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ENVIRONMENT-DEPENDENT SEXUAL SELECTION AND THE MAINTENANCE OF GENETIC VARIABILITY IN SEXUAL TRAITS

Direttore della Scuola: Ch.mo Prof. Paolo Bernardi

Coordinatore d'indirizzo: Ch.mo Prof. Andrea Pilastro

Supervisore: Ch.mo Prof. Andrea Pilastro

Dottorando: Silvia Cattelan

“No species is more prolific in the diversity of phenotypic and genetic responses to the environment than the guppy”

G. R. Kolluru

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Thesis abstract

The problem of the maintenance of the additive genetic variability underlying sexually selected traits has been typically investigated in a precopulatory context (see Radwan et al. 2015). However, whenever females are sexually promiscuous within the same reproductive cycle, sperm competition can also generate directional selection on traits involved in fertilisation success, in particular on sperm number (Parker and Pizzari 2010). The selection acting on traits associated with competitive fertilization success should erode their genetic and phenotypic variability. In contrast, observed genetic variability for postcopulatory traits is unexpectedly high (reviewed by Evans and Simmons 2008). Among the several hypotheses that have been proposed to explain this paradox, environmental variation may play a crucial role in preventing one genotype from producing the optimal phenotype across all the possible environments (the so-called genotype-by-environment interaction, GEI). Under this hypothesis, genetic variability may be maintained because different phenotypes are favoured in a continuously changing environment (reviewed by Hunt and Hosken 2014). While the role of GEI in maintaining the genetic variability in precopulatory traits has been investigated in a relatively large number of cases, yet postcopulatory traits have been very little studied in this context so far. The aim of my study was to investigate whether environmental variations contribute to the maintenance of the genetic variability in sperm number in the guppy (*Poecilia reticulata*). Guppies are particularly appropriate to investigate the effects of environmental variations on postcopulatory sexual traits for a number of reasons. First, their natural habitats vary along multiple environmental gradients (Magurran 2005). Secondly, postcopulatory mechanisms are relatively well understood in this species (Evans and Pilastro 2011). Sperm number was chosen as target trait, since it shows a surprisingly high additive genetic variability (Gasparini et al. 2013) although sperm competition leads to strong directional selection on this trait (Boschetto et al. 2011). I used males selected for producing high and low number of sperm (Di Nisio 2014) to investigate GEIs. The use of artificially selected lines is a powerful method to investigate GEIs as it allows me to estimate the average effect size of this interaction in a group of individuals, giving a good representation of the “average” genetic background in the population. I performed three experiments by exposing male guppies from the two selected lines to different environmental manipulations. No evidence of GEI was found under dietary restriction both in sperm production and in the expression of other covarying pre- and postcopulatory sexual traits. The occurrence of GEIs was also evaluated in the offspring of males and females from the selected lines, as GEIs might arise in a trans-generational context. Again, no significant effects were detected at this level. On the contrary, evidence for GEIs was found when I manipulated the social conditions in which males and females interacted. In particular, the reproductive success of males from the selected lines significantly differed accordingly to sex ratio variations, suggesting that social environments appear to be especially relevant in generating GEIs for reproductive fitness. Although this, I found no evidence of GEI for sperm production adjustment in response to different perceived mating opportunities showed by males of the two selected lines. An experimental manipulation of the perceived male mating opportunities was also used to investigate the investment in mating acquisition associated to an increase in sperm investment. I found a trade-off between sperm investment and courtship behaviour, highlighting the importance of social interactions in potentially maintaining genetic variability in sperm number. In the final experiment, I found that diet had a significant effect on social context by altering the mating rate and hence the variance in mating and reproductive success. Furthermore, my results indicate that harsh environmental conditions tend to reduce the relative importance of postcopulatory sexual selection relatively to precopulatory processes. This suggests a synergetic effect of multiple environmental factors that could prevent one genotype to be the best across the environments and, as a consequence, maintaining genetic variability. My study provides a comprehensive picture of mechanisms responsible for the maintenance of genetic variability in a trait, sperm number, which was supposedly exposed to continuous, strong directional selection. Moreover, I have shown that it is not necessary to investigate particularly harsh or stressful environmental conditions in order to reveal significant GEIs (Hoffmann and Merila 1999), since the environmental manipulations I employed are within the normal bounds that the guppy might encounter, especially those affect social interactions. My results therefore highlight the potential of environmental fluctuations in the maintenance of sexually selected traits and the importance of studying sexual selection across varying environmental conditions.

General Introduction

Few questions in evolutionary biology have received as much attention as the problem of the large observed genetic variability in sexual traits despite the strong directional selection acting on them represented by female choice. This paradox is particularly evident for those species in which males contribute nothing to reproduction apart from their genes (the so-called resource free mating systems). In this kind of systems, females can only take advantage from mating with a specific male through genetic benefits to the offspring (Iwasa et al. 1991). Genetic variability in sexual traits among males is a necessary prerequisite for the maintenance of female choice itself since, as already Darwin argued, "*variability is the necessary basis for the action of selection*" (Darwin 1871). However, under strong directional selection the genetic variability for preferred traits is expected to be rapidly depleted and hence the benefits of choice. On the contrary sexual traits usually show high genetic variability (Pomiankowski and Moller 1995), often higher than that observed in non-sexual traits and females continue to choose, a phenomenon known as the "lek paradox" (Borgia 1979). In search for an answer for this paradox, empirical work has largely focused on assessing the relationship between male ornamentation and genetic benefits for the offspring (Qvarnstrom 2001). As suggested above, this problem has been typically investigated from a mate choice perspective and thus only in a precopulatory context (see Radwan et al. 2015). Although mate choice often generates a strong directional selective force (Andersson and Simmons 2006), when females are sexually promiscuous, male reproductive success also depends on the outcome of sperm competition with other males (Parker 1970). Sperm competition therefore generates selection on traits involved in fertilisation success (Parker and Pizzari 2010). As for precopulatory traits, selection acting on traits associated with competitive fertilization success should erode their genetic and phenotypic variability, as for precopulatory traits (Kirkpatrick and Ryan 1991). In contrast, as for precopulatory traits, genetic variability for postcopulatory traits is unexpectedly high (reviewed by Evans and Simmons 2008). Theoretically, mutation-selection balance, antagonistic pleiotropy, heterozygote advantage, frequency-dependent selection, and environmental heterogeneity in the presence of genotype-by-environment interaction (GEI) may all retain genetic variability for traits subject to the reducing effects of directional selection (Falconer and Mackay 1996, Barton and Keightley 2002, Radwan 2008, Chenoweth and McGuigan 2010). Among the mechanisms proposed, this study is mainly focused on the last of the factors listed above, GEI, and examines its potential contribution to genetic variability of sexual traits by preventing one genotype from producing the optimal phenotype across all environments (Jia 2000, Danielson-Francois et al. 2006, Lewis et al. 2012). It was not until the 1980, however, that the role of GEI in the evolutionary process was considered (Gillespie and Turelli 1989), although the notion of GEI and their potential evolutionary importance has been introduced in sexual selection studies only much later (reviewed by Hunt and Hosken 2014). In particular, the investigation on GEIs has been focused mainly on precopulatory traits, and postcopulatory traits have been little studied so far (Engqvist 2008, Morrow et al. 2008, Vermeulen et al. 2008, Evans et al. 2015). GEIs may be particular relevant to solve "the genetic variability question" since environmental variations are known to influence the expression of sexual traits (Qvarnstrom 2001). For GEI to occur it is necessary that genotypes differ in their phenotypic plasticity (Ingleby *et al*. 2010). The range of environmental conditions also determines the phenotypic response pattern and thus, the occurrence and the intensity of GEI, but only experimental manipulations that encompass the natural environmental range are evolutionarily relevant. I therefore chose to test environmental conditions that individuals potentially experience throughout their life.

Environmental variation could affect genotype fitness ranks (and hence generate GEI) through two main, not mutually exclusive and interacting mechanisms: it can determine a variation in the expression of a sexual trait that differs across genotypes. This variation in trait expression is then translated in a difference in fitness within a certain phenotype-fitness function, which is not influenced by the environment. For example, let us image to have two genotypes, A and B, which produce two phenotypes in the environment 1 (A1 and B1) and two in the environment 2 (A2 and B2), where A1>B1 and B2>A2. If the phenotype-fitness function remains the same, the genotype A will have greater fitness in the environment 1 and the genotype B in the environment 2. Alternatively, the environment may have little or no effect on trait expression (i.e. different genotypes may have the same phenotypic plasticity), but the phenotype-fitness faction varies across environment and may generate GEI even if there is no variation in phenotypic plasticity among genotypes (i.e. all genotypes have the same reaction norm). For several reasons, we can expect that sexual traits show high phenotypic plasticity (they are typically condition-dependent, e.g. David et al. 2000) and that phenotype-fitness functions vary across different environmental conditions, in particular for social conditions, because the reproductive fitness of a male is clearly influenced by his phenotypic values in relation to that of the competitors (Hunt and Hosken 2014). Furthermore, phenotype-fitness function of male sexual traits strongly depends on female sexual behaviour and environmental conditions are likely to influence both female choice and mating rate. These two behaviours are extremely sensitive to costs, possibly more than to benefits, and we can expect that both environmental and social conditions have a large influence on the costs of female choice and mating rate (which differently affect selection on pre- and postcopulatory male traits). Clearly, all these distinctions are irrelevant when the fitness of different male genotypes across environments is measured directly (although in this case the sexual trait involved in the GEI may be difficult to be detected). However, when differences in reproductive fitness are indirectly derived from differences in trait expression, it is important to bear in mind these distinctions. In particular, the variation in the costs and benefits of female mate choice and remating behaviour could in turn vary the strength and the direction of the sexual selection forces acting on male traits (Siepielski *et al.*, 2011). Some studies indicate that the genetic benefits of choosing males expressing extreme sexual traits depend on the context (David et al. 2000, Jia 2000). Thus, genetic variability may be maintained through the relaxation of sexual selection or alternatively through trade-offs between genetic variants across environments (Gillespie and Turelli 1989). The aim of my study was to investigate whether environmental variations contribute to the maintenance of the genetic variability in sperm number in the guppy (*Poecilia reticulata*). Guppies are particularly appropriate to investigate the role of environments in influencing the expression of sexual traits. Firstly, because natural habitats of the guppy vary along multiple environmental gradients which include biotic factors, such as predation regime, population density and sex ratio, and physical factors, such as food availability and turbidity (Grether et al. 2001b, Pettersson et al. 2004). Secondly, because guppies can easily be maintained in the lab and most of these factors can be manipulated experimentally. Finally, male traits involved in postcopulatory processes are particularly well known in this species (Evans and Pilastro 2011). I chose sperm number as target trait, since the number of sperm delivered during copulation is the most important predictor of postcopulatory success in this species (Boschetto et al. 2011). Although sperm competition leads to strong directional selection on this trait, sperm production shows surprisingly high sire heritability and additive genetic variance (Gasparini et al. 2013).

A more general question in sexual selection, which can be indirectly investigated when attempting to solve the paradox of the maintenance of genetic variability for sexually selected traits, is the role of sexual selection in adaptation. A number of recent models have suggested that sexual selection in response to an environmental variation can have beneficial effects on nonsexual fitness (Rundle et al. 2006). These include increasing the rate and extent of adaptation (Lorch et al. 2003) and improving the purging of genetic load (Whitlock and Agrawal 2009). In some systems, for instance, has been demonstrated that a relaxation of sexual selection at the time of environmental change, could diminish the erosion of genetic variability and thus maintain the potential for adaptation (Candolin and Heuschele 2008). Such effects can protect species or populations from extinction (Lumley et al. 2015) and can ultimately provide an advantage to sexual reproduction (Agrawal 2001). Therefore, more empirical work is needed to evaluate the role of environmental heterogeneity in sexual selection processes.

Aim of the study

The aim of my study was to investigate whether environmental variations may contribute to the maintenance of the genetic variability in sperm number, a trait under strong directional selection. This issue was addressed using three experimental approaches. The variety of approaches used allowed me to simultaneously explore others fascinating and challenging questions in evolutionary biology.

1. Genotype-by-environment interactions when ecological and social factors vary

Producing large ejaculate represents a significant cost for males (Dewsbury 1982, Hayward and Gillooly 2011). Especially in species with high degree of sperm competition there is evidence that males can deplete their sperm reserves (Shapiro et al. 1994, Birkhead and Fletcher 1995, Warner et al. 1995, Matthews et al. 1997, Olsson et al. 1997, Preston et al. 2001, Rubolini et al. 2007) and, as a consequence, males are limited not only in the number of mating they can achieve (Borgia 1979), but also in the number of females (or eggs) they can successfully inseminate. Environmental stresses, such as diet restriction (Gage and Cook 1994, Vermeulen et al. 2008, Simmons 2012, Gasparini et al. 2013, Rahman et al. 2013, O'Dea et al. 2014) and temperature fluctuations (Breckels and Neff 2013, Vasudeva et al. 2014), have a negative effect on sperm production, suggesting the non-trivial energetic investment of males in sperm production. Genetic stresses, such as those caused by inbreeding, may cause a decreased in sperm production and quality (Konior et al. 2005, Gage et al. 2006, Zajitschek et al. 2009, Gasparini et al. 2013). Taken together this evidence corroborates the idea that sperm production is costly. As a consequence of costly sperm production, pattern of resource allocation is expected to vary under different scenario depending both on environmental factors and on socio-sexual interactions (Hosken and Ward 2001, Hunt et al. 2004a, Ramm and Stockley 2009). Theory predicts that investment in one component of sexual selection (e.g. sperm investment) will come at the cost of investment in other components, such as those involved in mating acquisition, leading to a trade-off in investment towards these successive episodes of sexual selection (Parker 1998). Although trade-offs in sexual selection are widely documented (Immler et al. 2011, Dowling and Simmons 2012) in the guppy the results are controversial. In the Australian feral populations some phenotypic (see my Manuscript 5: Cattelan et al. 2016) and evolutionary (Brooks 2000, Evans 2010) trade-offs are evident in the presence of environmental variations (Evans et al. 2015). On the contrary, studies on guppies native from rivers of Trinidad have revealed positive correlation within ejaculate traits (Skinner and Watt 2006) and between ejaculate traits or sperm competition success and traits involved in mating acquisition, such as the orange coloration and the courtship rate (Matthews et al. 1997, Evans et al. 2003, Locatello et al. 2006, Pitcher et al. 2007). Moreover, previous experiments on lines of guppies artificially selected for high and low sperm production failed to find genetic trade-offs with other sexual traits (Di Nisio 2014) suggesting a positive correlation between sperm number and the overall individual's genetic quality (Rowe and Houle 1996). However, genetic trade-offs may emerge when environmental conditions change (Reznick et al. 2000). Genes that are "good" in one environment might have a weaker or even negative impact on fitness in another environment (e.g. David et al. 2000, Jia 2000). To investigate the potential role of environmental variations in maintaining genetic variability in sperm number I determined whether GEIs existed such that various environments exerted different influences on trait expression among lines of guppies that were artificially selected for high and low sperm number. There is an abundant literature documenting the role of GEIs in maintaining additive genetic variance for naturally selected traits (reviewed by Hoffmann and Merila 1999). The principles applied on natural traits are the same for sexual traits and this allowed me to investigate whether sexual traits also exhibit substantial genetic variability in plasticity. I can predict three scenarios: i) the genotype fitness ranks (measured as trait expression or as reproductive fitness) do not change across harsh and favourable environments. However, the difference in relative fitness in the two environments will shed light on the strength of selection in different environments (see figure 1a and 1b). Alternatively, ii) an ecological crossover would reveal previously hidden genetic variability in male allocation to reproduction (Van Noordwijk and Dejong 1986). It is finally possible that iii) I do not find evidence of GEIs for postcopulatory traits, because they perform relatively equally well across environments. I compared the responses of different genotypes (selected lines) of guppies to various environmental conditions expected to be in the range encountered by natural populations.

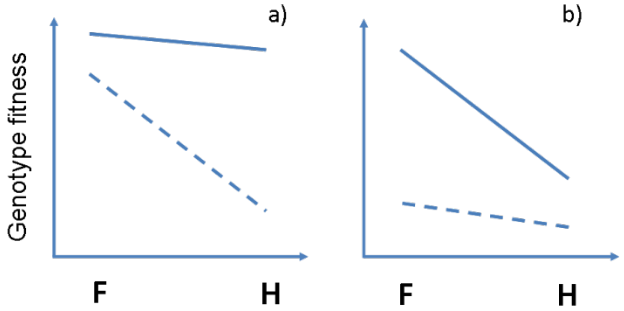


Figure 1a and 1b - A scenario in which the genotype fitness ranks do not change across environments. However, difference in genotypes' fitness is magnified either a) in harsh (H) or in b) favourable (F) conditions.

A. Firstly I determined whether (1) sperm number and other sexual traits of male guppies exhibit variation in their phenotypic plasticity in response to diet restriction both in a short and long-term scenario (**Manuscript 1**); (2) fitness-related traits (e.g. fecundity and condition) of females of the selected lines are influenced in different ways by diet restriction showing negative pleiotropy (**Manuscript 2**); and (3) effects of parental diet are different among selected lines, leading to trans-generational trade-offs (**Manuscript 2**). Overall my aim is to determine whether a variation in food availability may contribute to maintain genetic variability in sperm number because trade-offs (i.e. significant GEIs) exist between selected lines.

B. Secondly, another class of environmental factors that may cause GEIs in sexual traits is represented by social conditions. I evaluated whether (1) adaptive plasticity in "ready" sperm production in response to female presence varies between lines of guppies artificially selected for high and low sperm number (**Manuscript 3**) and, whether (2) the reproductive success of selected lines is influenced by the sex ratio (**Manuscript 4**). In particular I expected that the payoff of the cost of sperm investment and the paternity benefit should be higher when mating opportunities are high and should be lower when mating opportunities are low, potentially generating a GEI.

2. Trade-off between sperm investment and mating acquisition

Theory predicts that in polyandrous species males that invest more in traits enhancing postcopulatory success should have less energy to invest in mating acquisition, leading to trade-offs between pre- and postcopulatory episodes of sexual selection. The second question I have tried to answer is whether phenotypic trade-off between these two episodes of sexual selection. Although such trade-offs are well studied (Immler et al. 2011, Dowling and Simmons 2012), surprisingly little attention has been paid to individual-level changes in ejaculate expenditure according to variation in the costs incurred in acquiring mates (Parker and Pizzari 2010). I manipulated a component of social environment (namely, the presence of females) to experimentally elevate sperm production in males and to test whether individual-level changes in sperm expenditure cause rapid changes in male mating behaviour (**Manuscript 5**). Evidence for trade-offs among sexual traits, although only phenotypic, may shed light on how resources available for reproduction are allocated according to environmental conditions encountered by males (Van Noordwijk and Dejong 1986).

3. The strength of pre- and postcopulatory sexual selection when environment changes

Since the costs and the benefits arising from sexual selection could change with respect to environmental condition, it is necessary to incorporate ecological factors into sexual selection dynamics (Janicke et al. 2015). The last experiment I carried out consisted in a diet manipulation of two groups of replicated populations of six guppy males and females (**Manuscript 6**). Aim of this experiment was to investigate simultaneously the effect of food availability on male sexual traits and female mating behaviour and their interaction (Reznick et al. 2000). One of the most prominent ecological factors governing reproductive performance of males and females is food availability (Miller and Svensson

2014). Food availability are expected to modify the expression of male sexual traits (see for instance **Manuscript 1**) but it is also likely to affect the costs and benefits of female mate choice and remating behaviour. This, in turn, might affect the strength of the sexual selection forces acting on male traits. Experimental investigations of the interplay between food availability and strength of sexual selection are still scarce (e.g. Janicke et al. 2015) but several theoretical scenarios have been proposed (Candolin and Heuschele 2008). First, food availability typically affects phenotypic expression of condition-dependent traits in males (David et al. 2000, Jia 2000). For example, unfavourable environmental conditions may affect the effort in mating acquisition (Rosenthal and Hebets 2015) and this, in turn, may decrease the mating rate, resulting in different levels of polyandry (Gillespie et al. 2014, Janicke et al. 2015). Second, food availability may directly influence female mating and reproductive rate and affect their choosiness (Robinson et al. 2012, Judge et al. 2014) (Syriatowicz 2004). Finally, food availability is expected to affect female fecundity and therefore indirectly affect male success after the mating event (Rundle et al. 2006). Environmental variations influencing the strength of sexual selection are, intuitively, an appealing explanation for the maintenance of genetic variability in sexual traits, but they have received surprisingly little empirical attention. At present, the role of fluctuating selection in maintaining genetic variability is yet under debate (Hoffmann and Merila 1999, Robinson et al. 2008).

Study species

The guppy

The species object of my study is the guppy, *Poecilia reticulata*, a livebearing freshwater fish native to Central America (Houde 1997). In the last two decades *P. reticulata* has become a model species for studies in pre- and postcopulatory sexual selection (Magurran 2005, Evans and Pilastro 2011). Males and females are promiscuous and males contribute to reproduction only with sperm. Guppies show an elevated degree of sexual dimorphism (see Figure 2): females have mimetic coloration, whereas, males exhibit a polymorphic colour pattern, inherited via Y-chromosome (Houde 1992), that has a fundamental role in female choice (Pilastro et al. 2004). This species is an internal fertilizer and males have a modified anal fin, named gonopodium, used to transfer sperm during mating. Males attempt to mate by courting females (via sigmoid displays) or adopting a coercive mating tactic (gonopodial thrust) (Liley 1966). These tactics are interchangeable according to prevailing social conditions (Jirotkul 1999, Magellan and Magurran 2007) and male condition, since gonopodial thrusts appear to be less energetically demanding than courtship displays (Devigili et al. 2012, Rahman et al. 2013). From their part, females prefer to mate with males with high rates of courtship (Kodric-Brown and Nicoletto 2001) and actively avoid forced mating attempts (Houde 1997). Females are able to modulate mating duration and, as a consequence, the quantity of sperm transferred (Pilastro et al. 2007). This mechanism, termed cryptic female choice, explains why colourful males inseminate more sperm (Pilastro et al. 2004) and why gonopodial thrusts result in lower insemination efficiency than solicited copulations (Pilastro and Bisazza 1999). Moreover, female guppies are highly polyandrous (Neff et al. 2008), and among the traits contributing to male fertilisation success, the number of sperm inseminated has been identified as the most important predictor of paternity when two males mate with a female (Boschetto et al. 2011). Sperm are transferred in bundles, containing about 21000 sperm cells each bundle. Once in the female gonoduct, sperm cells are released from the bundles and stored in the ovary, where they are maintained for several months. Thus, females can produce several successive broods using the stored sperm (Winge 1937), often beyond a male's lifetime (Lopez-Sepulcre et al. 2013). Sperm production depends on resource acquisition and it is more affected by condition than other sperm quality traits such as sperm size and velocity (Devigili et al. 2012, Gasparini et al. 2013, Rahman et al. 2013, Rahman et al. 2014). Sperm number shows large phenotypic variability which is largely genetically additive (Gasparini et al. 2013). In the guppy population used in this study, from the lower part of the river Tacarigua in Trinidad (with the exception for Manuscript 5), phenotypically attractive males produce more sperm faster (Matthews et al. 1997, Pitcher and Evans 2001) and more viable sperm (Locatello et al. 2006). Attractive males inseminate more sperm than their less attractive counterparts (Pilastro et al. 2002) and, as a result, have higher success in sperm competition both after natural copulation (Evans and Magurran 2001) and after artificial insemination (Evans et al. 2003). Thus, precopulatory sexual selection seems to be reinforced also during postcopulatory episode, as demonstrated by the large covariance between pre- and postcopulatory success observed in this species (Devigili et al. 2015b).

Figure 2 - Two males (right and left) and a female (central). Body size and colour pattern sexual dimorphism is evident. Modified anal fin (gonopodium) is also visible. In this species the level of sexual harassment is high: females receive, on average, one coercive mating attempt per minute along the entire lifetime. Photo: Alessandro Devigili.

Environmental heterogeneity in the guppy habitats

The guppy system is a suitable model for studying the effect of the environmental heterogeneity on sexual selection processes. The major environmental variations experienced by guppies in Trinidad involve both abiotic and biotic factors such as: predation intensity, food availability, light characteristics, social environment and water fluctuations. Among these factors, my research is focused on food availability and social environment.

1. Food availability

Food availability is usually an important driver of variability in sexual traits because of its direct effects on energetically demanding traits. For guppies, the primary food source is algae and small invertebrates (Dussault and Kramer 1981). These two main categories represent also the primary source of carotenoid pigments, such that variation in resource availability also affects coloration and individuals' condition. Seasonal flooding may profoundly change streams architecture in Trinidad, resulting in a reduction for instance of algae available for guppies foraging (Grether et al. 2001a). The same authors demonstrated that more open-canopied streams receive greater levels of photosynthetically active radiation than closed-canopy streams within the same drainage system (Grether et al. 2001b). This difference results into greater algae availability per individual, such that guppies grow faster and males reach larger asymptotic sizes in the more open-canopied sites. Moreover there is also a strong association between predation regime and diet quality. Indeed guppies from high predation regime (the population used in this study) feed primarily on invertebrates (Zandona et al. 2011). Food is expected to be the primary limiting resource affecting the expression of sexual traits, which are dependent on individual condition. Hughes and colleagues addressed whether GEI maintains variation in guppy populations, focusing on the effect of food availability on body size and coloration (Hughes et al. 2005). They found evidence for GEI for body size across populations such that variation could be maintained with gene flow among populations. However, no GEI for coloration was detected and this study was also confirmed by another study (Miller and Brooks 2005). Lack of GEI for coloration may be characteristic of some guppy populations but not of others, as in an Australian feral population significant GEIs for male colour (and other sexual traits) in response to diet restriction has been demonstrated (Evans et al. 2015).

2. Social environment

Individual guppies appear to infrequently long-distance movements within a stream, as revealed by mark-recapture studies, probably because of geographic barriers such as waterfalls (Crispo et al. 2006). In the longer term, however, there is evidence that gene flow occurs and includes movement among predation risk gradients (Becher and

Magurran 2000). Movements caused by flooding are likely to carry males from a pool to another but, movements may also be active (Schwartz and Hendry 2010) and they are in general biased towards males (Croft *et al*. 2003). Adult sex ratio shows large variation among populations, from strong male-biased (0.9) to female-biased (0.15) (Pettersson et al. 2004). Several environmental factors also influence sex ratio in the guppy (McKellar et al. 2009). Variations in the sex ratio may affect sexual interactions among individuals within the same pool and among different pools. Sex ratio affects male mating behaviour and male aggressiveness (Jirotkul 1999, Magellan and Magurran 2007). Second, female mating behaviour is likely to be influenced by the sex ratio (Jirotkul 1999, but see Head et al. 2008). All these factors together occur to generate a complex scenario in which sexual and social interactions change over time and space suggesting a great potential for social environment to generate GEIs (Hunt and Hosken 2014). However, the only one study that has directly tested whether sex ratio is associated with GEI in sexual traits did not find any significant effect (Miller and Brooks 2005). Considering the importance of social interactions in driving the evolution of sexual traits, more studies are required to exclude that variation in the social conditions has minor or no effect on the maintenance of genetic variability in sexual traits.

Figure 3 – The natural habitat of guppies is characterized by waterfalls and pools. Here the low part of the Turure river in Trinidad. Photo: Silvia Cattelan

Summary of the results

Manuscript 1 and 2

Among the infinite environmental factors that could generate a GEI, variations in food availability seem to be one of the most relevant (Jia 2000, Engqvist 2008, Vermeulen et al. 2008). In natural population guppies live in a highly dynamic environment in which food availability varies over time both in quality and quantity (Reznick 1989). Indeed, periodic disturbances, such as floods or forest canopy cover, can decrease resource availability in guppy population (but see 'Study species' for more details). The experimental approach consisted in a diet manipulation (*ad libitum* and restricted diet) on the two selected lines for sperm production (High and Low) in order to evaluate the effect of food availability on the performances of males and females from the two lines. Experimental males (n=164, n=82 per selection line) and females (n=158, n=78 from High line and n=80 from Low line) were subjected to poor or rich feeding conditions in a split-family design: full-sibs individuals were assigned at random either to the *ad libitum* food regime or to the restricted food regime. *Ad libitum* diet consisted of ca. 140 fresh *Artemia salina* nauplii per fish twice daily, 6 days per week. Restricted diet consisted of ca. 40 nauplii per fish twice daily, 6 days per week, following established protocols (Devigili et al. 2012, Gasparini et al. 2013). Diet manipulation started when fish were 2 months old and lasted for two months. During this period, the amount of food in the restricted treatment was increased of 10% per week (Kolluru and Grether 2004). From the end of this period onwards, all fish were fed *ad libitum*. The effect of food availability on sexual traits and overall body condition (measured as performance in a evasion test from a simulated predator) was assessed in the males at the end of the treatment and repeated at one year of age. I have also analyzed the effect of diet in the two selection lines on female fecundity and on offspring quality, as trans-generational effects of parental diet on offspring traits have been shown in several taxa (Hunt 2000, Bonduriansky and Head 2007, Frost et al. 2010, Valtonen et al. 2012).

Manuscript 1

Several sexual traits other than sperm number differed significantly between the High and Low males, confirming previous results on the same artificially selected lines (Di Nisio 2014). High males had larger body size and body mass, higher sexual activity and they were better able to escape from a simulated predator. As expected, High males produced more sperm (the trait for which they were selected), but also sperm with Higher swimming velocity. To evaluate the occurrence of GEIs, I compared the relative expression of sexual traits between High and Low males by performing a Monte Carlo simulation in which the ratio between High and Low for each trait was bootstrapped 10,000 times (a ratio significantly $\neq 0$ indicates that one genotype performs relatively better than the other in one environment). Diet manipulations did not modify the rank order of the genotypes: High males had generally better performances (even if not significantly so for some of the traits) both in good and in harsh environmental conditions. When the same measures were taken at one year, the pattern remained substantially unmodified except for sexual activity for which I found evidence (although not significant) of faster senescence in High males. These results suggest that sperm production correlates with male overall genetic quality and that High sperm males are better able to cope with unfavourable conditions despite their higher investment in sperm production.

Manuscript 2

Males and females from the same artificial selection line and diet regime were randomly paired and were allowed to mate (n=34 pairs High *ad libitum*; n=23 High restricted pairs; n=30 pairs Low *ad libitum*; n=24 pairs Low restricted). After mating, females were isolated and the number of offspring delivered was recorded. Food limitation affected significantly females' fecundity but female performances from the two lines did not differ either within or between diet regimes. To investigate the occurrence of GEIs in a trans-generational context I have estimated offspring quality when they were five months old. Parents raised on poor food condition produced an increased proportion of males than females (logistic regression: $F_{1,98}=4.065$, $p=0.044$), in line with the hypothesis that females should produce more offspring of the sex with the higher survival and reproductive prospects (Nager et al. 1999, Rosenfeld and Roberts 2004). No sex-biased mortality occurred after parturition and the observed sex ratio bias has therefore occurred at fertilization or during gestation. Surprisingly, parents raised on poor food condition produced larger daughters (GLM: $F_{1,82}=4.711$, $p=0.033$) (Bashey 2006). Finally, no evidence for trans-generational effects of parental diet on sexual traits of offspring was detected. Taken together, my results indicate that selection for sperm production was not associated with a fecundity cost for females (in term of both number and quality), irrespective of food availability.

