UNIVERSITÀ DEGLI STUDI DI PADOVA

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Sexual selection in *Poecilia reticulata*: the maintenance of variability in male pre- and postcopulatory sexual traits

Selezione sessuale in *Poecilia reticulata*: il mantenimento della variabilità nei caratteri sessuali maschili pre- e postcopulatori

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3. Immunocompetence, condition-dependence and sexually selected traits in the guppy

Alessandro Devigili, Vincenzo Belluomo, Lisa Locatello, Maria Berica Rasotto, Andrea Pilastro

4. Condition-dependent expression of pre- and postcopulatory sexually selected traits in guppies

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Alessandro Devigili, Jennifer L. Kelley, Andrea Pilastro, Jonathan P. Evans

ABSTRACT

Sexual selection is a driving force in sexually reproducing organisms and strongly shapes their evolution. In the last three decades, sexual selection research has seen a rapid growth, and both theoretical and empirical work has clarified many components of pre- and postcopulatory sexual selection. Despite that, the coexistence of two basic observations still forms an unsolved evolutionary question: in natural populations genetic variation is found in almost all traits in the presence of strong natural and sexual selection. As selection should deplete variability those two observations are in direct conflict. This problem attracted the attention of many researchers, as it regards potentially most of the numerous traits describing an organism's phenotype, or at least all the traits under some selection. During my PhD I explored part of this field of study, focusing on sexually selected male traits. Most of the efforts done to understand this evolutionary contradiction have been done in a precopulatory context, with particular attention to the prominent case of the so called 'lek-paradox'. However, whenever females are sexually promiscuous, a directional selection for traits associated with sperm competition success is expected to arise. As ejaculate characteristics are expected, and actually known, to play a crucial role in determining the fitness outcome of males, selection acting on them should be strong and as a consequence their variability reduced. Yet, as for precopulatory traits, there are many experimental evidences that variability in postcopulatory traits is unexpectedly high.

Many hypotheses have been formulated to explain the maintenance of genetic variability of sexually selected traits. During my PhD I tested some prediction of three main models applicable to both pre- and postcopulatory traits: first that selection constrains and non linear selection are acting on the set of traits defining the male phenotype. Second, I verified that resource trade-offs are present between pre- and postcopulatory traits, as proposed by Parker's sperm competition theory. Third, I tested the fundamental assumption of the 'genic capture hypothesis' that sexually selected traits are condition dependent.

I performed four main experiments using the guppy, *Poecilia reticulata*. This small tropical fresh-water fish is well suited for my purposes as traits subject to both pre- (male ornamentation, size, and behaviour) and postcopulatory selection (sperm number, velocity, and viability) exhibit high levels of phenotypic and additive genetic variation. With the first experiment (manuscript 1) I characterized, for the first time, the selection acting in a whole on both pre- and postcopulatory traits. I then measured the long term cost of sperm production (manuscript 2) with the aim of determine the trade-offs present between pre- and postcopulatory traits. With the last two experiments (manuscripts 3 and 4) I tested condition dependence of a wide set of sexually selected traits.

My results suggest that in this species non linear selection may be more important than previously estimated and, in particular, that disruptive and correlational selection can contribute to maintain polymorphisms in sexually selected traits. Moreover investment in ejaculate is traded off with investment in obtaining mating, in agreement with sperm competition theory. Lastly, both pre- and postcopulatory sexually selected traits show a strong condition dependence, thus confirming one assumption of the 'genic capture hypothesis'.

GENERAL INTRODUCTION

Sexual selection

Two hundred years ago, in his most famous book, Darwin described the engine of evolution: the natural selection (Darwin, 1859). While natural selection is an important factor of adaptive evolution, he understood that this process was not the only one through which evolution takes form. He guessed that some traits avoid the control of natural selection and he argued that another process, sexual selection, takes place in sexually reproducing organisms (Darwin, 1871). Darwin recognised two forms of sexual selection: intra-sexual selection acts on traits (e.g. armaments used during fights) that allow one sex (typically males) to compete for the access to the other one. Inter-sexual selection acts on traits (e.g. ornaments) involved in the interaction between the two sexes. This latter mechanism has been controversial for nearly one century but has received experimental and theoretical support in the last three decades (Kirkpatrick & Ryan, 1991; Andersson, 1994; Jennions & Petrie, 1997). While female choice is not anymore an issue in evolutionary biology, it has been recognised that sexual selection does not stop with mating but, as a consequence of females mating promiscuously in many species, continue also after mating in the form of sperm competition (occurring when sperm of two or more males compete for fertilizing the eggs Parker, 1984) and cryptic female choice (when females are able to bias the fertilisation success towards a specific male Eberhard, 1996). The two processes of sexual selection, pre- and postcopulatory, can potentially act on the same or on different traits, and can reinforce the effect of each other or not (Birkhead & Pizzari, 2002), with the extreme possibility of having divergent direction. The determination of the interactions between different traits, the characterization of the selection(s) acting on them and the understanding of their expression are the means in order to answer one of the still unsolved big evolutionary questions: how variability of those traits is maintained.

What we expect and what we observe: a scientific contradiction

Despite the last three decades have seen rapid growth of sexual selection research (see for example Andersson, 1994; Andersson & Simmons, 2006), this field of study still retains some unresolved questions. One of these is how genetic variability is maintained despite strong directional sexual selection (Walsh & Blows, 2009). Following Darwin's theory on female choice for males with more elaborated ornaments (1859), increased mating success of individuals bearing such traits, generation after generation, should erode the genetic variability underlying these traits, leading selected traits to fixation (Tomkins *et al.*, 2004). When male genetic quality is the benefit for the choice, we have the so-called "lek paradox", in which female mate choice should deplete the variability of chosen male traits, reducing the benefit for the choice. Yet females continue to choose (Borgia, 1979). The problem of the maintenance of the additive genetic variance underlying sexually selected traits has been typically investigated in a precopulatory context (see Radwan, 2008). However, whenever females are sexually promiscuous, a directional selection for traits associated with sperm competition success is expected to arise. If success in sperm competition and male genetic quality are genetically correlated and females mate promiscuously to seek for such genetic benefits (Keller & Reeve, 1995; Yasui, 1997), the paradox should also apply to postcopulatory sexually selected traits. As pointed out before, in polyandrous species sexual selection acts after mating through

cryptic females choice and sperm competition. Ejaculate characteristics are therefore expected to play a crucial role in determining male fitness. If this is the case, selection acting on ejaculate traits should be strong and, as a consequence, their genetic and phenotypic variability reduced. In contrast, experimental evidence indicates that variability in those traits is unexpectedly high (reviewed by Evans & Simmons, 2008).

Several explanations have been put forward to explain the maintenance of genetic variability of sexually selected traits (Radwan, 2008; Chenoweth & McGuigan, 2010). Three models seems to particularly applicable to pre- and postcopulatory traits: condition dependence (Rowe & Houle, 1996), resource trade-offs (Parker, 1998), and non directional or contrasting selection (disruptive selection or correlative selection due to constraints in the genetic architecture of traits, Walsh & Blows, 2009; Chenoweth & McGuigan, 2010).

The last two mechanisms (resources trade-offs and non-directional and contrasting selection) are likely to occur in particular when pre- and postcopulatory traits are considered together: it is clear that resources allocated to mate acquisition cannot be invested in traits enhancing sperm competition success, assuming that both types of traits are costly. Despite these obvious implications, previous research has tended to focus mainly on pre- and postcopulatory traits separately, and relatively few studies have attempted to integrate the episodes of sexual selections occurring before and after gametes are released.

AIMS OF THE THESIS

The aim of my PhD project is to test some of the predictions of the theories presented above, by extending my analyses to both pre-and postcopulatory sexually selected traits in four experiments. My model organism is the guppy, *Poecilia reticulata*, a small tropical fresh-water fish. In this sexually dimorphic fish (Endler & Houde, 1995; Pilastro *et al.*, 2002; Evans *et al.*, 2003b; Locatello *et al.*, 2006; Pilastro *et al.*, 2007; Boschetto *et al.*, 2011) traits subject to both pre- (male ornamentation, size, and behaviour) and postcopulatory selection (sperm number, velocity, and viability) exhibit high levels of phenotypic and additive genetic variation (Brooks & Endler, 2001; Evans, 2010; 2011).

