reproductive success of the father and that of the daughters has been reported in the red deer (*Cervus elaphus*, Foerster et al. 2007) and may therefore be the result of sexually antagonistic genes. For example, a negative correlation between the success in coercive mating tactic and body size has been reported in the guppy (Pilastro & Bisazza 1999) and it seems a general phenomenon in poeciliids (Bisazza & Pilastro 1997; Pilastro et al. 1997). Male body size is heritable in the guppy (Brooks & Endler 2001a), but, as far as we know, whether sire body size affects daughter growth rate has not been investigated in the guppy. However, it has to be noted that the level of sexual harassment experienced by the mother did not affect body size of the sons, or at least not to the same extent as that of the daughters. We think, however, that this result does not completely dismiss the possibility that the reduced growth rate observed in the female offspring from the HC group was not influenced by sexually antagonistic genes. The reason is that male body size shows little variation in this population, less variation in males than in females (CV in body size of females = 8.0%, males = 5.5%), and male body size heritability if not significant (**paper II**).

Another, not mutually exclusive explanation is that mother condition was directly affected by sexual harassment and this was reflected in the growth rate of the daughters. Sexual harassment may have imposed a physiological stress on females, for example increasing their level of stress hormones, which are known to be passed into the eggs and affect the growth rate of the progeny in other vertebrates (e.g. Welberg & Seckl 2001; Andersson et al. 2004; Rubolini et al. 2005).

Apart from body size, sexual harassment affected several phenotypic traits associated to sons' reproductive success. First of all, sons of females from the LC group were more attractive than their HC counterparts, although the size of their colour spots on the body was not significantly different between treatments. In contrast we found that gonopodium was significantly longer in LC sons as compared with their HC counterparts. This result is somehow surprising, as long gonopodium is usually associated with coercive matings (Reynolds et al. 1993). Further evidence comes from studies comparing different guppy populations (Kelly et al. 2000) or different poeciliid species (Rosen & Tucker 1961). In addition, a recent work revealed that gonopodium shape is positively associated with successful coercive copulation (**paper IV**). Nothing is known about sire heritability in our guppy population, but if heritability is greater than 0 and possessing a long gonopodium increases a male's chances to obtain coercive copulations, one may expect the opposite pattern of variation in gonopodium length, that is LC males having shorter gonopodium than HC males. However, gonopodium length is likely to be under contrasting selective pressures. The difference in gonopodium length may be driven by female mate choice, as there is evidence that females prefer males with longer gonopodium in guppy (Brooks & Caithness 1995, **paper V**) and also in other poeciliids (Langerhans et al. 2005). It is therefore possible that females favoured males with longer gonopodium in the LC group, although to observe the effect of this choice in the progeny it is necessary to assume that sire heritability for this trait is greater than zero (see above). Alternatively, gonopodium length may be influenced by males' condition during development, as this is likely to be a costly trait subject to natural selection, as it has been shown in the genus *Gambusia* (Langerhans et al. 2005). If mothers exposed to high levels of sexual harassment produced progeny in lower condition (see above), this may have in turn affecting condition-dependent traits in male offspring.

Finally, we found that LC sons were at an advantage in precopulatory sexual selection when compared to their HC counterparts. LC males were preferred, in female mate choice tests, over their HC counterparts. This preference was independent to the size of the ornaments (colour spots) shown by males or on any other phenotypic trait we measured in these males. Furthermore, they were also more successful in obtaining coercive matings, as evidenced by the experiment with sexually unreceptive females. In this same experiment, males from the HC group did not preferentially adopt gonopodial thrusting as mating tactic, suggesting that HC males were not by any mean more 'persistent' than LC males. Finally, sons from the two experimental groups did not differ for any postcopulatory trait, such as number of strippable sperm and sperm velocity.

In conclusion, we found several female fitness costs associated with male sexual harassment, all of which were visible in the offspring upon sexual maturity. Sexual harassment reduced the expected reproductive success of the progeny of both sexes, i.e. body size of daughters and sexual attractiveness and success in gonopodial thrusts of the sons. The costs of sexual harassment in natural conditions are likely to be larger than those we found in our study, as foraging costs and predation-induced mortality are likely to disproportionally increase as male sexual harassment increases (Magurran & Seghers 1994b). These findings are particularly interesting for our understanding of the evolution of female resistance.

Our results allow us to conclude that there is no support for the idea that female resistance evolves through indirect benefits achieved by screening the more persistent males. At least in the guppy, the magnitude of costs of imposed by sexual conflict over mating rate and the evolution of coercive mating tactic in the male does not seem to be compensated by any indirect benefits.

PAPER II
Quantitative genetics of pre- and post-copulatory sexually selected traits in the guppy

Clelia Gasparini, Ryan Dosselli & Andrea Pilastro

Abstract

In sexual conflict studies the 'selective resistance' hypothesis is based on the assumption that female resistance evolves because direct costs are offset by indirect benefits. Females might resist to males in order to screen among them and to select the most persistent male that is in turn more efficient in achieving mating and fertilizing eggs. If these male abilities are heritable, sons of more resistant females will show the same higher-than-average reproductive success. To test this hypothesis and other evolutionary models involving the acquisition of indirect benefits via the 'sexy sons' mechanism, it is crucial to estimate the sire heritability of male traits involved in reproductive success. We used a quantitative genetic approach to measure sire heritability of male traits that are likely to determine the male reproductive success in the guppy (Poecilia reticulata). We estimated heritability of sexual behaviors (courtship and coercive attempts), sperm quality and sperm number and compared the heritability and the coefficient of additive genetic variation of these traits with those of sexually selected male body ornaments. We found that only sperm number shows a significant sire heritability, comparable to the heritability of orange coloration that has been previously demonstrated to be Y-linked. The observed low additive genetic variation in most of the considered traits suggests that the extent of genetic benefits, in terms of increased sons' fertilization success, even if present might be very low. In conclusion, our results do not support the theoretical prediction that female resistance evolves via indirect benefits.

Introduction

Divergence between males and females interests over mating rate and over fertilization success are two of the most common male-female interactions in which sexual conflict emerges (Parker 1979; Parker 2006). This conflict is triggered by the differences in reproductive investment between the sexes that lead to take the advantage of different strategies to maximize lifetime reproductive success. In particular, whereas males has been selected to optimize their fitness by increasing the number of sexual partners, females do not gain as much advantage from this strategy (Bateman 1948). Therefore, opposite evolutionary forces are acting on the two sexes, leading to sexually antagonistic coevolution (Rice & Holland 1997). Indeed, males are selected to evolve traits that allow them to obtain as many copulations as possible, even at the expenses of female fitness. On the other hand, females are predicted to evolve resistance traits against superfluous matings, with the aim of reducing the associated costs (e.g. Daly 1978; Pocklington & Dill 1995; Chapman et al. 1995; Rolff & Siva-Jothy 2002). It has been suggested an alternative scenario in which female resistance evolves to achieve indirect benefits rather than to minimize direct costs by means of the so-called 'sexy sons' mechanism, i.e. the selection of the most persistent male by resistant females would allow to produce offspring with enhanced reproductive success (Pizzari & Snook 2003; Cordero & Eberhard 2003). This hypothesis, also known as ‘selective resistance’ (Eberhard 2002) is based on the assumption that ‘persistence’ traits are heritable between sires and sons. Studying the sire heritability for male traits that are likely to influence reproductive success will therefore provide evidences for the evolution of female resistance via indirect benefits.

We studied the quantitative genetics of sexual behavior and sperm traits in the guppy (*Poecilia reticulata*), a freshwater livebearing fish with YX males, characterized by a polyandrous mating system in which males contribute only with gametes to reproduction (Constantz 1989). Therefore, the guppy is an ideal model to investigate the heritability of male traits involved in reproductive success and to shed light on the evolution of female resistance. The definition of ‘persistent traits’ embodied all those characteristics that enable males to leave as many descendants as possible. In this species they include both precopulatory traits, such as sexual behaviors that could advantage males in mating acquisition, and postcopulatory sperm traits that influence the fertilization outcome. In the guppy a high level of sexual harassment has been reported (Magurran & Seghers 1994b) as a consequence of female promiscuity, of the brevity of sexually receptive phase resulting in male-biased operational sex ratio (Liley 1966; Houde 1997) and of the strong discrimination between sexual partners operated by females (for an extensive review see Houde 1997; Magurran 2005). Indeed, the inter-sexual conflict over mating rate and fertilization success in this species lead to the evolution of a forced mating tactic (gonopodial thrust) that is alternative to courtship (sigmoid display). Gonopodial thrust allows males to inseminate reluctant females by forcibly insert the intromittent organ into the female genital opening (Liley 1966).

We used a half-sib/full-sib design to estimate the sire genetic additive variance of sexual behaviors and sperm traits (sperm velocity and number) in the guppy. We compared the results with the additive genetic variance of body coloration (in particular orange coloration) which is known to be under directional selection through female preference (Bischoff et al. 1985; Kodric-Brown 1985; Houde & Endler 1990). If female resistance evolves and is maintained through the acquisition of indirect benefits for sons, we will expect that the male traits influencing reproductive success in such sexual conflict scenario show a substantial sire heritability.

Materials and Methods

Study population and its maintenance

The guppies used in this experiment were descendents of wild-caught fish collected in 2002 from the Tacarigua River in Trinidad (Trinidad national grid reference: PS 787 804; coordinates: N10° 40.736’ W061° 19.168). This is a high-predation locality where guppies coexist with a variety of predators, including the pike cichlid *Crenicichla alta* (Magurran and Seghers 1994). Laboratory stock and all experimental fish were maintained under controlled temperature and lighting conditions (26°C±1°C; 12:12h light dark cycle provided by Philips TLD 36W fluorescent lamps) and fed twice daily on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplaRin). Virgin females (four months old) were reared for the experiment in single-sex groups under the same temperature, light and food regimes as the stock fish.

Breeding Design

Experimental design consisted of a half-sibs/full-sibs breeding design. Each male (sire) was used to inseminate several females (dams) and data derived from the sons were analyzed. Artificial insemination (see Clark 1950; Lodi 1981; Evans et al. 2003c) was used to inseminate 103 sexually naïve virgin female guppies with sperm collected from 34 males. Artificial insemination makes possible to control for differential maternal effects in relation to male phenotype (Burley 1988; Sheldon 2000), which may inflate sire heritability estimates (Kotiaho et al. 2003). Following the methods in Evans et al. (2003c), sperm were manually stripped from individual males that were previously isolated from females for 3-5 days. We randomly selected mature males from our stock populations. Males were aged between 4 and 6 months and exhibited a range of color phenotypes. We confirmed that each male was fully sexually mature, as evident by the maturational state of the gonopodium apical hood (Houde 1997). Sperm are packaged in bundles (spermatozeugmata), each containing approximately 27,000 individual sperm cells (Billard 1969; Evans et al. 2003c). For each insemination, a virgin female was anaesthetized in a water bath containing a mild dose of MS222 and placed in a polystyrene cradle with her genital pore exposed. A Drummond 3µl micropipette was used to inseminate 20 sperm bundles from each stripped ejaculate (suspended in 10µl of 0.9% NaCl) into the female's gonoduct (penetration depth approximately 2 mm). Immediately after insemination, females were revived in a 5l plastic container (containing conditioned freshwater, gravel, aquatic weed and an airstone) where they remained isolated until they produced their first broods. The same procedure was followed for the second female inseminated with the identical amount of sperm bundles collected from the same male. Within 12 h from birth, the offspring were isolated from the mother and kept in 5l containers until they could be sexed. Each container was equipped with gravel, natural and artificial weed, and an airstone. After sexing, male offspring from each brood were individually placed in 1l containers and maintained there until used for experiments. A total of 238 adult male offspring from 33 sires and 66 dam were examined in subsequent analysis (see below).

Measurement of sons' behavior

Behavioral tests were conducted on sons in four different replications. These trials were performed in sons at the age of 93, 100, 136 and 142 days. We recorded frequency of sigmoid displays (courtships) and gonopodial thrusts (forced mating attempts) (for description of behaviors see Houde 1997; Head & Brooks 2006). Trials were performed with non receptive females that were allowed to settle from the night before, while males were placed in experimental tank and left to acclimatize for 15 minutes before started the trials. After observation period (10 minutes) males were gently moved back to their original container.

Measurement of sons' sperm quality traits

Out of the 238 males tested in behavioral trials, we conducted sperm assays on 187 males available at seven months of age. To collect sperm each male was placed on a Petri dish under a low-power dissection microscope. The gonopodium was swung forward and gentle pressure was applied to the side of the abdomen, just anterior to the base of the gonopodium, to release the sperm bundles. Immediately after ejaculate collection, a sperm bundle one at a time was taken with a micropipette along with three µl of distilled water. Each bundle was individually placed on a slide, previously sprayed with rain coat (silicone spray, CRC Industries) to reduce adherence of sperm to the glass, and covered with a glass slide. Once the bundle was set, 10 µl of activating solution (250 mM of KCL and 4 mg/ml bovine serum albumin) was added, and gently covered with a cover slip. Sperm was analyzed using a Ceros Sperm Tracker (version 12.3; Hamilton Thorne Research, Beverly, MA, USA). Sperm velocity were measured on motile sperm (mean number of sperm: 260.97 ± 110.14 SD, range: 62-675) from two different bundles for each male and the average was used in the analysis. These measures include average path velocity (VAP); straight line velocity (VSL) and curvilinear velocity (VCL). These three measures provide an estimate of progressive velocity and have been shown to be the best predictor of fertilization success in different taxa (e.g. (Birkhead et al. 1999; Levitan 2000; Gage et al. 2004) and in the guppy (Boschetto C., Gasparini C. and A. Pilastro, in prep.). The threshold values defining static cells were predetermined at 25 µm/s for VAP and 20 µm/s for VSL.

Measurement of sons' sperm quantity

Estimation of sons' sperm quantity were performed following (Pilastro et al. 2004; Pilastro et al. 2008) Total number of sperm per ejaculate was estimated in 177 males. Free sperm solution was obtained by vortex all sperm bundles for 1 minute and the total number of sperm was counted with an 'improved Neubauer chamber' haematocytometer.

Measurement of sons' phenotypic pattern

To measure pigmentation, male offspring were anaesthetized in a water bath containing MS222 and photographed using a digital camera (Nikon Coolpix 4300) and a reference ruler. Image analysis software (Image Tool: http://ddsdx.uthscsa.edu/dig/download.html) was used to estimate the body area of each male (including caudal fin but excluding dorsal fin) as well as the surface area of orange, yellow and red spots (hereafter 'orange') black spots (excluding fuzzy black lines), and iridescent spots (combined measures of blue, green, purple and silver; for details see (Pilastro et al. 2004; Evans et al. 2004a). We also measured the standard length (distance from the snout to the end of the tail, SL).

Statistical analysis

We obtained data from sons of 33 sires and 66 dams (see above). The half-sibs/full-sibs breeding design permits the estimation of additive genetic variance (V_A) that are required for the calculation of heritability (h^2). Considering that the species is viviparous and several offspring are produced in the same brood, dam heritability estimates are likely to be inflated by dominance, maternal effects, and common gestational and early life environment, therefore we did not include dam heritability in this study. However, the lack of paternal contribution apart from sperm and the use of artificial insemination (which prevents differential maternal allocation) should provide an unbiased estimate of sire heritability, which is the aim of this study. Due to mortality of some of the males analyzed in behavior trials we obtained sperm (for velocity and quantity analysis) only for a subset of the sons (187 for velocity and 177 for quantity). For these reasons we have different sample sizes for different variables. Data derived from the offspring were analyzed as a nested analysis of variance with dams nested within sires.

We estimated coefficient of additive genetic variance and heritabilities following the methods described in (Lynch & Walsh 1998) : sire heritability (h^2) as four times the ratio of sire variance component to total phenotypic variance observed and coefficients of additive variation /CV_A) were calculated as 100 √Va /X , where Va equals additive genetic (VA) and X the mean of given trait (Houle 1992). Statistical analyses were performed using SPSS 14.0

Results

As shown in table 1 we found highly significant sire additive genetic variance for coloration (percentage of orange and iridescent). Color traits showed sire heritability values above 1, suggesting Y-linkage (Houde 1992). For behavioral traits (see table 2), although both number of displays and gonopodial thrusts shown significant repeatability (R=0.284 ± 0.07, P<0.001 and R=0.186 ± 0.05, P=0.007 respectively; repeatability estimated following Becker (1984), there was no evidence of sire heritability. Among sperm traits, only sperm number showed significant sire heritability while different measures of sperm velocity did not shown sire heritability (table 2).

trait	mean	SD	df	F	*P*	CV_A	h^2
% orange	13.34	4.11	32	4.377	<0.001	46.08	1.921
% black	2.77	0.93	32	1.295	0.236	15.12	0.201
% iridescent	4.30	2.48	32	3.732	<0.001	69.92	1.440
Standard length	17.91	1.12	32	1.203	0.306	2.55	0.165

Table 1. Coefficients of additive genetic variance (CV_A) and corresponding heritabilities (h^2) for somatic traits

trait	Mean	SD	df	F	*P*	CV_A	h^2
Courtship displays	6.70	3.76	32	1.264	0.252	21.85	0.151
Gonopodial thrusts	14.12	8.98	32	1.125	0.369	26.44	0.170
Sperm velocity (VAP)	104.24	17.99	32	1.396	0.178	10.08	0.342
Sperm velocity (VSL)	97.60	17.88	32	1.458	0.147	10.49	0.327
Sperm velocity (VCL)	128.36	16.68	32	1.698	0.069	11.26	0.336
Sperm number ($x10^6$)	13.52	8.43	32	3.321	<0.001	66.37	1.151

Table 2. Coefficients of additive genetic variance (CV_A) and corresponding heritabilities (h^2) for behavioral and sperm traits (behavioral data were measured over 10-min observation period, mean of four repetitions)

Discussion

Our results confirm previous quantitative genetic studies on male ornaments in guppies (size of color spots), which nearly invariably show heritability estimates greater than 1 (Houde 1992; Brooks 2000; Brooks & Endler 2001a), suggesting Y-linked inheritance (Angus 1989).

It has been suggested that high predation populations color pattern expression has mainly Y-linked inheritance (Haskins et al. 1961). Consistently with pattern, the fish used in this experiment are derived from a high predation population and showed heritabilities >1 for color pattern. The size of body orange spots is subject, in this population, to directional pre (Evans et al. 2004a) and postcopulatory sexual selection (Evans et al. 2003c; Pilastro et al. 2004). Despite very high heritability of orange spots, which should allow directional selection to rapidly erode additive genetic variance, we found substantial sire additive genetic variance for the area of the spots of orange and iridescent. This result indirectly support the importance of other non directional selection pressures, such as negative frequency-dependent survival (Olendorf et al. 2006), complex female preference functions (Brooks & Endler 2001b) and male fitness surface (Blows et al. 2003), and female preference for unfamiliar males (Hughes et al. 1999).

Reproductive behaviour, gonopodial thrusts and courtship displays, did not show significant sire heritability, even our analysis revealed significant repeatability within male. The results agree with previous studied in which has been demonstrated that behavioural patterns show low heritability compared to physiological and morphological traits (Mousseau & Roff 1987; Moore 1990; Lynch 1994). Our results have different possible, not mutually exclusive, explanations. First, although our experimental design was planned to minimize the extent of environmental components, we cannot rule out the possibility that environmental noises could have affect heritability estimation. More important, behavioural patterns have often been reported to have low additive genetic variance. Explanation for the low heritabilities could rest on genetic architecture at the bases of complex behaviours that could reflect genetic non additive effects of dominance or epistasis. Behaviours are strongly subjected to environmental influences. When individuals evolved to live in a unpredictable environment it is unlikely that a single phenotype confer the highest fitness in all the situations, and therefore phenotypic plasticity is one possible adaptive solution to heterogeneous environments (Via et al. 1995). In the guppy the use and the relative success of the two alternative tactics are strongly dependent on a number of biotic (female receptivity, presence of rival competitors, operational sex ratio) and abiotic (light, visibility, water flow) variables (reviewed in Houde 1997; Magurran 2005). Indeed, as discussed for ornamentations, there are time and space fluctuations in the success of males that could make a phenotype more successful in one situation than in one other, and in consequence, the outcome of displays or gonopodial thrusts could be different under different circumstances. Plasticity in the use of alternative tactics is therefore likely to be more favoured than a fixed behaviour in species like the guppy.

Results for sperm velocity traits suggests that the evolvability (sensu Houle 1992) of these traits is very low. The very low sire additive genetic variance in sperm quality contrasts with quantitative genetic studies on sperm traits conducted in other vertebrates and in invertebrates showing that sperm traits can respond to selection (Woolley 1971; Morrow & Gage 2001; Konior et al. 2005). There may be different explanations for the lack of evolvability of sperm velocity observed in the guppy. One possibility is that the species studied so far are characterized by a relatively lower level of sperm competition (e.g. Birkhead et al. 2005), compared to guppies (and in poeciliids in general). Indeed, female guppies mate with multiple partners during the sexually receptive phase (Liley 1966; Evans & Magurran 2000; Pitcher et al. 2003), receive several forced copulations during the unreceptive phase (Magurran & Seghers 1994a; Pilastro & Bisazza 1999; Evans et al. 2003b), and store sperm for months (Winge 1937; Constantz 1989). As a results, most broods are sired by multiple males (Winge 1937; Kelly et al. 1999; Evans & Magurran 2000; Pitcher et al. 2003; Becher & Magurran 2004). The intense directional selection may have eroded the genetic variance underlying sperm traits associated with sperm competition success, such as sperm velocity. Another, not mutually exclusive explanation is that the adaptive evolution of sperm design may be genetically less constrained in XY species (such as guppy and mammals, Roldan & Gomendio 1999) as compared to ZW species (for example birds and butterflies), in which exclusive paternal inheritance is prevented. Data from more species with different chromosomal sex determination mechanism and different level of sperm competition may allow to discriminate between these two possibilities. Finally, the observed low coefficients of additive genetic variation may be due to a secondary loss of genetic variability in our captive guppy population. Although this possibility cannot be completely ruled out, it seems unlikely for two reasons. First, the genetic variation observed at neutral microsatellite loci in stock from which the individuals used in this experiment were taken (A. Pilastro et al., unpublished results) was not lower than that observed in the founders of this captive population (Evans et al. 2003c). Second, the additive genetic variation observed for body ornaments in this population was comparable to that reported in other studies (e.g. Hughes et al. 2005). Thus it seems unlikely that genetic variance has been reduced by stochastic loss only at the loci controlling sperm quality.

Despite the very low coefficients of additive genetic variance we found for sperm velocity, that one for sperm number was high and comparable to value obtained for orange coloration. The high sire heritability for sperm number it is a not unexpected result. It has been suggested that traits related to sperm production (testes size, ejaculate volume and sperm number) may have relatively higher levels of evolvability than sperm morphology and performance. Our result is similar to what found by Moore and colleagues (2004), in which they found high heritability (and corresponding CV_A) for the number of sperm but not for sperm quality (viability) in the cockroach *Nauphoeta cinerea.* In several species sperm competitiveness may be influenced by the number of sperm a male is able to inseminate besides differences in sperm quality (Snook 2005). Indeed, in other species it has been shown that sperm numbers can be an important determinant of sperm competition success (Martin et al. 1974; Petersen et al. 2001; Neff et al. 2003; Stoltz & Neff 2006), that sperm production responds to selection (Hosken et al. 2001) and shows significant additive genetic variation (Handelsman 1997; Simmons & Kotiaho 2002; Moore et al. 2004). One may therefore argue that sires with the highest sperm production, and not with highest sperm quality, are favored, at least in a competitive scenario. Anyway, in this species it seems improbably that sperm production (in term of sperm number) is an important trait in determining fertilization success. In fact, it has been shown that female guppies actively limit, during cooperative copulations with unattractive males, the number of sperm transferred (Pilastro et al. 2004), by reducing the duration of the copulation (Pilastro et al. 2007). Moreover, the number of sperm transferred during forced copulations are much lower (about 50×103 sperm, Pilastro & Bisazza 1999) compared to the daily sperm production

(750 x10^3 sperm, Billard 1986) and therefore it is improbable for males to be sperm depleted. Although the number of sperm transferred could be important in a sperm competition scenario (Boschetto C., Gasparini C. and Pilastro A. in prep.), the sperm production it is unlikely to limit male fertilization success. This suggests that sperm production is not under a strong selective pressure and that genetic variance is not eroded by strong directional selection.

In conclusion, in this study we showed that the guppy males' traits which potentially influence mating acquisition and fertilization success are not associated with significant sire heritability, except for sperm number and body ornaments. Low sire heritability of sexual behaviors corroborate previous work that showed as behavioral patterns are usually scarcely heritable (Mousseau & Roff 1987; but see Lemon 1993). In spite of the high sire heritability for sperm number found in this study, it has been though that in the guppy sperm production is not under strong selection because do not influence a male reproductive success, due to the fact that the number of sperm inseminated in a given mating is probably more under female control and sperm depletion is unlikely to occurs. The very low sire additive genetic variance observed in sperm quality suggests that when sperm competition is intense, as in the guppy, the additive genetic variation in sperm quality traits may be rapidly eroded, reducing the potential for further selection. Therefore, our study does not support the 'selective resistance' hypothesis that suggests the evolution and maintenance of female's resistance via indirect benefits to produce successful sons.

PAPER III
Female presence affects sperm velocity in the guppy

Clelia Gasparini, Alfredo Peretti And Andrea Pilastro

Abstract

As sperm production is costly, males are expected to evolve strategy to strategically adjust their allocation to sperm production in response to mating opportunities, in order to minimise such costs. Indeed, adjustment in sperm number has been reported in several taxa, but only few study investigated whether sperm quality can show adaptive phenotypic plasticity. We tested this prediction in the guppy, *Poecilia reticulata*, a species in which males are known to allocate their sperm production in response to mating opportunities. Two groups of males were maintained isolated for three days either in visual contact with three females or not and in vitro sperm assays were performed after treatment. We found that males that were maintained in visual contact with females produced significantly faster sperm than their control counterpart. Sperm velocity is an important factor determining fertilization success and therefore is likely to be crucial for males' fitness and in the evolution of ejaculate traits. This result indicates a phenotypic plasticity in sperm velocity in response to variable cues, and that exposure to females has to be considered in experimental procedures.

Introduction

Reproductive effort involves all the investments necessary to offspring production, first of all the energetic investment in gamete production. Unitary cost of sperm production is usually smaller compared to that necessary to produce eggs (see Bjork & Pitnick 2006 for an exception) and this difference drives the often divergent strategies to maximize reproductive output in males and females (Trivers 1972). On the basis of the low unitary cost of producing sperm, it has long been assumed that sperm are cheap and males are rarely, if ever, sperm limited. In contrast to this view, there is now ample evidence that sperm production is costly (Dewsbury 1982; Nakatsuru & Kramer 1982; Shapiro et al. 1994; Olsson et al. 1997) and that sperm production is strategically adjusted according to mating opportunities and hence expected sperm utilization (e.g. Shapiro et al. 1994; Aspbury & Gabor 2004; Scharer et al. 2004; Pyter et al. 2005; Bjork et al. 2007).

Although the costs of sperm production are certainly influenced by the number of sperm produced, ejaculates vary not only in their numerical composition, but also in their quality. Recently, it has been recognized that not only numbers are involved in determining fertilization success, but also sperm quality, such as viability and velocity, has a great impact on male fitness (Snook 2005) and has been found to influence fertilization success in invertebrates (Levitan 2000; Kupriyanova & Havenhand 2002; Garcia-Gonzalez & Simmons 2005), fish (Vladic et al. 2002; Gage et al. 2004; Casselman et al. 2006) birds (Birkhead et al. 1999) and mammals (Malo et al. 2005). In particular elevated sperm velocity is associated with a higher fertilization success both in presence (Froman et al. 1999; Levitan 2000; Kupriyanova & Havenhand 2002) and in absence (Birkhead et al. 1999; Gage et al. 2004; Denk et al. 2005) of sperm competition. Although experimental estimates of the cost of producing faster sperm are, to our knowledge, still lacking, producing sperm with enhanced swimming performances is likely to be costly. For example, higher sperm speed (or sperm motility) is associated with higher sperm ATP content (Froman & Feltmann 1998; Lahnsteiner et al. 1998; Burness et al. 2004; Locatello et al. 2007). While there is evidence that sperm velocity and viability are adjusted to the perceived level of sperm competition (Kilgallon & Simmons 2005; Rudolfsen et al. 2006; Simmons et al. 2007), whether or not males adjust their sperm quality in response to mating opportunities has never been investigated so far.

