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Evolutionary consequences of producing competitive ejaculates: insights from an artificial selection study for sperm production in *Poecilia reticulata*

Conseguenze evolutive della produzione di eiaculati competitivi: evidenze da uno studio di selezione artificiale per la produzione di spermi in *Poecilia reticulata*

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Thesis Abstract

The past decades have seen an intense growth of the research interest in sexual selection. It is now definitely clear that mate choice occurs and a growing number of studies indicate that sexual selection via mate choice can be a strong evolutionary force. Sexual selection in animals in which males (generally) compete to mate with choosy (generally) females has generated traits that promote the transmission of one individual's genes at the expense of another (Eberhard 1996; Birkhead and Moller 1998; Simmons 2001). Researchers also began to study postcopulatory sexual selection and how this might influence other sexual traits' evolution, when the presumption that monogamy is the dominant female reproductive strategy has been progressively abandoned since Geoff Parker's studies on females sexual promiscuity in the early 1970's. Female sexual promiscuity leads to sperm competition (SC) and cryptic female choice, contributing to differential male reproductive success.

SC has been shown to be a pervasive and important source of selection on males (Birkhead 2000; Simmons 2001): it can result in the evolution of morphological, physiological and behavioural adaptations in both sexes. Typically, it results in the evolution of ejaculate characteristics that lead to increased success in postcopulatory male-male competition, such as sperm number or concentration, and sperm quality traits, such as sperm size and performance (viability, longevity, swimming speed, etc.). A recent review of quantitative genetics studies on ejaculate evolution suggests that the coefficients of additive genetic variation (evolvability) of sperm production is relatively high compared to sperm quality traits (Simmons and Moore 2009). The same pattern has recently been confirmed in the guppy, *Poecilia reticulata* (Gasparini et al. 2013). One of the most controversial topics in evolutionary biology is the maintenance of such elevated additive genetic variance for sexual traits undergoing strong directional selection and many hypotheses have been put forward to explain how genetic variation in sexually selected traits is maintained (Radwan 2008). Collectively, three different non-mutually exclusive processes can be recognized:

1) genic-capture through condition dependence (Rowe and Houle 1996): exaggerated male traits are costly and become linked to condition (the pool of resources available for reproduction and survival) and hence influenced by most of the genome. Virtually any new mutation is therefore likely to influence male condition and consequently the expression of condition-dependent traits. New deleterious mutations, occurring at each generation, will introduce new genetic variation despite purging by directional sexual selection. The expression of a sexually selected trait therefore signal a male's mutation load.

2) negative pleiotropy: these models predict that genetic variation on trait is maintained by trade-offs with other traits, subject to either sexual or natural selection. These models predict that negative genetic correlations should be observed between fitness related traits.

3) antagonistic sexual selection: traits that increase male reproductive fitness are detrimental when expressed in females. These models predict that daughters of most successful females have a lower fitness than the daughters of least successful males, or that most successful males impose a reproductive cost to the females they mate with, as a consequence of sexual conflict.

The aim of my study was to investigate the maintenance of variability for sperm production in the guppy, *P. reticulata*. Sperm number, which is the main determinant of SC outcome in the Trinidadian guppy population (from Lower Tacarigua river) studied in my lab (Boschetto et al. 2011), shows high additive genetic variance despite strong directional selection and very high sire heritability and stronger condition dependence than other ejaculate traits (Gasparini et al. 2013); furthermore, multiply mated females produce higher quality offspring, suggesting that SC, and hence sperm number, may mediate genetic benefits to the female (Kuckuck and Greven 1997; Pilastro et al. 2008; Barbosa and Magurran 2011). To clarify the evolutionary processes associated with sperm allocation, I used bi-directional artificial selection for sperm production to 1) estimate the genetic correlations between sperm production and other sexually and naturally selected traits in both males and females; 2) investigate whether sperm production is associated with the rate of senescence and longevity; 3) estimate the contribution of partially recessive deleterious mutations to phenotypic variation in sperm production .

Males selected for large sperm number showed only null or positive correlated responses with other fitness-related traits. However, I have highlighted a trade-off between sperm investment, longevity and senescence (a

"live fast-die young" strategy, Bonduriansky et al. 2008). These trade-offs may have the potential to maintain the genetic variance in sperm number, although they involve a limited number of traits among those considered. Because of its condition-dependence, sperm production is also expected to be targeted more easily by deleterious mutations, ultimately linking trait expression with male genetic quality (Rowe and Houle 1996; Wilkinson et al. 1998). My results also accord with the genic-capture hypothesis: I found evidence of more severe inbreeding depression, an estimate of mutation load (Charlesworth and Willis 2009), in both males and females selected for reduced sperm production. In contrast, I found no evidence of sexual antagonism, as female fecundity was not reduced in high sperm lines. Collectively, these results indicate that both deleterious mutations and negative pleiotropy, but apparently not antagonistic sexual selection, play a role in the maintenance of genetic variability for sperm number.

General Introduction

The main function of sperm is quite obvious: sperm deliver the male genome to the zygote, restoring a diploid state in sexually reproducing organisms. In general, eggs are big and immotile whereas sperm are small and motile, so that, in internal fertilizers, swimming ability is important for them to move through the gonoduct to ensure egg fertilization. Moreover, copulation may not coincide temporally with the availability of mature eggs, and sperm may therefore need to persist for long periods inside female genitalia. When females mate with more than one male during the same reproductive cycle, sperm competition (SC) occurs – gametes from two or more males compete for the fertilization of the same set of ova. Postcopulatory sexual selection, resulting from SC, is thought to be the evolutionary cause of the large variation in sperm number, morphology, and swimming ability observed across species.

Indeed, relative testes size has been shown to increase ubiquitously with SC across species in many different animal groups (Birkhead and Moller 1998; Simmons and Fitzpatrick 2012) and experimental evolution studies have confirmed an increased ejaculate investment in response to elevated SC (Hosken and Ward 2001; Simmons and Garcia-Gonzalez 2008; Firman and Simmons 2011). Although it is often assumed that sperm are cheap, and that males are limited in their reproduction only by the number of females they mate with (Borgia 1979), ejaculate production can represent a significant cost for males (Dewsbury 1982; Hayward and Gillooly 2011) and there is evidence that males can deplete their sperm reserves (Birkhead and Fletcher 1995; Olsson et al. 1997; Preston et al. 2001; Doyle 2011), thus limiting the numbers of effective matings they can obtain. If sperm are costly, we expect to observe evolutionary trade-offs between ejaculate production and other costly traits such as those involved in precopulatory competition over access to females. While empirical evidence generally seems to support this prediction, there are a few notable exceptions (table 1).

Table 1. A review of correlations involving male expenditure in postcopulatory traits and in precopulatory traits.

Species	Postcopulatory trait	Precopulatory trait	Correlation	Ref.
Insects				
Cyrtodiopsis dalmanni	Testes mass	Eyespan	-	Fry 2006
Teleopsis dalmanni	Testes size	Eyespan	+	Rogers et al. 2008
Gnatocerus cornutus	Testes mass	Mandible size	-	Yamane et al. 2010
Panorpa cognate	Sperm investment per mating	Attractiveness	-	Engqvist 2011
Photinus greeni	Fertilization success	Attractiveness	-	Demary et al. 2007
Hemideina crassidens	Testes size and ejaculate volume	Weapon size	-	Kelly 2008
Fishes				
Thalassoma bifasciatum	Sperm production	Mating success	-	Warner et al. 1995
Poecilia reticulata	Sperm quality	Attractiveness	-	Evans 2010
Poecilia reticulata	Sperm viability and velocity	Colouration	+	Locatello et al. 2006
Birds				
Gallus gallus domesticus	Ejaculate quality	Dominance status	-	Pizzari et al. 2007
Malurus melanocephalus	Sperm number	Plumage ornamentation	-	Rowe et al. 2010
Mammals				
Cervus elaphus	Testes size and sperm velocity	Antler size	+	Malo et al. 2005
Homo sapiens	Ejaculated sperm number	Voice attractiveness	-	Simmons et al. 2011

Costs of acquiring matings and producing ejaculates have recently been incorporated into Parker's game theoretic models of ejaculate expenditure (Parker et al. 2013): males are assumed to have a fixed energy budget to spend on reproduction (R), which can be spent either on mating acquisition or on ejaculate components favouring SC success (e.g. sperm number vs. sperm quality). Although pre- and postcopulatory expenditure are expected to trade-off within a fixed energy budget, they may both increase if variance in resource acquisition exceeds variance in allocation: males with greater ability to acquire resources will be able to allocate more

resources to both armaments and ejaculates (Reznick 1985; van Noordwijk and de Jong 1986). Indeed, positive associations between pre- and postcopulatory expenditure have been reported (table 1). In guppies, *Poecilia reticulata*, colourful males (more attractive to females) have faster and more viable sperm (Locatello et al. 2006), implying that some males have a greater investment in both attractiveness and fertilization capability. If sperm competitiveness is correlated with male genetic quality, females might thereby ensure good-genes benefits for their offspring not just by precopulatory mate choice, but also by polyandry (Yasui 1997). However, more attractive male guppies have been shown to sire sons with a reduced longevity (Brooks 2000), suggesting that increased male reproductive allocation may occur at the expenses of naturally-selected traits.

According to life-history theory, rather than a fixed reproductive energy budget R, it is often assumed that there is a fixed energy budget Q, from which resources are allocated to reproduction and somatic maintenance (S), that is: $Q = R + S$. Postcopulatory allocation is expected to increase with SC, and R cannot increase unless at the expense other trait affecting S (Parker et al. 2013). This is the so-called "live fast-die young" scenario, in which males investing more in reproduction pay a cost in survival due to a more rapid senescence (the deterioration in phenotype that occurs with advancing age) and/or a reduced longevity (Brooks 2000; Hunt et al. 2004; Miller and Brooks 2005; Preston et al. 2011). If we partition R into its two pre- (R_{pre}) and postcopulatory (R_{post}) components, then $Q = (R_{pre} + R_{post}) + S$. There are several complications here, however. One is that R_{pre} can affect SC level faced by a male - for instance males with large weaponry can limit the access of other males to the females, thus reducing female mating rate. The investment in R_{pre} will affect therefore the fitness return of R_{post}. Second, Q may actually vary among males, due to overall differences in genetic quality (due, for example, to the load of deleterious mutations). Quantifying R and S is therefore of crucial importance for our understanding of the evolutionary trajectories associated with sexual traits and these parameters have been studied in several taxa. Very few studies, however, have attempted a comprehensive analysis of the effect of the investment in ejaculate traits (R_{post}) on the other fitness components subject to sexual and natural selection.

The phenotypic expression of exaggerated sexual traits subject to directional selection is expected to evolve condition dependence, ultimately linking trait expression with male overall genetic quality (Rowe and Houle 1996; Wilkinson et al. 1998). Whether male genetic quality is determined by few loci with alleles with large effect at intermediate frequency (e.g. loci associated with parasite resistance) or by a reduced load of numerous, partially recessive, small-effect deleterious mutations has important implications for the solution of the lek paradox for sexually-selected traits, and more generally for the maintenance of sexual reproduction (Agrawal 2001). When the genetic variance in sexual traits under directional selection is due to few loci with large-effect alleles, directional selection is expected to rapidly fix the alleles with highest fitness, unless other mechanisms such as negative pleiotropy and/or antagonistic sexual selection are operating and generate trade-offs among fitness components (e.g. Johnston et al. 2013). Alternatively, the continuous influx of deleterious mutations can maintain the genetic variance in sexual traits because their cost depends on condition. Condition can be defined as the pool of resources available to an individual for its maintenance and reproduction (Rowe and Houle 1996). As virtually any trait, and hence the whole genome, is expected to influence condition, any new mutation will affect condition and hence the expression of sexual trait. Evidence that the effect of mutations is disproportionally deleterious on the expression of sexually selected traits has been found in several cases (Howie et al. 2013), including ejaculate traits (Simmons and Kotiaho 2002). There is no reason, however, to expect that a single mechanism is responsible for the maintenance of genetic variation in sexually selected traits, and a comprehensive approach aimed at estimating the relative contribution of different mechanisms is therefore warranted.

Aim of the Study

The aim of this study is to investigate the mechanisms involved in the maintenance of genetic variability in sperm production in the guppy, *Poecilia reticulata*. This species shows one of the highest levels of multiple paternity reported for any vertebrate (Neff et al. 2008) and the pre- and postcopulatory sexual selection processes have been studied in great detail (Houde 1997; Evans and Pilastro 2011). In particular, during precopulatory mate choice, females prefer to mate with males with larger colour spots on the body (Houde 1997; Evans et al. 2004a), but these ornamental traits are genetically associated with a reduced male survival (Brooks 2000) and a reduced sperm quality (Evans 2010), suggesting that genes enhancing sexual attractiveness may be associated with pleiotropic costs. Negative genetic correlations within ejaculate among different sperm quality traits have also been reported (Evans 2011), suggesting that different ejaculate components may not evolve independently (Moore et al. 2004; Birkhead et al. 2005). Evidence of these genetic trade-offs, however, have been obtained in a feral Australian population. Studies conducted on guppies from their original distribution area (Trinidad) have instead revealed a positive phenotypic correlation within ejaculate between sperm traits (Skinner and Watt 2006) and between ejaculate and precopulatory traits such as colour and courtship rate (Matthews et al. 1997; Locatello et al. 2006; Pitcher et al. 2008). Sperm number, which is the main determinant of sperm competition outcome in the Trinidadian guppy population (from lower Tacarigua river) studied in my lab (Boschetto et al. 2011), shows high additive genetic variance despite strong directional selection and very high sire heritability and stronger condition dependence than other ejaculate traits (Gasparini et al. 2013); furthermore, multiply mated females produce higher quality offspring, suggesting that SC (probably through sperm number) may mediate genetic benefits to the female (Evans and Magurran 2000; Ojanguren et al. 2005; Barbosa and Magurran 2011). To clarify the evolutionary processes associated with sperm allocation, I used bi-directional artificial selection for sperm production to answer to the following four questions.

1) Are there genetic correlations between sperm production and other sexually-selected traits in males at sexual maturation?

Polyandry promotes the overlap of ejaculates from different males, resulting in a competition for the fertilization of the same set of eggs, sperm competition (SC). SC favours adaptations in the ejaculate that ensure a higher fertilization success (Parker 1970). Evolutionary response to elevated sperm competition involves primarily the number of sperm produced/ejaculated, although ejaculate performance (e.g. sperm size, swimming velocity or viability, Snook 2005, and seminal fluids, Poiani 2006) is also often involved. Indeed, relative testes size has been shown to increase ubiquitously with SC across species in many different animal groups (Birkhead and Moller 1998; Simmons and Fitzpatrick 2012) and experimental evolution studies have confirmed an increased ejaculate investment in response to elevated SC (Hosken and Ward 2001; Simmons and Garcia-Gonzalez 2008; Firman and Simmons 2010; 2011).

Although it has often been assumed that sperm are cheap, and that males are limited in their reproduction only by the number of females they mate with (Borgia 1979), ejaculate production can instead represent a significant cost for males (Dewsbury 1982; Hayward and Gillooly 2011; Rahman et al. 2013; Gasparini et al. 2013) and males can deplete their sperm reserves (Birkhead and Fletcher 1995; Olsson et al. 1997; Preston et al. 2001; Doyle 2011), thus limiting the number of offspring they can sire. If sperm are costly, we expect to observe evolutionary trade-offs between ejaculate production and other costly traits such as those involved in precopulatory competition over the access to females, an assumption supported by empirical observations (see Kvarnemo and Simmons 2013 for a recent review). Ejaculate investment can also be traded-off among within ejaculate among different quality traits such as, for example, sperm number and size (e.g. Parker et al. 2010). Indeed, evidence that sperm number is traded offs with sperm size or performance has been found both at the interspecific and intraspecific level (Gage and Morrow 2003; Moore et al. 2004; Snook 2005; Immler et al. 2011). Negative genetic correlations among sexual traits associated with reproductive fitness would help explaining the maintenance of genetic variation in sperm number.

To clarify the evolutionary processes associated with sperm allocation, I performed a bidirectional artificial selection experiment for high (HS) and low (LS) sperm production in guppies. Correlated responses to selection

in traits related to mating acquisition and fertilization were investigated to assess whether trade-offs were present among these components.

2) Does male investment in sperm production affect life history traits in males or females?
Male investment in reproduction may be traded-offs with life history traits subject to natural selection (Reznick 1985; Lynch and Spitze 1994; Gustafsson et al. 1995; Roff 2000). Moreover, these trade-offs may become evident also in females, through antagonistic sexual selection (Arnqvist and Rowe 2005). There is cumulating evidence that sexual conflict increase with multiple mating (Rice 2000; Miller and Brooks 2005; Gasparini et al. 2012), as does the potential for sperm competition (Parker 1970). As a result, sperm competition should enhance sexual conflict and thus lead to the evolution of traits that increase SC success in one sex, even when they are costly to the other. One of the most important traits in SC in many species is the number of sperm produced (Parker 1970; Birkhead et al. 2009; Boschetto et al. 2011), but whether this trait is associated with sexual conflict or with costs in male life history traits is still unknown. Indeed 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 from being universally true (Dewsbury 1982; Olsson et al. 1997; Gasparini et al. 2013). Eggs are costly to produce because of their great unitary investment. Consequently, females are more careful in the choice of the mating partner than males, because sperm reserves could be restored more quickly. Thus, males are typically less limited than females in the number of gametes they can produce and their reproductive success is more strongly correlated with the number of mates (Bateman 1948). As a consequence, sexual selection acts differently on males and females. Males are usually selected to produce as many offspring as possible, whereas females to produce high quality offspring. Sexual conflict enters the scene whenever males and females evolutionary interests diverge. This conflict can take two forms: i) traits that enhance male reproductive success directly reduce female reproductive success. For example, in *Drosophila*, ejaculate characteristics associated with SC success are harmful for the females (Rice 1996). 2) traits selected in the males reduce female fitness when expressed in the daughters. This is expected when sex-limited expression is incomplete and sexes have different trait expression optima. For example, it has been hypothesized that in the red deer (*Cervus elaphus*) high levels of androgens make males more successful but decrease female fecundity (Foerster et al. 2007).

In this study, I used the guppy *Poecilia reticulata*, a model system in sexual selection studies (Evans and Pilastro 2011), to test whether males investing more resources in sperm production face trade-offs in fitness-related traits in both males and females. After one generation of artificial selection for sperm production I compared the expression of important life-history traits in both sexes in the two selection lines.

3) Is the opposite investment in sperm production associated with senescence rate and longevity?
Central to the understanding of the evolution of life histories is the fundamental concept that trade-offs among life-history traits involving reproduction and survival are expected (Stearns 1989; Roff 1992; 2000; 2002). The focus of attention has been on the trade-offs involving reproduction as number of matings, or sexual traits involved in mating acquisition, and survival (Kotiaho and Simmons 2003; Fedorka et al. 2004; Martin and Hosken 2004; Simmons and Kvarnemo 2006), but whether this cost is associated with sperm production remains untested. Life-history theory of senescence predicts that traits expressing late in life may accumulate more mutations because they are subject to weaker selection (due to extrinsic mortality) or because they are associated with early expression of fitness related traits (negative pleiotropy) (Reznick 1985; Stearns 1992; Roff 2002). I will therefore extend my investigation to the rate of senescence in fitness related traits and to survival. In particular, the aim of this study is to investigate whether male investment in sperm production affects longevity and the rate of senescence in sexual traits (pre- and postcopulatory) and condition in male guppies. To this end, I used males artificially selected for the high (HS) and low (LS) sperm number for three generations. A subset of these males was monitored until death, and traits affecting mating and sperm competition success were measured at the age of 10 and 14 months, which are comparable with previous studies of senescence in the guppy (Miller and Brooks 2005; Gasparini et al. 2010a). Life-history theory predicts that males selected for high sperm production should have shorter lifespan and more severe senescence compared to males selected for lower sperm investment.

4) Is sperm production correlated with deleterious mutations?

Directional selection on sexual traits should rapidly erode the underlying genetic variation (Borgia 1979; Andersson 1994). In striking contrast with this expectation, traits subject to sexual selection usually show large genetic variation than most naturally selected traits (Pomiankowski and Moller 1995). One mechanism invoked to explain this paradox is that trait expression depends on condition which, in turn, is influenced by most of the genome and hence by the overall load of deleterious mutations (Borgia 1979; Pomiankowski et al. 1991; Rowe and Houle 1996). New mutations occur at each generation, maintaining the genetic variation in the sexual trait despite directional selection. Empirical support for this relationship, however, has been proved difficult to obtain (e.g. Postma 2011) and direct, experimental evidence that males with higher expression of a sexually selected trait have a reduced load of deleterious mutations is still lacking.

I investigated this topic in guppies (*Poecilia reticulata*), a tropical livebearing fish with a resource-free mating system (Evans and Pilastro 2011), using an experimental approach specifically aimed at testing the correlation between genome-wide deleterious mutations and trait expression (Kelly 1999). In this species, females mate promiscuously and a male's sperm competition success is linearly and positively correlated with the number of sperm he transfers during copulation (Boschetto et al. 2011). Sperm number shows condition dependence, large genetic variance, and strong directional dominance (a signature of historical directional selection) (Gasparini et al. 2013). Multiply mated females produce better quality offspring (Evans and Magurran 2000), suggesting that sperm number may be associated with male overall genetic quality (i.e., a reduced mutation load) .

I artificially selected male guppies with high and low sperm production (two replicates). Using fish from the second generation of selection, I performed (within each selection/replicate) two generations of matings between full-sibs and unrelated mates. Mating with close relatives amplifies the negative effect of partially recessive deleterious mutations due to their increased homozygosity and trait expression in inbred individuals (relative to that of their outbred counterparts) gives an estimate of the mutation load (Charlesworth and Willis 2009). If sperm number is negatively correlated with mutation load, lines selected for low sperm number should show a stronger inbreeding depression (ID) in fitness than that observed in lines selected for high sperm number.

Study Species

Poecilia reticulata, a freshwater viviparous fish with internal fertilization, is a ideal species for sexual selection studies because it is particularly well suited for the investigation of pre- and postcopulatory processes: females are sexually promiscuous, with one of the highest levels of multiple paternity observed in nature (Neff et al. 2008); ejaculate collection and analysis is relatively easy and does not require male sacrifice (Gasparini and Pilastro 2011); natural copulation and sexual behaviour normally occurs under laboratory conditions (Houde 1997); generation time is three months, making experimental evolution experiments reasonably practical.
The guppy, *P. reticulata,* is an internal fertilizer species and presents a strong sexual dimorphism (Fig. 1). Females have a camouflaging gray colouration and a black spot in the anal region. Males are, on average, smaller than females and show an elaborated colour pattern on the body and the fins. Colour spots vary in size, shape and pigmentation: yellow and orange (determined by carotenoid and pteridine pigments), black (melanin pigments), and iridescent (white-blue-green structural colours). Males present an extremely high inter- and intra population variation in the colouration pattern, with strong Y-linkage (Brooks and Postma 2011).

Male guppies have a modified anal fin, called gonopodium, used as copulatory organ. This structure is formed with three rays of the anal fin fused to form a capillary-like structure through which sperm bundles are transferred into the female gonoduct. The gonopodium in provided with a distal cover part called 'hood' and a pair of hooks, both with sensory functions (Constantz 1989; Kwan et al. 2013). Sperm are transferred in packs (spermatozeugmata) called 'sperm bundles', containing about 21,000 sperm each (Gasparini et al. 2010a; Boschetto et al. 2011). Once in the female gonoduct, sperm bundles break up and release sperm cells that swim to the ovary where can be maintained for several months and fertilize several clutch of eggs (Constantz 1989).