With first experiment (manuscript 1) I wanted to characterize, for the first time, the selection acting in a whole on both pre- and postcopulatory traits (see Box 1 for a short description of methodology used for sperm analysis). I then measured the long term cost of sperm production (manuscript 2) with the aim of determine the trade-offs present between pre- and postcopulatory traits. In the last two experiments (manuscripts 3 and 4) I tested condition dependence of sexually selected traits.

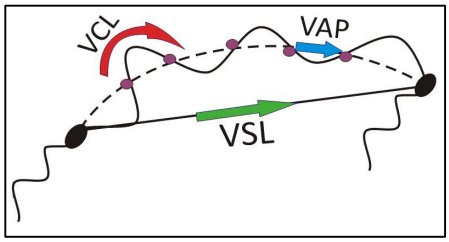
BOX 1. Ejaculate Analysis

My research takes into account many traits influencing male mating success, but it specifically focuses on postcopulatory traits. In all papers I present, I measured at least two ejaculate characteristics known to influence male fertilization success. I will quickly present here the methodology used to measure sperm quality traits.

Sperm collection. Sperm have been stripped following Matthews methodology (1997). After anaesthetization in a bath containing water and MS-222 (Tricaine Methanesulfonate), each male was placed on a Petri dish under a low-power dissection microscope with a drop of saline solution (0.9% NaCl). The gonopodium was rotated forward and gentle pressure was applied to the side of the abdomen. This action releases the sperm, packaged in bundles. Sperm bundles were collected with a Drummond micropipette.

Sperm Number. The number of sperm produced by males is often the primary factor influencing sperm competition (see Parker & Pizzari, 2010; Boschetto *et al.*, 2011; Kelly & Jennions, 2011). To obtain an estimate of the sperm reserves at rest, sperm were collected from males after at least 3 days of isolation. This allows males to replenish their sperm reserves after a copulation (Gasparini *et al.*, 2009). The whole ejaculate (except bundles needed for other measures) was collected and diluted in an appropriate volume of saline solution and then vortexed. Sperm were counted in an 'Improved Neubauer chamber' haematocytometer (Gasparini *et al.*, 2010).

Sperm Velocity. Sperm velocity is another important factor in sperm competition success (Boschetto *et al.*, 2011). Four (experiment 1) or six (experiments 2-4) sperm bundles were used to analyse sperm velocity using a CEROS sperm Tracker connected by a digital video-camera with a microscope. Measures were replicated twice using half of bundles each time. Bundles were placed on a multi-well slide and activated with 150 mM KCl and 4 mg ml-1BSA (Gasparini & Pilastro, 2011). This methodology provides seven standard measure of sperm velocity. We considered only the three most important and used:



VAP, average path velocity. Estimates the average velocity over a smoothed cell path.

VSL, straight line velocity. The velocity on a straight line between the start and the end point of the track.

VCL, curvilinear velocity. The actual velocity along the trajectory.

Sperm Morphology. Morphological sperm features are often highly variable across species (Jamieson, 1987) and in some cases are known to play a crucial role in sperm competition and cryptic female choice (Snook, 2005). In the paper no. 4 I measured the length of three sperm parts: sperm head, middle piece and flagellum. Photographs of each male's sperm were obtained under 1000x magnification (Leica DM1000 microscope) using a digital camera (Leica DFC320). ImageJ software was used to measure the length of the head, midpiece and flagellum of >20 sperm per male (Gasparini *et al.*, 2010).

Sperm Viability. This parameter has long been studied in animal reproductive sciences as indicators of fertility (Snook, 2005) and postcopulatory sexual selection appear to influence it (Hunter & Birkhead, 2002). Sperm viability has been measured in paper no. 4 using a sperm viability kit (Live/Dead, Invitrogen, Molecular Probes). Dead or damaged sperm are labelled red (with propidium iodide, a membrane-non-permanent stain) whereas live sperm are labelled green (with the membrane-permanent nucleic acid stain SYBR-14). Samples were observed under a fluorescence microscope and the proportion of live and dead spermatozoa were assessed from 200 sperm cells per male.

STUDY SPECIES

Poecilia reticulata, commonly named guppy, is a tropical livebearing fish. It has become a model species in evolutionary biology and has been largely used for field and laboratory studies in pre- and postcopulatory sexual selection (Houde, 1997; Magurran, 2005; Evans *et al.*, 2011b). It is characterized by a resource-free mating system in which the role of female mate choice and sexual coercion can vary across ecological gradients (in particular predation pressure). Females mate promiscuously and a high level of sperm competition has been reported. I have used fish descendant from a wild population (Lower Tacarigua River, Trinidad) which has been extensively studied in the context of pre- and postcopulatory sexual selection and life history traits.

The guppy is an internal fertilizer species and presents a strong sexual dimorphism (fig. 1). Females have a camouflaging gray colouration and a black spot in the anal region. Males are, on average, smaller than females and show an elaborated colour pattern composed by spots and stripes that can be distinguished in three main categories on the base of the mechanism that is responsible for the colouration: yellow and orange (determined by carotenoid and pteridine pigments), black (melanine pigments), and iridescent (white-blue-green; structural colours). Males present an extremely high inter- and intra population variation in the colouration pattern, with strong Y-linkage (Houde, 1992; 1997; Gordon *et al.*, 2012).

Male guppies have a modified anal fin, called gonopodium, used as copulatory organ. This structure is formed by three rays of the anal fin fused to form a capillary-like structure through which sperm bundles are transferred into the female gonopore. The gonopodium in provided with a distal cover part called 'hood' and a couple of hooks, both with sensory functions (Constantz, 1989; Cheng, 2004). Sperm are transferred in packs (spermatozeugmata) called 'sperm bundles', containing about 27000 sperm each (Evans *et al.*, 2004). Once in the female gonoduct, sperm bundles will release sperm cells that can be maintained in the ovary for several months and used by females to fecund later sets of ova (Hildemann & Wagner, 1954; Constantz, 1989).

During gestation, embryos develop in the uterine cavity for about one month, when most of the yolk is consumed and fully independent offspring are given birth. Broods are numerous (1-40) and number of offspring is correlated with season, mother size, condition, and age (Houde, 1997). After parturition, a new set of ova is ready to be fertilized and female will be receptive for two or three days. Males will reach sexual maturity after six to eight weeks but maturation and growth rate can vary between populations, when males fully develop gonopodium and ornamentation, and nearly stop body growth (Reznick, 1980).

When females are sexually receptive (2-4 days post-partum and virgin females), males court the females (performing a "sigmoid display") and try to obtain cooperative mating (Liley, 1966). When females are not receptive or refuse to mate, males adopt a coercive mating behaviour (gonopodial thrust) consisting in the furtive insertion of the copulatory organ into the genital opening of the female (the gonopore, see Box 2 and Liley, 1966). Female prefer to mate with males with larger colour spots and higher courtship rate (Endler & Houde, 1995). Attractive males, however, pay higher costs in terms of survival (Endler, 1983; Brooks, 2000). As females are highly polyandrous, sperm competition is intense in this species (Evans & Pilastro, 2011).

BOX 2. Male Sexual Behaviour

In all the experiments I dedicated some time to observe and measure male sexual behaviour. This sexually selected male trait is very important in this species as males in nature spend most of their time in the attempt to obtain matings (Clark & Aronson, 1951). It has been estimated that females receive in average (but it varies between populations) up to one mating attempt per minute during their entire life (Magurran & Seghers, 1994). There are two main tactics that males can adopt to inseminate females:

Males can court the female through an elaborated and stereotyped behaviour called 'sigmoid display'. During the courting the male moves slowly in front of the female, bends and shakes the body showing the colours and spreading all the fins (Baerends *et al.*, 1955). If the female is receptive, she will move toward the male eliciting the insertion of the gonopodium in to the gonopore (a behaviour called 'gliding'). Sperm transfer is fast: after males insert the gonopodium, fish move in a circle for one or two seconds and then separate themselves.