We tested the hypothesis that males vary their sperm velocity in response to the predicted mating opportunities in the guppy (*Poecilia reticulata*), a small freshwater fish with internal fertilization. In this species it has been demonstrated that males that were maintained subject to visual and possibly chemical contact with a female exhibited larger amount of strippable sperm when compared to isolated males (Bozynski & Liley 2003). This result suggests that male guppies adjust their sperm production to mating opportunities as a mean to save energy when no mates are available. To test whether male guppies can also adjust the quality of their sperm in response to presence or absence of females during treatment period we compared one trait of sperm quality, in vitro sperm swimming velocity, in males that were kept in visual contact with females with that of males isolated from females. If increasing sperm quality is costly and males possess the physiological basis to adjust sperm quality we may expect that males in presence of females will produce faster sperm than in their absence. We have chosen to measure sperm velocity because it is the main determinant of fertilisation success in other vertebrates (see citations above) and we have evidence that it is positively correlated with sperm competition success in the guppy (Chiara Boschetto, Clelia Gasparini and Andrea Pilastro, in prep.). In contrast, sperm viability (proportion of motile sperm), sometime used to correlated with fertilisation success (e.g. Garcia-Gonzalez & Simmons 2005), is virtually 100% in guppies (Locatello et al. 2006).

Materials and Methods

The study populations and its maintenance

We used guppies descendents of wild-caught fish from the lower part of Tacarigua River, Trinidad. Fish were maintained at the temperature regime of 26 ± 1° and under a 12 h light/dark cycle. All fish were fed a mixed diet of brine shrimp nauplii and commercially prepared flake food. Males were maintained in stock aquaria with roughly 1:1 sex ratio. Virgin females were reared in dedicate tanks visually and physically isolated from males since the moment they were sexable (on average after about 4 weeks).

Treatment

Experimental males were randomly chosen from stock population. Males were isolated from females for 3 days and stripped in order to completely remove sperm reserves before starting the treatment. Stripping procedure followed that described in Evans et al. (2003c): the male was anesthetized in a bath containing Tricaine Methanesulfonate (MS222) and then placed on a dark slide under a stereomicroscope. The gonopodium (male's intromittent copulatory organ) was gently swung forward three or four times to facilitate the release of the strippable sperm (that in this species are packaged in sperm bundles termed spermatozeugmata) following a soft pressure of abdomen which was applied just anterior to the gonopodium's base. We pressed each male three times to extrude all mature sperm bundles. After stripping the male was revived in a contenitor with fresh water. We allowed male to rest 24 hours before starting the treatment that consisted in the presence or absence of a visual stimulus represented by three females. To do so, an individual male was placed in a small transparent tank (1l), right next to an identical container which either contained three virgin females selected from dedicate tanks (exposed males) or was empty (isolated males). After three days, males were stripped as above and sperm were collected for subsequent analysis (see below). Then male was photographed with a digital camera (Nikon Coolpix 4300) and body size and coloration were analyzed with image software (UTHSCSA Image Tool). We measured body size (SL) and the area of colour spots, dividing colour spots into three categories: orange and yellow (hereafter orange coloration), black (melanistic coloration) and blue and green (iridescent coloration). The area of each spot was expressed as a percentage of total body area.

Sperm velocity assay

Immediately after ejaculate collection, a sperm bundle one at a time was taken with a micropipette along with three μl of distilled water. Each bundle was individually placed on a slide, previously sprayed with rain coat (silicone spray, CRC Industries) to reduce adherence of sperm to the glass, and covered with a glass slide. Once the bundle was set, 10 μl of activating solution (250 mM of KCL and 4 mg/ml bovine serum albumin) was added, and gently covered with a cover slip. Sperm was analysed using a Ceros Sperm Tracker (version 12.3; Hamilton Thorne Research, Beverly, MA, USA). Sperm velocity were measured on motile sperm (mean number of sperm: 178.80 ± 13.90) from two different bundles for each male and the average was used in the analysis. These measures include average path velocity (VAP); straight line velocity (VSL) and curvilinear velocity (VCL). These three measures provide an estimate of progressive velocity and have been shown to be the best predictor of fertilization success in different taxa (e.g. Birkhead et al. 1999; Levitan 2000; Gage et al. 2004) and in the guppy (Chiara Boschetto, Clelia Gasparini and Andrea Pilastro, in prep.). The threshold values defining static cells were predetermined at 25 μm/s for VAP and 20 μm/s for VSL. The within-male repeatability (between the two bundles) estimated following Becker (1984) was: 0.70 ± 0.08 SE ($P<0.001$) for VAP, 0.71 ± 0.08 SE ($P<0.001$) for VSL and 0.58 ± 0.11 SE ($P<0.001$) for VCL, repeatability values are comparable to analogous studies of repeatability of sperm velocity within same ejaculate (Denk et al. 2005; Rudolfsen et al. 2005; Locatello et al. 2006; Locatello et al. 2008)

Statistical Analysis

Statistical analyses were performed using SPSS v 16.0; colour percentages on body area were arcsine square root transformed prior to analysis. The three main parameters estimated for sperm swimming velocity were highly correlate (all $P<0.001$), therefore we showed results only for VAP.

Results

The average of sperm velocity (mean ± SE) was 115.08 ± 2.75 μm/sec. Males produced significantly faster sperm in the presence of females than in isolation (with females: 121.82 ± 3.35 n=23; no females: 108.35 ± 3.94, n=23; $F_{1,45}=6.784$; $P=0.012$; fig 1). Males that were randomly assigned to the two experimental groups, did not differ in any of morphological traits we considered, as body size and coloration (all differences between treatments, P>0.249). Sperm velocity remained significantly different between groups also after controlling for different male traits (ANCOVA, treatment: $F_{1,43}$=6.267; P=0.017; covariates: body size $F_{1,43}$=1.591; P=0.215, orange: $F_{1,43}$=0.929; P=0.341, black: $F_{1,43}$=0.493; P=0.487, iridescent: $F_{1,43}$=1.315; P=0.259).

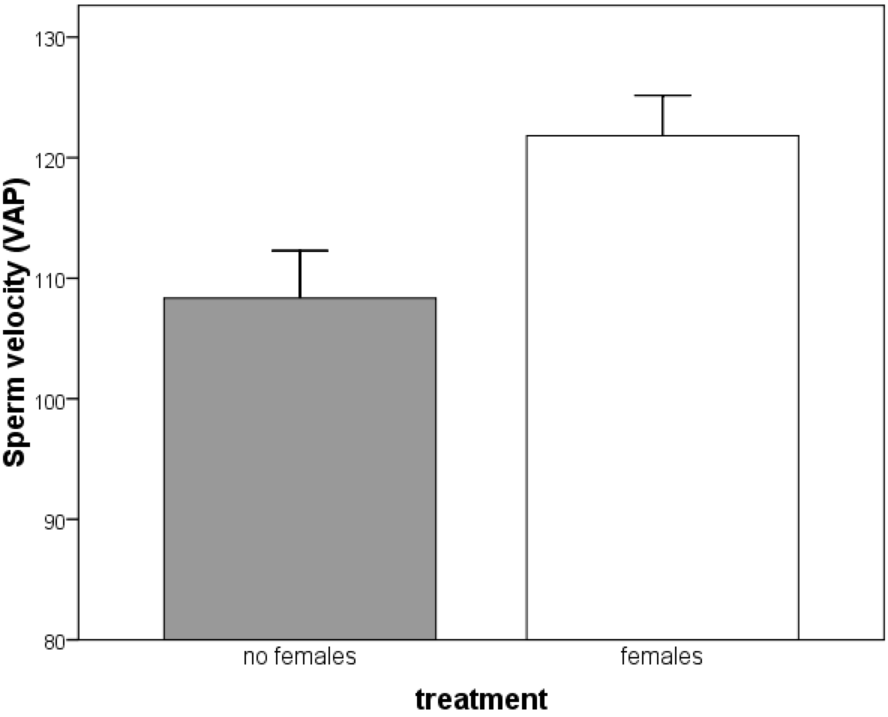

Fig. 1 Sperm swimming speed (mean ± SE) for males in the two treatments.

Discussion

Our results demonstrate that male guppies do not only vary the amount of strippable sperm quality in response to the availability of potential mates (Bozynski & Liley 2003), but also reduce the velocity of their sperm after three days of visual isolation from females. Adjustment of sperm production to mating opportunities has been found in other poeciliids (Aspbury & Gabor 2004), but this is the first study, to our knowledge, demonstrating that males can modulate their sperm's velocity. Our results, associated with those obtained by Bozynski and Liley (2003) suggest that male guppies reduce the number and the quality of their strippable sperm when isolated from females. Strategical adjustment of sperm velocity has been documented in other species in response to different levels of sperm competition (Kilgallon & Simmons 2005; Rudolfsen et al. 2006; Simmons et al. 2007). Our results demonstrated that this capability have evolved also in response to the presence of potential mates.

Interestingly, we also found that the visual stimulus represented by the presence/absence of females is sufficient to trigger a change in sperm quality in the guppy. This suggests that male's response is elicited without any chemical information regarding the reproductive condition (receptive/unreceptive) of the female. This is not surprising, and can have two not mutually exclusive explanations. First, females become sexually receptive soon after parturition (Houde 1997) and parturition may not be predictable for males, unless, probably, when females are at the very end of their gestation. It has to be noted, however, that we used virgin female as stimulus and virgin females, obviously, do not have a large belly. Virgin females are sexually responsive and it is possible that male are able to detect female sexual responsiveness based on their behaviour (e.g. through female "gliding" response, Liley 1966). It is worth noting that male sexual behaviour is influenced by subtle (i.e. not perceivable to human eye) differences in female behaviour (see for example Evans et al. 2002). It may be interesting to test if females with large belly and anal spot (i.e. visually close to parturition) elicit a stronger sperm priming response as compared to females. Second, it may pay for males to increase their investment in sperm production also in the presence of unreceptive females if sneaky copulations are expected to result in paternity. Whether or not this happens in guppies is debated, but it is at least known that forced copulations can result in large sperm transfer (Pilastro & Bisazza 1999), and may therefore represent an important contribution to male fitness.

Females produce relatively few eggs during each reproductive cycle and female fecundity (brood size) is not limited for ejaculate size as low as two bundles (about 50×10^3 sperm) (Pilastro et al. 2008). Considering that males have been estimated to produce about 750 $x10^3$ sperm per day (Billard 1986), males are in principle capable to fertilise several females. Furthermore, although admittedly there is no information about the effect of sperm velocity on fertilisation success, viability at stripping is virtually 100% in the guppy (Locatello et al. 2006) and seems unlikely that sperm velocity limits fertilisation rate. Thus, one may wonder why male guppies respond to the perceived level of mating opportunities by increasing the velocity of their sperm. One possible explanation is that, in the guppy, sperm competition is never absent. Indeed, multiple paternity is the rule in this species, with an average of 3 sires per brood and a proportion of multiply sired broods about 95% (Hain & Neff 2007). Therefore, it seems likely that female presence in the guppy is always associated with sperm competition situations, triggering strategy to enhance sperm competitiveness

An important corollary of our work concerns the consequences of experimental and isolation condition of males. It is indeed crucial to ensure that experimental males that has to be isolated (e.g. for sperm replenishment) would be not only physically keep out from females, but also have the same possibility to observe, or not, females. Therefore with our current work we demonstrated that males isolate in visual contact with females not only produce more sperm, as previously reported (Bozynski & Liley 2003), but also of better quality, in term of swimming speed. In conclusion visual stimulus of females has to be considered when discussing results from experiments with different isolation conditions, even if for brief periods. Future researches will be address to investigate if the adjustment of sperm quality includes other sperm traits (e.g. viability, longevity or morphology) and is affected also by other variables as the receptivity stage of females, the value of potential competitors or the risk or intensity of sperm competition.

PAPER IV
The influence of female population origin and male genital morphology on the success of forced copulations in Trinidadian guppies

Jonathan P. Evans, Clelia Gasparini, Indar W. Ramnarine, and Andrea Pilastro

Abstract

An increasing body of work has implicated sexual conflict in fuelling the evolution of male and female reproductive traits, potentially resulting in sexually antagonistic selection for adaptations in one sex and counter-adaptations in the other. Where traits shaped by sexual conflict diverge among populations, theory predicts that females will be more resistant to males from their own population, with whom they have coevolved, than hetero-population males. We test this prediction using the guppy *Poecilia reticulata*, a livebearing freshwater fish where males use a combination of courtship and forced mating to achieve copulations. In natural populations, the level of predation is associated with the prevalence of forced matings, which in turn results in greater mating costs endured by females inhabiting high-predation populations. Our recent comparative work has revealed highly significant divergence (and covariance) in male and female genital traits that reflect these differences in mating tactics and associated costs. Here we show that males from a focal high-predation population were more likely to transfer sperm during forced mating attempts with females from a low-predation population, compared to when they encountered females from their own, or a different high-predation (control) population. We also report a significant association between male genital shape and the success of forced matings, drawing an explicit link between male genital shape and the outcome of these antagonistic mating interactions. These findings, in conjunction with our previous comparative work, lead us to speculate that selection for male genital traits that increase the efficacy of forced matings will be balanced by counter-adaptations in females.

Introduction

Male copulatory structures are among the most rapidly evolving morphological traits in animals with internal insemination (Eberhard 1985). In many cases, such modifications exhibit far greater levels of structural complexity than is thought necessary for transferring sperm to females. Evidence that sexual selection is responsible for such diversity is now widespread (Eberhard 1985; Sirot et al. 2003; Hosken & Stockley 2004), coming from studies linking variation in male genital morphology to mating success (Brooks & Caithness 1995; Preziosi & Fairbairn 1996; Langerhans et al. 2005; Bertin & Fairbairn 2005) and to relative fertilization success (Arnqvist & Danielsson 1999; Danielsson & Askenmo 1999; House & Simmons 2005; Wenninger & Averill 2006). Evidence for sexual selection on genitalia also comes from comparative studies revealing evolutionary divergence in male genital shape among populations or strains that differ in the intensity of sexual selection acting on male genital traits (Arnqvist 1998; Ramm 2007).

An increasing body of work has focused on the potential for sexual conflict to fuel the evolution of animal genitalia. Sexual conflict occurs whenever the evolutionary interests of males and females differ at mating, and is expected to promote sexually antagonistic cycles of selection on male ('persistence') and female ('resistance') traits (Parker 1979; Rice & Holland 1997; Chapman et al. 2003; Arnqvist & Rowe 2005). Accordingly, several comparative studies have revealed correlated patterns of divergence in male genital shape and female reproductive traits (Arnqvist & Rowe 2002a; Arnqvist & Rowe 2002b; Huber 2003; Cordoba-Aguilar 2005; Ronn et al. 2007; Brennan et al. 2007). While such coevolutionary patterns are consistent with a history of sexually antagonistic selection, it is often difficult to distinguish this process from other (e.g. Fisherian) models of preference-trait coevolution (e.g. Cordero & Eberhard 2003). As a result, verbal models that aim to distinguish sexually antagonistic coevolution (SAC) from other forms of sexual selection have been proposed (Holland & Rice 1998; Parker & Partridge 1998). One basic prediction arising from these models is that where reproductive traits that are shaped by sexual conflict diverge among populations, females will be more resistant to males from their own population, with whom they have coevolved, than those from alien populations against whom they lack adaptations to resist superfluous matings (e.g. see Holland & Rice 1998). Empirical support for this basic prediction comes from experiments on animals and plants that have crossed genetically differentiated strains or populations and revealed interacting effects of population (or strain) on female mating rates or relative fertilization success (Clark et al. 1999; Andres & Arnqvist 2001; Knowles & Markow 2001; Hosken et al. 2002; Nilsson et al. 2002; Jolivet & Bernasconi 2007).

In this paper we use the guppy (*Poecilia reticulata*) to provide further experimental evidence that female population origin can influence male mating success. We also test for an association between male genital shape and insemination success, as we suspect that male genital morphology is the target of antagonistic selection in this species (see below). Guppies are livebearing poeciliid fish with internal fertilization and a promiscuous mating system (Houde 1997; Magurran 2005).

Males use both courtship and forced matings to obtain copulations, inserting the tip of their gonopodium (intromittent organ) into the female's gonopore to transfer sperm (Rosen & Gordon 1953). Although males use both mating strategies interchangeably, the extent to which they employ either tactic can vary according to the level of predation (Endler 1995). Under elevated predation risk, males tend to switch from highly visual courtship displays to less conspicuous forced matings (Godin 1995), probably because forced matings are more profitable when females are preoccupied with predator evasion (Evans et al. 2002). Consequently, females from high-predation localities typically endure relatively high levels of sexual harassment (and associated costs) compared to their low-predation counterparts (Magurran & Seghers 1994a; Magurran & Seghers 1994b). Our recent comparative work on ten Trinidadian populations has shown that the shape of the distal tip of the male's gonopodium varies predictably among populations according to the level of predation intensity (**paper VI**). These concordant patterns of sexual harassment, predation intensity and correlated patterns of divergence in both sexes lead us to predict that these patterns of variation in male genital shape reflect a history of SAC. In this paper we provide preliminary support for this prediction by documenting population differences in the male's ability to transfer sperm to females during forced (unsolicited) matings, and an association between the success of forced matings and male genital shape.

Our study had two broad aims. First, we determined whether males originating from a focal high-predation population exhibited higher insemination success during forced matings when paired with females from a population characterised by relatively low levels of predation (and therefore relatively low levels of sexual harassment). We compared the success of forced matings by focal males when paired with females originating from three natural populations: (i) ‘native high predation’ - the same high-predation population of focal males, (ii) ‘alien low-predation’ - a low-predation site within the same river, and (iii) ‘control high-predation’ - a high-predation population from a different river. Our expectation was that males from the focal high-predation population would be more successful at transferring sperm through forced matings when paired with low-predation females, compared to the other cases. This prediction is based on two assumptions. First, we assume that any female counter-adaptations (either behavioural or morphological) that thwart unsolicited male sexual advances will be better developed in high-predation populations, where this mating tactic is most common (Endler 1995). Second, we assume that females at the control high-predation site will have evolved the same degree of resistance to forced matings as those from the focal males’ native population (for discussion and criticisms of this assumption, see Pizzari & Snook 2003).

Our second aim was to establish whether variation in male genital shape in the focal population was associated with mating success through forced copulations. We used geometric morphometric techniques to characterize the shape of the gonopodium in males from the focal population and related these traits to the efficacy of sperm transfer during forced matings. We predicted that variation in the shape of the distal tip of the gonopodium would influence sperm transfer during forced matings because this is the portion of male’s genitalia that physically interacts with the female’s genital pore during copulations (Rosen & Gordon 1953; Clark et al. 1954). This prediction has already received some support from recent work revealing that the experimental removal of the paired hooks at the gonopodium distal tip impairs sperm transfer during forced matings (Cheng 2004). We therefore also characterized variation in hook shape and related this parameter to the success of forced matings. This study therefore asks whether intra-population variation in male genital traits can explain variation in the success of forced matings, thus offering a functional explanation for why these traits differ predictably among populations (**paper VI**).

Materials and Methods

Populations

Guppies were collected within a seven-day period in April 2005 from populations located in the southern flowing streams of the Oropouche drainage in Trinidad’s Northern Mountain Range. Our focal male population (total used in the experiment: *n*=108) came from the Lower Quaré River (N10° 40.418’ W061° 11.833’), a high-predation locality in which guppies coexist with numerous piscivorous predators. The experimental females came from three different populations: (1) the same high-predation (Lower Quaré) site from which focal males were collected (hereafter ‘native high-predation’, *n*=36); (2) the low-predation Upper Quaré site (N10° 40.553’ W061° 11.792’), located upstream from the focal male population (‘alien low-predation’, *n*=36); and (3) the high-predation Lower Turure (N10° 39.394’ W061° 10.059’), a site situated in a different river within the same drainage (‘control high-predation‘, *n*=36). The predation status of these three streams has been characterized in detail elsewhere (Magurran & Seghers 1991; Magurran et al. 1992; Endler & Houde 1995; Godin 1995; Evans et al. 2003b).

Mating trials

Following the field collections, all fish were returned the University of the West Indies (St Augustine, Trinidad & Tobago). Gravid females from each of the three populations (Lower Quaré, Upper Quaré and Lower Turure) were individually isolated in 5L containers (containing river weed and an airstone) until they produced their first brood. Offspring were immediately removed and each post-partum female was then maintained individually within the same container for a further 14 days before taking part in the mating trials. This ensured that all females (irrespective of population or the exact timing of collection) were at the same reproductive stage (approx. mid-way through their ca. monthly cycle) and sexually unreceptive (post-partum females are only sexually receptive to male courtship for 2-3 days after producing a brood, Houde 1997). This also allowed us to consider male mating success through forced copulations, because the use of unreceptive females excluded cooperative matings (Houde 1997). Males were maintained in mixed-sex groups until 3 days before the mating trials, at which point they were isolated from females to ensure that they had replenished their sperm stores prior to entering the mating trials (Kuckuck & Greven 1997). All fish were fed commercial flake food during the holding phase, and maintained at ambient light and temperature.

On the evening before each trial, females were placed individually in a 45 x 30 x 30 cm observation tank (filled to 20cm) and allowed to settle overnight. Observation tanks contained a small amount of river weed and natural stream gravel. On the following morning, a male was placed in the tank and left for 2h to interact with the female. We then observed the male's sexual behaviour for the following 15 min. During these observations, we recorded the number of 'sigmoid' displays (courtship) and gonopodial thrust attempts (no prior courtship, male swings his gonopodium forward at least 90° and attempts a forced mating). We also counted the number of gonopodial contacts (gonopodial thrusts that resulted in genital contact between the male and female). After this 15 min. observation, the two fish were left in the mating arena until 5h had elapsed since the trial commenced, at which point males and females were separated for the sperm recovery trials (see below). All behavioural and sperm analysis trials (see below) were performed blind of experimental treatment. That is, one of us set up treatments and issued each tank a unique code that subsequently identified the fish being tested (but was unknown to the observer during the trial).

Sperm recovery from males and females

We estimated each focal male's ability to transfer sperm to females during the mating trials by recovering and counting sperm both from males and females immediately after the 5h trial. To estimate male sperm reserves we manually stripped each male of recoverable sperm. This technique is described in detail elsewhere (Matthews et al. 1997) and provides an estimate of a male's available sperm load (which in turn would have been influenced by his mating activity during the trial). We also recovered sperm from the female's reproductive tract to estimate the number of sperm transferred during the mating trials, as well as the proportion of males that had successfully transferred sperm to the females. Again, the technique is described in detail elsewhere (Pilastro & Bisazza 1999), but briefly involves flushing the female's genital pore and oviduct five times with a 0.9% saline solution, which is then recovered for subsequent sperm counts (for experimental examples of this technique in poeciliid fishes see Evans et al. 2003a; Schlupp & Plath 2005). For both males and females, we counted the number of sperm in a sub-sample of each extracted ejaculate using an 'Improved Neubauer' Haemocytometer (Matthews et al. 1997). Total sperm counts were then estimated by multiplying the mean of five counts by the total volume of each sample. Immediately after sperm extractions, males were killed with an overdose of anaesthesia and digitally photographed (with a section of ruler for calibration). The size of each male was estimated from the photographs by measuring the distance between the snout and the tip of the caudal peduncle (= standard length). Gonopodium length was also estimated from these photographs (base of gonopodium to distal tip, not including the fleshy 'hood', see Kelly et al. 2000). Each male was then preserved in Dietrich's fixative (30% ethanol, 10% formalin, 2% glacial acetic acid and 58% DI H_2O) for the subsequent geometric morphometric analyses of gonopodial shape.

Geometric morphometric analyses of gonopodium shape

A digital image of the lateral (left) side of each male's gonopodium was captured with a Leica DFC320 fitted to a Leica MZ75 stereomicroscope under transmitted light and dark field illumination. This image was taken at x50 magnification and incorporated the distal tip of the gonopodium (Fig. 1a). We also took a second digital image (x50 magnification) of the dorsal surface of the gonopodium's paired 'hooks' (see Constantz 1989) (Fig. 1b). We characterized variation in the shape of both components of genital shape (distal tip and hooks) using geometric morphometric analyses (reviewed in Zelditch et al. 2007). For the analysis of the distal tip, we digitized eight landmarks around the peripheral edge of each specimen's image, placing each landmark at homologous points (see Fig. 1a). For hook shape, we digitized three landmarks: one at the base of both hooks and two at each of their respective tips (Fig. 1b). In both cases, landmarks were digitized using tpsDig2 software (Rohlf 2005a, available at http://life.bio.sunysb.edu/morph/). The raw coordinate data for these landmarks were superimposed using the Procrustes generalized least-squares procedure (Rohlf & Slice 1990) with tpsRelw v1.42 software (Rohlf 2005b).

For each male, landmark data were analysed with thin-plate spline relative warp analysis (TPSRW) using tpsRelw (v1.42) software (Rohlf 2005b). This generated partial warp scores, which describe shape variation as deviations from a consensus shape. Partial warp scores were subject to relative warp analysis, which corresponds to a principle components analysis and serves to reduce multivariate shape data to relative warps that describe most of the variation in shape. Variation in gonopodial shape and hook shape across our sample could then be visualized as deformations of the thin-plate spline (see bottom panel of Fig. 4). Each visualization plot displays deformations of the consensus configuration corresponding to a point in the space spanned by a particular pair of relative warps. The relative warp analysis returned five relative warp scores (RWS) explaining >90% of the variance in the distal tip, and two relative warps (71% & 29% for RWS1 & RWS2, respectively) for the genital hooks. In our subsequent analysis of genital shape, we use '$\mathrm{RWS}_{\mathrm{DISTAL\ TIP}}$' and '$\mathrm{RWS}_{\mathrm{HOOKS}}$' to refer to relative warp scores for the distal tip and hooks, respectively.

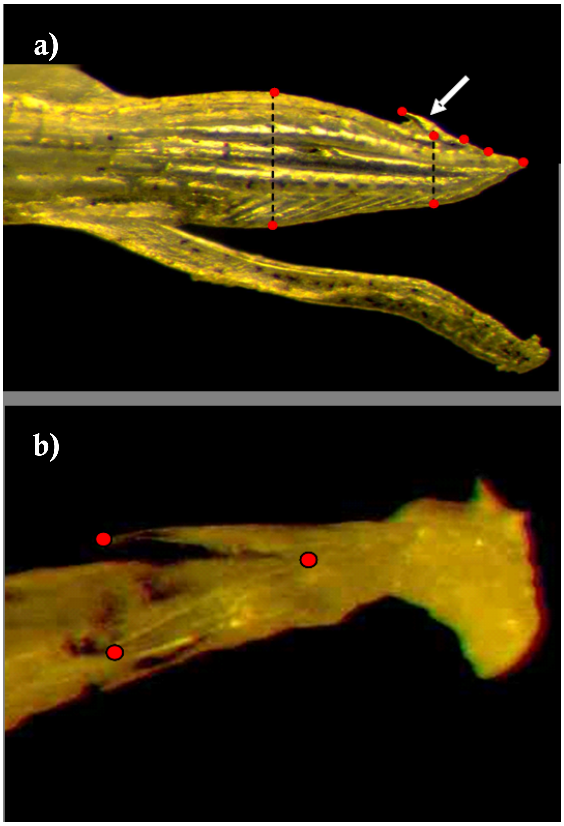

Fig.1 a) Lateral image of the gonopodium's distal tip. The with arrow depicts one of the paired hooks on the dorsal surface of the gonopodium's tip. b) Dorsal view of the paired gonopodial hooks. On both images, landmarks are depicted as red dots. These were superimposed on each image for the analysis of shape variation using geometric morphometric software (see *Materials & Methods* for details).