In conclusion I evaluated the occurrence of GEIs both in a short-term and a long-term scenario. As expected, food limitation affected the overall expression of male sexual traits and this effect was maintained for some traits also in a long-term scenario. As predicted by "good genes" models of sexual selection, I failed to find evidence of GEIs in sperm number and other sexual traits: the genotype ranks were maintained across environments. High males are better able to cope with unfavourable conditions despite their higher investment in sperm production. Variation in food availability does not seem contribute to the maintenance of the genetic variability for sperm number since sperm production correlates with male overall genetic quality also when diet is manipulated.

Manuscript 3

Costs associated with sperm production have been demonstrated in a number of species and come from both direct and indirect evidence (e.g. inbreeding depression, diet restriction, temperature fluctuations, sperm depletion). As a consequence, the pattern of resource allocation to ejaculate traits and the allocation of ejaculate itself across different mating episodes are expected to vary under different scenario (Hunt et al. 2004a). Indeed, males are able to allocate strategically their sperm accordingly to mating opportunities to maximise their reproductive success (Parker and Pizzari 2010, Kelly and Jennions 2011). An example of plastic sperm allocation is provided by sperm priming, a plastic and a rapid adjustment of the amount of "ready" sperm in response to social stimuli, often a female (Bozynski and Liley 2003). Sperm priming allows males to economize the investment in sperm production when mating opportunities are low or absent. Here, I explored if sperm priming varies between sperm selection lines. Considering that a high investment in sperm is expected to be costly, a stronger sperm priming effect should be observed in males with high sperm investment. To test this hypothesis I used males artificially selected for high and low sperm number in a paired experimental design.

Following 3 days of isolation from females I estimated the baseline of the number of sperm produced by High (n=32) and Low (n=38) males. After this first sperm estimation, each male was randomly allocated to these two treatments for 7 days: female-present or isolation. I used a paired experimental design in which each male experienced both treatments in a haphazard order. Males selected for high sperm production did not change their sperm production over the three sperm estimations (repeated measures ANOVA: $F_{1,30}=0.114$, $p=0.893$). On the contrary, males selected for low sperm production significantly changed their sperm production over time (repeated measures ANOVA: $F_{1,36}=12.010$, $p<0.001$). In particular, when Low males were in the presence of a female produced significantly more sperm than when female stimulus was absent. Despite this increase, their number of sperm was still significantly lower than those produced by High males in the same condition (t-test: $t_{1,68}=5.893$, $p<0.001$). However, the interaction between selection line and treatments (female presence and no female) was not significant (repeated measures ANOVA: $F_{1,68}=1.294$, $p=0.259$). Although the differences within selection line between treatments did not differ, we can exclude the High males pay a higher cost in the number of sperm produced when females are absent. Low showed a greater flexibility in sperm priming than High males. Low males may be able to better respond to an increased level of mating opportunities by reducing their initial disadvantage at the baseline sperm production. It remains unclear, however, why High males apparently lack the capability to adaptively adjust their sperm production in response to varying mating opportunities.

Manuscript 4

Among the possible aspects of the social environment to be considered, I have chosen to manipulate the sex ratio, as this may obviously affect both male-male competition and female mate choice. Indeed, a variation in the adult sex ratio will vary mating opportunities, which are known to influences pattern of pre- and postcopulatory episodes (Edward et al. 2011). *Narrow-sense* sex ratio is expressed as the number of sexually active males to the total number of receptive females and sexually active males (Kvarnemo and Ahnesjö 1996). In this study I tested if variation in OSR might explain part of the existing genetic variability in sperm number in the guppy. To test this hypothesis I compared the fitness response of the genotypes expressing high and low sperm number to two levels of mating opportunities (throughout OSRs variation). Considering the importance of sperm number in competitive fertilization success (Parker and Pizzari 2010), I expected that genotypes expressing the highest number of sperm fertilize a greater proportion of eggs when the risk of sperm depletion is higher (female-biased OSR). In contrast, the importance of sperm number should be lower as the risk of sperm depletion decreases (*equilibrium* OSR). If the difference in fitness between genotypes significantly varies among OSRs, GEI may contribute maintaining genetic variation for sperm number in this species.

Males from the two selection lines were randomly paired (N=21 pairs). Each pair's reproductive success was consecutively estimated at two different OSRs (paired experimental design): female-biased OSR (one week with 6 virgin females; OSR=0.25) and *equilibrium* OSR (one week with 2 virgin females; OSR=0.5). The two OSRs used were within the range of variation found in natural guppies population (Pettersson et al. 2004). At the end of the mating period females were isolated until parturition and tissue samples from adults and offspring were collected. DNA was extracted from a tail fin clip using Salting Out procedure for the adults (145 females and 42 males) (Miller et al. 1988), and by CHELEX extraction for the offspring (n=1058) (Walsh et al. 1991). We used two hypervariable microsatellite loci to assign paternity within each pair of males, following an established protocol (Devigili et al. 2015b). Fragments lengths were determined using Peak Scanner software and paternity was assigned using CERVUS 3.0. Paternity was assigned with 95% confidence in 1047 genotyped offspring (99%).

As expected, males from the two lines of selection significantly differ in their number of sperm and they did not differ for other three fitness-related traits. To test for difference in reproductive success (RS) between selection lines, I considered the proportion of offspring sired by each High male on the total offspring per pair/treatment. I performed a generalized linear mixed model (GLMM) with each pair of males as random factor and OSR treatment as fixed factor. There was a significant effect of OSR treatment on High males RS (GLMM: $F_{1,40}$=25.197, p<0.001). As expected, High males performed better in the female-biased treatment. However, contrary to prediction, Low males sired significantly more offspring than their High counterparts at the *equilibrium* sex ratio.

This result suggests that the social context has an important effect on the interaction between sperm number and RS and may contribute explaining the large genetic variation for sperm number observed in this fish population. The specific mechanism responsible for the higher RS of Low males in a balanced sex ratio remains presently unknown, and will require further investigation.

Manuscript 5

Theory predicts that in polyandrous species males that invest more in traits enhancing postcopulatory success should have less energy to invest in mating acquisition, leading to trade-offs between pre- and postcopulatory episodes of sexual selection. Although such trade-offs are well studied (Immler et al. 2011, Dowling and Simmons 2012), there has been surprisingly little attention paid to individual-level changes in ejaculate expenditure according to variation in the costs incurred in acquiring mates (Parker and Pizzari 2010), and this is especially so in the case of alternative mating tactics (ARTs). Male guppies can obtain matings by performing energetically costly courtship (via sigmoid displays) or by using less costly forced copulations (gonopodial thrusts). Moreover males are capable of rapid adjustment in ejaculate investment according to the presence or absence of females (Bozynski and Liley 2003). This plasticity in both mating behaviour and ejaculate traits, coupled with the demonstrated high costs of ejaculate production, provided the impetus for investigating trade-offs between these two components of pre- and postcopulatory sexual selection.

In this experiment I manipulated a component of social environment (namely, the presence of females) to experimentally elevate sperm production in males. Adult male guppies (*N*=35) were then randomly allocated to one of the following two treatments for seven days: (1) female-presence treatment and (2) no-female treatment. The timeframe we chose (seven days) is likely to be ecologically relevant in natural populations. To account for inter-individual variation in sperm production and sexual behaviour we used a paired experimental design in which each male experienced both treatments in a haphazard order (second treatment started immediately following the first). As expected, sperm production was significantly affected by treatment (treatment, LMM: $\chi^2_{1,66}$=5.286, *P*=0.022, *N*=70). Indeed, when males were assigned to the female-presence treatment produced significantly more sperm than when they were tested in the no-female treatment. I found that males performed significantly fewer sigmoid displays but more gonopodial thrusts in the female-present treatment compared to when they were tested in the no-female treatment (GLMM: $\chi^2_{1,67}$ =14.11, *P*<0.001, *N*=70). Moreover, the difference in sperm production between treatments was negatively correlated with the strength of corresponding change in courtship behaviour (Pearson's correlation: r_{30}=-0.369, *P*=0.043). In short, the larger the increase in sperm production, the more pronounced is the switch from courtship to coercive mating. My study reveals that a change in sperm investment causes a shift in the use of alternative tactics. Male guppies rapidly compensate by reducing their reliance on courtship in favour of forced matings, consistent with a trade-off between pre- and postcopulatory episodes of sexual selection.

Manuscript 6

The costs and the benefits arising from sexual selection could change with respect to environmental variations. Over the last decade there has been growing evidence that the strength of sexual selection varies in space and time, which highlights the need for incorporating ecological factors into sexual selection dynamics (Cornwallis and Uller 2010, Miller and Svensson 2014). First, ecological factors, such as for example food availability, may influence the strength of sexual selection, because environmental conditions typically affect the phenotypic expression of condition-dependent traits (David et al. 2000, Jia 2000). Second, food availability may differently influence the potential reproductive rate of males and females. For example, unfavourable environmental conditions may affect both the mean mating effort and the variance in mating effort (Rosenthal and Hebets 2015). In turn, females may decrease their mating rate, if polyandry is costly, and their choosiness, if mate evaluation is costly. Although the importance of environment-dependent effects of sexual selection is recognised, experimental studies are yet scarce (Janicke et al. 2015). One of the most prominent ecological factors governing reproductive performance of males and females is food availability (Miller and Svensson 2014). Although multiple indirect evidence suggest that food availability might have a profound effect on the strength of both pre- and postcopulatory sexual selection, experimental demonstrations still lack (Gillespie et al. 2014, Janicke et al. 2015). Here, sexual selection was decomposed in each part: expression of costly traits in response to environmental fluctuations; precopulatory sexual selection (both in the light of female mate choice and male sexual behaviour) and ultimately postcopulatory sexual selection. Variance in male reproductive success was partitioned for both diet treatments, in order to infer which selection episodes (i.e. pre- and postcopulatory sexual selection) were mainly affected by the food availability.

To do this, I established 20 replicated populations of 6 virgin females and 6 males. Each population was exposed to an *ad libitum* diet or a restricted diet for two weeks. At the end of the diet manipulation, females and males were allowed to freely interact in 90-min mating sessions over 5 consecutive days. During the mating sessions, sexual behaviour and mating success of males, and sexual responsiveness and choosiness of females, were assessed. After the last mating session the females were isolated until parturition. The 85% of the females fed *ad libitum* produced a litter, compared to the 65% of the food-restricted females. We genotyped the females that mated with more than one male (n=58) and their offspring (n=573) and all the possible fathers (n=116). Paternity was assigned with 95% confidence in the 89% of the genotyped offspring. As expected, food-restricted males had a reduced body size, a reduced area of body covered by orange spots (important pre-copulatory trait) and they produced significantly less sperm than their *ad libitum* counterparts. Moreover, during the interaction period with females, food restricted males showed lower courtship behaviour and a reduction in the number of sneaky attempts. On the other hand, females from the restricted treatment had a reduced body size and a reduced reproductive output, suggesting that reproductive resources were successfully manipulated. Food restricted individuals mated on average less frequently and with a lower number of partners than *ad libitum* individuals. Although the mating rate was reduced, I found a higher variance in mating success meaning that females preferentially mate with some specific males. I also found a higher variance in male reproductive success of food-restricted individuals, although not significantly different from that of the *ad libitum* individuals. My variance-partition analysis revealed that variation in sperm competition success was the largest source of variation in male reproductive success, but its relative importance is greater in the *ad libitum* treatment (39% of the variance in reproductive success) compared to that in the restricted treatment (34%). Interestingly the relative contribution of variation in mating success in determing reproductive success is significantly higher in the restricted treatment (32%) compared to that in the *ad libitum* treatment (13%). Thus, food restriction increases the variance in mating success, suggesting stronger precopulatory selection under restricted food conditions, is in line with the idea that environmental stress should promote stronger sexual selection (David et al. 2000). This study provides experimental evidence for environment-dependent sexual selection. Overall, these results indicate that the strength of sexual selection is increased in a harsh environment and, to the extent to which sexual selection has a beneficial effect on female fitness (e.g. through a good genes process), sexual selection may contribute accelerating adaptation when populations face new environments in this species. This may have important consequences on the evolution and on the maintenance of sexual traits since sexual selection could facilitate the fixation of some beneficial alleles under the new conditions (Candolin and Heuschele 2008).

Discussion

The aim of this study was to investigate whether environmental variations, both at the ecological and at the social level, may contribute to the maintenance of the genetic variability in sperm number, a trait with large additive genetic variability despite the strong directional selection (Boschetto et al. 2011, Gasparini et al. 2013). To this aim, I evaluated the impact of environmental variations on males artificially selected for high and low sperm production. Previous experiments on the same selection lines failed to evidence genetic trade-offs with other sexual traits (Di Nisio 2014), suggesting a positive correlation between sperm number and male overall genetic quality (Rowe and Houle 1996). However, genetic trade-offs may emerge when environmental conditions change (Reznick et al. 2000), thus genes that are “good” in one environment might have a weaker or even negative impact on relative fitness in another environment (e.g. David et al. 2000, Jia 2000). I therefore estimated GEIs in sperm number expression (and other fitness-related traits) among the selection lines at varying environmental conditions.

My first experiment demonstrated that food restriction negatively affects the expression of sexual traits but the phenotypic plasticity in sperm number and other fitness-related sexual and non-sexual traits did not vary between selection lines (**Manuscript 1**). High males show a relatively higher trait expression for sperm number and other sexual traits also under food restriction, as compared to their Low counterparts. Similarly, condition (capability to escape a simulated predator) and female fecundity were equally affected by diet restriction in the two selection lines. This allows me to exclude a role of GEI in the expression of sexual traits (and other fitness related traits possibly associated through negative pleiotropy with sexual traits). Finally effects of parental diet were not significant different among offspring of males and females from the selection lines. Thus I am able to exclude also the occurrence of GEI in a trans-generational context (**Manuscript 2**). My results are largely in agreement with previous experiments conducted on these selection lines. As predicted by “good genes” models of sexual selection: the genotype fitness ranks (measured as trait expression) were maintained across environments. High males are better able to cope with unfavourable conditions despite their higher investment in sperm production than Low males. Variation in food availability does not seem to contribute to the maintenance of the genetic variability for sperm number as far as this can be assessed on the sole basis of the phenotypic plasticity.

Although genetic variability in sperm production is unlikely to be maintained through trade-offs between genetic variants across food availability gradients, other environmental factors have to be considered. Social environments appear to be especially relevant in generating GEIs for reproductive fitness. I found that Low males significantly increased their sperm production when they were allowed to see females (perceived mating opportunities), whereas High males maintained their sperm production more constant (**Manuscript 3**). However, the difference among selected lines was not enough large to generate a GEI for plasticity in sperm production. Moreover, variation in the social environment has the potential to have strong and complex effects on the intensity of sexual selection, since social interactions are intrinsic to sexual selection through competition for mates, mate choice and mating itself. Indeed, manipulating the number of females I showed a significant interaction between sperm production genotype, male mating opportunities and male reproductive fitness (**Manuscript 4**). In particular, Low males sired a significantly greater proportion of offspring than High males when mating opportunities were low (*equilibrium* sex ratio). On the contrary, when the sex ratio was female biased the reproductive success of males from the selection lines did not significantly differ. This GEI is potentially crucial in the maintenance of genetic variability for sperm number, considering the strong temporal and spatial fluctuations in the sex ratio that male guppies commonly experience. Importantly, this result is based on a direct measure of male reproductive fitness, and not, as usually occurs, on the assumptions that a GEI in sexual traits expression will result into a GEI in fitness. Although the mechanism responsible for the higher reproductive success of Low males is unknown, it seems reasonable that it involves genetic trade-offs with traits associated with mate acquisition. This study emphasizes the importance of taking social environment into account in studies of sexual selection, and the paucity of research on this subject indicates that there is clearly scope for future research in this area. Furthermore, I have shown that it is not necessary for environments to be particularly harsh or stressful in order to identify significant GEIs (Hoffmann and Merilä 1999). All the significant effects I found were identified within the range of environmental variations normally experienced by *P. reticulata* in natural conditions. The importance of male-female interactions in generating a trade-off between pre- and postcopulatory episodes was also demonstrated in a short-term scenario (**Manuscript 5**). Experimental manipulation of the perceived male mating opportunities was used to investigate the investment in mating acquisition associated with an increased sperm investment. As predicted by theory, individual-level increase in sperm investment caused a rapid change in the investment in mating acquisition, with a shift from costly courtship displays to less costly coercive mating tactics. Further, although I failed to find evidence of significant genetic variation for phenotypic plasticity in male sexual traits and female fecundity in response to food availability, the results of my final experiment indicate that diet may significantly alter male-female interactions. These, in turn, may affect the strength of sexual selection on male traits associated with mate acquisition and competitive fertilization success (**Manuscript 6**). Aim of this experiment was to estimate the relative importance of pre- and postcopulatory success in groups of males and females whose condition was experimentally manipulated in a food restriction experiment. This experimental setting allowed to simultaneously test the effect of temporary food restriction on male sexual traits and female mating strategy, and their interaction. My results showed that, while the overall strength of sexual selection was not affected by condition, the relative importance of precopulatory selection increases under temporary food restriction, ultimately reducing the importance of postcopulatory episode. Recently it has been suggested that it is unlikely that a single phenotype would achieve the maximum of the reproductive success in this species (Devigili et al. 2015b). Here we provide evidence that relatively small changes in resources availability (two weeks of reduction in food availability) may increase the strength of the selection on pre- or postcopulatory traits. These results suggest that the strength of sexual selection might fluctuate substantially in nature at a fine temporal scale, providing an explanation for the large genetic variability in sperm number.

Conclusions

This research was carried out with a particular interest in the role of the environment in the maintenance of genetic variability in traits under directional sexual selection. The role of GEI in sexual selection has been the subject of an increasing research interest in recent years. My first four manuscripts fits in the rapidly growing literature on this subject and concur in suggesting that the variation in food availability (which represent a proxy for favourable vs. unfavourable environmental conditions) does not reveal large GEIs when variation in trait expression is considered. In other words, the genetic variation in the reaction norm is usually small, and rarely significant. This conclusion held when a large number of sexual traits and trans-generational effects were considered. In contrast, the effect of the variation in social conditions are more likely to expose large GEIs in sexual traits, either through their competing effects on the plastic allocation between reproductive investment and maintenance, and among pre- and postcopulatory traits, or through a change in the fitness function across environments. This latter effect was particularly evident from the results of my last experiment, which demonstrated that selection on pre- and postcopulatory traits show a significant variation in response to food availability. This was largely due to the effect of condition on female mating behaviour, which affected the variance in male mating success and the opportunities for postcopulatory sexual selection. From a strictly methodological point of view, my results highlight the importance of i) including the effect of the environment on female sexual traits and their interaction with male traits, and ii) to measure the reproductive fitness consequences of the environmental conditions, rather than limiting the analyses on sexual traits phenotypic expression.

My results challenge the classical view of sexual selection as a static force. Together, the six manuscripts I present here emphasize the importance of taking into account environmental variations in sexual selection studies, in particular for those aimed at understanding the maintenance of genetic variability for sexually selected traits. There are several potentially useful directions for future research and some empirical challenges to overcome. I think that future research should be based on measures of reproductive fitness, rather than on sexual trait expression. This implies manipulating the environmental conditions in both sexes and not only in males. Furthermore, the recognition that male-female interactions play a crucial role in determining GEIs, requires to establish laboratory experiments that mimic natural conditions both for the environmental factor manipulated, and for the socio-sexual context. Clearly, research into the role of environment in sexual selection have useful implications for conservation biology; for instance, future works could investigate how breeding plans or reintroduction projects should take into consideration sexual selection across environments.

Manuscripts

Manuscript 1

The interaction between diet and sperm production genotype in shaping the expression of male sexual traits in the guppy

Silvia Cattelan and Andrea Pilastro
Department of Biology, University of Padova, 35131, Padova, Italy

Abstract

Under directional sexual selection, the genetic variability underlying the preferred trait is expected to be rapidly depleted. In contrast, sexual traits usually show higher genetic variability than most ordinary traits. Among the several mechanisms that have been proposed to explain this paradoxical observation, it has been suggested that genotype-specific, environmental-dependent expression (the so-called genotype by environmental interaction, GEI) may play a major role in maintaining the genetic variability in sexual traits. Guppies (*Poecilia reticulata*) are characterized by a resource-free mating system with high levels of polyandry. Sperm number is subject to strong directional selection in this species, yet it shows very high levels of genetic variability. We explored the effect of food availability on sperm production and other sexual traits in male guppies that were artificially selected for high and low sperm production. As expected, food limitation affected the overall expression of sexual traits. However, in contrast with predictions, we failed to evidence GEIs in sperm number and in other sexual traits. These results suggest that sperm production correlates with male overall genetic quality since high sperm males are better able to cope with unfavourable conditions despite their higher investment in a costly trait.

Introduction

The maintenance of additive genetic variability in sexual traits subjected to strong directional selection represents a central question in evolutionary biology (Barton and Turelli 1989). However this problem has been typically investigated in a precopulatory context (Prokuda and Roff 2014, Radwan et al. 2015). However, sperm competition, the competition between sperm from two or more males for the fertilization of a given set of ova, has been recognized as an important evolutionary force causing strong selection on many male reproductive traits (Simmons 2001). In many polyandrous mating systems, male reproductive success will be determined by males' success in the subsequent competition between sperm for fertilizations (Birkhead and Moller 1998). Among ejaculate traits, sperm number has been widely recognized as the most important trait affecting the outcome of sperm competition (Parker and Pizzari 2010). Under strong directional selection phenotypic and genetic variability for traits conferring strong reproductive advantage is expected to be rapidly depleted: genes providing high expression for those traits are expected to be fixed in populations due to the strong selection (Kirkpatrick and Ryan 1991). Contrary to this prediction, ejaculate traits usually show high genetic variability, even higher than no-sexually selected traits (reviewed by Evans & Simmons, 2008). The process by which this genetic variability is maintained remains largely unresolved, although several mechanisms have been proposed to explain this paradox (Radwan 2008). In search for an answer, much empirical work has focused on assessing the relationship between male sexual traits and heritable condition, because this correlation lies at the heart of all good genes models. Since, producing large ejaculate represents a significant cost for males (Dewsbury 1982, Hayward and Gillooly 2011), they will therefore reflect the overall condition of the individual (i.e. the heritable component of the resources available to an individual for reproduction (Rowe and Houle 1996)) meaning that they have evolved condition dependence. Condition dependence is expected to arise when only the males in good condition can afford the cost of expressing such exaggerated trait (Zahavi 1975, Hunt et al. 2004b). However condition is also likely to be strongly influenced by environmental effects (Hill 2011) and condition-dependence theory (Andersson 1986, Houle 1991, Rowe and Houle 1996) is widely interpreted as predicting that the effects of environmental and genetic quality on trait expression should have the similar direction (i.e. aligned) because both are mediated through condition. An adverse environment (e.g. nutritional stress) is recognised to depress condition and thereby reduce the expression of condition-dependent traits (Bonduriansky et al. 2015). However, the strength and the sign of the correlation between sexual traits and condition might change when environmental variation occurs in the population (Qvarnstrom 2001). Genetic trade-offs may emerge when environmental conditions change (Reznick et al. 2000) since genes that are “good” to possess in one environment might have a weaker or even negative impact on relative fitness in another environment (e.g. David et al. 2000, Jia 2000).

Environmental variations may play a crucial role in preventing one genotype from producing the optimal phenotype across all the possible environments (the so-called genotype by environment interaction-GEI). For instance, in the presence of GEI some genotypes are better able to maintain optimal phenotypic expression under relatively poor conditions than others (Hunt et al. 2004b). Thus, the existing genetic variability for traits subjected to directional selection may be explained whether genetic variability exists for plasticity (Gillespie and Turelli 1989, Kokko and Heubel 2008). GEIs, largely studied for naturally selected traits, are likely to be especially relevant in the context of sexual selection, where there is increasing recognition that individual plasticity in sexual trait expression can have a genetic basis (Evans et al. 2015). The evidence that ejaculate traits, including sperm number, have environmental-dependent expression (Gage and Cook 1994, Blount et al. 2001, Morrow et al. 2008, Lewis 2012, Rahman et al. 2013) suggest that GEIs may occur through environmental variations on multiple gradients.

Environmental variations may influence both the extent of the phenotypic variability and the rank order of genotypes' fitness (Ingleby et al. 2010). The occurrence and the relative strength of a GEI will be depended on the nature of the genetic variability underlying the trait(s) considered. Change in the scale of variability corresponds to a reaction norm for relative fitness that may not modify the rank order of the genotypes, then some genotypes are always better and some always worse at producing condition-dependent traits across the studied environments (Via and Lande 1985). This scenario is in line with the classical view of "good genes" models, but the difference in relative fitness of genotypes across environments may reveal the strength of selection on those genes in different environments. Selection on those genes may be relaxed or enhanced under the new environmental conditions. If, however, environmental variation affects the sign of genes' effect, "good genes" in one environment could be convert into a "bad genes" in another environment (Kondrashov and Houle 1994). This causes genotype reaction norms to cross, a situation where there is no single best genotype (Qvarnstrom 1999, Jia 2000, Danielson-Francois et al. 2006). This scenario suggests that the classical view of "good genes" is likely to be overly simplistic when GEIs are present and may go some way to explaining why their effects, although widespread, appear to be relatively minor. Both the scenarios would reveal the complex architecture of sexual traits, providing insights into the nature of good genes variation (Tomkins et al. 2004). Each of type of interaction resulted from a significant GEI can influence the effect of a gene on fitness and so that the effect of an allele can change from positive to negative or vice-versa. These types of interactions have largely been ignored in sexual selection theory, and especially in good genes theory (Hunt and Hosken 2014). GEIs could maintain additive genetic variability in traits subject to directional selection, as they could prevent the fixation of a single genotype (Lewis 2012).

Although there are an increasing number of studies trying to investigate the role of GEI in the maintenance of genetic variability in sexual traits, postcopulatory traits have surprisingly received little attention. However, from the few studies conducted emerge that for postcopulatory traits exist some genetic variability for plasticity, although without producing any ecological cross-over (with the exception of sperm velocity in the guppy, (Evans et al. 2015). For instance in the scorpionfly, *Panorpa cognata*, in which number of sperm transferred is the most important postcopulatory trait, has been shown that sperm transfer rate is not influenced by larval food availability. However, there was evidence that different genetic background may generate different reaction norms for sperm transfer rate (Engqvist 2008). In spite of the important role of postcopulatory sexual selection (Birkhead and Pizzari 2002), the occurrence of GEI in postcopulatory traits has received little attention as far (Arnqvist and Thornhill 1998, Engqvist 2008, Morrow et al. 2008, Vermeulen et al. 2008, Lewis 2012, Evans et al. 2015). There are ample evidence of GEI generated through a diet manipulation (Hunt and Hosken 2014). For instance in the stalk-eyed fly *Cyrtodiopsis dalmanni,* male eye span, a trait strongly subjected to directional female choice, showed an increased phenotypic variability under food quality manipulation. However, although there is a significant GEI, the genotypes ranks were maintained across the three tested environments, suggesting that the eye span expression is strongly condition-dependent (David et al. 2000). Another example comes from males of the waxmoth *Achroia grisella* that emit ultrasonic signals to attract females. Jia and colleagues reared males from two artificially selected lines for signal rate to various environmental conditions. The lines exhibited significant GEI and also ecological cross-overs occur for some fitness-related traits under high larval density (i.e. limited food resources). This suggests that environmental variations may maintain genetic variability among males for the signal rate (Jia 2000).

In the present study we used *Poecilia reticulata*, a livebearing freshwater fish native to Central America that in the last two decades the guppy has become a model species for studies in pre- and postcopulatory sexual selection (Houde 1997, Magurran 2005). Males and females are promiscuous and the mating system does not provide material resources: males contribute only with sperms, which are transferred in bundles. Guppies show an elevated degree of sexual dimorphism: females have a mimetic coloration, whereas, males exhibit a polymorphic colour pattern, that has a fundamental role in female choice (Pilastro et al. 2004). Males attempt to mate by courting females (performing a “sigmoid display”) or adopting a coercive mating tactic (termed “gonopodial thrust”) (Houde 1997), however this alternative mating strategy, allows to transfer a lower number of sperms (Pilastro and Bisazza 1999). Males that perform intensely courtship displays are preferred by females, because this behavior is more energetically costly than gonopodial thrust (Abrahams 1993) and therefore, is a good proxy of male condition. Females copulate with more than one male over the same reproductive cycle and, as a result, broods are often sired by multiple males (Neff et al. 2008). From multiple mating females obtain genetic benefits: they produce larger broods, larger offspring and offspring with higher schooling and predator-evading abilities (Evans and Magurran 2000, Ojanguren et al. 2005). Polyandry leads to high level of competition among sperm of several males and both sperm number and quality contribute to sperm competition success although the number of sperm inseminated is the most important predictor of paternity (Boschetto et al. 2011). Sperm production is costly for males and condition dependent, as suggested by the positive correlation between sperm production and females presence (Gasparini et al. 2009), and by the effect of diet restriction on sperm production (Gasparini et al. 2013). Moreover, in the same study has been shown that sperm number has large additive genetic variability and heritability close to 1 (*Y*-linkage) (Gasparini et al. 2013). Studies on guppies native from Trinidad have revealed some positive correlation within ejaculate traits (Skinner and Watt 2006) and between ejaculate traits and traits involved in mating acquisition, such as the orange coloration and the courtship rate (Matthews et al. 1997, Locatello et al. 2006, Pitcher et al. 2007). Moreover, previous experiments on selected lines for sperm production failed to find genetic trade-offs with other sexual traits (Di Nisio 2014) suggesting a positive correlation between sperm number and the overall individual's genetic quality (Rowe and Houle 1996). However, genetic trade-offs may emerge when environmental conditions change (Reznick et al. 2000). Genes that are "good" to possess in one environment might have a weaker or even negative impact on relative fitness in another environment (e.g. David et al. 2000, Jia 2000). We took advantage from an artificial selection experiment for high and low sperm production (Di Nisio 2014) to explore the potential for GEI to occur under food restriction treatment.

Guppies are particularly appropriate to investigate the role of GEI in influencing sexual traits expression because their natural habitats vary along multiple environments gradients (Magurran 2005). Environmental heterogeneity may allow the opportunity for GEI to maintain genetic variability in sexual traits (Hunt and Hosken 2014). Among the infinite environments that could generate a genotype-by-environment interaction, variations in food availability seem to be one of the most relevant (Jia 2000, Engqvist 2008, Vermeulen et al. 2008, Evans et al. 2015). In the guppy food availability may vary over time in natural populations both in space and in time, also in a short period (Reznick 1989, Grether et al. 1999, Grether et al. 2001b). Indeed, periodic disturbances, such as floods or forest canopy cover, can decrease resource availability in guppy population (Grether et al. 2001b). The experimental approach consists in a food availability manipulation (*ad libitum* and restricted diet treatment) on the two lines artificially selected (High and Low) for sperm production. Studies in guppies have revealed that food manipulation influences both pre- and postcopulatory sexual traits (Grether 2000, Kolluru et al. 2008, Auer et al. 2012, Devigili et al. 2012, Rahman et al. 2013). The logistic of carrying out a selection experiment may be more costly in labor and time than a classical half-sibling breeding design but they may shed light on the nature of genetic variation underlying the selected trait (Scheiner 2002). Moreover, we combined a selection experiment with a full-sibs/half-sibs breeding design to sample the entire variability present in the selection lines and to control both for the maternal and paternal effects (Evans et al. 2015).