Males can also force females to mate using a coercive mating tactic called 'gonopodial thrusting'. If the female is not receptive, the male approaches the female without any display, swings his gonopodium forward and tries to insert it into the genital pore of the unwilling female Liley 1966 (Liley, 1966).

After a successful sperm transfer, both cooperative or coercive, males perform another stereotyped behaviour, called 'jerking': males jerk the body up and forwards, several times at first rapidly and then with decreasing frequency (sometimes for some minutes). The meaning of the behaviour is not clear but it has been shown that the number of jerks performed correlates with the number of sperm transferred during copulation (Pilastro *et al.*, 2007). Cooperative copulations are, in average, more successful that sneaky copulations and allow the male to inseminate more sperm (Luyten & Liley, 1991; Pilastro & Bisazza, 1999; Evans *et al.*, 2003a). The choice of the tactic is driven by different factors (Endler, 1987; Abrahams, 1993; Bisazza & Pilastro, 1997; Evans & Magurran, 1999; Evans *et al.*, 2002): predation risk, environmental physic conditions, male condition, operational sex ratio, female receptivity and male phenotype.

Besides the two main coercitive and cooperative behaviours described above, there are other behaviours commonly used to measure male sexual activity (see for example Rodd & Sokolowski, 1995; Head & Brooks, 2006; Head *et al.*, 2008). Here I reported a list of behaviours measured in the experiments presented in the thesis.

Sexual interest\activity: males normally spent most of the time following or chasing the females. In papers no. 2, 3 and 4 I measured the proportion of time spent by males in all the sexual behaviours performed towards females. This includes the time spent performing display and gonopodial thrusts (see above), but also following or chasing the female, jockeying (see below) or nipping (see below).

Physical contacts: males often bite the females near the gonopore (genital nipping) or on other body parts. In paper one I measured the times males performed those behaviours.

Jockeying: it is a common behaviour when two or more males are interested in the same female. In this case males chase together the same female 'jockeying' for position, to attain a closer position to the female. In paper one I measured how many times males jockeyed during observations.

Aggressive behaviour: when numerous males are present in the same tank they can interrupt other males' sexual behaviour, typically sigmoid display, attacking them. In the first experiment, I recorded the number of attacks performed.

Sperm competition success is influenced by relative differences in sperm number and sperm velocity between males (Boschetto *et al.*, 2011), but it has been shown that, once sperm number is controlled for, also sperm viability strongly determine competitive fertilization success when artificial insemination is used to compete ejaculates from rival males (Fitzpatrick, J. L. & Evans, J. P.; unpublished data). This is not surprising, as sperm viability determines the number of sperm that actually compete for the eggs.

In part, pre- and postcopulatory traits appear to be subject to directional sexual selection in the guppy (Endler & Houde, 1995; Pilastro *et al.*, 2002; Evans *et al.*, 2003b; Pilastro *et al.*, 2004; Locatello *et al.*, 2006; Pilastro *et al.*, 2007; Boschetto *et al.*, 2011). Quantitative genetic studies, however, have revealed that both pre- and postcopulatory sexually selected traits exhibit relatively high levels of additive genetic variation (Brooks & Endler, 2001; Evans, 2010; 2011).
This species is therefore appropriate to study how variability is maintained on traits despite strong (apparently) directional sexual selection.

Figure 1. Male (left) and female (right) guppies. Body size and colour pattern sexual dimorphism is evident. Modified anal fin (gonopodium) is also visible.

MANUSCRIPTS EXTENDED ABSTRACTS

I summarise below the content of the four studies that I have conducted and that are described in more detail in the manuscripts 1-4.

Multivariate selection gradients in sexually selected male traits

(See Manuscript 1 for more details)

A key issue in order to determine how variability is maintained despite selection is to characterize the strength, direction and shape of the selection itself. In fact, it is now well recognized that, nonlinear components (disruptive or stabilizing) of selection have been largely underestimated in the past years (Kingsolver *et al.*, 2001; Blows & Brooks, 2003). Moreover selection (both natural and sexual) seldom operates on a single trait, rather acts on the whole organism (Lande & Arnold, 1983; Phillips & Arnold, 1989; Schluter & Nychka, 1994). One way to account for both phenomena is to use a multivariate method to determine the fitness surface (that describes the relationship between fitness and all the traits under consideration Shaw & Geyer, 2010). This approach has the potential to describe how all traits interact at once to determine fitness and to visualize multivariate selection (Phillips & Arnold, 1989; Blows *et al.*, 2003), allowing to understand if constraints between different traits are present and, if this is the case, how these constraints orientate the selection.

Male guppies present an extreme variety of traits that have been shown to influence their reproductive success by influencing their mating success and their sperm competition success. These traits have a significant heritability (Brooks & Endler, 2001; Evans, 2010; 2011). However, information about multivariate selection on this species is limited. While selection's strength and shape have been well characterized for precopulatory traits (Blows *et al.*, 2003), only linear selection has been tested in one postcopulatory traits, i.e. sperm number (Head *et al.*, 2008). I filled this gap determining both linear and nonlinear sexual selection operating on pre- and postcopulatory male traits simultaneously. To do so, I set up 10 guppy populations, each composed by six males and eight females which freely interacted for one week in a 60l tanks. Sexual behaviour of males has been recorded (6 h obs. each, see Box 2). At the end of this period I measured male traits (size, ornamentation and ejaculate characteristics, Box 1), and I isolated all the females. Offspring born from first and second parturition have been collected (females can retain sperm in the oviduct and use them for fertilize eggs up to six months after mate). I used polymorphic microsatellites to assign paternity to offspring. Sixty males, 74 females, and 1003 offspring have been genotyped. Data obtained from paternity analysis have been used to determine male reproductive success (data obtained from first and second parturition were pooled).

I used multivariate selection analysis and fitness surface methodology (Lande & Arnold, 1983; Phillips & Arnold, 1989; Reynolds *et al.*, 2010) to determine the form, intensity and direction of selection acting on male traits. I estimated linear selection gradients (β) and the matrix of quadratic and correlational selection gradients (γ) using multiple regression on the following (standardized) eight traits: behaviour (2 principal components, PC1 and PC2, obtained by the records of male

behaviour) male size (body area), gonopodium length, orange and iridescent spot area (% of body size), sperm number at rest, and sperm curvilinear velocity (VCL). When testing for nonlinear selection, I used canonical rotation of the matrix of non linear selection gradients (γ matrix) to find the major axes of the fitness surface. This method generate an M matrix of new composite trait scores (eigenvectors, m1, m2, …m8), each describing a major axis of the fitness surface (see manuscript 1, tab. 3). The strength of selection along each eigenvector is given by its eigenvalue (λ), and the shape by its sign (positive: disruptive selection, negative: stabilizing selection). The significance of the model was assessed using double regression method (Bisgaard & Ankenman, 1996). This methodology simplifies the interpretation of gamma matrix reducing the number of variables and removing the effect of correlational selection gradients of the gamma matrix (Phillips & Arnold, 1989). Fitness surface on the two stronger M vectors (m1 and m8) was visualized fitting thin-plate spline using Tps function in the '*fields*' package of R (fig. 2).

Results

I did not find any significant linear selection gradients (β). The γ matrix did not reveal significant non linear selection on quadratic traits (the diagonal of the matrix), although gonopodial length showed a high positive value of γ (1.032), suggesting disruptive selection. Five correlational selection gradients were significant. Positive values indicate, in a vector space, that the sense of vector (=selection) is the same in the two correlated traits and that traits are selectively positively correlated, while the opposite occurs for negative values (e.g. sperm number and velocity, tab. 2 in first manuscript; see:Phillips & Arnold, 1989). Interpretation of this result in a fitness surface is not simple and should be achieved after canonical rotation procedure (Phillips & Arnold, 1989; Blows & Brooks, 2003). After the application of this procedure, non linear selection has been detected on four m vectors, revealing both disruptive (m1 and m2) and stabilizing (m6 and m8) selection. I considered only the highest load of each trait in significant m vectors. Vector m1 was primary loaded by gonopodium length and orange colouration whereas m2 was mainly loaded by PC2 of behaviour (in turn, loaded in the PCA by sigmoid display performed first day of observation and aggressive behaviour of males). Other two vectors were loaded by body size and sperm production (m6) and sperm velocity (m8).