Statistical analysis

We checked the distributions of our data for normality and for homogeneity of variance and used appropriate transformations where necessary. To determine the factors influencing the success of gonopodial thrusts, we first examined how male and female morphological traits influenced the proportion of thrust attempts that resulted in genital contact. To do this we used a logistic regression with a logit link function and a correction for overdispersion (using the EXTRABINOMIAL procedure in Genstat 7.0). In this analysis, male and female body length (log transformed) and the residuals of gonopodium length on body size (see below) were entered as predictor variables. The sample size for this analysis was n=91, as not all males performed at least one GT attempt during the observation (n=8) and data were unavailable for some females (n=9), males (n=2) and gonopodium measures (n=3). To determine the factors influencing insemination success we used a logistic regression in which the dependent variable was either 0 (no sperm retrieved from the female's reproductive tract after mating trials) or 1 (sperm retrieved). Predictors in this model were female population origin (factor, with three levels), male and female body size, relative gonopodium length, the relative warp scores (RWS) derived from the geometric analysis on gonopodium shape (shape of the distal tip and claw) and the number of sperm retrieved from the males after the experiment (log transformed) (all entered as covariates in the model). Our analysis of gonopodium size revealed hypo- (negative) allometry in this trait (slope ± S.E. from the regression of log gonopodium length on log body length = 0.52 ± 0.12; observed slope tested against a null expectation of one; t_{103}=4.06,

$P<0.001$) (see also Jennions & Kelly 2002). We therefore used the residuals from the regression of log-gonopodium length on log-standard length to estimate relative gonopodium length for inclusion in the model. All predictors were initially included into the model. We used a backward stepwise method to obtain the best model, based on the adjusted R-squared criterion (procedure: RSEARCH; METHOD=bstepwise; CRITERION=adjusted], Genstat 7.0). All tests are two-tailed.

Results

Comparison of male genital traits among treatments

Although males were randomly assigned to the three experimental groups, we found that relative gonopodium size varied significantly among groups of males that were assigned to the different (female) populations; those assigned to females from the low-predation (Upper Quaré) population had relatively shorter gonopodia than those in the other groups (residual gonopodium size: Upper Quaré = 4.00±.047, $n=33$; Lower Quaré = 4.22±.047, $n=36$; Lower Turure =4.21±.059, $n=33$; General Linear Model: $F_{2,101}=6.43$, $P=0.002$). Although unfortunate, this finding is conservative with respect to our prediction, as we show that the success of forced matings (proportion of thrusts resulting in genital contact and the average number of sperm recovered from females) was higher in the low-predation treatment group (see below). Moreover, we also show that relative gonopodium length was significantly positively associated with the proportion of gonopodial thrusts that resulted in successful genital contact (see below). Hence, the observation that males assigned to the low-predation treatment (where we predicted higher forced mating success) had relatively shorter gonopodia actually strengthens our conclusion that males were relatively more successful when paired with low-predation females

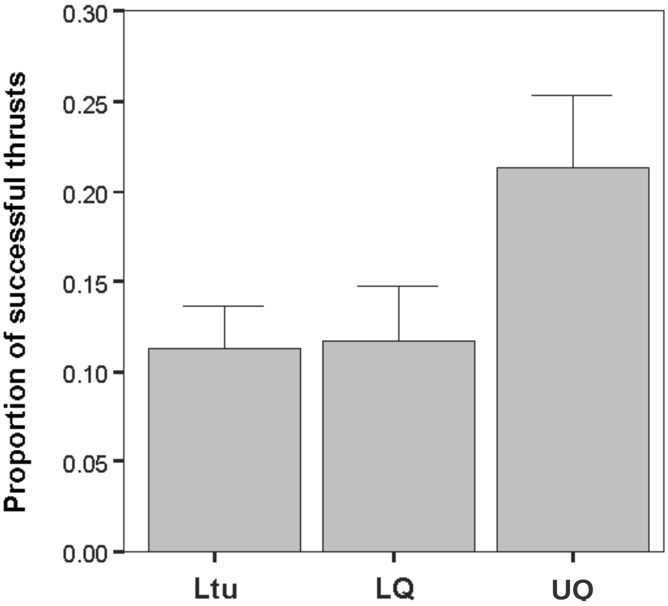

Fig. 2 Proportion of GT that ended with a contact between male and female genitalia

(see below). None of the other male morphological traits, or female standard length, differed significantly among treatment groups (RWS for genital morphology and male and female body size; all $P>0.11$).

Comparison of the success of forced matings among treatments

For this analysis we obtained data from $n=102$ (out of 108) mating trials (Female population origins: Lower Quaré = 36, Lower Turure = 33, Upper Quaré = 33). On average, males performed 12.89 ± 1.11 s.e. sigmoid displays and 8.37 ± 0.77 s.e. gonopodial thrust attempts during the observation period. The number of sigmoid displays and gonopodial thrusts did not differ significantly among experimental groups (sigmoid displays: $F_{2,101}=1.10$, $P=0.34$; gonopodial thrusts: $F_{2,101}=2.11$, $P=0.13$). However, consistent with our prediction, we found that the proportion of gonopodial thrust attempts that resulted in genital contact tended to be higher in the trials involving low-predation females (Generalized Linear Model: deviance $\text{ratio}_{2,88}=3.41$, $P=0.033$; LQ, $b=-0.746\pm0.305$ S.E., $t_{88}=2.44$, P=0.015, LTu, $b=-0.555\pm0.282$ S.E., $t_{88}=1.97$, P=0.049, [parameters for factors are differences compared with the reference level, QT]; Fig. 2). When residual gonopodium size (which varied across treatments – see above) was entered as a covariate in this analysis, our analysis revealed a significant effect of both population and relative gonopodium length on the proportion of thrust attempts that resulted in male-female genital contact (deviance $\text{ratio}_{3,87}=4.34$, $P=0.005$, LQ, $b=-0.880\pm0.303$ S.E., $t_{87}=2.90$, P=0.004, LTu, $b=-0.802\pm0.294$ S.E., $t_{87}=2.73$, P=0.006; gonopodium length, $b=9.75\pm4.09$ S.E., $t_{1,87}=2.38$, $P=0.017$). Male and female body sizes were not significant predictors of the proportion of thrust attempts that resulted in genital contact between males and females.

Of the 102 females assayed for the presence of sperm, 50 were demonstrably inseminated during the mating trials. Although the proportion of females that were successfully inseminated

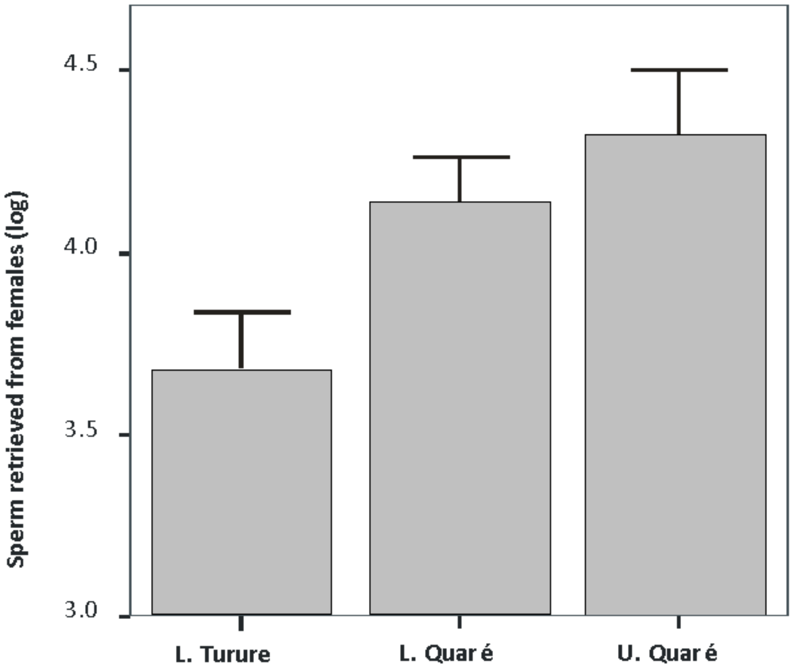

Fig. 3 Comparison of the mean (±SE) number of sperm extracted from female oviducts among three populations.

did not differ among treatments (high predation = 17, low predation = 19, control = 14; chi square=1.59, *P*=0.48), our analysis revealed a significant difference among treatments in the mean number of sperm recovered from the females' oviducts ($F_{2,\ 47}$=3.58, *P*=0.036, Fig. 3); females from the low-predation (Upper Quaré) population had higher numbers of recoverable sperm in their oviducts than those from the control (Lower Turure) population, while those from the focal high-predation population (Lower Quaré) had intermediate values (Tukey's post-hoc test).

Morphological predictors of insemination success

Our geometric morphometric analyses revealed a significant effect of male genital morphology on insemination success, estimated as the presence (success) or absence (failure) of recoverable sperm from the female's reproductive tract (Table 1). In these analyses we converted our metric of insemination success into a binomial variable (0=no sperm, n=52; 1=sperm retrieved, n=50) and used logistic regression to analyse how male genital shape variation influenced insemination success. We used a stepwise backward procedure (based on the adjusted R-squared) to select among the potential predictors of insemination success (female population origin, male and female body size, relative gonopodium length, gonopodium geometric morphometrics, and the number of sperm stripped from the male). The best model included three predictors, among which two were significantly associated with insemination success, namely $\mathrm{RWS2_{HOOKS}}$ and $\mathrm{RWS3_{DISTAL\ TIP}}$ (Table 1). Similar results were obtained when we included only these two predictors in the final model ($\mathrm{RWS2_{HOOKS}}$, P=0.003 and $\mathrm{RWS3_{DISTAL\ TIP,}}$ P=0.023, Fig. 4).

Table 1 Generalized linear model analysing the relationship between male genital shape and insemination success.

<u>*Model summary*</u>

	d.f.	Mean deviance	Deviance ratio	*P*
Regression	5	4.716	3.90	0.003
Residual	87	1.210		
Total	92	1.400		

<u>*Estimates of parameters**</u>

	b	s.e.**	t(87)	*P*
Constant	0.522	0.460	1.14	0.259
LQ***	-0.929	0.645	1.44	0.153
LTu***	-0.877	0.655	1.34	0.184
$\mathrm{RWS1_{DISTAL\ TIP}}$	-4.37	3.10	1.41	0.162
$\mathrm{RWS3_{DISTAL\ TIP}}$	16.94	8.42	2.01	0.047
$\mathrm{RWS2_{HOOKS}}$	11.42	3.56	3.20	0.002

* Full model included: Female SL, male SL, residual gonopodium length, RWS1, RWS2, RWS3 scores, RWS1_topview, RWS2_topview, and number of sperm stripped from males. Final model included only the variables selected with a stepwise backward procedure based on the adjusted R-squared.
** s.e.s are based on the residual deviance.
*** parameters for factors are differences compared with the reference level, QT.

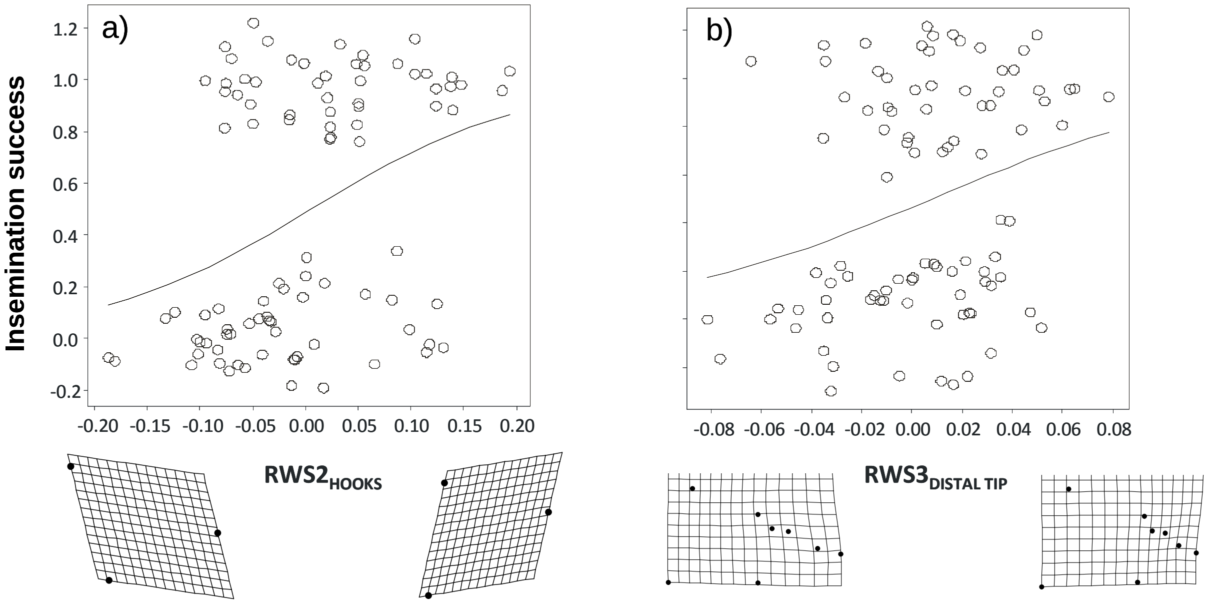

Fig. 4 The relationships between male genital shape and insemination success during forced matings in guppies. Upper panels are fitted and observed relationships from logistic modeling of insemination success (modeled as the binomial response, where 0 = no sperm present in female's reproductive tract and 1 = sperm present) on (a) the shape of gonopodial hooks at the distal tip of the gonopodium, and (b) the shape of the gonopodium's distal tip. Lower panels depict deformations of the thin-plate splines for each relative warp score (RWS) for positive and negative values See *Materials & Methods* for further details.

Discussion

Our results reveal that female population origin influences the success of forced matings. Males from the focal high-predation site had higher mating success (as estimated by the proportion of gonopodial thrust attempts that resulted in genital contact and the proportion of females that were successfully inseminated) when paired with females from the low-predation population than either females from their own population or the control (high-predation) site. We also show that male genital shape influences mating success, suggesting that this trait mediates the outcome of forced matings and is therefore a potential target of antagonistic selection.

Our results may be explained by differences in the level of sexual conflict over mating rate among populations. Such conflict is likely to dominate the guppy's mating system for several reasons (Magurran 2005). For example, females are able to store sperm for several months and fertilise consecutive batches of eggs following a single copulation (Constantz 1989). Although females can gain substantial direct and indirect benefits from mating polyandrously (Evans & Magurran 2000; Ojanguren et al. 2005), optimal mating rates for males are still likely to be far higher than those for females. Indeed, males perform up to one mating attempt per minute in natural populations (Magurran & Seghers 1994b), with potentially important impacts on female fitness (Constantz 1984; Magurran & Seghers 1994a; Ojanguren & Magurran 2007). Superfluous matings may make females more vulnerable to predators (Pocklington & Dill 1995) and may undermine female choice, potentially reducing any genetic benefits associated with mate choice (Reynolds & Gross 1992; Nicoletto 1995). As Magurran and Seghers (1994b) have previously noted, some (if not all) of these costs are likely to be elevated in high-predation populations where females are subject higher levels of forced matings than their low-predation counterparts.

We predict that the elevated mating costs in high-predation populations will generate antagonistic selection for female traits that serve to resist superfluous matings. Our recent comparative study on ten natural populations in Trinidad (encompassing the focal populations used here) supports this prediction by revealing that males in high-predation populations have gonopodia with less elongated distal tips with reduced hook angles than males from low-predation populations (**paper VI**) This same study revealed correlated patterns of genital trait divergence in females (the relative width of the female's oviduct), suggesting a possible role for sexually antagonistic selection favouring female counteradaptations. Evidence for similar correlated patterns of genital trait divergence comes from interspecific comparative work on the poeciliid genus *Gambusia*, where the occurrence of relatively long and pointed (and potentially damaging) gonopodia occur in species where females have large urogenital papillae, in contrast to those where males have blunt or rounded gonopodia where the females' papilla is either reduced or absent (Peden 1972; but see Langerhans et al. 2005 for evidence of female choice on male genital size in this genus). Constantz (1984) has argued that the function of such female modifications is to deflect gonopodia during forced mating attempts, thereby improving the female's control of paternity.

Our findings add to an increasing body of work reporting interacting effects of male and female population origin on mating success. Much of this work has addressed verbal theoretical arguments proposed to test sexual conflict theory (e.g. see Holland & Rice 1998; Parker & Partridge 1998). One basic prediction arising from these discussions is that when reproductive traits that are shaped by sexual conflict diverge among populations, females will be more resistant to males from their own population, with whom they have coevolved, than those form alien populations (Clark et al. 1999; Andres & Arnqvist 2001; Knowles & Markow 2001; Hosken et al. 2002; Nilsson et al. 2002; Jolivet & Bernasconi 2007). Our work similarly reveals an effect of population origin on mating success, thereby offering preliminary evidence for a history of sexually antagonistic selection acting on male genital morphology and female traits that function to thwart unwanted male mating advances. Nevertheless, our study is restricted to just one focal male population (lower Quaré) and to test the generality of this finding further work is required that encompasses a range of populations.

We also report that male genital shape is associated with the success of forced matings. We found that variance in the shape of both the gonopodium distal tip (RWS3, explaining approximately 9% of the variance in overall shape of the distal tip) and its genital hooks (RWS2, explaining 29% in hook shape) predicted the likelihood of sperm transfer during coercive mating attempts. These findings accord with early work on this species and other poeciliid fishes showing that this region of the gonopodium plays an important role in sperm transfer. For example, Rosen and Gordon (1953) observed that in the genera *Poecilia* and

Xiphophorus, only the tip of the gonopodium was inserted into the female's gonopore. They suggested that contact between the male and female was maintained by holdfast devices that project from rays 3 and 4 of the gonopodium. These findings were subsequently corroborated in xiphophorin fishes by Clark et al. (Clark et al. 1954), who demonstrated experimentally that that these structures were necessary for successful insemination. Consistent with this observation, a recent study that experimentally removed the paired hooks from the male guppy's gonopodial tip revealed that this processes impairs subsequent sperm transfer, although interestingly this effect was only apparent during forced (unsolicited) matings (Cheng 2004). Again, this finding is consistent with our results showing that variance in the shape of these hooks is associated with insemination success during gonopodial thrusts.

In conclusion, our results offer preliminary experimental support for verbal models of sexual conflict that predict that females will be more resistant to males from their own population (or species) with whom they have coevolved (Holland & Rice 1998). Our study therefore adds to a small, but increasing body of literature revealing similar effects in animals and plants populations (Clark et al. 1999; Andres & Arnqvist 2001; Knowles & Markow 2001; Hosken et al. 2002; Nilsson et al. 2002; Jolivet & Bernasconi 2007). We also report a significant association between male genital shape and the success of forced matings, confirming the potential for sexual selection to act on male genital morphology.

PAPER V
Does male genital size and shape influence female mate choice and sperm transfer in the guppy?

Clelia Gasparini, Andrea Pilastro And Jonathan P. Evans

Abstract

In internal-fertilizing species male genitalia show a high degree of diversification that mainly results from sexual selection processes. Indeed, female preference has been proposed to account for the association between the morphology of male genitalia and the increase of their fertilization success. Nevertheless the incredible elaboration of male genital traits may be driven also by sexual conflict, in which interests over mating rate and fertilization differ between the two sexes. The mechanisms underlying the evolution of male genitalia still need to be clarified and this aim is further complicated by the possibility that different mechanisms, such as sexual selection and sexual conflict, could act on different aspects of male genital morphology. We therefore evaluated the influence of male genital morphology on the outcome of cooperative matings in terms of female latency to mate and number of sperm transferred, in the guppy (*Poecilia reticulata*). The results showed that females mated more quickly with those males possessing a gonopodium with increased linear length and with a larger shape. Moreover, we also found that males with a shorter hook on the distal region of the gonopodium transferred a higher number of sperm. The discrepancy between female preference for longer gonopodium and male insemination success associated with shorter hooks suggests an influence of sexual conflict in the evolution of male genitalia in *P. reticulata*.

Introduction

In animals with internal fertilization male genitalia can exhibit extreme morphological divergence, even among closely related species. Understanding the evolutionary basis for this variation has been a key goal in evolutionary biology (Eberhard 1985; Arnqvist & Danielsson 1999; Hosken & Stockley 2004). Among the hypotheses proposed to explain such extraordinary patterns of divergence, the sexual selection hypothesis has gained the most empirical support (see Eberhard 1985; Hosken & Stockley 2004). The sexual selection hypothesis predicts that genital morphology evolves via selection for increased fertilization success via several non mutually exclusive mechanisms (Eberhard 1985; Arnqvist 1997). Although the majority of studies examining genital evolution via sexual selection emphasise the role of postmating mechanisms (sperm competition and cryptic female choice) (e.g. see (Eberhard 1985; Hosken & Stockley 2004), other studies have uncovered a role for premating mechanisms of sexual selection, and in particular female choice (Brooks & Caithness 1995; Preziosi & Fairbairn 1996; Langerhans et al. 2005; Bertin & Fairbairn 2005)

The evolution of male genitalia may be a consequence of cryptic female choice, where females where differential sperm use by females is triggered by the stimulatory capabilities of the males' genitalia in the form of tactile courtship (Thornhill 1983; Eberhard 1994). By contrast, male genitalia may be favoured through the avoidance of sperm competition, where genital adaptations facilitate the displacement of previously inseminated sperm or induce non receptivity in females (Smith 1984) or function as an 'anchor' to secure the male inside the female in spite of takeover from rivals (Simmons 2001). Alternatively, the elaboration of male genital traits may be driven by sexual conflict, which in turn results from differences in the reproductive interests of males and females (Parker 1979). According to the sexual conflict hypothesis, the evolution of traits that are beneficial to males but harmful to the females will fuel sexually antagonistic selection for adaptations in one sex and counter-adaptations in the other (Arnqvist & Rowe 2005).

Male genitalia may also be the target of precopulatory sexual selection. For example, in species where genitalia are not hidden in males' body (i.e. non retractile), they may additionally serve as ornaments (Preziosi & Fairbairn 2000; Fairbairn et al. 2003; Langerhans et al. 2005). Despite increasing evidence that male genital morphology is targeted by sexual selection, in many cases the selective mechanisms have yet to be determined (Simmons 2001). The scenario is further complicated by the fact that different selective pressures might act on different aspects of male genital morphology (Werner & Simmons 2008).

The guppy, *Poecilia reticulata*, is an ideal model system to study the evolution of male genitalia. Guppies are livebearing poeciliid fish with internal fertilization and a promiscuous mating system (Houde 1997). Males inseminate females through a modified anal fin that serves as the intromittent organ (the gonopodium). Three fin rays are differentially elongated to form a channel that allows the passage of sperm into female genital opening. Male guppies exhibit two different mating tactics: they can court females performing a display to expose body ornamentation (courtship) or they try to insert sneakily the gonopodium into the gonopore (coercive mating). During the female's receptive period (approx. 2-3 days after producing a brood) males are able to use elaborate courtship displays to achieve solicited copulations (Houde 1997), while in the non-receptive period, the remainder of the female's *ca.* monthly brood cycle (Liley 1966), copulations can only be achieved through coercive matings.

In guppies differences in gonopodium shape between natural populations are explained, at least in part, by sexual conflict: gonopodium length and the distal region's shape of the male gonopodium varies predictably among populations according to the level of predation intensity that it is associated with level of sexual conflict (Kelly et al. 2000, **paper VI**). In another work authors showed a significant association between the distal region of the gonopodium and the success of forced matings (**paper IV**). Taken together, these results suggest that sexual conflict is involved in the evolution of gonopodium morphology. However, due to the great variability in genital morphology found in natural populations it can not be rule out the possibility that other evolutionary forces take part on its evolution. In *Poecilia reticulata* another selective pressure we suppose to act on gonopodium evolution is sexual selection driven by the outcome of cooperative mating. Indeed, in this species the relative contribution of alternative mating strategies are not exactly known. On average, sneaky attempts result in a low insemination success (Matthews & Magurran 2000; Russell et al. 2006) although sometimes large ejaculates are delivered (Pilastro & Bisazza 1999; Evans et al. 2003b). Moreover guppy system appears predominate by sexual conflict with females receive up to one sneaky attempt per minute along all their life (Magurran & Seghers 1994b). Nevertheless in the guppy females' choice plays a central role with evidences of strong pre and post copulatory female choice (for an extensive review see Houde 1997; Magurran 2005). Therefore in order to understand the different evolutionary components acting on male genitalia, its role in determine success in cooperative mating has to be examined.

In this study we investigated the importance of gonopodium morphology in male-female consensual interactions in a series of experiments involving only cooperative matings. In particular, we examined both female precopulatory preference and the number of sperm transferred in relation to gonopodium length and shape.

Materials and Methods

Origin and maintenance of experimental fish

All animals used in this experiment were laboratory born descendents of wild-caught fish collected from Alligator Creek (30 km south of Townsville) in Queensland, Australia. Recent genetic analyses indicate that this population was founded *ca.* 1910 from a source of wild guppies from Guyana (Lindholm et al. 2005). Fish were maintained at a constant 26°C on a 12h:12h day-night cycle and fed live brine shrimp and commercial flake.

Mating trials

All experimental males were taken from stock aquaria and isolated from females for at least 3 days to ensure that they entered the mating trials with fully replenished sperm stores (Kuckuck & Greven 1997). On the evening before each mating trial, a sexually mature virgin female (approx.18 month-old) guppy was placed individually into the mating tank (43 x 23 x 25 high, filled to a depth of 21 cm) and left overnight to settle. On the following morning, a male was taken from the isolated stock males and placed in the mating arena. From this time, we observed the focal pair until they successfully copulated. Virgin female guppies are generally receptive to male courtship (Houde 1997) and in this study we only considered copulations that were preceded by a courtship display (termed ‘sigmoid’ display), involved female cooperation (the so-called 'glide' response, see Liley 1966) and were followed by male postcopulatory jerks (hereafter ‘PCJs’), which are known to reliably signal successful sperm transfer (Liley 1966). We also considered ‘copulation latency’ (time to mate) as a measure that is inversely correlated with female responsiveness. We also counted the total number of courtship displays and gonopodial swings (rapid movement of gonopodium, Liley 1966) as well as the number of attempted forced copulation attempts (although none of the gonopodial thrusts we recorded resulted in successful insemination, as evident by the lack of PCJs). As soon as the focal pair successfully copulated, we counted the number of PCJs, as this measure correlates positively with the number of sperm transferred during cooperative copulations (Pilastro et al. 2007). We then removed the first male for subsequent morphological analyses (see below) and the female was left in the mating arena for 5 min. before introducing the second male to the tank. We scored mating behaviour and PCJs as before, but additionally noted the time interval between successful first and second copulations (i.e. the time elapsed since male 1 had mated and male 2 achieved copulation, see Evans & Magurran 2001). As soon as the second male had successfully mated with the female, we removed the male for morphological analyses (see below). In total, we performed 79 mating trials, of which 41 resulted in successful successive matings between the female and each of the two males. If a male did not copulate or exhibit any sexual behaviour within 10 min, we removed it from the experimental tank and added a different male.

Estimating male ornamentation

We used UTHSCSA Image Tool (University of Texas Health Science Center, San Antonio, TX, available at http://ddsdx.uthscsa.edu/dig/itdesc.html) to estimate the body area of each male (including caudal fin but excluding dorsal fin), body size (snout to tip of caudal peduncle = standard length), and the surface area of the various colour spots. These colour spots were measured on the left side of each male's body from digital photographs, and included the relative area of carotenoid and pteridine pigments (orange, yellow and red spots, summed as 'orange'), structural colours such as iridescent blue, green, purple, silver and white (summed as 'iridescence') and melanin spots (summed as 'black'). Hence, in our analysis we included three separate colour measures: orange, iridescence and black, as well as body area.

Male genital morphology

Following photography, males were killed with an overdose of the anaesthetic benzocaine and immediately fixed in Dietrich's solution (30% pure ethanol, 10% formalin, 2% glacial acetic acid, 58% H_2O) where they remained for at least two months. Two digital images of the lateral (left) side of each male's gonopodium were subsequently captured using a Leica DFC320 fitted to a Leica MZ75 stereomicroscope under transmitted light and dark field illumination. The first image was taken at x16 magnification and included the entire gonopodium. The second image was captured x50 magnification and included the distal tip of the gonopodium.