During gestation, embryos develop in the uterine cavity for about one month, when most of the yolk is consumed and fully independent offspring are given birth. Brood size (1-40 offspring) varies with season, mother size, condition, and age (Houde 1997). After parturition, a new set of ova is ready to be fertilized and female will be sexually receptive to males for 2-5 days. Males reach sexual maturity after 6-8 weeks, when males have a fully develop gonopodium, produce sperm and develop their colour spots (Evans et al. 2002). Body growth is strongly reduced after sexual maturation (Reznick 1980).

Sexually receptive female guppies prefer to mate with males with large body size, and bright colouration and high rate of courtship display (Liley 1966; Houde 1997). These three precopulatory traits show a positive phenotypic correlation with sperm reserves in guppies from the lower part of the Tacarigua River, Trinidad, from where derived the fish used in my experiment (Matthews et al. 1997; Pitcher and Evans 2001). Males present three major types of colour spots, orange, iridescent and black, which all show a high, Y-linked heritability (Postma et al. 2011). Female preference for these colour components can vary across populations, but the preference for orange spots, which are due to carotenoid and pteridine pigments, seems universal as it has been found in all populations investigated so far (Magurran 2005). Preference for males with large orange spots has also been confirmed in the population I have used for this study (Evans et al. 2004a). In some populations a preference for gonopodium length has also been evidenced (Brooks and Caithness 1995). Gonopodium length is also associated with the success in obtaining forced copulations (Evans et al. 2011, see also below). When the female is unreceptive, males attempt to obtain matings through a coercive mating tactic called gonopodial thrusting (GT) (Houde 1997). Forced matings have a lower insemination success than cooperative copulations (Pilastro and Bisazza 1999) but are thought to contribute to a male's reproductive success given the high frequency with which this mating tactic is adopted (up to one GT attempt per minute, Magurran and Seghers 1994).

Sperm competition success is influenced by relative differences in sperm number and sperm velocity between males (Boschetto et al. 2011), but it has been shown that, once sperm number is controlled for, also sperm viability strongly determine competitive fertilization success when artificial insemination is used to compete

ejaculates from rival males (Fitzpatrick, J. L. and Evans, J. P.; unpublished data). This is not surprising, as sperm viability determines the number of sperm that actually compete for the eggs.

In part, pre- and postcopulatory traits appear to be subject to directional sexual selection in the guppy (Endler and Houde 1995; Pilastro et al. 2002; Pilastro et al. 2004; Locatello et al. 2006; Pilastro et al. 2007; Boschetto et al. 2011). Quantitative genetic studies, however, have revealed that both pre- and postcopulatory sexually selected traits exhibit relatively high levels of additive genetic variation (Brooks and Endler 2001; Evans, 2010; 2011).

This species is therefore appropriate to study how variability is maintained on traits despite strong (apparently) directional sexual selection.

Fig. 1. Male (above) and female (below) guppies. Body size and colour pattern sexual dimorphism is evident. Modified anal fin (gonopodium) is also visible.

Summary of the Results

I summarize below the sections of my study.

I. Correlated responses in pre- and postcopulatory traits to artificial selection on sperm production in the guppy (*Poecilia reticulata*)

(see Manuscript I for details)

Sperm production readily responded to selection already at the 1st generation. My results show that sperm production is positively, genetically correlated with several other sexually-selected traits, operating both at pre- and postcopulatory level. Males selected for high sperm number attain a larger body size, are more colourful and more attractive to females. Sperm velocity and morphology are not affected, but sperm viability and the number of sperm transferred per copulation increase with sperm number.
The positive correlations with orange colouration and male attractiveness in particular, suggest that males with increased sperm production can afford to invest more resources also in mating acquisition traits. Indeed in this species, females prefer to mate with males bearing larger orange spots (Evans et al. 2004a), and allow them to transfer more sperm during cooperative matings (Pilastro et al. 2002; 2004; 2007). Males with larger sperm reserves transfer more sperm during natural copulations, even after including male body size and orange colouration as covariates, which confirms results from previous experiments (Pilastro et al. 2002; 2004; 2007). This result indicates that males selected for increased sperm production transfer more sperm even when their sexual attractiveness is controlled for. Moreover, their ejaculate contains a greater proportion of viable sperm, indicating that these males produce more sperm of higher quality. These results, combined with the absence of trade-offs with other measured traits, suggest that male genotypes selected for increased sperm production are expected to have striking advantages in both mating acquisition and SC, increasing their reproductive fitness.

II. Consequences of male investment in sperm production on life-history traits in males and females of *Poecilia reticulata*

(see Manuscript II for details)

I found that sons of males selected for increased sperm production have a faster sexual maturation. compared to their lower sperm (LS) counterparts. Thus, high sperm (HS) males can start breeding earlier than LS males. This is an important advantage, considering that the original population of our experimental fish is subject to high extrinsic mortality due to predation in its natural environment (Endler 1987). Because fitness depends on the age at sexual maturation through faster generation time (early maturation) and reduced mortality in the early stages (delayed maturation), then when extrinsic mortality is high, faster sexual maturation is favoured (Reznick et al. 1996). Age at maturation is particularly important in species with continuous breeding during their whole lifetime, such as *P. reticulata*. Indeed, a shortening of the birth-to-maturation interval may result in a conspicuous increase in the population growth, which is an index of fitness (Reznick et al. 1990).

Despite earlier maturation (two weeks on average) HS males attained the same body size at maturation as LS males, because their growth rate was faster as compared to that of LS males. In many species there is a trade-offs between growth rate and size at maturation (Gibbons et al. 1981; Stearns 1989; 1992; Nylin and Gotthard 1998), but in my experiment evidence goes in the opposite direction: not only males selected for increased sperm production do not pay a cost in terms of somatic development, but there is also a positive genetic covariation with both growth rate and sexual maturation. In contrast, I found not evidence of correlated response in female life-history traits, as HS and LS females grew at the same rate, matured at the same age and showed similar fecundity.

Altogether these results suggest that male genotypes selected for increased sperm production, a costly trait (Dewsbury 1982; Hayward and Gillooly 2011; Gasparini et al. 2013; Devigili et al. 2013), show better performance also in other fitness-related traits and apparently no costs for traits associated with natural selection. These data confirm the results of the previous study in which same most pre- and postcopulatory sexual traits

were positively correlated with sperm number, without obvious negative effects on male performances (see Manuscript I). The presence of positive rather negative correlations in traits undergoing both sexual and natural selection indicate that sperm production is associated with male condition (Gasparini et al. 2013). This implies that the resources available for reproduction and survival (at least up to sexual maturity) covary with sperm number, suggesting that, at least at this stage of the life-cycle, the variance in resource acquisition exceeds the variance. Why, then, HS males do not invade the population, being a sort of Darwinian demon (Reznick 1985; van Noordwijk and de Jong 1986). Two mechanisms may explain this paradoxical result: negative pleiotropy and deleterious mutations. The next two chapters will deal, in succession, with these two mechanisms.

III. Male guppies (*Poecilia reticulata*) selected for increased sperm production age more rapidly and die young

(see Manuscript III for details)

I measured senescence rate and longevity in males from the third generation of selection. High sperm line (HS) showed a reduced lifespan compared to their low sperm counterparts (LS). Moreover, old males from the HS line also showed a reduced sperm velocity, suggesting a potential trade-off between sperm quantity and quality during senescence. Moreover, while sperm number increased throughout the entire life of males, according to previous results (Gasparini et al. 2010a), I observed a higher increment in LS males, although their sperm production remained higher, in absolute values, in HS males. Finally, the difference in orange and black colour spots observed at 5 months of age (HS males were more colourful) (see Manuscript I), disappeared with age, suggesting that the potential advantage in mating acquisition highlighted in young HS males was not permanent. Altogether these aspects suggest that resource acquisition and allocation pattern vary during male lifespan and that guppy males "live fast and die young" (Brooks 2000; Hunt et al. 2004; Preston et al. 2011). It is difficult to extrapolate the total fitness of the two selected genotypes starting from the phenotypic expression of the traits I have measured in this experiment, because the relative contribution of each trait to total fitness is hard to be estimated. Nonetheless, my results clearly demonstrate that the positive associations between sperm production and other sexual traits observed around sexual maturation become null or negative later in life, suggesting a negative pleiotropic effect of sperm number on male senescence and longevity. Genetic fitness costs associated with SC may be either direct (pleiotropic) consequences of sperm production (e.g. energetic costs), or due to linkage disequilibrium between sperm production genes and deleterious alleles expressed late in life.
Sexually-selected traits show higher genetic variance than expected for traits constantly exposed to directional sexual selection (the so-called lek paradox, Andersson 1994; Pomiankowski and Moller 1995; Rowe and Houle 1996). A number of hypotheses relate trait expression to multiple genes of small effect that underlie male's condition, capturing genome-wide variance through condition dependence for costly sexual traits (Rowe and Houle 1996; Tomkins et al. 2004; Radwan 2008; Chenoweth and McGuigan 2010). Altogether these data suggest that negative pleiotropy contribute to the maintenance of the genetic variance in sperm number. In the last part of my study (see below) I estimated the role of partially recessive deleterious mutations in the expression of sperm number.

IV. Negative correlation between deleterious mutations and sperm number in guppies

(see Manuscript IV for details)

If the expression of a sexual trait is influenced by the load of partially recessive, deleterious mutations, the negative effect of inbreeding (which increases the number of homozygous mutations) on this trait and on other condition-dependent traits should be larger in individuals with low sexual trait expression. I therefore conducted two generations of full-sib and unrelated matings within each family obtained from the second generation of selection line. After one generation of inbreeding I found significant ID in sperm number in LS, but not in HS males. Consistent with this result, ID was larger in LS males than in HS males also in the size of the body colour spots (area of orange and iridescent spots) and in capture time. The pattern of female fecundity further supported the prediction that sperm number is correlated with the load of genome-wide deleterious mutations in guppies. When the same experimental males were mated with outbred, unrelated virgin females from the stock

population (i.e. unselected), I did not observe any significant difference in female fecundity among experimental groups, indicating that the reduced brood size observed in the inbred LS families was not due to sperm limitation. The decline in fecundity was due to the direct effect of inbreeding on embryo viability and/or to its indirect effect on female condition and the initial number of eggs per clutch. My results demonstrated that sperm number reflects the load of deleterious mutations in guppies and that a significant part of the genetic variance exhibited by this condition-dependent trait could therefore be maintained through a mutation-selection balance (Pomiankowski et al. 1991; Rowe and Houle 1996; Houle and Kondrashov 2002). Previous studies on the same population demonstrated that multiply mated females produce fitter offspring (Evans and Magurran 2000) and that sperm number mediates male competitive fertilization success (Boschetto et al. 2011). It seems therefore reasonable to hypothesize that postcopulatory processes responsible for the genetic benefits associated with polyandry are at least partly mediated by the reduced load of deleterious mutations associated with high sperm production (Rowe and Houle 1996).

Conclusions

I used an artificial selection approach to manipulate male investment in a costly postcopulatory trait, sperm production (HS=high sperm, LS=low sperm), subjected to directional selection in guppies. Indeed, the number of inseminated sperm is the main determinant of SC outcome in *P. reticulata* (Boschetto et al. 2011) as in most vertebrates (Birkhead et al. 2009). The high level of polyandry observed in this species (Houde 1997; Magurran 2005) generates a strong selective pressure on this ejaculate trait, and should deplete its genetic variance. In contrast, genetic variance for sperm number is actually larger than for any other ejaculate trait in guppies (Gasparini et al. 2013). There are three, non-mutually exclusive explanations for this apparent paradox: evolutionary trade-offs with other male costly traits, antagonistically sexual selection traits and condition dependence (Radwan 2008). Trade-offs are expected to arise whenever resources available for traits' expression are limited (Stearns 1989). Antagonistic sexual selection car arise because of sexual conflict and can be caused by a direct negative effect of male sexual trait on female fitness (for example when males with larger sexual traits are more harmful to the females) or indirectly when genes responsible for male trait exaggeration have negative effect on females fitness due to incomplete sex-limited gene expression. Condition dependence is expected to arise for traits closely related to fitness (Houle 1992) and could contribute maintaining genetic variance in sexual traits, as condition (the pool of resources available to an individual for allocation on survival and reproduction) is likely to be influenced by large part of the genome. If a trait, because of its association with condition, is affected by most of the genome, it is more likely to be affected, directly or indirectly (Chenoweth and McGuigan 2010), by any deleterious mutations (Rowe and Houle 1996). New mutations are expected to arise at any generation, thus introducing new genetic variation for the sexual trait. I tested the predictions of these three models using guppies artificially selected for opposite investments in sperm production. This experimental design offers the possibility to investigate the evolutionary constraints between sperm number and other fitness-related traits subject to both sexual and natural selection, and to assess whether loci with intermediate allele frequency or partially recessive deleterious mutations contribute to the maintenance of sperm production variance.

Males selected for large sperm number showed only null or positive correlated responses with other fitness-related traits (table 2), suggesting a substantial variance in resource acquisition (van Noordwijk and de Jong 1986; Reznick et al. 2000), as expected for a trait with strong condition dependence (Gasparini et al. 2013). However, I have also highlighted a trade-off between sperm investment, longevity and senescence (a "live fast-die young" strategy, Bonduriansky et al. 2008). These trade-offs have the potential to contribute to the maintenance of the genetic variance in sperm number, although they involve a limited number of traits among those considered (table 2). It would be interesting to extend this scenario also to females, because sexual conflict has the potential to displace the sexes from their sex-specific optima for life span and senescence (Promislow 2003). At least in early stages of life and for the limited number of traits measured, however, I could not find evidence of negative effects of sperm production on female fitness, suggesting that variance in sperm number may not be maintained through negative effects on female fitness.

Because of its condition-dependence, sperm production is also expected to be targeted more easily by deleterious mutations (see above). The results of the inbreeding experiment accord with the genic-capture hypothesis: I found evidence of more severe inbreeding depression in both LS males and females (table 2). As far as now, this is the first evidence that a sexual trait's expression reflects the mutation load, suggesting new perspectives for the resolution of the evolutionary questions related to the maintenance of genetic variability in traits undergoing strong directional sexual selection (Rowe and Houle 1996) and for the advantage of sexual reproduction itself (Agrawal 2001). The strength of sexual selection in reducing the mutation load in the offspring will depend on the rate of female sexual promiscuity, the association between sperm production and SC success, and the relative strength of selection on deleterious mutations in males and females.

Table 2. Summary of HS performance relative to LS in SSTs (associated with both mating acquisition and fertilization success) and NSTs (in males, M, or females, F) considered during the selection experiment (see results for details) and their relative senescence in a subset of traits; "+" marks traits in which HS individuals perform significantly better than LS, the contrary if traits are marked with "-". Traits with no significant difference are marked with "=". Traits with lower inbreeding depression (ID) in the HS line are marked with "-".

	Sexually-selected traits (SST)										Naturally-selected traits (NST)				
	Mating acquisition success					Fertilization success									
Traits measured	Colour spots	Standard length	Gonopodium length	Sexual behaviour	Attractiveness	Sperm transferred	Sperm number	Sperm velocity	Sperm viability	Sperm size	Growth rate (M+F)	Sexual maturation (M)	Capture time (M+F)	Female fecundity	Longevity (M)
Selection	+	+	=	=	+	+	+	=	+	=	+	+	=	=	-
Senescence	=	=	=	=			+	-					=		
ID	-	=	=				-						-	-	

Collectively my results show that sperm production was positively, genetically correlated with several other sexual traits, operating both at the pre- and postcopulatory level, and naturally-selected traits, reinforcing selection for higher male quality and reduced mutation load. Despite these several short-term advantages, males with higher investment in sperm production suffered from a more severe senescence and a reduced longevity, probably reducing their lifetime fitness. Also intuitively, the mutation-selection balance is unlikely to be the only source of variation for sperm production: if its variation depends mostly on new mutations introduced at every generation, this trait should have a relatively low heritability and should not respond to directional selection so quickly, whereas my heritability estimates (see Manuscript I) confirmed previous data from this population (Gasparini et al. 2013).

It is difficult to compare the relative importance of mutation/selection balance and life-history trade-offs in maintaining the genetic variance for sperm production in guppies. It is known that the strength of selection on late-expressing alleles is reduced, thus suggesting that early advantages of HS males may not be completely offset by late costs. The effect of deleterious mutations is expected to increase with age (Charlesworth and Hughes 1996; Hughes 2010). Although the inbreeding depression experiment revealed that LS had a larger load of deleterious mutations expressed at early life stages (before sexual maturation up to 5 months), the effect of the life-history trade-off masked the age-specific effect of deleterious mutations late in life (from 10 to 14 months), indicating that the effects of the former override the effects of the latter as males age.

Any conclusion about the relative importance of these two processes, however, needs also to keep into account the interactions between genotype and environment. My experimental fish were maintained in optimal conditions (unlimited food, absence of competition for reproduction, etc.), but it is unlikely that harsher environmental conditions have the same effect on both genotypes. For example, it may be that the costs of the "live-fast" strategy revealed by my ageing experiment are stronger in harsh environmental conditions. Whether or not this actually occurs also depends on the interaction between mutation load and the cost of producing large sperm numbers with the environmental conditions (e.g. food availability). The interaction between environmental conditions and the high or low sperm production genotype clearly warrants further investigation.

Manuscripts

Correlated responses in pre- and postcopulatory traits to artificial selection on sperm production in the guppy (*Poecilia reticulata*)

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Abstract

Sperm competition has been recognized as a widespread phenomenon, leading to the evolution of ejaculates and of their competitive abilities. Evolutionary response to elevated sperm competition involves primarily the number of sperm produced, indeed, relative testes size has been shown to increase ubiquitously with sperm competition across species in many different animal groups. Because ejaculate production can represent a significant cost for males, it is expected to be negatively correlated with other costly sexual traits. Genetic relationships between reproductive traits have often involved mating acquisition traits, but the role of sperm production in this pattern is still unexplored. To clarify the evolutionary processes associated with sperm production, we performed a bidirectional artificial selection experiment for sperm number in *Poecilia reticulata*. Correlated responses to selection in traits related to mating acquisition and fertilization were investigated to assess whether trade-offs were present among these components. Collectively our results showed that sperm production was positively, genetically correlated with several other sexually-selected traits, operating both at pre- and postcopulatory level, suggesting a differential variance in resource acquisition across selection lines. Males selected for high sperm number attained a larger body size, were more colourful and more attractive to females. Sperm velocity and morphology were not affected, but sperm viability and the number of sperm transferred per copulation increased with sperm number. These results suggest that some guppy genotypes perform better in most reproductive traits, and may also explain the maintenance of polyandry, despite the costs associated with promiscuity.

Introduction

Polyandry promotes the overlap of ejaculates from different males, resulting in a competition for the fertilization of the same set of eggs, sperm competition (SC), and favouring adaptations in ejaculates that ensure a higher fertilization success (Parker 1970). Evolutionary response to elevated sperm competition involves primarily the number of sperm produced/ejaculated, although performance (e.g. swimming velocity or viability, Snook 2005) and seminal fluids (Poiani 2006) are also often involved. Indeed, relative testes size has been shown to increase ubiquitously with SC across species in many different animal groups (Birkhead and Moller 1998; Simmons and Fitzpatrick 2012) and experimental evolution studies have confirmed an increased ejaculate investment in response to elevated SC (Hosken and Ward 2001; Simmons and Garcia-Gonzalez 2008; Firman and Simmons 2010; 2011).

Although it is often assumed that sperm are cheap, and that males are limited in their reproduction only by the number of females they mate with (Borgia 1979), ejaculate production can instead represent a significant cost for males (Dewsbury 1982; Hayward and Gillooly 2011; Rahman et al. 2013; Gasparini et al. 2013) and males can deplete their sperm reserves (Birkhead and Fletcher 1995; Olsson et al. 1997; Preston et al. 2001; Doyle 2011), thus limiting the number of offspring they can sire. If sperm are costly, we expect to observe evolutionary trade-offs between ejaculate production and other costly traits such as those involved in precopulatory competition over access to females, an assumption supported by empirical observations (see Kvarnemo and Simmons 2013 for a recent review). Ejaculate investment can also be traded-off among within ejaculate among different quality traits such as, for example, sperm number and size (e.g. Parker et al. 2010). Indeed, evidence that sperm number is traded offs with sperm size or performance has been found both at the interspecific and intraspecific level (Gage and Morrow 2003; Moore et al. 2004; Snook 2005; Immler et al. 2011).

Costs associated with mate acquisition traits and with ejaculate production have recently been incorporated into game theoretic models of ejaculate expenditure (Parker et al. 2013). Results from this model substantially confirm previous conclusions: expenditure per ejaculate should increase and then decrease, and total postcopulatory expenditure should increase, as the level of sperm competition increases (Parker et al. 1996; Parker and Pizzari 2010). In all these models, however, males are assumed to have a fixed energy budget that can be spent on reproduction (R), and trade-off are therefore expected between mating acquisition and ejaculate investment (e.g. sperm number and sperm quality).

Even though pre- and postcopulatory expenditure may trade-off within the fixed energy budget, both may increase with R if variance in resource acquisition exceeds variance in allocation: males with greater ability to acquire resources will be able to allocate more resources to both armaments/ornaments and ejaculates (Reznick 1985; van Noordwijk and de Jong 1986). Indeed, positive associations between pre- and postcopulatory expenditure have been reported (Rogers et al. 2008; Hosken et al. 2008), suggesting that SC may act concordantly with

precopulatory sexual selection. In guppies, *Poecilia reticulata*, colourful males have faster and more viable sperm (Locatello et al. 2006), implying that some males can invest heavily in both attractiveness and fertility. Females might thereby ensure good-genes benefits for their offspring not just by precopulatory mate choice, but also by polyandry and the SC success of good quality males (Yasui 1997).

The balance between producing a large ejaculate and high-quality sperm may therefore represent a common evolutionary constraint on males. Nonetheless, models assuming positive genetic correlations in case of higher variance for resource acquisition than allocation (see above) can equally be applied also within postcopulatory traits. Results from artificial selection experiments suggest that selection on one sperm trait results in positive correlated response in other fertility related traits (Birkhead et al. 2009). For example, Pitnick and Miller (Pitnick and Miller 2000) selected upward and downward on testis size in *Drosophila hydei* and found a positive correlated response on sperm length. In a recent comparative study (Rowe and Pruett-Jones 2011), a positive correlation was observed between increasing sperm competition and both sperm number and sperm performance (motility and viability) in eight species of Australian *Maluridae*.

To understand the evolutionary consequences of directional selection on ejaculate traits it is therefore necessary to explore the quantitative genetics of sperm production and its correlations with other sperm traits potentially relevant in SC and mating acquisition.

The guppy is an ideal species to investigate the potential trade-offs between these fitness components: male guppies heavily invest in reproduction, with one mating attempt every minute, on average, during all the year (Houde 1997), leading to very high level of polyandry (Houde 1997; Magurran 2005). The pre- and postcopulatory sexual selection processes have been studied in detail (Houde 1997; Evans and Pilastro 2011). In particular, traits associated with fertilization success (sperm number and, to a lesser extent, sperm velocity) have been identified (Boschetto et al. 2011). This species is characterized by a resource-free mating system in which males provide no direct benefits to females (Pilastro et al. 2008), yet females mate with several males at each breeding cycle (Houde 1997) , resulting in one of the highest levels of multiple paternity reported for any vertebrate species (Neff et al. 2008). In this species, during precopulatory mate choice, females prefer to mate with colourful males with high rates of courtship (sigmoid display). In particular, the area of carotenoid coloration (including orange, red and yellow) consistently influences female mating decisions (Endler and Houde 1995; Houde 1997; Evans et al. 2004a). Negative genetic correlations among different sperm quality traits have also been reported (Evans 2011), suggesting that different ejaculate components may not evolve independently (Moore et al. 2004; Birkhead et al. 2005), and between sperm quality traits and precopulatory traits (Evans 2010). Evidence of these genetic trade-offs, however, have been obtained in a feral Australian population, and sperm production, the main predictor of SC outcome in this species (Boschetto et al. 2011), as in most vertebrates (Birkhead et al. 2009), was not included. Sperm production is the most costly ejaculate trait in the guppy, and strongly depends on resource acquisition, compared to other ejaculate traits (Gasparini et al. 2013), although not in all populations (Devigili et al. 2013), suggesting potential trade-offs associated with a greater investment in this trait. The positive correlation between sperm investment and mating opportunities (Bozynski and Liley 2003; Gasparini et al. 2009) further highlights the potential for a negative correlation among reproductive traits. Studies conducted on guppies from their original distribution area (Trinidad) have instead revealed a positive phenotypic and genetic correlations within ejaculate between sperm traits (Skinner and Watt 2006; Gasparini et al. 2013) and between ejaculate and precopulatory traits such as colour and courtship rate (Matthews et al. 1997; Locatello et al. 2006; Pitcher et al. 2007).