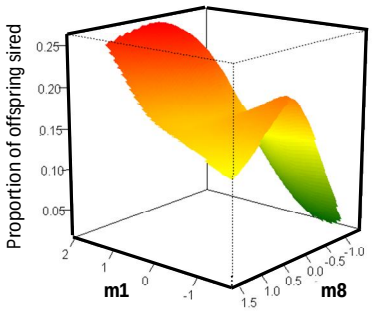


Figure 2. Fitness surface obtained with the thin-plate spline method. The surface describes the relationship between fitness and the m1 and m8 major vectors of selection. M1 is primary loaded by gonopodium length and orange coloration and is characterized by disruptive selection (positive λ), whereas on m8, loaded by sperm velocity and number, selection appear to be stabilizing (negative λ).

My results agree with those of previous studies (Blows *et al.*, 2003) revealing a non linear disruptive selection on male ornaments (orange colouration), but also on gonopodium length and different aspects of behaviour (courtship display and aggressive male behaviour. Vectors m1 and m2). More importantly, post-copulatory traits (sperm number and sperm velocity) were characterized by non-linear stabilizing selection (vectors m6 and m8). This result is partially in contrast with a previous study that found a negative linear selection acting on sperm production (Head *et al.*, 2008). I detected significant correlational selection in five gradients which were all negative, suggesting contrasting selective pressures between those traits (see manuscript 1, tab. 2).

Costs and trade-offs associated with sperm production

(See Manuscript 2 for more details)

If resources are limited, an increased investment in reproduction should result in a reduced investment in other life-history (i.e. naturally selected) traits (Roff, 1993). In polyandrous species, sexual selection continues after mating (Parker, 1970) and trade-offs can occur between ejaculate and mate acquisition traits (Parker, 1998). Pre- and postcopulatory episodes of sexual selection can therefore counteract each other (Danielsson, 2001; Simmons & Emlen, 2006). Whereas there are many correlative and comparative evidences that sperm quantity or quality are traded off with other traits (see for exampleHosken, 2001; Pitcher *et al.*, 2009; Rowe *et al.*, 2010; Klaus *et al.*, 2011; Lewis *et al.*, 2011), fewer empirical works tested experimentally if an increased investment in precopulatory traits results in a reduced investment in postcopulatory traits (e.g.', Simmons & Emlen, 2006).

In the guppy, postcopulatory sexual selection appears to favour males that are also successful at the precopulatory stage (Matthews *et al.*, 1997; Evans *et al.*, 2003b; Pilastro *et al.*, 2004; Locatello *et al.*, 2006; Pilastro *et al.*, 2007), supporting the phenotype-linked fertility hypothesis. This suggests that selection should work in the same direction in both trait types. However, recent studies suggest that attractiveness is genetically traded off with ejaculate quality (Evans, 2010).

In this experiment I investigated the effect of a long term investment in sperm production on other sexually selected traits in male guppies. To do so, I experimentally manipulated mating opportunities in two groups of males. Male guppies increase sperm investment (both quantity and quality) in response to perceived mating opportunities (i.e. within 1-3 days; Bozynski & Liley, 2003; Gasparini *et al.*, 2009) and both males and females prefer to mate with non familiar individuals (Mariette *et al.*, 2010), I manipulated male-female encounter rate to increase male mating rate and to force a greater reproductive investment. This allowed to compare the allocation to pre- and postcopulatory traits and highlight possible phenotypic trade-offs between these functions.

I set up two experimental groups, each composed by ten replicates. Each replicate consisted of randomly chosen fish (4 males and 6 females), placed in a 60l tanks and allowed to freely interact for 16 weeks. In the High Mating Rate (HMR) tanks the females were changed every two days. In the Low Mating Rate (LMR) tanks, the females were changed every 10 days (range 9-11). To equalize treatments, LMR females were manipulated (i.e. captured and released) every two days as HMR females, but were moved to another tank only when planned. Males which died during the treatment (12 in each treatment) were immediately replaced with new adult males collected from stocks, but were not considered for the subsequent analyses (males were individually recognisable through their unique colour pattern).

Before the beginning of the experiment and after three days of physical isolation from females, we analyzed males morphology (body size and colouration) and ejaculate characteristics (sperm number at rest and sperm velocity, see Box 1). At the end of the treatment males were isolated in singular 1l tanks for 2 days. The third day of isolation males have been moved to a new tank for a measure of post-treatment sexual behaviour with one unfamiliar female (see Box 2 for a description

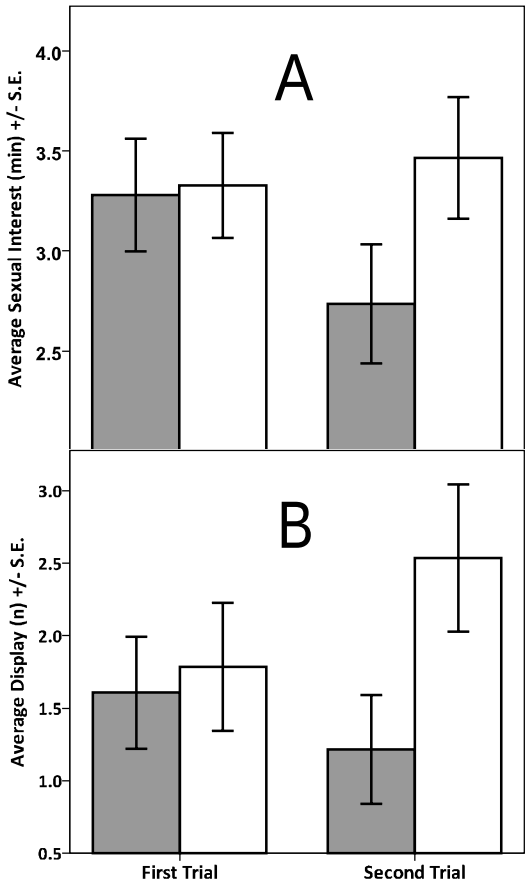


Figure 3. Change in male sexual interest (A) and sexual display performed (B). In gray HMR and in white LMR groups. On the left, behaviour recorded during the first five minutes. On the right behaviour recorded after 10 minutes.

of sexual behaviour). After 5 min of acclimatization, each male was allowed to interact with the female for 20 min and his behaviour was recorded during the initial and the final 5 min. I recorded number of sigmoid display, number of gonopodial thrust, and time spent by the male following the female (an index of sexual interest), (modified from Head & Brooks, 2006). At the end of the test, males were moved back in their singular tanks and the day after I analysed ejaculate, morphology, and colour pattern (Cuthill *et al.*, 1999; and Griggio *et al.*, 2009 for details on methodology; see experiment 1, Box 1, and Young *et al.*, 2011).

Results

Groups were compared using a repeated measures GLM (Time: 2 levels factor within subject= measure before and after treatment; factor between subjects= treatment; dependent variables: morphological male traits). In both groups, males body size increased during the experiment ($p<0.001$). No difference was observed in the relative colour spot area (parcentage of totale body area) before and after the treatment and between groups ($p>0.06$). Sperm production and sperm velocity increased during the experiment but not differently between the treatment groups ($p>0.143$). No differences in spectral properties of colour spots have been found between the two treatments (Student-t test, $p>0.223$). Males behaviour after the treatment has been analysed using a repeated measures GLM (Time: 2 levels factor within subject, measure at the beginning of the observation and after 10 min; factor between subjects= treatment). HMR males progressively reduced their courtship rate during time, while the opposite did the LMR males (see fig. 3 A and B and manuscript 2).

To analyse the relationship between sperm production and sexual behaviour after the treatment we considered the increment in ejaculate production (i.e. number of sperm after the treatment minus number of sperm before the treatment) and the sexual interest measured in the two periods of observation. There was a negative correlation between the time spent following the female and the increment in sperm production but this correlation was significant only for HMR males, in which there was a greater increase in sperm number (Pearson correlation= -0.508, $p=0.006$, $N=28$).

In summary, HMR males showed a reduced endurance in courtship rate and a negative correlation between investment in sperm production (sperm number before–after treatment) and sexual activity. Collectively, these results suggest that sperm production in *Poecilia reticulata* is costly and traded-off with mating effort.