We estimated variation in the shape of the whole gonopodium (x16 images) and the gonopodium distal tip (x50 images) using geometric morphometric analyses (reviewed in Zelditch et al. 2007). In the x16 images (whole gonopodium) we digitized 40 landmarks around the peripheral edge of each specimen's image. In the x50 (close-up) images, eight fixed landmarks were superimposed at homologous points on each image. Since whole gonopodia have relatively smooth edges along much of their lateral surface, and therefore few homologous points on which to attach fixed landmarks, we used a combination of fixed and sliding semilandmarks to quantify variation in shape of whole gonopodial (Bookstein 1997). In these images, 8 fixed landmarks were positioned over homologous points on each specimen, while sliding semilandmarks were placed approximately equidistantly between them. In the subsequent analysis of shape, the eventual position of each sliding semilandmark was adjusted along the line tangent to the curve to minimize the amount of shape change between each specimen and the average of all specimens. Landmarks were digitized using tpsDig2 software (ref. Rohlf 2005a, software available at http://life.bio.sunysb.edu/morph/) and defined as sliders or fixed landmarks with tpsUtil (Rohlf 2004). The raw coordinate data for both fixed landmarks and sliding semilandmarks were superimposed using the Procrustes generalized least-squares procedure (Rohlf & Slice 1990) with tpsRelw v1.42 software (Rohlf 2005b).

For each male, landmark data for x50 and x16 images were analysed with thin-plate spline relative warp analysis (TPSRW) using tpsRelw v1.42 software (Rohlf 2005b). This generated partial warp scores, which describe shape variation as deviations from a consensus shape. Partial warp scores were subject to relative warp analysis, which corresponds to a principle components analysis and serves to reduce multivariate shape data to relative warps that describe most of the variation in shape. Variation in gonopodial shape across our sample could then be visualized as deformations of the thin-plate spline. Each visualization plot displays deformations of the consensus configuration corresponding to a point in the space spanned by a particular pair of relative warps. The relative warp analyses returned 3 relative warps, explaining >75.36% and 70.43% of the variance in gonopodial shape in x50 and x16 images, respectively. We will refer to relative warps of the entire gonopodium (x16) as RWSw1, RWSw2 and RWSw3 and to relative warps of the distal tip of the gonopodium (x50) as RWSt1, RWSt2 and RWSt3.

Finally, we estimated linear measures for gonopodium length using photographs. Image analysis software (UTHSCSA Image Tool v.3.0) was used to estimate the distance from the base of the gonopodium to the distal tip, not including the fleshy 'hood' (see Kelly et al. 2000).

Statistical analysis

We used a general linear mixed model (GLMM) to test the effect of male genitalia on both copulation latency, as a measure of female preference, and number of postcopulatory jerks 'PCJs', as an estimation of sperm transfer success.

To control for the fact that there were non-independent measurements (two males were tested with a single female) female identity was included as a random factor in the model and the order of mating (1st or 2nd) as fixed factor. Variables enter in the model as predictors were reported for each analysis. Area of coloured spots and gonopodium length were expressed in their absolute values and to control for males body size we entered also body area as one of the predictors. From an initial model we used a backward elimination to remove non significant variables, both models (full and reduced) are provided. Statistical analyses were performed using SPSS v16.0. Data were log-transformed to achieve normal distribution when necessary; mean are presented ± SE.

Results

We obtained data from a total of 82 males that were paired to 41 virgin females. Males were randomly chosen from stock tanks, and subsequent analysis revealed no significant differences in any of morphological traits we considered for males presented to females as first or second mates (see Material and methods; all P>0.161). The frequency of courtship displays was strongly positively correlated with the frequency of gonopodial swings (r=0.818, P<0.001, N=76) and less strongly with body size (r=0.286, P=0.012, N=76).

Copulation latency

Copulation latency, measured as time taken for females to copulate following the introduction of the male into the tank (mean: 297.67s ± 25.28), did not significantly differ between the first and the second males (paired *t*-test: t_{40}=1.409, P=0.167). We included courtship rate (display min^{-1}), body area, coloration, gonopodium length and gonopodium shape (RWSw) as predictors of copulation latency. As shown in table 1, the number of displays, gonopodium length and gonopodium shape were significant predictors of copulation latency. The third relative warp (RWSw3 explaining 11.09 % of the variance in male genital shape) was marginally non-significant in the full model (P=0.051), but significantly associated with copulation latency in the final restricted model (P=0.021). Females mated more quickly with males that performed more courtship displays and with a longer (linear length, see fig. 1) and larger (as indicate from RWSw3, see fig. 2) gonopodia

(a)

Predictor	*F*	*P*	*b*	*SE*(*b*)
Order of mating	0.902	0.545	0.116	0.076
Display (min^{-1})	32.488	0.000	-0.164	0.026
area	1.848	0.180	0.011	0.005
Orange	0.457	0.502	-0.006	0.010
Black	0.608	0.439	0.024	0.034
Iridescent	2.765	0.102	-0.022	0.015
Gonopodium length	9.102	0.004	-0.343	0.126
RWSw1	0.069	0.793	-0.440	1.187
RWSw2	0.276	0.602	0.069	1.364
RWSw3	3.982	0.051	5.176	2.143

(b)

Predictor	*F*	*P*	*b*	*SE*(b)
Order of mating	2.746	0.102	0.119	0.072
Display (min-1)	51.606	0.000	-0.170	0.024
Body area	4.546	0.037	0.009	0.004
Gonopodium length	6.286	0.015	-0.287	0.114
RWSw3	5.619	0.021	4.954	2.090

Table 1. Results of GLMM analysis testing the influence of male gonopodium on copulation latency. (a) full model (b) reduced model.

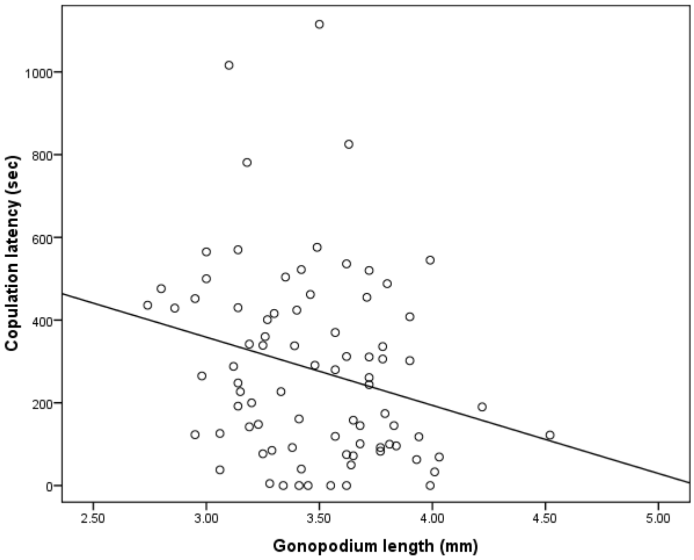

Fig. 1 The relationship between gonopodium length and copulation latency

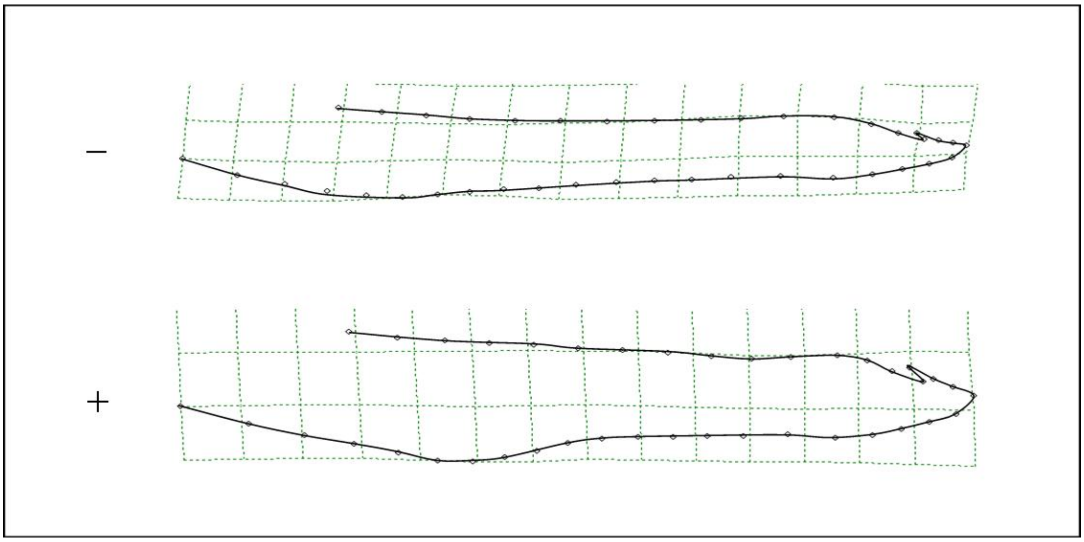

Fig. 2 Changes in the shape of whole gonopodium along the third relative warp (RWSw3). Negative values for the third relative warps are associated with longer female copulation latency, while positive values are associate with shorter copulation latency.

Number of jerks

The number of postcopulatory jerks (average mean: 18.46 ± 1.36, 1st male: 18.32 ± 1.88; 18.61 ± 1.98) did not differ between the first and the second male presented to the females (paired *t*-test: t_{40}=0.118, P=0.907). Results showed that there was slightly non significant negative association (full model: P=0.094; reduced model: P=0.081) between RWSt3 (explaining 8.32 % of the variance in distal tip shape, see fig. 3) and the number of postcopulatory jerks (see table 2). More jerks were performed by males possess a shorter hook on the distal region of gonopodium.

a)

Predictor	*F*	*P*	*b*	*SE*(*b*)
Order of mating	0.010	0.921	0.007	0.072
Body area	0.002	0.969	0.000	0.004
Gonopodium length	0.786	0.378	-0.112	0.127
RWSt1	0.015	0.904	-0.080	0.655
RWSt2	1.711	0.195	1.337	1.022
RWSt3	2.873	0.094	-2.647	1.562

(b)

Predictor	*F*	*P*	*b*	*SE*(*b*)
Order of mating	0.044	0.836	0.014	0.067
RWSt3	3.117	0.081	-2.668	1.511

Table 2. Results of GLMM analysis testing the influence of male gonopodium on number of postcopulatory jerks. (a) full model (b) reduced model.

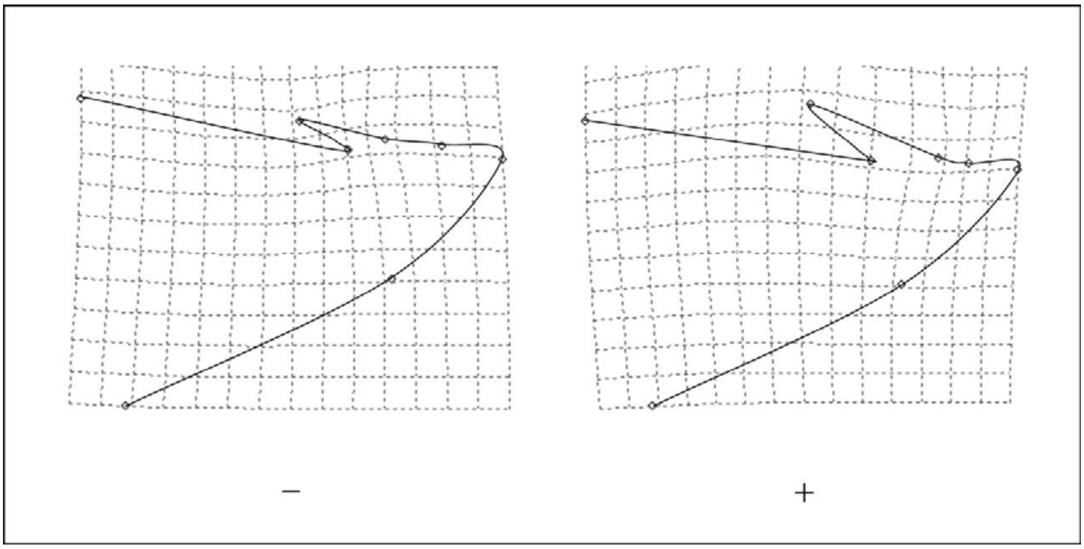

Fig. 3 Changes in the shape of the distal region of the gonopodium along the third relative warp (RWSt3). Negative values for the third relative warps are associate with a higher number of postcopulatory jerks performed by the males after matings, while positive values are associated with smaller number of jerks.

Discussion

In this study we demonstrated that females copulate more quickly with males that posses a larger gonopodium and, as expected, that had higher courtship display rate. We also provide evidence that the male's genital shape, in particular that referred to the distal tip of the gonopodium, influences the number of sperm transferred, as indicated by the number of postcopulatory jerks performed after mating (Pilastro et al. 2007). These results suggest that females exhibit sexual preferences for males with relatively longer gonopodia, independent of body size. We found preferences for both length and shape (Relative Warps whole gonopodium #3 which indicate a larger gonopodium). Preferences for longer genitalia have been reported for other animal species (Preziosi & Fairbairn 1996; Langerhans et al. 2005; Bertin & Fairbairn 2005). Our findings also confirm previous work on guppies revealing preferences for longer gonopodia, even they not control for body size (Brooks & Caithness 1995). Furthermore, previous work has shown that males from high predation populations have longer gonopodia than their low-predation counterparts (Kelly et al. 2000), confirming our own recent unpublished observations (**paper VI**). Since the level of forced copulations by males is greater in high-predation populations (Magurran & Seghers 1994b), and male genital size positively covaries with the frequency of forced matings both in guppies (Reynolds et al. 1993) and more generally in poeciliid fishes (Rosen & Tucker 1961), male genital length may also respond to selection through forced matings. Female preferences based on male genital size and shape might also help to clarify the function of gonopodial swings, which are rapid movements of the gonopodium that are often performed by males (called 'gonopodial swing' Liley 1966). This behaviour has never been explained, but our finding that the frequency of such swings was strongly positively correlated with courtship intensity suggests that this behaviour may serve as a form of 'gonopodial display'. Therefore our findings corroborate previous findings of female preference for longer gonopodia in poeciliids. Further researches has to be perform to understand the evolutionary origin of such preference.

Our results reveal a tendency for the length of the gonopodial hook at the gonopodium distal tip (RWSt3) to be associated with the number of postcopulatory jerks performed by males, suggesting that males with shorter hooks transfer more sperm than their counterparts with longer hooks. Interestingly, our recent unpublished work (**paper IV**) revealed that gonpopodial tip morphology significantly predicts the likelihood of sperm transfer during coercive matings. Both studies therefore highlight the importance of distal tip morphology in transferring sperm. A further conclusion from our study is that female preferences favour increased gonopodial length, but insemination success (transfer of sperm) depends on variation in gonopodium morphology. These two traits were not correlated each other (r=0.136, P=0.222, N=82) suggesting that different traits are involved in determining premating success and insemination success. As consequence of our results it seems unlikely that the higher insemination success of males with shorter hook is determined by a female decision, given that these are not the same males preferred during premating choice. A possible alternative explanation could be that tip characteristics enable a sort of stimulation of the female genital tract, favouring males by the mechanism of cryptic female choice, as suggested by Eberhard (1985). Finally, insemination could be more under male control and this particular tip morphology might ensure a better interaction between genitalia, allowing a larger sperm transfer. This may suggest the occurrence of sexual conflict. Nevertheless these explanations are far from being conclusive and future studies are needed to ascertain the proximate causes of higher sperm transfer due to genital morphology.

In conclusion, our work provides evidence for female premating preference for larger gonopodium and association between gonopodium tip morphology and success in sperm transfer during cooperative matings. Therefore our results shed light on the mechanisms of sexual selection acting on male genitalia and help to improve our understand of genital evolution.

PAPER VI
Intraspecific evidence for correlated patterns of male and female genital trait diversification

Jonathan P. Evans, Clelia Gasparini, Gregory I. Holwell, Indar W. Ramnarine, Trevor E. Pitcher, and Andrea Pilastro

Abstract

The role of sexual selection in fuelling genital evolution is becoming increasingly apparent from comparative studies revealing interspecific divergence in male genitalia and evolutionary associations between these structures and female reproductive traits. While these studies offer compelling insights into past evolutionary events, identifying contemporary evolution on genitalia has remained elusive. We address this issue using a single-species comparative approach, focusing on natural populations of the livebearing fish *Poecilia reticulata*. This species exhibits divergent patterns of male sexual behavior among populations differing in predation intensity; under elevated predation risk males switch from courtship to forced mating attempts, with consequent increases in the level of sexual harassment endured by females. Our survey of ten natural populations in Trinidad revealed consistent patterns of divergence in both male and female genital morphologies that reflect these divergent mating tactics. Our subsequent analysis implicated sexual selection in fuelling the evolution of these traits by revealing correlated patterns of trait divergence in both sexes. This correlated pattern of genital trait diversification across ecological gradients that govern the level of sexual harassment is consistent with a history of sexually antagonistic selection acting on these traits.

Introduction

Sexual selection is a key force driving the rapid evolutionary divergence of genitalia in animals with internal fertilization (Eberhard 1985) and several studies have established associations between male genital morphology and reproductive success (Hosken & Stockley 2004). For example, male genital traits can stimulate females during mate choice (Brooks & Caithness 1995; Preziosi & Fairbairn 1996; Langerhans et al. 2005; Bertin & Fairbairn 2005) but may also function to influence the relative fertilization success of competing ejaculates during postcopulatory episodes of sexual selection (Waage 1979; Arnqvist & Danielsson 1999; Danielsson & Askenmo 1999; House & Simmons 2005; Wenninger & Averill 2006) Further evidence for sexual selection on male genitalia comes from comparative studies that report evolutionary associations between male genital traits and female reproductive morphology or behaviour. These studies emphasize the role of cryptic female choice (Eberhard 1985) and sexual conflict (Alexander et al. 1997; Arnqvist & Rowe 2002a; Arnqvist & Rowe 2002b) in generating selection on reproductive traits in both sexes. On the one hand, cryptic female choice will favour female traits that bias fertilization towards males with (preferred) elaborated genitalia (Eberhard 1985), while on the other, sexual conflict will favour female adaptations that mitigate the costs of superfluous matings (Arnqvist & Rowe 2005). While evidence for such covariance in male and female reproductive traits is accumulating from interspecific comparative work (Arnqvist & Rowe 2002a; Arnqvist & Rowe 2002b; Huber 2003; Cordoba-Aguilar 2005; Ronn et al. 2007; Brennan et al. 2007), surprisingly few studies have focused on patterns of intraspecific variance in male and female genitalia (Arnqvist & Rowe 1995; Polihronakis 2006), and none has tested for their predicted covariance among populations exhibiting divergent genital traits. This is surprising because intraspecific studies have the potential to illuminate current evolutionary processes (Arnqvist 1997; Holland & Rice 1998).

Guppies (*Poecilia reticulata*) are livebearing poeciliid fish with internal fertilization and a highly promiscuous mating system (Houde 1997; Magurran 2005). In the poeciliid family, the relative length of the male intromittent organ (the gonopodium) covaries predictably with patterns of male mating behaviour. In species with relatively long gonopodia, males predominantly employ forced (unsolicited) matings, while those with relatively short gonopodia tend to use conspicuous courtship displays to solicit matings with cooperative females (Constantz 1989). Although guppies belong to the latter class (in terms of relative gonopodium size), individual males use a combination of both courtship and forced matings (Houde 1997), and the relative frequency of both male mating strategies varies according to predation intensity (see Endler 1995 for a review) Kelly et al. (Kelly et al. 2000), in turn, reported that males inhabiting high-predation sites have relatively longer gonopodia than their counterparts from less dangerous environments (but see ref. 29), which was interpreted as an adaptation to increased forced mating activity under elevated predation risk in accordance with the general interspecific pattern for the family.

In this paper we estimate intraspecific divergence in male and female genital traits in Trinidadian guppies and relate these patterns to differences in the intensity of predation, which is known to have resulted in a myriad of morphological and behavioral adaptations in these livebearing fish (Endler 1995). Guppies that inhabit lowland river populations typically endure high levels of predation from a variety of fish predators, while those inhabiting upstream sites are usually exposed to far lower levels of predation (Houde 1997). Across these ecological gradients, males use both forced mating attempts and courtship to acquire copulations, but the extent to which they employ either tactic is influenced by the level of predation. Under elevated predation risk, males tend to switch from highly visual courtship displays to less conspicuous forced matings (Godin 1995), possibly because forced matings are more profitable when females are preoccupied with predator evasion (Evans et al. 2002). Consequently, females from high-predation localities typically endure higher levels of sexual harassment (and associated costs) than their low-predation counterparts (Magurran & Seghers 1994a; Magurran & Seghers 1994b). Recent work suggests that the structures of the male guppy's intromittent organ (the gonopodium), including the presence or absence of the genital claws at the distal tip, influence the male's ability to transfer sperm during forced mating attempts (Cheng 2004). This finding corresponds with our ongoing work showing that the angle of genital claws in relation to the gonopodial tip affects the male's ability to transfer sperm during unsolicited mating attempts (**paper IV**). Given the importance of these gonopodial traits in influencing the success of forced copulations, we predicted that male genital morphology would reflect the divergent male mating tactics observed across predation gradients in Trinidad. To test this prediction we sampled guppy populations from upstream and downstream sites within each of five river systems in Trinidad's Northern Mountain Range. Four of these rivers (Aripo, Tacarigua, Quaré and Turure) exhibited the typical upstream-downstream predation gradient (upstream sites = low predation, downstream sites = high predation) seen in many Trinidadian rivers (Houde 1997), while the fifth (Oropouche) was characterized by high levels of predation both in the upstream and downstream sites (Endler & Houde 1995). By incorporating the Oropouche River in our sampling design we therefore controlled for variation in physical and ecological factors other than predation intensity that may drive inter-population diversification of phenotypic traits.

Materials and Methods

Populations

We sampled guppies from 10 populations located in southern flowing streams in Trinidad's Northern Mountain Range (for biological, physical and geographical information on each site, see Magurran & Seghers 1994b; Endler & Houde 1995; Evans et al. 2003b). Fish were returned to aquarium facilities at the University of the West Indies (UWI), St Augustine, Trinidad and Tobago, where they were maintained until needed. The populations came from five different river systems located in two river drainages. Within each river system, guppies were sampled from one upstream and one downstream site. All downstream sites were characterised by high levels of predation, as documented elsewhere (Evans et al. 2003b), while upstream sites tended to be largely devoid of large predatory fishes, with the exception of the Oropouche River where the distribution of predators extends into upstream sites (see Introduction). Fish from each population were maintained in 30 x 30 x 45 cm tanks under ambient lighting and temperature conditions and fed on brine shrimp nauplii until required for morphological analyses (see below).

Male genital morphology

At *least* 20 males from each of the 10 populations were killed with an overdose of the anaesthetic benzocaine and immediately fixed in Dietrich's solution (30% pure ethanol, 10% formalin, 2% glacial acetic acid, 58% DI H_2O) where they remained for at least two months. Two digital images of the lateral (left) side of each male's gonopodium were subsequently captured with a Leica DFC320 fitted to a Leica MZ75 stereomicroscope under transmitted light and dark field illumination. The first image was taken at x16 magnification and included the entire gonopodium (Fig 1a). The second image was captured x50 magnification and included the distal tip of the gonopodium, including the genital claw (see Fig. 1b).

We examined variation in the shape of the whole gonopodium (x16 images) and the gonopodium distal tip (x50 images) using geometric morphometric analyses (reviewed in Zelditch et al. 2007). In the x16 images (whole gonopodium) we digitized 40 landmarks around the peripheral edge of each specimen's image (Fig. 1a). In the x50 (close-up) images, eight fixed landmarks were superimposed at homologous points on each image (Fig. 1b). Since whole gonopodia have relatively smooth edges along much of their lateral surface, and therefore few homologous points on which to attach fixed landmarks, we used a combination of fixed and sliding semilandmarks to quantify variation in shape of whole gonopodia (Bookstein 1997). In these images, 8 fixed landmarks were positioned over homologous points on each specimen, while sliding semilandmarks were placed approximately equidistantly between them. In the subsequent analysis of shape, the eventual position of each sliding semilandmark was adjusted along the line tangent to the curve to minimize the amount of shape change between each specimen and the average of all specimens. Landmarks were digitized using tpsDig2 software

(Rohlf 2005a, software available at http://life.bio.sunysb.edu/morph/) and defined as sliders or fixed landmarks with tpsUtil (Rohlf 2004). The raw coordinate data for both fixed landmarks and sliding semilandmarks were superimposed using the Procrustes generalized least-squares procedure (Rohlf & Slice 1990) with tpsRelw v1.42 software (Rohlf 2005b).

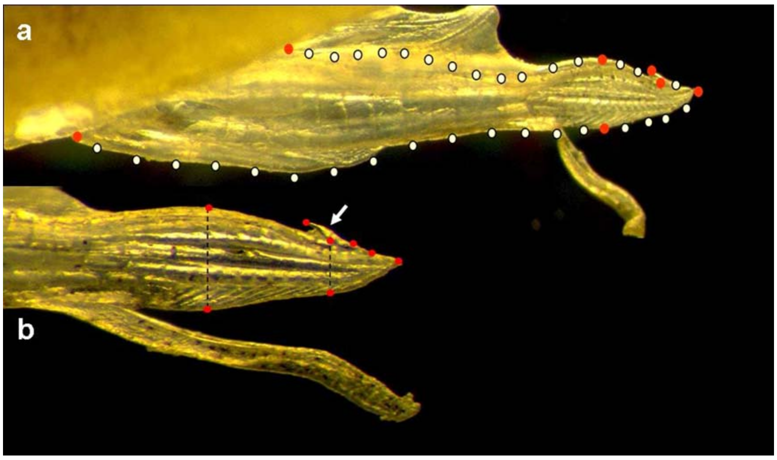

Fig. 1 Image of whole gonopodium (a) and the gonopodium's distal tip (b). Fixed landmarks (red dots) and sliding semilandmarks (white dots in upper panel) were superimposed on each image using Geometric Morphometrics software (see text for details). The genital claw at the distal tip is clearly visible from the images taken at x50 magnification (top of [b]), indicated by white arrow.

For each male, landmark data for x50 and x16 images were analysed with thin-plate spline relative warp analysis (TPSRW) using tpsRelw (v1.42) software (Rohlf 2005b). This generated partial warp scores, which describe shape variation as deviations from a consensus shape. Partial warp scores were subject to relative warp analysis, which corresponds to a principle components analysis and serves to reduce multivariate shape data to relative warps that describe most of the variation in shape. Variation in gonopodial shape across our sample could then be visualized as deformations of the thin-plate spline. Each visualization plot displays deformations of the consensus configuration corresponding to a point in the space spanned by a particular pair of relative warps. The relative warp analyses returned 5 relative warps explaining >86% and 92% of the variance in gonopodial shape in the x50 and x16 images, respectively. The TPSRW analysis also yielded centroid size for each structure, providing a measure of size that is independent of shape. Centroids were calculated as the square root of the summed square distances between each landmark and the centroid of each specimen.

Finally, we estimated linear measures for gonopodium length and width using photographs of the preserved specimens. Image analysis software (UTHSCSA Image Tool v.3.0) was used to estimate the distance from the base of the gonopodium to the distal tip, while gonopodium width was measured at the widest lateral surface of the gonopodium between the genital palp and the distal tip.

Female Genital Morphology

Approximately twenty females from each of the ten populations were included in our analysis of female genital tract morphology (sample sizes in Table 1). Immediately after collection, females were isolated individually in a 4L container until they produced their first brood. At this stage we counted the number of offspring produced by each female and preserved each brood for subsequent measures of offspring size. We measured maximal width of each offspring's head (using the eye as a landmark) to within 0.05mm for five offspring per brood. Following parturition, the female was isolated for a further 3-4 days before being killed with an overdose of anaesthetic (Benzocaine). In this way all females were preserved at approximately the same gestational stage (3-4 days postpartum) to minimise variation in reproductive tract anatomy among females. Females were then preserved individually in Dietrich's fixative until required for the morphological analyses.