Furthermore, multiply mated females produce higher quality offspring, suggesting that SC (probably through sperm number) may mediate genetic benefits to the female (Evans and Magurran 2000; Ojanguren et al. 2005; Barbosa and Magurran 2011).

To clarify the evolutionary processes associated with sperm allocation, we performed a bidirectional artificial selection experiment for sperm production in guppies. Correlated responses to selection in traits related to mating acquisition and fertilization were investigated to assess whether trade-offs were present among these components.

Materials and Methods

The fish used in this experiment were descendants of wild-caught fish collected in 2002 from the lower part of Tacarigua River in Trinidad (Trinidad national grid reference: PS 787 804; coordinates: N10° 40.736' W061° 19.168). Laboratory stock and all experimental fish were maintained under controlled temperature and lighting conditions (26°C ± 1°C; 12:12h light dark cycle) and fed twice daily on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplaRin). Males and females used in the experiment derived from large stock tanks (150 l), each of which containing approx. 50 individuals of each sex that were allowed to breed freely. Initial population size consisted of about 400 adults (approx 200 males and females), but the effective founding population size was larger because the females were gravid and the average number of sires per brood in this population is about 3 (Neff et al. 2008). Population size was subsequently maintained constantly above 1000 individuals distributed in large tanks. In order to maintain the original genetic variation, twice a year fish were mixed among stock tanks and at least once a year a substantial number of males (usually > 100, randomly sampled from the stock tanks) were allowed to mate monogamously and their offspring was subsequently distributed among stock tanks in order to reduce skewness in male reproductive success. Indeed, observed heterozygosity and number of alleles did not change in three paternity studies conducted, with the same

microsatellite markers, soon after the establishment of the stock population (Evans et al. 2003a) and about 20 generations later (Gasparini et al. 2010b; Gasparini and Pilastro 2011).

Selection Protocol

Artificial selection has several advantages for determining genetic variation and covariation over the alternative methods of offspring-parent regression and sibling analysis. First and foremost, artificial selection directly answers whether the trait can evolve in response to selection, whereas a single-generation method is indirect. Artificial selection has greater statistical power than sib methods. This is primarily because artificial selection tests differences between line means, whereas sib analysis relies on variance and covariance components (Falconer and Mackay 1996). Another important advantage of artificial selection experiments is that they can provide more robust estimates of the genetic parameters for the trait targeted by the selection. Artificial selection involves repeated episodes of expression of the consequences of any interactions with other traits, and therefore yields more convincing descriptions of the potential roles of genetic coupling among traits. Any genetic correlations of the target trait with other traits are likely to manifest themselves in correlated responses to the selection applied to the target itself (Brakefield 2003).

We performed a bi-directional artificial selection for sperm number. Initially, 150 males were screened for sperm production, and the 20 males with the highest sperm number and the 20 with the lowest sperm number were allowed to reproduce with 2 random virgin females from the stock population to establish the parental generation (P). Each treatment (high sperm production, HS, and low sperm production, LS) was replicated twice, and an unselected control treatment was included. In the first and subsequent generations, the female HS or LS genotype was incorporated into the selection protocol: each selected male was individually housed with two unrelated virgin females from the same selection line and replicate. Females were checked daily until their first brood was delivered. Male offspring were individually raised (up to 3 randomly chosen per brood, i.e. 6 males per sire) with a randomly chosen companion female from our stock population and screened for sperm production and other sexual traits at 5 months of age. The large majority of the males produce sperm by the age of three months and sperm production afterward increases with age (Evans et al. 2002; Gasparini et al. 2010a). In a subset of F1 males from the two lines (31 HS, 27 LS) we measured sperm number at 4, 5 and 6 months. Sperm number increased between 4 and 5 months ($F_{1,57}$=46.69, p<0.001), but not between 5 and 6 months ($F_{1,57}$=1.20, p=0.28). Sperm number significantly differed between lines, but the interaction between age and line was not significant ($F_{1,56}$=1.35, p=0.25), indicating that sperm increase did not differ between lines. The repeated measure of sperm count at 4 and 5 months of age was used to determine sperm number repeatability (repeatability = 0.79±0.05, $F_{58,115}$=8.50, p<0.001).

Sperm count

The presence of females prime sperm production in guppies (Bozynski and Liley 2003). Prior to sperm extraction, males were isolated for 3 days to ensure the complete replenishment of sperm reserves (Pilastro et al. 2004) and sperm were collected from each male following an established procedure (Evans et al. 2003b).

Briefly, each male was anaesthetized in a water bath containing tricaine mesylate (MS-222) and placed on a slide under a stereomicroscope. A gentle abdominal pressure allowed the release of sperm in a drop of saline solution (NaCl 0.9%). Sperm in this species are packaged in discrete units, called sperm bundles, each containing about 21000 individual sperm cells (Pilastro et al. 2008; Boschetto et al. 2011), which were photographed on a black background and counted using a digital image analysis software (ImageJ, http://rsbweb.nih.gov/ij/). Although the number of sperm per bundle can vary across males, it is not associated with male body size, colour and age (Pilastro et al. 2008; Gasparini et al. 2010a), nor with the total number of bundles produced (r=0.072, p=0.56 estimated on 36 HS and 39 LS males). From the number of sperm per bundle (estimated in a subset of 17 randomly selected males from F1), we calculated the total number of sperm produced (mean number of sperm per bundle: 22,005±663.6, t_{16}=33.159, r^2=0.986, p<0.001). The number of sperm cells per bundle, estimated in a subsample of F2 males did not differ between selection lines ($F_{1,69}$=0.375, p=0.54).

Mating acquisition traits

Female guppies are sexually receptive for 3-5 days after parturition (i.e. on average every 20-30 days) and, during this phase, base their mating preferences on male body size and colouration and the intensity of courtship display (Liley 1966; Houde 1997), which is phenotypically positively correlated with sperm reserves in this guppy population (Matthews et al. 1997; Pitcher and Evans 2001). Males present three major types of colour spots, orange, iridescent and melanistic, which all show a high, Y-linked heritability (Postma et al. 2011). Female preference for these colour components can vary across populations, but the preference for orange spots, which are due to carotenoid and pteridin pigments, seems universal as it has been found in all populations investigated so far (Magurran 2005; Evans and Pilastro 2011). Preference for colourful males, in particular for those with large orange spots, has also been confirmed in this population (Evans et al. 2004a). In some populations a preference for gonopodium length has also been evidenced (Brooks and Caithness 1995). Gonopodium length is also associated with the success in obtaining forced copulations (Evans et al. 2011, see also below). When the female is unreceptive, males attempt to obtain matings through a coercive mating tactic called gonopodial thrusting (GT) (Houde 1997). Forced matings have a lower insemination success than cooperative copulations (Pilastro and Bisazza 1999) but are thought to contribute to a male's reproductive success given the high frequency with which this mating tactic is adopted (up to one GT attempt per minute, Magurran and Seghers 1994). We therefore estimated the expression of the following traits associated

with male mating success: body size, gonopodium length, relative size of three types of colour spots, gonopodium length, male sexual behaviour (no. of courtship displays and GT) and male sexual attractiveness according to the following procedures.

After extracting sperm bundles, all males were photographed on their left side using a Canon EOS 450D digital camera. Photos were then analysed with ImageJ software (http://rsbweb.nih.gov/ij/download.html). The following measures were recorded: standard length (SL, distance from the snout to the tip of the caudal peduncle), body area (including caudal fin but excluding dorsal fin), gonopodium length (following Evans et al. 2011) and the area of black, iridescent and orange spots (Pilastro et al. 2008; Evans 2010; Devigili et al. 2013). To control for the effects of body size, the relative area of each colour pigment was used in the analyses. Using absolute spot area and total body area as covariate in the analyses below did not change the results.

Male sexual behaviour was measured as follows: 15 F_1 males for each line and replicate (total n=60) were placed in a 25 l tank (40x29x32 cm) with 2 unreceptive females previously acclimated for 30'. Unreceptive females were used to exclude any effect of female preference on male behaviour. Then we observed male sexual behaviour for 20', recording the number of sigmoid displays (SD = courtship behaviour) and gonopodial thrusts (GT = sneaky behaviour, following Evans 2010).

To estimate male attractiveness to females, we performed a female choice test on the same 60 males used for the behaviour experiment. In each of the trials we allowed a sexually receptive virgin female to observe a HS and a LS male in a dichotomous mating chamber where males were in 2 sectors one next to the other and in front of female's sector. An opaque screen blocked visual access between the two males. Focal males were assigned at random to the left or the right sector of the tank. Following a 30' settlement period, during which visual access into the male compartments was obscured, female was allowed to watch males and her position was recorded for 20' every 5". Possible female positions were three: a non-choice area, a choice area 3 cm long in front of HS male sector, and a choice area in front of LS male sector. The total amount of time (sec.) the focal female spent in front of each male was used as estimate of male's sexual attractiveness (Evans et al. 2004a).

Sperm quality assays

To assess the interaction between sperm quantity and quality, after sperm extraction we analysed for each male of generation 2 (F_2) sperm velocity, viability and size. Sperm velocity or viability has been shown to be associated with fertilization success in sea urchins (Levitan 2000), fish (Burness et al. 2004; Gage et al. 2004; Rurangwa et al. 2004), including *P. reticulata* (Boschetto et al. 2011), birds (e.g. Birkhead et al. 1999) and mammals (e.g. Malo et al. 2005). Evidence that sperm morphology affects sperm competition success is controversial (see Snook 2005), but evidence suggests that sperm size may influence sperm competition success in internal fertilizers (e.g. Briskie and Montgomerie 1992; Anderson et al. 2005).

Sperm velocity was analysed *in vitro* immediately after sperm collection. 5 sperm bundles were collected and activated with 4 µl of 150 mM KCl and 4 mg/ml of bovine serum albumin [see (Gasparini et al. 2009) for details]. We used a Hamilton-Thorne CEROS sperm tracker (v. 12.3; Hamilton Thorne Research, Beverly, MA, USA). We recorded the average path velocity (VAP, $\mu m \cdot s^{-1}$) which is the average velocity of a sperm cell over a smoothed path, and the curvilinear velocity (VCL, $\mu m \cdot s^{-1}$). The two measures are highly correlated (Boschetto et al. 2011) and, for brevity, we present here only the VAP data. Two ejaculate aliquots from the same male were analysed and the mean was used for statistical analysis. *In vitro* measure of sperm velocity is highly repeatable in this population (Gasparini et al. 2009).

To measure sperm size, an ejaculate aliquot was stored in 100 µl of physiological solution at +4 °C until analysis was performed to avoid sperm deterioration (up to 4 hours). Sperm bundles were broken by vortexing them and 3 slides were prepared for each male with 7 µl of solution containing sperm. Samples were then viewed under ×1000 magnification with a Leica DMI400 microscope and photographed with a digital camera (Olympus DP10, Japan). Mean head length, midpiece length, and total sperm length (all measures in µm) were obtained from 20 sperm per male using image analysis software (ImageJ).

Sperm competition success is influenced by relative differences in sperm number and sperm velocity between males (Boschetto et al. 2011), but it has been shown that, once sperm number is controlled for, also sperm viability strongly determine competitive fertilization success when artificial insemination is used to compete ejaculates from rival males (Fitzpatrick, J. L. & Evans, J. P.; unpublished data). This is not surprising, as sperm viability determines the number of sperm that actually compete for the eggs. To this end, immediately after sperm extraction, 20 bundles for each male were collected in 10 µl of 0.9% NaCl solution and broken with a vortex. We assessed sperm viability, using the live⁄dead Sperm Viability Kit (L-7011; Molecular Probes Inc., OR, USA). A membrane-permeant nucleic acid stain (SYBR14) labelled live sperm green and a membrane-impermeant stain (propidium iodide) labelled dead or damaged sperm red. Samples were digitally photographed using a 40× magnification (DFC480; Leica Microsystems, USA) and images were stored using Leica IM500 image-manager software (v. 4.0, Leica Microsystems, USA). The proportions of live and dead spermatozoa (coloured green and red respectively) were then assessed from images of at least 100 sperm for each male. After sperm stripping, each male was photographed as described below.

Sperm insemination success

Because the number of sperm inseminated is the most important predictor of SC outcome (Boschetto et al. 2011), we analysed whether HS males inseminate more sperm than LS males during cooperative matings with receptive females. 37 HS males and 31 LS males from F_2 were used in the mating trials: each male was allowed to freely

interact with a virgin female from stock population in a 15 l mating tank for 30'. In case of cooperative mating, female was removed and isolated for 30' in a small tank. After this period, the female was anesthetized in MS222 solution, her standard length was recorded and sperm were retrieved, following an established protocol (Pilastro et al. 2007). Briefly, the tip of a plastic micropipette (Drummond) was inserted for 2 mm into female's gonoduct and 3 µl of 0.9% NaCl solution were gently ejected into the gonoduct. Without removing the micropipette from the gonoduct the solution was subsequently retrieved and collected in a plastic vial. This process was repeated five times, and the female were afterwards revived in a 5 l tank. The solution recovered from female's gonoduct was then diluted and the sperm concentration was assessed using an improved Neubauer haematocytometer. The reliability and repeatability of our sperm recovery technique was tested on females that were previously artificially inseminated with known sperm numbers, showing that sperm inseminated and retrieved were strongly positively correlated (r=0.83, p<0.001, n=25).

Statistical analysis

Statistical analyses were performed using SPSS version 21.0. If not otherwise stated, means ± SE are reported. All probabilities are two-tailed. Data were checked for normality and appropriate transformation was adopted when necessary. Proportion data were arcsine square root transformed before analysis. In the ANOVA analyses, we first tested for homogeneity of variances. Realized heritability (h^2) was calculated as twice the regression of cumulative response over cumulative selection coefficient (Hill 1972; Falconer and Mackay 1996). To assess 95% CIs we generated 10000 datasets using resample function in PopTools 3.0 (http://www.cse.csiro.au/poptools). We corrected for multiple comparisons using the false discovery rates (FDRs) method (Benjamini and Hochberg 1995; Benjamini et al. 2006)

Results

Response to selection

After one generation of artificial selection, sperm number differed significantly between HS and LS lines ($F_{1,191}$=45.51, p<0.001) and remained consistent in F_2 ($F_{1,337}$=81.87, p<0.001) and F_3 ($F_{1,269}$=117.02, p<0.001)(Fig. 1). Including body size (SL) in the model, differences remained significant ($F_{1,770}$=33.76, p<0.001).

Estimated realized heritability of sperm number was similar to that obtained in a previous quantitative genetics experiment (Gasparini et al. 2013) 1.06 (95% C.I. 0.92 — 1.21, based on 10000 replications). Replicates within line did not significantly differ one from the other for the selected trait at any generation (Table 1), and they were therefore pooled in following analyses.

Values and relative statistics for each male trait separated between replicate are reported on Table 1.

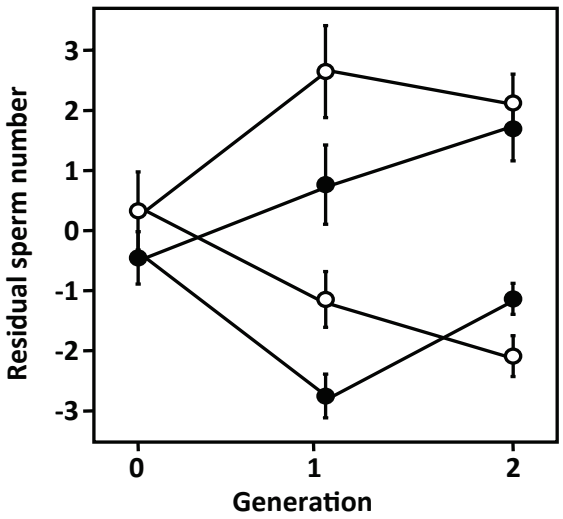

Fig. 1. Response to artificial selection for sperm number. Different symbols refer to the two independent replicates per selection regime. Sperm number (± SE) is expressed as the residual number of sperm ($\times 10^6$) on body size (standard length, SL, mm: sperm number = SL · 0.90±0.14 – 7.32±2.21, $F_{1,770}$=33.76, p<0.001).

Correlated responses in traits associated with fertilization success

In the sperm quality analysis, no difference was found between HS and LS lines after two generations of selection in both sperm velocity (VAP: $F_{1,203}$=0.12, p=0.74; Fig. 2.a) and neither total sperm length ($F_{1,160}$=0.04, p=0.84) nor the size of its components (head: $F_{1,160}$=0, p=0.995; midpiece: $F_{1,160}$=3.516, p=0.063; flagellum: $F_{1,160}$=0.516, p=0.474; Fig. 2.b). On the other hand, sperm viability in HS males was significantly higher than in LSP males ($F_{1,173}$=14.47, p<0.001; Fig. 2.c). All differences were consistent in both replicates, as reported on table 1.

Moreover, not only HS males had greater sperm reserves, but they also inseminated four times more sperm, on average, than LS males in cooperative (natural) copulations with a virgin, unselected female ($F_{1,43}$=11.45, p=0.002; Fig. 2.d), including male body size and colouration as covariates, with a significant effect of SL (p=0.03) and orange (p=0.01).

Correlated responses in mating acquisition traits

After two generations of selection, HS males were bigger than LS males (SL, $F_{1,337}$=8.09, p=0.01)(Fig. 3.a), but this difference was not consistent across replicates (Table 1), they also showed higher relative extension of orange ($F_{1,337}$=50.57, p<0.001) and black ($F_{1,337}$=32.99, p<0.001)(Fig. 3.b) and remained consistent in both replicates (Table 1). Iridescent spots ($F_{1,337}$=10.37, p=0.001)(Fig. 3.a, b) were larger in HS males (Fig. 3.b), but not in both replicates (Table 1). Finally no difference was found in gonopodium length ($F_{1,337}$=0.385, p=0.536).

In dichotomous choice experiment females showed a higher preference for HS males from the 1st generation of selection ($F_{1,27}$=5.99, p=0.02; Fig. 3.c), including as covariates male orange colouration ($F_{1,27}$=0.61, p=0.44) and body size ($F_{1,27}$=2.42, p=0.13), even after controlling for replicates (Table 1). In the behavioural observation trials, males from F1 of the two lines did not differ in the degree of courtship (SD,

$F_{1,59}$=1.266, p=0.265) or sneaky mating attempts (GT, $F_{1,59}$=1.66, p=0.20; Fig. 3.d), nor in their preference for one tactic over the over (t_{58}=0.17, p=0.86).

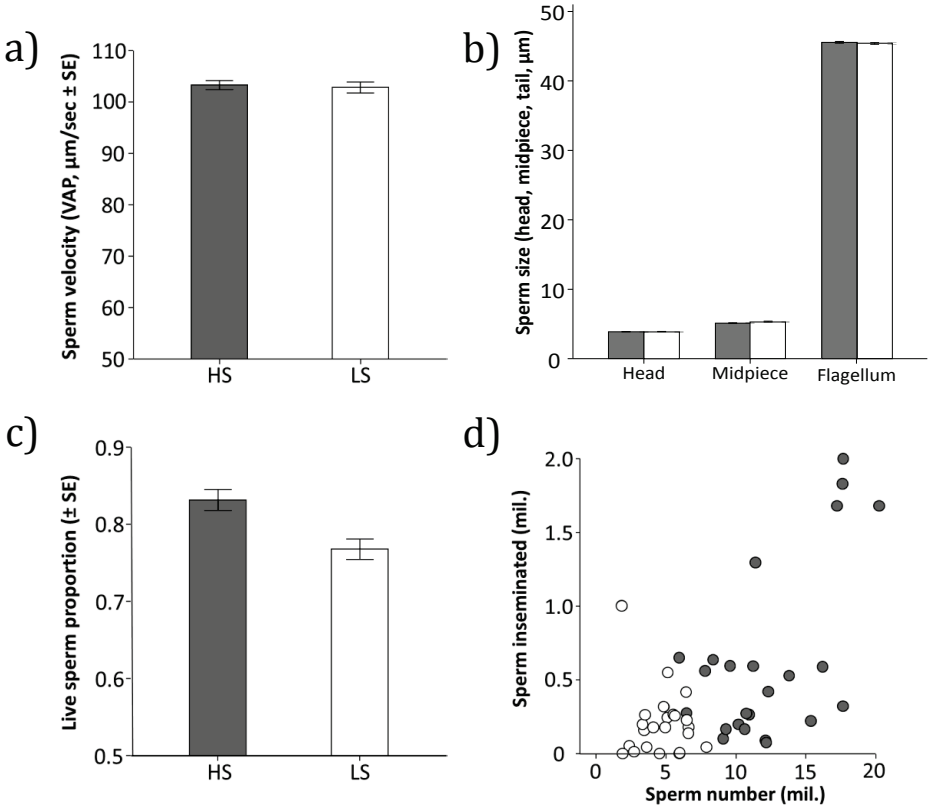

Fig. 2. A) Mean sperm velocity (average path velocity, VAP, µm/sec ± SE) in HS (grey bar) and LS (empty bar) selection lines. **B)** Mean size of sperm components in HS (grey bars) and LS (empty bars) males. **C)** Live sperm proportion (mean ± SE) in HS (grey bar) and LS (empty bar) males. **D)** Linear regression between the number of sperm produced (x10^6) and the number of sperm inseminated (x10^6) in cooperative matings for HS (grey circles) and LS (empty circles) males.