Condition dependence of sexually selected traits

(See Manuscript 3 and 4 for more details)

To explain how genetic variability underlying sexually selected traits can be maintained, Rowe and Houle (1996) proposed the "genic capture hypothesis", which predicts that male traits under directional sexual selection, as become exaggerated, progressively increase their cost and hence become condition-dependent. Condition (i.e. the quantity of resources an individual can use for development and maintenance) depends on many loci (possibly the entire genome), linking the expression of sexually selected traits (SST) to a large proportion of the genome. Given this large mutational target, new mutations (which usually have deleterious effects) will arise as rapidly as selection removes inferior genotypes, thus maintaining variability in condition-dependent SSTs despite persistent directional selection (Tomkins *et al.*, 2004). One of the predictions of this model is that sexually selected traits are more condition-dependent than traits subject to stabilising selection.

I experimentally test for condition dependence in pre- and postcopulatory SSTs in the guppy. In this species, some behavioural and ornamental sexually selected traits have been shown to exhibit condition dependence (Grether, 2000; van Oosterhout *et al.*, 2003; Karino & Haijima, 2004; Kolluru & Grether, 2005; Kolluru *et al.*, 2009), but the possibility that postcopulatory traits also reflect male condition has received less attention (but seeZajitschek *et al.*, 2009). I thus manipulated male condition in two different and separated experiments (manuscript 3 and 4, in collaboration with Maria Berica Rasotto, University of Padova, and Jonathan Evans, from the university of Western Australia). In the first case I experimentally manipulated condition in a group of males by an immunological challenge (injection with LPS); in the second case (manuscript 4, submitted to *Journal of Evolutionary Biology*) by manipulating male diet. Metods used for behaviour, morphology, colour and ejaculate analysis are similar to those used in previuos experiments (see also Box 1 nad Box 2).

Immunological Challenge and Condition

(Manuscript 3)

I used an intramuscular injection of LPS (lipopolysaccarides from E. coli cellular membrane) to challenge males' immune system. This antigen is effective in eliciting an immune response in other fish species (M.B. Rasotto, pers. comm.). LPS is a potent activator of the immune system as it induces an inflammatory response that is followed by the production of specific antibodies, without simultaneously introducing a metabolically active, replicating pathogen. In this way, the cost of immune system activation can be disentangled from the cost of infection.

After preliminary tests (aimed to discover the appropriate dose of antigen), I randomly allocated 40 male guppies to two experimental groups: 20 males were injected with 1,25µl of LPS in saline solution, corresponding to 10mg antigen/Kg fish body mass (hereafter LPS group), and 20 males were injected with the same volume of saline solution (hereafter FISIO group). Males were

measured before and after the treatment (see manuscript 2). After the treatment, sexual behaviour of males (see Box. 2) has been recorded for 10 min in a tank containing 2 unreceptive females. Capture time (an index of fish ability to escape pradators) has also been recorded after the behavioural observation.

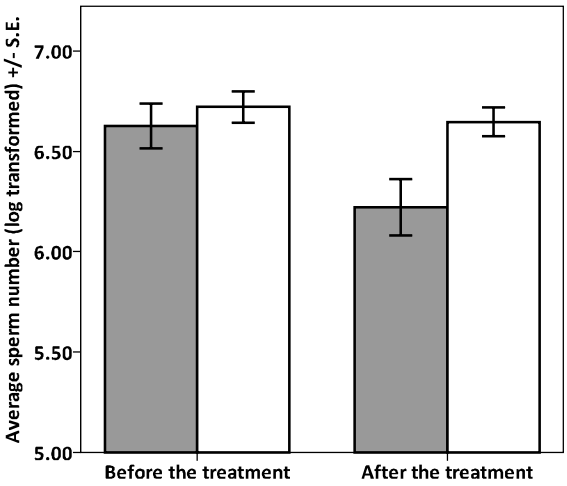


Figure 4. Average sperm number in the two experimental groups (white bars:FISIO; gray bars: LPS) before and after the treatment.

Results

No differences in sexual behaviour have been observed between the two experimental groups ($p>0.218$). However, capture time was significantly shorter in LPS males (Student-t test Dependent variable: Log-transformed 'capture time'. $T_{35}= 2.494$; $p=0.018$).

Male ornamentation (orange and black spot size, and orange chroma) was reduced after the treatment but did not differ between groups (see manuscript 3, tab. 1). In contrast, sperm number and velocity was significantly lower in the LPS group males (fig. 4, and manuscript 3, tab. 1).

Both groups showed a significant reduction of orange and black coloration in the second measure, but this change was similar in the two groups. In contrast, LPS treatment negatively affected postcopulatory traits. These results suggest that postcopulatory traits are more sensitive to immunological challenges than precopulatory traits, suggesting that ejaculate traits have a strong link with actual male condition.

Food restriction and Condition

(Manuscript 4)

In this experiment I focused on two precopulatory (sexual behaviour and colour ornamentation, including total area of colour spots and their spectral characteristics, see Box 2) and four postcopulatory (sperm number, velocity, viability and size, see Box 1) traits. I tested for condition dependence in these traits by comparing their expression between groups of guppies fed different diets. In the first group, 45 males were fed *ad libitum* while in the second group 45 males were fed a restricted diet (limited food quantity). Treatment lasted one month, to test the effect of condition manipulation in the short-medium term.

Pre- and postcopulatory traits were measured after the treatment period as in previous studies. Univariate General Linear Models (GLMs) were used to test for an effect of diet treatment (fixed factor) on body area, total area of orange, black and iridescent spots and number of sperm at rest. As these traits are typically linked to variation in male body size, we controlled for the effect of this parameter by entering standard length as a covariate in these models. Multivariate GLMs (Cuthill *et al.*, 1999) were used to test for an effect of diet treatment on the spectral characteristics of orange

and iridescent spots by entering the principal components obtained from spectrum data (orange: OR-PC1-2; iridescent: IR-PC1-4) as dependent variables. The remaining behavioural and sperm traits were analysed using either Student t-tests (parametric data) or Mann-Whitney U-tests (for non-parametric data), depending on whether the data were normally distributed.

Results

Food-limited males performed fewer sexual displays and exhibited a reduction in the time spent following females. By contrast, no significant difference in the number of gonopodial thrust attempts between the groups was detected (manuscript 4, tab. 1).

I found a significant reduction in the area of orange spots in males fed a restricted diet compared to their well-fed counterparts. By contrast, I found no significant effect of treatment on the area of iridescent or black spots (manuscript 4, tab. 1). Interestingly, despite the effect of diet treatment on the area of (orange) colour spots, my multivariate analysis of spot spectral characteristics revealed no overall differences in mean percentage reflectance (brightness), hue or saturation between the two experimental groups (manuscript 4, tab. 1).

My analysis revealed a significant reduction of sperm viability in low-diet males, while no effect was visible on sperm velocity, sperm length and sperm number (manuscript 4, tab. 1).

These results reveal that short-medium term dietary manipulation can have dramatic effects on the expression of pre- and postcopulatory sexually selected traits. Reductions in ornament and sexual display were accompanied by concomitant reductions in sperm viability, confirming that at least one component of ejaculate quality also exhibits condition dependence.

Condition dependence in the guppy

I showed that diet and immunological stress influence the expression of both precopulatory (sexual behaviour and ornamentation) and postcopulatory sexually selected traits (sperm number, velocity, viability). These results support the assertion from previous studies that precopulatory traits exhibit condition dependence in this species (Grether, 2000; van Oosterhout *et al.*, 2003; Karino & Haijima, 2004; Kolluru & Grether, 2005; Kolluru *et al.*, 2009), but also show that traits under postcopulatory sexual selection can exhibit concomitant declines in response to dietary and immunological stress. The results of the two experiments (LPS and diet), however, showed also some interesting differences. All sperm traits measured were particularly sensitive to immunological challenges, whereas the effect of diet restriction was limited to sperm viability. An opposite effect was found in male sexual behaviour. However, in the two experiments I used two different guppy populations and more work is necessary to disentangle the effect of treatment from that associated with differences among populations.