Materials and methods

Experimental individuals and artificial selection experiment design

All fish were descendants of wild-caught guppies collected in 2002 from the Lower Tacarigua River in Trinidad, a high-predation site where guppies coexist with several predator species. The fish were maintained in stock aquaria (ca. 100 fish/tank) as outbred population to avoiding inbreeding. The water temperature was maintained between 25°C and 27°C and illumination was set on a 12 h⁄12 h light⁄dark cycle. Fish were fed on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplarinS). Experimental individuals came from an artificial selection experiment for high and low sperm production. For the artificial selection protocol was initially screened for sperm number 400 males to found the two selection lines. Each line consisted of 50 males with highest (High) or lowest (Low) sperm number out of 200 males per line. Each male was individually housed with two virgin females from the same line and then the females were isolated until parturition. Offspring were allowed to grow in 8-L tanks and at the age of 5 months 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 40 males per line (selected for highest or lowest sperm number) were used to found the next generation.

Diet treatments

A full-sibs/half-sibs breeding design was established with families from the fourth generation of the artificial selection experiment. Experimental males (n=164, 82 per selection line) came from 82 families (41 per selection line), of which 62 families full-sibs/half-sibs (31 pairs with the same founder male) and 20 families only full-sibs. Males were subjected to poor or rich feeding conditions with a split-family design: full-sibs individuals was assigned at random to *ad libitum* food treatment (n=82) and to restricted food treatment (n=82). Experimental females (n=158, 78 from High line and 80 from Low line) were randomly chosen from 83 families and assigned to *ad libitum* food treatment (n=80) and to restricted food treatment (n=78). The treatment started when fish had 60 days, which coincides with a critical period for fish: males reach sexual maturity, starting sperm production and expressing definitive colour pattern (Houde 1997). The diet trials lasted two months and all fish were maintained in standard condition of pH, temperature, light, with a density of 2 fish (one male and one female) per 8-L tank in a Tecniplast ZebraTank system. Fish in the same tank were separated to avoid mating interaction and to permit an accurate food giving but they had visual interaction to allow a normal sexual development and sperm production (Bozynski and Liley 2003). Individuals assigned to the *ad libitum* treatment were fed ca. 140 fresh *Artemia salina* nauplii twice daily, 6 days per week. This amount was chosen following preliminary trials, which confirmed that fish fed this quantity of nauplii within 8–10 minutes but rarely finished all nauplii during this time. Fish assigned to the restricted treatment were fed to fewer of one-third of the amount of the *ad libitum* treatment (ca. 40 nauplii) twice daily, 6 days per week (Devigili et al. 2012). The food amount of the restricted treatment was increased by 10% every week (Grether et al. 2004) until the sixth week, when the food amount was increased to ca. 100 nauplli twice daily, 6 days per week (see table1). We standardized the concentration of nauplii each day and adjusted the volume using a micropipette to ensure that food quantities did not differ among males within each group throughout the feeding treatments.

Weeks	**restricted treatment**	***ad libitum* treatment**
1 week	40 nauplii	140 nauplii
2 week	(+10%) 44 nauplii	
3 week	(+20%) 48 nauplii	
4 week	(+30%) 52 nauplii	
5 week	(+40%) 56 nauplii	
6-7-8 weeks	(+150%) 100 nauplii	

Table1- Food amount for the two treatment during the diet treatments (8 weeks).

Morphological analysis

Morphological analysis (weight, body size and body coloration) was made both before (at age of 60 days) and at the end of the treatment period (at age of 120 days). Males and females were anaesthetized in a water solution of MS-222 (0.15 g/L) following Chambel's protocol (Chambel et al. 2013), then weighed with an analytical balance AND HR-120 (0.1 mg precision) and finally digitally photographed on the left side of body, using a Canon 450D placed on ZEISS Stemi 2000-C stereomicroscope. Measurements were made from the digital images using image analysis software (ImageJ: http://rsbweb.nih.gov/ij/download.html) and were included the total area of the body (including head and caudal fin), the distance between the snout and the base of the tail (standard length, SL), and the total area of colour spots (relating to males). In particular was considered orange and yellow carotenoid and pteridine spots (hereafter "orange") and structural iridescent spots (blue, green, violet, hereafter "iridescent"). The relative area of colour spots was calculated as the ratio between the area of spots and total body.

Sperm collection and count

Sperm in this species are packaged in discrete units, called sperm bundles, each containing about 21.000 individual sperm cells (Boschetto et al. 2011). To collect sperm bundles the anaesthetized male was placed under a stereomicroscope slide. Gentle pressure was then applied to the side of his abdomen, just anterior to the base of the gonopodium, to release sperm bundles in a drop of saline solution (NaCl 0.9%) (Matthews et al. 1997). Afterward sperm bundles were photographed on a black background and counted using ImageJ (see above). 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 first generation of selection. The two measures are highly correlated (mean number of sperm per bundle: 2.005±663.6, $t_{16}=33.159$, $r^2=0.986$, $p<0.001$).

Sperm velocity analysis

Sperm velocity was analyzed immediately after sperm collection, bundles were activated with 40 μL of 150 mM KCl solution in 2 mg/L bovine serum albumin (Billard and Cosson 1990) and placed in a 12-cell multiset slide (MP Biomedicals) coated with a 1% polyvinyl alcohol to prevent sperm from sticking to the glass slide. The swimming velocity was measured with a Hamilton-Thorne CEROS Sperm Tracker (for the setting parameters see (Gasparini et al. 2013) for three standard measures. These measures included: average path velocity (VAP), which estimates the average velocity of sperm cells over a smoothed cell path; straight line velocity (VSL), the average velocity on a straight line between the start and the end point of the track and curvilinear velocity (VCL), the actual velocity along the trajectory. These measures provide an estimate of progressive velocity and have been shown to positively correlate with fertilization success in this guppy population (Boschetto et al. 2011). Sperm velocity was estimated from a minimum of 100 sperm cell tracks. For each male, the motility analyses were performed on two subsamples (each containing 4 bundles) of the same ejaculate and the mean was used in final analysis.

Behavioral observations

After sperm collection, males were allowed to replenish their sperm reserves for 7 days (Kuckuck and Greven 1997). Sexual behavior was observed between 9.00 and 13.00 (which corresponds with the peak period of sexual activity (Houde 1997). Each male was placed individually in a 30-L tank with a non-virgin female and observations began after 5 minutes to permit the acclimatization. The trial consist in 5 minutes of observation, 15 minutes of no observation and other 5 minutes of observation (adapted from Devigili et al., unpublished observations). During the observations, were recorded the time spent by male interacting with female (hereafter "sexual activity" (Head and Brooks 2006), the number of sigmoid displays (courtship behaviours) and the number of forced mating attempts via gonopodial thrusts (Liley 1966). Courtship behavior (sigmoid displays) in the guppy consists of bending the body in a characteristic 'S' shape and quivering, in front of a female. Gonopodial thrusting is an alternative mating strategy that is not preceded by any courtship (Houde 1997). At the end of each trial, males were returned to their individual tanks and after 4 hours they were subjected to a predator-evasion test.

Predator-evasion test

A predator-evasion test (adapted from (Evans and Magurran 2000)) was used to the capability of males to escape from a simulated predator. Each male was put into a 45-L tank (same tank of the behavioral test) and after 90 seconds of acclimatization the male was captured using a small hand net (blindly to treatment). Capture procedure consisted of chasing the male with the net at a constant speed, which was kept as constant as possible. The test started inserting the net in the tank when the fish was in a central position in the tank and proceeded until the fish was captured. The escape time was recorded using a chronometer by another observer. It has been showed that the time spent to escape showed is repeatability within individual (R~0.70) and related to the condition of individuals (Gasparini et al. 2013).

Statistical analysis

Statistical tests were performed using SPSS 21. All data were normally distributed except the number of gonopodial thrusts, the time of evasion capability and the sexual activity. For the number of gonopodial thrusts a normal distribution was not attained even after transformation, therefore was performed a generalized linear mixed model with a poissonian distribution, in which selection line and food treatment were the fixed factors, and family was the random factor. The others non-normal traits were log10 transformed prior to analysis. For these traits and for the remaining normal traits was performed a linear mixed model with selection line and food treatment as fixed factors, and family as random factor. N=15 males died die before the end of the diet treatment: n= 12 males from the restricted treatment (n=6 High, n=7 Low) and the others n=3 from the *ad libitum* treatment (n=1 High, n=2 Low). At the moment of sperm assays n=12 males (equally distributed across the selection line and the treatments) were not sexually mature and they were excluded from the further behavioural observations. Within the sperm collection and the behavioural observations 27 males die, for a total amount of 36 males (n=6 High al libitum males, n=8 High restricted males, n=6 Low ad libitum males, n=16 Low restricted males) that die before the behavioral observations and the capture test. To measure the effects of diet treatment and genotype (selection line) we performed a mixed-model ANOVAs (Scheiner 2002) for each of the life-history traits, sexually selected traits and a measure of condition. Each model included diet treatment, genotype, sire and dams effects. Sire and dams effects were treated as random effects. To calculate GEI a MonteCarlo simulation analysis was performed (Thomas and Bazzaz 1993, Winding 1997) as implemented in PopTools (Hood 2011). Data set for each selection line within each environment was resampled with replacement. We calculated the ratio between High and Low line of traits expression for each treatment. Monte Carlo simulations (10,000 bootstraps) were performed to calculate confidence intervals (Winding 1997). No difference in traits expression between selection lines gave an estimate equal to unity and deviations from this expectation was used as a test criterion of interaction between selection line and the environments (GEI) (Dingemanse et al. 2007). Confidence intervals were used to estimate the occurrence of ecological cross-overs. Longevity was analysed using Kaplan-Meier survival analysis with diet and selected line as factor (Smith and Blumstein 2010) to test for differences due to diet and selection line.

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). The permit 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 (SC) following established procedures, was minimized and completed under 5 min.

Results

Diet treatment effects

As expected, we found that food limitation affected significantly most of the measured traits immediately after the diet treatment, at the fourth month of age irrespective of selected lines (see table 2 for descriptive analysis). Mixed-model ANOVAs showed a significant negative effect of diet on each fitness-related traits, such as body size, mass and the evasion ability (see table 2). Furthermore, diet restriction negatively affected the expression of precopulatory traits but we failed to detect effect on postcopulatory traits, including for sperm number that is the selected trait. The same analysis was performed also at one year of age (after eight months from the end of the diet trial). At one year of age, negative effect of diet remained only for the evasion ability from a simulated predator (see table 3). Diet has an impact on the probability of an individual to survive and Kaplan-Meier curves for diet treatment show a net difference between the two trends (see Supplementary figure S1). Diet-restricted individuals significant lived less than their ad libitum counterparts and this occurs both in the earlier time (Generalized Wilcoxon: χ^2=7.600, p=0.006), in the middle time (Tarone-Ware: χ^2=7.459, p=0.006) and in the later time (Mantel-Cox: χ^2=7.478, *p*=0.006).

	Ad libitum treatment		**Restricted treatment**				
Trait	**Mean ± s.e.m.**	**N**	**Mean± s.e.m.**	**N**	**F**	**df**	***p***
Body size (mm^2)	53.49 ± 0.64	82	47.50 ± 0.83	71	58.615	75	**<0.001**
Mass (g)	0.06 ± 0.01	82	0.04 ± 0.01	72	181.604	72	**<0.001**
Ability to escape (sec)	39.99 ± 3.85	61	21.39 ± 3.04	48	20.901	68	**<0.001**
Orange spots (%)	5.75 ± 0.39	82	3.70 ± 0.37	70	31.993	102	**<0.001**
Iridescent spots (%)	8.00 ± 0.31	82	7.00 ± 0.29	70	6.249	100	**0.014**
Gonopodial thrusts	2.34 ± 0.52	61	0.40 ± 0.20	47	13.327	106	**<0.001**
Sexual activity (sec)	109.21 ± 17.79	63	18.45 ± 5.14	49	16.264	60	**<0.001**
Sperm number (x10^6)	6.63 ± 0.50	81	6.01 ± 0.43	69	1.002	65	0.324
Sperm velocity (VAP μm*s-^{1})	92.08 ± 1.35	55	93.72 ± 1.31	50	0.372	57	0.544

Table 2- Mean, standard errors (s.e.m.), number of tested individuals (N) for all traits measured in the two diet treatments and effect of diet treatment on traits (Mixed-model ANOVAs) at the fourth month. Significant differences in bold.

	Ad libitum treatment		Restricted treatment				
Trait	**Mean ± s.e.m.**	**N**	**Mean ± s.e.m.**	**N**	**F**	**df**	***p***
Body size (mm^2)	67.48 ± 1.35	38	68.00 ± 2.18	26	0.171	38	0.681
Ability to escape (sec)	73.41 ± 13.04	29	28.18 ± 7.64	22	9.199	43	**0.004**
Orange spots (%)	7.30 ± 0.51	38	6.90 ± 0.5	26	0.033	40	0.857
Iridescent spots (%)	9.39 ± 0.55	38	8.66 ± 0.52	26	1.883	24	0.117
Gonopodial thrusts	2.41 ± 0.47	29	1.77 ± 0.38	22	1.029	49	0.315
Sexual activity (sec)	343.69 ± 41.05	29	350.41 ± 51.65	22	0.039	36	0.844
Sperm number ($x10^6$)	5.34 ± 0.55	38	6.05 ± 0.69	26	0.746	41	0.393
Sperm velocity (VAP $\mu m*s^{-1}$)	94.15 ± 1.31	30	90.26 ± 1.57	22	2.911	23	0.102

Table 3- Mean, standard errors (SE), number of tested individuals (N) for all traits measured in the two diet treatments and effect of diet treatment on traits (Mixed-model ANOVAs) at one year. Significant differences in bold.

Genotype-by-environment interactions

To evaluate the occurrence of GEIs we performed MonteCarlo simulation analysis (Thomas and Bazzaz 1993, Winding 1997) as implemented in PopTools (Hood 2011). Data set for each selection line within each environment was resampled with replacement. We calculated the ratio between High and Low line of traits expression foreach treatment. Monte Carlo simulations (10,000 bootstraps) were performed to calculate confidence intervals (Winding 1997). No difference in traits expression between selection lines gave an estimate equal to unity and deviations from this expectation was used as a test criterion of interaction between selection line and the environments (GEI) (Dingemanse et al. 2007). Confidence intervals were used to estimate the occurrence of ecological cross-overs (see supplementary table S3). Under restricted food treatment differences between High and Low are reduced but High males maintain higher relative trait expression than Low males for most traits (see Figure 1 and 2). Descriptive statistics of all male traits for each selection line and diet treatment are reported in supplementary table S1 for the fourth month measures and in supplementary table S2 for the measures at one year. Comparing the probability to survive among groups (after clustering each diet treatment with each selected line) we found that Low males from the restricted diet significantly died before the males from the other groups (Generalized Wilcoxon: χ^2=8.561, p=0.036), but the difference become marginally no significant both in the middle time (Tarone-Ware: χ^2=7.769, p=0.051) and in the later time (Mantel-Cox: χ^2=7.668, p=0.053) (see figure 3)

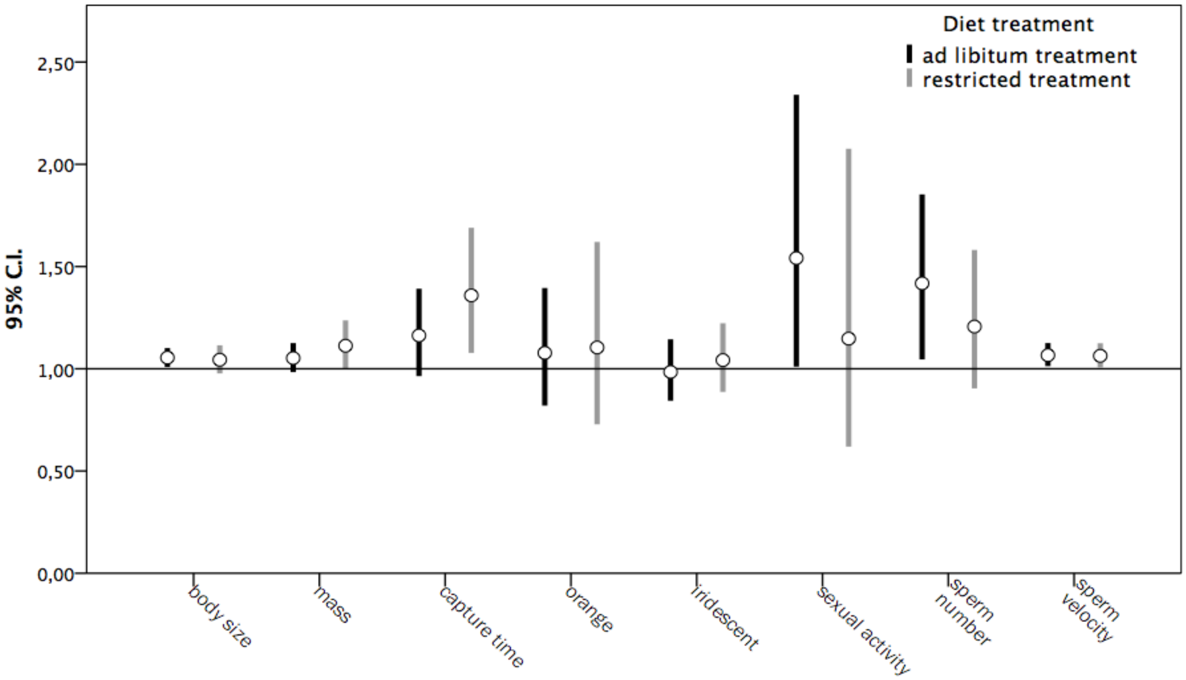


Figure 1- Mean of the ratio High/Low (white dots) of eight fitness-related traits expression for each treatment at the fourth month by creating 10,000 bootstrap replicates (95% CI).

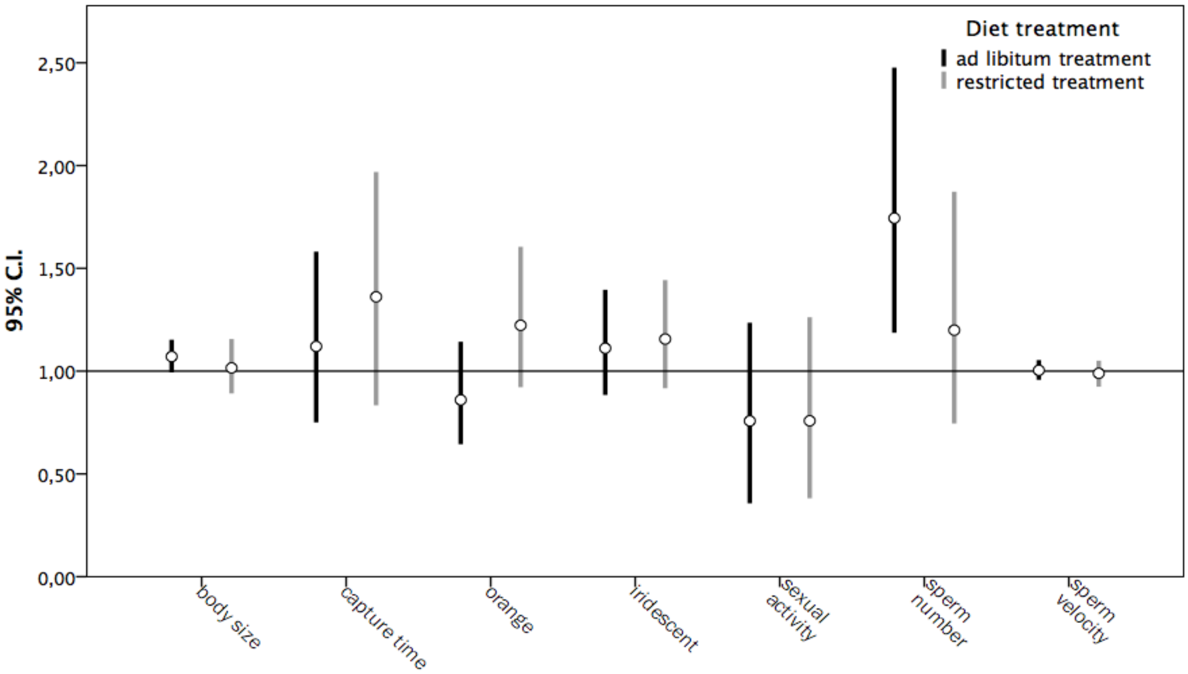


Figure 2- Mean of the ratio High/Low (white dots) of seven fitness-related traits expression for each treatment at one year by creating 10,000 bootstrap replicates (95% CI).

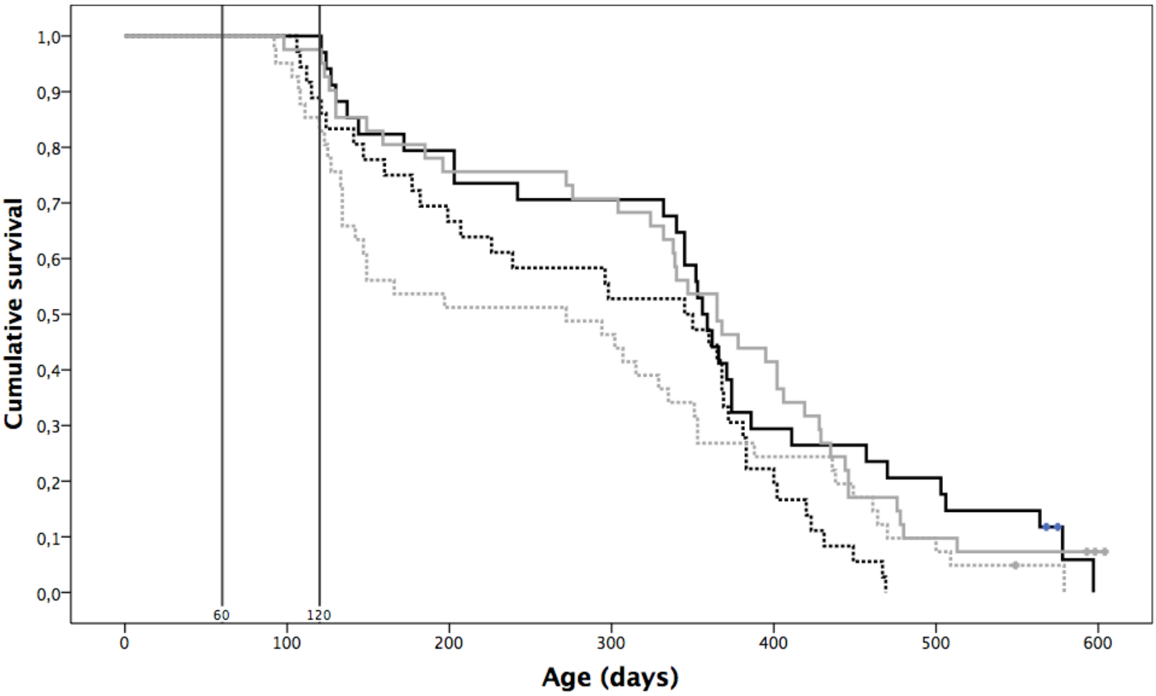


Figure 3- Kaplan–Meier curves of male survival probability during the diet treatments (from 60 to 120 days of life) and during the subsequent months until day 600. Dashed lines represent the restricted treatment and solid lines represent the ad libitum treatment. High males are showed in black and Low males in grey.

Discussion

Fourth-month measures

As expected, males assigned to the restricted treatment exhibited significantly reduced expression of most of the traits we measured, including body size, weight, ability to escape from a simulate predator and pre- and postcopulatory traits compared to the ad libitum counterparts. *Indeed* all the precopulatory traits we measured, showed a significant effect of food limitation, suggesting their strong dependence to condition (Cotton et al. 2004). Our finding that orange coloration was affected by diet treatment is largely in agreement with previous studies (Grether 2000, Devigili et al. 2012, Rahman et al. 2013, Rahman et al. 2014, Evans et al. 2015). On the other hand there is much debate over whether iridescent-structured colours are condition dependent (McGraw et al. 2002). In a similar study on the same species found that iridescent colorations were less responsive to diet manipulation than orange coloration (Devigili et al. 2012). On the contrary we found that the areas of these colours were significantly reduced in the males fed restricted compared to those fed *ad libitum*. However, our study was conducted on young males, exactly at two months of life, which coincides with a critical period for males since they start sperm production and express the definitive colour pattern (Houde 1997). Thus our food manipulation exerted during the developmental of colour patterns in males may explain the significant effect of diet restriction on both these colours. Previous studies in other species have revealed that diet stress in early developmental stages significantly affected iridescent coloration suggesting their dependence to condition (Kemp and Rutowski 2007, Griggio et al. 2009). Sexual behaviour of males was measured in two of its components: the time spent by the male following the female (Head and Brooks 2006) and the number of coercive sneaky attempts via gonopodial thrusts (Liley 1966). Both oh the components were affected from restriction of diet, in agreement with results found in previous studies (Devigili et al. 2012, Rahman et al. 2013, Rahman et al. 2014). Although, gonopodial thrusts appear to be less energetically demanding than courtship displays (Devigili et al. 2012, Rahman et al. 2013), our results suggest that we successfully manipulated condition of males. Interestingly, we found no evidence that ejaculate traits were significantly affected by diet treatment although both sperm number and sperm velocity exhibited downward trends in the restricted group. Our results were in line with another study conducted in the same population (Devigili et al. 2012). An explanation for this finding comes from the fact that we started diet manipulation at an early stage of males' life, as said above, and this could have caused a differential resource allocation between pre- and postcopulatory traits (Van Noordwijk and Dejong 1986). It is reasonable to suppose that young males have strategically allocated resources in traits involved in fertilization success over those involved in mating acquisition. However, this hypothesis clearly needs further investigations.

Genotype-by-environment interactions

Previous experiments on selection lines suggested that males form the two line differ for other sexual traits in addition to sperm number (Di Nisio 2014). In this study we found that High males seem to have larger body size and body mass, higher sexual activity and they were better able to escape from a simulated predator. Moreover, as expected, High males produced more sperm, that is the trait for which they were selected, and also sperm with higher swimming velocity. To evaluate the occurrence of GEIs, we compared the relative sexual traits expression between High and Low males and we found no evidence of significant interaction between selection lines and diet treatments, meaning that the reduced phenotypic expression of a trait due to diet restriction was not different among High and Low males. Two previous studies utilizing full-sibling breeding designs have tested for GEI for coloration in male guppies without finding any significant interaction (Hughes et al. 2005, Miller and Brooks 2005). However Evans and colleagues recently showed that significant GEIs for orange and iridescent coloration occur under food restriction in the Australian feral population of guppies (Evans et al. 2015). Differences in population origin may account for the different results between studies, indeed population native from Trinidad and from Australia are recognised to be different for the some phenotypic correlations among sexual traits (e.g. Locatello et al. 2006, Evans 2010). However, in our study, males selected for a higher investment in sperm production have generally better performances than males selected for low sperm production even in harsh environment, although not significantly different. Therefore these results suggest that High males did not pay a major cost despite the high investment in sperm production, recognized a highly costly trait and strongly dependent by male condition (Gasparini et al. 2013). Our results perfectly fit with previous experiments on the selection lines (Di Nisio 2014) that failed to find genetic trade-offs with other sexual traits. Sperm production seems to be correlated with male overall genetic quality as predicted by “good genes” models of sexual selection (Pomiankowski 1991, Rowe and Houle 1996). Genetic quality is likely to be correlated to the number of mildly harmful, partially recessive mutations in the entire genome (Houle 1998, Barton and Keightley 2002) rather than to the classical definition of “genetic load” (the number of fully recessive mutations that tends to be phenotypically invisible). These partially recessive mutations of small effect are widespread in the genome and may be responsible for the maintenance of the additive genetic variability in sperm production (Houle 1998, Charlesworth and Willis 2009). If this hypothesis is corrected, our results suggest that is likely that these mutations are unconditionally deleterious in the two environments we tested because the ranks of the genotypes (measured as trait expression or as reproductive fitness) were maintained across environments. However, contrary to our initial expectation and to other studies (David et al. 2000, Kemp and Rutowski 2007, Dmitriew and Blanckenhorn 2014) we found a trend for phenotypic variability to be reduced under diet restriction, although not significant. Thus, we can suspect that the differences between selection lines are magnified when food is unlimited and are reduced when the environment becomes harsh. This possible scenario needs further investigations, but whether confirmed may reveal a relaxation of the strength of selection on mutations in harsh environment.

One-year measures

At one year of age the effects of diet restriction persisted only for the ability to escape form a simulated predator. The fact that at one year of age males subjected to diet restriction in early stage of life were still less able to escape from a simulated predator compared to those raised with unlimited food suggests that this measure acts as honest signal of individual’s condition as observed also in previous studies (Evans and Magurran 2000, Gasparini et al. 2013) and suggests that our food manipulation had an effect on condition of males during their entire life. On the contrary, other traits we measured did not significantly differ among diet treatments. For instance, orange coloration is based on carotenoid pigments (although not exclusively) that cannot be synthetized by males and thus their availability ultimately depends on dietary sources (Endler 1980, Grether 2000, Grether et al. 2001a). We found that the negative impact of early diet manipulation on the area of orange spots coloration disappeared at one year of age, probably as a result of the increased intake of carotenoid pigments after the end of food restriction.

Genotype-by-environment interactions

At one year of age differences in sexual traits expression among males of the two selection lines were reduced compared to the differences at four months of life. Although Low males seems to have reduced their initial disadvantage with their High counterparts, GEIs did not occur. However, the reduced differences among selection lines could suggest a more rapid senescence for High males than for Low males, a result in line with previous experiments on the selection lines (Di Nisio 2014). Moreover, the trend observed in the longevity analysis indicates that High males tend to live shorter than Low males, although sample size was not sufficient to detect a significant difference. There is increasing evidence demonstrating that trade-offs between reproduction and senescence or longevity occur in many taxa (Hunt et al. 2004a, Simmons and Kotiaho 2007). Under a “live fast-die young” scenario, males pay their investment in reproduction with a reduction in their lifetime reproductive success (Bonduriansky et al. 2008, Jordan and Brooks 2010). A study conducted in a feral Australian guppy population supports this hypothesis demonstrating a negative correlation between males attractiveness and offspring survival (Brooks 2000). The net fitness of an individual depends on i) his early-life reproductive rate, ii) on the pattern of change in resource allocation to reproductive traits with age (senescence) and finally iii) on his life-span. Males are likely to vary in life-span and senescence rate, and this variation may reflect sexual traits expression, whether genes affecting reproduction have pleiotropic effects, positive or negative, on senescence and longevity, as expected under a life history scenario. Altogether our results seem to indirectly support a “live fast-die young” strategy associated with a strong investment in sperm production in early life. Furthermore, Miller & Brooks explored the effects of age and social environment on the expression of male sexual traits, including orange and iridescent coloration. Although they found no significant GEIs attributable to male social environment, significant age-by-treatment interactions for both traits suggested that genotypes vary in the way that colour is expressed with age (Miller and Brooks 2005).