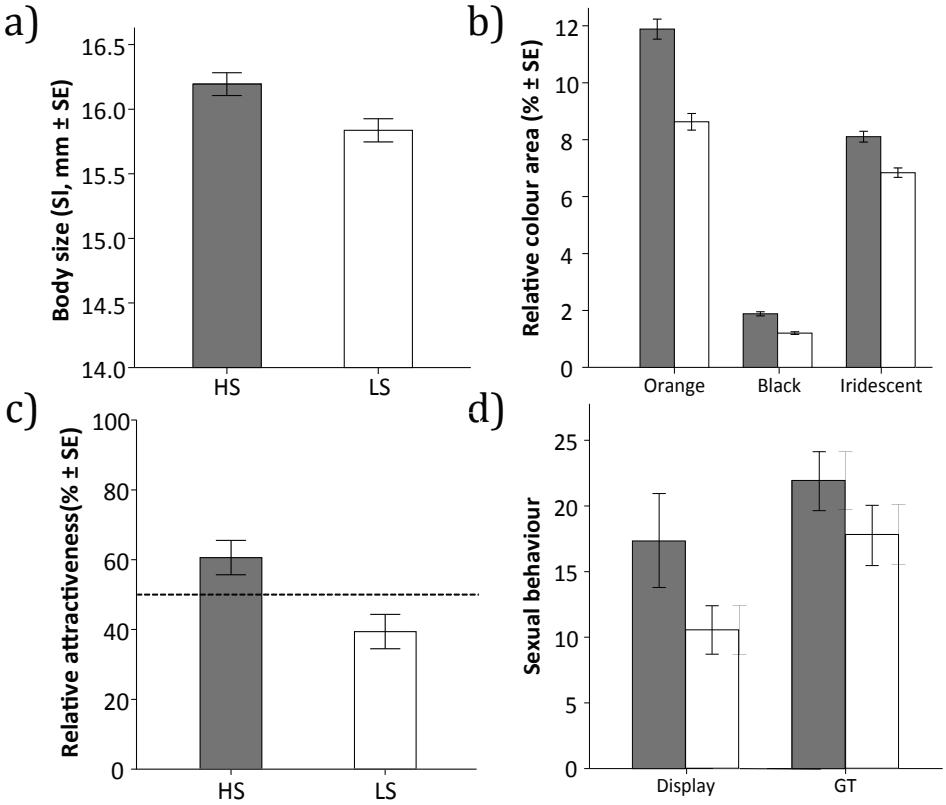

Fig. 3. A) Mean body size (SL ± SE) of HS (grey bars) and LS (empty bars) males. **B)** Mean relative extension (% ± SE) of colour spots (orange, black and iridescent) in HS (grey bars) and LS (empty bars) lines. **C)** Female preference (%± SE) of HS (gray bar) or LS (empty bar) in a dichotomous choice test. **D)** Mean sexual behaviour attempts ± SE for courtship behaviour (sigmoid display) and sneaky attempts (GT) for HS (grey bar) and LS (empty bar) males.

Table 1. Means ± standard errors (SE) and relative statistics for all male traits measured in the two selection lines (HS and LS) and in each replicate (A and B). Significant differences in bold.

Trait	Gen.	Replicate	HS	LS	Statistic	D df	*p*
Standard length (mm)	F_2	A	16.04±0.15	15.90±0.13	0.52	157	0.47
		B	16.32±0.11	15.78±0.12	11.44	179	0.001
Orange spots relative area (%)	**F_2**	**A**	**10.18±0.54**	**6.75±0.41**	**26.18**	**157**	**<0.001**
		B	**10.83±0.46**	**7.99±0.34**	**22.94**	**179**	**<0.001**
Black spots relative area (%)	**F_2**	**A**	**1.30±0.07**	**1.02±0.07**	**10.61**	**157**	**0.001**
		B	**1.39±0.06**	**0.96±0.07**	**22.77**	**179**	**<0.001**
Iridescent spots relative area (%)	F_2	A	9.46±0.28	7.14±0.26	36.10	157	<0.001
		B	6.80±0.35	6.67±0.33	0.01	179	0.94
Gonopodium length (mm)	F_2	A	3.51±0.02	3.50±0.02	0.08	157	0.78
		B	3.47±0.02	3.45±0.02	0.55	179	0.46
Courtship rate	F_1	A	18.60±4.10	11.20±2.32	1.2	29	0.28
		B	16.07±5.99	9.87±2.92	0.32	29	0.58
Sneaky attempts	F_1	A	23.87±3.93	15,00±3.55	4.62	29	0.04
		B	20,00±2.21	20.60±2.86	0.06	29	0.81
Attractiveness (choice proportion)	**F_1**	**A**	**0.59±0.07**	**0.41±0.07**	**4.28**	**29**	**0.05**
		B	**0.62±0.08**	**0.38±0.08**	**5.24**	**29**	**0.03**
Sperm number (x10^6)	**F_1**	**A**	**8.13±0.62**	**6.08±0.45**	**7.56**	**119**	**0.01**
		B	**9.82±0.76**	**3.33±0.31**	**69.62**	**71**	**<0.001**
	F_2	**A**	**9.00±0.52**	**5.32±0.33**	**36.66**	**157**	**<0.001**
		B	**9.92±0.51**	**5.97±0.29**	**33.91**	**179**	**<0.001**
	F_3	**A**	**7.07±0.47**	**3.49±0.32**	**40.32**	**96**	**<0.001**
		B	**9.76±0.65**	**3.58±0.29**	**85.47**	**172**	**<0.001**
Sperm transferred (x10^6)	**F_2**	**A**	**0.50±0.15**	**0.14±0.03**	**10.11**	**23**	**0.005**
		B	**0.73±0.19**	**0.34±0.11**	**3.53**	**19**	**0.08**
Sperm velocity (VAP, μm · s^{-1})	F_2	A	104.16±1.08	104.48±1.48	0.01	102	0.93
		B	102.40±1.37	101.08±1.47	0.78	100	0.38
Proportion of live sperm	**F_2**	**A**	**0.82±0.02**	**0.76±0.02**	**6.02**	**95**	**0.02**
		B	**0.85±0.01**	**0.78±0.02**	**9.87**	**77**	**0.002**
Total sperm length (μm)	F_2	A	54.78±0.14	54.66±0.16	0.05	73	0.82
		B	54.31±0.15	54.47±0.14	2.34	86	0.13

Discussion

Collectively our results indicate that sperm production was positively, genetically correlated with several other sexually-selected traits, operating both at pre- and postcopulatory level. Males selected for high sperm number attained a larger body size, were more colourful and more attractive to females. Sperm velocity and morphology were not affected, but sperm viability and the number of sperm transferred per copulation increased with sperm number.

These results suggest that, at least in lab conditions, some guppy genotypes perform better in most reproductive traits, involved in both pre- and postcopulatory processes. This is compatible with two, non mutually exclusive, explanations: 1) variance in resources acquisition is larger than variance in resource allocation (van Noordwijk and de Jong 1986; Reznick et al. 2000), as expected, for example, if sperm production is condition dependent (Gasparini et al. 2013). According to Parker et al. (2013) if Q is the total amount of resources available to an individual, S is the fraction of Q invested in survival and R is the fraction of Q invested in reproduction (with its two components, pre- and postcopulatory, R_{pre} and R_{post}), males in better condition have a larger Q and can increase both their R_{post} and R_{pre} investment (without the need to decrease S). 2) Alternatively, variance in resources allocation is larger than that in resource acquisition. Our results would imply, in this case, that the observed increased expression of sexual traits (R_{post} + R_{pre}) occurs at the expenses of survival (S), i.e. Q=costant.

We will discuss the two scenario in sequence. Producing larger and more viable ejaculates is likely to be costly and it is expected to be attained at the expenses of traits involved in mate acquisition or sperm quality (e.g. Olsson et al. 1997; Gage and Morrow 2003; Simmons and Emlen 2006; Klaus et al. 2011), we can expect to observe a positive

genetic correlation between fitness related traits when individuals differ (genetically) in their degree of resource acquisition (van Noordwijk and de Jong 1986; Reznick et al. 2000). When the genetic variance for resource acquisition is larger than the genetic variance in resource allocation, a positive genetic correlation between mating acquisition and fertilization success could be observed. Under this scenario, variance in condition is expected to lead to positive genetic covariance between condition and costly sexual signals, providing an avenue for females to gain indirect genetic benefits for their offspring via mate choice for males with exaggerated sexually-selected traits (Madsen et al. 1992; Jennions and Petrie 2000; Simmons 2005). Indeed, it has been recently shown that resource acquisition and allocation are genetically correlated, and that the plasticity in the allocation trade-offs between fitness components is largely dependent on the genetic variance for total resource acquisition rather than allocation (Robinson and Beckerman 2013), suggesting that variance for resource acquisition can generate positive covariance among life-history traits. Indeed several studies suggest that pre- and postcopulatory traits are positively correlated in *P. reticulata* (Matthews et al. 1997; Evans et al. 2003a; Locatello et al. 2006; Pitcher et al. 2007). Our results demonstrate that there is a positive genetic correlation between the same traits. In contrast, it has recently been evidenced a genetic trade-off in a different guppy population between sperm quality traits and male courtship rate (Evans 2010) and within ejaculate, among sperm quality traits (Evans 2011). Unfortunately, sperm number was not included in these studies for a direct comparisons of the two populations.

In our experiment we found that males with higher sperm investment do not pay any trade-off in either sperm velocity or length, instead they show higher sperm viability, which combined with the higher number of sperm they inseminated in cooperative matings suggests a striking advantage for them in SC (Boschetto et al. 2011). The positive correlations with orange colouration and male attractiveness in particular, suggest that males with increased sperm production can afford to invest more resources also in mating acquisition traits. Indeed in this species, females prefer to mate with males bearing larger orange spots (Evans et al. 2004a), and allow them to transfer more sperm during cooperative matings (Pilastro et al. 2002; 2004; 2007). Indeed, males with greater sperm reserves also transferred more of them during natural copulations, even including male body size and orange colouration as covariates, which confirmed the results from previous experiments (Pilastro et al. 2002; 2004; 2007) and show that males selected for increased sperm production transfer more sperm even when their attractiveness is controlled for. Moreover, their ejaculate contained a greater proportion of viable sperm, indicating that even a greater quantity of their sperm could actually compete for eggs fertilization.

These results, combined with the absence of trade-offs with the other traits considered, suggest that male genotypes selected for increased sperm production are expected to be advantaged in both mating acquisition and SC. This is concordant with the idea that heritable variation in traits associated with sperm competitiveness can promote positive, reinforcing selection on traits involved in the competition for mates. For example, in *Drosophila simulans* there is a positive correlation between male attractiveness and sperm competitiveness (Hosken et al. 2008); in *Drosophila hydei* males selected for bigger testes have longer sperm, are larger and developed faster, Pitnick and Miller 2000); and in the dung beetle soma weight, an estimate of male condition, is positively correlated with attractiveness and with testes weight (Simmons and Kotiaho 2002). Sperm production seems therefore to be a good indicator of males' ability to allocate more resources to other traits associated with reproductive success.

Altogether these results exacerbate the question of the maintenance of genetic variance in a trait subject to directional selection: if males that produce more numerous and viable sperm are at an advantage also in precopulatory stages of sexual selection, why this trait show a large genetic variance (Gasparini et al. 2013)?

It is possible that our estimate of the relative contribution of both pre- and postcopulatory traits in determining male fitness is still unknown. For example in the guppy females can retain sperm viable months in the oviduct (Evans and Pilastro 2011), so it is possible that the number of viable sperm inseminated at the moment of copulation does not reflect the proportion of sperm competing for eggs fertilization in the following days/months. We have no idea whether sperm production is associated with fertilization success of stored sperm. Moreover male guppies perform up to one mating attempt every minute and show no seasonality in breeding (Houde 1997), so we can expect that sperm depletion may play an important role in determining male's lifetime reproductive success (Birkhead and Fletcher 1995; Preston et al. 2001; Doyle 2011), with males that have grater mating success and transfer more sperm being more exposed to the risk of sperm depletion. So, the advantage of HS males might even be larger when forced matings are kept into account.

While our results collectively indicate that HS males are at a reproductive advantage as compared to LS males, further work is necessary to clarify the mechanisms contributing to the maintenance of genetic variation in sperm number. For example, the relative contribution of loci with intermediated allele frequency versus partially recessive deleterious mutations at low frequency to the observed genetic variance in sperm number is still unknown. Condition dependence is expected to arise for costly traits closely related to fitness (Houle 1992) and could contribute to the maintenance of variance in sperm production, as condition is likely to be influenced by large part of the genome. If a trait, because of its association with condition, is affected by most of the genome, it is more likely to be affected, directly or indirectly (Chenoweth and McGuigan 2010), by any deleterious mutations (Rowe and Houle 1996). New mutations are expected to arise at any generation, introducing new genetic variation in the sexually selected trait. The comparison of the response to artificial selection for high and low sperm number with the inbreeding depression in the selected lines (Kelly 1999)

will allow to directly test the assumption that the expression of condition-dependent, sexually selected, traits reflects the mutational load of males (Rowe and Houle 1996).

Another explanation for the observed positive covariance between sperm number and other costly sexual traits is that resource allocation in male guppies occurs between reproduction and survival (scenario 2). There are indications that experimentally elevated sperm production results into a reduced survival in guppies. Negative correlations can affect other fitness components we did not measure. For instance, male attractiveness in guppies has been shown to be associated with reduced survival (Brooks 2000), suggesting that genes enhancing mating acquisition may be associated with pleiotropic costs. Other empirical evidence, however, goes in the opposite direction. For example, studies on Trinidadian guppy populations have revealed a positive genetic correlation between male ornamentation and offspring survival traits (Evans et al. 2004b). It is therefore necessary to extend this scenario also to naturally-selected traits, in both males and females. Moreover, the positive correlations between sperm production and other sexual traits were found when males were 5 months old. In laboratory conditions, guppy males can live up to 15 months (Gasparini et al. 2010a) and trade-offs between reproduction and senescence or longevity have been demonstrated in other taxa (Hunt et al. 2004; Jacot et al. 2007; Simmons and Kotiaho 2007b; Preston et al. 2011; Lee et al. 2013). We can expect resource acquisition and allocation pattern to vary during male lifespan. Therefore under a "live fast-die young" scenario, males investing a lot in reproduction could pay a cost in their senescence and longevity, reducing their lifetime reproductive success (Brooks 2000; Hunt et al. 2004; Preston et al. 2011), an hypothesis that has not been tested yet for male investment in ejaculate traits in vertebrates. There is an indication that experimentally elevated sperm production reduces survival in guppies (Jordan and Brooks 2010), but whether intrinsic sperm production is associated with survival and senescence rate (Hunt et al. 2004; Robinson et al. 2006) need to be investigated.

Finally, interactions between genotype and environment could explain why the HS genotype does not spread to fixation in natural populations, despite better condition and many positive correlations with traits related to mating acquisition and fertilization. Our experimental fish were maintained in optimal conditions (unlimited food, absence of competition for reproduction, etc.), but it may be possible that harsher environmental conditions have different effects on the two genotypes. HS males may better than LS males when resource availability is high, but this advantage may be reduced when resource availability is low. In the field cricket *Teogryllus oceanicus* it has been demonstrated that male expenditure on the ejaculate can depend strongly on the availability of nutrients, when combined with an immunitary challenge (Simmons 2011). In addition, nutrient availability has been found to affect male allocation to testes growth (Ward and Simmons 1991; Knell and Simmons 2010), ejaculate size (Gage and Cook 1994; Perry and Rowe 2010), and competitive fertilization success (Simmons and Parker 1992).

Collectively our results are in agreement with the hypothesis of a differential resource acquisition ability for males, rather than a differential resource allocation pattern driven by pleiotropic costs associated with greater investment in sperm production, leading males with higher condition to perform better in many different tasks, both at a pre- and postcopulatory level. To our knowledge, this is the first evidence, in vertebrates, that a strongly-selected ejaculate trait is a reliable indicator of male's genetic quality, and may also explain the maintenance of polyandry, despite the costs associated with promiscuity (Keller and Reeve 1995; Yasui 1997; Simmons 2001).

Further investigations is needed to test whether sperm allocation affects senescence rate and longevity in both sexes, and whether the sign and magnitude of the genetic correlations among fitness related traits change in different environmental condition (food scarcity, stronger mating competition, immunitary challenge).

Consequences of male investment in sperm production on life-history traits in males and females of *Poecilia reticulata*

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Abstract

Male investment in sexually selected traits is expected to be traded-off against maintenance and survival. If sex-limited expression of these traits is incomplete or directly harmful to the female, the evolution of male traits can further be constrained by counter selection on females. A typical, nearly universal evolutionary response to sperm competition is an increased sperm production, but whether increasing sperm production has a negative effect on male and female traits that are expected to be associated with survival and/or female fecundity is still unknown. In our study, we used the guppy *Poecilia reticulata*, to test this hypothesis. We used artificially selected males for high and low sperm production to compare growth rate, body size at maturation and condition (estimated from a predator evasion test) in males and females from the two selection lines. Our results revealed that males selected for increased sperm production did not apparently pay any cost in the measured traits, but had instead a significantly earlier sexual maturation and faster growth rate. We did not find any difference between female from high and low sperm production lines in fecundity, body size, growth rate and condition. Altogether our results suggest that sperm production may be a good indicator of overall male genetic quality, as it was positively associated with other survival-related traits in males. Moreover, male investment in sperm production was not associated with any detectable decrease in female fitness, suggesting that it was not correlated with traits that were harmful for the females. The possible role of other mechanisms responsible for the maintenance of the genetic variance in sperm number are discussed.

Introduction

Polyandry remains one of the most controversial topics in evolutionary biology (see Parker and Birkhead 2013 for a recent review). This is primarily because in several species females derive no direct benefits from mating with many males, but frequently incur direct costs (Magurran and Seghers 1994). One of the numerous hypotheses proposed to explain the evolution of polyandry in the absence of direct benefits for females is the good sperm hypothesis (Madsen et al. 1992; Yasui 1997), which suggests that a male's success in sperm competition (SC) correlates with other aspects of his genetic quality which is passed to the progeny (a postcopulatory version of the good genes models of female choice evolution).

Theory also predicts that when SC is fundamentally analogous to a raffle, the relative numbers of sperm in competition will be the primary determinant of success (Parker 1970; 1990). This should select for increased investment in spermatogenesis since larger testes typically produce ejaculates containing more sperm (Moller 1988, but see Pitnick 1996) and comparative evidence is abundant (Harcourt et al. 1981; Gage 1994; Hosken 1997; Simmons et al. 1999).

The good sperm hypothesis makes the prediction that polyandrous females should produce offspring with higher fitness. Studies conducted so far have obtained contrasting results: Madsen et al. (1992) reported an association between polyandry and offspring survival and recently Firman (2011) showed that female mice benefit from polyandry by producing sons that achieve increased fitness in a semi-natural environment; similarly, Hosken et al. (2003) found that males that were more successful in SC also had offspring that developed faster. On the contrary, Simmons (2001) failed to find any association between sperm competitiveness and offspring quality.

An alternative scenario is expected under a life-history theory approach: evolution of a costly trait is constrained by trade-offs with other fitness-related, costly traits as a consequence of the limited resources. Investment in sperm production must be maintained at some minimum requirement to ensure fertility, but with limited resources available to reproduction, there are likely to be trade-offs between sperm production and either sperm quality or other fitness-related traits (Stearns 1989; Roff 1992; 2000).

Game theory has provided a theoretical framework within which to assess the evolutionary consequences of SC for male expenditure on the ejaculate (Parker et al. 2013). At the core of sperm competition game theory lies the assumption that males face a life history trade-off between expenditure on the ejaculate and expenditure on gaining matings. Although there is evidence to suggest that males finely tune their investment in sperm production (Wedell et al. 2002), implying that ejaculate production is costly, sperm production has rarely been studied within a life history context. If, for example, male offspring sired by males with higher investment in sperm production are smaller at birth or grow slower than male offspring from males with reduced sperm investment, the advantage of polyandry may be reduced and the trade-off between investment in sperm competitiveness and non-sexually selected fitness traits could explain the maintenance of genetic variance for sperm production. Consequently, an estimate of female reproductive success should be complemented with information on how this is affected by other life-history traits known a priori to be correlated with fitness (Bolund et al. 2011).

Unitary cost in sperm production is smaller compared to that necessary to produce eggs (Trivers 1972), although the traditional view of 'cheap' sperm has been demonstrated far to be universally true (Dewsbury 1982; Olsson et al. 1997; Gasparini et al. 2013). 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 lifetime 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.

Empirical evidences of the existence of sexual conflict in nature are ever increasing (see Arnqvist and Rowe 2005 for an extensive review). As defined originally by Parker (1998), "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 favour one sex but have harmful effect on the fitness of the other sex.

Any deviation from monogamy increases sexual conflict because individuals' lifetime reproductive interests will not coincide (Rice 2000). Therefore, sexual conflict should increase with multiple mating (Rice 2000), as does the potential for SC (Parker 1970). As a result, sperm competition should enhance sexual conflict and thus lead to the evolution of traits that increase reproductive success in one sex even when they are costly to the other. Frequent matings, which are usually advantageous for males, are known to reduce female fecundity and reproductive success in a number of species (Arnqvist 1989; Byrne and Roberts 1999; 2000; Stutt and Siva-Jothy 2001; Maklakov and Lubin 2004; Maklakov et al. 2005). In the guppy, the intense male sexual activity has shown to be costly for the females (Gasparini et al. 2012), but whether this cost is associated with a postcopulatory trait remains unclear.

Poecilia reticulata is one of the species with the highest levels of polyandry (Houde 1997; Evans and Magurran 2000; Pitcher et al. 2003; Magurran 2005) and SC outcome is in larger part determined by the number of sperm inseminated (Boschetto et al. 2011), which generates a strong selective pressure on sperm production, and should deplete its genetic variance. In contrast, genetic variance for sperm number is actually larger than for any other ejaculate trait in guppies (Gasparini et al. 2013). Although females can gain substantial direct and indirect benefits from mating polyandrously (Evans and 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 and Seghers 1994), with potentially important impacts on female fitness (Ojanguren and Magurran 2007; Gasparini et al. 2012). Finally, guppy life history patterns are very similar to those expected from life history theory under high predation regimes, where high mortality favours earlier maturity, higher fecundity and greater reproductive allocation (Endler 1995).

The aim of this study is to evaluate the evolutionary consequences of male investment in sperm production, the main predictor of SC success in *P. reticulata* (Boschetto et al. 2011) and in most vertebrate species (Birkhead et al. 2009), on traits subject to natural selection. An artificial selection approach has been used to manipulate male investment in sperm production in two opposite directions, thanks to the high heritability and evolvability shown for this trait in a recent half-sib/full-sib experiment (Gasparini et al. 2013). Indirect responses in fitness-related traits (sexual maturation in males, growth rate and condition in both males and females and female fecundity) were investigated to assess whether the good genes hypothesis or the trade-off theory are associated with SC mechanisms in this species.

Methods

All the fish used in this experiment were descendants of wild-caught fish collected in 2002 from the lower part of Tacarigua River in Trinidad (Trinidad national grid reference: PS 787 804; coordinates: N10° 40.736' W061° 19.168). Laboratory stock and all experimental fish were maintained under controlled temperature and lighting conditions (26°C ± 1°C; 12:12h light dark cycle) and fed twice daily on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplaRin).

Males and females used in this experiment were 1st generation descendants of males artificially selected for sperm high and low sperm production (HS and LS lines respectively, for details on the artificial selection protocol and sperm counting see manuscript I). Briefly, we initially screened for sperm number 400 males to found the two replicated selection lines. Each replicate consisted of 25 males with highest or lowest sperm number (see below) out of 100 males per replicate. Each male was individually housed with two virgin females from the stock population in the initial generation. When visibly pregnant, females were individually isolated and checked three times per day until parturition. After brood delivery, mothers were removed, date of parturition was recorded, and offspring were allowed to grow in 8-L tanks. Once males became morphologically distinguishable from females (approx. at 5 weeks, Houde 1997), they were isolated from the rest of the

brood and kept separately (with a companion, unrelated female).

To assess sperm production, each male was anaesthetized in a water bath containing tricaine mesylate (MS-222) and placed on a slide under a stereomicroscope. A gentle abdominal pressure allowed the release of sperm in a drop of saline solution (NaCl 0.9%). Sperm in this species are packaged in discrete units, called sperm bundles, each containing about 21,000 individual sperm cells (Pilastro et al. 2008; Boschetto et al. 2011), which were photographed on a black background and counted using a digital image analysis software (Image J: http://rsbweb.nih.gov/ij/download.html).

From the number of sperm per bundle (estimated in a subset of 17 randomly selected males), we calculated the total number of sperm produced (mean number of sperm per bundle: 22,005±663.6, t_{16}=33.159, r^2=0.986, p<0.001).