DISCUSSION

In this section I will briefly discuss the results of the four studies. A more detailed presentation and discussion of the results will be found in the attached manuscripts.

My PhD thesis focused on the problem of how variability is maintained in male sexually selected traits, with particular emphasis on postcopulatory traits.
I first characterized the strength, the shape and the direction of sexual selection acting on male guppy SSTs (manuscript 1). I then measured (manuscript 2) whether pre- and postcopulatory traits are phenotypically traded-off ones against the others when reproductive investment is experimentally manipulated. Finally, I tested condition dependence of sexually selected traits as predicted by the "genic capture hypothesis" (papers 3 and 4, Rowe & Houle, 1996).
With first experiment, I provided a more complete scenario of selection acting on the whole set of traits influencing reproductive success in male guppies, suggesting that non linear selection may be more important than previously estimated. This means that considering only linear selection is not sufficient to fully understand the selection processes underlying the evolution of pre- and postcopulatory sexually selected traits. Disruptive and correlational selection have the potential to maintain polymorphisms in these traits (see for example: Brodie, 1992 for snakes; and Smith, 1993 for birds) and this may be the case in our fish population. While purely postcopulatory sexual selection on sperm traits appears to be directional (Boschetto *et al.*, 2011), overall sexual selection on postcopulatory traits, which shape has never been explored before in this species, appears to have a prevalent non linear (stabilizing component) when the whole set of sexually selected traits is analysed in a multivariate approach. Stabilising selection is expected to reduce variability (Walsh & Blows, 2009), but this process appears to be slowed by the constraint due to the negative correlational gradients in the γ matrix. This result indicates that selection forces acting on male phenotype are counteracting each other, possibly contributing to the maintenance of genetic variability underlying sexually selected traits. This is the first time, to our knowledge, in which multivariate selection analysis and fitness surface techniques are used in order to determine the shape and strength of selection acting simultaneously on pre- and postcopulatory traits.

My second experiment tested a key assumption of sperm competition theory which has been experimentally rarely tested (Parker, 1998): that investment in ejaculate is phenotypically traded off against investment in obtaining mating. Ejaculate production is likely to be energetically costly (Olsson *et al.*, 1997; Snook, 2005; Parker & Pizzari, 2010) and our results confirm this observation for the guppies. More importantly we demonstrated that, when reproductive effort of males is experimentally elevated, ejaculate investment is traded off with precopulatory mating effort. In my experiment, in fact, males investing in postcopulatory traits (sperm production) are apparently forced to decrease investment in precopulatory traits (sexual behaviour). This suggests that increasing allocation to postcopulatory fitness components cannot be attained without reducing the investment in precopulatory SSTs. This is the first case, to our knowledge, in which the effects of experimental increase in sperm production are tested in a vertebrate.

In last two experiments I tested how male condition simultaneously influences mate acquisition and competitive fertilization success. Ornamentation, behaviour, sperm production, velocity and viability were all affected by a reduction in male condition. My results add to a growing number of studies that test critical components of the genic capture hypothesis (Rowe & Houle, 1996; Tomkins *et al.*, 2004), thus potentially explaining the high level of variability observed in sexually selected traits, by a selection/mutation balance. Future works would consider manipulation of condition during different developmental stages, a full and deep evaluation of condition itself, and, more importantly a measure of the effect of condition manipulation on sexual fitness (Kotiaho, 2001).

Conclusions

All the three, not mutually excluding hypotheses I tested during my PhD are potentially able to contribute explaining the high variability observed in sexually selected traits. However, some cautions are necessary when interpreting these results. First, exploring selection pressure acting on individuals, even if essential, is not a simple task. Multivariate selection analysis allows us to better comprehend the interaction between different traits and the overall strength of selection. However, considering many traits together makes data analysis and interpretation more difficult. Using fitness surface visualization I described in detail the non-linear features of selection acting on sexually selected traits, uncovering the constrains on selections acting on pre- and postcopulatory traits, possibly explaining part of the variability observed. Second, I confirmed, as proposed by Parker's 'sperm competition theory' (1998), that sperm production is costly and energetically traded-off with precopulatory investment. Although surprisingly not often tested, resource-related trade-offs among traits are likely to contribute explaining how variability is maintained despite directional or stabilizing selection. My results confirm that, once reproductive effort is experimentally elevated by manipulating male mating opportunity, sperm investment and mating investment are negatively correlated. Finally I successfully tested Houle and Rowe (Rowe & Houle, 1996) hypothesis that sexually selected traits are condition-dependent, thus potentially explaining the variability of those traits by the selection/mutation balance proposed in the 'genic capture hypothesis'. My two experiments of condition manipulation revealed that postcopulatory traits are condition-dependent and often they have been affected more strongly than precopulatory traits. However, the type of manipulation and/or, possibly the differences between populations, have an effect on which specific sperm trait is affected. Clearly, this point will require further investigation.

MANUSCRIPTS

Multivariate selection analysis of pre- and postcopulatory traits in the guppy

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Abstract
Describing how selection operates on traits is essential in order to determine how they can evolve. One general expectation is that, under strong selection, variability of traits should decrease, leading to a reduced evolvability. However, selection operates on the whole organism, rather than on a single trait and there is increasing evidence that its shape is rarely linear, possibly explaining why genetic variation is maintained despite apparently directional selection. Multivariate selection analysis and fitness surface visualization allow us to determine the strength, shape and direction of selection, accounting for selection operating on the multidimensional space represented by "multi-traits" individuals. In polyandrous species sexual selection continues after mating and simultaneously operates on all traits involved in mating and fertilization success. Here we simultaneously assessed how selection operates on pre- (ornamentation, behaviour, and morphology) and postcopulatory (sperm number and velocity) sexually selected traits in the guppy, *Poecilia reticulata*. Analysis of the reproductive success (our estimation of fitness) of sixty males revealed that selection acting on precopulatory traits is mainly disruptive (gonopodium length, orange colouration and behaviour) whereas postcopulatory traits are subject to both stabilizing and non linear selection (sperm velocity and number). Correlational selection on pair of traits was also significant, revealing the presence of constraints in the evolutionary response of several sexually selected traits. Our results agree with previous studies on guppies which also revealed non linear selection on ornamentation. Furthermore, this study shed light on the selective forces operating in postcopulatory traits, uncovering numerous constraints between traits associated with mating and fertilization success.

Introduction
As pointed out by numerous reviews (see for example: Johnson & Barton, 2005; Walsh & Blows, 2009), there are two main observation in nature that, taken together, form an unsolved problem in evolutionary biology: traits which are tightly associated with fitness, despite being subject to strong selection, are characterised by a large genetic variation (Endler, 1989; Lynch & Walsh, 1998; Kingsolver *et al.*, 2001). This problem has been primarily addressed for traits under natural selection but it clearly applies also to sexually selected traits (Borgia, 1979; Tomkins *et al.*, 2004; Radwan, 2008). One essential step in order to solve this problem is to evaluate the strength, the direction and the shape of selection acting on different traits (Travis, 1989; Kingsolver *et al.*, 2001; Blows & Brooks, 2003), considering that both natural and sexual selection operate on the whole individual and not on single traits (Lande & Arnold, 1983; Phillips & Arnold, 1989; Schluter & Nychka, 1994). Non linear (disruptive) and correlative selection potentially reduce the opportunities for selection to decrease trait variability (Walsh & Blows, 2009). To test this hypothesis it is necessary to use a multivariate selection analysis which will determine the fitness surface associated with individual phenotypes (Shaw & Geyer, 2010). This approach has the potential to describe how all traits interact at once to determine fitness and to visualize multivariate selection (Phillips & Arnold, 1989). This approach has largely been used for traits subject to natural (Kingsolver *et al.*, 2001; Kingsolver & Pfennig, 2007; e.g. Gimenez *et al.*, 2009; Ritz & Kohler, 2010;

Crean *et al.*, 2011) or precopulatory sexual selection (see for example Blows *et al.*, 2003; Blais *et al.*, 2004; Brooks *et al.*, 2005; Rundle *et al.*, 2008; Brooks *et al.*, 2010; Punzalan *et al.*, 2010; Ritz & Kohler, 2010; Thomas & Simmons, 2010; Rundle & Chenoweth, 2011; Steele *et al.*, 2011), but very few studies attempted to also analyse postcopulatory traits (see below). Yet, in most sexual reproducing species, sexual selection continue after mating trough criptical female choice (Eberhard, 1996) and sperm competition (Parker, 1984), and strong selection on these traits is therefore expected (Simmons & Kotiaho, 2002; Moore *et al.*, 2004; Birkhead *et al.*, 2005; Simmons & Moore, 2009; Snook *et al.*, 2010; Evans, 2011; reviewed by Evans & Simmons, 2008). Surprisingly, we found very few articles in literature that exploit multivariate regression techniques and fitness surface visualization in order to determine selection forces acting on postcopulatory traits (Wojcieszek & Simmons, 2011) and none focusing directly on sperm characteristics.