In conclusion, in this study we tested the role of GEI in the maintenance of genetic variability for a trait highly associated with male sperm competition success: sperm production (Boschetto et al. 2011). As expected under a “good genes” model of sexual selection we did not find evidence of GEIs both in sperm number and in other fitness-related traits. High males were better able cope with unfavourable conditions despite their higher investment in sperm production and thus the ranks of genotypes were maintained across environments. Although our results suggest that the variability in sperm production should be negatively correlated with the number of deleterious mutations, it is likely that other mechanisms are involved. First, the lack of GEIs under dietary manipulations does not exclude that GEIs might occur under other environmental conditions. Among the possible factors that may affect sexual traits expression and thus, sexual selection dynamics, social environment is likely to be especially relevant (Hunt and Hosken 2014). Since the role of social environment in driving the evolution of sexual traits, more studies are required for understanding whether variations in the social environment may generate GEIs and thus, contribute to the maintenance of genetic variability in sexual traits. Second, there are multiple evidence, both from previous experiments and from our results here, that indicate a more rapid senescence in males selected for high sperm production. Therefore it is likely that males investing more resources in early reproductive life, pay a major cost in a long-term period in terms of somatic maintenance (Jordan and Brooks 2010). Further studies need to investigate whether genes affecting reproduction have pleiotropic effects, positive or negative, on senescence and longevity, as expected under a life-history scenario.

Supplementary Materials

Trait	Selected line	Diet treatment	Mean ± s.e.m.	N
Body size (mm^2)	High	ad libitum	54.91±0.97	41
		restricted	48.47±1.22	37
	Low	ad libitum	52.07±0.77	41
		restricted	46.43±1.10	34
Mass (g)	High	ad libitum	0.06±0.01	41
		restricted	0.05±0.01	36
	Low	ad libitum	0.06±0.01	41
		restricted	0.04±0.01	36
Ability to escape (sec)	High	ad libitum	46.12±6.18	39
		restricted	26.28±4.78	28
	Low	ad libitum	34.44±4.61	32
		restricted	14.30±2.01	20
Orange spots (%)	High	ad libitum	5.94±0.62	41
		restricted	3.84±0.48	37
	Low	ad libitum	5.56±0.49	41
		restricted	3.56±0.56	33
Iridescent spots (%)	High	ad libitum	7.93±0.46	41
		restricted	7.12±0.43	37
	Low	ad libitum	8.08±0.43	41
		restricted	6.86±0.39	33
Gonopodial thrusts	High	ad libitum	3.52±0.82	31
		restricted	0.57±0.33	28
	Low	ad libitum	1.13±0.57	30
		restricted	0.16±0.12	19
Sexual activity (sec)	High	ad libitum	143.70±26.68	31
		restricted	18.94±6.82	28
	Low	ad libitum	75.79±22.50	32
		restricted	17.77±8.00	21
Sperm number (x10^6)	High	ad libitum	7.72±0.75	41
		restricted	6.53±0.66	36
	Low	ad libitum	5.52±0.63	40
		restricted	5.47±0.54	33
Sperm velocity (VAP, $\mu m*s^{-1}$)	High	ad libitum	95.26±1.26	25
		restricted	96.20±1.43	28
	Low	ad libitum	89.42±2.13	30
		restricted	90.56±2.22	22

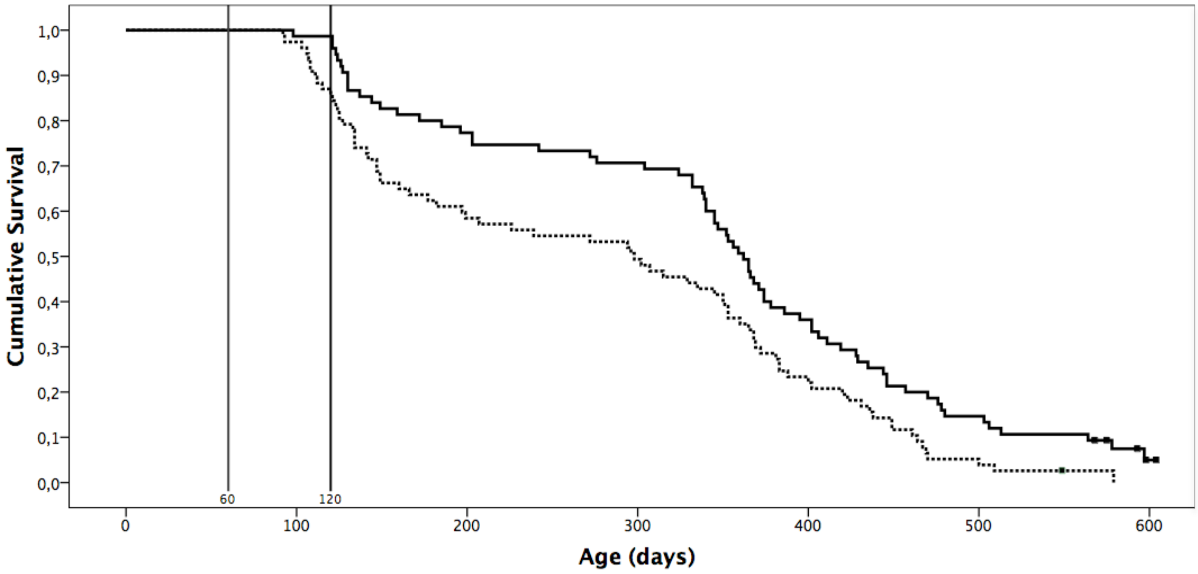
Supplementary table S1- Mean, standard errors (s.e.m.), number of tested individuals (N) for all traits measured in the two diet treatment and in the two selection lines (High and Low) at the fourth month.

Trait	Selection line	Diet treatment	Mean± s.e.m.	N
Body size (mm^2)	High	ad libitum	70.28±1.99	15
		restricted	68.38±2.66	15
	Low	ad libitum	65.66±1.76	23
		restricted	67.46±3.80	11
Mass (g)	High	ad libitum	84.2±24.62	10
		restricted	38.27±14.47	11
	Low	ad libitum	67.74±15.47	19
		restricted	18.09±3.88	11
Ability to escape (sec)	High	ad libitum	6.62±0.82	15
		restricted	7.44±0.69	15
	Low	ad libitum	7.74±0.65	23
		restricted	6.16±0.68	11
Orange spots relative area (%)	High	ad libitum	2.92±0.27	15
		restricted	2.43±0.19	15
	Low	ad libitum	3.21±0.24	23
		restricted	2.48±0.30	11
Iridescent spot relative area (%)	High	ad libitum	276.80±76.86	10
		restricted	301.00±70.92	11
	Low	ad libitum	378.89±47.40	19
		restricted	399.82±75.40	11
Gonopodial thrusts (n.)	High	ad libitum	2.70±0.90	10
		restricted	2.27±0.66	11
	Low	ad libitum	2.26±0.56	19
		restricted	1.27±0.30	11
Sexual activity (sec)	High	ad libitum	7.14±0.88	15
		restricted	6.44±0.95	15
	Low	ad libitum	4.17±0.61	23
		restricted	5.52±1.03	11
Sperm number ($x10^6$)	High	ad libitum	94.36±1.16	12
		restricted	89.81±2.60	12
	Low	ad libitum	94.01±2.07	18
		restricted	90.80±1.63	10
Sperm velocity (VAP, $\mu m*s^{-1}$)	High	ad libitum	70.28±1.99	15
		restricted	68.38±2.66	15
	Low	ad libitum	65.66±1.76	23
		restricted	67.46±3.80	11

Supplementary table S2- Mean, standard errors (s.e.m), number of tested individuals (N) for all traits measured in the two diet treatments and in the two selection lines (High and Low) at one year.

		4 months			12 months		
			95% Confidence Interval			95% Confidence Interval	
Trait	**Diet**	**Mean**	**Lower Bound**	**Upper Bound**	**Mean**	**Lower Bound**	**Upper Bound**
Body size	ad libitum	1.05	1.01	1.10	1.07	0.99	1.15
	restricted	1.04	0.98	1.11	1.02	0.89	1.16
Predator-evasion time	ad libitum	1.16	0.96	1.39	1.12	0.75	1.58
	restricted	1.36	1.08	1.69	1.36	0.83	1.97
Orange spots	ad libitum	1.08	0.82	1.39	0.86	0.64	1.14
	restricted	1.10	0.73	1.62	1.22	0.92	1.60
Iridescent spots	ad libitum	0.98	0.84	1.14	1.11	0.88	1.40
	restricted	1.04	0.89	1.22	1.16	0.92	1.44
Sexual activity	ad libitum	1.54	1.01	2.34	0.76	0.36	1.24
	restricted	1.15	0.62	2.08	0.76	0.38	1.26
Sperm number	ad libitum	1.42	1.05	1.85	1.74	1.19	2.48
	restricted	1.21	0.90	1.58	1.20	0.75	1.87
Sperm velocity	ad libitum	1.07	1.01	1.13	1.00	0.96	1.05
	restricted	1.06	1.01	1.12	0.99	0.92	1.05

Supplementary table S3- Mean and confidence intervals (95%) of High/Low ratio for each traits (both at 4 and 12 months) in both diet treatments resulted from the MonteCarlo simulations.



Supplementary figure S1- Kaplan–Meier curves of male survival probability during the diet treatments (from 60 to 120 days of life) and during the subsequent months until day 600. Lines represent males form the two diet treatments, irrespective of selection lines. Dashed lines represent the restricted treatment and solid lines represent the ad libitum treatment.

Manuscript 2

Exploring genotype-by-parental environment interactions in the guppy

Silvia Cattelan and Andrea Pilastro
Department of Biology, University of Padova, 35131, Padova Italy

Abstract

Under a life- history scenario, the evolution of a trait is constrained by trade-offs with other traits. However, these trade-offs could also occur in homologous traits of female and in life-history and sexual traits of offspring. Indeed, genetic trade-offs may emerge as a reduction in fitness of offspring, as a consequence of negative pleiotropy in genes associated with sexual antagonism. Here we tested this hypothesis in the guppy (*Poecilia reticulata*) manipulating the food availability of males and females from an artificial selection experiment for high and low sperm production. We determined whether genotype-by-parental environment interaction existed such that food restriction exerted different influences on female reproductive performances and on offspring quality (measured through life-history and sexual traits expression) among selected lines of guppies that were artificially selected for high and low sperm number. Overall we investigated whether a variation in food availability may contribute to maintain genetic variability in sperm number because genetic trade-offs (i.e. significant genotype-by-parental environment interactions) exist in females and in offspring. We found no evidence of negative pleiotropy that may reduce fitness of males with high investment in sperm production. This suggests that genes affecting reproduction in females are not negatively related with genes enhancing investment in reproduction in males. Thus, contrary to what expected under a life-history scenario, sperm number seems to be positively correlated to the overall quality of males without incurring in any costs for females and offspring.

Introduction

Environmental stresses are recognised to have detrimental effect on the expression of sexual traits, on fecundity and on survival probability (Robinson et al. 2008, Auer et al. 2012, Rosenthal and Hebets 2012, Vergara et al. 2012, Fricke et al. 2015). Among the stress factors, food availability is one of the most important influencing individual’s fitness (Maklakov et al. 2008, Devigili et al. 2012, Simmons 2012, Zikovitz and Agrawal 2013, Gillespie et al. 2014). Conditions experienced during early development may affect reproductive performance in late life and could affect also the subsequent generation, influencing for instance offspring size, offspring growth rate and offspring fecundity. In the last two decades the increasing use of quantitative genetic design have allowed to extensively test the influence of environmental experience of parents on offspring performance (Donelson et al. 2009) and trans-generational effects have been shown to be more common than previously thought (Hunt 2000, Naguib and Gil 2005, Bonduriansky and Head 2007, Vijendravarma et al. 2010, Franzke and Reinhold 2012, Triggs et al. 2012, Valtonen et al. 2012, Garbutt et al. 2014). However, trans-generational effects of environment experienced by parents are not necessarily detrimental. Indeed, environmental heterogeneity over a coarse-grain timescale may select for environment-dependent parental effects that enhance offspring fitness. Indeed, whether the nutritional status that parents experienced predicts the environment that offspring will encounter, parents may be selected to optimize offspring phenotype for that environment ("the adaptive hypothesis") (Mousseau and Fox 1998). In this perspective, according to life-history theory, natural selection could be expected to favour parents that produce fewer but better offspring in response to cues indicating offspring food stress (Bonduriansky and Head 2007, Valtonen et al. 2012, Garbutt et al. 2014). No less important is the effect of parents condition on the determination of offspring sex ratio (Rosenfeld and Roberts 2004). In sex allocation theory one of the most fascinating issue was developed by Trivers and Williard and predicts that parents should adjust offspring sex ratio accordingly to maximize fitness (Trivers 1973). Biased sex ratio in response to food restriction and thus to due reduction of maternal condition) has been demonstrated in some species of birds (Nager et al. 1999, Komdeur et al. 2002, Sutherland 2002). However, very little is known about the genetic variation in the maternal and paternal effects on offspring. Most of the studies have focused on the interaction between genotype and abiotic factors, such as food availability and temperature, in influencing offspring resistance to infection (Garbutt et al. 2014). This process has the potential to promote the maintenance of genetic variability in traits, if genotypes for a given trait differ in the extent to which parental environments affects offspring survival and reproduction. This genotype-by-parental environment interaction would provide a mechanism for the maintenance of genetic polymorphism in the immune response. The same principles applied for the immune-system could be adopt also for any quantitative traits, even for those involved in mating acquisition and fertilization success, often subjected to a strong directional selection that should erode their genetic and phenotypic variability (Kirkpatrick and Ryan 1991). These traits act as reliable information about the quality of the signaler, since they are costly to produce (Zahavi 1975). Most studies that examine the reliability of signals have focused on performances of sons and ignored the fitness of daughters (Hunt et al. 2004b). However, negative genetic correlations may arise also in homologous traits in females, as reproductive success, due to sexual antagonism. Thus, if genes affecting reproduction have negative pleiotropic effects on female survival and reproduction this could lead to the unreliability of sexual signals.

In the guppy, *Poecilia reticulata,* sperm number the number of sperm inseminated is the most important predictor of paternity (Boschetto et al. 2011). Sperm production is costly for males and condition dependent, as suggested by the positive correlation between sperm production and females presence (Gasparini et al. 2009), and by the effect of diet restriction on sperm production (Gasparini et al. 2013). Moreover, in the same study has been shown that sperm number has large additive genetic variability and heritability close to 1 (*Y*-linkage) (Gasparini et al. 2013). Previous studies have revealed both phenotypic and genetic correlations between sperm number and other sexual traits, both pre- and postcopualtory (Matthews et al. 1997, Locatello et al. 2006, Skinner and Watt 2006, Pitcher et al. 2007, Di Nisio 2014). This suggests a positive correlation between sperm number and the overall individual's genetic quality (Rowe and Houle 1996). Moreover, previous experiments failed to find costs for females whom mated with males with high investment in sperm production (Di Nisio 2014).However, this hypothesis was tested only under optimal condition and it is likely that negative pleiotropy in female reproductive performances emerge when environmental conditions change become harsher. Genes that are "good" to have in one environment might have a weaker or even negative impact on female reproductive fitness in another environment (Reznick et al. 2000). This would result in a breakdown of the relation between sperm number and honest indicator of male quality and hence of "good genes" for offspring. For instance In the bank vole, it has been shown that male dominance is an unreliable signal for females when resources become limited and thus competition among individuals increases (Mills et al. 2007). This process is thought to be responsible for the elevated variability in the male dominance advertisement observed in nature.

Thus to investigate the potential role of environmental variations in maintaining genetic variability in sperm number we determined whether genotype-by-parental environment interaction existed such that food restriction exerted different influences on female reproductive performances and on offspring quality (measured through life-history and sexual traits expression) among lines of guppies that were artificially selected for high and low sperm number. Firstly we raised a parental generation of males and females on either an ad libitum diet or on a restricted diet (see Manuscript 1). Then we assessed the reproductive performance of females and measured the trans-generational effects of the early developmental conditions of parents on their own offspring. We investigated how parental nutrition influences reproductive allocation in terms of offspring number, inter-brood time, number of brood cycle but also offspring fitness traits (such as offspring size at maturity, sex ratio and the degree of sexual traits' expression. We chose to manipulate food availability since there is evidence that it varies over time in natural populations (Reznick 1989). Experimental reduction in food availability produced trans-generational effects on life-history traits in the guppy. Indeed female guppies grow less, reproduce less, and bear fewer, larger offspring in response to food limitation (Reznick and Yang 1993, Zandona et al. 2011). The fecundity cost of producing less offspring is outweighed by the increased fitness of producing larger offspring (Bashey 2006), suggesting that in this species the adaptive hypothesis of maternal effects occurs (Mousseau and Fox 1998). Overall we investigated whether a variation in food availability may contribute to maintain genetic variability in sperm number because genetic trade-offs (i.e. significant GEIs) exist in females and in offspring.

Material and methods

Experimental individuals

All fish were descendants of wild-caught guppies collected from the lower part of Tacarigua river in Tinidad, in 2002. Fish were maintained in mixed-sex stock aquaria (ca.100 fish/tank) as outbred population to avoid inbreeding. Stock populations were maintained at 26 °C on a 12:12h light–dark cycle. Fish were fed on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplarinS). Experimental individuals, both females and males came from the fourth generation of artificial selection for high and low sperm production (see Di Nisio 2014). N=57 individuals per sex from High line and n=58 individual per sex from Low line were randomly assigned to either restricted and ad libitum diet treatment and then used to found the next generation.

Diet trials

We chose to manipulate the number of *Artemia salina* since small invertebrates seem to be the most inmportant source of food for guppy populations that live in the lower part of the rivers (Zandona et al. 2011). The treatment started when fish had 60 days, moment in which differences between the sex arise but individuals didn’t reach sexual maturity yet (Houde 1997). The diet trials lasted two months and all fish were maintained in standard condition of pH, temperature, light, with a density of 2 fish (a male and a female were kept separated by a divisor in order to avoid matings) per 8-L tank in a Tecniplast ZebraTank system. Individuals assigned to the *ad libitum* treatment were fed ca. 140 fresh *A. salina* nauplii twice daily, 6 days per week. Fish assigned to the restricted treatment were fed to fewer of one-third of the amount of the *ad libitum* treatment (ca. 40 nauplii) twice daily, 6 days per week (Devigili et al. 2012). The food amount of the restricted treatment was increased by 10% every week (Kolluru and Grether 2004) until the sixth week, when the food amount was increased to ca. 100 nauplli twice daily, 6 days per week. We standardized the concentration of nauplii each day and adjusted the volume using a micropipette to ensure that food quantities did not differ among males within each group throughout the feeding trials. At the end of the trials period, the individuals were fed ca. 140 nauplii twice daily, 6 days per week.

Breeding design

After two months from the end of the trials was established a breeding design to evaluate the effect of diet on females' fecundity and the trans-generational effects on offspring. We randomly paired a male and a female from the same diet treatment with respect to the selection lines and we avoided to pair male and female related each other. Thus we generated 4 groups: High line-ad libitum diet group (High-A), High line-restricted diet (High-R), Low line-ad libitum diet (Low-A) and Low line-restricted diet (Low-R). Overall we formed 115 pairs, n=34 High-A, n=23 High-R, n=31 Low-A and n=27 Low-R. Males and females were freely to interact in a 8-L tank for two weeks. Food was provided ad libitum during all the mating trials. After mating trials, females were isolated for three months and the number of offspring produced at each brood cycle was recorded. For logistic reason we raised only the first brood from each female therefore, all the results regarding sexually mature offspring concern only the first brood from each female.

Morphological analyses

Offspring morphological analysis (body size and body coloration) was made at five months of life. Males and females were anaesthetized in a water solution of MS-222 (0.15 g/L) and digitally photographed on the left side of each individual's body, using a Canon 450D placed on ZEISS Stemi 2000-C stereomicroscope. Measurements were made from the digital images using image analysis software (ImageJ: http://rsbweb.nih.gov/ij/download.html) and were included the total area of the body (including head and caudal fin), and the total area of colour spots for males. In particular orange and yellow carotenoid and pteridine spots were considered as orange coloration, structural spots such as blue, green, violet colors as iridescent coloration and melanic spots as black coloration. The relative area of colour spots was calculated as the ratio between the area of spots and total body.

Sperm collection and count

Sperm in this species are packaged in discrete units, called sperm bundles, each containing about 21.000 sperm cells (Boschetto et al. 2011). To collect sperm bundles the anaesthetized male was placed under a stereomicroscope slide. Gentle pressure was then applied to the side of his abdomen, just anterior to the base of the gonopodium, to release sperm bundles in a drop of saline solution (NaCl 0.9%) (Matthews et al. 1997). Afterward sperm bundles were photographed on a black background and counted using ImageJ (see above). 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 (unpublished data). The two measures are highly correlated (mean number of sperm per bundle: 2.005 ± 663.6, t_{16}=33.159, r^2=0.986, p<0.001).

Predator evasion test

A capture test (adapted from Evans and Magurran 2000) was used to estimate predator evasion capability of individuals, a condition-dependent trait. Each fish was individually put into a 45-L tank and after 90 seconds of acclimatization the male was captured using a small hand net (blindly to treatment). Capture procedure consisted of chasing the male with the net at a constant speed, which was kept as constant as possible. The test started inserting the net in the tank when the fish was in a central position in the tank and proceeded until the fish was captured. The escape time was recorded using a chronometer by another observer. Capture test showed a significant repeatability within individual (R~0.70) (Gasparini et al. 2013).

Statistical analyses

Statistical tests were performed using SPSS 21. Where not normally distributed, data were appropriately transformed (log-transformation was adopted for sperm number). Proportion data were arcsin-square root transformed prior to analysis. We analyzed sex ratio and number of females giving birth as a function of diet treatment by using a binary logistic regression. Number of broods was analyzed by performing an ordinal regression. For all the others traits we performed general linear models (Scheiner 2002) in which diet treatment and selected lines were included as fixed factors. Where necessary, dame body size and number of sisters were included in the model as covariates. Whether the covariates were not significant, they were removed from the models.

Ethical Notes

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). The permit 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 of fish was conducted by an expert operator (SC) and once fish were anesthetized, the manipulation was completed under 5 min.

Results

The effects of early diet treatment in parents were firstly measured as reproductive performances of females (see Table 1). As expected, diet affected the probability to give birth: the proportion of females from the restricted treatment was significantly higher than the proportion of the ad libitum counterparts. Moreover diet affected females of selected lines differently: the 91% of females from the High-R group gave birth to at least one brood, whereas only the 74% from Low-R group produced offspring. Number of offspring delivered at first birth was affected by diet in which parents were raised but not significantly different between selected lines (see Table 1). The effect of diet on the number of offspring produced persisted also after controlling for dame body size and for different effects of dame body size on selected line and diet treatment, therefore dame body size was excluded from the final model (GLM, body size effect: $F_{1,104}=2.984$, $p=0.087$; body size*diet: $F_{1,102}=0.126$, $p=0.724$; body size*line $F_{1,102}=0.267$ $p=0.607$). Moreover, diet significantly affected the probability of offspring to survive until the fifth month of life (when offspring were measured and then released) and this effect was significantly higher in the Low-R group (significant Diet*Line interaction) (see Figure 1). Indeed 16% of offspring from High-A died, 9% from High-R, 11% from Low-A and 34% from Low-R. This effect was not significantly influenced by dame body size (GLM, body size effect: $F_{1,104}=0.062$, $p=0.804$; diet * body size: $F_{1,102}=0.094$, $p=0.759$; and line*SL: $F_{1,102}=0.449$, $p=0.505$). The effect of diet restriction on the number of broods produced was marginally not significant (but see Figure 2). Finally restricted groups produced significantly more offspring males than females, resulting in a skewed sex ratio toward males. Although sex ratio was estimated at sexual maturity, the bias must have occurred at the time of embryo development, because no sex-biased mortality was detected during offspring development. This result probably suggests that the general pattern of allocation of resources into male and female offspring differed between diet treatments. Female body size at sexual maturity was affected by diet restriction. Females which parents were raised with diet restriction were larger than their ad libitum counterparts but this effect did not differ between selected lines. The number of sisters significantly affected body size at maturity (GLM, number of females: $F_{1,82}=25.882$, $p<0.001$) but this effect was not different neither between diet treatments (GLM, number of females*diet: $F_{1,80}=0.956$, $p=0.003$) and selected lines (GLM, number of females*line: $F_{1,80}=0.204$, $p=0.653$). Moreover, no effect of parental diet was detected for the capability to evade from a simulated predator in both the sexes (Table 2) and for pre- and postcopulatory traits of male offspring (respectively body coloration and sperm number).

		Number of families	Brood size at birth ± s.e.m.	Unviable newborn (%)
High line	Ad libitum	34	5.09 ± 0.40	16.70 ± 3.51
	Restricted	21	4.43 ± 0.48	8.74 ± 3.23
Low line	Ad libitum	30	5.07 ± 0.46	11.47 ± 2.90
	Restricted	20	3.75 ± 0.61	34.22 ± 9.39

Table 1- Female fecundity for each selected line and each diet treatment. Means ± s.e.m. are given for brood size at birth.

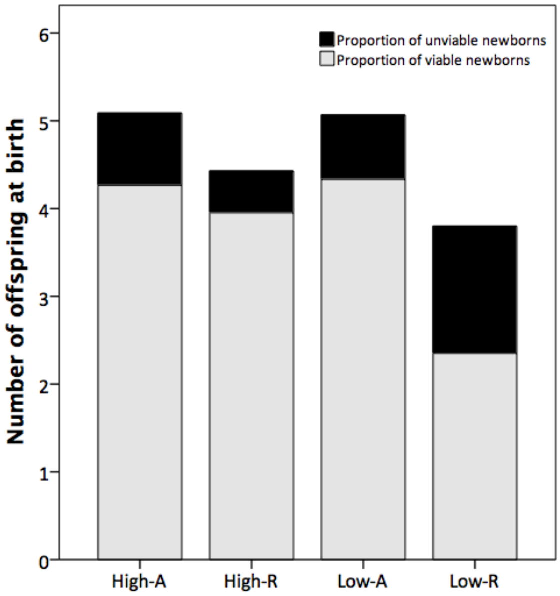


Figure 1- Number of offsrping produced at the first brood as a function of parental diet distinguishing in unviable and viable offspring.

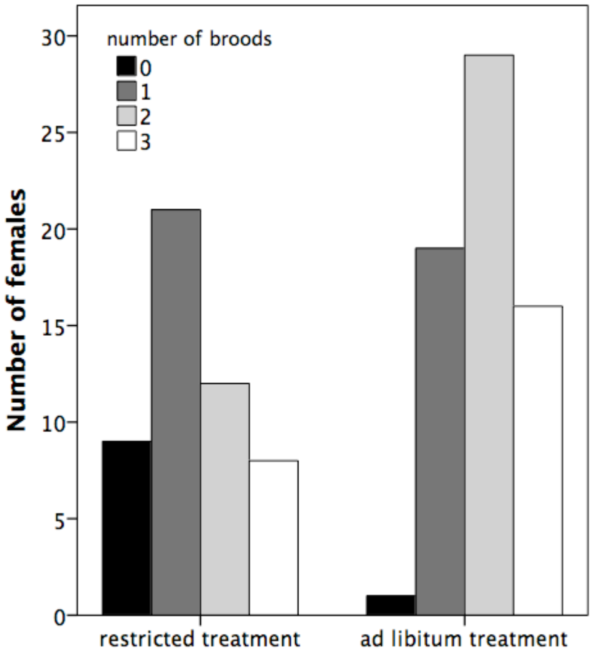


Figure 2- Number of broods produced in three months as a function of diet of dams.

Trait		Statistics	df	*p*
Number of females that produced offpsring (%)[a]	Line	3.830	2	**0.050**
	Diet	9.646		**0.002**
	Line * Diet	12.765		**0.002**
Brood size at birth	Line	0.733	105	0.394
	Diet	4.710		**0.032**
	Line * Diet	0.659		0.419
Unviable newborns (%)	Line	4.088	105	**0.046**
	Diet	1.798		0.183
	Line * Diet	9.165		**0.003**
Number of broods[b]	Line	0.048	1	0.825
	Diet	3.586		0.058
Sex ratio[a]	Line	1.198	2	0.274
	Diet	4.065		**0.044**
	Line * Diet	5.798		0.055

Table 2 - Effect of diet treatments, selection lines and their interaction on reproductive performances of females (GLMs). [a] logistic regression; [b] ordinal regression. Significant differences are reported in bold.

		Males			Females		
Trait		**F statistics**	**df**	***p***	**F statistics**	**df**	***p***
Body size (mm^2)	Line	0.198	69	0.658	1.195	82	0.278
	Diet	0.852		0.359	4.711		**0.033**
	Line * Diet	0.472		0.495	1.242		0.268
Capture test (sec)	Line	1.401	87	0.240	0.218	82	0.642
	Diet	1.181		0.280	1.998		0.161
	Line * Diet	0.992		0.322	0.303		0.583

Table 3- GLMs results for two fitness-related traits in both females and males. Effects of diet treatments, selected lines and their interaction. Significant differences in bold.

Trait		F statistics	df	*p*
Orange spots relative area (%)	Line	0.028	88	0.868
	Diet	0.001		0.981
	Line * Diet	1.083		0.301
Black spots relative area (%)	Line	1.007	88	0.318
	Diet	0.312		0.578
	Line * Diet	0.348		0.557
Iridescent spots relative area (%)	Line	0.257	88	0.614
	Diet	1.300		0.257
	Line * Diet	0.844		0.361
Sperm number ($x10^6$)	Line	1.388	88	0.242
	Diet	0.087		0.769
	Line * Diet	0.657		0.420

Table 4- Results from GLMs for pre- and postcopulatory traits in males. Effects of diet treatments, selected lines and their interaction. Significant differences reported in bold.

Discussion

In this study we investigated whether the investment in sperm production in males is traded-off by costs for females and offspring under harsh food condition. In particular we tested i) whether reproductive performances of females of the selected lines are influenced in different ways by diet restriction showing negative pleiotropy and ii) whether effects of parental diet are different among selected lines, leading to trans-generational trade-offs.