Male traits

We randomly selected one male from each artificial selection sire when possible (see below). We obtained 35 males from F_1 HS line (25 from replicate A and 10 from replicate B) and 36 males from LS line (21 from replicate A and 15 from replicate B). For logistic reasons, we had to exclude 29 sires did not produce male offspring, or their dams were pregnant yet at the moment we started the experiment (mainly from replicate B that was started one month after replicate A).

In the population of *P. reticulata* used in this study, under natural conditions, predation is very high e only few individuals survive until sexual maturation occurs (Magurran 2005). For this reason, a faster development, which includes growth and sexual maturation, may represent an important factor in determining male fitness (Barbosa et al. 2012). Males were individually monitored from birth until sexual maturation occurred. We considered a male fully mature when the gonopodium's apical hood is longer than the tip of the gonopodium (for details see Houde 1997). To this end the length of the gonopodiumwas checked every other day. The date of maturation was recorded and the male was anesthetized in a water bath containing tricaine mesylate (MS-222) and placed on a slide under a stereomicroscope to confirm gonopodium maturation. Then the male was photographed using a Canon EOS 450D digital camera and revived in a small tank until fully recovered. Photos were then analysed with ImageJ software. Standard length (SL, distance from the snout to the tip of the caudal peduncle) and body area (including caudal fin but excluding dorsal fin) were recorded. Male growth rate was estimated as the ratio between male SL at sexual maturity (mm) over age (days) at sexual maturity. We estimated growth rate at sexual maturation because male growth in this species is determinate, with little growth after sexual maturation, while females grow continuously and, hence, attain larger sizes (Reznick 1980; Houde 1997).

A capture test, adapted from an established protocol used for newborn guppies (Evans and Magurran 2000; Evans et al. 2004b) and adults (Gasparini et al. 2013), was used to estimate predator evasion capability of males. An individual performance in this test is highly repeatable and depends on its condition (Gasparini et al. 2013).

Briefly, after 10-min acclimatization in the test tank, one of us (ADN), blind to male identity, captured the male using a small hand net (7 × 10 cm). Capture procedure consisted of chasing the male with the net at a speed which was kept as constant as possible. The test started when the fish was in a central position in the tank, and the time until the fish was captured was recorded by another observer (AP) using a chronometer. This measure is significantly repeatable within individual (Gasparini et al. 2013). We excluded 11 males (5 HS and 6 LS) from the analysis because after the acclimation period they were still frozen in a corner of the experimental tank and for this reason the capture test could not be performed. This test was performed two weeks after sexual maturation.

Female traits

In organisms with indeterminate growth, such as *P. reticulata,* the relationship between growth and reproduction is important because size is usually positively correlated with fecundity (Reznick et al. 1990). For this reason we tested whether daughters of males artificially selected for opposite investment in sperm production have differential growth rate and fecundity.

We tested a total of 44 HS virgin females (25 from replicate A and 19 from replicate B) and 41 virgin females from LS line (26 from replicate A and 15 from replicate B). When possible, we randomly selected one virgin female from each sire of the selection experiment as for males (see above).

When 60 days old, each female was anesthetized and photographed as above, standard length (SL) and body area were recorded. For 5 females from the LS line we were not able to collect body measures at this age because they were not yet univocally distinguishable as females rather than males. We repeated the same procedure as above when females were exactly 3 months old (90 days). We did not estimate growth rate at sexual maturity because the exact moment of sexual maturation is not visually recognizable in females as it is in males (see above). One week later, we performed the capture test on female following the same procedure described for males. The test was not performed on 3 LS females because they did not resume a normal swimming in the test tank after the acclimation period remaining immobile on the bottom of the tank.

One week after the capture test, each female was housed with a single unrelated male from the same selection line and replicate in a 1-l tank. After 10 days the male was removed and each female was checked three times per day until parturition. After brood delivery, mothers were removed, date of parturition was recorded, and offspring were counted. We obtained a brood from 33 HS and 28 LS females.

Statistical analysis

Statistical analyses were performed using SPSS-version 21.0 (SPSS Inc., Chicago, IL, USA). When we compared multiple data collected from the same female (such as body size), we used repeated measures ANOVA. Data were

tested for homogeneity of variance and normality, and appropriate transformation was adopted when necessary. If not otherwise stated, means are reported with their standard errors (SE).

Results

Males from the 1st generation of selection significantly differed for sperm production between HS and LS lines (mean sperm number: HS=8.70±0.49, LS=4.94±0.33; $F_{1,191}$=45.51, p<0.001), and the difference was consistent also within replicates: replicate A, HS=8.13±0.62, LS=6.08±0.45, $F_{1,119}$=7.56, p=0.01; replicate B, HS=9.82±0.76, LS=3.33±0.31, $F_{1,71}$=69.62, p<0.001. Since both replicates showed a comparable response to selection, they were pooled in the following analysis.

Male traits

Males from the two selection lines significantly differed in their age at sexual maturation, with HS males being sexually mature, on average, 9 days earlier than their LS counterparts (Fig. 1a, Table 1). Body size, measured as male standard length (SL), did not differ across selection lines at sexual maturation, but since HS males mature earlier that LS males, growth rate (mm/days) was significantly higher in HS males than in LS males (Fig. 1b, Table 1). Moreover, there was a significant, positive correlation between SL at maturation and time to sexual maturation (r=0.58, p<0.001, n=71), which was consistent in both lines (HS: r=0.56, p<0.001, n=35; LS: r=0.66, p<0.001, n=36), suggesting that larger males required more time for sexual maturation and have a slower growth rate in both lines (r=-0.92, p<0.001, n=71). Finally, male condition did not differ between HS and LS males at sexual maturation (Table 1).

Female traits

Female body size, measured as standard length (SL), did not differ across selection lines either at 2 months or 3 months of age (Table 2). SL increased significantly over time in both lines (repeated measure ANOVA: $F_{1,75}$=565.88, p<0.001), but did not differ between lines (interaction age*line: $F_{1,75}$=0,04, p=0.836, Fig. 2a). Female fecundity, measured as number of offspring produced at first parturition, did not differ between HS and LS selection lines (Fig. 2b), including female body size (SL measured at 3 months of age) as a covariate (Table 2). Finally, we found no difference across lines in female ability to evade a simulated predator (Table 2).

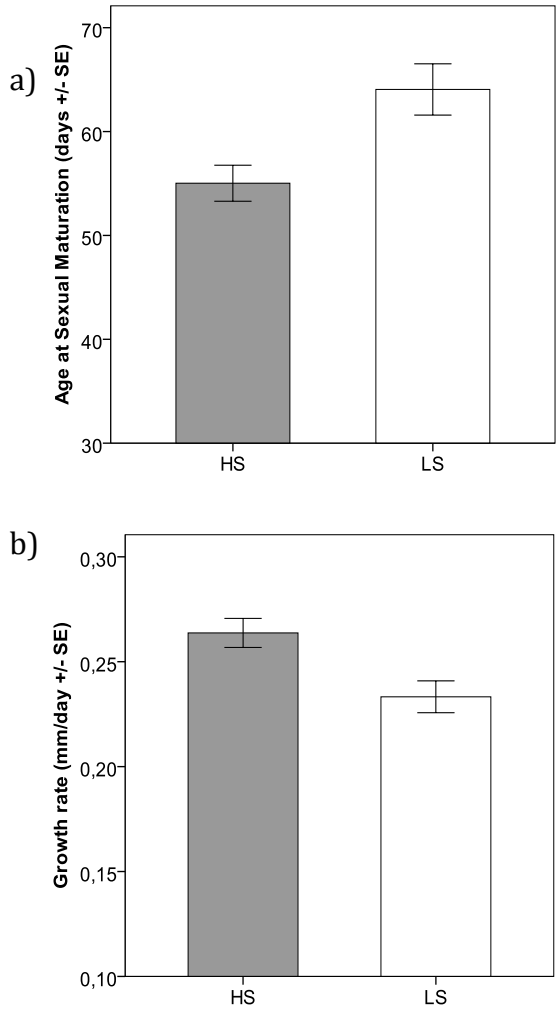

Figure 1. A) Mean male age at sexual maturation (days ± SE) in the HS (grey bar) and LS (blank bar) selection lines. **B)** Mean growth rate (mm · days^{-1} ± SE) in males from HS (grey bar) and LS (blank bar) selection lines

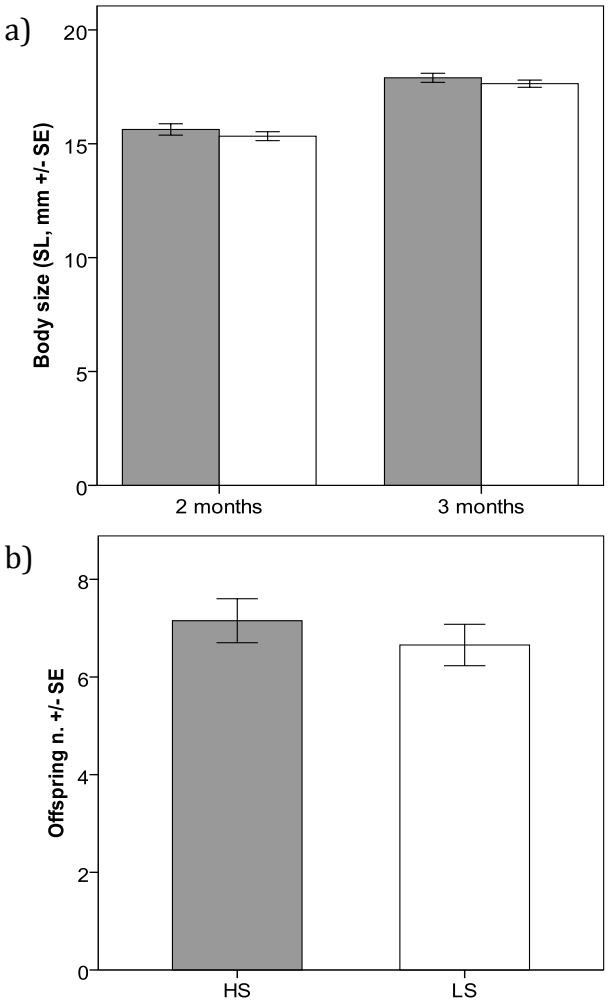

Figure 2. A) Mean female body size (SL, mm ± SE) in the HS (grey bars) and LS (blank bars) selection lines at 2 and 3 months of age. **B)** Number of offspring (mean ± SE) produced by females from HS (grey bar) and LS (blank bar) selection lines.

Table 1. Mean and standard errors for male traits at sexual maturation in high sperm (HS) and low sperm (LS) selection lines. One-way ANOVA statistics are reported. Significant differences across lines in bold. Male condition was log-transformed in the analysis.

Trait	HS		LS		ddf	*F*	*p*
	Mean	SE	Mean	SE			
Body size (SL, mm)	14.39	0.18	14.34	0.19	70	0.003	0.960
Age at maturity (days)	**55.03**	**1.73**	**64.06**	**2.47**	**70**	**8.88**	**0.004**
Growth rate (mm/day)	**0.26**	**0.01**	**0.23**	**0.01**	**70**	**8.77**	**0.004**
Condition (sec.)	3.93	0.47	5.34	1.28	59	1.25	0.267

Table 2. Mean and standard errors for female traits at 2 and 3 months of age in high sperm (HS) and low sperm (LS) selection lines. One-way ANOVA statistics are reported. Female condition was log-transformed in the analysis.

Trait	HS		LS		ddf	*F*	*p*
	Mean	SE	Mean	SE			
Body size at 2 months (SL_2, mm)	15.63	0.25	15.33	0.16	76	0.83	0.364
Body size at 3 months (SL_3, mm)	17.90	0.20	17.54	0.16	81	1.91	0.171
Fecundity (offspring n.)	7.15	0.45	6.66	0.43	61	1.25	0.740
Condition (sec.)	6.22	0.81	6.32	0.86	78	0.14	0.708

Discussion

Collectively, our results failed to find any negative genetic correlation between sperm production and the measured traits that under natural selection in male and female guppy. In particular, female growth rate and fecundity was comparable across selected lines and both sexes performed equally well in the capture evasion test. In contrast, we found that HS males grow significantly faster and mature at a significantly earlier age than their LS counterparts. Our results are therefore in contrast with the predictions of the life-history theory, which assumes that an increased investment in reproduction should result into a reduced somatic investment. Furthermore, I failed to highlight any negative correlated response of sperm production in female life-history traits, suggesting that antagonistic sexual selection may not play an important role in the evolution of this postcopulatory trait in males.

Life-history traits such as body size, age at sexual maturation, growth rate and fecundity are involved in survival and reproduction. Even though the theoretical framework underlying these traits is well established (Stearns 1982; Hillesheim and Stearns 1992), experimental tests of the theory are often difficult (Stearns and Koella 1986). The recognition that sexual promiscuity is a widespread phenomenon (Birkhead and Moller 1998) and has important consequences on the evolutionary processes mediated by sexual selection has given new perspectives to the study of relationships between reproductive traits undergoing sexual selection and traits undergoing natural selection such as life-history traits. These two components are expected to be traded-off because resources are limited and their allocation to reproduction and maintenance or survival cannot proceed independently (Stearns 1989; Roff 1992; 2000; 2002). Empirical evidence agrees with theoretical predictions for traits associated with male mating success (Reznick 1985; Lynch and Spitze 1994; Gustafsson et al. 1995; Roff 2000), but whether the cost of an enhanced investment in postcopulatory traits such has started to be investigated only recently (Hunt et al. 2004; Jacot et al. 2007; Simmons and Kotiaho 2007b; Preston et al. 2011; Lee et al. 2013).

In contrast with the predictions of the life-history theory, our results showed that sons of males selected for increased sperm production had a faster sexual maturation and growth rate compared to their lower sperm counterparts. Earlier sexual maturation is favoured because it minimizes pre-reproductive mortality, which is important in our guppy population which is subject to a high predation rate in its natural environment (Endler 1987). Shorter generation time (early maturation) and reduced mortality in the juvenile stages are favoured whenever extrinsic mortality (mainly due to predation) is high. In the guppy both juvenile and adult mortality are very high, suggesting that early maturation may represent an important fitness advantage in our (and most) guppy population (Reznick et al. 1996). Age at maturation is particularly important in species with continuous breeding during their whole lifetime, such as *P. reticulata*, and a shortening of the birth-to-maturation interval may result in a conspicuous increase in the population growth (Reznick et al. 1990).

Furthermore, earlier maturation comes at no cost, as it was not associated with a reduced body size at maturity. Male body size at sexual maturation was similar in the two groups thanks to the higher growth rate of HS males as compared to their LS counterparts. Growth rate and size at maturation are expected to be negatively correlated and empirical support for this prediction has been found in several species (Gibbons et al. 1981; Stearns 1989; 1992; Nylin and Gotthard 1998). Our experimental evidence goes, however, in the opposite direction: not only males selected for increased sperm production did not pay a cost in terms of somatic development, but there was also a positive genetic covariation between growth rate and sperm number, and a negative correlation between age at sexual maturation and sperm number.

Although sperm production is costly (Dewsbury 1982; Hayward and Gillooly 2011; Gasparini et al. 2013; Devigili et al. 2013), HS males therefore showed an enhanced performance in an important fitness trait such as body

growth rate and sexual maturation. These results are in agreement with previous evidence, from the same artificial selection experiment, showing that sperm number is positively correlated with pre- and postcopulatory sexual traits that are strongly associated with reproductive fitness (see Manuscript I). The presence of positive, rather than negative correlations among traits undergoing sexual and natural selection confirms that sperm production is associated with male condition or, in other words, with the resources available for reproduction and survival (Gasparini et al. 2013). These results suggest that the variance in resource acquisition may exceed the variance in resource allocation, determining a positive genetic correlation between reproductive and somatic investment (Reznick 1985; van Noordwijk and de Jong 1986).

A genotype that grows faster, matures earlier and has a greater reproductive success should rapidly invade a population. Instead, genetic variation in sperm number is very high in this population (Gasparini et al. 2013) and other types of trade-offs must be in place. For example, mating with HS males may reduce female fecundity (due to sexual conflict) or alleles associated with sperm number may reduce fecundity when expressed in daughters (due to incomplete sex-limited expression and antagonistic sexual selection). For example, since sperm number *per se* is unlikely to directly harm the female, sperm production may be associated with other harmful traits such as seminal fluids or increased male harassment. 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 is thought to be the result of sexually antagonistic genes controlling androgen levels.

We tested this hypothesis in daughters of males selected for divergent sperm production. We measured female fecundity, growth rate and condition, but found no evidence of sexual conflict that may lead to a reduction of fitness for males with higher sperm production, thus balancing the many positive correlations with male fitness-related traits associated with this trait (see above and Manuscript I). Our experimental design was not specifically designed to distinguish between inter- and intralocus sexual conflict. Instead, we allowed the male to freely interact with the females for 10 days, possibly incorporating the effect of all these components of sexual conflict on female fecundity. Clearly, absence of evidence is not evidence of absence and antagonistic sexual selection may be elusive for several reasons.

For example, sperm production is Y-linked and therefore artificial selection on sperm number for one generation may be effective in producing a response in the selected trait but may have a have a limited effect on somatic loci (Keller and Reeve 1995; Gasparini et al. 2013). This is equivalent to say that this trait has an effective sex-limited expression, which reduces intralocus conflict. In evolutionary terms, the result of *intralocus* sexual conflict is generally transient and it results in the sex-specific expression of a trait, even though the extent to which this occurs is still debated (Harano et al. 2010). In a previous study on the same population has demonstrated that male sexual harassment does not reduce female fecundity, but has detrimental effects on offspring quality (Gasparini et al. 2011). A full evaluation of the effect of HS genotype on female fitness would therefore require to measure female fecundity after more generations of artificial selection.

In conclusion, we found that sons of males selected for higher investment in a strongly selected ejaculate trait, sperm production (Boschetto et al. 2011; Gasparini et al. 2013), showed faster sexual maturation and growth rate. These results seem to confirm a good genes process associated with sperm number. Sperm production may therefore be correlated with the overall male genetic quality, as predicted by good genes models of sexual selection (Yasui 1997). It has been recently shown that sperm number is strongly condition dependent (Gasparini et al. 2013) and hence probably affected by most of the genome. It is therefore likely that any deleterious mutation will affect the expression of sperm number (Chenoweth and McGuigan 2010) (Rowe and Houle 1996). New mutations are expected to arise at any generation, thus introducing new genetic variation for the condition-dependent sexual trait, possibly maintaining a positive genetic covariation between all (or most) costly, fitness-related traits involved in reproduction and survival.

Although we did not find any negative effect of sperm number on somatic and reproductive investment during juvenile stages and around sexual maturation, sperm number may be associated with a reduced post-maturation survival and/or increased senescence rate. Evidence of "live fast-die young" trade-off has been found for precopulatory traits (Hunt et al. 2004; Jacot et al. 2007; Simmons and Kotiaho 2007b; Preston et al. 2011; Lee et al. 2013). Experimentally enhanced sperm production reduces longevity in guppies (Jordan and Brooks 2010) and whether HS males pay a senescence and longevity cost it is therefore worth to be investigated.

Male guppies (*Poecilia reticulata*) selected for increased sperm production have faster senescence rate and reduced lifespan

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Abstract

The net fitness of an individual depends on his reproductive rate, the pattern of change in resource allocation to reproductive traits with age (senescence) and longevity. Individuals vary in life span and senescence rate, and this variation has been shown to reflect the expression of male secondary sexual trait associated with mate acquisition. In particular, males with an enhanced investment in display traits and weaponry have an increased senescence rate and a reduced longevity. In principle, this should also apply to postcopulatory traits, such as sperm number and quality, because these traits are costly and under directional selection in species with high levels of sperm competition. However, the evolutionary consequence of postcopulatory investment on senescence rate and longevity has rarely been investigated. We used artificial selection to manipulate male investment in sperm production in the guppy, *Poecilia reticulata*. Sperm number is the main determinant of sperm competition outcome in this species and responds readily to artificial selection. We found that males from the high sperm line (HS) had a reduced lifespan compared to their low sperm counterparts (LS). Moreover, males from the HS line also showed a reduced sperm velocity with aging, suggesting a potential trade-off between sperm quantity and quality during senescence. Finally, sperm number increased with age but differentially in the two lines, with a stronger increment in LS males. Although sperm production was higher in senescing HS males than in their LS counterparts, the difference between lines attenuated with age. Altogether our results indicate that negative pleiotropy may contribute maintaining the large genetic variance in sperm production observed in this species.

Introduction

Central to the understanding of the evolution of life histories is the fundamental concept that trade-offs in resource allocation among reproduction and survival are expected (Stearns 1989; Roff 1992; 2000; 2002). In the case of males, the focus of attention has been on the trade-offs involving traits associated with mating acquisition, whereas trade-offs with traits involved in sperm competition success have been, with a few exceptions, overlooked. Sperm competition (Parker 1970; 1998) is now widely accepted as a strong selective pressure favouring adaptations in male behaviour, morphology and physiology that contribute to competitive fertilization success.

In particular, theoretical (Parker 1982; 1998), comparative (Parker et al. 1997) and experimental studies (Gage and Morrow 2003; Boschetto et al. 2011) have shown that sperm number is often the main determinant of sperm competition outcome, and males tend to increase their investment in testes size and sperm quantity with increasing sperm competition level (Parker 1970; Hosken and Ward 2001). Sperm numbers are therefore expected to be under directional selection in species in which females are highly promiscuous. Under these conditions, genetic variance for sperm production should rapidly be eroded. Experimental evidence contradicts this prediction, in line with what is observed for ornaments and weapons involved in mate acquisition. For example, in a recent study (Gasparini et al. 2013) sperm production resulted in the highest coefficient of additive genetic variance among ejaculate traits in *Poecilia reticulata*, in accordance with the paradoxical observation that sexual traits subject to directional selection often have higher genetic variance than more ordinary traits (Pomiankowski and Moller 1995), an evolutionary conundrum known as "the lek paradox" (Rowe and Houle 1996; Tomkins et al. 2004). Nonetheless producing sperm is costly (Dewsbury 1982; Olsson et al. 1997; Birkhead and Pizzari 2002) and it can be attained at the expenses of other traits involved in reproduction and/or survival.

Game theory has provided a theoretical framework within which to assess the evolutionary consequences of sperm competition for male expenditure on the ejaculate (Parker 1998; Parker et al. 2013). At the core of sperm competition game theory lies the assumption that males face a life history trade-off between expenditure on the ejaculate and expenditure on gaining matings. Although there is evidence to suggest that males finely tune their investment in sperm production (Wedell et al. 2002), implying that ejaculate production is costly, sperm production has rarely been studied within a life history context. Mating is known to reduce male lifespan in a number of species (Kotiaho and Simmons 2003; Fedorka et al. 2004; Martin and Hosken 2004; Simmons and Kvarnemo 2006), but whether this cost is associated with sperm production remains unclear.

In addition, life history theory predicts a trade-off between current and future reproduction (Reznick 1985; Stearns 1992; Roff 2002), extending the previous scenario also to the pattern of change in resource allocation to reproductive traits with age (senescence) and the relative mating acquisition and/or sperm competition success.

Senescence is the reduction of phenotype, physiological functions e residual reproductive value with increasing age (Roff 1992; Stearns 1992). This happens because selection on genes expressed late in life is weak, permitting the

accumulation of late-acting deleterious mutations and, in particular, genes that enhance early-life reproductive performance while contributing to somatic deterioration (Bonduriansky et al. 2008). Senescence occurs because resources allocated to reproductive traits are unavailable for investment in somatic repair, as predicted by the optimality theory of ageing (Partridge and Barton 1993): individuals, or populations, allocating more resources to sexual traits will face a trade-off with somatic maintenance, resulting in shorter lifespans and faster ageing, the so-called live fast-die young scenario.