We focused on linear measures of the female's oviduct, extending from the urogenital sinus to the posterior end of the egg sac. Prior to dissection, we measured each female's body length to within 0.5 mm. Dissections were subsequently carried out under a stereomicroscope. Each female was placed with her ventral side exposed and a scalpel was used to make an incision from the gills to the anus. The ventral abdomen muscles and skin were then removed to expose the peritoneal cavity. We carefully removed visceral organs and fat within the cavity, leaving the ovary and the oviduct intact. At this stage an image of each female's oviduct was captured using a digital camera (DFC480 Leica) fitted to the stereomicroscope. Images were stored and analysed using Leica IM500 image manager software. Oviduct length was taken from the point closest to the egg sac to the urogenital sinus (oviduct opening, situated between the anus and the anal fin) while oviduct width was estimated mid-way along this tract (Fig. 2). We tested for repeatability (Lessells & Boag 1987) in these measures by taking two linear measures of both

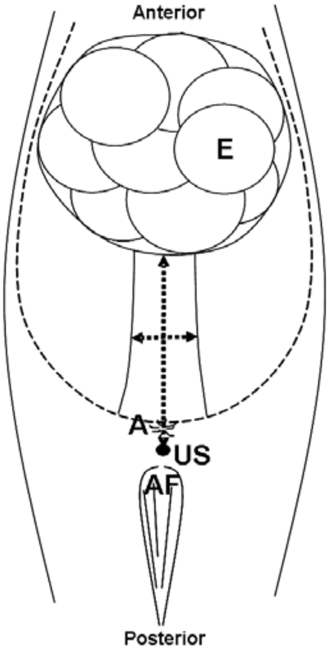

Fig. 2 Estimates of gonoduct length and width following dissection of females. Ventral view shows shows E=egg sac, A=anus, US=urigenital sinus, AF=anal fin. Dotted arrows depict length (vertical arrow from urogenital sinus to posterior end of egg sac) and width (horizontal arrow, measured midway between the gonoduct opening and E) of the gonoduct.

oviduct length and width from a sub-sample of 45 females. The two measures were performed by the same operator (CG) working blind of the identity of each sample. Intraclass correlation coefficients (*R*) for both traits were extremely high (oviduct length: ANOVA $F_{42,85}=38.2$. $P<0.001$, $R = 0.95$; oviduct width: $F_{43,87}=107.8$. $P<0.001$, $R = 0.98$).

Statistical Analysis

A General Linear Model (GLM) was used to compare linear measures for both male and female genitalia (length, width and ratios) among populations. In these analyses, predation was entered as the fixed factor (2 levels) and river as a random effect (5 levels). We controlled for variation in body size among individuals by including body length as a covariate. We ensured homogeneity of slopes in these analyses by testing the significance of the interaction between predation and the covariate (body size). These were all non-significant ($P>0.20$), satisfying the assumptions of ANCOVA. Geometric Morphometric data were also analysed with GLM, where relative warps scores (RWS) from the x50 and x16 images were entered as response variables in a multivariate model, with predation as the fixed factor and river as a random effect. To analyse the covariance between male and female genital morphology, we calculated the mean value for each trait in each population (overall, $n=10$) and used Pearson correlations to estimate correlation coefficients for each trait.

Results

Our analysis of gonopodial morphology focused on both the entire gonopodium (Fig. 1a) and the gonopodium distal tip (Fig. 1b). These analyses revealed differences in the size and shape of the gonopodium across predation gradients. In accord with previous work (Kelly et al. 2000; but see Cheng 2004), we found that males captured from high-predation populations had relatively longer gonopodia than their low-predation counterparts, when controlling for variation in body size among males (river, $F_{4,211}=5.12$, $P=0.001$; predation, $F_{1,211}=6.96$, $P=0.009$; body size [covariate], $F_{1,211}=30.80$, $P<0.0001$). Furthermore, our geometric morphometric analyses (see *Materials and Methods*) revealed highly significant variation in gonopodial shape among populations, both in whole gonopodia and the gonopodial tip (Table 1). Our primary analyses of male genital morphology focused on variation in the shape of the gonopodial tip because this is the region of the male's intromittent organ that physically interacts with females during sperm transfer. The thin plate spline relative warp (TPSRW) analysis revealed highly significant differences among populations in the first (RWS1) and third (RWS3) relative warp scores attributable to differences in predation regime (Table 1). On average, males from high-predation populations exhibited gonopodia with less elongated distal tips (Fig. 3a) and reduced gonopodial claw angles (Fig. 3b) compared to their low-predation counterparts.

Whole gonopodium with sliding semilandmarks (x16 images)

River	Population		TPSRW_1	TPSRW_2	TPSRW_3	TPSRW_4	TPSRW_5
Aripo	upstream	N=20	0.003 ± 0.034	-0.020 ± 0.016	-3.99 ± 0.015	-0.009 ± 0.011	0.0005 ± 0.010
	downstream	N=27	0.008 ± 0.042	0.023 ± 0.021	0.005 ± 0.015	0.003 ± 0.013	-0.004 ± 0.013
Oropouche	upstream	N=22	0.005 ± 0.043	-0.016 ± 0.015	0.006 ± 0.012	0.002 ± 0.009	-0.002 ± 0.007
	downstream	N=22	0.003 ± 0.023	0.009 ± 0.016	0.002 ± 0.014	0.005 ± 0.012	-0.010 ± 0.010
Quare	upstream	N=22	0.007 ± 0.040	-0.016 ± 0.014	-0.005 ± 0.014	-0.001 ± 0.010	0.002 ± 0.010
	downstream	N=20	-0.015 ± 0.022	0.017 ± 0.021	-0.006 ± 0.014	0.012 ± 0.015	0.002 ± 0.014
Tacarigua	upstream	N=21	-0.004 ± 0.034	-0.011 ± 0.015	0.006 ± 0.017	-0.001 ± 0.009	0.0001 ± 0.009
	downstream	N=23	-0.032 ± 0.046	-0.005 ± 0.017	-0.016 ± 0.016	-0.002 ± 0.010	0.005 ± 0.009
Turure	upstream	N=22	0.013 ± 0.035	0.002 ± 0.016	0.011 ± 0.012	-0.004 ± 0.010	-0.004 ± 0.007
	downstream	N=23	0.012 ± 0.050	0.015 ± 0.018	-0.001 ± 0.021	-0.003 ± 0.011	0.011 ± 0.010
	River	*F*	4.89	10.03	6.66	3.97	8.06
		P	0.0009	<0.00001	0.00005	0.004	<0.00001
	Predation	*F*	4.05	67.9	11.39	14.33	6.19
		P	0.045	<0.00001	0.0009	0.0002	0.014

Gonopodium tip with fixed landmarks (x50 images)

River	Population		TPSRW_1	TPSRW_2	TPSRW_3	TPSRW_4	TPSRW_5
Aripo	upstream	N=27	-0.014 ± 0.043	-0.005 ± 0.037	-0.012 ± 0.030	-0.002 ± 0.019	0.003 ± 0.021
	downstream	N=23	0.007 ± 0.037	-0.018 ± 0.038	0.012 ± 0.027	0.003 ± 0.019	-0.003 ± 0.018
Oropouche	upstream	N=22	0.011 ± 0.044	-0.009 ± 0.043	-0.005 ± 0.023	0.003 ± 0.019	0.0002 ± 0.013
	downstream	N=21	0.006 ± 0.039	0.005 ± 0.027	-0.001 ± 0.032	-0.011 ± 0.018	-0.002 ± 0.014
Quare	upstream	N=21	-0.009 ± 0.039	0.005 ± 0.036	-0.009 ± 0.017	-0.009 ± 0.021	0.003 ± 0.018
	downstream	N=22	0.044 ± 0.048	0.009 ± 0.028	0.008 ± 0.024	-0.002 ± 0.013	-0.010 ± 0.020
Tacarigua	upstream	N=23	-0.012 ± 0.044	-0.006 ± 0.045	-0.018 ± 0.031	0.007 ± 0.021	-0.002 ± 0.017
	downstream	N=21	0.023 ± 0.054	0.014 ± 0.022	0.017 ± 0.029	-0.007 ± 0.021	0.003 ± 0.015
Turure	upstream	N=23	-0.034 ± 0.054	0.003 ± 0.031	0.003 ± 0.036	0.009 ± 0.023	0.003 ± 0.019
	downstream	N=22	-0.016 ± 0.043	0.007 ± 0.028	0.007 ± 0.019	0.007 ± 0.014	0.004 ± 0.015
	River	F	7.75	2.041	0.639	3.087	1.185
		P	<0.00001	0.09	0.64	0.017	0.32
	Predation	F	22.01	0.349	23.1	0.072	1.864
		P	<0.00001	0.56	<0.00001	0.79	0.17

Table 1 Partial warp scores (mean ± SD) generated from thin-plate spline relative warp analysis. Populations were sampled from five river systems in Trinidad with their position within rivers being designated as either upstream or downstream. The F and P values come from a GLM model in which river was entered as the random factor and predation (high/low) as a fixed factor.

Because of the expected sexual asymmetry in optimal mating rates in polyandrous species (Parker 1979), coupled with the potential costs to females of elevated sexual harassment in high-predation populations (Magurran & Seghers 1994a), we anticipated that counter-selection would operate on female traits that may limit the success of forced matings, and that these traits would show similar (co)variance to the patterns observed in males. We specifically focused on the internal anatomy of the female's genital tract, since this is the portion of the females' reproductive system that interacts with the males' intromittent organ during copulation. In these analyses, we used linear measures to estimate the width and length of the oviduct (Fig. 2; see *Materials and Methods*).

These two measures were correlated with female body size (oviduct length, $r=0.60$, $P<0.0001$, $n=191$; oviduct width, $r=0.37$, $P<0.0001$, $n=186$), which in turn was positively correlated with mean offspring body length at birth ($r=0.30$, $P<0.0001$, $n=192$). We therefore used the residuals from a (log-log) regression of oviduct length and width on body size, and those of the oviduct width on oviduct length, as indices of oviduct shape (hereafter referred to as relative oviduct width).

Furthermore, since offspring size may influence the anatomy of female oviduct, we included mean body size of the offspring at birth as a covariate in our analyses. We found that the length of the oviduct varied significantly among populations, but this variation was due to differences in body size among populations rather than differences in the intensity of predation (river, $F_{4,184}$=3.98, P=0.004; predation, $F_{1,184}$=2.97, P=0.09; body size [covariate], $F_{1,184}$=98.45, P<0.0001). Similarly, variation in the width of the oviduct among populations was due to

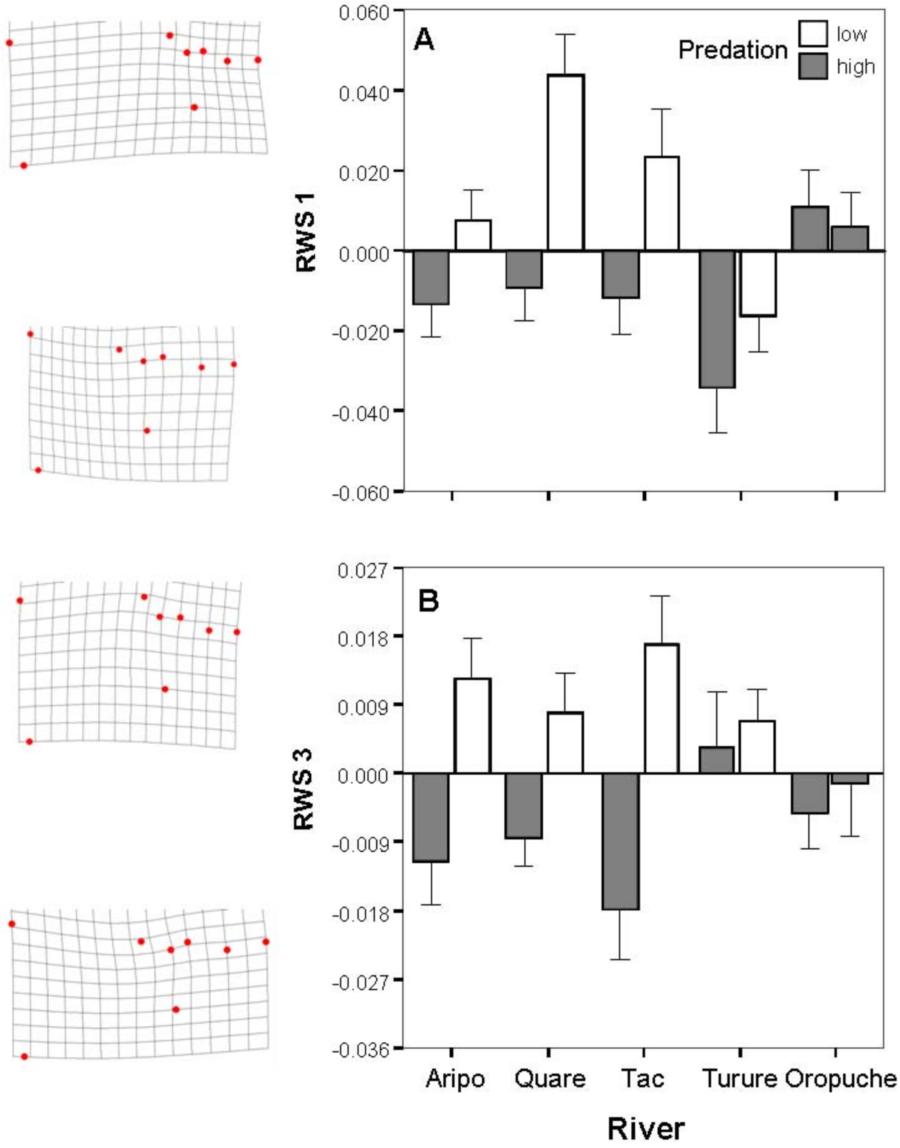

Fig. 3 Variation in gonopodial shape among natural guppy populations. Mean (± SE) values for RWS 1 (a) and RWS 3 (b) in relation to predation regime in 10 guppy populations. Deformations of the thin plate splines for each RWS are shown (panels on left hand side) for positive and negative values.

differences in body size and not predation regime (river, $F_{4,179}$=5.52, P<0.0001; predation, $F_{1,179}$=1.63, P=0.20; body size[covariate], $F_{1,179}$=39.14, P<0.0001). However, our analysis of relative oviduct width revealed a significant difference between predation regimes when controlling for variation in mean offspring size. Specifically, females from high predation populations had relatively wider oviducts than their low predation counterparts (river, $F_{4,178}$=6.84, P<0.001; predation, $F_{1,178}$=8.26, P=0.005; offspring size(covariate), $F_{1,178}$=11.44, P=0.001). Similar results were obtained when we used the residuals of oviduct width on oviduct length to compare relative oviduct width among populations (analysis not shown).

The higher relative oviduct width in high-predation females is unlikely to be due to the production of larger offspring in these populations, since females from high-predation streams tend to produce smaller, not larger offspring (Reznick & Endler 1982). Indeed, our analysis of mean offspring size among populations confirmed that high-predation females produced significantly smaller offspring than their low-predation counterparts (river $F_{4,185}$=7.516, P<0.001; predation, $F_{1,177}$=57.813, P<0.001, log transformation; predation $F_{1,177}$=30.262,

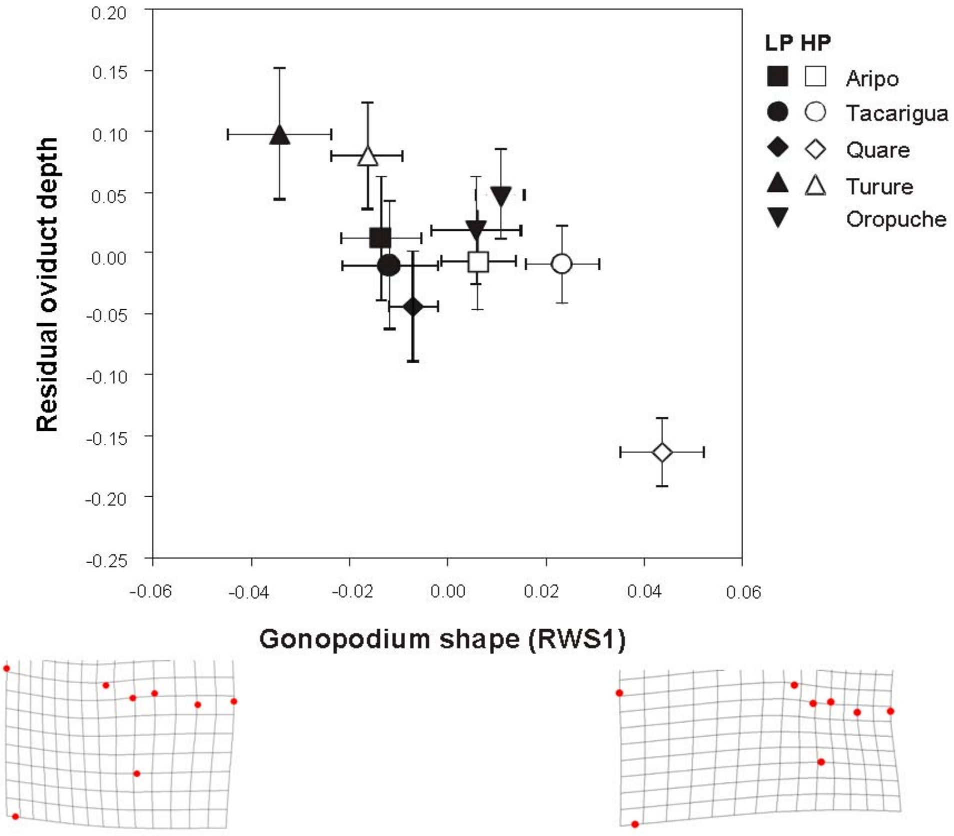

Fig. 4 The relative width of the females' oviduct in relation to the shape of male gonopodium tip. Relative oviduct width is estimated from the residuals of the regression of oviduct width on female body size. Plotted values are population means with standard errors for male (x error bars) and female (y error bars) traits. Key: Symbols depict high predation (HP) and low predation (LP) sites. Bottom panels are visualization plots for negative (x-axis, left hand side) and positive values (x-axis, right hand side) of RWS1.

$P<0.001$). Interestingly, our analyses revealed no overall significant relationship between relative oviduct width and the mean size of offspring at birth ($r=0.08$, $P=0.26$, $n=185$, log transformation), which was evident only when differences among populations in female body size and mean offspring size were accounted for (see above).

Given the highly-significant divergence among populations in both male and female genital traits, we looked for evidence of coevolutionary patterns of selection in these traits by correlating sex-specific mean values for each trait in each of the ten populations. Our analysis revealed a significant negative correlation between relative oviduct width (residuals of oviduct width on female body length) and RWS1 – the principle source of variation in male genital shape ($r=-0.769$, $n=10$, $P=0.009$); relatively wide oviducts values were associated with low RWS1 scores (i.e. with relatively shorter gonopodium tip and lower claw angle; Fig. 4).

Similar results were obtained when we used the residuals of oviduct width on oviduct length to estimate oviduct shape in the covariance analysis ($r=-0.675$, $n=10$, $P=0.032$). Relative oviduct width was also significantly and positively correlated with RWS5 (oviduct width relative to body size, $r=0.747$, $P=0.013$; oviduct width relative to oviduct length, $r=0.821$, $P=0.004$, respectively), suggesting that in populations in which males have longer claws, females tend to have relatively wider oviducts. RWS4 was significantly correlated with relative oviduct length ($r=-0.738$, $P=0.015$; residuals of oviduct length on female size, log transformation). By contrast, our analysis of relative warp scores from the images of whole gonopodia revealed no significant associations between male and female genital shape (all $P>0.11$).

Interestingly, when we calculated the ratio between gonopodium depth (male) and relative oviduct width (female), we found that the ratio differed significantly according to predation regime, but not river (GLM, Predation: $F_{1,8}=8.54$, $P=0.043$; River: $F_{4,8}=2.27$, $P=0.22$). This

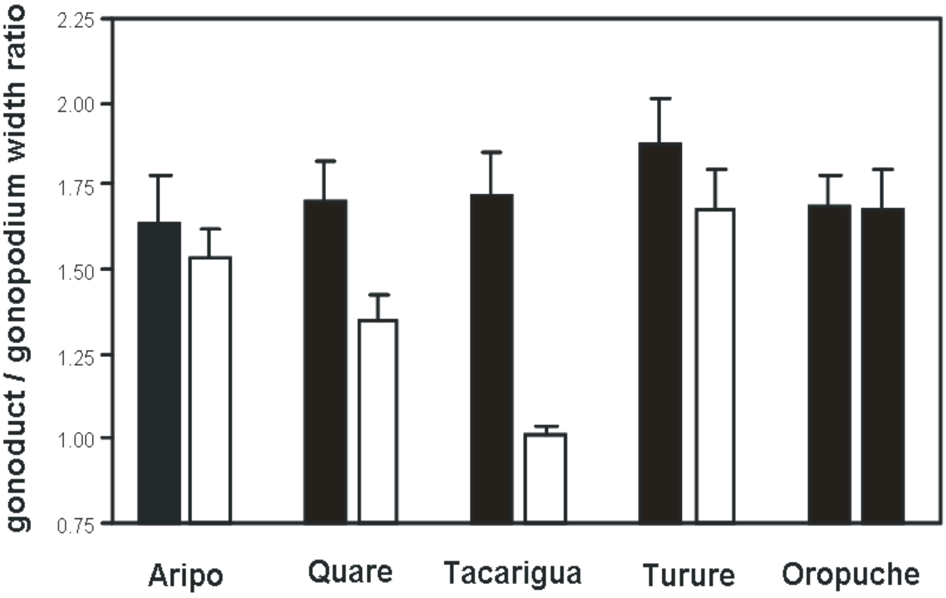

Fig. 5 The ratio of oviduct width to gonopodium width among natural guppy populations. Mean oviduct/gonopodium width ratios and their SEs were calculated by randomly pairing males and females within population over 1000 randomizations. Black bars represent high-predation populations.

latter result is consistent with our finding that these two traits covary among populations. On average, males from the high predation populations had thinner gonopodia in relation to the depth of the females' oviduct than their low predation counterparts, a pattern that was predictably absent in the two Oropouche sites that differed in stream elevation but not predation intensity (Fig. 5). Similar results were evident when we used female oviduct depth as the dependent variable, excluded river as a random effect (which was not significant), entered predation as the fixed factor, and included gonopodium depth as a covariate (predation, $F_{1,10}$=9.30, P=0.019; gonopodium depth[covariate], $F_{1,10}$=14.54, P=0.007).

Discussion

Our results, coupled with a detailed understanding of the behavior and ecology of guppies in their natural habitats, lead us to predict that males inhabiting high-predation populations exhibit adaptations that reflect their higher levels of forced mating activity, and that counter-selection on female traits may serve to reduce the success of forced matings in these populations. In concert with the observed inter-population divergence in male genital size and shape (see also Kelly et al. 2000; Cheng 2004), we found that females from high-predation populations had relatively wider oviducts than their low-predation counterparts, a finding that could not be attributed to the production of larger offspring in these populations. In high-predation streams, where the prevalence of forced matings is relatively high (Endler 1995), wider oviducts may reduce the males' ability to effectively clasp onto the female's internal reproductive tract during forced matings. Such an adaptation may therefore enable greater control by females over the duration of copulations, which has recently been shown to covary with the number of sperm transferred during matings (Pilastro et al. 2007). Furthermore, increased oviduct width may be an adaptation that reduces the damage inflicted by gonopodial claws during forced matings (Constantz 1989). Both of these possibilities warrant further investigation.

An increasing body of work suggests that male and female traits evolve in response to sexual conflict over optimal mating rates (Arnqvist & Rowe 2005). Such conflicts are likely to be pervasive in all but sexually monogamous animals where there are direct costs associated with mating, and are predicted to generate coevolutionary patterns of divergence in male (persistence) and female (resistance) traits (Arnqvist & Rowe 2005). While evidence for such coevolutionary patterns of selection is accumulating from interspecific comparative work (Arnqvist & Rowe 2002a; Arnqvist & Rowe 2002b; Huber 2003; Cordoba-Aguilar 2005; Ronn et al. 2007; Brennan et al. 2007), we know of no other study that has explicitly tested for patterns of covariance in male and female genital traits where they diverge among natural populations of the same species. Our speculation that variation in male and female genital shape reflects a history of sexually antagonistic selection on these traits is bolstered by our recent finding (**paper IV**) that males from high-predation populations are more successful at transferring sperm during forced matings when paired with females from low-predation sites, compared to when they encounter females either from their own population or those originating from a different high-predation population. Nevertheless, in order to provide further support for sexually antagonistic selection it is necessary to demonstrate that variation in male genital shape influences female fitness, and that there is sufficient additive genetic variance underlying the expression of these traits to permit a response to selection.

Riassunto generale

Il conflitto sessuale è un fenomeno ubiquitario in natura e interessa molte delle interazioni maschio-femmina portando alla evoluzione di caratteristiche sessuali antagonistiche. Per definizione, nel conflitto sessuale una caratteristica che avvantaggia un sesso comporta dei costi per l'altro sesso, per cui di conseguenza in quest'ultimo viene favorita la comparsa di controadattamenti in una sorta di 'corsa agli armamenti', in cui ogni cambiamento evoluzionistico che appare in un sesso è seguito da un cambiamento nell'altro sesso volto a minimizzarne i costi. La frequenza degli accoppiamenti è uno degli aspetti cruciali in cui il conflitto sessuale si manifesta e porta alla selezione sessuale antagonistica perché in generale i maschi hanno una frequenza ottimale di accoppiamenti che è maggiore di quella delle femmine. La mia tesi di dottorato ha avuto lo scopo di studiare alcuni aspetti del conflitto sessuale utilizzando *Poecilia reticulata* come modello di studio. Questa specie è particolarmente adatta per lo studio di queste tematiche, dal momento che è evidente la presenza del conflitto sessuale per quanto riguarda il numero di accoppiamenti, come si evince chiaramente dall'alto livello di sexual harassment (i continui tentativi di copula da parte dei maschi) subito dalle femmine. Le ricerche che ho effettuato per la mia tesi di dottorato si possono suddividere in due filoni. Primo: costi e benefici del conflitto sessuale. Secondo: coevoluzione sessuale antagonistica.

Costi e benefici del conflitto sessuale. Studiare costi e benefici legati al conflitto sessuale è importante per capire l'evoluzione della resistenza femminile che si assume si sia evoluta con lo scopo di ridurre i costi legati agli accoppiamenti superflui. Di recente tuttavia si è avanzata l'ipotesi che la resistenza evolva attraverso il meccanismo dei benefici indiretti ottenuti selezionando i maschi più persistenti. Il primo lavoro ha riguardato lo studio degli effetti del sexual harassment sulla fecondità life-time delle femmine, ma anche degli effetti sulla prole. I risultati indicano che il costo del sexual harassment è visibile sulla prole, sia nei maschi che nelle femmine. Questi risultati non supportano perciò l'ipotesi che la resistenza femminile evolva attraverso l'acquisizione di benefici indiretti. Inoltre lo studio sull'ereditabilità di tratti maschili legati al successo riproduttivo ha dimostrato che tali caratteristiche sono poco ereditabili per via paterna, presupposto essenziale per questa ipotesi. La plasticità adattativa dei caratteri ne può spiegare la scarsa ereditabilità paterna riscontrata. Ho testato questa possibilità per un tratto legato al successo riproduttivo, la velocità spermatica, a seconda delle opportunità di accoppiamento percepite dal maschio. Si è dimostrato che i maschi sono in grado di modulare la velocità spermatica, confermando perciò la possibilità di un certo grado di plasticità che può spiegare i bassi valori di ereditabilità.

Coevoluzione sessuale antagonistica. Lo studio della coevoluzione sessuale antagonistica si è concentrato sulla morfologia dei genitali, sia maschili che femminili. I risultati indicano che la morfologia dell'organo copulatore influenza il successo nel maschio sia negli accoppiamenti cooperativi che in quelli forzati. In particolare la dimensione e la forma generale dell'organo copulatore sono sessualmente selezionati attraverso la scelta femminile, e le femmine si accoppiano più velocemente con maschi dotati di gonopodi più lunghi e di forma allargata. Anche la forma della parte più distale del gonopodio risulta importante nel determinare il trasferimento degli spermi.

La coevoluzione sessuale antagonistica prevede che quando una caratteristica modellata dal conflitto sessuale differisce tra popolazioni diverse, le femmine siano più resistenti ai maschi della propria popolazione, con cui si sono coevolute, rispetto a quelli di popolazioni diverse contro cui esse non hanno gli adattamenti necessari per resistere ad accoppiamenti non voluti.