Diet effects on female reproductive performances and offspring phenotype

It is recognised that environmental conditions that individuals experience during early life could affect their reproductive performance and phenotype of their offspring (Qvarnström and Price 2001) and, as expected parental diet had strong effects on female fecundity but only partially on offspring phenotype (summary of the results are reported in Table 5). Females that experienced food restriction in their early life had a significant lower probability to produce offspring than females that did not experience such stress. Moreover females from restricted diet that were able to give birth, produced significant less offspring than their ad libitum counterparts, in line with two previous studies (Reznick and Yang 1993, Bashey 2006). Reproductive performances of females are affected by diet treatments also in a long-term scenario since the probability to produce a second birth is low in those females that experienced diet restriction, although marginally no significant. This suggests that a diet restriction in early life affects reproductive performances even after six months from the end of diet. According to life-history theory, we found that parents that experienced diet restriction in early life produced fewer but better provisioned offspring in response to cues indicative that offspring will experience nutritional stress (Smith and Fretwell 1974). Our setting did not allow us to estimate the maternal and paternal contributions separately since both parents are subjected to the same diet treatment. However, maternal effects are typically considered more important than paternal effects due to the tendency for mothers to invest more resources in offspring (Mousseau and Fox 1998, but see Crean and Bondurianský 2014). Thus it is likely that the offspring size was mainly influenced by mother contribution on eggs rather than by epigenetic effects of the sire. The optimal offspring size should be larger in more competitive environments (Parker and Begon 1986) in which larger offspring should have an increased advantage over smaller (Mousseau and Fox 1998, Bashey 2008, Valtonen et al. 2012). However, this adaptive plasticity is not widespread among taxa (Donelson et al. 2009, Franzke and Reinhold 2012). Here, it is likely that the cost of producing less offspring is outweighed by the benefit of producing large female since female body size is positively correlated with fecundity in this species (Herdman et al. 2004). Alternatively it is possible that optimal condition of parents raised on ad libitum diet has favoured a slower growth rate of their offspring. Thus, we are not able to exclude whether the difference in female body size between parents diet treatments is due to a different growing rate of offspring (Arendt and Reznick 2005). Our results showed a skewed sex ratio toward males in broods produced by parents raised under food restriction. Sex ratio skewed in response to environmental variation, such as food availability, has been reported in many species of mammals and birds (Nager et al. 1999, Komdeur et al. 2002, Rosenfeld and Roberts 2004). Sex allocation theories predict that parents should invest more in the sex that could have more survival and reproductive probability (Trivers 1973) and under this scenario, we expect that parents would invest in the sex requiring less resource for somatic development and maintenance. Our result seems in line with this prediction since once males guppies become sexual mature they stop grow (Houde 1997) and therefore a biased sex ratio toward males should maximize the fitness of parents. Another hypothesis for the male-biased sex ratio in the food restricted families comes the from population structure of guppies. In their natural habitat, female guppies tend to form shoals of varying sizes, whereas males seeking mating opportunities often decide which group to join (Griffiths and Magurran 1998). In the guppy the percentage of males that emigrated from release pools is higher than the percentage of females (respectively 27% and 7%) leading an accentuated male-biased movement (Croft et al. 2003). Male dispersal tendency could also be further influenced by a male-biased sex ratio in the population of origin. In addition, movement allows individuals to exploit change in resources both in time and in space. Under this scenario, producing more males than females may represent an adaptive strategy to maximize the probability for offspring to encounter a favourable environment. The analysis of o control for potential trade-offs arising from producing more males we measured traits related to condition (body size and capability to escape from a simulated predator), although more abundant did not pay cost of parental diet both in term of condition and of expression of sexual traits compared to males produced by parents raised ad libitum. Thus we showed that producing more males than females was not traded-off with the expression of traits strictly related to male fitness (i.e. sexual traits). Indeed, the expression of such traits was not affected by the diet treatment in which their parents were raised. This results in line with one previous study on guppy (Grether et al. 2008) in which authors failed to find any effect of maternal diet on offspring coloration.

Traits	**Food availability**
Proportion of parturient females	yes
Number of offspring at birth	yes
Number of broods	yes
Offspring mortality	yes
Sex ratio	yes*
Offspring size at maturity	yes
Offspring condition (predator-evasion test)	no
Offspring sexual traits	no

Table 5- Summary of the effects of parental diet treatment. Asterisk indicates that the effect food availability affected only female offspring.

Genotype-by-parental environment interactions

Under the "good genes" scenario, males that possess exaggerated sexual traits should also show higher survival and, more generally, perform better than their less ornamented counterparts (Rowe and Houle 1996). An alternative hypothesis is expected under a life- history scenario since the evolution of a trait is constrained by trade-offs between that and other traits, occurring potentially as a consequence of either the partitioning of resources or genetic constraints (Stearns 1989). However, these trade-offs could also occur in homologous traits of females and in life-history and sexual traits of later generations (Garcia-Gonzalez and Dowling 2015). Indeed, genetic trade-offs may emerge as a reduction in fitness of offspring, as a consequence of negative pleiotropy in genes associated with sexual antagonism (Arnqvist and Rowe 2005). Here we tested this hypothesis under a genotype-by-environment interaction scenario in which both parents experienced food restriction in their early life. In line with the previous study (see Manuscript 1) we found no evidence of negative pleiotropy that may reduce fitness of males investing in sperm (High line). This suggests that genes affecting reproduction in females are not negatively related with genes enhancing investment in reproduction in males. Thus, contrary to what expected under a life-history scenario, sperm number seems to be positively correlated to the overall quality of males without incurring in any costs for females and offspring. Moreover in this study, we highlighted a negative correlation between poor investment in sperm production and fitness offspring. Interestingly we found that offspring produced by Low-R pairs had a lower probability to reach sexual maturity, resulting in a higher percentage of unviable newborns. This certainly has detrimental consequences for fitness of parents. Previous experiments on selected lines failed to find reproductive costs for Low males in standard lab condition but our results suggest that ecological factors may have a crucial role in revealing genetic correlations previously hidden (Hunt and Hosken 2014). An adverse environment (e.g. food restriction) is recognised to increase variance in condition-dependent traits expression (David et al. 2000). However, the strength and the sign of the correlation between sexual traits and condition might change when environmental variation occurs in the population (Qvarnstrom 2001). Here, the genotype fitness ranks (measured as sexual trait expression and as reproductive performance) did not change across harsh and favourable environments, but Low-R group suffered of higher offspring mortality than High-R group. Therefore the difference in relative fitness of genotypes is magnified under harsh environment, suggesting that selection for genes associated to sperm number is even stronger in harsh environmental condition. Thus, genetic variability is likely to be maintained through the relaxation of selection under favourable conditions rather than through trade-offs between genetic variants across environments (Gillespie and Turelli 1989). In this scenario, genotype-by-parental environment interaction may partially contribute to the maintenance of genetic variability for sperm number through a fine-grained environmental variation in food availability.

Altogether these results suggest that genotypes selected for increased sperm production showed no trade-offs associated with investment in a highly costly trait

(Dewsbury 1982, Gasparini et al. 2013). The presence of positive rather negative correlations in sexual traits and in life-history traits (in females) strongly suggests that sperm production act as honest index of male condition (Gasparini et al. 2013). It remains obviously possible that parental diets in High line have negative effects on fitness-related traits of offspring that we did not measure (e.g. fecundity, sperm velocity and viability, sexual behaviour) or that such effects depend on environmental factors that we did not test (e.g. parasites, predators, temperatures). For this reason, further studies are clearly needed to clarify both the potential mechanism(s) that maintain variability in sperm number and the genetic architecture underlying this trait.

Manuscript 3

The effect of perceived mating opportunities on male guppies selected for different levels of sperm production

Silvia Cattelan and Andrea Pilastro

Department of Biology, University of Padova, 35131, Padova, Italy

Abstract

Costs associated with sperm production have been demonstrated in a number of species and come from both direct and indirect evidence. As a consequence, the pattern of resource allocation to ejaculate traits and the allocation of ejaculate itself across different mating episodes are expected to vary under different scenario. Indeed, males are able to allocate strategically their sperm accordingly to mating opportunities to maximise their reproductive success. In this study we manipulated a component of social environment (namely, the presence of females) to experimentally elevate sperm production in males of guppy (*Poecilia reticulata*) artificially selected for high and low sperm number. We investigated whether males selected for different investment in sperm vary their sperm allocation according to mating opportunities. We found that that low sperm males significantly increased their sperm production when they were allowed to see females (perceived mating opportunities), whereas high sperm males maintained their sperm production more constant. However, the difference among selected lines was not enough large to generate a GEI for plasticity in sperm production suggesting that it is unlikely that suggesting that it is unlikely that genetic variation in sperm production plasticity could explain the maintenance of genetic variability in sperm number.

Introduction

In species where females mate with multiple males, the investment in reproduction for a male is not limited to mate acquisition but continues after mating as sperm compete with rival sperm to fertilize eggs (Parker 1998). Therefore, males face energy expenditure in both episodes of sexual selection: precopulatory (mate acquisition traits) and postcopulatory (ejaculate traits). Depending on the species, obtaining a mate can involve different activities for males, as for example searching for females, fighting with other males, and courting females. Costs associated with these activities have been extensively studied so far (Andersson 1994). Costs associated with ejaculate production, instead, have been often overlooked, despite evidence that sperm production represents a significant cost in many species (e.g. Dewsbury 1982, Hayward and Gillooly 2011). Investment in the ejaculate includes sperm production (number) and quality (e.g.

sperm size, velocity and viability) (Pizzari et al. 2008, Parker and Pizzari 2010). Costs associated with sperm production have been shown in a number of species and come from both direct and indirect evidence. For example, males often have a refractory period between ejaculations and when mating frequency is high males can deplete their sperm reserves (Birkhead and Fletcher 1995, Matthews et al. 1997, Olsson et al. 1997, Preston et al. 2001, Rubolini et al. 2007). Environmental stresses, such as diet restriction (Gage and Cook 1994, Vermeulen et al. 2008, Simmons 2012, Rahman et al. 2013, O'Dea et al. 2014) and temperature fluctuations (Breckels and Neff 2013, Vasudeva et al. 2014) have a negative effect on sperm production, suggesting the non-trivial energetic investment of males in sperm production. A decreased in sperm production and quality in many species has also been reported following the genetic stress associated with inbreeding (Konior et al. 2005, Gage et al. 2006, Zajitschek et al. 2009, Gasparini et al. 2013). Taken together this evidence corroborates the idea that sperm production is costly. As a consequence, the pattern of resource allocation to ejaculate investment is expected to vary under different environmental factors and social conditions (e.g. Hosken and Ward 2001, Hunt et al. 2004a).

In many taxa males are able to allocate strategically their sperm accordingly to the mating context (e.g. number of rival males and number and quality of available mates) in order to maximise their reproductive success (Parker and Pizzari 2010, Kelly and Jennions 2011). An example of plastic sperm allocation is provided by sperm priming, a rapid adjustment of the amount of “ready” sperm in response to the number of potential mates (Bozynski and Liley 2003). The capacity of adjusting sperm production or sperm quality in response to female presence has been observed in some species (Shapiro et al. 1994, Bozynski and Liley 2003, Aspbury and Gabor 2004, Scharer and Vizoso 2007, Gasparini et al. 2009). Sperm priming allows males to economize their investment in sperm production when mating opportunities are low or absent. Although there is extended evidence of plasticity in sperm adjustment accordingly to mating opportunities, little attention has been paid to the genetic differences in sperm allocation plasticity. Genetic approaches for studying sperm evolution are essential, and especially those that focus on the genetic covariances among traits, in order to shed light on the genetic architecture of ejaculate traits (e.g. Simmons and Kotiaho 2002, Moore et al. 2004, Birkhead et al. 2005, Snook et al. 2010). Ejaculate traits can exhibit very high levels of additive genetic variance (reviewed by Simons and Moore, 2009), yet most of these traits also show adaptive plasticity. For instance, sperm size is a highly heritable trait and it is subjected to some level of plasticity in at least three species (Immler et al. 2010). In *Drosophila melanogaster,* sperm size varies plastically according to socio-sexual conditions and this plasticity of sperm size has a significant genetic component, as revealed by a significant genotype-by-environment interaction (Morrow et al. 2008). Plasticity in sperm transfer rates show evidence of genetic variability in male scorpionflies (Engqvist 2008).

Here, we experimentally evaluated within-individual plasticity in the number of sperm primed by male guppies (*Poecilia reticulata*) selected for high and low sperm production in varying social contexts. Guppy is an ideal model to look for genetic difference in sperm adjustment under social and sexual stimuli since guppies live in a highly dynamic environment in which social context may vary over time and space (Magurran 2005). Moreover, male guppies show adaptive plasticity both in the quantity and in the quality of the ‘ready sperm’ in response to the perceived mating opportunities (Bozynski and Liley 2003, Gasparini et al. 2009). Although in natural populations males typically encounter multiple females concurrently (Houde 1997), males may become temporarily isolated in pools over a period of days to weeks (Pettersson et al. 2004). For their part, female guppies are highly polyandrous (Houde 1997, Magurran 2005, Neff et al. 2008) and among the traits contributing to male fertilization success, the number of sperm inseminated has been identified as the most important predictor of paternity when two males mate with a female (Boschetto et al. 2011). Multiple evidence confirm that sperm number is the most costly trait among ejaculates traits in the guppy (Gasparini et al. 2013, Rahman et al. 2013, Rahman et al. 2014) and it is likely that males are forced to economize their investment when mating opportunity are absent. Taking advantage from the high heritability of sperm number (Gasparini et al. 2013), we selected males for high and low sperm number (Di Nisio 2014) to investigate if different genetic investments in sperm are associated with different patterns in sperm priming. Considering the high cost of sperm production, we predict that high sperm males show a stronger reduction in the number of ‘ready’ sperm as compared to their low sperm counterparts. To our knowledge, this is the first study that aims to shed light on the genetic correlation between sperm investment and the capability of adjusting sperm production in a short-term scenario. We explored whether a high investment in sperm production (expected to be costly) is associated with a stronger reduction in sperm production when mating opportunities are absent.

Material and Methods

Experimental fish

The guppies used in this experiments were all descendants of wild-caught guppies collected in 2002 from the Lower Tacarigua River in Trinidad. The fish were maintained in stock aquaria (ca. 100 fish/tank) as outbred population to avoiding inbreeding. The water temperature was maintained between 25°C and 27°C and illumination was set on a 12 h⁄12 h light⁄dark cycle. Fish were fed on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplarinS). Males used in this study were 5th and 6th generation descendants of a bidirectional artificial selection experiment for sperm production. Experimental males were kept in large tanks (115L), each containing the same proportion of each sex (approx. 1:1 sex ratio) until required for this experiment. We used 70 males, n=32 from the High line and n=38 from the Low line. At the beginning of the experiment males were 5±1 months old and no differences of age between the selection lines were detected.

Experimental protocol

Experimental males were individually housed in a 8-L tank for 3 days (i) in which males were unable to see any other conspecific male or female for this period. Following 3 days of isolation, males were stripped from their sperm to determine the baseline of the number of sperm. Males were, then, allocated to one of the following treatments for 7 days: (ii) female-present treatment and (iii) no-female treatment. I used a paired experimental design in which each male experienced all the treatments to account for inter-individual variation for sperm production. Indeed, males started the last treatment immediately following the second sperm estimation. Each experimental tank was divided in two sectors by a transparent perforated slide to separate the male from the female in the female-present treatment. One side of the tank was left empty in the no-female treatment. The slide was perforated to allow the exchange of water and any associated chemicals between the fish. We used pregnant, sexually not receptive females to minimize possible differences among males attributable to variation in female responsiveness (Meyer and Liley 1982). On day seven of the experiment we stripped males from their sperm and we repeated the same procedure at end of the second treatment (three sperm strippings were performed in total). At the end of the experiment, males were photographed to measure body size.

Sperm collection and count

Ejaculates were collected three times during the entire experiment. In guppies, sperm are packaged in discrete bundles (spermatozeugmata), each containing about ~21,000 individual sperm cells (Boschetto et al. 2011). To collect sperm bundles, males were anaesthetized in a water bath containing 0.15 g/litre Tricaine methanesulfonate (MS-222) and placed on a black slide under a dissecting microscope. Gentle pressure was then applied to the side of his abdomen, just anterior to the base of the gonopodium, to release sperm bundles in a drop of saline solution (NaCl 0.9%) (Matthews et al. 1997). Afterward sperm bundles were photographed with a digital camera (CANON 40s) to allow the following count using ImageJ analysis software (http://rsbweb.nih.gov/ij/download.html). Sperm number was subsequently calculated from these data to facilitate comparisons with previous work.

Body size measurement

Anaesthetized males were photographed on their left side (along with a scale for calibration) using a Nikon D70s camera (Nikon Corporation, Tokyo, Japan). The distance between the snout and the base of the tail (standard length, SL in mm) was obtained from digital images using ImageJ software.

Statistical analysis

All analyses were performed using SPSS 21.0. All data were normally distributed. To test for an effect of social environment on sperm number, we ran a repeated-measures ANOVA, with the number of sperm across the environments as the response variable, environment, selection line and their interaction as fixed factors. Male size (standard length) and trial order were included in the model as covariates. Male size was included since bigger males are expected to produce more sperm (Pitcher and Evans 2001).

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). The permit 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 (SC) following established procedures, was minimized and was completed under 5 min.

Results

Males from the two selection lines significantly differed in their baseline number of sperm (t-test: $t_{1,68}$=12.261, p<0.001) and the difference remained significant also over the three sperm estimations (repeated measures ANOVA: $F_{1,66}$=79.031, p<0.001) and there was no effect of male body size or trial order on patterns of sperm production (SL: $F_{1,66}$=1.754, p=0.190; order: $F_{1,66}$=0.056, p=0.814). However, males selected for high sperm production did not change their sperm production over the three sperm estimations (repeated measures ANOVA: $F_{1,30}$=0.114, p=0.893) (Figure 1). On the contrary, males selected for low sperm production significantly changed their sperm production over time (repeated measures ANOVA: $F_{1,36}$=12.010, p<0.001). In particular, when Low males were in the presence of a female they produced significantly more sperm than when female stimulus was absent (repeated measures ANOVA: $F_{1,37}$=10.086, p=0.003). Instead, the presence of a female stimulus did not affect number of sperm produced by High males (repeated measures ANOVA: $F_{1,37}$=0.158, p=0.694) . Despite this increase, their number of sperm was still significantly lower than those produced by High males in the same condition (t-test: $t_{1,68}$=5.893, p<0.001). However, the interaction between selection line and treatments (no-female and female-present) was not significant (repeated measures ANOVA: $F_{1,68}$=1.294, p=0.259).

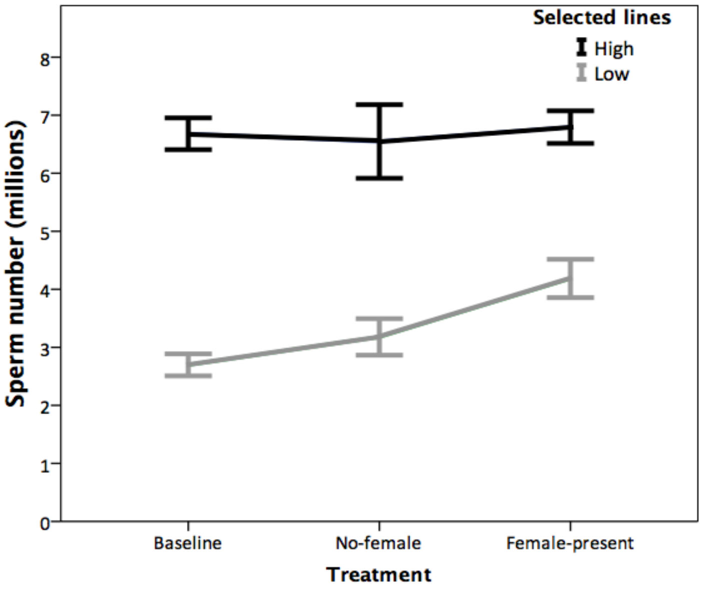


Figure 1 – Number of sperm produced by the males of two selection lines over the three sperm estimations. Bars indicate the standard error of the mean.

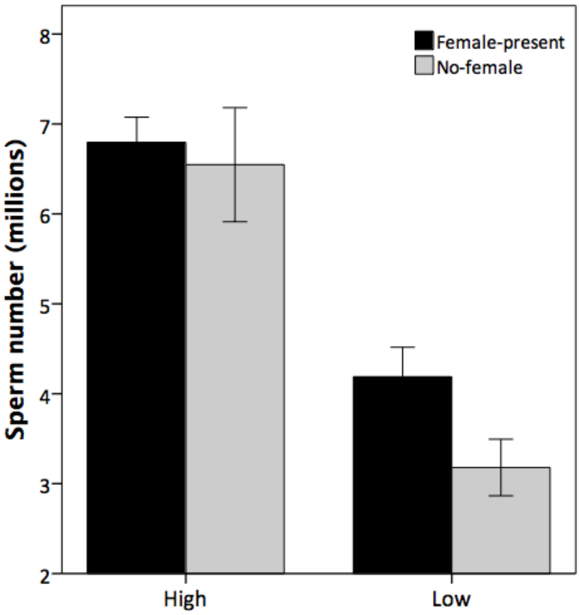


Figure 2 – Number of sperm produced by the males of two selection lines in the two experimental treatments: female-present and no-female. Bars indicate the standard error of the mean.

Discussion

As expected, males from the selected lines strongly differed in their baseline number of sperm produced (Di Nisio 2014). In this species males do not seem to respond to different level of sperm competition (Evans 2009, Barrett et al. 2014) despite the high degree of polyandry in the species (Neff et al. 2008). However, male guppies are able to adjust the number (and velocity) of their “ready” sperm in the presence of female stimulus (Bozynski and Liley 2003, Gasparini et al. 2009). Although this, High males did not show an increase in their sperm production when they were able to see females. On the contrary, Low males significantly increased the number of sperm primed, suggesting a stronger sperm priming effect in the Low line rather than in the High line. Low males seem to be more able to adjust the amount of their "ready" sperm according to mating opportunities (i.e. the presence of female). Therefore, they respond to an increased level of mating opportunities by reducing their initial disadvantage in the baseline sperm production. Although this, we failed to find a significant genotype-by-environment interaction for sperm production, suggesting that it is unlikely that genetic variation in plasticity could explain the maintenance of genetic variability in sperm number. It remains unclear, however, why High males apparently lack the capability to adaptively adjust their sperm production in response to varying mating opportunities.

There are some hypothesis, no mutually exclusive, to explain this result. First, the difference in sperm priming effect between lines could be attributable to a differential increase in hormone levels between males from the two selection lines in response to female presence. Indeed, there is evidence that presence of females promotes an increase in circulating levels of testosterone (Pinxten et al. 2003), which in turn may stimulate a change in both sperm production and social interactions. Testosterone (or more generally gonadal androgens) is associated with spermatogenesis in male guppies (Pandey 1969, Billard 1986), while in other species, is associated with aggressive and dominance behaviour (Batty 1978, Wingfield et al. 1987, Mills et al. 2007, Buchanan et al. 2010). The significantly increase in the number of available sperm in Low males may be associated with a larger increase in the level of circulating testosterone. This in turn may also affect the tendency of low males to behave aggressively to achieve a dominance status. It is reasonable to suppose that Low males could reduce their initial disadvantage in sperm production both increasing their availability of sperm in the presence of females and enhancing their effort in precopulatory male-male competition. Clearly this hypothesis needs further investigations. Secondly, plasticity is expected to be advantageous depending on environmental conditions that males may encounter throughout their life. In highly dynamic environment with fine-grained environmental variations (i.e. environmental variations within a generation) selections should favour males with high level of plasticity that can adopt the optimal phenotype across environments. Thus, when the optimal phenotype varies with environmental conditions experienced, phenotypic plasticity is predicted to be advantageous. However, there are some benefits associated with the increase of sperm number by Low males, this plasticity may also have some costs. In this species we have evidence of a reduction in the number of live sperm in males that increased their sperm number after being exposed to female presence (Cardozo et al., in prep). Moreover, male guppies adjust their mating tactics according to short-term changes in sperm production triggered by a change in socio-sexual conditions. Indeed, males in presence of a female stimulus relied relatively more on the less costly mating tactic via gonopodial thrusts than on courtship displays for mating acquisition compared to when they were in the no-female treatment resulting in a trade-off between pre- and postcopulatory episodes of sexual selection (Cattelan et al. 2016). A similar study conducted in a longer period (four months) showed that when mate encounter rates are experimentally heightened there is a concomitant investment in postcopulatory traits (sperm number) and a decline in courtship in favour of forced matings (Devigili et al. 2015a).

In a long-term scenario, we may expect to observe evolutionary trade-offs between ejaculate production and other costly traits such as those involved in precopulatory competition or in traits involved in survival and somatic maintenance (e.g. Simmons 2012). Indeed, individuals vary in life span and senescence rate and the pattern of this variation may be influenced by the investment in reproduction in term of both sexual traits and reproductive effort (Bonduriansky et al. 2008). The variation in sexual traits investment may generate different life-trajectory strategies: under a “live fast-die young” scenario, for instance, males investing in reproduction in they early life should pay a cost in their senescence and longevity, ultimately reducing their lifetime reproductive success (e.g. Hunt et al. 2004a). In the guppies a negative pleiotropic correlation between attractiveness and offspring survival has been found (Brooks 2000) and we have also evidence that differential investment in a highly costly ejaculate trait reflects substantial variation in longevity and senescence rate among individuals (Di Nisio 2014).

As we mentioned above, plasticity itself may respond to natural or sexual selection whether there is genetic variability in plasticity among individuals. When high plasticity confers a benefit that outweighs the cost of plasticity, positive directional selection can operate on plasticity. Selection favours individuals that flexibly adjust their optimal behaviour in response to changing environments. On the contrary selection may also favour the lack of plasticity whether it is expected that a single phenotype to be optimal across environments. Indeed, although the literature on plasticity has shown that one of the most important selective factors for phenotypic plasticity is environmental variation (Snell-Rood 2013), it is unlikely that producing a low number of sperm could be adaptive in some environments. It is reasonable that the capability to adjust the number of “ready” sperm was favoured since males are unable to sustain the cost of producing high sperm number over time. On the contrary High males seem to be able to sustain this cost, suggesting the positive correlation between sperm production and the genetic quality of males, as predicted by good genes model of sexual selection (Neff and Pitcher 2005). Future works will need to further investigate whether sperm production may be a good indicator of male genetic quality (as observed until now) and how genetic variability in this highly costly trait is maintained in our population since genetic variation in plasticity does not seem crucial for the maintenance of genetic variability in the number of sperm.

Manuscript 4

Genotype-by-social environment interactions and the maintenance of genetic variability in sperm number in the guppy

Silvia Cattelan*[1] - Alessandro Devigili*[1], Francesco Santi[1], Elia Gatto[2] and Andrea Pilastro[1]

[1] Department of Biology, University of Padova, 35131, Padova, Italy

[2] Department of Psychology, University of Padova, 35131, Padova, Italy

** These authors contributed equally to the manuscript*

Abstract

Genotype-by-environment interaction (GEI) occurs whenever the relative performance of different genotypes depends on the environment in which they are expressed. GEI theories have an outstanding importance in evolutionary biology as they could potentially explain how genetic variability in sexual traits is maintained, despite strong directional selection. Among the possible environments, social environment is expected to generate large GEIs since sexual selection trajectories are particularly sensitive to variations in male-male competition and female mate choice. Here we tested whether variations in sex ratio may explain part of the existing genetic variability in sperm number in the guppy, *Poecilia reticulata*. In this highly polyandrous species, sperm competition leads to strong directional selection on sperm number, a trait that shows large phenotypic and genetic variability. To test whether the large genetic variability in sperm production observed in guppies may be maintained if mating opportunities vary, we compared the fitness response of genotypes expressing high and low sperm number to two levels of mating opportunities (throughout sex ratio variations). As expected, High males performed better in the female-biased treatment. However, contrary to prediction, Low males sired significantly more offspring than their High counterparts at the *equilibrium* sex ratio. This result suggests that the social environment has an important effect on the interaction between sperm number and reproductive success, generating a GEI that may potentially contribute to explain the large genetic variability for sperm number observed in this fish population. Here we discussed the possible mechanisms responsible for the higher reproductive success of Low males.

Introduction

The problem of the maintenance of the additive genetic variability underlying sexual traits has been typically investigated in a precopulatory context (Radwan et al. 2015). However, whenever females are sexually promiscuous, sperm competition leads to strong directional selection on traits involved in fertilisation success. Among ejaculate traits, the number of sperm produced, typically increases in response to sperm competition pressure (Parker and Pizzari 2010). The strong directional selection acting on ejaculate traits and in particular on sperm number should erode their genetic and phenotypic variability. In contrast, empirical evidence indicates that genetic variability for sperm number is usually unexpectedly high (reviewed by Evans & Simmons, 2008). This is surprising also because producing large ejaculate represents a significant cost for males (Dewsbury 1982, Hayward and Gillooly 2011), especially in species with high degree of sperm competition and an optimal sperm investment should be selected. Indeed, growing evidence indicates that males can deplete their sperm reserves (Birkhead and Fletcher 1995, Matthews et al. 1997, Olsson et al. 1997, Preston et al. 2001, Rubolini et al. 2007) and, as a consequence, males of highly polyandrous species are limited not only in the number of mating they can achieve (Borgia 1979), but also in the number of females they can successfully inseminate. As a consequence of costly sperm production, pattern of resource allocation is expected to vary under different scenario depending both on abiotic (e.g. food availability) and biotic (e.g. predation, intraspecific social interactions) environmental factors (Hunt et al. 2004a). Environmental factors are known to influence on the expression of sexual traits (Jia et al., 2000) and may also play a crucial role in preventing one genotype from producing the optimal phenotype across all the possible environments (the so-called genotype by environment interaction-GEI). Therefore, it has been suggested that the genetic variability for traits subject to directional (sexual) selection may be explained by GEIs (Gillespie and Turelli 1989, Kokko and Heubel 2008).

While largely studied in a natural selection context, the studies of GEIs in a sexual selection context are much less numerous, and largely limited to GEIs resulting from ecological environmental heterogeneity, such as that deriving from variations in food availability and ambient temperature (Ingleby et al. 2010). These factors are certainly of central importance in many species, in particular in a global change context. However, sexual selection trajectories are known to be particularly sensitive to variations in the social context, since male-male competition and female mate choice are typically affected by factors like sex ratio and population density (Shuster and Wade 2003). Social environment is therefore expected to generate large GEIs associated with reproductive fitness (Hunt and Hosken 2014). The relatively few studies that have attempted to identify GEIs associated with the social environment in sexually selected traits have usually found quite strong GEIs (Jia 2000, Danielson-Francois et al. 2006, Mills et al. 2007, Kent et al. 2008, Morrow et al. 2008). For instance, sex ratio could affect both male-male competition and male mating opportunities, on one hand, and female choice and female mating rate, on the other hand, influencing pre- and postcopulatory sexual selection episodes (Edward et al. 2011). For instance, the decline in male mating opportunities for males may be accompanied by an increase in female choosiness and male-male competition, leading to an increased variance in male mating success and ultimately in a stronger precopulatory selection. In contrast, a male biased-sex ratio may determine an increase in female mating rate, moving the focus of sexual selection from the precopulatory to the postcopulatory male traits. When the sex ratio is calculated as the number of sexually active males to the total number of receptive females and sexually active males (Kvarnemo and Ahnesjö 1996) is known as operational sex ratio (OSR) and ranges from 0 to 1.

In this study, we tested whether variations in OSR might explain part of the existing genetic variability in sperm number in the guppy, *Poecilia reticulata*. In this highly polyandrous species, sperm competition leads to strong directional selection on ejaculate traits. Among the ejaculate traits associated with fertilisation success, the number of sperm delivered during copulation is the most important predictor of paternity (Boschetto et al. 2011). Sperm number (i.e. the number of sperm available for copulation in a rested male) shows large phenotypic variability that it is also associated with a large additive genetic variance (Gasparini et al. 2013). Sperm production is the most costly ejaculate trait in this species, and strongly depends on resource acquisition, compared to sperm quality traits, such as sperm size, viability and velocity (Devigili et al. 2012, Gasparini et al. 2013, Rahman et al. 2013, Rahman et al. 2014). Considering the cost of producing sperm, and the postcopulatory advantage of producing numerous sperm, the large genetic variability in sperm production observed in guppies may be maintained if mating opportunities vary unpredictably during a male's lifetime and the postcopulatory advantage of producing large sperm numbers is evident only when mating opportunities are high (i.e. the OSR is female-biased). Indeed, mean sperm reserves of low sperm males (Di Nisio 2014) are larger than the mean number of sperm delivered by one male in a single copulation, but may not be sufficient to avoid sperm depletion when more copulations occurs over a short time (Pilastro and Bisazza 1999, Pilastro et al. 2007). In contrast, a high sperm male may successfully inseminate several females without incurring in sperm depletion. Therefore, we predicted that when mating opportunities (i.e. females) are high, males with higher sperm production have a higher reproductive fitness as compared to their low sperm counterparts. On the contrary, when mating opportunities are scarce, we expect a decline in the importance of sperm production in determining male's reproductive fitness. If a male's sperm allocation is traded-off against his precopulatory male-male competitive capability, we may even expect that low sperm males have a higher reproductive fitness than high sperm males when the OSR is more balanced. To test this hypothesis we compared the reproductive fitness of different genotypes producing respectively high and low sperm numbers to two levels of OSR: female biased (OSR=0.25) and *equilibrium* (OSR=0.5). These two OSR values were within the range of variation found in natural guppies population (Pettersson et al. 2004). In particular, we paired males from two artificially selected lines for high and low sperm number and we established a paired experimental design in which each pair of males experienced the two OSRs consecutively (in haphazard order). Considering the importance of sperm number in postcopulatory success (Parker and Pizzari 2010), we expected that the genotype producing high sperm number fertilized a greater proportion of eggs when the risk of sperm depletion was higher (female-biased OSR). Symmetrically, we expected that the importance of sperm number decreased as the risk of sperm depletion declines (*equilibrium* OSR).