In this manuscript we used multivariate selection analysis to describe selection forces acting on a set of both pre- and postcopulatory traits in the guppy, *Poecilia reticulata*. In this species female are sexually promiscuous and sperm competition is very intense (Houde, 1997; Evans & Pilastro, 2011). Guppies are sexually dimorphic, with males exhibiting complex colour patterns composed of orange (carotenoid and pteridine based), iridescent (structural), and black (melanin based) spots. Females are sexually receptive only few days after parturition. During this period males can obtain cooperative mating and females prefer more colourful and courtship males (Endler & Houde, 1995). When females are not receptive males can obtain copulation only through forced mating attempts, behaviour named gonopodial thrusts (Houde, 1997). Male fitness is therefore determined both by precopulatory traits (mainly colouration, gonopodium size and sexual behaviour) and by postcopulatory traits (ejaculate traits. See Endler & Houde, 1995; Pilastro *et al.*, 2002; Evans *et al.*, 2003b; Pilastro *et al.*, 2004; Locatello *et al.*, 2006; Pilastro *et al.*, 2007; Boschetto *et al.*, 2011). Moreover these traits have a significant genetic component and often show an extreme intra-population variation (Brooks & Endler, 2001; Evans, 2010; 2011). In this species selection's strength and shape have been well characterized for precopulatory traits (Blows *et al.*, 2003) whereas only linear selection has been tested in one postcopulatory traits, sperm number (Head *et al.*, 2008).

We used multivariate selection analysis and fitness surface methodology to determine the form, intensity and direction of selection acting on the following (standardized) male traits: behaviour, male body size, gonopodium length, colors, sperm number, and sperm velocity. Fitness of 60 males has been estimated trough paternity analysis (allele sharing) of 1003 offspring obtained by 74 females in two subsequent breeding cycles (females can retain sperm in the oviduct and use them for fertilize eggs up to six months after mate Constantz, 1989).

We found that nonlinear (both stabilizing and disruptive) and correlational selection acting on male traits are strong, improving our understanding of selection pressure acting in this species and offering an explanation of the high variability observed.

Methods

Study population and fish maintenance

Fish used for this experiment were descendant of wild-caught fish from low Tacarigua river in Trinidad (national grid reference PS 787 804). Males and females are reared in stock tanks with a sex ratio of 1:1. Virgin females are kept in separate single-sex tanks. All tanks are maintained 26±1°C with 12:12 h light-dark cycle and fed twice a day with commercial flake food (Duplarin) and fresh food (*Artemia salina* nauplii). For body size and colour analyses, fish have been anesthetized with MS-222, then photographed, stripped for ejaculate analyses and finclipped for DNA extraction and paternity assignment.

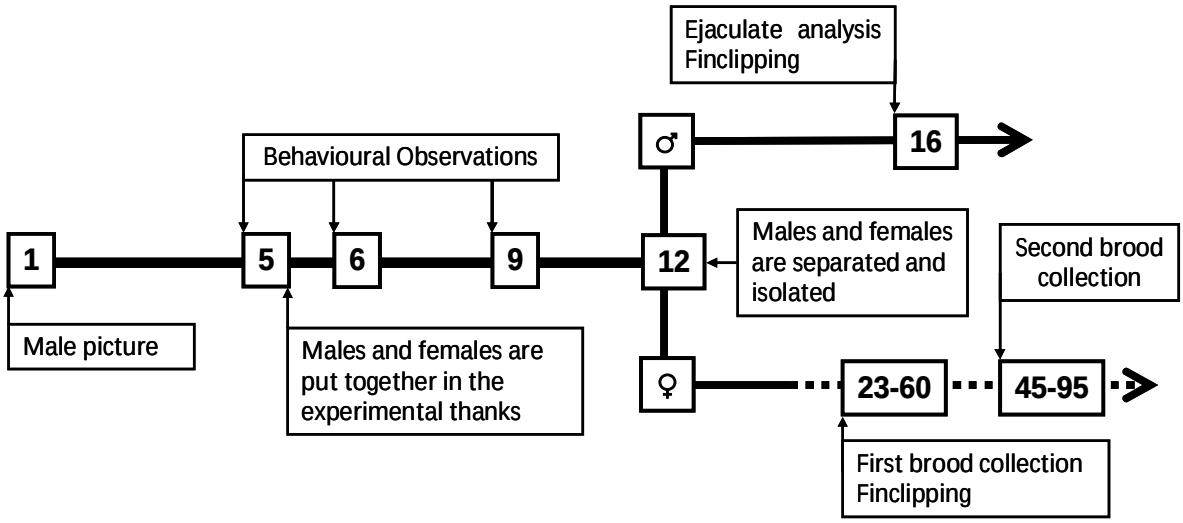

Figure 1. Timescale of the experiment. Each male undergo the same treatment, schematized in the figure. Squares represents the days. After day twelve operation on both males and females are shown.

General experimental design

We formed 10 experimental tanks (60 l) in which six sexually mature males (age 3 to 4 months) and eight mature virgin females (age 4 to 5 months) could freely interact for one week. Four days before this period male size and ornamentation have been measured (see fig. 1 for the timescale). This rest period, in which males were kept in isolation, ensured that anesthetization effect would not have effects on male condition during the period of interaction with females. Sexual behaviour of males has been monitored in the experimental tank during the week of interaction (see below). At the end of the week females were isolated in singular 2l tanks provided with artificial vegetation and allowed to give birth. Males were isolated in singular tanks with visual access to females for 3 days and then the ejaculate was collected for further analyses (see below). The 3-days isolation period ensures a fully replenishment of sperm supplies (Bozynski & Liley, 2003; Gasparini *et al.*, 2009). After ejaculate analysis (males) and after first parturition (females), a small portion of caudal fin was collected from the adults (finclipping). This tissue was subsequently used for DNA extraction. Female tanks were checked at least twice a day in order to detect and collect any fry. One day after birth, offspring from first and second brood were euthanatized in iced water for DNA extraction.

Male size and ornamentation

Males were anesthetized and excess of water on their body was removed using blotting paper. A digital photograph of the fish placed on a ruler was taken (Nikon Coolpix 4300). Digital images were analysed using UTHSCSA ImageTool software (http://ddsdx.uthscsa.edu /dig/itdesc.html): we measured, on the left side of each male, the body area, the total area of carotenoid and pteridine spots (hereafter "orange") and iridescent spots (blue, green, violet, hereafter "iridescent"). Colour spot areas were subsequently standardized to male's body area (%).

Behavioural observations

Behaviour of each male (individually recognized on the basis of their unique colour pattern) has been observed three times during the experiment (fig. 1).

The first behavioural observation (1 h) took place when males were put into the tank, after five min of acclimatization, and the number of sigmoid displays performed by each male was recorded. Each male has been observed again, in random order, for 10 min, in the following day and after five days. We recorded the number of sigmoid displays (courting behaviour), gonopodial thrusts (sneaky attempts), aggressive interactions, and jockeying performed by the male (for description see Head *et al.*, 2008). Male behaviours were standardized per min of observation and summarized, using PC analysis, in two PC axes that describe 60% of total variability (tab. 1).

Table 1. Principal components analysis of male behaviour. Only PCs with eigenvalue greater than one have been accepted. Behaviours have been recorded in 2 different days. Loading factors and percentage of variability described by PC are given.