Questa previsione è stata confermata attraverso uno studio su maschi e femmine di popolazioni a diverso conflitto sessuale, in cui è stato dimostrato che femmine poste con maschi provenienti da una popolazione con un maggiore grado di conflitto sessuale hanno un minor controllo sul trasferimento degli spermi, rispetto a femmine che hanno avuto la possibilità di coevolvere con tali maschi. Infine uno studio comparativo fra popolazioni diverse indica l'esistenza di coevoluzione tra genitali maschili e femminili, che varia a seconda del grado di conflitto sessuale a cui le popolazioni sono soggette. Per cui l'evoluzione dei genitali sembra essere il risultato di una corsa agli armamenti tra maschi e femmine per il controllo della frequenza degli accoppiamenti e del successo di inseminazione.

Riassunto esteso

Introduzione

La visione tradizionale della riproduzione è quella di un'armoniosa cooperazione fra i due sessi con lo scopo comune di lasciare il maggior numero di discendenti alla generazione successiva. In realtà, gli interessi evolutivi dei genitori divergono almeno in parte nel momento in cui l'investimento riproduttivo di un genitore riduce le sue prospettive riproduttive future (Trivers 1972). Dato che maschi e femmine hanno un diverso investimento nella produzione dei gameti, ne risulta che le strategie che massimizzano il successo riproduttivo generalmente differiscono tra i due sessi generando un conflitto sessuale (Parker 1979).

Per definizione i maschi producono gameti, gli spermi, il cui costo unitario è minore rispetto a quelli femminili, le uova. Questa situazione di partenza fa sì che i maschi possano aumentare il successo riproduttivo aumentando il numero di partner sessuali, mentre le femmine, limitate nella loro fecondità, possono massimizzare il successo riproduttivo aumentando la qualità della prole, per esempio scegliendo un partner di qualità genetica elevata. Nei maschi la selezione sessuale favorirà quindi individui in grado di assicurarsi il maggior numero di partner sessuali, mentre nel sesso femminile saranno avvantaggiati gli individui in grado di accoppiarsi con i partner di migliore qualità. Tra tutte le interazioni tra i due sessi legate alla riproduzione, tipicamente sfociano nel conflitto il numero degli accoppiamenti, l'utilizzo degli spermi e la suddivisione delle cure parentali laddove queste esistono (Arnqvist & Rowe 2005). Ad esempio, in *Drosophila* durante l'accoppiamento i maschi trasferiscono con l'eiaculato un cocktail di sostanze che influenzano diversi aspetti della riproduzione, come il trasferimento degli spermi ed il loro utilizzo, la recettività della femmina, l'ovulazione e l'oogenesi (riassunte in Wolfner 1997). Queste sostanze avvantaggiano la fitness del maschio che le produce, ma causano nelle femmine una diminuzione della durata della vita e del successo riproduttivo (Chapman et al. 1995). Questa ridotta fecondità viene compensata, nel maschio, dall'accoppiamento con altre femmine, ma determinano nelle femmine l'evoluzione di controadattamenti volti a limitare il costo degli accoppiamenti; questo meccanismo che prevede una serie di adattamenti seguiti da controadattamenti, viene definito coevoluzione sessuale antagonistica.

Il risultato atteso della coevoluzione sessuale antagonistica è una riduzione della fitness media delle femmine, e quindi della popolazione (Arnqvist & Rowe 2005). È stato però suggerito (Cordero & Eberhard 2003; Pizzari & Snook 2004) che le femmine, aumentando la resistenza agli accoppiamenti selezionino i maschi più persistenti, compensando, se non superando, in questo modo i costi diretti del conflitto. Infatti, se le caratteristiche di persistenza maschile sono ereditabili, le femmine resistenti produrrebbero figli maschi a loro volta più persistenti e con un successo riproduttivo atteso maggiore, secondo un meccanismo simile a quello proposto da Fisher per l'evoluzione degli ornamenti maschili attraverso la scelta femminile (modello del *sexy sons*, (Fisher 1930). Anche se i modelli teorici sembrano escludere la possibilità che un tale effetto *sexy sons* possa mantenere una scelta femminile costosa all'equilibrio (Cameron et al. 2003), pochi tentativi sono stati finora condotti per quantificare i costi diretti e gli eventuali benefici indiretti della coevoluzione antagonistica sessuale.

Scopo

Il lavoro della mia tesi di dottorato si può suddividere in due filoni principali volti a studiare diverse tematiche legate al conflitto sessuale:

A) Costi e benefici del conflitto sessuale: studio del costo del conflitto sessuale in termini di fecondità *life-time* delle femmine ed effetti diretti ed indiretti sulla prole (articolo I), stima dell'ereditabilità di caratteri maschili come misura indiretta dei potenziali benefici indiretti della resistenza femminile (articolo II) e studio della plasticità nella qualità spermatica in relazione alle opportunità di accoppiamento (articolo III).

B) Coevoluzione sessuale antagonistica nell'evoluzione dei genitali: studio del ruolo delle caratteristiche morfologiche dei genitali maschili in copule forzate (articolo IV) e in copule cooperative (articolo V) e stima tra popolazioni della variazione morfologica dei genitali maschili e femminili in relazione al livello di conflitto sessuale (articolo VI).

Specie utilizzata

Il modello scelto per studiare queste tematiche è un piccolo pesce teleosteo a fecondazione interna, il guppy (*Poecilia reticulata*). Questo pesce è una specie modello per studi di selezione sessuale (Houde 1997) ed è caratterizzato da un sistema di accoppiamento non basato su risorse materiali (i maschi contribuiscono alla riproduzione con i soli spermatozoi) e da un intenso grado di competizione spermatica dovuta al fatto che le femmine si accoppiano con più di un maschio durante il periodo fertile e possono conservare gli spermi nelle pliche dell'ovario per oltre sei mesi (Constantz 1989). Le femmine sono sessualmente recettive per due/tre giorni dopo il parto o quando sono vergini, mentre non sono più interessate all'accoppiamento per il resto del ciclo riproduttivo. La fecondazione è interna: il maschio trasferisce gli spermi nel gonodotto della femmina mediante il gonopodio, un organo copulatore derivante dalla modificazione della pinna anale. La gestazione dura in media 20-30 giorni, durante i quali gli embrioni si sviluppano completamente all'interno dell'ovario della femmina (Constantz 1989).

Il dimorfismo sessuale è particolarmente evidente nella colorazione, mimetica nelle femmine e vistosa nei maschi, composta da macchie arancioni e gialle (formate per lo più da carotenoidi, ma anche da altri pigmenti), melaniche, e iridescenti, di colore blu-verde. I maschi presentano due tattiche riproduttive alternative (Liley 1966): corteggiamento ('sigmoid display' SD) o copula forzata ('gonopodial thrust' GT). Un individuo può adottare una o l'altra tattica a seconda, per esempio della recettività della femmina, del rischio di predazione, delle condizioni di visibilità e della sex ratio. I maschi molestano in continuazione le femmine nel tentativo di accoppiarsi e il livello di 'sexual harassment' (i continui tentativi di copula da parte dei maschi) è molto elevato, pari a circa un tentativo di copula forzata al minuto (Magurran & Seghers 1994a). Anche se le copule forzate normalmente risultano in un basso numero di spermi inseminati, occasionalmente questo numero è simile a quello osservato durante le copule cooperative (Pilastro & Bisazza 1999). Le femmine mostrano una preferenza per i maschi più colorati (principalmente in relazione all'estensione delle macchie arancioni) e che esibiscono un elevato tasso di corteggiamento (Houde 1997). Maschi più colorati hanno inoltre un maggior successo nella competizione spermatica (Evans & Magurran 2001; Evans et al. 2003c), determinato dal fatto che sono in grado di inseminare più spermi (Pilastro et al. 2002; Pilastro et al. 2004) e di produrre eiaculati più competitivi (Locatello et al. 2006).

A) Costi e benefici del conflitto sessuale

In *Poecilia reticulata* il conflitto sessuale è in particolar modo evidente per quanto riguarda il numero di accoppiamenti. I maschi cercano di massimizzare il numero di femmine con cui si accoppiano, mentre le femmine non necessitano di molti accoppiamenti per assicurasi la fecondazione delle uova, dato che un singolo accoppiamento è normalmente sufficiente (Pilastro et al. 2008). Al contrario, un elevato tasso di accoppiamento comporta dei costi per la femmina in termini di aumento del rischio di predazione, di trasmissione di parassiti e di riduzione del tempo di foraggiamento. I costi del sexual harassment, in termini di conseguenze sull'abilità di foraggiamento, sono stati dimostrati in diverse specie di pecilidi come in *Gambusia holbrooki* (Pilastro et al. 2003), la stessa *P. reticulata* (Magurran & Seghers 1994a) e in altre specie dello stesso genere (Plath et al. 2007). La riduzione del tasso di foraggiamento dovrebbe risultare in una minore fecondità, ma gli studi che hanno finora esaminato il costo del sexual harassment in termini di successo riproduttivo si sono limitati allo studio di uno o pochi cicli riproduttivi e hanno dato risultati discordanti (Smith & Sargent 2006; Head & Brooks 2006; Ojanguren & Magurran 2007).

Per questa ragione ho condotto uno studio (articolo I) sugli effetti del conflitto sessuale che si è esteso alla fecondità *life-time* delle femmine. Diversamente dai lavori sopra citati, per la prima volta, non sono stati considerati solo pochi cicli riproduttivi ma tutta la durata della vita della femmina, per avere un'idea completa degli effetti *life-time* del sexual harassment. Gli effetti potrebbero però manifestarsi non solo sulla fecondità della femmina, ma anche, o solo, sulla qualità della prole, sia come conseguenza di effetti diretti sulla femmina che si ripercuotono sulla prole (ad esempio una femmina nutrita male potrebbe investire meno risorse nella formazione delle uova con conseguente scarsa qualità dei figli) ma anche come conseguenza di effetti genetici legati a una diminuzione della capacità delle femmine di esercitare la scelta sessuale, con conseguente riduzione dei benefici genetici legati alla scelta femminile (Nicoletto 1995). In contrasto con il punto di vista che nelle femmine la resistenza si sia evoluta per diminuire i costi diretti imposti dal conflitto sessuale, è stato suggerito da alcuni autori che la resistenza femminile sia mantenuta da una selezione indiretta per i benefici genetici derivanti da tale comportamento (Eberhard 1996; Cordero & Eberhard 2003). Secondo questa ipotesi la resistenza femminile rappresenterebbe un modo per le femmine di selezionare i maschi con caratteristiche migliori. Maschi più persistenti, cioè più ostinati nel tentare di accoppiarsi, dovrebbero essere anche quelli di migliore qualità e più abili nel fecondare le uova, per cui le femmine, aumentando il livello di resistenza, dovrebbero beneficiare del fatto di produrre figli maschi di qualità altrettanto buona, e/o altrettanto abili nel fecondare le uova. Valutare la qualità della prole, alla nascita e dopo lo sviluppo sessuale ha avuto lo scopo di testare entrambe le ipotesi. Se i costi del sexual harassment si manifestano sulla prole, la previsione è di trovare degli effetti negativi sui figli delle femmine che subiscono un livello di harassment maggiore. Al contrario, se le femmine ricavano qualche tipo di beneficio dal selezionare maschi più persistenti, ci si aspetta di trovare di migliore qualità i figli di femmine sottoposte ad alto harassment; in particolare i figli maschi potrebbero aver ereditato dai padri più persistenti alcuni tratti importanti nel determinare il successo riproduttivo, come comportamento sessuale e qualità dell'eiaculato.

Quest'ultima ipotesi si basa sul fatto che i maschi persistenti generino figli maschi altrettanto persistenti e che quindi i tratti che regolano il successo riproduttivo siano ereditabili per via paterna. Per cui ho stimato (articolo II) l'ereditabilità paterna del comportamento sessuale (ovvero l'utilizzo di sigmoid display e di gonopodial thrust) e di altre caratteristiche legate all'eiaculato (come velocità e quantità). I valori di ereditabilità di queste caratteristiche sono stati rapportati a quelli trovati per la colorazione della livrea, che sono stati dimostrati essere legati al cromosoma Y (Houde 1992). La stima dell'ereditabilità di queste caratteristiche però è complicata da diversi fattori, come la componente materna, l'erosione genetica dovuta alla selezione stabilizzante e la plasticità dei caratteri. Per quanto riguarda la velocità degli spermi è stato suggerito da numerosi studi che questo carattere di qualità spermatica sia associato con la capacità di fecondazione (Snook 2005), ma non è stato dimostrato se questo carattere possa essere modulabile dall'individuo in relazione alla possibilità di accoppiamento. Per cui ho condotto un esperimento (articolo III) per valutare se i maschi allochino l'investimento nella produzione di spermi di elevata velocità in relazione alle possibilità di accoppiamento.

Articolo I

Il disegno sperimentale ha previsto l'utilizzo di 62 femmine che sono state divise in due gruppi e mantenute, per tutta la loro vita, a due opposti livelli di harassment: 'basso conflitto' (LC) ed 'alto conflitto' (HC). È stato misurato il successo riproduttivo *life-time* espresso come numero dei piccoli partoriti nell'arco della vita, la durata delle gestazioni, l'accrescimento corporeo (peso e lunghezza) e il tasso di mortalità.

Le femmine del gruppo a basso conflitto (LC) sono state in contatto con i maschi per circa 24 ore durante il periodo in cui erano sessualmente recettive (cioè nel giorno successivo al parto); le femmine appartenenti al gruppo alto conflitto (HC) sono invece state esposte all'harassment dei maschi per otto giorni consecutivi nel periodo in cui non erano sessualmente recettive. Questi trattamenti sono stati ripetuti ad ogni ciclo riproduttivo fino alla morte della femmina. Il trattamento non ha avuto effetto sull'accrescimento e sulla mortalità delle femmine. Per quanto riguarda la fecondità, le femmine HC hanno prodotto un numero maggiore di piccoli fino al quinto parto, ma dal sesto parto in poi vi è stata un'inversione di tendenza e le femmine LC sono risultate più prolifiche. Complessivamente la fecondità delle femmine non è risultata diversa tra i due gruppi, ma è risultato significativamente diverso il pattern temporale di allocazione riproduttiva (wald chi square=6.30, P=0.012). Dai risultati, perciò, si evince che le femmine allocano differenzialmente il loro investimento riproduttivo in relazione al livello di harassment. Questo può essere spiegato dal fatto che il sexual harassment rende più visibili, in natura, a eventuali predatori, aumentando perciò il rischio di mortalità o la percezione di tale rischio in condizioni di laboratorio. Se una femmina perciò percepisce un maggiore rischio di predazione è portata ad investire più risorse nei primi parti, diminuendo poi l'investimento nei cicli successivi. Le femmine in condizioni di poco harassment mantengono invece l'investimento riproduttivo linearmente, aumentando il numero di figli con l'aumentare delle dimensioni corporee.

I figli nati dopo quattro cicli di trattamento sono stati allevati in condizioni standard per determinarne il tasso di accrescimento. I piccoli sono stati misurati in tre diversi momenti e non sono state trovate differenze tra i due gruppi per quanto riguarda le dimensioni alla nascita e dopo un mese, mentre dopo quattro mesi (quando sono sessualmente maturi) le figlie delle femmine LC risultano più grandi rispetto a quelli delle femmine HC (F=4.911, P=0.027). Le figlie delle femmine LC presentano perciò dimensioni maggiori a parità di condizioni di crescita, indicando un maggior successo riproduttivo atteso (in questa specie, infatti, il numero di uova prodotte è positivamente legato alle dimensioni corporee (Reznick & Endler 1982).

Una volta raggiunta la maturità sessuale sui figli maschi sono stati condotti diversi test per valutarne il successo riproduttivo (per comodità i figli delle femmine appartenenti al gruppo LC e al gruppo HC sono stati a loro volta denominati nello stesso modo). Per prima cosa è stata valutata la preferenza femminile pre-copulatoria attraverso un test di scelta dicotomica, quindi è stato valutato il comportamento sessuale. E’ stato misurato anche lo sviluppo dei caratteri sessuali secondari, misurando la colorazione presente nella livrea in relazione alle dimensioni del maschio. Infine, su un sottocampione, sono state misurate alcune caratteristiche dell’eiaculato: il numero e la velocità degli spermatozoi. Non sono state trovate differenze nel fenotipo per quanto riguarda la colorazione, ma i maschi LC presentano gonopodi più lunghi (F=4.882, P=0.029), inoltre vengono preferiti a livello precopulatorio (F=5.717, P=0.018) e risultano più abili nell’ottenere copule cooperative (Wald chi square=4.86, P=0.028). In conclusione i maschi HC risultano avere un successo riproduttivo atteso minore dei maschi LC, suggerendo anche qui come per le femmine costi del sexual harrasment visibili a livello della prole.

Questi risultati suggeriscono che in *Poecilia reticulata* è poco plausibile l’ipotesi che le femmine attraverso la resistenza possano ottenere benefici indiretti che superino i costi diretti derivanti dal sexual harassment.

Articolo II

Questo esperimento si è proposto di valutare l’ereditabilità per via paterna di: comportamento sessuale (preferenza nell’utilizzo di copule cooperative o coercitive) e caratteristiche dell’eiaculato (qualità e quantità). L’approccio di genetica quantitativa utilizzato ha permesso di calcolare il coefficiente di variazione genetica additiva (CVa) chiamato ‘evolvability’ che indica la potenzialità di un carattere di rispondere alla selezione, quindi di evolvere (Houle 1992). L’esperimento è consistito in un breeding design half-sibs/ full-sibs, basato sul confronto della varianza fenotipica per i caratteri interessati in linee di fratelli e fratellastri ottenuti dall’incrocio di 34 maschi ciascuno con due femmine non imparentate. I figli maschi ottenuti (in totale 238) sono stati allevati in condizioni standard. Una volta raggiunta la maturità sessuale i soggetti sono stati sottoposti a test ripetuti in cui è stato misurato il comportamento sessuale con femmine non recettive. I risultati mostrano che i maschi presentano una significativa ripetibilità individuale nella frequenza di adozione delle due tattiche alternative di accoppiamento (display: r= 0.284, P<0.001; gonopodial thrust: r=0.186, P=0.007, proporzione display su GT: r=0.293, P<0.001), ma questa non ha una significativa ereditabilità paterna (vedi tabella). I risultati ottenuti confermano altri studi analoghi volti a indagare l’ereditabilità dei pattern comportamentali, in cui si conclude che ai caratteri comportamentali sono associati i livelli più bassi di ereditabilità, che spesso risulta assente o comunque molto bassa.

Discutendo i risultati trovati si possono trovare più spiegazioni non mutualmente esclusive. Primo, sebbene la selezione stabilizzante tenda ad eliminare la varianza genetica additiva, altri processi concomitanti possono intervenire nel mantenere un certo grado di variabilità come selezioni frequenza-dipendente che premiano il fenotipo raro (Mousseau & Roff 1987). Secondo, in laboratorio sono presenti meno variazioni ambientali che in un ambiente naturale, ed essendo l'ereditabilità di un carattere legata alla variabilità dell'ambiente e alla struttura genetica della popolazione (Lynch & Walsh 1998), in laboratorio possono vedersi pattern diversi rispetto a popolazioni naturali. Terzo, potrebbe risultare più adattativo per gli individui un comportamento plastico, piuttosto che un comportamento rigido e fissato, per rispondere in maniera ottimale alle variazioni dell'ambiente e/o alle proprie condizioni. Utilizzando i medesimi esemplari è stata stimata l'ereditabilità delle caratteristiche dell'eiaculato (quantità e velocità spermatica). Nessuna delle caratteristiche prese in considerazione presenta una significativa ereditabilità per via paterna, ad eccezione della quantità di spermi prodotti (vedi tabella) .

Variabile	**df**	**F**	***P***	**CV_A**	**h^2**
Display	32	1.264	0.252	21.85	0.151
Gonopodial thrust	32	1.125	0.369	26.44	0.170
% arancio	32	4.377	<0.001	46.08	1.921
Lunghezza standard	32	1.203	0.306	2.55	0.165
VAP	32	1.396	0.178	10.08	0.342
Numero spermi	32	3.321	0.001	66.37	1.151

Tabella: stima dell'ereditabilità paterna per alcuni dei caratteri considerati

Il valore di CV_A (66.37) per la quantità di spermi prodotti è risultato superiore ai valori di CV_A riscontrati per le caratteristiche di colorazione della livrea, come l'estensione delle macchie arancioni (46.08), che sono legate al cromosoma Y (Houde 1992). Questo risultato è in linea con altri lavori in cui è stato suggerito che la dimensione dei testicoli e la quantità di spermi prodotti hanno un'ereditabilità maggiore rispetto ad altri caratteri, come la velocità spermatica, che sono più importanti nel determinare il successo di fecondazione, e che presentano valori molto bassi di ereditabilità (Roff & Mousseau 1987).

Il basso valore di varianza genetica additiva e la mancata ereditabilità paterna per la velocità spermatica potrebbero dipendere dall'intensa pressione selettiva direzionale dovuta all'elevata competizione spermatica presente in questa specie, che agisce su tali caratteristiche portando ad un'erosione della variabilità genetica. Un'altra spiegazione plausibile, come discusso per la parte di ereditabilità del comportamento sessuale, potrebbe identificarsi in una plasticità adattativa del carattere. Un individuo potrebbe beneficiare della plasticità di questo carattere per massimizzare il suo successo riproduttivo, allocando strategicamente le risorse per la produzione di spermi di elevata qualità (in questo caso con elevata velocità spermatica). Se produrre spermi di elevata qualità è costoso, la previsione è che ad un individuo convenga allocare meno risorse quando le probabilità di accoppiamento sono scarse.

Articolo III

Lo scopo di questo esperimento è valutare se esista una differenza nell'allocazione della qualità dell'eiaculato fra maschi in presenza o in assenza di femmine. È già stato dimostrato che i maschi di *P. reticulata* producono una maggiore quantità di spermi quando sono in presenza delle femmine rispetto a quando non lo sono (Bozynski & Liley 2003). Questo indica che i maschi possono regolare lo sforzo riproduttivo in termini di spermi prodotti, e l'innovazione di questo esperimento sta nel verificare se questa plasticità esiste anche per la qualità degli spermi. Il disegno sperimentale ha previsto l'utilizzo di 46 maschi, divisi in due gruppi sperimentali. Inizialmente tutti gli individui sono stati ripuliti dagli spermi disponibili, e sono quindi stati sottoposti al trattamento, per tre giorni consecutivi, che prevedeva la presenza (solo visiva) o l'assenza delle femmine. Dopo il trattamento i maschi sono stati fotografati e sono stati prelevati gli spermi per le analisi di velocità. Il trattamento, presenza o assenza delle femmine, influenza significativamente la velocità di nuoto degli spermi, con i maschi esposti alla presenza delle femmine che presentano spermi più veloci (VAP: $F_{1,44}=6.784$, $P=0.012$).

Questi risultati ci portano perciò alla conclusione che i maschi in questa specie allocano strategicamente le risorse investite nella produzione degli spermi, riducendo la qualità degli spermi quando le opportunità di accoppiamento sono scarse.

Conclusioni

Attraverso lo studio degli effetti *life-time* e degli effetti sulla qualità della prole si è dimostrato un costo indiretto del sexual harassment a livello di successo riproduttivo atteso sia per le figlie femmine che per i figli maschi, ma nessun effetto indiretto che possa dare supporto all'ipotesi che la resistenza evolva grazie all'acquisizione di benefici indiretti per la prole (Pizzari & Snook 2003; Cordero & Eberhard 2003). I benefici indiretti perciò non rappresentano, almeno in questa popolazione, una forza selettiva paragonabile, e tanto meno maggiore, all'evoluzione della resistenza per ridurre i costi diretti. Questo è inoltre dimostrato, indirettamente, dal fatto che le caratteristiche coinvolte nel determinare il successo di fecondazione di un maschio non sono ereditabili per via paterna. Né il comportamento sessuale, né le caratteristiche dell'eiaculato (ma si veda la discussione nell'articolo per quanto riguarda la produzione di spermi) hanno, infatti, rivelato significativi valori di varianza genetica additiva. Fra le varie spiegazioni esiste la possibilità che queste caratteristiche non presentino forte ereditabilità in quanto risulti più conveniente mantenere certe caratteristiche plastiche, che sono più adattabili all'ambiente ed al contesto. A riguardo è stato dimostrato che i maschi possono modulare la qualità degli spermi in relazione alle possibilità di accoppiamento, che pertanto non è compatibile con un'elevata ereditabilità.

B) Coevoluzione sessuale antagonistica

Caratteri legati al successo riproduttivo sembrano evolvere molto più velocemente di altri, anche in specie filogeneticamente molto vicine tra loro (Andersson 1994). La morfologia dei genitali, in particolare quella degli organi copulatori maschili, mostra una diversificazione enorme e la loro variabilità è spesso usata dai tassonomi per distinguere fra loro specie altrimenti difficilmente riconoscibili (Mayr 1963). Il motivo per cui questi tratti tendano ad evolvere così rapidamente è ancora oggetto di dibattito (Hosken & Stockley 2004), ma una delle possibili spiegazioni è una conseguenza del conflitto sessuale: la coevoluzione sessuale antagonistica, ovvero una continua 'corsa agli armamenti' evolutiva, in cui ogni cambiamento in un sesso provoca un cambiamento nell'altro (Rice & Holland 1997). Nonostante ci siano esempi di adattamenti e contro adattamenti a tutti i livelli (morfologici, fisiologici e comportamentali, (Arnqvist & Rowe 2005), in particolare sono stati dimostrati pattern di coevoluzione tra genitali maschili e femminili.

Nella famiglia dei pecilidi i maschi trasferiscono gli spermi inserendo la punta del gonopodio, dotata di una serie di uncini, all'interno del gonoporo femminile (Rosen & Gordon 1953). La lunghezza del gonopodio varia notevolmente fra specie, misurando dal 20% fino al 70% della lunghezza del corpo. Sono sicuramente numerosi i fattori che influenzano la lunghezza del gonopodio, fra cui la selezione sessuale e il conflitto sessuale. La lunghezza del gonopodio è positivamente legata alla preferenza femminile in *Gambusia affinis*, anche se comporta uno svantaggio nell'evitare i predatori, dal momento che riduce la velocità di nuoto (Langerhans et al. 2005). La lunghezza e la morfologia del gonopodio potrebbero essere caratteristiche modellate dal conflitto tra i sessi perché un gonopodio più lungo si pensa favorisca i maschi nelle copule sneaky. A rinforzo di questa ipotesi, analisi comparative dimostrano che le specie in cui l'accoppiamento avviene solo con la modalità coercitiva possiedono un gonopodio relativamente più lungo rispetto a specie che presentano anche il corteggiamento (Rosen & Tucker 1961). Ho quindi valutato l'importanza della morfologia del gonopodio in *P. reticulata* sia a livello di copule forzate (articolo IV) che di copule cooperative (articolo V).

Se la morfologia dell'organo copulatore è modellata da una situazione di conflitto sessuale, una previsione è che questa si diversifichi in relazione ai diversi livelli di conflitto sessuale, a riprova della peculiare coevoluzione tra i sessi. *Poecilia reticulata* è un modello di studio ideale da questo punto di vista in quanto le popolazioni presenti nei diversi corsi d'acqua dell'isola di Trinidad (di cui sono originari questi pesci) si sono evolute separatamente, differenziandosi in relazione all'ecologia ed, in particolare, alla diversa pressione predatoria che le caratterizza. Quest'ultima influenza il comportamento sessuale dei maschi, i quali nelle popolazioni ad alta predazione tendono a fare più copule sneaky; di conseguenza le femmine in queste popolazioni sono soggette a livelli di harassment più elevati (Magurran 2005). Ho studiato quindi (articolo VI) la diversificazione nella morfologia dei genitali maschili in relazione al grado di conflitto sessuale nelle diverse popolazioni e la variazione nella morfologia dei genitali femminili per verificare la presenza di coevoluzione sessuale antagonistica fra questi due tratti.