Materials and Methods

Experimental fish

All fish were descendants of wild-caught guppies collected in 2002 from the Lower Tacarigua River in Trinidad, a high-predation site where guppies coexist with several predator species. The fish were maintained in stock aquaria (ca. 100 fish/tank) as outbred population. The water temperature was maintained between 25°C and 27°C and illumination was set on a 12 h⁄12 h light⁄dark cycle. Fish were fed on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplarinS). Males used in this study were 4th and 5th generation descendants of a bidirectional artificial selection experiment for sperm production (see Di Nisio 2014). We established 21 experimental pairs of males (n=11 from the 4th generation and n=10 from the 5th generation) composed by one male from high sperm production line (High) and the other from the low sperm production line (Low). Each selection line consisted in two replicates (A and B) and we used an equal number of males from each replicate (n=22 males from replicate A and n=20 from replicate B). Before starting the experiment, males were individually housed in 8-L tank with a non-virgin female to maintain male sexual interest. At the beginning of the experiment males were 7 ± 1 month old and no difference of mean age between the selected lines was detected.

Experimental protocol

Males were initially isolated for ten days to ensure the full replenishment of sperm reserves (Pilastro et al. 2004). Afterwards, two males from the same generation and the two selection lines were randomly paired (i.e. each pair was formed by one High and one Low male from the same generation of artificial selection) resulting in 21 pairs. Each pair of males was tested in two different OSR conditions: female-biased (each pair with 6 females; OSR=0.25) and *equilibrium* (each pair with 2 females; OSR=0.5). We used virgin females to avoid paternity deriving from stored sperm of previous matings and to ensure that females were sexually receptive. Each pairs of males was randomly allocated to one of the two OSR treatments and was housed into 125-L tank with the respective number of virgin females (2 or 6). 10 of the 21 pairs started first with the female-biased treatment and the others 11 with the *equilibrium* treatment. Males and females were free to interact for the following 11±3 days (no differences in fitness due to time spent with females were detected). During the mating period each tank was daily monitored to assess the sexual interest for the other sex. At the end of the interaction period females were individually isolated. After giving birth offspring were collected and euthanized for later paternity analysis. Each pair of males started the second mating round in the alternative OSR condition immediately following the first one. After completing both OSR mating tests males were individually isolated for ten days and then were photographed for subsequent morphological analyses. Males were stripped of sperm to perform ejaculate quality assays (sperm count and sperm velocity). Morphological analyses (see below for the traits considered) were performed to control for differences among males that may account for differences in their reproductive fitness other than differences in sperm number. Sperm number was assessed to ensure the actual difference between males from the two selection lines. Fin clips were collected from females and males and kept in ethanol for later molecular paternity analysis.

Morphological analysis

Males were anaesthetized in a water solution of MS-222 (0.15 g/L) and digitally photographed on the left side of each individual's body, using a Canon 450D placed on ZEISS Stemi 2000-C stereomicroscope. Measurements were made from the digital images using image analysis software (ImageJ: http://rsbweb.nih.gov/ij/download.html). We measured the body area (fin excluded) and the fin area, the distance between the snout and the base of the tail (standard length, SL), and the total area covered by three different colours. In particular we considered orange and yellow coloration (hereafter "orange"), structural iridescent coloration (blue, green, violet, hereafter "iridescent"), and melanic black spots (hereafter "black"). The relative area of each coloration was calculated as the ratio between the area covered by each coloration and male's total body area.

Sperm collection and count

In guppies, sperm are packaged in bundles, each containing about 21.000 individual sperm cells (Boschetto et al. 2011). To collect sperm bundles, the anaesthetized male (see above) was placed on a black slide under a dissecting microscope. Gentle pressure was then applied to the side of his abdomen, just anterior to the base of the gonopodium, to release sperm bundles in a drop of saline solution (NaCl 0.9%) (Matthews et al. 1997). Afterward sperm bundles were photographed to the following count using ImageJ (see above). Sperm number was subsequently calculated from these data to facilitate comparisons with previous work.

Sperm velocity analysis

Sperm velocity was analyzed immediately after sperm collection, bundles were activated with 40 µL of 150 mM KCl solution in 2 mg/L bovine serum albumin (Billard and Cosson 1990) and placed in a 12-cell multiset slide (MP Biomedicals) coated with a 1% polyvinyl alcohol to prevent sperm from sticking to the glass slide. The swimming velocity was measured with a Hamilton-Thorne CEROS Sperm Tracker (for the setting parameters see Gasparini et al. 2013) for three standard measures. These measures included: average path velocity (VAP), which estimates the average velocity of sperm cells over a smoothed cell path; straight line velocity (VSL), the average velocity on a straight line between the start and the end point of the track and curvilinear velocity (VCL), the actual velocity along the trajectory. These measures provide an estimate of progressive velocity and have been shown to positively correlate with fertilization success in this guppy population (Boschetto et al. 2011).

Molecular paternity analysis

DNA was isolated from the tail for adults by Salting Out extraction (Miller et al. 1988). We genotyped all the mothers (n=39 from the *equilibrium* treatment and n=106 from the female-biased treatment) and all the pairs of males (21 pairs, n=42 in total). We isolated the offspring DNA from the whole body of offspring (n=1058) by CHELEX extraction (Walsh et al. 1991). We used two microsatellite loci to assign paternity within each pair of males. The loci used in this experiment were highly variable loci and the primers were labeled with two fluorescent markers (see Table 1 for details). PCRs were performed through an established cycling protocol (Devigili et al. 2015a) and using the GeneAmp PCR System 9700 Thermocycler. PCR products were run on ABI PRIMS DNA Analyzer 3100/3700 sequencer (ABI PRISM, Applied Biosystems), using filter 400 HD ROX (Perkin-Elmer, Applied Biosystems) as a size standard. Fragments lengths were determined using Peak Scanner software (http://www.appliedbiosystems.com) and paternity was assigned using CERVUS 3.0 (http://www.fieldgenetics.com). Paternity was assigned with 95% confidence in 1047 genotyped offspring (99%).

Statistical analysis

Statistical tests were performed using SPSS 21. We used general linear models (GLMs) to test for differences in morphological and ejaculate traits among males from the selected lines. When data were not normally distributed, we used appropriate transformations. All models included the pair of males as random factor, and selection line as fixed factor. To test for differences in fitness between selection lines we considered the proportion of offspring sired by each male from the High lines on the total offspring produced by the females (within pair) in each OSR treatment. We used a generalized linear mixed model (GLMM) with a binomial error distribution in which the number of offspring sired by High males was the binomial response and the brood size the binomial total. The model included the pair of males as random factor and OSR treatment as fixed factor.

Microsatellite locus	Bp range	No. of alleles	GenBank accession no.	Ta (°C)	Reference
TTA	102-163	15	AF368429	52	(Taylor et al. 1999)
Agat11	240-371	21	BV097141	56	(Olendorf et al. 2004)

Table 1 - Microsatellite loci used to assign paternity.

Ethical note

This experiment was conducted according to the Italian legal requirements and was approved by the Ethics committee of the University of Padova (permit no. 36/2011 to AP). The fish were fully anaesthetized before sperm extraction and phenotypic measurement. Manipulation, which was conducted by expert operators (AD and SC) following established procedures, was minimized and was completed under 5 min. Fish were humanly killed with an overdose of MS-222 following the dosage reported for the guppy (Chambel et al. 2013).

Results

Pre- and post-copulatory traits

As expected, the males from the two artificial selection lines differed significantly for the number of sperm at rest (Di Nisio 2014) (GLM: $F_{1,15}$=17.885; *p*=0.001, see table 2). There was no correlation between male standard length and the number of sperm produced (SL, GLM: $F_{1,15}$=0.371; *p*=0.552). Sperm velocity did not differ between High and Low males, as well as standard length and body area (see table 2). Moreover, we failed to detect significant difference in the relative area of orange coloration (calculated as the ratio between area of orange coloration and body size). Overall, the males from the selection lines used in this experiment differed significantly only for sperm number (Di Nisio 2014).

	High males	**Low males**	**df**	**F**	***p***
Standard length (mm)	17.49 ±0.36	17.06 ±0.29	37	1.067	0.309
Body size (mm^2)	72.99 ± 2.18	70.58 ± 2.52	37	0.521	0.475
Orange relative area (%)	7.96 ± 0.85	8.31±0.54	37	0.117	0.734
Sperm velocity µm/s	94.06±2.35	97.13 ±1.23	37	1.067	0.308
Sperm number[a]	12.03±1.95	6.16±1.23	37	9.262	**0.004**

Table 2. Results from the multivariate GLM testing the difference between High and Low. Significant values are shown in bold. [a] Sperm number was square-root transformed to meet normal distribution assumption.

Reproductive success

We quantified the reproductive success of the males from the two selection lines only in the females that produced a brood (*equilibrium* OSR: 39 out of 42, 92%; female biased OSR: 106 out of 126, 84%). Females that successfully gave birth did not differ between OSR treatments in their body size (GLM: $F_{1,57}$=-0.977, p=0.327) and neither the time to the first brood (GLM: $F_{1,119}$=0.388, p=0.534). However, females differed in the number of offspring produced between OSR treatments (GLM: $F_{1,115}$=3.514, p=0.015), probably due to the cost imposed by the interactions with other females (Borg et al. 2006). High males did not sire offspring in 4 of the 21 pairs in both the OSR treatments, whereas in only one pair they sired all the offspring produced by females in both the OSR treatments. In 2 pairs High males did not sire offspring in the *equilibrium* OSR, whereas in one the High male sired all the offspring in the *equilibrium* OSR (see Figure 1). There was a significant effect of OSR on the reproductive success of High males (see Table 3). As expected, High males performed better than Low males at the female-biased sex ratio (see figure 2). In contrast, High males sired significantly fewer offspring than Low at *equilibrium* OSR (GLMM: $ß_1$=-0.816, p<0.0001). There was a significant effect of the pair of males but not of the treatment order, on the reproductive success of males (see table 3).

	Statistics	**df**	***p***
OSR	25.197	40	<0.001
Pair	2.584	40	0.010
OSR order	1.963	39	0.169

Table 3 – Results of the GLMM testing the effect of OSR variations on High males reproductive success. In the model was included also the pair of males and the order in males experienced the two OSRs.

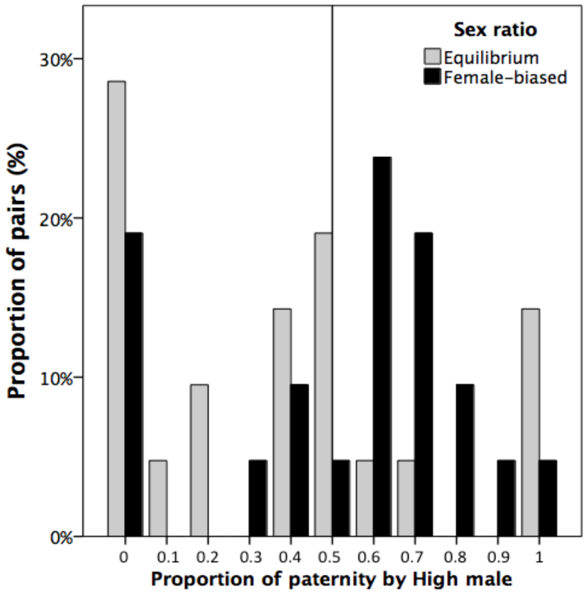


Figure 1 – Distribution of paternity sired by the High males, expressed as the proportion of offspring sired in each OSR treatment.

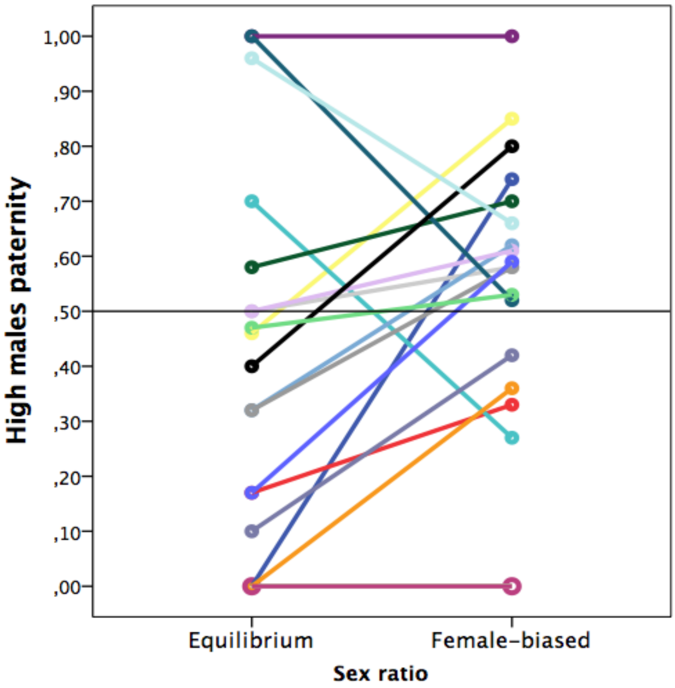


Figure 2 - Reaction norms for reproductive success of High males expressed as the proportion of offspring sired in each OSR treatment. Each line represents a pair of males replicate. 4 replicates are combined in the lowest line since High males had the same reproductive success.

Discussion

We chose to manipulate a component of social interactions since the mechanisms driving sexual selection are based on interactions between individuals (Wolf et al. 1999). The social context typically affects both male-male competition and female mate choice, all of which influence sexual selection (Andersson 1994). Indeed, it is expected that a phenotype's reproductive performance is influenced by the social context and that different phenotypes may vary in their relative performance across different social contexts. If these phenotypes are heritable, significant GEIs should therefore be expected. However, only a few studies have attempted to test for GEIs in sexual selection through experimental manipulation of social environments. These studies investigated the effect of early competition through a manipulation of larval density, litter size and sexual environment (Jia 2000, Miller and Brooks 2005, Danielson-Francois et al. 2006, Mills et al. 2007, Morrow et al. 2008). Collectively, the occurrence of GEIs seems to be common but in general ecological-crossovers among treatments appear to be rare (Jia 2000, Mills et al. 2007). Here, we manipulated the OSR, as it affects both the degree of male-male competition and potentially all the traits involved in female choice (i.e. preference function and choosiness) (Jirotkul 1999). Furthermore, sex ratio also potentially affects the postcopulatory success, as, for instance, sperm number benefits and sperm depletion risks are expected to vary according to the OSR and the sperm production genotype. In this species large adult sex ratio fluctuations occur over time and space (Pettersson et al. 2004) and the two sex ratio conditions considered in our experiment are within the range of natural variation.

We found that when mating opportunities are lower (*equilibrium* OSR), High males sired a significantly lower proportion of offspring than Low males. On the contrary High males significantly increased their reproductive success when females are abundant (female-biased OSR). These results indicate that the reproductive fitness of genotypes producing high and low sperm number significantly changes across social environments. To our knowledge this is the first evidence showing a genotype-by-environment interaction in the realized reproductive success rather than based on the expression of sexual traits (Hunt and Hosken 2014). The measure of GEI in the expression of sexual traits may be a poor estimate of reproductive fitness, as this is influenced by the interaction of phenotypes. Although in a simplified experimental condition (2 males with 2 / 6 females is not an usual condition in nature since guppy shoal usually contain several males), we were able to identify a difference in reproductive fitness across OSR conditions that was, at least partly, in the direction predicted on the basis of the characteristics of the males from the two selection lines (Di Nisio 2014) and the known pre- and postcopulatory processes occurring in this species (Evans and Pilastro 2011). Indeed, we expected that High males had on average a higher reproductive fitness than Low males, and that they performed relatively better in a female-biased OSR. Our results partly confirmed this prediction since High males reproductive success was relatively higher in female-biased OSR than at the *equilibrium*. However, Low males had an overall higher reproductive success. The specific mechanism responsible for the higher reproductive success of Low males is unknown. The stronger increase in sperm production by Low males in response to female presence (see manuscript 3) suggests that Low males may reduce their initial numerical sperm disadvantage. In this guppy population (from the lower part of the river Tacarigua in Trinidad) phenotypically attractive males produce more sperm faster (Matthews et al. 1997, Pitcher and Evans 2001) and more viable sperm (Locatello et al. 2006). Moreover, attractive males inseminate more sperm than their less attractive counterparts (Pilastro et al. 2002) since the number of sperm inseminated during solicited copulations is influenced by the female’s perception of male attractiveness (Pilastro et al. 2004). As a result, attractive males have higher success in sperm competition both in natural (Evans and Magurran 2001) and in artificial insemination (Evans et al. 2003). Thus, precopulatory sexual selection is reinforced also during postcopulatory episode. Although the number of sperm inseminated by males is known to be the most important predictor of sperm competition (Boschetto et al. 2011) we found that males producing more sperm had an equal or lower success in sperm competition compared to males producing less sperm. However, Boschetto and colleagues’ experiment controlled for precopulatory effects that could interfere with sperm competition output by artificially inseminating the females. In our setting males could interact with each other and the matings could be influenced by female choice.

During precopulatory mate choice, females prefer large (Reynolds and Gross 1992) and colourful males (in particular those with large orange spots) (Evans et al. 2004) with high rates of courtship (Houde 1997). Males from the selection lines used in this experiments did not significantly differ in their orange coloration and in their body size. We did not quantify male courtship activity during the mating trials, but previous observations suggest that High males are sexually more active and perform more courtship displays (Di Nisio 2014). Although it is generally considered that inter sexual selection (via female choice) plays a major role in the guppy mating system, it has also been shown that also male-male competition, under some conditions, may affect male mating success (Kodric-Brown 1992, 1993, Price and Rodd 2006). Guppies do not defend territories but antagonistic behaviours, such as jockeying for position behind females and chasing and biting the male competitor (Kodric-Brown 1992), can be observed. In contrast, female mate preference for dominant males seems unimportant (Kodric-Brown 1993). However, aggressive and dominant males may have a higher mating success (Price and Rodd 2006) by monopolizing mating opportunities (Kodric-Brown 1993) and this effect seems more important under male-biased OSR conditions (Jirotkul 1999). Previous observations on male-male interaction did not reveal any difference in dominance between the selection lines (Di Nisio 2014). However, in our experiment, pairs of males interacted for 11 days, and it could be hypothesized that one of the two males in the pair (more often the Low male) attained a dominant status over the other male, monopolizing mating opportunities and excluding the competitor from accessing to females. This could explain the greater reproductive success of Low males. Moreover another possible explanation could come from the difference in sexual behaviours among males of the selected lines. It has been observed that High males tend to perform more coercive mating attempts via gonopodial thrust than Low males (Di Nisio 2014) and it is possible that we overlooked the importance of gonopodial thrusts in determing the reproductive success of High males. Our setting is likely to be favoured the adoption of courtship behaviours instead of gonopodial thrusts since females were all sexual receptive (Pilastro and Bisazza 1999), unintentionally disfavouring those males that mainly gain mating by adopting gonopodial thrusts. Whether such an explanation applies to selected lines awaits further attention.

Another hypothesis comes from the post-meiotic senescence of sperm cell (Reinhardt 2007) as sperm ageing may deleteriously affect a range of fitness traits, including fertilization ability (Jones and Elgar 2004). In the guppy, sperm storage by males compromises sperm velocity (Gasparini et al. 2014), a predictor of sperm competitiveness in this species (Boschetto et al. 2011). The time-frame we chose (11 days ca) allowed males to replenish their sperm reserves with fresh sperm when sperm depletion occurs (Kuckuck and Greven 1997). Since High males had significantly higher sperm reserves than Low males they were unlikely to incur in sperm depletion. On the contrary, Low males are expected to use a larger part of their sperm reserves and may be therefore have a higher turnover of their sperm reserves. This may have resulted in a more balanced fitness among genotypes under the experimental condition considered.

These two hypotheses, male-male competition and faster turnover of sperm reserves, may concur explaining our results. Clearly, these explanations need to be tested. Whatever the explanation for the observed overall pattern in male reproductive success across selection lines, our results suggest that the strength of directional selection on sperm number may vary in different social environments. Thus, one explanation of the maintenance of the genetic variability in sperm number may the strong variation in the sex ratio with its associated effects on sexual selection dynamics (Grether et al. 2001b, Pettersson et al. 2004). Guppy populations live in highly dynamic habitats, in which resource availability, predation regime, water level and sex ratio fluctuations occurring at a fine-grained scale, may continuously vary the fitness of different genotypes and maintain the additive genetic variability underlying sperm number.

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The effect of sperm production and mate availability on patterns of alternative mating tactics in the guppy

Silvia Cattelan [a, *], Jonathan P. Evans [b], Andrea Pilastro [a], Clelia Gasparini [a, b]

[a] *Department of Biology, University of Padova, Padova, Italy*
[b] *Centre for Evolutionary Biology, School of Animal Biology, University of Western Australia, Nedlands, Australia*

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Theory predicts that in polyandrous species males that invest more in traits enhancing postcopulatory success should have less energy to invest in mating acquisition, leading to trade-offs between pre- and post-copulatory episodes of sexual selection. Although such trade-offs are well studied, the potential constraints that postcopulatory sexual selection imposes on alternative mating tactics are rarely considered. In guppies, *Poecilia reticulata*, individual males can obtain matings by performing energetically costly courtship displays or by using less costly forced copulations. In this study we manipulated a component of social environment (namely, the presence of females) to experimentally elevate sperm production in males. We found that male guppies rapidly compensated by reducing their reliance on courtship in favour of forced matings. As both tactics differ in mating and fertilization success, the consequences of this trade-off may have important ramifications for male reproductive fitness.

The energetic costs associated with mating are well documented for males (Dewsbury, 1982; Hayward & Gillooly, 2011). However, as females typically mate with several males during a single reproductive period (polyandry; Simmons, 2005), males must also devote energy towards traits that maximize fertilization success in the light of sperm competition, in which ejaculates from different males compete to fertilize eggs (Parker, 1970). Theory therefore predicts that investment in one component of sexual selection (e.g. in acquiring mates) will come at the cost of investment in other components (competing for fertilizations), leading to a trade-off in investment towards these successive episodes of sexual selection (Parker, 1998).

Although trade-offs in sexual selection are widely documented (e.g. Dowling & Simmons, 2012; Immler et al., 2011), there has been surprisingly little attention paid to individual level changes in ejaculate expenditure according to variation in the costs incurred in acquiring mates (Parker & Pizzari, 2010), and this is especially so in the case of alternative mating tactics (ARTs). Among individual males, different ARTs are often associated with distinct patterns of ejaculate investment, for example where small sneaker males invest more in sperm production than their larger territorial counterparts (e.g. Locatello, Poli, & Rasotto, 2013). In such cases, ARTs are typically 'fixed', meaning that they are determined genetically, morphologically or physiologically. Males exhibiting fixed ARTs will therefore have limited flexibility in adjusting their behaviour and are consequently likely to be constrained in their patterns of ejaculate investment. However, in some species males are able to adjust mating tactics according to environmental, social (e.g. female quality or responsiveness) or condition-related (e.g. parasitic state, food or energy availability) factors, and this may provide the opportunity for concomitant rapid changes in ejaculate expenditure to balance these changing energetic demands in acquiring mates (e.g. Pizzari, Cornwallis, & Froman, 2007; Rudolfsen, Figenschou, Folstad, Tveiten, & Figenschou, 2006; Smith & Ryan, 2011).

In this study we tested whether individual level changes in sperm expenditure triggered by a manipulation of mate availability cause rapid changes in male mating tactics in the guppy, *Poecilia reticulata*, a livebearing fish in which males use either courtship (via sigmoid displays) or forced matings (gonopodial thrusts) interchangeably according to prevailing social conditions (Jirotkul, 1999; Magellan & Magurran, 2007). Male guppies are capable of rapid adjustment in ejaculate investment according to the presence or

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* Correspondence: S. Cattelan, Department of Biology, University of Padova, Via U. Bassi 58/B, 35131 Padova, Italy.
E-mail address: silvia.cattelan.2@studenti.unipd.it (S. Cattelan).

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absence of females, with female presence promoting increased investment in ejaculates, in terms of both sperm production and sperm quality (Bozynski & Liley, 2003; Gasparini, Peretti, & Pilastro, 2009). For their part, female guppies are highly polyandrous (Neff, Pitcher, & Ramnarine, 2008), and among the traits contributing to male fertilization success, the number of sperm inseminated has been identified as the most important predictor of paternity when two males mate with a female (Boschetto, Gasparini, & Pilastro, 2011). Experiments involving different diet levels also confirm that there is a high cost of sperm production in this species (Gasparini, Devigili, Dosselli, & Pilastro, 2013; Rahman, Kelley, & Evans, 2013; Rahman, Turchini, Gasparini, Norambuena, & Evans, 2014). This plasticity in both mating behaviour and ejaculate traits, coupled with the demonstrated high costs of ejaculate production, provide the impetus for investigating trade-offs between these two components of pre- and postcopulatory sexual selection.

We used an established experimental design that promotes an increase in sperm investment (see Bozynski & Liley, 2003; Gasparini et al., 2009) to determine whether male guppies compensate for increased investment in ejaculates (and the concomitant availability of females) by adjusting mating tactics over a 7-day treatment period. Our study builds on recent work by Devigili, Doldan-Martelli, and Pilastro (2015) revealing trade-offs among precopulatory, postcopulatory and somatic investment in males exposed to familiar or unfamiliar females over a far longer duration (4 months). Furthermore, we assessed male swimming performance to test for potentially confounding effects of female exposure on male activity levels (i.e. independent of sperm investment) which may in turn influence male sexual behaviour (Nicoletto, 1993). We accounted for the natural variability among males in sperm production (Gasparini et al., 2013; Rahman, Gasparini, Turchini, & Evans, 2015) and the genetic propensity to preferentially adopt one mating tactic over the other (Evans, 2010) by employing a paired experimental design, in which each male experienced both treatments consecutively (in haphazard order). We predicted that males assigned to the female-present treatment would increase sperm production (as reported by Bozynski & Liley, 2003) and decrease their reliance on costly courtship in favour of forced mating attempts, with the reverse pattern expected in the no-female treatment.

METHODS

Experimental Fish

The guppies used in this experiment were laboratory-reared descendants of fish captured in 2006 from Alligator Creek, Queensland (Australia). Guppies were kept in large stock tanks (115 litres), each containing the same proportion of each sex (approximately 1:1 sex ratio) until required for this experiment. The bottom of the tanks was covered with mixed-colour gravel, mimicking the natural environment. The tanks were subject to a controlled photoperiod (12:12 h light:dark cycle) and were maintained at 26 ± 1 °C. All fish were fed ad libitum twice a day a diet of fresh *Artemia salina* nauplii supplemented with commercial dry food.

Treatment Regime

Adult male guppies ($N = 35$) were randomly chosen from the stock population (aged approximately 6–8 months) and individually maintained in a glass tank (35 × 19 cm and 22 cm deep) for 1 week to standardize recent social history and acclimatize the fish. After this 7-day period, males were stripped to deplete sperm reserves before the beginning of the experiment. Each male was then randomly allocated for 7 days to either a (1) female-present or a (2) no-female treatment. The time frame we chose (7 days) is likely to be ecologically relevant in natural populations. Indeed, water flows and levels in many natural populations are typically highly dynamic and males have been reported to often become temporarily isolated in pools over a period of days to weeks (Houde, 1997; Magurran, 2005). To account for interindividual variation in sperm production and sexual behaviour we used a paired experimental design in which each male experienced both treatments in a haphazard order (second treatment started immediately following the first). Each experimental tank contained a centrally positioned transparent perforated plastic drinks bottle (12 cm diameter) to house a female in the female-present treatment. The bottle was left empty in the no-female treatment. The bottles were perforated to allow the exchange of water and any associated chemicals between the fish. Females were moved between males within the same treatment twice a week to prevent familiarity that might otherwise reduce the male's sexual interest in the females (Kelley, Graves, & Magurran, 1999). The use of nonvirgin (and therefore probably nonreceptive) females coupled with the rotation of females between males was done to minimize possible differences between males attributable to variation in female responsiveness. On day 6 of the experiment we assessed male swimming performance (see below) before assaying each male for sexual behaviour and sperm production on day 7. Following these trials, the males were photographed to measure body size.

Swimming Performance

On day 6 of the experiment we assessed the swimming performance of males in each treatment in order to determine whether our prescribed experimental treatments may have inadvertently altered male condition. For example, we might expect that males assigned to the female-present treatment would be more sexually active during the treatment period and thus exhibit signs of exhaustion during the subsequent courtship trials. If so, we would expect to see impaired swimming performance when males were assigned to the female-present treatment. We used a predator evasion trial to assess male swimming performance (modified from Evans & Magurran, 2000). Briefly, this assay tests a male's ability to escape a simulated threat and is known to both be repeatable and reflect an individual's condition (Gasparini et al., 2013). Each male was taken from its experimental tank and gently placed into an empty 30-litre tank; after at least 90 s of acclimatization the male was chased and captured in a standardized way using a small hand net. The procedure was done by one of us (S.C.) who was not aware of the treatment status of the fish (i.e. the procedure was carried out blind to experimental treatment). Briefly, the procedure consisted of chasing the male with the net at a constant speed, starting when the fish was positioned in the centre of the tank and proceeded until the fish was captured. The time (s) taken to catch the male was recorded using a chronometer.

Sexual Behaviour

Sexual behaviour was observed on day 7 of the experiment between 0900 and 1200 hours (which corresponds with the peak period of sexual activity Houde, 1997). Each male was placed individually in a 30-litre tank and allowed to interact freely with a nonvirgin female (approximately 6–8 months old, mean standard length ± SE: 21.5 ± 2.5 mm). These stimulus females were matched for size among trials to avoid possible differences in male sexual interest attributable to variation in receptivity and/or fecundity, which can vary with female body size (Herdman, Kelly, & Godin, 2004). Each behavioural trial lasted 10 min, during which we recorded the number of sigmoid displays (where the male positions himself in front of the female in an s-shaped posture and quivers) and forced mating attempts (termed ‘gonopodial thrusts’, where the male attempts to forcibly inseminate the female without prior display; Liley, 1966). We also recorded the time that each male spent interacting and swimming within two body lengths of the female as a measure of the male's overall sexual interest in the female (modified from Head & Brooks, 2006). No successful copulation attempts were recorded during the behavioural trials.