A growing number of studies seem to confirm the negative correlation between male investment in gaining matings and longevity (Cordts and Partridge 1996; Kotiaho 2001; Hunt et al. 2004; Bonduriansky and Brassil 2005; Miller and Brooks 2005; Hunt et al. 2006). In addition, male attractiveness has a negative pleiotropic effect also on offspring survival in the guppy, *P. reticulata* (Brooks 2000). Nonetheless, under some circumstances, selection could act concordantly on both longevity and reproduction. Indirect selection on male condition (the total pool of resources available for allocation to reproduction and survival, Rowe and Houle 1996) may favour genes with positive pleiotropic effects on longevity and somatic maintenance (Abrams 1993; Williams and Day 2003; Bronikowski and Promislow 2005). For example, although an increase in the abundance of predators (and concomitant reduction in life expectancy) is generally assumed to permit the evolution of accelerated ageing, the opposite effect has been observed for some indices of ageing in guppies (Reznick et al. 2004). Because also sexual selection can generate positive selective pressures on male condition through selection on condition-dependent sexual traits, positive genetic correlations between reproduction and longevity can arise. Indeed some individuals may be able to acquire more resources than others individuals, and if variance for condition is greater than variance for resource allocation, positive covariances are expected between reproduction and survival (van Noordwijk and de Jong 1986).

In conclusion, the net fitness of an individual depends on his early-life reproductive rate, the pattern of change in resource allocation to reproductive traits with age (senescence) and life span. Individuals are likely to vary in life span and senescence rate, and this variation may reflect secondary sexual trait expression, if genes affecting reproduction have pleiotropic effects, positive or negative, on senescence and longevity, as expected under a life history scenario. Whether differential investment in an ejaculate trait reflects substantial variation in longevity and senescence rate is still unknown.

The aim of this study is to investigate how male investment in a strongly-selected ejaculate trait, sperm production, affects longevity and the intensity of senescence in sexual traits (pre- and postcopulatory) and condition in male guppies.

P. reticulata is a polyandrous livebearing freshwater fish that is emerging as an important model in linking pre- and postcopulatory sexual selection. During courtship, male guppies show their highly polymorphic colour patterns to females using sigmoid displays, a ritualized courtship posture in which males attempt to persuade sexually receptive females to mate (Houde 1997). Males can also switch from courtship to a coercive mating tactic, termed gonopodial thrusting (GT). In the wild, females can be subjected to up to one sneaky mating attempt per minute (Magurran and Seghers 1994), which may account for the high levels of multiple paternity reported in natural guppy populations (Neff et al. 2008). Forced matings have a lower insemination success than cooperative copulations (Pilastro and Bisazza 1999) but are thought to contribute to a male's reproductive success given the high frequency with which this mating tactic is adopted (up to one GT attempt per minute, Magurran and Seghers 1994). Both the intensity of male's courtship displays and the area of orange in the male's colour patterns have been shown to be positively phenotypically correlated with ejaculate quality (Matthews et al. 1997; Locatello et al. 2006; Pitcher et al. 2007), and these relationships are thought to explain why sperm competition favours males with relatively high levels of orange (Evans et al. 2003a) and correspondingly high levels of courtship (Evans and Magurran 2001).

To investigate whether sperm investment affects male senescence and longevity, we artificially selected males with the highest and lowest values of sperm numbers at rest. After three generations of bidirectional selection, a subset of males was monitored until death occurred, and traits affecting mating and sperm competition success in this species were measured at the age of 10 and 14 months, equivalent to male ages considered in previous studies of senescence in the guppy (Miller and Brooks 2005; Gasparini et al. 2010a). Game theory predicts that males selected for higher sperm production should have shorter lifespan and more severe senescence compared to males selected for lower sperm investment.

Methods

General methods

The fish used in this experiment were 3rd generation descendants of a bidirectional artificial selection experiment for sperm production. Each selection line (high sperm production, HS, and low sperm production, LS) consisted of 20 males scored for highest or lowest sperm production values and was replicated twice (see Manuscript I for details). Briefly, each selected male was individually housed with two unrelated virgin females from the same selection line and replicate. Females were checked daily until their first brood was delivered. Male offspring were individually raised (up to 3 randomly chosen per brood) and screened for sperm production at 5 months of age.

Parental individuals were descendants of wild-caught fish collected in 2002 from the lower part of Tacarigua River in Trinidad (Trinidad national grid reference: PS 787 804; coordinates: N10° 40.736' W061° 19.168). Laboratory stock and all experimental fish were maintained under controlled temperature and lighting conditions (26°C ± 1°C; 12:12h light dark cycle) and fed twice daily on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplaRin).

Males of known age that were selected as founders for the 4th generation of selection (44 HS and 40 LS), were housed individually in constant conditions in 2 l tanks to record longevity. A new companion female was provided to each male every 15 days to maintain a sustained male reproductive investment (Bozynski and Liley 2003).

Previous studies investigated the effect of age on sexual traits in old male guppies at the age of 10 (Miller and Brooks 2005) and 14 months (Gasparini et al. 2010a). In order to estimate senescence rate in HS and LS males, we screened each male at the age of 10 (mean age: HS = 299.91±0.20 days, LS = 300.76±0.13 days) and 14-months for the following traits: body size, body colouration, sexual behaviour, sperm velocity and condition. These traits have previously been shown to be differentially affected by ageing in *P. reticulata* (Miller and Brooks 2005), (Gasparini et al. 2010a), so we wanted to investigate whether there is a differential senescence for these traits in two lines artificially selected for sperm production. For the artificial selection protocol (see above), sperm production was measured also at the age of 5 months (mean age: HS = 152.86±0.16 days, LS = 150.95±0.30 days). Of the 84 males measured at 5 months of age, 9 HS and 4 LS died before being 10 months old, whereas only 24 males (10 HS and 14 LS) survived until the age of 14 months.

Sperm count and sperm velocity assay

Males were kept in isolation for at least 3 days before sperm collection in order to ensure that they had fully replenished sperm reserves (Pilastro et al. 2004), and that sperm number and velocity were not influenced by the differences in the social context. This is because male guppies respond quickly (i.e. 3 days) to the perceived mating opportunities by priming more sperm with higher swimming speed (Bozynski and Liley 2003; Gasparini et al. 2009). Sperm were manually stripped following Matthews et al. (Matthews et al. 1997) and Evans et al. (Evans et al. 2003b). Briefly, each male was anaesthetized in a water bath containing tricaine mesylate (MS-222) and placed on a slide under a stereomicroscope. A gentle abdominal pressure allowed the release of sperm in a drop of saline solution (NaCl 0.9%). Sperm in this species are packaged in discrete units, called sperm bundles, each containing about 21,000 individual sperm cells (Pilastro et al. 2008; Boschetto et al. 2011), which were photographed on a black background and counted using a digital image analysis software (Image J, http://rsb.info.nih.gov/ij). To transform the number of sperm bundles into the actual number of sperm, we regressed the total number of sperm on the number of sperm bundles in a subsample of 17 randomly chosen males from the 1st generation of selection. The two measures are highly correlated (mean number of sperm per bundle: 22,005±663.6, t_{16}=33.159, r^2=0.986, p<0.001). Furthermore, we compared the number of sperm per bundle between a subsample of randomly selected HS (n=36) and LS (n=34) males from 2nd generation of selection and did not find any significant difference in the number of sperm cells per bundle between selection lines ($F_{1,69}$=0.375, p=0.54).

After sperm counting, we measured sperm velocity, which is the second determinant of sperm competition outcome in this species (Boschetto et al. 2011) and it suffers from severe reduction with increasing age (Gasparini et al. 2010a). Sperm velocity was analysed *in vitro* immediately after sperm collection. 5 sperm bundles were collected and activated with 4 µl of 150 mM KCl and 4 mg/ml of bovine serum albumin (see Gasparini et al. 2009 for details). We used a Hamilton-Thorne CEROS sperm tracker (v. 12.3; Hamilton Thorne Research, Beverly, MA, USA). We recorded the average path velocity (VAP, $\mu m \cdot s^{-1}$), which is the average velocity of a sperm cell over a smoothed path, and the curvilinear velocity (VCL, $\mu m \cdot s^{-1}$). The two measures are highly correlated (Boschetto et al. 2011) and, for brevity, we present here only the VAP data. Two ejaculate aliquots from the same male were analysed and the mean was used for statistical analysis. *In vitro* measure of sperm velocity is highly repeatable in this population (Gasparini et al. 2009).

Male body size and colour pattern

After sperm extraction, all males were photographed on their left side using a Canon EOS 450D digital camera. Photos were then analysed with ImageJ software (http://rsbweb.nih.gov/ij/download.html). The following measures were recorded: standard length (SL, distance from the snout to the tip of the caudal peduncle), body area (including caudal fin but excluding dorsal fin) and the area of black, iridescent and orange spots (Pilastro et al. 2008; Evans 2010; Devigili et al. 2013). Black spots (encompassing fuzzy black lines) and iridescent spots (combined measures of blue, green, purple, and white) were included in the analysis because these colours are known to influence female mating preferences in some populations (e.g. Kodric-Brown and Nicoletto 1996; Brooks 1996a). In the population used for this study, however, only the area of the orange spots is positively correlated with sperm competition success and sperm quality (Evans et al. 2003a; Locatello et al. 2006). Male colouration has shown to increase from 5 to 10 months of age (Miller and Brooks 2005), whereas another study has found no different between 5 and 14 months (Gasparini et al. 2010a). Moreover, in a previous study based on the same artificial selection experiment, males selected for increased sperm production have shown a greater proportion of orange and black colouration at the age of 5 months, compared to their lower sperm counterparts (see Manuscript I). To control for the effects of body size, the relative area of each colour pigment was used in the analyses.

Male condition

A capture test, adapted from an established protocol used for newborn guppies (Evans and Magurran 2000; Evans et al. 2004b) and adults (Gasparini et al. 2013), was used to estimate predator evasion capability of males, a condition-dependent trait (Gasparini et al. 2013).

We performed capture tests 7 days after sperm extraction, to ensure males' complete recovery. Briefly, after 10' acclimatization in the test tank, C.C., blind to male identity, captured the male using a small hand net (7 × 7 cm).

Capture procedure consisted of chasing the male with the net at a speed which was kept as constant as possible. The test started when the fish was in a central position in the tank, and the time until the fish was captured was recorded by A.D.N. using a digital chronometer. This measure is significantly repeatable within individual (Gasparini et al. 2013). 3 HS and 4 LS males died prior to capture test, and for this reason sample size is lower for this trait.

Statistical analysis

Statistical analyses were performed using SPSS version 21.0. If not otherwise stated, means ± SE are reported. All probabilities are two-tailed. Data were checked for normality and appropriate transformation was adopted when necessary. Proportion data were arcsine square root transformed before analysis.

Because sample size is very low at the age of 14 months, those males were excluded from our analyses because a longitudinal analysis on 10 and 14 months old males would have dramatically reduced our statistical power and sample size. To test for the effect of age on the artificially selected trait (sperm production) we used repeated measures ANOVA with age and treatment as fixed factors. A univariate ANOVA with selection treatment as a fixed factor was used to compare traits' expression in the two selection lines in 10 months old males and male longevity.

For those males survived until the age of 14 months (N=24, see above), a repeated measures ANOVA was used to test whether in this subset of males the senescence pattern observed when males were 10 months old remained consistent also at the age of 10 months.

Results

Response to selection and effect of age on sperm production

After three generations of selection for high (HS) and low (LS) sperm production, males from the two lines significantly differed for the selected trait ($F_{1,269}$=117.02, p<0.001). The males used in this experiment were selected as founders of the 4th generation of artificial selection, in particular we used 43 males from the HS lines, (mean number of sperm at rest of 12.8±0.76 millions of sperm) and 40 males form the LS lines (sperm at rest: 1.13±0.10 millions of sperm). For the artificial selection experiment, sperm production was measured at the age of 5 months (see Materials and Methods), so we tested whether the selected trait differentially changes between lines from 5 to 10 months of age. We found that sperm number increased with age in both lines ($F_{1,66}$=14.56), p<0.001), but the low sperm line showed a stronger increase for the selected trait (age*line: $F_{1,66}$=8.426, p=0.005, Fig. 1).

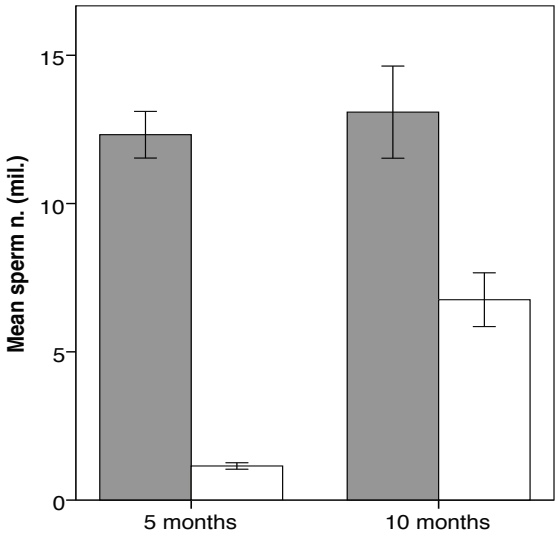

Figure 1. Mean sperm number ± SE at 5 and 10 months of age in the HS (grey bars) and LS (blank bars) selection lines

Male longevity

We found that males from the LS line had a significantly higher longevity compared to their HS counterparts ($F_{1,76}$=6.33, p=0.014; Fig. 2), with a mean lifespan of 414.94±17.23 days, opposed to a mean longevity of 358.83±14.43 days for HS males.

Sperm velocity, male body size and colouration

For what concerns male sperm velocity assay, males from the HS line had significantly slower sperm as compared to their LS counterparts at the age of 10 months: $F_{1,64}$=6.54, p=0.013 (Fig. 3). Within the precopulatory traits we have measured, neither body size nor colour extension (orange, black and iridescent) differed across selection lines in 10 months old males (Table 1).

Condition

We found no difference in the condition of males from the two selection lines at the age of 10 months ($F_{1,60}$=0.01, p=0.907), estimated as time spent evading a simulated predator (HS: 60.24±7.70 s.; LS: 59.67±9.70 s.).

Senescence male traits at 10 and 14 months

Analyses on the subset of males survived until the age of 14 months show a strong senescence for sperm production, sperm velocity, orange and black colouration and condition (Table 2). For the selected trait, a differential increase between lines in the number of sperm was confirmed, with LS males having a grater increase in sperm production (Table 2).

Table 1. Means and SE for body size and colouration in the two selection lines (HS and LS) at the age of 10 months. Univariate ANOVA statistics are reported. For orange colouration Welch statistics is reported because homogeneity of variances was not met for this trait.

Trait	HS		LS		ddf	F	p
	Mean	SE	Mean	SE			
Standard length (mm)	16.80	0.17	16.47	0.17	76	1.99	0.162
Relative area of orange spots*	11.12	0.73	12.09	1.02	65.16	0.11	0.745
Relative area of black spots*	2.61	0.15	2.91	0.17	76	1.57	0.214
Relative area of iridescent spots*	5.56	0.52	5.51	0.50	76	0.01	0.918

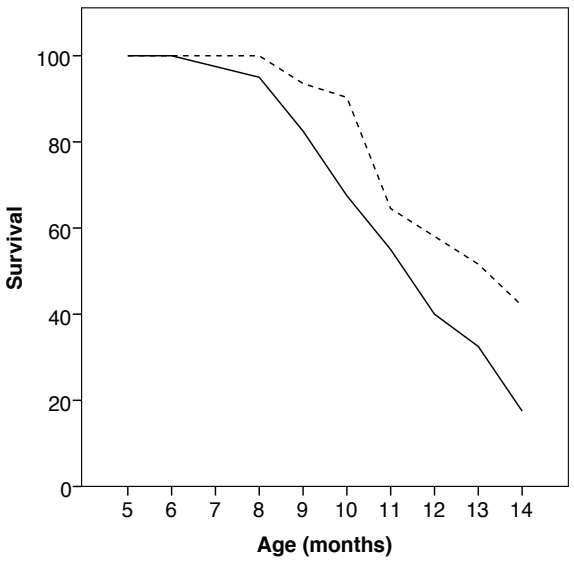

Figure 2. Proportion of survival males for HS (solid line) and LS (broken line) lines with increasing age.

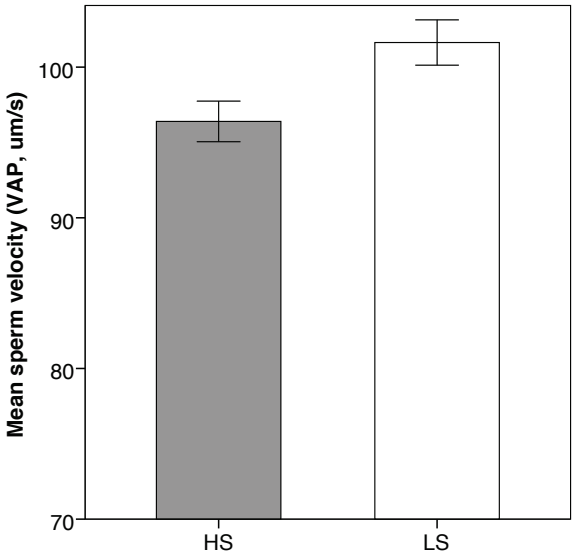

Figure 3. Mean sperm velocity ± SE (VAP, μm · s^{-1}) in 10 months old males from HS (grey bar) and LS (blank bar) selection lines.

Table 2. Means and standard errors (SE) at different ages in the two selection lines (HS and LS). Repeated measures ANOVA statistics are reported.

Trait	Age	HS		LS						
	(Months)	Mean	SE	Mean	SE	ddf	Age	*p*	Age* Line	*p*
Sperm n. (mil.)	5	12.80	0.76	1.13	0.10	22	22.17	<0.001	14.83	0.001
	10	13.08	1.55	6.76	0.91					
	14	15.53	3.23	10.18	1.66					
VAP (μm · s^{-1})	10	96.40	1.35	101.63	1.50	22	105.8	<0.001	0.05	0.823
	14	75.36	2.37	80.44	2.06					
Standard length (mm)	10	16.80	0.17	16.47	0.17	22	3.97	0.06	0.24	0.633
	14	16.53	0.25	16.05	0.26					
Orange %*	10	11.12	0.73	12.09	1.02	22	13.00	0.002	0.55	0.466
	14	9.76	1.39	2.38	0.24					
black %*	10	2.61	0.15	2.91	0.17	22	13.11	0.002	0.06	0.810
	14	1.81	0.17	2.38	0.24					
Iridescent %*	10	5.56	0.52	5.51	0.50	22	0.03	0.867	0.11	0.740
	14	4.94	0.88	4.22	0.81					
Condition (sec.)	10	60.24	7.70	59.67	9.70	22	6.78	0.017	0.18	0.675
	14	19.71	4.88	30.63	7.96					

Discussion

We compared male longevity and senescence in two lines artificially selected for high and low sperm production and we found evidence of negative genetic correlation between male investment in this strongly selected ejaculate trait (Gasparini et al. 2013) and male longevity. Moreover, the selected trait has shown to differentially increase with age in the two lines, with a stronger increase in the low sperm line, although sperm production was still higher in the high sperm line. An important sperm quality trait, sperm velocity, was negatively correlated in old males selected for increased sperm production, suggesting a potential trade-off between sperm quantity and quality in old males. Indeed, a previous experiment investigated correlated responses to artificial selection for sperm production in pre- and postcopulatory traits (see Manuscript I) and found no evidence of trade-off between sperm quantity and velocity in young males after sexual maturation (5 months old). Moreover young males selected for high sperm production also showed larger orange and black colour spots, normally involved in mating success in this species (Brooks 1996b; Evans et al. 2003a), compared to their low sperm counterparts (see Manuscript I). In this study we found no difference between selection lines in colour extension, suggesting that the potential advantage in mating acquisition highlighted in young males selected for increased sperm production disappeared with increasing age. Finally, male condition did not differ across selection lines, suggesting that a differential resource acquisition ability may not explain the differential senescence pattern that we observed. The pattern we observed at 10 months of age was also confirmed in the subsample of males survived until the age of 14 months, with a role of age on the expression of sperm number and velocity confirming the results from a previous study (Gasparini et al. 2013),

whereas the effect of age on male colouration and condition clearly requests further investigation, because of the low sample size obtained at 14 months of age.

Our results extend any potential for trade-offs involving male guppies life-histories to a lifetime time scale, suggesting that increased reproductive investment in strongly selected ejaculate trait in the early life stages is associated with costs late in life and, on the whole, with reduced lifespan.

Models of sexual selection suggest that females should prefer to mate with older males because old age is evidence of heritable high viability (Zahavi 1975). Good genes models have used age as indicator of genetic quality and suggest that females will benefit from choosing an old rather than a young male (Kokko 1998). It has been argued that, simply by surviving, males provide information about their genetic quality in terms of viability. Selection should therefore favour mate choice decisions by females that incorporate traits indicating older age, because such traits would intrinsically reveal a male's overall ability to cope within a given environment. Selection for old age, however, may come with costs in terms of genetically correlated responses that affect mating acquisition (Jacot et al. 2007). In the guppy, females do not discriminate between males of different age at the precopulatory stage (Gasparini et al. 2010a), so it is unlikely that male age is an important cue in female mate choice and it is associated with male genetic quality. Indeed, despite living longer and having faster sperm, low sperm males still produced less sperm than their short-living counterparts. Moreover, in early adulthood, high sperm males did not pay any trade-off, but conversely they show positive correlations between sperm production and body colouration (see Manuscript I). Altogether these aspects suggest that resource acquisition and allocation pattern to vary during male lifespan. Therefore under a "live fast-die young" scenario, males investing a lot in reproduction could pay a cost in their senescence and longevity, reducing their lifetime reproductive success (Brooks 2000; Hunt et al. 2004; Preston et al. 2011). It is difficult to estimate the global fitness of the two selected genotypes starting from the phenotypic expression of the traits we have measured in this experiment, because the relative contribution of each trait to global fitness is hard to be estimated. Nonetheless we can clearly assess that positive associations between sperm production and other sexual traits in early adulthood become null or negative with increasing age, suggesting a negative pleiotropic effect between sperm number and male's senescence and longevity.

Previous studies on ageing and longevity associated with reproductive investment mainly focused on costly sexual advertisement: a greater investment in ornaments often results in higher mortality and more severe senescence. In *Teleogryllus commodus*, for example, Hunt and colleagues (2004) manipulated male condition by altering the protein content of their diet: high-quality males invested more energy in calling effort during early adulthood, but died sooner and aged more rapidly than males reared on a low protein diet. This trade-off was confirmed in the same species with an artificial selection experiment for longevity, with short-living adults having a higher calling rate (Hunt et al. 2006).

The same pattern would be expected for other costly sexual traits, related to both mating acquisition and sperm competition success. Although it is often assumed that sperm are cheap, and that males are limited in their reproduction only by the number of females they mate with (Borgia 1979), ejaculate production can instead represent a significant cost for males (Dewsbury 1982; Hayward and Gillooly 2011; Rahman et al. 2013; Gasparini et al. 2013) and males can deplete their sperm reserves (Birkhead and Fletcher 1995; Olsson et al. 1997; Preston et al. 2001; Doyle 2011), thus limiting the number of offspring they can sire. If sperm are costly, we expect to observe evolutionary trade-offs between ejaculate production and other costly traits such as those involved in precopulatory competition (see Kvarnemo and Simmons 2013 for a recent review) or traits involved in survival and somatic maintenance (Parker et al. 2013).