Articolo IV

Scopo di questo esperimento è di valutare il ruolo della morfologia dei genitali nel successo delle copule sneaky e di stimare il successo, relativamente alle copule coercitive, di maschi provenienti da una popolazione ad alta predazione (con livelli maggiori di conflitto sessuale) nei confronti di femmine provenienti da popolazioni ad alta e bassa predazione. Visto dal punto di vista femminile, se si sono evoluti controadattamenti per minimizzare i costi di accoppiamenti non voluti ci si aspetta che le femmine siano più resistenti a questa tattica in popolazioni in cui le femmine sono più soggette al conflitto sessuale e viceversa. In altre parole ci si aspetta che le femmine siano più adattate a maschi della propria popolazione (con cui si sono coevolute) rispetto che a maschi di popolazioni diverse. Sono stati testati maschi provenienti da una popolazione ad alta predazione (n=108) con: (a) femmine provenienti dalla stessa popolazione (n=36), (b) femmine da una popolazione dello stesso corso d'acqua ma caratterizzato da basso regime predatorio (n=36) e (c) femmine provenienti da un altro corso d'acqua ma con lo stesso elevato regime predatorio della popolazione d'origine dei maschi (n=36). Le femmine sono sempre state testate nel medesimo momento del ciclo riproduttivo (14 giorni dopo il parto) per assicurarsi la non recettività delle stesse. Un maschio ed una femmina per volta sono stati lasciati interagire per un totale di 5 ore durante le quali sono stati osservati per 15 minuti, per stimare, in particolare, il tasso di 'genital contact', cioè gonopodial thrusts che risultano in un contatto fra i genitali, con probabile trasferimento di spermi. Sono stati prelevati gli spermi presenti nel gonodotto femminile, valutando la loro presenza o assenza (successo di inseminazione) e il loro numero. Sono state analizzate sia le dimensioni lineari (lunghezza e larghezza) che la forma del gonopodio, analizzata mediante la morfometria geometrica (Rohlf & Marcus 1993) usando il programma TPS. I risultati indicano che i maschi hanno più successo, stimato nel numero di genital contact ($F_{2,88}$=3.41, P=0.033) e nel numero di spermi trasferiti ($F_{2,47}$=3.58, P=0.036), con femmine provenienti dalla popolazione a bassa predazione. Questo risultato può quindi essere spiegato nell'ottica dell'evoluzione sessuale antagonistica, date le differenze nell'incidenza di copule sneaky fra le popolazioni. Inoltre c'è una relazione fra forma del gonopodio e probabilità di inseminazione ($F=_{5,87}$=3.90, P=0.003), in particolare hanno più successo i maschi dotati di gonopodio con l'estremità distale più stretta e con un uncino terminale che forma un angolo minore con l'asse longitudinale del gonopodio.

Articolo V

Obiettivo di questo esperimento è stato quello di valutare il ruolo della morfologia del gonopodio nel determinare il successo nelle copule cooperative, che potrebbe dipendere da una preferenza femminile a livello pre-copulatorio, come è stato dimostrato in *Gambusia affinis* (Langerhans et al. 2005) e/o da una relazione tra morfologia e abilità nel trasferimento degli spermi. Sono stati eseguiti 41 esperimenti in cui femmine vergini venivano fatte accoppiare cooperativamente con due maschi. Sono state analizzate diverse componenti comportamentali, come la latenza alla copula della femmina (misura della preferenza pre-copulatoria), ed il tasso di corteggiamento. È stato anche considerato il numero di jerks, piccoli sobbalzi effettuati dal maschio dopo la copula, il cui numero correla con il numero di spermi trasferiti (Pilastro et al. 2007). I risultati sono stati messi in relazione con la morfologia del gonopodio, le analisi sono state effettuate come descritto sopra. Le femmine si accoppiano più velocemente con maschi con possiedono un gonopodio più lungo (dimensioni lineari, F=6.286, P=0.015) e di forma allargata (F=5.619, P=0.021), e che corteggiano di più (F=51.606, P<0.001). Il numero di spermi trasferiti, indicato dal numero di jerks, è associato (sebbene il risultato non sia del tutto significativo) alla forma della parte distale del gonopodio, in particolare riferito alla lunghezza dell'hook, dove trasferisce più spermi il maschio con uncini più corti. Le caratteristiche del gonopodio intero e della morfologia dell'hook non sono però correlate.

Articolo VI

In questo esperimento si è valutata la differenziazione dei genitali maschili in popolazioni con diversi livelli di conflitto sessuale (legato al diverso grado di predazione) e la loro eventuale coevoluzione con i genitali femminili. Diversi studi dimostrano che i contro adattamenti femminili alle strategie dei maschi possono coinvolgere anche la morfologia dei genitali (Arnqvist & Rowe 2002a; Cordoba-Aguilar 2005; Brennan et al. 2007), sebbene in generale la morfologia dei genitali femminili sia meno variabile di quella dei genitali maschili (Eberhard 1985). Sono perciò stati analizzati maschi e femmine di 10 popolazioni appartenenti a 5 diversi corsi d'acqua dell'isola di Trinidad (circa 20 individui per sesso per popolazione). Per ogni corso d'acqua i prelievi sono stati effettuati in due zone caratterizzate da regime predatorio opposto (a bassa o ad alta predazione). Le femmine sono state dissezionate ed è stata misurata la lunghezza e la larghezza del gonodotto. Nei maschi il gonopodio è stato analizzato come descritto precedentemente. I risultati evidenziano che maschi di popolazioni ad alta predazione possiedono un gonopodio più lungo, con l'estremità distale più stretta e caratterizzata da un uncino terminale che forma un angolo minore con l'asse longitudinale del gonopodio rispetto a quanto si osserva nei maschi delle popolazioni a bassa predazione. Dall'analisi del pattern di covariazione tra la morfologia dei genitali di maschi e femmine si nota una relazione significativa tra il rapporto larghezza/lunghezza del gonodotto e la forma della parte distale del gonopodio; in particolare nelle popolazioni ad alta predazione (dove i maschi tendono a scegliere maggiormente la tattica delle copule forzate) i maschi presentano un gonopodio più stretto rispetto al gonodotto se confrontati con le popolazioni a bassa predazione. Una spiegazione per quest'ultimo risultato è che la variazione nel gonodotto femminile rifletta un contro adattamento per ridurre il successo delle copule forzate; un gonodotto più largo potrebbe ridurre l'abilità del maschio di trattenere il gonopodio attaccato al gonoporo, oppure potrebbe servire per ridurre eventuali danni provocati dagli uncini durante la copula.

Conclusioni

Con i risultati del lavoro sulle copule cooperative si è dimostrata l'importanza della selezione sessuale a livello precopulatorio del gonopodio maschile. Inoltre, la morfologia del gonopodio del maschio, in particolare la sua parte distale, gioca un ruolo importante nel determinare sia il successo nelle copule cooperative che in quelle coercitive, sebbene i dettagli della morfologia del gonopodio implicati non siano gli stessi. Questo suggerisce un *trade-off* tra successo nelle copule forzate e nelle copule cooperative che può spiegare la variabilità presente a livello naturale, modellata sia dalla selezione sessuale che dal conflitto sessuale. L'importanza della forma del gonopodio nelle copule forzate si estrapola anche dal lavoro comparativo intraspecifico che ha messo in evidenza sia la variazione morfologica che rispecchia il livello di conflitto sessuale nelle diverse popolazioni prese in considerazione, sia la coevoluzione sessuale antagonistica fra genitali maschile e femminili. A conferma dell'esistenza di tale coevoluzione si è anche dimostrato che maschi provenienti da una popolazione caratterizzata da livelli elevati di sexual harassment hanno più successo nelle copule forzate con femmine provenienti da popolazioni con livello minore di sexual harassment, come previsto dal processo di coevoluzione sessuale antagonistica.

Conclusioni generali

Un crescente numero di lavori hanno evidenziato l'importanza del conflitto sessuale nel modellare l'evoluzione delle specie. Il conflitto sessuale è particolarmente evidente in specie, come in *Poecilia reticulata*, in cui il sistema riproduttivo è promiscuo e maschi e femmine differiscono nel numero ideale di accoppiamenti. Il conflitto sessuale ha profonde conseguenze nelle interazioni tra i sessi e grosse implicazioni per il processo di speciazione. I risultati ottenuti dalla mia tesi confermano l'importanza del conflitto sessuale in *Poecilia reticulata*, date le conseguenze sulla *life-history* delle femmine e i costi, dimostrati per la prima volta a livello indiretto, sulla qualità della loro prole. Implicazione del conflitto sessuale è la coevoluzione sessuale antagonistica, processo che ho dimostrato avvenire a livello dei genitali, che può favorire una rapida differenziazione delle popolazioni e costituire un inizio di speciazione.

References

Alexander,R.D., Marshall D.C. & Cooley J.R. 1997. Evolutionary perspectives on insect mating. In: *The Evolution of Mating Systems in Insects and Arachnids* (Ed. by C.Cambridge University Press), pp. 4-31.

Andersson,M. 1994. *Sexual Selection*. Princeton, New Jersey: Princeton University Press.

Andersson,S., Uller,T., Lohmus,M. & Sundstrom,F. 2004. Effects of egg yolk testosterone on growth and immunity in a precocial bird. *Journal of Evolutionary Biology* **17,** 501-505.

Andres,J.A. & Arnqvist,G. 2001. Genetic divergence of the seminal signal-receptor system in houseflies: the footprints of sexually antagonistic coevolution? *Proceedings of the Royal Society of London Series B, Biological Sciences* **268,** 399-405.

Angus,R.A. 1989. A genetic overview of Poeciliid fishes. In: *Ecology and Evolution of Livebearing Fishes (Poeciliidae)* (Ed. by G.K.Meffe & F.F.Snelson, Jr.), pp. 51-68. Englewood Cliffs, NJ, Prentice Hall.

Arnqvist,G. 1989. Multiple mating in water strider: mutual benefit or intersexual conflict? *Animal Behaviour* **38,** 749-756.

Arnqvist,G. 1997. The evolution of animal genitalia: Distinguishing between hypotheses by single species studies. *Biological Journal of the Linnean Society* **60,** 365-379.

Arnqvist,G. 1998. Comparative evidence for the evolution of genitalia by sexual selection. *Nature* **393,** 784-786.

Arnqvist,G. 2004. Sexual conflict and sexual selection: Lost in the chase. *Evolution* **58,** 1383-1388.

Arnqvist,G. & Danielsson,I. 1999. Copulatory behavior, genital morphology, and male fertilization success in water striders. *Evolution* **53,** 147-156.

Arnqvist,G., Edvardsson,M., Friberg,U. & Nilsson,T. 2000. Sexual conflict promotes speciation in insects. *Proceedings of the National Academy of Sciences of the USA* **97,** 10460-10464.

Arnqvist,G. & Nilsson,T. 2000. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour* **60,** 145-164.

Arnqvist,G. & Rowe,L. 1995. Sexual conflict and arms races between the sexes: a morphological adaptation for control of mating in a female insect. *Proceedings of the Royal Society of London Series B-Biological Sciences* **261,** 123-127.

Arnqvist,G. & Rowe,L. 2002a. Antagonistic coevolution between the sexes in a group of insects. *Nature* **415,** 787-789.

Arnqvist,G. & Rowe,L. 2002b. Correlated evolution of male and female morphologies in water striders. *Evolution* **56,** 936-947.

Arnqvist,G. & Rowe,L. 2005. *Sexual Conflict*. Princeton, New Jersey: Princeton University Press.

Aspbury,A.S. & Gabor,C.R. 2004. Differential sperm priming by male sailfin mollies (*Poecilia latipinna*): Effects of female and male size. *Ethology* **110,** 193-202.

Basolo,A.L. 1990. Female preference predates the evolution of the sword in swordtail fish. *Science* **250,** 808-810.

Bateman,A.J. 1948. Intra-sexual selection in *Drosophila*. *Heredity* **2,** 349-368.

Becher,S.A. & Magurran,A.E. 2004. Multiple mating and reproductive skew in Trinidadian guppies. *Proceedings of the Royal Society of London Series B, Biological Sciences* **271,** 1009-1014.

Becker,W.A. 1984. *A Manual of Quantitative Genetics*. Washington: Pullman, Academic Enterprises.

Berry,J.F. & Shine,R. 1980. Sexual dimorphism and sexual selection in turtles (order Testudines). *Oecologia* **44,** 185-191.

Bertin,A. & Fairbairn,D.J. 2005. One tool, many uses: precopulatory sexual selection on genital morphology in *Aquarius remigis*. *Journal of Evolutionary Biology* **18,** 949-961.

Billard,R. 1969. La sperm spermatogenese de *Poecilia reticulata*. *Ann. Biol. Anim. Bioch. Biophys.* **9,** 251-271.

Billard,R. 1986. Spermatogenesis and spermatology of some teleost fish species. *Reproduction Nutrition Development* **26,** 877-920.

Birkhead,T.R., Martinez,J.G., Burke,T. & Froman,D.P. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society B: Biological Sciences* **266,** 1759-1764.

Birkhead,T.R., Pellatt,E.J., Brekke,P., Yeates,R. & Castillo-Juarez,H. 2005. Genetic effects on sperm design in the zebra finch. *Nature* **434,** 383-387.

Bisazza,A. 1993. Male competition, female mate choice and sexual size dimorphism in Poeciliid fishes. *Marine Behaviour and Physiology* **23,** 257-286.

Bisazza,A., Manfredi,S. & Pilastro,A. 2000. Sexual competition, coercive mating and mate assessment in the one-sided livebearer, *Jenynsia multidentata*: Are they predictive of sexual dimorphism? *Ethology* **106,** 961-978.

Bisazza,A. & Pilastro,A. 1997. Small male mating advantage and reversed size dimorphism in poeciliid fishes. *Journal of Fish Biology* **50,** 397-406.

Bischoff,R.J., Gould,J.L. & Rubenstein,D.I. 1985. Tail size and female choice in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology* **17,** 253-255.

Bjork,A., Dallai,R. & Pitnick,S. 2007. Adaptive modulation of sperm production rate in *Drosophila bifurca*, a species with giant sperm. *Biology Letters* **3,** 517-519.

Bjork,A. & Pitnick,S. 2006. Intensity of sexual selection along the anisogamy-isogamy continuum. *Nature* **441,** 742-745.

Blanckenhorn,W.U., Hosken,D.J., Martin,O.Y., Reim,C., Teuschl,Y. & Ward,P.I. 2002. The costs of copulating in the dung fly *Sepsis cynipsea*. *Behavioral Ecology* **13,** 353-358.

Blanckenhorn,W.U., Muhlhauser,C., Morf,C., Reusch,T. & Reuter,M. 2000. Female choice, female reluctance to mate and sexual selection on body size in the dung fly *Sepsis cynipsea*. *Ethology* **106,** 577-593.

Blows,M.W., Brooks,R. & Kraft,P.G. 2003. Exploring complex fitness surfaces: Multiple ornamentation and polymorphism in male guppies. *Evolution* **57,** 1622-1630.

Bonduriansky,R. & Rowe,L. 2005. Intralocus sexual conflict and the genetic architecture of sexually dimorphic traits in *Prochyliza xanthostoma* (Diptera : Piophilidae). *Evolution* **59,** 1965-1975.

Bookstein,F.L. 1997. Landmark methods for forms without landmarks: morphometrics of group differences in outline shape. *Medical Image Analysis* **1,** 225-243.

Bozynski,C.C. & Liley,N.R. 2003. The effect of female presence on spermiation, and of male sexual activity on 'ready' sperm in the male guppy. *Animal Behaviour* **65 Part 1,** 53-58.

Brennan,P.L.R., Prum,R.O., McCracken,K.G., Sorenson,M.D., Wilson,R.E. & Birkhead,T.R. 2007. Coevolution of male and female genital morphology in waterfowl. *PLoS One* **2,** e418.

Brooks,R. 2000. Negative genetic correlation between male sexual attractiveness and survival. *Nature* **406,** 67-70.

Brooks,R. & Caithness,N. 1995. Female choice in a feral guppy population: Are there multiple cues? *Animal Behaviour* **50,** 301-307.

Brooks,R. & Endler,J.A. 2001a. Direct and indirect sexual selection and quantitative genetics of male traits in guppies (*Poecilia reticulata*). *Evolution* **55,** 1002-1015.

Brooks,R. & Endler,J.A. 2001b. Female guppies agree to differ: Phenotypic and genetic variation in mate-choice behavior and the consequences for sexual selection. *Evolution* **55,** 1644-1655.

Burley,N.T. 1988. The differential-allocation hypothesis: an experimental test. *Am. Nat.* **132,** 611-628.

Burness,G., Casselman,S.J., Schulte-Hostedde,A.I., Moyes,C.D. & Montgomerie,R. 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). *Behavioral Ecology and Sociobiology* **56,** 65-70.

Byrne,P.G. & Roberts,J.D. 1999. Simultaneous mating with multiple males reduces fertilization success in the myobatrachid frog *Crinia georgiana*. *Proceedings of the Royal Society of London Series B-Biological Sciences* **266,** 717-721.

Byrne,P.G. & Roberts,J.D. 2000. Does multiple paternity improve fitness of the frog *Crinia georgiana*? *Evolution* **54,** 968-973.

Cameron,E., Day,T. & Rowe,L. 2003. Sexual conflict and indirect benefits. *Journal of Evolutionary Biology* **16,** 1055-1060.

Carayon,J. 1966. Tramatic insemination and paragenital system. In: *Monograph of Cimicidae (Hemiptera, Heteroptera)* (Ed. by R.L.Usinger), pp. 81-166. College Park, Md, Entomological Society of America.

Casselman,S.J., Schulte-Hostedde,A.I. & Montgomerie,R. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). *Canadian Journal of Fisheries and Aquatic Sciences* **63,** 2119-2125.

Chapman,T., Arnqvist,G., Bangham,J. & Rowe,L. 2003. Sexual conflict. *Trends in Ecology & Evolution* **18,** 41-47.

Chapman,T., Liddle,L.F., Kalb,J.M., Wolfner,M.F. & Partridge,L. 1995. Cost of mating in *Drosophila melanogaster* females Is mediated by male accessory-gland products. *Nature* **373,** 241-244.

Chapman,T. & Partridge,L. 1996. Female fitness in Drosophila melanogaster: An interaction between the effect of nutrition and of encounter rate with males. *Proceedings of the Royal Society of London Series B-Biological Sciences* **263,** 755-759.

Cheng,Y.Y. 2004. *Sexual Selection and the Evolution of Genitalia in the Guppy (Poecilia Reticulata).* Toronto: University of Toronto.

Chippindale,A.K., Gibson,J.R. & Rice,W.R. 2001. Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proceedings of the National Academy of Sciences of the USA* **98,** 1671-1675.

Choe,J.C. & Crespi,B.J. 1997. *The Evolution of Mating Systems in Insects and Arachnids*. Cambridge: New York: Cambridge University Press.

Clark,A.G., Begun,D.J. & Prout,T. 1999. Female x male interactions in *Drosophila* sperm competition. *Science* **283,** 217-220.

Clark,E. 1950. A method for artificial insemination in viviparous fishes. *Science* **112,** 722-723.

Clark,E. & Aronson,L.R. 1951. Sexual behavior in the guppy, *Lebistes reticulatus* (Peters). *Zoologica* **36,** 49-66.

Clark,E., Aronson,L.R. & Gordon,M. 1954. Mating behavior patterns in two sympatric species of *Xiphophorus* fishes: their inheritance and significance in sexual isolation. *Bulletin of the American Musuem of Natural History* **103,** 139-225.

Clutton-Brock,T.H. & Parker,G.A. 1995. Sexual coercion in animal societies. *Animal Behaviour* **49,** 1345-1365.

Constantz,G.D. 1984. Sperm competition in Poeciliid fishes. In: *Sperm Competition and the Evolution of Animal Mating Systems* (Ed. by R.L.Smith), pp. 465-485. London, Academic Press.

Constantz,G.D. 1989. Reproductive biology of Poeciliid fishes. In: *Ecology and Evolution of Livebearing Fishes (Poeciliidae)* (Ed. by G.K.Meffe & F.F.Snelson), pp. 33-50. Englewood Cliffs, Prentice Hall.

Cordero,C. & Eberhard,W.G. 2003. Female choice of sexually antagonistic male adaptations: a critical review of some current research. *Journal of Evolutionary Biology* **16,** 1-6.

Cordoba-Aguilar,A. 2005. Possible coevolution of male and female genital form and function in a calopterygid damselfly. *Journal of Evolutionary Biology* **18,** 132-137.

Crudgington,H.S. & Siva-Jothy,M.T. 2000. Genital damage, kicking and early death - The battle of the sexes takes a sinister turn in the bean weevil. *Nature* **407,** 855-856.

Daly,M. 1978. The cost of mating. *American Naturalist* **112,** 771-774.

Danielsson,I. & Askenmo,C. 1999. Male genital traits and mating interval affect male fertilization success in the water strider Gerris lacustris. *Behavioral Ecology and Sociobiology* **46,** 149-156.

Davis,N.T. 1965. Studies of the reproductive physiology of the Cimicidae (Hemiptera). II. Artificial insemination and the function of the seminal fluid. *Journal of Insect Physiology* **11,** 355-366.

Denk,A.G., Holzmann,A., Peters,A., Vermeirssen,E.L.M. & Kempenaers,B. 2005. Paternity in mallards: effects of sperm quality and female sperm selection for inbreeding avoidance. *Behavioral Ecology* **16,** 825-833.

Dewsbury,D.A. 1982. Ejaculate cost and male choice. *American Naturalist* **119,** 601-610.

Eberhard,W.G. 1985. *Sexual Selection and Animal Genitalia*. Cambridge: Harvard University Press.

Eberhard,W.G. 1994. Evidence for widespread courtship during copulation in 131 species of insects and spiders, and implications for cryptic female choice. *Evolution* **48,** 711-733.

Eberhard,W.G. 1996. *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton, New Jersey: Princeton University Press.

Eberhard,W.G. 2002. The function of female resistance behavior: intromission by male coercion vs. female cooperation in sepsid flies (Diptera: Sepsidae). *Revista De Biologia Tropical* **50,** 485-505.

Endler,J.A. 1987. Predation, light intensity and courtship behaviour in *Poecilia reticulata* (Pisces: Poeciliidae). *Animal Behaviour* **35,** 1376-1385.

Endler,J.A. 1995. Multiple-trait coevolution and environmental gradients in guppies. *Trends in Ecology & Evolution* **10,** 22-29.

Endler,J.A. & Houde,A.E. 1995. Geographic variation in female preferences for male traits in *Poecilia reticulata*. *Evolution* **49,** 456-468.

Evans,J.P., Bisazza,A. & Pilastro,A. 2004a. Female mating preferences for colourful males in a population of guppies subject to high predation. *Journal of Fish Biology* **65,** 1154-1159.

Evans,J.P., Gasparini,C. & Pilastro,A. 2007. Female guppies shorten brood retention in response to predator cues. *Behavioral Ecology and Sociobiology* **61,** 719-727.

Evans,J.P., Kelley,J.L., Bisazza,A., Finazzo,E. & Pilastro,A. 2004b. Sire attractiveness influences offspring performance in guppies. *Proceedings of the Royal Society of London Series B, Biological Sciences* **271,** 2035-2042.

Evans,J.P., Kelley,J.L., Ramnarine,I.W. & Pilastro,A. 2002. Female behaviour mediates male courtship under predation risk in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology* **52,** 496-502.

Evans,J.P. & Magurran,A.E. 2000. Multiple benefits of multiple mating in guppies. *Proceedings of the National Academy of Sciences of the USA* **97,** 10074-10076.

Evans,J.P. & Magurran,A.E. 2001. Patterns of sperm precedence and predictors of paternity in the Trinidadian guppy. *Proceedings of the Royal Society of London Series B, Biological Sciences* **268,** 719-724.

Evans,J.P., Pierotti,M. & Pilastro,A. 2003a. Male mating behavior and ejaculate expenditure under sperm competition risk in the eastern mosquitofish. *Behavioral Ecology* **14,** 268-273.

Evans,J.P., Pilastro,A. & Ramnarine,I.W. 2003b. Sperm transfer through forced matings and its evolutionary implications in natural guppy (*Poecilia reticulata*) populations. *Biological Journal of the Linnean Society* **78,** 605-612.

Evans,J.P., Zane,L., Francescato,S. & Pilastro,A. 2003c. Directional postcopulatory sexual selection revealed by artificial insemination. *Nature* **421,** 360-363.

Fairbairn,D.J. 1993. Costs of loading associated with mate carrying in the water strider, *Aquarius remigis*. *Behavioral Ecology* **4,** 224-231.

Fairbairn,D.J., Vermette,R., Kapoor,N.N. & Zahiri,N. 2003. Functional morphology of sexually selected gentalia in the water strider *Aquarius remigis*. *Canadian Journal of Zoology* **81,** 400-413.

Farr,J.A. 1989. Sexual selection and secondary differentiation in poeciliids: determinants of male mating success and the evolution of female choice. In: *Ecology and Evolution of Livebearing Fishes (Poeciliidae)* (Ed. by G.K.Meffe & F.F.Snelson), pp. 91-123. Englewood Cliffs, Prentice Hall.

Fisher,R.A. 1930. *The Genetical Theory of Natural Selection*. Oxford: Clarendon Press.

Foerster,K., Coulson,T., Sheldon,B.C., Pemberton,J.M., Clutton-Brock,T.H. & Kruuk,L.E.B. 2007. Sexually antagonistic genetic variation for fitness in red deer. *Nature* **447,** 1107-11U9.

Froman,D.P. & Feltmann,A.J. 1998. Sperm mobility: A quantitative trait of the domestic fowl (*Gallus domesticus*). *Biology of Reproduction* **58,** 379-384.

Froman,D.P., Feltmann,A.J., Rhoads,M.L. & Kirby,J.D. 1999. Sperm mobility: A primary determinant of fertility in the domestic fowl (*Gallus domesticus*). *Biology of Reproduction* **61,** 400-405.

Gage,M.J.G., Macfarlane,C.P., Yeates,S., Ward,R.G., Searle,J.B. & Parker,G.A. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. *Curr. Biol.* **14,** 44-47.

Garcia-Gonzalez,F. & Simmons,L.W. 2005. Sperm viability matters in insect sperm competition. *Current Biology* **15,** 271-275.

Gavrilets,S., Arnqvist,G. & Friberg,U. 2001. The evolution of female mate choice by sexual conflict. *Proceedings of the Royal Society of London Series B-Biological Sciences* **268,** 531-539.

Godin,J.-G.J. 1995. Predation risk and alternative mating tactics in male Trinidadian guppies (*Poecilia reticulata*). *Oecologia* **103,** 224-229.

Hain,T.J.A. & Neff,B.D. 2007. Multiple paternity and kin recognition mechanisms in a guppy population. *Molecular Ecology* **16,** 3938-3946.

Handelsman,D.J. 1997. Estimating familial and genetic contributions to variability in human testicular function: a pilot twin study. *International Journal of Andrology* **20,** 215-221.

Haskins,C.P., Haskins,E.F., McLaughlin,J.J.A. & Hewitt,R.E. 1961. Polymorphism and population structure in *Lebistes reticulatus*, an ecological study. In: *Vertebrate Speciation* (Ed. by W.F.Blair), pp. 320-395. Austin, University of Texas Press.

Head,M.L. & Brooks,R. 2006. Sexual coercion and the opportunity for sexual selection in guppies. *Animal Behaviour* **71,** 515-522.

Head,M.L., Lindholm,A.K. & Brooks,R. 2008. Operational sex ratio and density do not affect directional selection on male sexual ornaments and behavior. *Evolution* **62,** 135-144.

Holland,B. & Rice,W.R. 1998. Perspective: Chase-away sexual selection: Antagonistic seduction versus resistance. *Evolution* **52,** 1-7.

Holland,B. & Rice,W.R. 1999. Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proceedings of the National Academy of Sciences of the USA* **96,** 5083-5088.

Hosken,D.J., Blanckenhorn,W.U. & Garner,T.W.J. 2002. Heteropopulation males have a fertilization advantage during sperm competition in the yellow dung fly (*Scathophaga stercoraria*). *Proceedings of the Royal Society of London Series B, Biological Sciences* **269,** 1701-1707.

Hosken,D.J., Garner,T.W.J. & Ward,P.I. 2001. Sexual conflict selects for male and female reproductive characters. *Current Biology* **11,** 489-493.