Sperm Collection and Count

Ejaculates were collected from males shortly after the behaviour trials (day 7). In guppies, sperm are packaged in discrete bundles (spermatozeugmata), each containing about 21 000 individual sperm cells (Boschetto et al., 2011). To collect sperm bundles, males were anaesthetized in a water bath containing 0.15 g/litre of tricaine methanesulphonate (MS-222) and placed on a slide under a dissecting microscope LEICA MZ7.5 (Leica Microsystems GmbH, Wetzlar, Germany). Gentle pressure was then applied to the side of the male's abdomen, just anterior to the base of the gonopodium, to release sperm bundles in a drop of saline solution (NaCl 0.9%; Matthews, Evans, & Magurran, 1997). Sperm bundles were then photographed on a black background using a digital camera (LEICA DFC320) attached to the microscope. We used ImageJ analysis software (http://rsbweb.nih.gov/ij/download.html) to count the sperm bundles; sperm counts were subsequently calculated from these data (hereafter referred to as sperm production) to facilitate comparisons with previous work.

Body Size Measurement

After the various assays described above, anaesthetized males were photographed on their left side (along with a scale for calibration) using a Nikon D70s camera (Nikon Corporation, Tokyo, Japan). The distance between the snout and the base of the tail (standard length, SL in mm) was obtained from digital images using ImageJ software.

Statistical Analyses

All analyses were performed using ‘R’ software version 3.1.2 (R Development Core Team, 2014). We used mixed-effects models to test for an effect of treatment (female-present or no-female treatment) on sperm production, male condition and sexual behaviours. All models included male ID as a random factor to account for the nonindependence of the data collected from the same male in the two treatments and treatment order as a fixed factor. Q–Q plots of residuals were examined to confirm normality of error terms. The χ^2 and *P* values for the fixed effects were obtained from the univariate ANOVA function of the full model using a Wald test. Degrees of freedom were obtained by running the models without the random factor; sample size is reported for every model. To analyse sperm production we used linear mixed-effects models using the lme4 package of R, including body size (SL) as a covariate, as bigger males are expected to produce more sperm (Pitcher & Evans, 2001). Sigmoid display count was analysed using the glmmADMB package, which models zero inflation with a negative binomial distribution to account for overdispersion. The other count data were fitted with a Poisson distribution. The proportion of gonopodial thrusts over the total behaviours performed (indicating a shift in tactic usage) was tested using a binomial distribution.

Ethical Note

This research was approved by the University of Western Australia's Animal Ethics Committee (approval number: RA/3/100/1376). Fish used were descendants of wild-caught fish, so no transport of the experimental fish was necessary. The measures of sexual behaviour did not involve any invasive manipulations, and behavioural tests were performed in conditions that mimic, as best as possible, natural conditions (e.g. a gravel substrate, full spectrum lighting and aeration providing some water flow in the tank). To estimate sperm production, fish were anaesthetised through immersion in a water bath containing an approved fish anaesthetic (MS-222) at a concentration of 0.15 g/litre. This concentration allows a short induction and recovery time and reduces the risk of mortality (Chambel et al., 2013). Anaesthesia rendered the fish immobile during subsequent procedures (sperm extraction and photography). Sperm extraction from anaesthetized males is a technique widely used in guppies with no impairment of the individuals' health. Manipulation was conducted by an expert operator (S.C.) and was usually completed in under 3 min. No mortality was recorded in our experiment; after the study all fish were returned to nonexperimental aquaria where we continued to monitor them carefully for signs of stress or ill-health (no adverse effects were noted).

RESULTS

Sperm production was significantly affected by treatment (treatment, LMM: $\chi^2_{1,66} = 5.286$, $P = 0.022$, $N = 70$) but there was no effect of male body size or trial order on patterns of sperm production (SL, LMM: $\chi^2_{1,66} = 0.541$, $P = 0.462$; order, LMM: $\chi^2_{1,66} = 0.209$, $P = 0.648$, $N = 70$). When males were assigned to the female-present treatment they produced significantly more sperm (sperm number: $2.98 \times 10^6 \pm 0.32$) than when they were tested in the no-female treatment ($2.31 \times 10^6 \pm 0.22$). The male's sexual interest (i.e. the time that each male spent swimming within two body lengths of the female) was not significantly different between treatments (Table 1). We found that males performed fewer sigmoid displays but more gonopodial thrusts in the female-present treatment than when they were tested in the no-female treatment (see Table 1). The sum of the two behaviours (sigmoids and gonopodial thrusts) did not differ between treatments (GLMM: $\chi^2_{1,68} = 0.642$, $P = 0.423$, $N = 70$). As expected, however, the proportion of gonopodial thrusts to sigmoid displays did differ significantly between treatments (GLMM: $\chi^2_{1,67} = 14.11$, $P < 0.001$, $N = 70$): males performed proportionally more gonopodial thrusts in the female-present treatment (proportion of gonopodial thrusts: female-present: 0.52 ± 0.06; no-female: 0.37 ± 0.05). The male swimming performance test (assessed through the predation evasion test) revealed that males performed better (i.e. took longer to catch) when they were tested in the female-present treatment than when they were tested in the no-female treatment (female-

Table 1
Results from the GLMM testing the effect of treatment (presence or absence of females) on male sexual behaviours

	Female-present	No-female	Estimate (SE)	*N*	χ^2	*P*
Sexual interest (s)	399.5±14.3	428.34±14.50	27.54 (19.10)	70	2.079	0.145
Courtship behaviour (number of sigmoid displays per 10 min)	4.60±0.91	6.14±0.76	0.376 (0.18)	70	4.611	0.032
Sneaky attempts (number of gonopodial thrusts per 10 min)	3.80±0.55	2.86±0.45	−0.289 (0.13)	70	4.874	0.027

Mean ± SE are reported. See text for details of the analysis.

present: 20.74 ± 2.55 s; no-female: 15.37 ± 1.52 s, LMM: $\chi^2_{1,67} = 5.016$, $P = 0.025$, $N = 70$).

Finally, we found that the difference in sperm production between treatments was negatively correlated with the strength of corresponding change in courtship behaviour (Pearson correlation: $r_{30} = -0.369$, $P = 0.043$). In short, individual males that exhibited the strongest increase in sperm production when moved from the no-female to the female-present treatment showed the most pronounced decrease in courtship behaviour in favour of gonopodial thrusts (see Fig. 1).

DISCUSSION

Our study shows that male guppies adjust their mating tactics according to short-term changes in sperm production brought on by a change in sociosexual conditions. Using a paired design we showed that males in the presence of a stimulus female produced more sperm and relied relatively more on gonopodial thrusts than on courtship displays for mating acquisition compared with when they were in the no-female treatment. Our findings support recent evidence from a longer-term (4-month) study of guppies, which showed that experimentally increased mate encounter rates promote increased investment in postcopulatory traits (sperm number) and a concomitant decline in courtship in favour of forced matings (Devigili et al., 2015). Our present study showed that similar changes in sperm production and behaviour occur over far shorter timescales and are independent of the mating events themselves, as, in our experiment, males were not in physical contact with the stimulus females. The short period we used (1 week) is likely to be ecologically relevant in natural populations, which frequently encounter highly dynamic sociosexual conditions (Magurran, 2005).

Our results are consistent with a trade-off between pre- and post-copulatory episodes of sexual selection. According to this idea, a male that allocates more to sperm production is expected to have less energy available to invest in mate acquisition. Our findings support this possibility: gonopodial thrusts appear to be less energetically demanding than courtship displays (Devigili, Kelley, Pilastro, & Evans, 2012; Rahman et al., 2013) and males can therefore strategically adopt the less costly mating tactic without adjusting the overall number of mating attempts (as we showed here). However, alternative explanations for our findings that do not invoke a trade-off are also possible. One possibility is that males optimize both sperm production and mating behaviour under the different mate availability treatments. According to this idea, it may be optimal for males both to increase sperm production and to decrease courtship when females are present, especially when those females are sexually unreceptive (i.e. nonvirgin; see also Devigili et al., 2015). Although we cannot discount this possibility entirely, the explanation seems unlikely in the light of the fact that male guppies tend to increase their reliance on courtship when sex ratios are biased towards females (reviewed by Magurran, 2005) suggesting that, all else being equal, it is more profitable to engage in courtship when the availability of females is high (see also below). Moreover, support for the trade-off hypothesis comes from the finding that the choice to rely on gonopodial thrusts is proportional to the increase in sperm production at the individual level.

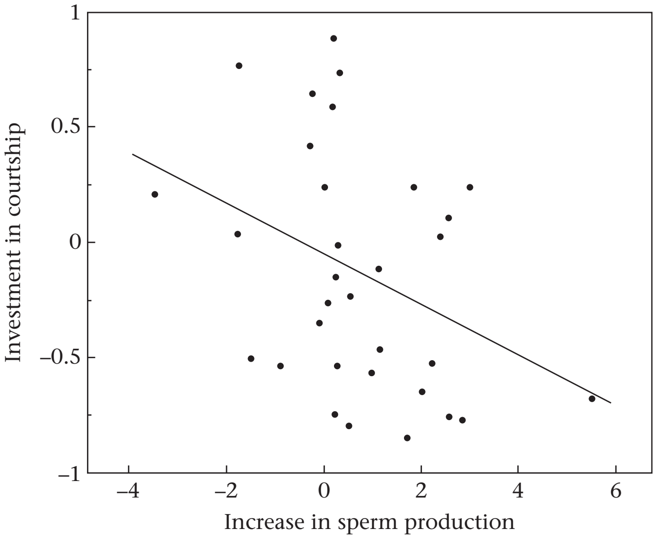

Figure 1. The relationship between a male's investment in sperm production (difference between female-present and no-female treatment) and in courtship (proportion of sigmoid displays).

The observed switch in male mating tactics according to female presence may generate costs for male reproductive fitness. Such impacts may occur at the pre- and post-copulatory levels. For example, at the precopulatory stage, females prefer to mate with males with high rates of courtship (Kodric-Brown & Nicoletto, 2001) and actively avoid forced mating attempts (Houde, 1997). It is also possible that the increased reliance on gonopodial thrusts may compromise male reproductive success during postcopulatory episodes of sexual selection. This is because gonopodial thrusts result in lower insemination efficiency than solicited copulations (Pilastro & Bisazza, 1999; Pilastro, Mandelli, Gasparini, Dadda, & Bisazza, 2007). Given these costs, we expect males to switch to gonopodial thrusts only because the energetic cost of producing sperm makes the cost of performing courtship too high to endure.

We used a predator evasion test to assess male swimming performance. In this way we attempted to account for the possibility that any changes in sexual behaviour could have been attributable to the possible confounding effects of treatment on male condition and activity levels that were independent of any adjustment in postcopulatory investment. In the event, we did not anticipate a difference in swimming performance between treatments, although any predicted difference would have involved males in the female-present treatment performing less well in this test. This is because such males were presumably more active during the preceding week, thus making them more tired during the evasion tests (possibly exacerbated by the expected trade-off between reproductive investment and somatic maintenance). Surprisingly, we found that males in the female-present treatment performed better in the predator evasion test. Although this result was unanticipated, it is actually conservative in terms of the interpretation of our results. The intensity of sigmoid displays has been shown to be positively correlated with a male's swimming ability (Nicoletto, 1993), while males in better condition tend to display more to females (Kolluru, Grether, Dunlop, & South, 2008). According to this evidence, males that performed better in the predator evasion test should also perform more displays, while we found the opposite. We can only speculate on the proximate basis for the difference in swimming performance between treatments, although one factor that may account for both this finding and the difference in sperm production may be a change in hormone levels between treatments. For example, it is likely that presence of females promotes an increase in circulating levels of testosterone (Pinxten, de Ridder, & Eens, 2003), which in turn may stimulate a change in both sperm production and physical activity levels. Indeed, testosterone (or more generally gonadal androgens) is associated with spermatogenesis in guppies (Billard, 1986; Pandey, 1969), while in other species testosterone levels are associated with physical activity and endurance (Lynn, Houtman, Weathers, Ketterson, & Nolan, 2000). For example, in the side-blotched lizard, *Uta stansburiana*, high levels of testosterone in males are associated with an increase in orange coloration (a sexually selected trait) and physical endurance (Sinervo, Miles, Frankino, Klukowski, & DeNardo, 2000). Similarly, changes in the level of circulating testosterone are thought to increase metabolic rate in the house sparrow, *Passer domesticus*, and ultimately account for the increase in overall physical activity (Buchanan, Evans, Goldsmith, Bryant, & Rowe, 2001). Whether such an explanation applies to guppies awaits further attention.

In conclusion, our study reveals that a change in sperm investment causes a shift in the use of alternative tactics by male guppies. We also show that the larger the increase in sperm production, the more pronounced is the switch from courtship to coercive mating attempts. Clearly, we need to understand the evolutionary mechanisms that link patterns of sperm production with a switch in ARTs in this species. For example, the mechanism linking the change in male reproductive tactics in response to sperm investment may not reflect a simple energetic trade-off, as male swimming performance tested in a nonsexual context (evasion from a simulated predator) actually revealed that males in the female-present treatment performed significantly better. We await further studies that quantify the relative fitness payoffs associated with these observed changes in sperm production and behaviour to better understand their evolutionary importance in natural populations.

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Manuscript 6

The strength of pre- and postcopulatory sexual selection under varying levels of food availability

Silvia Cattelan, Elisa Morbiato and Andrea Pilastro
Department of Biology, University of Padova, 35131, Padova, Italy

Abstract

The costs and the benefits arising from sexual selection could change with respect to environmental variations. Over the last decade there has been growing evidence that the strength of sexual selection varies in space and time. One of the most prominent ecological factors governing reproductive performance of males and females is food availability that might have a profound effect on the strength of both pre- and postcopulatory sexual selection. Here we investigated whether and how the strength of both the episodes of sexual selection change under varying level of food availability in the guppy (*Poecilia reticulata*). Our variance-partition analysis revealed that variation in sperm competition success was the largest source of variation in male reproductive success, but its relative importance is greater in the *ad libitum* treatment compared to that in the restricted treatment. On the contrary the relative contribution of variation in mating success in determing reproductive success was significantly higher in the restricted treatment compared to that in the *ad libitum* treatment, suggesting a stronger precopulatory selection under restricted food conditions. Our results suggest that the relaxation of selection alternatively on pre- and postcopulatory traits through small-temporal scale variation may maintain genetic variability in those traits.

Introduction

Sexual selection is one of the most effective evolutionary forces shaping not only sexual traits (Andersson 1994), but also driving speciation/extinction (Ritchie 2007), protecting from inbreeding (Lumley et al. 2015) and maintaining sexual reproduction (Agrawal 2001). While the strength of sexual selection clearly varies across species and populations (along with differences in ecology and population characteristics), it is often assumed to be constant selective force within population. Therefore, fluctuations in the strength, direction and form of selection on sexual traits in response of environmental variations within population have been largely ignored and attempts to understand how sexual selection varies in response of these fluctuations remain scarce (Candolin and Heuschele 2008, Cornwallis and Uller 2010, Miller and Svensson 2014). Indeed, we are only beginning to understand how sexual selection varies in response to variations in ecological factors, such as, for example, temperature or food availability. Ecological factors can affect the expression of sexual traits and the reproductive performance of the individuals, potentially shifting the phenotypic optima (Robinson et al. 2012, Henneken et al. 2015). This can occur because of the effects that environmental factors can have at the individual level, by modifying the expression of their sexual traits, and at the inter-individual level, by modifying the interaction dynamics that occur among males and between males and females during reproduction. There is an infinite number of environmental factors that could be ecologically relevant, each of which may have its own specific effect on sexual traits (Miller and Svensson 2014). However, we can assume that any environmental variation that moves the ecological conditions away from a population optimum has a negative effect on individual condition, i.e. on the resources an individual can invest into costly, fitness-related traits (Cotton et al. 2004). A limitation in the resources an individual can acquire will potentially affect i) its allocation strategy between reproduction and maintenance and ii) its interactions with others individuals. This could be directly triggered by resource competition, enhancing aggressive interactions among individuals, and by modifying sexual behaviour and reproductive effort of males and females, ultimately affecting each sex reproductive strategy and success. Probably the most direct way to experimentally manipulate the resources available to an individual is to manipulate its diet (Cotton et al. 2004).

Food is expected to be the primary limiting resource affecting the expression of sexual traits and in reproductive performance of both the sexes (Miller and Svensson 2014). Experimental investigations of the interplay between food availability and strength of sexual selection are still scarce (e.g. Janicke et al. 2015), although several theoretical scenarios have been proposed (Qvarnstrom 2001, Bussière et al. 2008, Candolin and Heuschele 2008, Cornwallis and Uller 2010, Miller and Svensson 2014). First, food availability typically affects phenotypic expression of condition-dependent sexual traits in males (David et al. 2000, Jia 2000). For example, unfavourable environmental conditions may affect the effort in mating acquisition (Candolin et al. 2007, Rosenthal and Hebets 2015) and this, in turn, may decrease the mating rate, resulting in different levels of polyandry (Gillespie et al. 2014, Janicke et al. 2015). Second, food availability may directly influence female mating rate and female choosiness (Syriatowicz 2004, Robinson et al. 2012, Judge et al. 2014). The variation in the costs and benefits of female mate choice and remating behaviour could in turn vary the strength and the direction of the sexual selection forces acting on male traits (Siepielski et al. 2011). There is evidence that genetic benefits to females choosing males with an extreme expression of sexual traits depend on the context (David et al. 2000, Jia 2000). This may lead females to adjust their mate choice depending on context (Qvarnstrom 2001). Alternatively, phenotypic variability in male sexual traits may be reduced or enhanced under adverse environmental conditions (depending on the type of gene-by-environment interaction, see Hunt and Hosken 2014), influencing the capability of female to discriminate male quality from precopulatory traits (Janicke et al. 2015). Finally, female condition itself could affect her choice by reducing or increasing her choosiness (Candolin 2003). The relative strength of precopulatory selection is therefore expected to vary according to male and female condition, but the direction and the strength of this change is difficult to be predicted, being influenced by a complex interaction between male sexual traits and female preference and choosiness. In polyandrous species individual condition may also influence the relative importance of pre-and postcopulatory episodes in determining male reproductive success if also female multiple mating is affected (see above). As for precopulatory traits, condition could affect the variance in male postcopulatory success and its covariance with mating success. Finally, food availability is expected to affect female fecundity and therefore indirectly affect male success after the mating event (Rundle et al. 2006). Environmental variations influencing the strength of sexual selection are, intuitively, an appealing explanation for the maintenance of genetic variability in sexual traits, but they have received surprisingly little empirical attention (but see Robinson et al. 2008). In particular, the relative importance of pre- and postcopulatory sexual selection has been poorly investigated in this context.

Here, we manipulated food availability in replicated populations of six males and six females of *Poecilia reticulata* to evaluate the strength of sexual selection when food is scarce. The guppy is a model vertebrate species for studies in pre- and postcopulatory sexual selection (Magurran 2005, Evans and Pilastro 2011). Males and females are promiscuous, with one of the highest multiple paternity recognised in nature (Neff et al. 2008), and males contribute to reproduction only with their sperm. Males attempt to mate by courting females, via sigmoid displays, or adopting a coercive mating tactic (Liley 1966). These tactics are interchangeable according to male condition, since coercive mating attempts appear to be less energetically demanding than courtship displays (Devigili et al. 2012, Rahman et al. 2013, Cattelan et al. 2016). Females prefer to mate with males with high rates of courtship (Kodric-Brown and Nicoletto 2001) and with colourful males (Evans et al. 2004). Attractive males inseminate more sperm than their less attractive counterparts (Pilastro et al. 2002) and thus they have higher success in sperm competition (Evans and Magurran 2001) since the number of sperm inseminated is the most important predictor of paternity (Boschetto et al. 2011). Thus, precopulatory sexual selection seems to be reinforced also during postcopulatory episode (Pilastro et al. 2002, Pilastro et al. 2004, Pilastro et al. 2007), as demonstrated by the large covariance between pre- and postcopulatory success observed in this species (Devigili et al. 2015b). Food availability is recognised to be an important driver of phenotypic variability in sexual traits because of its direct effects on energetically demanding traits. In guppies, diet restriction affects both precopulatory sexual traits, such as orange coloration and sexual behaviour, and postcopulatory traits, such as sperm number and performance (Kolluru and Grether 2005, Devigili et al. 2012, Gasparini et al. 2013, Rahman et al. 2013). Moreover, it has been recently showed that there is genetic variation for plasticity in sexual trait expression, providing evidence for different genetic covariation among traits when environmental conditions change b. Female guppy mating behaviour is also influenced by food availability. Archard *et al*. (2006) found no effect of short-term (5-day) food restriction on female preference for male coloration (Evans et al. 2015). However, females exposed to a longer period of diet restriction (4 weeks) showed a reduced responsiveness to male displays (Archard et al. 2006). Condition-dependent variation in female responsiveness may relax the strength of sexual selection on males (Syriatowicz & Brooks, 2004). Thus, taken all these evidence together, the guppy seems to be a suitable model system for disentangling pre- and postcopulatory episodes of sexual selection (Syriatowicz 2004) in changing environment. Aim of this experiment was to investigate simultaneously the effect of food availability on male sexual traits and female mating behaviour and their interaction, in order to provide a more comprehensive understanding on how environmental conditions might affect the strength of the pre-and postcopulatory selection forces acting on male traits.

Materials and Methods

Experimental fish

All fish were descendants of wild-caught guppies collected in 2002 from the Lower Tacarigua River in Trinidad, a high-predation site where guppies coexist with several predator species. The fish were maintained in mixed-sex aquaria (ca. 100 fish/tank) and individuals were periodically rotated among tanks to maintain an outbred population. The water temperature was maintained between 25°C and 27°C and illumination was set on a 12 h⁄12 h light⁄dark cycle. Fish were fed on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared dry food (DuplarinS). Experimental males were randomly chosen from mixed-sex aquaria. Experimental females were reared in single-sex tanks to ensure that they were virgin at the beginning of the experiments. We used virgin females to avoid any potential paternity out of our experimental males and to ensure sexual interest of females (virgin females are receptive and thus more interested in mating). At the beginning of the experiment both females and males were sexually mature and 6 ± 1 month old. Experimental design is illustrated in Figure 1.

Diet manipulation

We established 20 replicated populations of guppies, each composed of six adult males and six virgin females. Each replicate was housed in a 120 litres tank, but males and females were kept separated by an opaque divisor during diet treatment. Each population replicate was randomly assigned to one of the two diet treatments: in the *ad libitum* treatment guppies were fed with a unrestricted diet of dry flake food in the morning and with *Artemia salina* nauplii in the afternoon (ca. 150 nauplii/fish). In the restricted treatment guppies were fed once a day ca 40 nauplii/female and 57 nauplli/male according to male and female body size. We standardized the concentration of *Artemia* nauplii each day and adjusted the volume using a micropipette to ensure that food quantities did not differ among replicates within each treatment throughout the feeding trials. The diet treatment lasted 15 days (see Figure 1).

Predator-evasion test

On the last day of diet treatment the individuals were subject to capture test (adapted from Evans and Magurran 2000) to assess their predator evasion capability, a condition-dependent trait. Each individual was put into a 45-L tank (40 x 29 x 31 cm), and after 90 seconds of acclimatization the fish was captured using a small hand net (blindly to treatment). The capture procedure consisted of chasing the fish with the net at a speed, which was kept as constant as possible. The test started inserting the net in the tank when the fish was in a central position in the tank and proceeded until the fish was captured. Time spent to escape from the net was recorded using a chronometer. Individual guppies show a significant repeatability (0.70) in their evasion performance (Gasparini et al. 2013).

Morphological analysis

The day after the end of the diet treatment males and females were digitally photographed to assess whether diet manipulation successfully affected the condition of fish. Each fish was individually placed into 1-L tank and gently immobilized close to the front glass using a small net. Fish were photographed on the left side of body using a Canon 450D. Morphological traits were measured from the digital images (which included a reference scale) using image analysis software (ImageJ: http://rsbweb.nih.gov/ij/download.html) and included the total area of the body (including head and caudal fin, both sexes), and the total area of the orange spots (only in males). The relative area of colour spots was calculated as the ratio between the area of each type of spots and total body area.

Mating behavioural trials

At the end of morphological analysis fish were returned in their experimental tanks to start the mating trials. For each mating trial, the opaque divisor between males and females was removed and fish were free to interact for 90 minutes, after which the sexes were separated with the divisor until the next trial. This procedure was repeated once a day between 9.00 a.m. to 2.00 p.m. for 5 consecutive days. Each mating event was recorded and assigned to the individual male and female involved in the mating. Males were recognised by their color pattern and females by the differences in size. We recorded a mating as successful when it was followed by male jerking, a behaviour associated with successful sperm transfer (following Pilastro et al. 2007). On day 4 of the observation trials, we also recorded the number of sigmoid displays (stereotyped courtship behaviours and the number of forced attempts (hereafter “gonopodial thrusts”) performed by each male during the 90-min trial. At the end of the fifth mating trial, females were isolated from males and kept individually for the following 60 days.

Females were daily checked and the number of offspring produced was recorded. At birth, offspring were humanly killed with an overdose of MS222 and stored in a -80°C freezer until DNA extraction. Males were individually isolated for 5 days following the last mating trial to allow the full replenishment of sperm reserves before sperm extraction (see Figure 1)**.**

Sperm collection and count

In guppies, sperm are packaged in discrete units, called sperm bundles, each containing about 21.000 individual sperm cells (Boschetto et al. 2011). To collect sperm bundles, each male was anaesthetized in a water solution of MS-222 (0.15 g/L) (Chambel et al. 2013) and placed on a black slide under a ZEISS Stemi 2000-C stereomicroscope. A gentle pressure was then applied to the side of his abdomen, just anterior to the base of the gonopodium, with a rounded pipette tip until sperm bundles were released in a drop of saline solution, NaCl 0.9% (Matthews et al. 1997). Afterwards, the male was removed from the glass and sperm bundles were digitally photographed. The sperm bundles were subsequently counted from the digital photo using ImageJ and transformed into sperm number to facilitate comparisons with previous studies.

Sperm velocity analysis

Immediately after collection, sperm bundles were placed in a 12-cell multiset slide (MP Biomedicals) coated with a 1% polyvinyl alcohol to prevent sperm from sticking to the glass slide. Sperm were activated using a 40 µL of 150 mM KCl solution in 2 mg/L bovine serum albumin (Billard and Cosson 1990). The sperm swimming velocity was measured with a Hamilton-Thorne CEROS Sperm Tracker (for the setting parameters see Gasparini et al. 2013) and expressed as: average path velocity (VAP), which estimates the average velocity of sperm cells over a smoothed cell path; straight line velocity (VSL), the average velocity on a straight line between the start and the end point of the track and curvilinear velocity (VCL), the actual velocity along the trajectory. These measures provide an estimate of progressive velocity and are positively correlated with competitive fertilization success in this guppy population (Boschetto et al. 2011).

DNA extraction and amplification

DNA was isolated from the tail for adults by Salting Out extraction (Miller et al. 1988).. We genotyped females that mated with more than one male (n=58) and their offspring (n=573) and all the possible fathers (n=116). Offspring produced by females that were observed to mate with only one male (n=35, n=15 from the *ad libitum* treatment and n=20 from the restricted treatment) were 239 and were assigned to the mated male. We isolated offspring DNA from the whole body of offspring by CHELEX extraction (Walsh et al. 1991). We used three hypervariable microsatellite loci to assign paternity within each population. The loci used in this experiment were highly variable loci and the primers were labeled with two fluorescent markers (see Table 1 for details). PCRs were performed through an established cycling protocol (Devigili et al. 2015b) and using the GeneAmp PCR System 9700 Thermocycler. PCR products were run on ABI PRIMS DNA Analyzer 3100/3700 sequencer (ABI PRISM, Applied Biosystems), using filter 400 HD ROX (Perkin-Elmer, Applied Biosystems) as a size standard. Fragments lengths were determined using Peak Scanner software (http://www.appliedbiosystems.com/) and paternity was assigned using CERVUS 3.0 (http://www.fieldgenetics.com/). Paternity was assigned with 95% confidence in the 89 % of the genotyped offspring.

Microsatellite locus	Bp range	No. of alleles	GenBank accession no.	Ta (°C)	Reference
Agat11	240-371	21	BV097141	56	(Becher et al. 2002)
TTA	102-163	15	AF164205	52	(Olendorf et al. 2004)
Pr 80	142-168	10	AF467905	54	(Taylor et al. 1999)

Table 1- Microsatellite loci used to paternity and their annealing temperature (Ta).

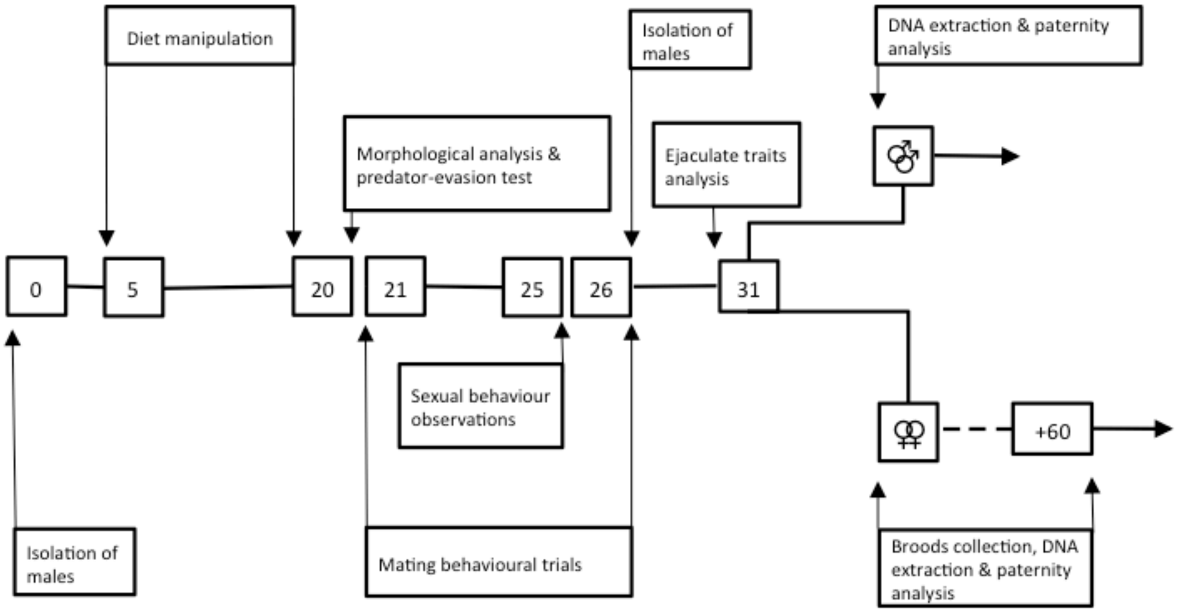

Figure 1 – Graphic illustration of the experimental design. Unit of measure is in days.