Evidence suggests a longevity cost for males with high mating rates, which can be indirectly associated with both mating success and sperm competition intensity. In *Protopiophila litigata,* high mating rate was associated with reduced longevity (Bonduriansky and Brassil 2005), and in *Ontophagus binodis* mating populations suffered a longevity cost compared with non-mating males (Simmons and Kotiaho 2007a), but ejaculate expenditure was not measured, so we do not know whether negative correlations between longevity and reproduction are caused by increased ejaculate investment or mating acquisition effort, or maybe both. A recent study on suicidal reproduction in mammals seems to confirm that increased sperm competition leads to higher postmating mortality: in species with shortened breeding seasons as a result of strong prey seasonality, competition for reproduction increases at the expenses of survival; species with this die-off have larger testes in relation to body size (Fisher et al. 2013), suggesting a greater investment in sperm production associated with increased sperm competition risk.

Genetic fitness costs associated with sperm competition may be either direct (pleiotropic) consequences of sperm competitiveness, or due to linkage disequilibrium between sperm production genes and deleterious alleles.

Sexually-selected traits show higher genetic variance than expected for traits constantly exposed to directional sexual selection (the so-called lek paradox, Andersson 1994; Pomiankowski and Moller 1995; Rowe and Houle 1996). A number of hypotheses relate trait expression to multiple genes of small effect that underlie male condition, capturing genome-wide variance through condition dependence for costly sexual traits (Rowe and Houle 1996; Tomkins et al. 2004; Radwan 2008; Chenoweth and McGuigan 2010). A recent study has found that horn size genetic variance is maintained through a negative association between reproductive success and survival in the soay sheep (Johnston et al. 2013). Going further, authors have shown that most of the genetic variation in horn size is explained by a single locus, suggesting that an evolutionary trade-off between longevity and reproduction operating at a single locus can maintain genetic variance in

a sexually-selected trait. This result goes in the opposite direction of previous models of genic capture for secondary sexual trait, where variation is expected to be maintained by numerous partially recessive mutations of small effect. Empirical evidence however is still scarce and it is unlikely that overdominance at single loci plays a large role in explaining the lek paradox, or that genic capture play no part (Howie et al. 2013).

Our results suggest a complex interaction with ageing and longevity at a multiple level involving both ejaculate traits and precopulatory traits, suggesting that it is unlikely that genetic variance for sperm production could be maintained by multiple trade-offs operating at a single locus. A similar pattern was observed in a different population of *P. reticulata*, where male attractiveness, determined by a range of morphological and behavioural traits, was negatively associated with offspring survival (Brooks 2000). Our results extend this pattern also to a postcopulatory level, suggesting that an increased reproductive effort, in terms of both mating acquisition (Miller and Brooks 2005) and sperm competition, leads to a reduced survival in the guppy. Altogether these data, combined with a strong condition-dependence for sperm production (Gasparini et al. 2013), suggest that genetic variance in sexually-selected traits is likely to be maintained by negative pleiotropy and/or numerous alleles with small effect. Further investigation is required to assess the mechanisms underlying genetic variation for this trait and other sexually selected traits. Estimation of complex genetic correlations taking into account as many fitness traits as possible is needed and the relative contribution of loci with intermediated allele frequency versus partially recessive deleterious mutations at low frequency to the observed genetic variance in sperm number is still unknown.

Any conclusion about the relative importance of these processes in maintaining genetic variance for sexual traits, however, needs to keep into account the interactions between genotype and environment. Our experimental fish were maintained in optimal conditions (unlimited food, absence of competition for reproduction, etc.), but it is unlikely that harsher environmental conditions have the same effect on both genotypes. For example, it may be expected that the costs of the "live-fast" strategy revealed by our ageing experiment are even stronger in harsh environmental conditions. Whether or not this actually occurs also depends on the interaction between mutation load and environmental conditions in early life.

Finally, it would be interesting to extend this scenario also to females, because sexual conflict has the potential to displace the sexes from their sex-specific optima for life span and senescence (Promislow 2003). Indeed sexually antagonistic selection on alleles that increase the fitness of one sex while decreasing that of the other may explain the high genetic variance in sexual traits (Bonduriansky and Chenoweth 2009). Moreover, considering that individual's fitness depends not only on his lifetime reproductive success, but also on his offspring survival and reproductive success, it is necessary to consider how selection for increased sperm production affects female longevity and fecundity in association with offspring survival and performance.

Negative correlation between deleterious mutations and the expression of a postcopulatory trait in guppies

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The maintenance of genetic variation for sexual traits under directional sexual selection remains a major paradox in evolutionary biology. Among the several mechanisms hypothesized to resolve this paradox, the most notably are those surrounding the balance between new deleterious mutations and selection: i) the cost of exaggerated sexual traits depends on a male' condition, i.e. the pool of resources available to an individual for maintenance and reproduction; ii) condition is expected to be influenced by large part of the genome; iii) the genome harbours a large mutational load despite selection due to continuous influx of new deleterious mutations and iv) the expression of sexual traits is negatively correlated with the number of deleterious mutations. While there is abundant evidence that sexual traits are condition dependent, direct evidence that deleterious mutations are negatively correlated with the expression of sexual traits, is still lacking. We compared the fitness decline associated with inbreeding (inbreeding depression, ID) in lines of guppies (*Poecilia reticulata*) artificially selected for high (HS) and low (LS) sperm production, a condition-dependent trait under directional postcopulatory selection. LS guppies showed larger ID in female fecundity and in three fitness-related male traits such as body colouration, sperm number and predator evasion capability, as expected if sperm number reflects the load of genome-wide deleterious mutations. These results support the prediction, postulated by pre- and postcopulatory good genes models, that the phenotypic variation in sexual traits is correlated with the number of deleterious mutations, and have important implications for the evolution of sexual reproduction.

The maintenance of additive genetic variation underlying traits under strong, directional selection is one of major unresolved issues in evolutionary genetics (Barton and Keightley 2002). A paradigmatic example is represented by exaggerated male traits resulting from sexual selection by female mating preference (Darwin 1871) which are observed in species in which males contribute nothing to reproduction apart from sperm. In these cases, genetic benefits sustaining female preference for male traits should be rapidly eroded due to strong directional selection and females preference should disappear, yet females continue to choose (Borgia 1979). Good genes models of sexual selection hypothesize that the expression of sexually selected traits must correlate negatively with the load of deleterious, partially recessive mutations (Pomiankowski et al. 1991; Rowe and Houle 1996). This is because costly traits inevitably become dependent on condition, i.e. the amount of resources available to individual males for reproduction. Condition, in turn, is likely to be determined by large proportion of the genome and hence any deleterious mutation is likely to affect condition and hence the expression of sexually selected traits. Although selection removes deleterious mutations, at each generation new mutations will occur, maintaining the genetic variance underlying condition and sexually selected traits (Rowe and Houle 1996). Partially recessive, deleterious mutations of small effect are thought to explain a large part of the additive genetic variation in traits that are closely associated with fitness (Houle 1998; Charlesworth and Willis 2009). However, direct, empirical evidence that the expression of sexually selected traits is negatively correlated with the load of deleterious mutations is still lacking. While traditionally applied to male ornaments, good genes models also apply to ejaculate traits subject to directional postcopulatory cryptic female choice (Eberhard 1996). If ejaculate traits associated with sperm competition success, such as sperm number and velocity, are costly and become genetically linked to male condition, polyandrous females may increase their reproductive success because genetically superior males are more likely to fertilize their eggs (Yasui 1997). Indeed, evidence that postcopulatory traits can be genetically correlated with condition has been found in insects (Arnqvist and Thornhill 1998; Simmons and Kotiaho 2002).

The guppy, a livebearing fish with internal fertilization, is characterized by a resource free mating system and high levels of sperm competition and multiple paternity (Evans and Pilastro 2011). When sexually receptive, female guppies actively seek multiple matings with different males, which are associated with genetic benefits to the offspring (Evans and Magurran 2000). These benefits must be mediated by sperm competition success (Yasui 1997), and competitive fertilization success in guppies is largely determined by the number of sperm inseminated (Boschetto et al. 2011). As expected for traits under directional selection (Charlesworth and Willis 2009), sperm number at rest (a measure of sperm production) is characterized by large directional dominance (i.e. shows larger inbreeding depression) and condition-dependence (Gasparini et al. 2013). Together, these data indirectly suggest that postcopulatory sexually selected processes mediated by sperm number may result in a lower load of deleterious mutations in the progeny.
To test this prediction, we conducted an inbreeding experiment in two lines of guppies that were subject to artificial selection for high (HS) and low (LS) sperm number. Inbreeding depression (ID) is the reduction in

fitness observed in the offspring of related (compared with unrelated) parents and is believed to be predominantly caused by the increased homozygosity of numerous, at least partially recessive, deleterious mutations of small effect (Charlesworth and Willis 2009). If sperm number is influenced by partially recessive deleterious mutations, we expect to observe a relatively higher ID in LS guppies, as compared to their high sperm number counterparts. Inbred and outbred crossings were all conducted within selection lines, which controlled for differences in the overall genetic background of outbred and inbred families (Supplementary Fig. S2) (Postma 2011). We compared between artificially selected lines the ID in sperm number and three traits related to male fitness, namely the size of orange and iridescent spots on the body (used by female in their mate choice Evans et al. 2004a), and the ability to escape a simulated predator, a highly repeatable, condition-dependent trait in this guppy population (Gasparini et al. 2013). We further compared the ID in female fecundity across the selection lines for high and low sperm production.

Sperm number readily responded to artificial selection, and HS and LS lines started to diverge after one generation of selection (Fig. 1a, Supplementary Table 1), as expected given the high sire heritability estimated for this sperm trait in a previous half-sib/full-sib experiment (Gasparini et al. 2013). The cumulative heritability of sperm number estimated from the artificial selection was 1.08 (0.93 — 1.23, 95% C.I., based on 10000 replications, Falconer and Mackay 1996). Fish from the second generation of artificial selection were used for the inbreeding experiment. After one generation of inbreeding we observed significant ID in sperm number in LS, but not in HS males (Fig. 1b, Table 1). Consistent with this result, ID was larger in LS males than in HS males also in the size of the body colour spots (area of orange and iridescent spots) and in capture time (Fig. 1b). The pattern of female fecundity further supported the prediction that sperm number is correlated with the load of genome-wide deleterious mutations in guppies (Fig. 1b,c). Firstly, ID in brood size was larger after the first full-sib mating, although the difference was not significant, as indicated by the 95% C.I.s overlapping with 0 (HS = -2.2%, 95% C.I. -25.8—16.0; LS = 10.1%, 95% C.I. -11.8—28.1, Supplementary Table 2). However, full-sib matings resulted in a higher incidence of unviable newborn offspring (41/355, 11.6%) [a figure similar to that reported for other fish species (McCune et al. 2002)], as compared to the outbred families (5/370, 1.4%). Unviable newborns were significantly less frequent in the inbred HS families (6.8% of the total offspring) than in their LS inbred counterparts (15.0%, $F_{1,37}$=5.33, p=0.027, logistic regression), in accordance with the higher mutation load expected in the line artificially selected for low sperm number. Secondly, brood size at birth significantly declined after a second generation of full-sib matings in the LS females (ID = 37.4%, 95% C.I. 18.4—52.7) but not in their HS counterparts (ID = 7.5%, 95% C.I. -13.3—25.8; Fig. 1c, Supplementary Table 2). When the same experimental males were mated with outbred, unrelated virgin females from the stock population (i.e. unselected), we did not observe any significant difference in female fecundity among experimental groups, indicating that the reduced brood size observed in the inbred LS families was not due to sperm limitation (Supplementary Table 3). Indeed, all males used in this experiment had reserves of viable sperm which largely exceeded those necessary to impregnate a female (Pilastro et al. 2008). The decline in fecundity is therefore likely to be determined by the direct effect of inbreeding on embryo viability and/or its indirect effect on female condition and the initial number of eggs per clutch. Overall, ID was significant in four (sperm number, area of orange spots, capture time and female fecundity) of the five fitness-related traits measured in the LS line, whereas it was significantly greater than zero for none of these traits in the HS line (Table 1). In all traits ID was larger in the LS line than in the HS line and the difference in ID between selection lines was significant for orange (p=0.025) and fecundity (p=0.045, based on 10000 permutations). Average ID depression for the five traits was 21.19% (95% C.I. = 14.15—27.76) in the LS line, as compared to 3.44% (95% C.I. = -3.62—10.20, bootstrapped C.I.'s based on 10000 replications) in the HS line (Fig. 1c). A permutation test (within each selection line) confirmed that the difference in overall ID for the five traits considered was significantly larger in LS males as compared to that observed in HS males (Fig. 1c). Taken together, our inbreeding depression results confirm previous studies that revealed a substantial load of deleterious mutations for this guppy population (van Oosterhout et al. 2003; Gasparini et al. 2013). They further indicate that in this guppy population the phenotypic variance in sperm number is negatively correlated with the genome-wide load of partially recessive mutations, which affect condition and other fitness related traits.

Good genes models of sexual selection assume that the mutation load of males determines the expression of sexual traits (Pomiankowski et al. 1991; Houle and Kondrashov 2002). Our results demonstrated that sperm number reflects the load of deleterious mutations in guppies and that a significant part of the genetic variance exhibited by this condition-dependent trait is maintained through a mutation-selection balance (Pomiankowski et al. 1991; Rowe and Houle 1996; Houle and Kondrashov 2002). Previous studies on the same population demonstrated that multiply mated females produce fitter offspring (Evans and Magurran 2000) and that sperm number mediates male competitive fertilization success (Boschetto et al. 2011). It seems therefore reasonable to hypothesize that postcopulatory processes responsible for the genetic benefits associated with polyandry are at least partly mediated by the reduced load of deleterious mutations associated with high sperm production (Rowe and Houle 1996). Although we found that variation in sperm number is associated with the number of deleterious mutations, it is unlikely that this is the only source of genetic variation in sperm number, also given its high sire heritability and significant Y-linkage (Gasparini et al. 2013). Variable selection (i.e. multiple female preference, Brooks and

Endler 2001) and frequency dependent selection (Hughes et al. 2013) have been shown to contribute to the maintenance of the large variance in male guppy colour pattern, which is, like sperm number, Y-linked and condition dependent (van Oosterhout et al. 2003). These mechanisms are unlikely to affect the genetic variance for sperm number, as postcopulatory sexual selection for these traits is likely to be unconditionally directional. Other mechanisms, such as negative pleiotropy and sexually antagonistic selection, are more likely to be associated with sperm production.

In conclusion, our results have important implications not only for sexual selection theory, but also for the evolution of sexual reproduction. When mutation load affects the expression of sexually selected traits, sexual selection can contribute to overcoming the two-fold cost of sex (Agrawal 2001; Siller 2001), providing that deleterious mutations are more strongly selected when expressed in males than in females.

METHODS

Experimental animals and artificial selection. Experimental fish were laboratory-reared descendants of wild-caught fish from the Lower Tacarigua River (Trinidad). Fish were fed twice daily, stock and experimental tanks were maintained at a constant temperature of 26±1°C and with a cycle of 12 L : 12 D h. We established independent populations of guppy that were selected for either increased (high sperm, HS) and decreased (low sperm, LS) (two replicates each) sperm number, plus one unselected, control population. At each generation, 20 males and females were mated monogamously for each replicate and all offspring from each family were raised to maturity. Details of the artificial selection protocol are given in the Supplementary Information (Supplementary Fig. S1).

Inbreeding experiment. After two generations of selection, 20 sires per selection line (in total 20 HS and 20 LS, 7 from replicate A and 13 from replicate B for each selection line) were mated with a full sib, virgin female and with an unrelated virgin female from the same selection line (see Supplementary Fig. S2). Brood size was estimated at birth and offspring raised to maturity. Once mature (4 months), we estimated sperm number at rest, body colour spots and the ability to escape a simulated predator in males (Gasparini et al. 2013), and repeated the mating schedule as above to assess the effect of inbreeding on female fecundity. In total we obtained 78 families of inbred and outbred guppies (HS, n=40, LS, n=38). For further details see the Supplementary Information.

Full Methods and any associated references are available in the supplementary information.

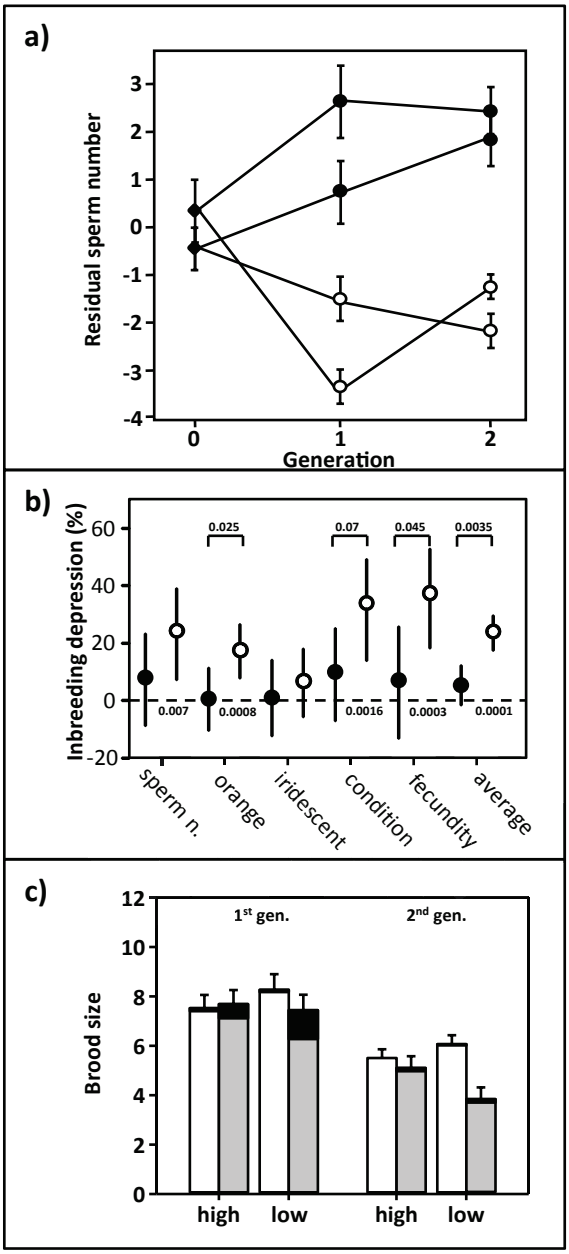


Fig. 1. A) Response to bidirectional artificial selection for sperm number in guppies (two replicates). Sperm number ($\times 10^6 \pm$ s.e.m.) is expressed as its unstandardized residual on body size (linear regression: sperm number = standard length · 0.903±0.138 – 7.320±2.214, $F_{1,770}$=33.76, p<0.001). HS males produced significantly more sperm than LS males (1st gen., selection: $F_{1,188}$=41.60, p<0.001, replicate (random factor): $F_{1,188}$=1.98, p=0.16, standard length: $F_{1,188}$=0.42, p=0.52; 2nd gen., selection: $F_{1,334}$=62.41, p<0.001, replicate (random factor): $F_{1,334}$=3.53, p=0.06, standard length: $F_{1,334}$=14.59, p<0.001). **B)** Inbreeding depression (ID, ±95% bootstrapped C.I., 10000 replications) for five fitness-related traits and their average in HS (black dots) and LS (open dots) lines. Significant probabilities that ID>0 and that LS ID > HS ID are given below and above the 0 line, respectively (2-tailed probabilities based on 10000 permutations within selection line). **C)** Female fecundity (brood size ± s.e.m.) after one and two generations of full-sib matings (grey bars) as compared to their outbred counterparts (white bars). The black portion within each bar represent the mean number of unviable offspring.

Supplementary Information

1. Supplementary Methods

Experimental animals and artificial selection

Sperm number

Males were isolated from females 3 days prior to sperm extraction to ensure that their sperm reserves were replenished, following an established protocol (Pilastro et al. 2007). Briefly, each male was anaesthetized in a water bath containing MS-222 and placed on a slide under a stereomicroscope. A gentle abdominal pressure allowed the release of sperm in a drop of saline solution (NaCl 0.9%). Sperm in this species are packaged in discrete bundles, called spermatozeugmata, each containing about 21000 individual sperm cells, which were photographed on a black background and counted using the automatic cell-counter function with digital image analysis software (Image J, http://rsb.info.nih.gov/ij/). To transform the number of sperm bundles into the actual number of sperm, we regressed the total number of sperm on the number of sperm bundles in a subsample of males. The two measures are highly correlated (mean number of sperm per bundle: 22,005±663.6 s.e.m., t_{16}=33.159, r^2=0.986, p<0.001). Furthermore, we compared the number of sperm per bundle between a subsample of randomly selected males HS and LS males and did not find any significant difference in the number of sperm cells per bundle between selection lines (t_{68}=0.61, p=0.54). In a subset of 58 males, sperm number was estimated twice one month apart to determine sperm number repeatability (repeatability = 0.79±0.05 s.e., $F_{58,115}$=8.50, p<0.001). In total, we estimated sperm number for 1227 male guppies (1029 and 198 for the artificial selection and the inbreeding experiment, respectively).

Artificial selection protocol

We initially screened for sperm number 400 males to found the two replicated selection lines. Each replicate consisted of 25 males with highest (HS) or lowest (LS) sperm number out of 100 males per replicate. Each male was individually housed with two virgin females from the stock population in the initial generation and from the same replicate afterwards. Mating between relatives was avoided by forming mating pairs with fish from different families. When visibly pregnant, females were individually isolated and checked three times per day until parturition. After brood delivery, mothers were removed, date of parturition was recorded, and offspring were allowed to grow in 8-L tanks. Once males became morphologically distinguishable from females (approx. at 6 weeks), they were isolated from the rest of the brood and kept separately (with a companion, unrelated female) until 5 months of age, when they were screened for sperm production as above. In the following generations, all sexually mature males obtained from the previous generation were screened for sperm number and 20 males per replicate (selected for highest or lowest sperm number) were used to found the next generation. A control population (one replicate) was founded by randomly selecting 25 males from the stock population. In the following generations, at each generation 25 males were randomly selected and allowed to reproduce (Supplementary Table 1, Supplementary Fig. S1).

Our sample size (2 replicates of 20 sires for each high and low line, n_{tot}=80) was comparable to that previously used in artificial selection experiment in guppies. Two studies investigated the evolution of precopulatory traits by artificially selecting males for their orange colouration (Houde 1994) (4 replicates of 20 sires for each high and low line, n_{tot}=160) and for sexual attractiveness (Hall et al. 2004) (2 replicates of 35-50 sires per line, according to the generation, n_{tot}=140-200). Both studies, however, used mass mating to generate the subsequent generation and only measured a subset of the offspring. In contrast, we mated each sire with two females and raised and measured all their offspring. There is a strong variance in male mating success in guppies (Neff et al. 2008). In a paternity study conducted in our lab on this population in which males competed in mass mating, the 20% least successful of 60 males sired 0 offspring, whereas the 20% most successful males sired about 50% of the total offspring (A. Devigili, A. Di Nisio and A. Pilastro, unpublished results). Thus, despite the smaller number of sires we used, the effective population size in our study is comparable to that of these previous studies. Our experimental design has the advantage that we could exclude that other sexually selected traits were inadvertently implicated in the selection of the sires at each generation. The need to raise to maturity each family in isolation, however, posed logistic limits to the number of sires and replicates that we could run.