Hosken,D.J. & Stockley,P. 2003. Benefits of polyandry: A life history perspective. *Evolutionary Biology* **33,** 173-194.

Hosken,D.J. & Stockley,P. 2004. Sexual selection and genital evolution. *Trends in Ecology & Evolution* **19,** 87-93.

Houde,A.E. 1992. Sex-linked heritability of a sexually selected character in a natural population of *Poecilia reticulata* (Pisces: Poeciliidae) (guppies). *Heredity* **69,** 229-235.

Houde,A.E. 1997. *Sex, Color, and Mate Choice in Guppies*. Princeton, New Jersey: Princeton University Press.

Houde,A.E. & Endler,J.A. 1990. Correlated evolution of female mating preferences and male color patterns in the guppy *Poecilia reticulata*. *Science* **248,** 1405-1408.

Houle,D. 1992. Comparing evolvability and variability of quantitative traits. *Genetics* **130,** 195-204.

House,C.M. & Simmons,L.W. 2005. Relative influence of male and female genital morphology on paternity in the dung beetle *Onthophagus taurus*. *Behavioral Ecology* **16,** 889-897.

Huber,B.A. 2003. Southern African pholcid spiders: revision and cladistic analysis of *Quamtana* gen. and Spermophora Hentz (Araneae: Pholcidae), male-female covariation. *Zoological Journal of the Linnean Society* **139,** 477-527.

Hughes,K.A., Du,L., Rodd,F.H. & Reznick,D.N. 1999. Familiarity leads to female mate preference for novel males in the guppy, *Poecilia reticulata*. *Animal Behaviour* **58,** 907-916.

Hughes,K.A., Rodd,F.H. & Reznick,D.N. 2005. Genetic and environmental effects on secondary sex traits in guppies (*Poecilia reticulata*). *Journal of Evolutionary Biology* **18,** 35-45.

Jennions,M.D. & Kelly,C.D. 2002. Geographical variation in male genitalia in *Brachyphaphis episcopi* (Poeciliidae): is it sexually or naturally selected? *Oikos* **97,** 79-86.

Jennions,M.D. & Petrie,M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews of the Cambridge Philosophical Society* **75,** 21-64.

Jolivet,C. & Bernasconi,G. 2007. Within/between population crosses reveal genetic basis for siring success in *Silene latifolia* (Caryophyllaceae). *Journal of Evolutionary Biology* **20,** 1361-1374.

Kelly,C.D., Godin,J.G.J. & Abdallah,G. 2000. Geographical variation in the male intromittent organ of the Trinidadian guppy (*Poecilia reticulata*). *Canadian Journal of Zoology* **78,** 1674-1680.

Kelly,C.D., Godin,J.G.J. & Wright,J.M. 1999. Geographical variation in multiple paternity within natural populations of the guppy (*Poecilia reticulata*). *Proceedings of the Royal Society of London Series B, Biological Sciences* **266,** 2403-2408.

Kilgallon,S.J. & Simmons,L.W. 2005. Image content influences men's semen quality. *Biology Letters* **1,** 253-255.

Kirkpatrick,M. 1985. Evolution of female choice and male parental investment in polygynous species: the demise of the "sexy son". *American Naturalist* **125,** 788-810.

Kirkpatrick,M. 1996. Good genes and direct selection in evolution of mating preferences. *Evolution* **50,** 2125-2140.

Kirkpatrick,M. & Barton,N.H. 1997. The strength of indirect selection on female mating preferences. *Proceedings of the National Academy of Sciences of the USA* **94,** 1282-1286.

Knowles,L.L. & Markow,T.A. 2001. Sexually antagonistic coevolution of a postmating-prezygotic reproductive character in desert *Drosophila*. *Proceedings of the National Academy of Sciences of the USA* **98,** 8692-8696.

Kodric-Brown,A. 1985. Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology* **17,** 199-205.

Konior,M., Keller,L. & Radwan,J. 2005. Effect of inbreeding and heritability of sperm competition success in the bulb mite *Rhizoglyphus robini*. *Heredity* **94,** 577-581.

Kotiaho,J.S., Simmons,L.W., Hunt,J. & Tomkins,J.L. 2003. Males influence maternal effects that promote sexual selection: A quantitative genetic experiment with dung beetles *Onthophagus taurus*. *American Naturalist* **161,** 852-859.

Kuckuck,C. & Greven,H. 1997. Notes on the mechanically stimulated discharge of spermiozeugmata in the guppy, *Poecilia reticulata*: A quantitative approach. *Zeitschrift für Fischkunde* **4,** 73-88.

Kupriyanova,E. & Havenhand,J.N. 2002. Variation in sperm swimming behaviour and its effect on fertilization success in the serpulid polychaete *Galeolaria caespitosa*. *Invertebrate Reproduction and Development* **41,** 21-26.

Lahnsteiner,F., Berger,B., Weismann,T. & Patzner,R.A. 1998. Determination of semen quality of the rainbow trout, Oncorhynchus mykiss, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. *Aquaculture* **163,** 163-181.

Langerhans,R.B., Layman,C.A. & DeWitt,T.J. 2005. Male genital size reflects a tradeoff between attracting mates and avoiding predators in two live-bearing fish species. *Proceedings of the National Academy of Sciences of the USA* **102,** 7618-7623.

Lemon,W.C. 1993. Heritability of Selectively Advantageous Foraging Behavior in A Small Passerine. *Evolutionary Ecology* **7,** 421-428.

Lessells,C.M. & Boag,P.T. 1987. Unrepeatable repeatabilities: A common mistake. *Auk* **104,** 116-121.

Levitan,D.R. 2000. Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. *Proceedings of the Royal Society B: Biological Sciences* **267,** 531-534.

Liley,N.R. 1966. Ethological isolating mechanisms in four sympatric species of poeciliid fishes. *Behaviour Supplements* **13,** 1-197.

Lindholm,A.K., Breden,F., Alexander,H.J., Chan,W.K., Thakurta,S.G. & Brooks,R. 2005. Invasion success and genetic diversity of introduced populations of guppies *Poecilia reticulata* in Australia. *Molecular Ecology* **14,** 3671-3682.

Locatello,L., Pilastro,A., Deana,R., Zarpellon,A. & Rasotto,M.B. 2007. Pattern of variation of sperm quality traits in two gobies with alternative mating tactics. *Functional Ecology* **21,** 975-981.

Locatello,L., Rasotto,M.B., Adriaenssens,B. & Pilastro,A. 2008. Ejaculate traits in relation to male body size in the eastern mosquitofish *Gambusia holbrooki*. *Journal of Fish Biology* **73,** 1600-1611.

Locatello,L., Rasotto,M.B., Evans,J.P. & Pilastro,A. 2006. Colourful male guppies produce faster and more viable sperm. *Journal of Evolutionary Biology* **19,** 1595-1602.

Lodi,E. 1981. Competition between pall and normal bearing spermatozoa of *Poecilia reticulata* (Pisces: Poeciliidae). *Copeia* **1981,** 624-629.

Lynch 1994. Evolutionary inferences from genetic analyses of cold adaptation in laboratory and wild populations of the house mouse. In: *Quantitative Genetic Studies of Behavioral Evolution* (Ed. by University of Chicago Press), pp. 278-301.

Lynch,M. & Walsh,J.B. 1998. *Genetics and Analysis of Quantitative Traits*. Sunderland, MA: Sinauer.

MacKay,T.F.C. 2002. The nature of quantitative genetic variation for *Drosophila* longevity. *Mechanisms of Ageing and Development* **123,** 95-104.

Magurran,A.E. 2005. *Evolutionary Ecology: The Trinidadian Guppy*. Oxford: Oxford University Press.

Magurran,A.E. & Nowak,M.A. 1991. Another battle of the sexes: The consequences of sexual asymmetry in mating costs and predation risk in the guppy, *Poecilia reticulata*. *Proceedings of the Royal Society of London Series B, Biological Sciences* **246,** 31-38.

Magurran,A.E. & Seghers,B.H. 1990. Risk sensitive courtship in the guppy (*Poecilia reticulata*). *Behaviour* **112,** 194-201.

Magurran,A.E. & Seghers,B.H. 1991. Variation in Schooling and Aggression Amongst Guppy (*Poecilia reticulata*) Populations in Trinidad. *Behaviour* **118,** 214-234.

Magurran,A.E. & Seghers,B.H. 1994a. A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proceedings of the Royal Society of London Series B, Biological Sciences* **258,** 89-92.

Magurran,A.E. & Seghers,B.H. 1994b. Sexual conflict as a consequence of ecology: Evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proceedings of the Royal Society of London Series B, Biological Sciences* **255,** 31-36.

Magurran,A.E., Seghers,B.H., Carvalho,G.R. & Shaw,P.W. 1992. Behavioural consequences of an artificial introduction of guppies (*Poecilia reticulata*) in N. Trinidad: Evidence for the evolution of anti-predator behaviour in the wild. *Proceedings of the Royal Society of London Series B, Biological Sciences* **248,** 117-122.

Maklakov,A.A., Bilde,T. & Lubin,Y. 2005. Sexual conflict in the wild: Elevated mating rate reduces female lifetime reproductive success. *American Naturalist* **165,** S38-S45.

Maklakov,A.A. & Lubin,Y. 2004. Sexual conflict over mating in a spider: Increased fecundity does not compensate for the costs of polyandry. *Evolution* **58,** 1135-1140.

Malo,A.F., Garde,J.J., Soler,A.J., Garcia,A.J., Gomendio,M. & Roldan,E.R.S. 2005. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. *Biology of Reproduction* **72,** 822-829.

Martin,O.Y. & Hosken,D.J. 2004. Copulation reduces male but not female longevity in *Saltella sphondylli* (Diptera: Sepsidae). *Journal of Evolutionary Biology* **17,** 357-362.

Martin,P.A., Reimers,T.J., Lodge,A. & Dziuk,P.J. 1974. The effect of ratios and numbers of spermatozoa mixed fromtwo males on proportions of offspring. *Journal of Reproduction and Fertility* **39,** 251-258.

Matthews,I.M., Evans,J.P. & Magurran,A.E. 1997. Male display rate reveals ejaculate characteristics in the Trinidadian guppy *Poecilia reticulata*. *Proceedings of the Royal Society of London Series B, Biological Sciences* **264,** 695-700.

Matthews,I.M. & Magurran,A.E. 2000. Evidence for sperm transfer during sneaky mating in wild Trinidadian guppies. *Journal of Fish Biology* **56,** 1381-1386.

Mayr,E. 1963. *Animal Species and Evolution*. Cambridge, Mass: Belknap Press of Harvard University Press.

Møller,A.P. & Jennions,M.D. 2001. How important are direct fitness benefits of sexual selection? *Naturwissenschaften* **88,** 401-415.

Moore,A.J. 1990. The inheritance of social dominance, mating behavior and attractiveness to mates in male *Nauphoeta cinerea*. *Animal Behaviour* **39,** 388-397.

Moore,P.J., Harris,W.E., Montrose,V.T., Levin,D. & Moore,A.J. 2004. Constraints on evolution and postcopulatory sexual selection: trade-offs among ejaculate characteristics. *Evolution* **58,** 1773-1780.

Morrow,E.H. & Arnqvist,G. 2003. Costly traumatic insemination and a female counter-adaptation in bed bugs. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270,** 2377-2381.

Morrow,E.H. & Gage,M.J.G. 2001. Sperm competition experiments between lines of crickets producing different sperm lengths. *Proceedings of the Royal Society of London Series B, Biological Sciences* **268,** 2281-2286.

Mousseau,T.A. & Roff,D.A. 1987. Natural selection and the heritability of fitness components. *Heredity* **59,** 181-197.

Nakatsuru,K. & Kramer,D.L. 1982. Is sperm cheap? Limited fertility and female choice in the lemon tetra (Pisces, Characidae). *Science* **216,** 753-755.

Neff,B.D., Fu,P. & Gross,M.R. 2003. Sperm investment and alternative mating tactics in bluegill sunfish (*Lepomis macrochirus*). *Behavioral Ecology* **14,** 634-641.

Newberry,K. 1989. The effects on domestic infestation of *Cimex lectularius* bedbugs of interspecific mating with *Cimex hemipterus*. *Medical and Veterinary Entomology* **3,** 407-414.

Nicoletto,P.F. 1995. Offspring quality and female choice in the guppy, *Poecilia reticulata*. *Animal Behaviour* **49,** 377-387.

Nilsson,T., Fricke,C. & Arnqvist,G. 2002. Patterns of divergence in the effects of mating on female reproductive performance in flour beetles. *Evolution* **56,** 111-120.

Ojanguren,A.F., Evans,J.P. & Magurran,A.E. 2005. Multiple mating influences offspring size in guppies. *Journal of Fish Biology* **67,** 1184-1188.

Ojanguren,A.F. & Magurran,A.E. 2007. Male harassment reduces short-term female fitness in guppies. *Behaviour* **144,** 503-514.

Olendorf,R., Rodd,F.H., Punzalan,D., Houde,A.E., Hurt,C., Reznick,D.N. & Hughes,K.A. 2006. Frequency-dependent survival in natural guppy populations. *Nature* **441,** 633-636.

Olsson,M., Madson,T. & Shine,R. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society of London Series B, Biological Sciences* **264,** 455-459.

Parker,G.A. 1979. Sexual selection and sexual conflict. In: *Sexual Selection and Reproductive Competition in Insects* (Ed. by M.S.Blum & N.A.Blum), pp. 123-166. New York, Academic Press.

Parker,G.A. 2006. Sexual conflict over mating and fertilization: an overview. *Philosophical Transactions of the Royal Society of London Series B Biological Sciences* **361,** 235-259.

Parker,G.A. & Partridge,L. 1998. Sexual conflict and speciation. *Philosophical Transactions of the Royal Society of London Series B Biological Sciences* **353,** 261-274.

Peden,A.E. 1972. The function of gonopodial parts and behavioral pattern during copulation by *Gambusia* (Poeciliidae). *Canadian Journal of Zoology* **50,** 955-968.

Petersen,C.W., Warner,R.R., Shapiro,D.Y. & Marconato,A. 2001. Components of fertilization success in the bluehead wrasse, *Thalassoma bifasciatum* . *Behavioral Ecology* **12,** 237-245.

Pilastro,A., Benetton,S. & Bisazza,A. 2003. Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Animal Behaviour* **65,** 1161-1167.

Pilastro,A. & Bisazza,A. 1999. Insemination efficiency of two alternative male mating tactics in the guppy (*Poecilia reticulata*). *Proceedings of the Royal Society of London Series B, Biological Sciences* **266,** 1887-1891.

Pilastro,A., Evans,J.P., Sartorelli,S. & Bisazza,A. 2002. Male phenotype predicts insemination success in guppies. *Proceedings of the Royal Society of London Series B, Biological Sciences* **269,** 1325-1330.

Pilastro,A., Gasparini,C., Boschetto,C. & Evans,J.P. 2008. Colorful male guppies do not provide females with fecundity benefits. *Behavioral Ecology* **19,** 374-381.

Pilastro,A., Giacomello,E. & Bisazza,A. 1997. Sexual selection for small size in male mosquitofish (*Gambusia holbrooki*). *Proceedings of the Royal Society of London Series B, Biological Sciences* **264,** 1125-1129.

Pilastro,A., Mandelli,M., Gasparini,C., Dadda,M. & Bisazza,A. 2007. Copulation duration, insemination efficiency and male attractiveness in guppies. *Animal Behaviour* **74,** 321-328.

Pilastro,A., Simionato,M., Bisazza,A. & Evans,J.P. 2004. Cryptic female preference for colorful males in guppies. *Evolution* **58,** 665-669.

Pitcher,T.E., Neff,B.D., Rodd,F.H. & Rowe,L. 2003. Multiple mating and sequential mate choice in guppies: females trade up. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270,** 1623-1629.

Pitnick,S., Miller,G.T., Reagan,J. & Holland,B. 2001. Males' evolutionary responses to experimental removal of sexual selection. *Proceedings of the Royal Society of London Series B, Biological Sciences* **268,** 1071-1080.

Pizzari,T. & Snook,R.R. 2003. Perspective: Sexual conflict and sexual selection: Chasing away paradigm shifts. *Evolution* **57,** 1223-1236.

Pizzari,T. & Snook,R.R. 2004. Sexual conflict and sexual selection: Measuring antagonistic coevolution. *Evolution* **58,** 1389-1393.

Plath,M., Makowicz,A.M., Schlupp,I. & Tobler,M. 2007. Sexual harassment in live-bearing fishes (Poeciliidae): comparing courting and noncourting species. *Behavioral Ecology* arm030.

Pocklington,R. & Dill,L.M. 1995. Predation on females or males: Who pays for bright male traits? *Animal Behaviour* **49,** 1122-1124.

Polihronakis,M. 2006. Morphometric analysis of intraspecific shape variation in male and female genitalia of *Phyllophaga hirticula* (Coleoptera : Scarabaeidae : Melolonthinae). *Annals of the Entomological Society of America* **99,** 144-150.

Pomiankowski,A., Iwasa,Y. & Nee,S. 1991. The evolution of costly mate preferences I. Fisher and biased mutation. *Evolution* **45,** 1422-1430.

Presgraves,D.C., Baker,R.H. & Wilkinson,G.S. 1999. Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. *Proceedings of the Royal Society of London Series B-Biological Sciences* **266,** 1041-1047.

Preziosi,R.F. & Fairbairn,D.J. 1996. Sexual size dimorphism and selection in the wild in the waterstrider Aquarius remigis: Body size, components of body size and male mating success. *Journal of Evolutionary Biology* **9,** 317-336.

Preziosi,R.F. & Fairbairn,D.J. 2000. Lifetime selection on adult body size and components of body size in a waterstrider: Opposing selection and maintenance of sexual size dimorphism. *Evolution* **54,** 558-566.

Pyter,L.M., Neigh,G.N. & Nelson,R.J. 2005. Social environment modulates photoperiodic immune and reproductive responses in adult male white-footed mice (*Peromyscus leucopus*). *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **288,** R891-R896.

Ramm,S.A. 2007. Sexual selection and genital evolution in mammals: A phylogenetic analysis of baculum length. *American Naturalist* **169,** 360-369.

Reinhardt,K., Naylor,R. & Siva-Jothy,M.T. 2003. Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270,** 2371-2375.

Reynolds,J.D. & Gross,M.R. 1992. Female mate preference enhances offspring growth and reproduction in a fish, *Poecilia reticulata*. *Proceedings of the Royal Society of London Series B, Biological Sciences* **250,** 57-62.

Reynolds,J.D., Gross,M.R. & Coombs,M.J. 1993. Environmental conditions and male morphology determine alternative mating behaviour in Trinidadian guppies. *Animal Behaviour* **45,** 145-152.

Reznick,D.N. & Endler,J.A. 1982. The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). *Evolution* **36,** 125-148.

Rice,W.R. 1996. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature* **381,** 232-234.

Rice,W.R. & Chippindale,A.K. 2001. Intersexual ontogenetic conflict. *Journal of Evolutionary Biology* **14,** 685-693.

Rice,W.R. & Holland,B. 1997. The enemies within: intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen. *Behavioral Ecology and Sociobiology* **41,** 1-10.

Rodd,F.H., Reznick,D.N. & Sokolowski,M.B. 1997. Phenotypic plasticity in the life history traits of guppies: Responses to social environment. *Ecology* **78,** 419-433.

Rodd,F.H. & Sokolowski,M.B. 1995. Complex origins of variation in the sexual behaviour of male Trinidadian guppies, *Poecilia reticulata*: Interactions between social environment, heredity, body size and age. *Animal Behaviour* **49,** 1139-1159.

Roff,D.A. & Mousseau,T.A. 1987. Quantitative genetics and fitness: lessons from *Drosophila*. *Heredity* **58,** 103-118.

Rohlf,F.J. 2004. *TpsUtil, Version 1.33.* Stony Btook, NY: Department of Ecology and Evolution, State University of New York.

Rohlf,F.J. 2005a. *TpsDig2, Digitize Landmarks and Outlines, Version 2.10.* Stony Brook, NY: Department of Ecology and Evolution, State University of New York.

Rohlf,F.J. 2005b. *TpsRelw, Relative Warps Analysis, Version 1.42.* Stony Brook, NY: Department of Ecology and Evolution, State University of New York.

Rohlf,F.J. & Marcus,F. 1993. A revolution in morphometrics. *Trends in Ecology & Evolution* **8,** 129-132.

Rohlf,F.J. & Slice,D. 1990. Extensions of the procrustes method for the optimal superimposition of landmarks. *Systematic Zoology* **39,** 40-59.

Roldan,E.R.S. & Gomendio,M. 1999. The Y chromosome as a battle ground for sexual selection. *Trends in Ecology & Evolution* **14,** 58-62.

Rolff,J. & Siva-Jothy,M.T. 2002. Copulation corrupts immunity: A mechanism for a cost of mating in insects. *Proceedings of the National Academy of Sciences of the USA* **99,** 9916-9918.

Ronn,J.L., Katvala,M. & Arnqvist,G. 2007. Coevolution between harmful male genitalia and female resistance in seed beetles. *Proceedings of the National Academy of Sciences of the USA* **104,** 10921-10925.

Rosen,D.E. & Gordon,M. 1953. Functional anatomy and evolution of male genitalia in poeciliid fishes. *Zoologica* **38,** 1-47.

Rosen,D.E. & Tucker,A. 1961. Evolution of secondary sexual characters and sexual behavior patterns in a family of viviparous fishes (Cyprinodontiformes: Poeciliidae). *Copeia* **1961,** 202-212.

Rowe,L. 1994. The Costs of Mating and Mate Choice in Water Striders. *Animal Behaviour* **48,** 1049-1056.

Rubolini,D., Romano,M., Boncoraglio,G., Ferrari,R.P., Martinelli,R., Galeotti,P., Fasola,M. & Saino,N. 2005. Effects of elevated egg corticosterone levels on behavior, growth, and immunity of yellow-legged gull (*Larus michahellis*) chicks. *Hormones and Behavior* **47,** 592-605.

Rudolfsen,G., Figenschou,L., Folstad,I., Nordeide,J.T. & Soreng,E. 2005. Potential fitness benefits from mate selection in the Atlantic cod (*Gadus morhua*). *Journal of Evolutionary Biology* **18,** 172-179.

Rudolfsen,G., Figenschou,L., Folstad,I., Tveiten,H. & Figenschou,M. 2006. Rapid adjustments of sperm characteristics in relation to social status. *Proceedings of the Royal Society B-Biological Sciences* **273,** 325-332.

Russell,S.T., Ramnarine,I.W., Mahabir,R. & Magurran,A.E. 2006. Genetic detection of sperm from forced copulations between sympatric populations of *Poecilia reticulata* and *Poecilia picta*. *Biological Journal of the Linnean Society* **88,** 397-402.

Ryckman,R.E. & Ueshima,N. 1964. Biosystematics of the *Hesperocimex* complex (Hemiptera: Cimicidae) and avian hosts. *Annals of the Entomological Society of America* **57,** 624-638.

Scharer,L., Ladurner,P. & Rieger,R.M. 2004. Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm *Macrostomum* sp. *Behavioral Ecology and Sociobiology* **56,** 420-425.

Schlupp,I., McKnab,R. & Ryan,M.J. 2001. Sexual harassment as a cost for molly females: Bigger males cost less. *Behaviour* **138,** 277-286.

Schlupp,I. & Plath,M. 2005. Male mate choice and sperm allocation in a sexual/asexual mating complex of *Poecilia* (Poeciliidae, Teleostei). *Biology Letters* **1,** 169-171.

Shapiro,D.Y., Marconato,A. & Yoshikawa,T. 1994. Sperm economy in a coral-reef fish, *Thalassoma bifasciatum*. *Ecology* **75,** 1334-1344.

Sheldon,B.C. 2000. Differential allocation: tests, mechanisms and implications. *Trends in Ecology & Evolution* **15,** 397-402.

Simmons,L.W. 2001. *Sperm Competition and Its Evolutionary Consequences in the Insects* . Princeton: Princeton University Press.

Simmons,L.W., Denholm,A., Jackson,C., Levy,E. & Madon,E. 2007. Male crickets adjust ejaculate quality with both risk and intensity of sperm competition. *Biology Letters* **3,** 520-522.

Simmons,L.W. & Kotiaho,J.S. 2002. Evolution of ejaculates: Patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution* **56,** 1622-1631.

Sirot,L.K., Brockmann,H.J., Marinis,C. & Muschett,G. 2003. Maintenance of a female-limited polymorphism in *Ischnura ramburi* (Zygoptera : Coenagrionidae). *Animal Behaviour* **66,** 763-775.

Skinner,A.M.J. & Watt,P.J. 2007. Phenotypic correlates of spermatozoon quality in the guppy, Poecilia reticulata. *Behavioral Ecology* **18,** 47-52.

Smith,C.C. 2007. Independent effects of male and female density on sexual harassment, female fitness, and male competition for mates in the western mosquitofish Gambusia affinis. *Behavioral Ecology and Sociobiology* **61,** 1349-1358.

Smith,C.C. & Sargent,R.C. 2006. Female fitness declines with increasing female density but not male harassment in the western mosquitofish, *Gambusia affinis*. *Animal Behaviour* **71,** 401-407.

Smith,R.L. 1984. *Sperm Competition and the Evolution of Animal Mating Systems*. London: Academic Press.

Snook,R.R. 2005. Sperm in competition: not playing by the numbers. *Trends in Ecology & Evolution* **20,** 46-53.

Stoltz,J.A. & Neff,B.D. 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. *Journal of Evolutionary Biology* **19,** 1873-1881.

Stutt,A.D. & Siva-Jothy,M.T. 2001. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. *Proceedings of the National Academy of Sciences of the USA* **98,** 5683-5687.

Thornhill,R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *American Naturalist* **122,** 765-788.

Trivers,R.L. 1972. Parental investment and sexual selection. In: *Sexual Selection and the Descent of Man* (Ed. by B.Campbell), pp. 136-179. Chigago, Aldine.

Usinger,R.L. 1966. *Monograph of Cimicidae (Hemiptera, Heteroptera).* College Park, Md: Entomological Society of America.

Via,S., Gomulkiewicz,R., Dejong,G., Scheiner,S.M., Schlichting,C.D. & Vantienderen,P.H. 1995. Adaptive Phenotypic Plasticity - Consensus and Controversy. *Trends in Ecology & Evolution* **10,** 212-217.

Vieira,C., Pasyukova,E.G., Zeng,Z.B., Hackett,J.B., Lyman,R.F. & MacKay,T.F.C. 2000. Genotype-environment interaction for quantitative trait loci affecting life span in *Drosophila melanogaster*. *Genetics* **154,** 213-227.

Vladic,T.V., Afzelius,B.A. & Bronnikov,G.E. 2002. Sperm quality as reflected through morphology in salmon alternative life histories. *Biology of Reproduction* **66,** 98-105.

Waage,J.K. Dual function of the damselfly penis: sperm removal and transfer. Science 203, 916-918. 1979.
Ref Type: Journal (Full)

Watson,P.J. 1998. Multi-male mating and female choice increase offspring growth in the spider *Neriene litigiosa* (Linyphiidae). *Animal Behaviour* **55,** 387-403.

Welberg,L.A.M. & Seckl,J.R. 2001. Prenatal stress, glucocorticoids and the programming of the brain. *Journal of Neuroendocrinology* **13,** 113-128.

Wenninger,E.J. & Averill,A.L. 2006. Influence of body and genital morphology on relative male fertilization success in oriental beetle. *Behavioral Ecology* **17,** 656-663.

Werner,M. & Simmons,L.W. 2008. The evolution of male genitalia: functional integration of genital sclerites in the dung beetle *Onthophagus taurus*. *Biological Journal of the Linnean Society* **93,** 257-266.

Winge,O. 1937. Successions of broods in *Lebistes*. *Nature* **140,** 467.

Wolfner,M.F. 1997. Tokens of love: Functions and regulation of *Drosophila* male accessory gland products. *Insect Biochemistry and Molecular Biology* **27,** 179-192.

Woolley,D.M. 1971. Selection for the length of the spermatozoan midpiece in the mouse. *Genetical Research* **16,** 261-275.

Zelditch,M.L., Swiderski,D.L., Sheets,H.D. & Fink,W.L. 2007. *Geometric Morphometrics for Biologists*. London: Elsevier Academic Press.

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