Statistical analyses

General linear model (GLM) was used to test for a significant effect of diet treatment (fixed factor) and replicate (random factor) on condition measures. When data were not normally distributed we log10-transofmed prior to analysis. Sexual behaviour counts (sigmoid displays and gonopodial thrusts) were separately analyzed using a generalized linear mixed model (GLMM) with a Poisson distribution. We analyzed the number of females giving birth as a function of diet treatment by using a binary logistic regression and number of brood produced by females by performing an ordinal regression. We calculated the parameters of sexual selection for each replicate (following Jones 2009) and compared them between diet treatments using two-sample *t-tests* and GLMs. Then we calculated fitness components affecting male reproductive success. Male reproductive success (RS) was calculated as the number of offspring produced by each male; sperm competition success (SCS) as the mean of the proportion of the offspring sired by the focal male with each female he successfully mated and the mating success (MS) as the number of females he mated with. Finally, we calculated for each male the average female fecundity as the mean number of offspring produced by the females he successfully mated with. This allowed to account for the effect of the fecundity of the female partners on a male's RS and to highlight differences in female fecundity due to diet treatment. We estimated the role of these fitness components (MS, SCS, FF), and their covariances, in determining the variance in RS using a linear regression approach. To do so, we followed Webster's variance decomposition methods (Webster et al. 1995). to partition variance in RS among effects attributable to MS, SCS, FF and their covariance:

$$\begin{aligned} VAR(\mathrm{RS}) = VAR(\mathrm{SCS\ x\ MS\ x\ FF}) \\ &= \overline{\mathrm{MS}}^2\overline{\mathrm{SCS}}^2 VAR(\mathrm{FF}) + \overline{\mathrm{MS}}^2\overline{\mathrm{FF}}^2 VAR(\mathrm{SCS}) + \overline{\mathrm{SCS}}^2\overline{\mathrm{FF}}^2 VAR(\mathrm{MS}) \\ &\quad + 2\overline{\mathrm{MS}}^2\overline{\mathrm{SCS}}\,\overline{\mathrm{FF}}\, COV(\mathrm{SCS,FF}) + 2\overline{\mathrm{MS}}\,\overline{\mathrm{SCS}}^2\overline{\mathrm{FF}}\, COV(\mathrm{MS,FF}) \\ &\quad + 2\overline{\mathrm{MS}}\,\overline{\mathrm{SCS}}\,\overline{\mathrm{FF}}^2\, COV(\mathrm{SCS,MS}) + D \end{aligned}$$

where RS, MS, SCS and FF are defined as previously and *D* is an error term. Variance and covariances were calculated for each diet treatments.

We considered two measures of the strength of sexual selection, the "opportunity for selection", *I*, and the Bateman selection gradient, β. *I* is a standardized variance in a fitness-related trait, such as MS, RS and FF (*I*) and is expressed as the trait variance divided by the square of the trait mean (Jones 2009). The Bateman gradient is the standardized slope of the regression between the mating success (or the fertilization success) and the reproductive success (Bateman 1948). Statistical analyses were carried out using IBM SPSS Statistic version 21.0.

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). The research was approved by the ethic committee of the University of Padova (Permit n. 36/2011 to AP). Fish were humanly killed with an overdose of MS-222 following the dosage reported for the guppy (Chambel et al. 2013).

Results

Diet effects on males

As expected, food manipulation had a significant negative effect on male's condition (see Table 3). Restricted males had a smaller body area and a reduced evasion capability in the simulated predator test, performed less courtships displays and coercive mating attempts. Furthermore, they showed a reduced area of orange spots. Moreover, diet restricted males produced significantly less sperm than their *ad libitum* counterparts. No male was found without sperm, however, suggesting that all the males were potentially able to inseminate at least one female. Although we successfully modified male condition, no mortality was recorded during the diet treatment and the mating trials. Results are summarized in table 2.

	Ad libitum	**Restricted**	**df**	**F**	***p***
Body size (mm^2)	63.25 ±1.50	56.66 ± 1.04	18	4.532	**0.047**
Predator-evasion capability*	54.80 ±6.52	26.96 ± 4.20	18	5.742	**0.028**
Courtship behaviour[a]	17.20 ± 1.50	10.80 ± 1.43	106	18.870	**<0.001**
Sneaky attempt[a]	22.35 ± 1.90	5.78 ± 0.84	106	5.511	**0.021**
Orange coloration (mm^2)	11.17 ± 0.73	8.23 ± 0.54	18	7.724	**0.012**
Sperm number*	5.17 ± 0.50	2.54 ± 0.21	18	26.974	**<0.001**
Sperm velocity um/sec	88.01 ± 1.83	90.91 ± 1.65	18	1.325	0.265

Table 2- Results from GLMs testing the effect of diet treatment on male fitness-related traits. Significant values are reported in bold. [a] GLMM with a Poissoin distribution. * Traits log10-transformed prior to analysis. Significant values are reported in bold.

Diet effects on females

Food limitation significantly affected female body size (body area), with females fed *ad libitum* had fuller body shapes than those on a restricted diet. Diet manipulation had an effect on the probability to reproduce: 83% of females fed *ad libitum* produced at least a brood, compared to the 65% of the food-restricted females. Among those females, 72% of the *ad libitum* females produced a second brood, compared to 85% of restricted females. On average, the number of broods produced during the 60d-days period did not differ between diet treatments (see table 3). *Ad libitum* females produced on average 2.28 more offspring in their first brood than restricted females (GLM: $F_{1,88}$=4.853, *p*=0.030, only females that produced at least one brood were considered). The difference in fecundity was larger after considering all the offspring produced within 60 days after matings (mean difference = 5) (GLM: $F_{1,88}$=6.134, *p*=0.015, only females that produced at least one offspring are considered). No significant effect of diet was detected on time to first brood and interbrood interval.

	Ad libitum	**Restricted**	**df**	**Statistics**	***p***
Body size (mm^2)	119.88 ± 1.68	95.55 ± 1.36	18	57.770	**<0.001**
Predator-evasion capability*	60.54 ± 7.15	47.34 ±4.72	25	2.021	0.167
First brood offspring	10.08 ±0.76	7.82 ± 0.64	18	14.355	**0.001**
Total offspring	14.94 ±1.01	11.59 ±0.81	18	11.041	**0.004**
Proportion of parturient females^b	0.83 ± 0.05	0.65± 0.06	1	5.075	**0.024**
Number of broods^c	1.42 ± 0.07	1.34± 0.07	1	0.092	0.762
Time to first brood (days)	35.94 ±1.39	39.82 ±1.59	20	2.122	0.161
Interbrood interval (days)	26.10 ±0.81	26.17 ± 1.15	19	0.383	0.355

Table 3- Diet effect on female traits and reproductive (GLMs). ^{b} logistic regression; ^{c} ordinal regression. * Traits log10-transformed prior to analysis. Significant values are reported in bold.

Effects of diet on pre-and postcopulatory sexual selection dynamics

Females from the restricted treatment were less polyandrous than their *ad libitum* counterparts (mean mating rate ± s.e.m: *ad libitum* 2.96 ± 0.20, restricted 2.15 ± 0.19; t-test: $t_{1,18}$=9.711 *p*=0.006). The standardized variance in female mating rate did not vary between diet treatments (t-test: $t_{1,18}$=0.359 *p*=0.724), whereas a significant increase in male mating success was observed in the restricted group (t-test: $t_{1,18}$=2.160 *p*=0.045). This suggests that under our experimental conditions of limited resources the opportunity for precopulatory selection increases. The standardized variance in reproductive success did not differ either in females (t-test: $t_{1,18}$=1.409 *p*=0.176) or in males (t-test: $t_{1,18}$=0.642 *p*=0.529) with respect of diet treatment. Bateman gradients were calculated from the slope of the regression between MS and RS for each sex. *Ad libitum* males showed a greater positive Bateman gradient than restricted males, although this difference was marginally no significant (t-test: $t_{1,18}$=2.095 *p*=0.051). In females, as expected, Bateman gradient was significant lower that in males (GLM, sex effect: $F_{1,18}$=18.646 p<0.001) and it was not affected by diet treatment ($t_{1,18}$=0.040 *p*=0.969). Male reproductive success was influenced by his pre- and postcopulatory performance (MS and SCS), and by the fecundity of the females (FF) he mated with. To measure the effect of diet on each trait of sexual selection we considered the number of offspring produced by each male (RS) as the dependent variable of the models, and SCS, MS and FF were included as covariates in the GLM. When all components of the male RS were considered simultaneously, we found a significant interaction between diet and SCS but not between diet and MS (table 4, Figure 2). The mean polyandry (P), calculated as the mean mating rate of the females with whom the focal male successfully mated, negatively predicted male SCS in both diet groups (Figure 3), and this effect was stronger in the *ad libitum* males.

Dependent variable: male RS				**95% Confidence Interval**	
	df	F	*p*	Lower Bound	Upper Bound
Diet	100	2.323	0.131	-	-
SCS	100	99.431	**<0.001**	17.503	27.754
MS	100	42.040	**<0.001**	2.057	3.762
FF	100	25.885	**<0.001**	0.408	0.930
Diet * SCS	100	10.787	**0.001**	-18.019	-4.441
Diet * MS	100	0.651	0.422	-2.218	0.936

Table 4 – GLM testing the effect of each components of fitness on male RS with respect to diet treatment. Diet was included as fixed factor, MS, SCS and FF as covariates in the model. Significant values are reported in bold.

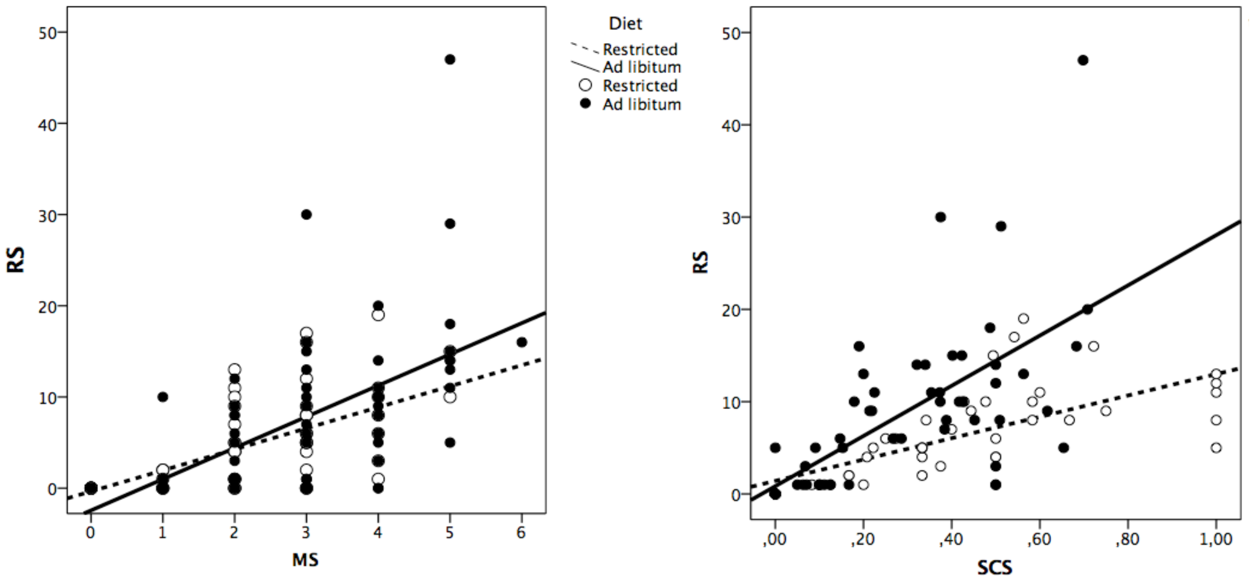

Figure 2- Male reproductive success (RS) in relation to mating success (MS) in each diet treatment (left panel). Restricted treatment: slope=2.469, t=6.466, R^2=0.394, *p*<0.001; *ad libitum* treatment: slope=2.894, t=5.527, R^2=0.302, *p*<0.001. Male reproductive success (RS) in relation to sperm competition success (SCS) in each diet treatment (right panel). Restricted treatment: slope=11.504, t=9.254, R^2=0.450, *p*<0.001; *ad libitum* treatment: slope=22.821, t=7.241, R^2=0.459, *p*=0.002.

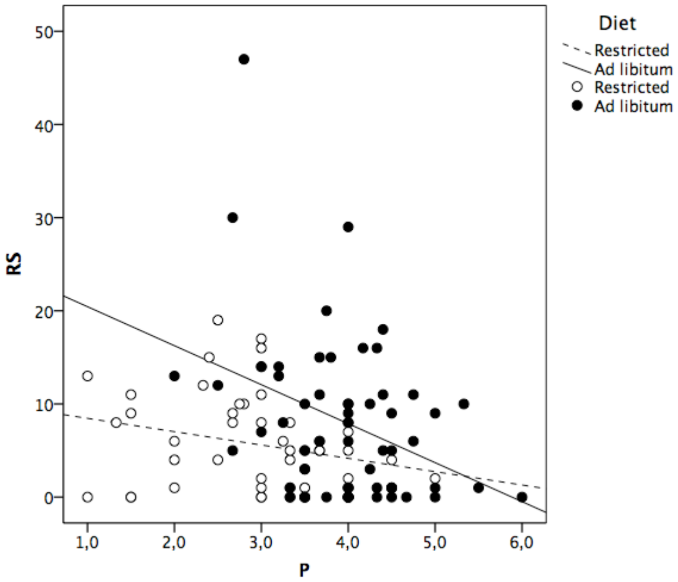

Figure 3 – Male reproductive success (RS) in relation to polyandry (P). Restricted treatment: slope=-2.661, t=-4.238, R^2=0.070, *p*<0.001. *Ad libitum* treatment: slope=-4.369, t=-3.251, R^2=0.136, *p*=0.002.

Our variance-partition analysis revealed that variation in SCS was the largest source of variation in male reproductive success, but the relative importance of SCS was similar across diet treatments (*ad libitum* = 39%; restricted = 34%). In contrast, the relative contribution to RS variation explained by MS was much larger in the restricted treatment (32%) as compared to the *ad libitum* treatment (13%). As expected, variation in female fecundity (FF) was a larger source of variation in RS in the restricted treatment than in the *ad libitum* treatment (28% compared to the 11% of the *ad libitum*; table 5).

	Restricted		***Ad libitum***	
	Unstandardized	Explained %	Unstandardized	Explained %
RS	25.843	100	76.379	100
MS	8.329	32.230	9.620	12.595
SCS	8.885	34.383	29.441	38.546
FF	7.244	28.030	8.680	11.364
Cov(MS, SCS)	0.784	3.035	6.075	7.954
Cov(SCS, FF)	-0.754	-2.917	3.424	4.483
Cov(MS, FF)	1.357	5.250	-0.578	-0.757
D	-0.003	-0.010	19.717	25.814

Table 5 – Variance in RS explained by variance in MS, SCS, FF and their covariances in each diet treatment. N=60 per each diet treatment. *D* represents an error term.

Discussion

This study provides experimental evidence for environment-dependent sexual selection. Our results indicate that, while the overall strength of sexual selection on males did not change between diet treatments, the reduction in food availability increases the potential for precopulatory sexual selection and simultaneously reduces the contribution of the postcopulatory episode. As a result, the relative importance of pre- and postcopulatory episodes of sexual selection varied in the two food quantities tested. We experimentally manipulated food availability because we had evidence of significant phenotypic plasticity in response to this factor (see Manuscript 1 and 2). A recent study has established the relative importance of pre- and postcopulatory processes in determining male reproductive fitness in the guppy (Devigili et al. 2015b). The study of Devigili *et al*. was based on fish that were fed *ad libitum*, and it is similar to the present experiment, even if small, but potentially important differences in the design of the mating trials prevent a direct comparison of their results. This point will be discussed later. Importantly, we demonstrated that the variation in food availability can affects the relative importance of the two subsequent episodes of sexual selection in determining male reproductive fitness. This result is likely to be due to a series of different, interacting factors.

As expected, food manipulation affected the expression of sexual traits of males and decreased also their general condition, in line with previous studies (Devigili et al. 2012, Gasparini et al. 2013, Rahman et al. 2013). In particular, we found a reduced performance in predator escaping capability and a decline in male courtship rate. The decline in the rate of courtship may have been caused by a reduced female responsiveness. Indeed, females tend to reduce their responsiveness to males when food is limited (Syriatowicz 2004). While we did not measure female responsiveness to male, we found that food restricted females had a lower mating rate. Most of the copulations in our study were cooperative (data not shown), and hence largely under female control. Despite the lower mating rate observed in the restricted group, the variance in male mating success was higher, indirectly suggesting that the reduced female mating rate was not accompanied by a reduced choosiness and/or a reduced variance in male capability to obtain matings. The variance in mating success we observed in the restricted replicates is higher than that observed in their *ad libitum* counterparts, suggesting two alternative, but not mutually exclusive, explanations: i) diet restriction increased male phenotypic variance for traits associated with female choice, and females were facilitated in their choice; ii) females in poor condition copied more the mate choice of the others, because the cost of choosing is too high (Wade and Pruett-Jones 1990). Studies of mate choice in female guppies have demonstrated significant levels of repeatable variation among females within populations (Godin and Dugatkin 1995, Brooks 1996, Brooks and Endler 2001). Although some differences among females may occur, most of the phenotypic variation (and also the heritable variation) was detected in responsiveness (Brooks and Endler 2001). Here, we found that the consistency in female mate choice resulted in a higher variance in male mating success. Copying behaviour is an adaptive alternative to random choice when there is a cost of choice (Wade and Pruett-Jones 1990) but, although we cannot completely exclude mate copying as mechanism for the observing high variance in male success, it is more likely that phenotypic differences among males have driven female consistency in mate choice. Indeed, it has recently recognised that traits expression of different genotypes is context-dependent (Evans et al. 2015), meaning that under some environmental circumstances some genotypes are more favoured than others. Thus, it is reasonable to suppose that females were able to detect male quality from precopulatory evaluation (Gillespie et al. 2014) contrary to what found in a similar study on a water snail (Janicke et al. 2015). Clearly, although the precise mechanism remains unknown, what is important to highlight is that food restriction increases the opportunity for precopulatory selection and thus males investing in traits involved to mating acquisition seem to be more favoured under these circumstances. Guppy sexual traits have a significant heritable component (Gasparini et al. 2013), which often remains significant across dietary conditions (Evans et al. 2015). Pre- and postcopulatory traits are characterized by negative genetic correlations (Evans 2010), suggesting that some males may be better than others according to how sexual selection acts on pre- and postcopulatory traits. Indeed, multivariate phenotypic selection analyses has revealed that linear and nonlinear selection simultaneously act on pre- and postcopulatory traits, and interact to generate multiple phenotypes with similar fitness (Devigili et al. 2015b). The results of the present experiment suggest that environmental variation is expected to further increase the range of genotypes that have similar reproductive fitness. As a consequence of food restriction, female body area and fecundity were significantly reduced, indicating that our food treatment succeeded in manipulating the reproductive resource of females, ultimately reducing their fecundity (Reznick and Endler 1982). This indirectly influenced male reproductive success (RS). Indeed the relative contribution of female fecundity in male RS appears to be more important under food restriction than under *ad libitum* condition. Moreover, the partitioning of the variance in male RS suggests a shift from postcopulatory toward precopulatory sexual selection under food restriction. More precisely, although in both diet treatments variation in sperm competition success (SCS) was the most important source of variation in male reproductive success, but in the restricted treatment its relative contribution is lower (although the difference is very small and probably not significant *per se*). For mating success (MS), the second most important component in RS variance, the pattern is the opposite: the proportion of RS explained by MS increased under food restriction. This could be attributable, as suggested above, by the increasing in the phenotypic variability in traits related to male attractiveness and sperm competitiveness.

Whatever the reasons of the differences in mating rate (a change in male effort, in the variance in male phenotypic quality, in female preference and/or choosiness), under *ad libitum* food condition, females mated on average with more males, shifting the importance of sexual selection from precopulatory to postcopulatory processes. Previous studies have demonstrated that increased polyandry is associated with a reduction in the opportunity for sexual selection (Collet et al. 2012, Pelissie et al. 2014). Our results indicate that this may not always be the case, and suggest that, although polyandry indubitably increases the opportunities for postcopulatory sexual selection, overall variation in the opportunities for sexual selection may not change in response to varying levels of polyandry, at least when the latter is accompanied by a change in female fecundity. Collectively, our results predict that sexual selection on male traits involved in mating acquisition is increased under harsh food condition and it is reduced when condition are favourable. In contrast, under favourable food condition sexual selection is disproportionally driven by traits involved in fertilization success, in contrast with a similar study conducted in the water snails *Physa acuta* (Janicke et al. 2015). Here we provide evidence that a relatively small change in resources availability, two weeks of food restriction at the adult stage in organisms that can live many years (Reznick et al. 2004), may significantly alter the selection balance between pre- or postcopulatory traits. Food availability is subjected to both temporal and spatial variation in the guppy habitat: for instance, seasonal flooding may profoundly change the availability of algae and invertebrates that are the two main food sources for this species (Dussault and Kramer 1981, Grether et al. 2001a). We suspect that the strength of sexual selection might fluctuate substantially in nature also at small temporal-scales, providing a possible explanation to the large genetic variability in sexual traits observed in this species. Our result suggests that when food is unlimited, selection on traits involved in mating acquisition may be relaxed. On the contrary, selection on traits involved in fertilization success may be relaxed when food is scarce. Thus, the relaxation of selection alternatively on pre- and postcopulatory traits can maintain genetic variability in those traits. In particular, sperm number seems to be the most important trait in determing postcopulatory success in this species and it is reasonable to suppose that fine-grained environmental variations might contribute to the maintenance of the genetic variability observed in this trait.

Finally, a number of recent models have suggested that sexual selection in response to an environmental variation can have beneficial effects on nonsexual fitness (Rundle et al. 2006). These include increasing the rate and extent of adaptation (Lorch et al. 2003) and improving the purging of genetic load (Whitlock and Agrawal 2009). Such effects can protect species or populations from extinction (Lumley et al. 2015) and can ultimately provide an advantage to sexual reproduction (Agrawal 2001). Variations in the patterns of sexual selection may ultimately influence the rate of adaptation of a population but the role of sexual selection in adaptation is still debated (Candolin and Heuschele 2008):

theoretical models have argued that sexual selection might or may not increase the rate of adaptation, and the few empirical data are controversial (Rundle et al. 2006, Fricke and Arnqvist 2007, Robinson et al. 2012, Gillespie et al. 2014, Chenoweth et al. 2015, Janicke et al. 2015). The only study providing genomic evidence has revealed that sexual selection impedes the adaptation to a novel environment, although this result strongly depends on *Drosophila serrata* mating system (Chenoweth et al. 2015). Our results indirectly suggest that under harsh environmental conditions, sexual selection may accelerate the adaptation rate of a population, but clearly we are far from its formal demonstration. Therefore, more empirical work is needed to shed light on the impact of sexual selection in the adaptive potential of a population.

Riassunto

Uno dei grandi problemi irrisolti nell’ambito della biologia evoluzionistica consiste nello spiegare come possa essere mantenuta variabilità genetica in tratti che sono sottoposti a selezione direzionale. Il problema è stato inizialmente investigato solo per quei tratti sottoposti a selezione naturale ma recentemente la problematica è stata applicata anche per quei caratteri sottoposti a selezione sessuale. Nelle specie in cui le femmine possono accoppiarsi con più di un maschio nello stesso ciclo riproduttivo, le femmine sono cioè poliandriche, si creano le condizioni per cui gli spermi di due o più maschi competano per fecondate lo stesso gruppo di uova. La competizione spermatica genera quindi una forte pressione selettiva in tutti quei caratteri che possono aumentare in qualche modo la probabilità di un maschio di fecondare le uova della femmina, vincendo quindi la competizione spermatica con gli altri maschi (Parker 1970). Tra questi, il numero di spermi prodotti è uno dei caratteri che maggiormente risponde alla forte pressione selettiva esercitata dalla competizione spermatica (Birkhead and Moller 1998). La selezione che agisce sul numero di spermi dovrebbe, generazione dopo generazione, eroderne la variabilità genetica ma, al contrario, la variabilità genetica di tale carattere, così come di altri caratteri postcopulatori, è elevata (Evans and Simmons 2008). Numerosi sono i meccanismi proposti per spiegare questo paradosso evolutivo (Radwan 2008) e la mia ricerca si è focalizzata principalmente su uno di questi: le interazioni tra il genotipo e l’ambiente (GEI). Le variazioni ambientali possono generare cambiamenti molto evidenti nell’espressione di molti caratteri sottoposti a selezione sessuale e, inoltre, genotipi diversi possono rispondere in maniera diversa a condizioni ambientali diverse (Ingleby et al. 2010). Secondo questa ipotesi, la *performance* relativa di diversi genotipi dipende dalle condizioni ambientali nelle quali sono espressi. E se l’ambiente è sufficientemente variabile nello spazio e/o nel tempo la variabilità genetica in caratteri sessuali può essere mantenuta a dispetto della selezione direzionale che agisce su questi, in quanto non vi è un genotipo con una *performance* relativa migliore in tutti gli ambienti (si veda il recente libro di Hunt and Hosken 2014). Lo scopo del mio lavoro, quindi, è stato quello di indagare se e come i cambiamenti ambientali, sia ecologici che relativi al contesto sociale, possano contribuire a mantenere la variabilità genetica in un tratto sottoposto a selezione direzionale quale il numero di spermi.

Il lavoro è stato svolto usando un piccolo pesce tropicale d’acqua dolce, il guppy (*Poecilia reticulata*). Questa specie è particolarmente appropriata per studiare gli effetti di cambiamenti ambientali sul numero di spermi, un carattere coinvolto nella selezione sessuale postcopulatoria. Innanzitutto l’habitat naturale dei guppies, originari di Trinidad and Tobago (nei Caraibi), è soggetto a modificazioni ambientali che comprendono molteplici fattori, tra i quali la disponibilità di cibo, la torbidità delle acque, la sex ratio e la densità (Magurran 2005). Inoltre in questa specie sono stati studiati approfonditamente i meccanismi coinvolti nella selezione postocopulatoria (Evans and

Pilastro 2011). Il numero di spermi trasferiti alla femmina è stato identificato come il responsabile più importante del successo postcopulatorio di un maschio, cosa che fa del numero di spermi un tratto sottoposto ad elevata pressione selettiva direzionale (Boschetto et al. 2011). Nonostante ciò, questo carattere possiede un'elevata variabilità genetica (Gasparini et al. 2013), il che rende tale carattere un candidato ideale per studiare i meccanismi che ne mantengono la variabilità. A questo scopo ho svolto sei esperimenti, descritti dettagliatamente nei sei manoscritti presentati in questa tesi. Buona parte degli esperimenti è stata svolta utilizzando maschi provenienti da due linee di selezione artificiale presenti nel mio laboratorio, in cui i maschi sono stati selezionati per un'elevata (High) e una bassa (Low) produzione di spermi (Di Nisio 2014).

Nel corso del primo esperimento ho indagato se un'alterazione nella disponibilità di cibo possa modificare l'espressione del carattere "numero di spermi" e di altri caratteri importanti per la fitness riproduttiva dei maschi in maniera diversa tra i maschi delle due linee di selezione. Ho quindi sottoposto i maschi delle due linee in età giovanile ad una manipolazione della dieta, creando un gruppo sottoposto a dieta *ad libitum* e l'altro sottoposto ad una dieta ristretta per poi valutare l'espressione dei caratteri nelle due condizioni e nelle due linee (**Manuscript 1**). Tuttavia in questo primo esperimento non ho evidenziato alcuna interazione tra le linee di selezione (il genotipo) e la disponibilità di cibo (l'ambiente), confermando quanto già trovato in precedenti esperimenti: il numero di spermi prodotti sembra essere correlato alla qualità genetica dei maschi. Inoltre, ho indagato se eventuali GEI emergessero nelle femmine derivate dalle linee di selezione (sottoposte anch'esse a manipolazione della dieta) e nella prole nata da maschi e femmine delle linee di selezione precedentemente sottoposti a manipolazione della dieta. Anche per quanto riguarda questo secondo esperimento non ho riscontrato interazioni tra il genotipo e l'ambiente significative che possano contribuire al mantenimento della variabilità genetica nel numero di spermi (**Manuscript 2**). Se quindi, la disponibilità di cibo non sembrerebbe essere implicata nel mantenimento della variabilità genetica, così non sembra per il contesto sociale. Infatti i maschi delle due linee di selezione hanno dimostrato un diversa risposta alla presenza di una femmina stimolo: i maschi Low innalzano la loro produzione di spermi maggiormente dei maschi High, anche se questa differenza non è abbastanza elevata da generare una significativa interazione genotipo-ambiente (**Manuscript 3**). Al contrario il successo riproduttivo dei maschi delle due linee è risultato essere significativamente diverso al variare della sex ratio. Infatti sottoponendo coppie di maschi delle due linee a due diverse sex ratio, una in cui potevano interagire con solo due femmine (*equilibrium*) e l'altra con ben sei femmine (female-biased), ho riscontrato un maggior successo riproduttivo dei maschi Low quando le femmine erano in numero minore. L'interazione genotipo-ambiente che ho riscontrato tramite questo esperimento sottolinea come il contesto sociale in cui i maschi si trovano possa contribuire a mantenere il mantenimento della variabilità genetica nel numero di spermi. Il mio quinto esperimento è stato svolto a Perth, in collaborazione con l'Università della Western Australia. Questa collaborazione mi ha permesso di effettuare uno studio sulla popolazione di guppies australiana, su cui ho indagato come l'investimento nel numero di spermi (questa volta fenotipico anziché genetico) possa alterare l'investimento nel comportamento sessuale, importante carattere precopulatorio. Infatti maschi che plasticamente aumentano la loro produzione di spermi in risposta alla presenza di una femmina stimolo, adottano la tattica di accoppiamento meno dispendiosa dal punto di vista energetico. Viceversa un minor investimento in spermi è accompagnato da un maggior investimento in corteggiamento, carattere sessuale dimostrato essere molto costoso (**Manuscript 5**). Infine nell'ultimo esperimento ho indagato come la modificazione di un parametro ecologico, quale la disponibilità di cibo, possa ripercuotersi sulla selezione sessuale nel suo complesso. Nonostante nei primi due esperimenti non abbia riscontrato alcuna interazione genotipo-ambiente significativa in risposta ad un cambiamento della disponibilità di cibo, in quest'ultimo esperimento ho riscontrato come un cambiamento della disponibilità di cibo possa influenzare il contesto sociale, modificando quindi le interazioni maschio-femmine con importanti ripercussioni sull'importanza relativa della selezione pre- e postcopulatoria (**Manuscript 6**). Ho sottoposto a dieta *ad libitum* e ristretta popolazioni composte da sei maschi e sei femmine e poi analizzato, non solo l'espressione dei loro tratti, ma anche il loro successo di accoppiamento e riproduttivo. I miei risultati indicano che in condizioni di ambientali sfavorevoli (poco cibo) gli individui si accoppiano meno ma aumenta significativamente la varianza nel successo di accoppiamento. Nel complesso ho riscontrato come l'importanza relativa dei due episodi di selezione sessuale, pre- e postcopulatorio, cambi relativamente alle condizioni ambientali. Questo processo potrebbe essere cruciale nel mantenere la variabilità genetica nei tratti sessuali in quanto la selezione è più forte alternativamente sui tratti pre- o postcopulatori al variare delle condizioni ambientali.

In conclusione questa tesi fornisce un quadro più completo dei meccanismi coinvolti nel mantenimento della variabilità genetica nei tratti sottoposti a selezione sessuale, in particolare il numero di spermi, carattere sottoposto a forte selezione direzionale. I miei risultati sottolineano come sia sufficiente generare condizioni ambientali "reali" per riscontrare delle interazioni genotipo-ambiente significative (Hoffmann and Merila 1999). Concludendo quindi, penso sia necessario che i fattori ambientali, siano essi ecologici o sociali, vengano presi in considerazione nei futuri studi di selezione sessuale visto il loro potenziale ruolo nel mantenimento della variabilità genetica dei tratti sessuali.

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