Inbreeding experiment

Families were selected according to the following criteria: we minimized the age differences between families and selected families that contained at least two females in order to balance the representation of each family in the inbred and outbred matings. Seven males from replicate A and 13 from replicate B for each selection line were mated with a virgin full sister and with a virgin, unrelated female from the same replicate to obtain one outbred and one inbred line from each sire. The number of sires per replicate was unbalanced because the two replicates were not completely synchronized (replicate A was slightly late) and we avoided to use males and females that were younger than 4 months when the experiment started. The 20 males per selection line, which were used as sires in the inbreeding experiment, did not differ from their non-used counterparts (n=20) in sperm number (HS: $t_{1,43}$=0.51, p=0.62; LS: $t_{1,41}$=0.21, p=0.84), orange (HS: $t_{1,43}$=0.99, p=0.33, LS: $t_{1,41}$=1.95, p=0.06) and iridescent colouration (HS: $t_{1,43}$=0.04, p=0.97, LS: $t_{1,41}$=0.90, p=0.38). Similarly, the size of the family from which the fish used in the inbreeding were originated did not differ from those that were not used (HS: $t_{1,43}$=0.53, p=0.60; LS: $t_{1,41}$=-0.61, p=0.87). It has to be noted that fish from each family were used in both the inbred and outbred matings, so any differences among families was balanced between experimental groups. The sample size used for this experiment (40 inbred and 40 outbred families) was larger than that used in two previous guppy studies aimed at estimating the inbreeding depression in postcopulatory traits (17 inbred and 16 outbred families) [8], and precopulatory traits (12 inbred and 11 outbred) (Mariette et al. 2006).

Each male was allowed to freely interact with two virgin females, one related (same family) and one unrelated (same replicate but different family). The females were allowed to mate with the male for three days each, in sequence. The order was randomized with respect to kinship between male and female. This procedure was repeated three times, in order to maximise the probability that all females mated with the male (i.e. each female spent in total 9 days with the male, alternating every 3 days with the other female). Females were subsequently isolated until they delivered their first brood. Brood size did not differ between HS and LS lines, as expected given that artificial selection for sperm number was not associated with any difference in female fecundity in the F_2 generation, which was used for the inbreeding experiment (selection: $F_{1,155}$=0.28, p=0.60; replicate: $F_{1,155}$=0.71, p=0.40). The number of viable and unviable newborn (i.e. the number of offspring that died with 24 h from the parturition) was determined and all offspring were subsequently raised to maturity in large tanks (40 l). In total we obtained 78 families of inbred and outbred guppies (HS, n=40, LS, n=38), because we failed to obtain inbred and outbred offspring from the same LS male (that was likely to be infertile).

Once mature (age 122 ± 9 days), all males per family, up to three, were anaesthetized, digitally photographed, and sperm bundles were stripped and counted as above. When there were more than three males per family, three males were randomly chosen (i.e. the first three males that were fished from their tank were measured). Male photographs, taken using a digital camera (Canon EOS 450D, Canon, Japan) were analysed, blind to selection line and family, using Image J to measure the total area of the body and the area of orange and iridescent male colour spots, a sexually selected ornament in this guppy population (Evans et al. 2004a). Colour extension was expressed as the proportion of body area covered by colour spots. About 10 days after stripping (age 131 ± 10 days) male condition was estimated from his ability to escape a simulated predator in a capture test (Gasparini et al. 2013). Briefly, after 10-min acclimatization in the test tank, one of us, blind to male identity, captured the male using a small hand net (7 × 10 cm). Capture procedure consisted of chasing the male with the net at a speed which was kept as constant as possible. The test started when the fish was in a central position in the tank, and the time until the fish was captured was recorded by another observer using a chronometer. This measure is significantly repeatable within individual (Gasparini et al. 2013). Six inbred and one outbred males died before the capture test was performed, and sample size is therefore smaller for this trait. Finally, in order to determine female fecundity, one inbred male, randomly chosen from each family as above, was allowed to reproduce with a randomly chosen sister. One outbred male, randomly chosen within each family, was allowed to reproduce with a unrelated female, randomly chosen from an outbred family of the same selection line. The number of viable and unviable newborn was determined at birth (Supplementary Table 2). A graphic representation of the experimental design is given in Supplementary Fig. S2.

Fecundity control experiment

Although inbred males had reserves of viable sperm which largely exceeded those that are in principle necessary to impregnate a female (Pilastro et al. 2008), we controlled for any differential effect of male ejaculate on female fecundity by allowing the experimental males to mate with a randomly chosen, unrelated virgin female from our population stocks. Each experimental male was allowed to freely interact with the virgin female for a week as above. Afterwards, females were

isolated until a brood was produced. The number of offspring did not differ between selection lines ($F1.32 = 0.407$, $p=0.528$) and between inbred and outbred groups ($F1.32 = 1.481$, $p=0.233$, Supplementary table 3). Some of the males died before this experiment was completed and not all the females produced a brood within 50 days of mating. Sample size is therefore smaller than the initial one.

Statistical analyses

The data from the inbreeding experiment were analysed using non parametric procedures (bootstrapping and permutation), which do not require to meet the assumptions of the parametric tests. Sperm number form the selection experiment did not met the assumption of the homogeneity of variance, high sperm males having consistently higher variances than low sperm males. This is expected under directional selection for trait exaggeration. The homogeneity of the variance was attained only after rank transformation. Using ranks instead of original sperm number values gave substantially the same results as the parametric analyses (data not shown).

Bootstrapped confidence intervals for point estimators (ID) were calculated using Monte Carlo simulations as implemented in PopTools (version 3.4, http://www.poptools.org). Differences in ID among experimental groups were tested by comparing the observed differences with 10,000 randomized distributions (Monte Carlo simulation).

Ethical Note

The experiments were carried out in conformity with the relevant Italian laws governing the care of animals in research (D.L. 116/27-01-92, C.M.S. 8/22-04-94) and was approved by the ethic committee of the University of Padova (Permit n. 36/2011 to AP). The fish were fully anaesthetized before sperm extraction and phenotypic measurement. Manipulation, which was conducted by an expert operator following established procedures, was minimized and was usually completed in less than 5 min. For the same reasons, we used the lowest number of individuals necessary to achieve the aims of the experiment. All individuals recovered fully from anaesthesia and were returned to post-experimental tanks.

2. Supplementary tables

Supplementary Table 1. Means (± s.e.m.) and relative sample size (n) for sperm production ($x10^6$) in the two selection lines (high sperm and low sperm) and in the control group during two generations of selection. For each generation the mean value for all the male offspring measured and for individuals selected as founders is reported.

Selection line	1st Generation		2nd generation	
	Total measured	Selected	Total Measured	Selected
High sperm	8.70 ± 0.49 (100)	13.10 ± 0.69 (40)	9.52 ± 0.37 (175)	15.54 ± 0.70 (40)
Low sperm	4.94 ± 0.33 (92)	2.70 ± 0.24 (40)	5.65 ± 0.22 (163)	3.12 ± 0.17 (40)
Control	6.49 ± 0.83 (22)*	6.49 ± 0.83 (22)*	6.28 ± 0.52 (25)	6.28 ± 0.52 (25)

* three males died before measure

Supplementary Table 2. Inbreeding experiment: female fecundity (first and second generation). Sample size (n) refers to the number of inbred and outbred families. Means ± s.e.m are given.

		1st full-sib/outbred mating			2nd full-sib/outbred mating		
		Brood size at birth	Unviable newborn (%)	Families with unviable newborn (%)	Brood size at birth	Unviable newborn (%)	Families with unviable newborn (%)
High sperm	Inbred	4.31 ± 0.45 (20)	6.8 ± 2.1	45	6.11±0.55 (19)	3.0 ± 1.7	15.8
	Outbred	4.55 ± 0.48 (20)	5.0 ± 5.0	5	6.60±0.43 (20)	0	0
Low sperm	Inbred	4.36 ± 0.60 (19)	15.0 ± 2.9	73.7	4.56±0.58 (18)	5 ± 3.9	11.1
	Outbred	5.24 ± 0.69 (19)	1.3 ± 1.3	5.3	7.28±0.48 (18)	1 ± 0.6	5.6

Supplementary Table 3. Fecundity control experiment: brood size (mean ± s.e.m.) and number of family (n)

Selection line	Treatment	Mean ± s.e.m (n)
High sperm	Inbred	4.31 ± 0.45 (16)
	Outbred	4.55 ± 0.48 (20)
Low sperm	Inbred	4.36 ± 0.60 (14)
	Outbred	5.24 ± 0.69 (17)

3. Supplementary figures

Figure S1. Schematic representation of the artificial selection protocol. High and low sperm lines were replicated twice, and males selected as founders were housed separately with 2 virgin females each. In the control group, males were housed altogether in a 125 l tank with 50 virgin females.

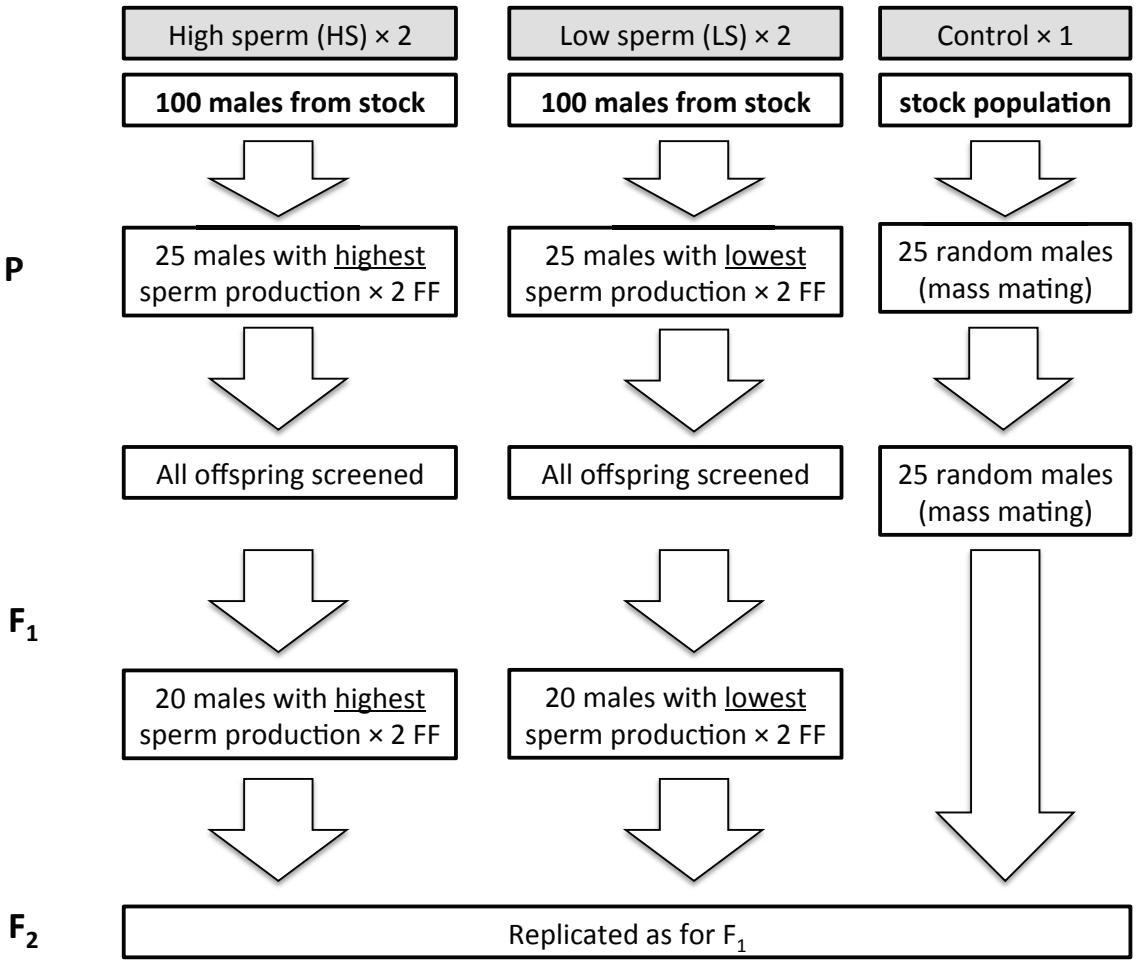
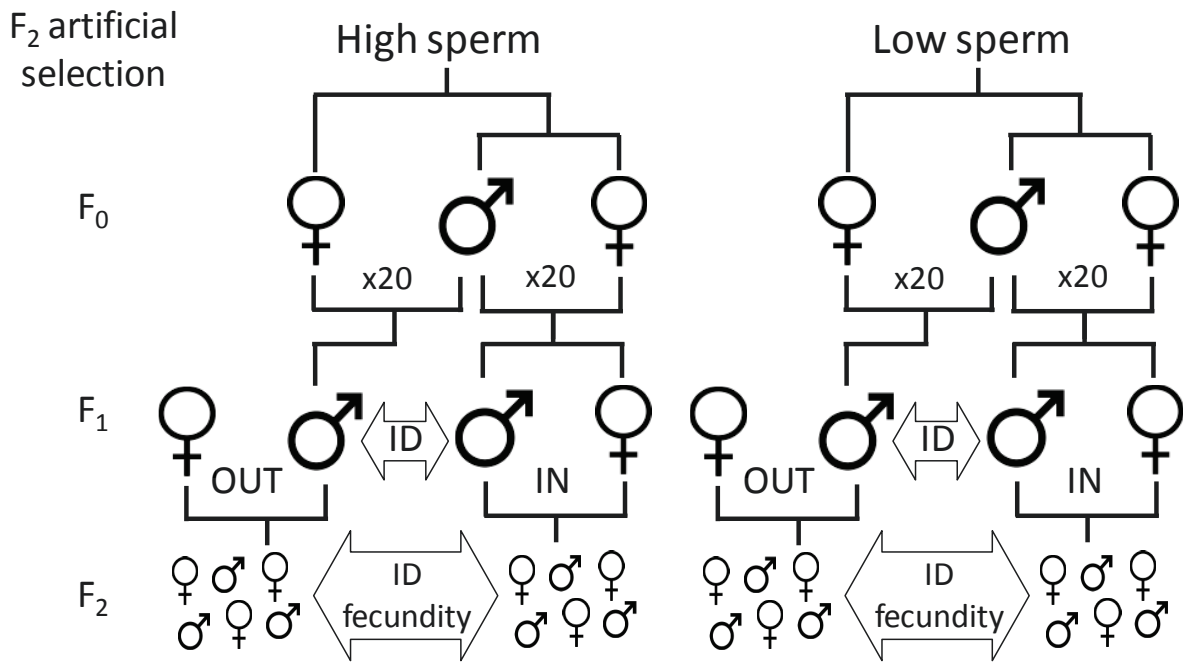


Figure S2. Graphic representation of the inbreeding depression (ID) experimental design. ID in male fitness-related traits was estimated by comparing mean trait expression of inbred and outbred families within selection line at F1. Female fecundity ID was estimated from the brood size produced by F1 females when mated with unrelated, outbred F1 males and related, inbred F1 males.



Riassunto

La poliandria è ormai riconosciuta come un fenomeno quasi universale nel regno animale (si veda la recente *review* di Parker and Birkhead, 2013), che porta alla competizione di due o più eiaculati di maschi diversi per la fecondazione dello stesso gruppo di uova (competizione spermatica), favorendo quindi tutti quegli adattamenti negli eiaculati che conferiscono al maschio un maggior successo di competizione spermatica. Tra questi, il numero di spermi prodotti è uno dei caratteri che mostra il maggior incremento in risposta alla competizione spermatica (Birkhead and Moller 1998).

Nonostante le forti pressioni selettive direzionali cui è sottoposto questo carattere in contesti di elevata competizione spermatica, la produzione di spermi non presenta un calo della variabilità genetica come atteso per caratteri sottoposti a selezione direzionale, ma al contrario risulta avere una varianza genetica additiva più elevata di altri carattere dell'eiaculato (Gasparini et al. 2013). Da questa osservazione nasce uno dei più importanti quesiti nel campo della biologia evoluzionistica applicata a studi di selezione sessuale: come è mantenuta la variabilità genetica in quei caratteri sottoposti a forti pressioni selettive direzionali? Originariamente applicato a quei caratteri sessuali che conferiscono un maggior successo precopulatorio, questo problema biologico può essere ugualmente esteso anche a quei caratteri post-copulatori importanti nel determinare il successo di fertilizzazione. Ad oggi mancano tuttavia evidenze sperimentali che indaghino i meccanismi sottesi al mantenimento della variabilità genetica in caratteri dell'eiaculato. Per la comprensione di questi meccanismi è necessario indagare quali siano le basi genetiche della produzione di spermi, in particolare se sussistono correlazioni genetiche tra questo e altri caratteri, sottoposti sia a selezione sessuale (pre- e postcopulatoria) che naturale, determinanti nella fitness dell'individuo. Eventuali correlazioni negative dovute ad esempio ad effetti pleiotropici o a *linkage disequilibrium* potrebbero spiegare il mantenimento della variabilità genetica in questo carattere. Produrre spermi è infatti energicamente dispendioso per un maschio (Dewsbury 1982; Hayward and Gillooly 2011) e poiché le risorse disponibile per un individuo sono limitate, ci si aspetta che a un maggior investimento in un carattere costoso sia associata una riduzione nell'espressione di uno o più caratteri ad esso correlati.

Un ulteriore meccanismo che potrebbe spiegare il mantenimento della variabilità in caratteri sessuali prevede che se il tratto è costoso, questo dipende allora dalla condizione complessiva dell'individuo (ovvero dalla capacità di acquisire risorse), che a sua volta è attesa essere influenzata da gran parte del genoma: la condizione è infatti un tratto multi genico che coinvolge la performance di un individuo su molteplici livelli (sistema cognitivo, locomozione, sistema immunitario, ecc…), pertanto se un carattere costoso diventa dipendente dalla condizione, ci si aspetta che ne catturi la varianza. Questa ipotesi, denominata *genic-capture* (Rowe and Houle 1996), prevede quindi che le mutazioni, principalmente quelle recessive deleterie non rimosse dalla selezione, introdotte a livello del genoma ad ogni generazione possano potenzialmente influire sulla varianza del carattere condizione-dipendente. Il carattere sarebbe quindi legato alla qualità genetica complessiva: individui con caratteri più sviluppati dovrebbero quindi presentare un minor carico di mutazioni recessive deleterie. Mancano tuttora evidenze a supporto di questa ipotesi, ma recenti prove sperimentali hanno confermato la forte condizione-dipendenza per la produzione di spermi in *Poecilia reticulata* (Gasparini et al. 2013), suggerendo quindi che questo carattere possa giocare un ruolo determinante nel collegare la competizione spermatica al carico di mutazioni deleterie, fornendo così anche una soluzione al problema del mantenimento della riproduzione sessuale nonostante il costo ad essa associato (Agrawal 2001).

Lo scopo di questa tesi è quello di valutare, in una specie nella quale sono noti in modo dettagliato i meccanismi di selezione sessuale pre- e postcopulatoria (Evans and Pilastro 2011), le conseguenze evolutive dell'investimento maschile nella produzione di spermi, il principale carattere che predice il successo di competizione spermatica in questa specie (Boschetto et al. 2011). Considerazioni teoriche suggeriscono che l'allocazione differenziale in caratteri pre e post-copulatori costituisca un *constraint* evolutivo cruciale nell'ambito della selezione sessuale (si veda Parker et al. 2013 per una *review* recente). A dispetto di questo mancano studi nei quali l'analisi di questi gruppi di caratteri sia valutata contemporaneamente in modo

integrato. A questo scopo è stato effettuato un esperimento di selezione artificiale bidirezionale per la produzione spermatica al fine di:

1) Determinare se la selezione artificiale per la produzione di spermi ha un effetto correlato sull'espressione degli altri caratteri post-copulatori (velocità, vitalità e morfologia degli spermi).

2) Determinare se la selezione artificiale per la produzione di spermi ha un effetto correlato sull'espressione dei caratteri pre-copulatori, in particolare del pattern di colorazione del maschio, del comportamento sessuale (frequenza di corteggiamento e di tattiche riproduttive alterative (*sneaky matings*) e del grado di preferenza esercitato dalle femmine sul maschio.

3) Determinare se la selezione artificiale per la produzione di spermi ha un effetto correlato sulla fitness non riproduttiva del maschio, stimata sul tasso dl accrescimento, sulla taglia a maturità, e sulla sopravvivenza.

4) Determinare se la selezione artificiale per la produzione di spermi ha un effetto correlato sulla fitness delle femmine. A questo scopo sarà determinato il tasso di accrescimento, la fecondità e la condizione nelle femmine.

5) Determinare se un maggior investimento riproduttivo in età giovanile comporta dei costi in età avanzata a causa di una maggior senescenza e una ridotta longevità (come atteso per strategie riproduttive del tipo "*live fast – die young*").

6) Determinare se una maggior produzione di spermi riflette una maggior qualità genetica del maschio attraverso un minor carico di mutazioni recessive deleterie espresse in importanti caratteri della fitness sia maschile (produzione di spermi, colorazione corporea, condizione) che femminile (fecondità), portate in omozigosi attraverso un esperimento di inbreeding sulle linee selezionate artificialmente (Postma 2011).

Biologia della specie

L'evoluzione del comportamento riproduttivo dei maschi e dei caratteri implicati nella competizione spermatica quali velocità, vitalità e numero di spermi prodotti sono fortemente influenzati dalla frequenza con la quale le femmine si accoppiano con due o più maschi durante lo stesso episodio riproduttivo. Una delle specie meglio studiate da questo punto di vista è *Poecilia reticulata*, un piccolo pesce tropicale viviparo d'acqua dolce a fecondazione interna, che rappresenta una specie modello per lo studio della selezione sessuale pre- e postcopulatoria (Evans and Pilastro 2011). Tra questi ultimi, il numero di spermi inseminati è probabilmente il fattore più importante (Boschetto et al. 2011), un pattern comune alla maggior parte delle specie (Immler et al. 2011). A dispetto dell'importanza della produzione di spermi nella selezione sessuale post-copulatoria, le nostre conoscenze sui pattern selettivi e sui cambiamenti microevolutivi associati a questo carattere sono relativamente scarse. Sappiamo infatti molto poco di come i caratteri precopulatori evolvono in risposta ad una pressione selettiva direzionale sulla produzione di spermi. Non solo, infatti, sono pochi i dati sperimentali che permettano di chiarire l'importanza relativa dei caratteri pre- e postcopulatori, ma sono poco noti i *constraints* genetici associati all'evoluzione di questo carattere per effetto di una selezione direzionale. Recenti studi hanno studiato le basi ereditarie e le correlazioni genetiche tra caratteri pre- e postcopulatori in guppy (Evans 2010), ma non hanno esaminato la produzione di spermi.

Risultati e conclusioni

I risultati del mio dottorato hanno mostrato che la produzione di spermi è positivamente e geneticamente correlata in maschi giovani (5 mesi di età) con diversi caratteri sessuali, sia a livello pre-copulatorio (colorazione della livrea e attrattività) che post-copulatorio (vitalità degli spermi e numero di spermi trasferiti), e anche con caratteri sottoposti a selezione naturale come l'età a maturazione sessuale e il tasso di accrescimento corporeo. Non vi sono invece differenze nelle femmine, mentre all'aumentare dell'età (10 mesi), maschi selezionati per un'elevata produzione spermatica presentano una ridotta velocità degli spermatozoi e le differenze nella colorazione emerse a 5 mesi sono annullate, lasciando quindi intendere che un maggior investimento riproduttivo in età giovanile comporti dei costi in età più avanzata. Un altro dato emerso dal mio

studio è infatti che maschi che producono più spermi vivono in media di meno rispetto a maschi selezionati per una bassa produzione di spermi. Infine i miei risultati mostrano per la prima volta che a un carattere dell'eiaculato determinante nel successo di competizione spermatica è correlato il carico di mutazioni deleterie recessive, come atteso dalla teoria della *genic-capture*.

Nell'insieme i risultati del mio studio mostrano come la variabilità genetica nella produzione di spermi è mantenuta sia da *trade-off* a lungo termine che dal bilanciamento tra mutazioni e selezione, tuttavia l'importanza relativa di questi meccanismi non è ancora nota. Questo lavoro rappresenta comunque il primo passo nella comprensione di un sistema integrato altamente complesso che incorpori molteplici aspetti della *life-history* di un individuo.

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