Behaviour		PC1 (34.7%)	PC2 (25.30%)
First day	Sigmoid display	0.341	0.604
Second day	Sigmoid display	0.739	0.177
	Gonopodial thrust (GT)	0.733	-0.420
	Aggressions	0.408	-0.712
	Jockeying	0.607	0.430

Sperm analysis

Sperm were stripped (Matthews *et al.*, 1997; Gasparini *et al.*, 2010) three days after the end of the experiment for sperm velocity assay and baseline sperm counts. Each male was placed on a Petri dish under a dissection microscope with 0.5ml of physiological solution (0.9% NaCl). A gentle pressure was applied to the side of the abdomen to release all sperm bundles (sperm packages). Four sperm bundles were collected with a Drummond Micropipette and immediately used for computer assisted sperm analysis (CASA, Hamilton Thorne CEROS). Sperm velocity assay was repeated twice using two bundles for each analysis which were placed on a multi-well slide and activated with 150 mM KCl and 4 mg ml^{-1}BSA (Gasparini & Pilastro, 2011). Three standard measures of sperm velocity: average path velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL). These three measures are highly correlated (*Pearson correlation*>0.717, p<0.001), and for simplicity only VCL has been reported. Results obtained with the other three measures were substantially identical (not shown). The mean of the two measures was used for each male. All other bundles were vortexed in a known volume of physiological solution for sperm count. Sperm number was obtained using an *improved Neubauer haemocytometer* under a 400x magnification. The average of ten counts per male was used as an estimate the number of sperm at rest.

Paternity analysis

The tissue samples obtained from the mothers (n=74), the potential fathers (n=60) and all of the offspring (n = 1003) were collected and stored in a freezer at -80°C until analysis. Genomic DNA was extracted using CHELEX (Walsh *et al.*, 1991) for newborns and Salting out for adults fins (Miller *et al.*, 1988). Paternity has been assigned using three microsatellite markers, including TTA (Genbank accession numbers: AF164205, Taylor *et al.*, 1999), AGAT11 (BV097141, Olendorf *et al.*, 2004) and KonD15 (AF368429, Seckinger *et al.*, 2002) which allow >99% paternity assignment in this population (Gasparini *et al.*, 2010). Polymerase chain reaction amplifications were performed on a GeneAmp® PCR System 9700 Thermocycler (Applied Biosystems, CA, USA). The PCR was performed with 7.635 µl BDH, 1 µl $MgLl_2$, 3 µl Taq buffer, 0.525 µl dNTPs, 0.38 µl primers (*forward* + *reverse*), 0.08 µl Taq DNA polymerase (Promega) and 2 µl DNA template. The cycling protocol included initial denaturation step at 95°C for 1 min, 30 cycles of 10 s denaturation at 95°C, 30 s annealing (TTA and AGAT11= 52°C, KonD15= 56°C),

Table 2. Linear (β) and non linear (γ) selection coefficients on male traits obtained from two separate regressions. Quadratic coefficients (the diagonal in γ matrix, values in italic) represent stabilizing (negative) or disruptive (positive) selection and have been doubled in order to be compared to other coefficients. Coefficients under the diagonal represent correlational selection. The response variable is the proportion of offspring sired by each male in the tank. In bold statistically significant values ($p<0.05$).

Male traits	β	γ matrix							
		Male size	**Gon. length**	**Orange %**	**Irid%**	**Sperm number**	**VCL**	**Behav. PC1**	**Behav. PC2**
Male size	-0.024	-0.408							
Gonopodium length	0.225	-0.078	1.032						
Orange%	-0.042	0.555	**-0.752**	0.182					
Iridescent%	-0.166	0.175	-0.082	-0.078	-0.116				
Sperm numb. (10^6)	-0.262	0.049	0.410	-0.559	**-0.717**	-0.482			
VCL	-0.129	0.566	**-1.440**	-0.023	**-0.464**	**-1.049**	0.534		
Behav. PC1	0.092	-0.123	0.059	-0.225	0.241	0.196	0.104	-0.014	
Behav. PC2	-0.081	0.108	-0.533	0.262	0.444	-0.550	-0.423	0.265	-0.188

extension at 72° C for 30 s, and a final extension for 5 min at 72° C.

Amplified fragments were separated by electrophoresis on an ABI PRIMS DNA Analyzer 3100/3700 sequencer (ABI PRISM, Applied Biosystems), using 400 HD ROX (Perkin-Elmer, Applied Biosystems) as a size standard. PCR products were visualized using Peack Scanner software (www.appliedbiosystems.com).

Paternity was assigned to offspring using Cervus 3.0 (http://www.fieldgenetics.com Marshall *et al.*, 1998; Kalinowski *et al.*, 2007).

Multivariate selection analysis

Male traits have been standardized to have mean of zero and standard deviation of one (Lande & Arnold, 1983). Proportion of offspring produced by each male over the total offspring produced in his tank has been used as fitness measure. Linear selection gradients (β), which describes directional selection, and the matrix of non linear (quadratic and correlational) selection gradients (γ) have been determined using a multiple regression approach (Lande & Arnold, 1983) on the following eight traits: behaviour (2 principal components, PC1 and PC2), male body size (body area), gonopodium length, orange and iridescent spot area (% of body size), sperm number, and sperm curvilinear velocity (VCL). Quadratic selection gradients have been doubled in order to obtain accurate estimates of non linear selection gradients (Stinchcombe *et al.*, 2008). Canonical rotation of gamma matrix has been performed when testing for nonlinear selection. This method generates a matrix of new composite trait scores (eigenvectors, m1, m2, …m8), each describing a major axis of the fitness surface (Phillips & Arnold, 1989 ; Blows & Brooks, 2003). The strength of non linear selection along each eigenvector is given by its eigenvalue (λ), and the shape by its sign (positive: disruptive selection, negative: stabilizing selection). The significance of the model was assessed using double regression method (Bisgaard & Ankenman, 1996). This methodology simplifies the interpretation of gamma matrix by reducing the number of variables and removing the effect of correlational selection gradients (Phillips & Arnold, 1989). To visualize fitness surface we fitted thin-plate splines (Green & Silverman, 1994; Blows *et al.*, 2003) using Tps function in the '*fields*' package of R (version 2.12.1, http://www.r-project.org/). This is a nonparametric approach that provides a less

constrained view of surface than the best quadratic approximation (Blows *et al.*, 2003).
Statistical analyses have been performed using PASW Statistic 18 (IBM SPSS) unless differently specified.

Results

Multivariate selection analysis

We did not find any significant linear selection gradients (β, tab. 2). The γ matrix did not reveal significant non linear selection on quadratic traits (the diagonal of the matrix), although gonopodial length showed a high positive value of γ (1.032), suggesting disruptive selection. Five correlational selection gradients were significant (tab. 2). After the application of this procedure, non linear selection was detected on four m vectors, revealing both disruptive (m1 and m2) and stabilizing (m6 and m8) selection (tab. 3). We considered only the highest load of each trait in significant m-vectors. Vector m1 was primarily loaded by gonopodium length and orange colouration whereas m2 was mainly loaded by behaviour PC2 (associated with sigmoid displays performed during first day of observation and aggressive behaviour). The other two vectors were loaded by body size and sperm production (m6) and sperm velocity (m8).

Fitness surface visualization

Fitness surface obtained fitting a thin-plate spline on the two major axes of selection (m1 and m8) revealed a saddle form (fig. 2) in which the two peaks corresponded to an average value in m8 (stabilizing selection) and extreme values in m1 (disruptive selection). This indicates that successful males had long gonopodium and small orange spots, or short gonopodium and large orange spots (m1), and an average sperm velocity and number (m8).

Discussion

Our study showed that selection forces acting on different male traits in *P. reticulata* are mainly non linear. In particular we found that selection on precopulatory traits (gonopudium length, orange colouration, courtship display and aggressive behaviour) was mainly disruptive. In contrast, selection on postcopulatory traits (sperm number and velocity) showed a stronger stabilizing component. Constraints between traits were also present as indicated by the negative correlative selection gradients. We will discuss in turn the results of the different selection types.

Linear selection

Linear selection, described by β coefficients in table 2, was not significant and less strong than the non linear selection components. Sign and magnitude of β coefficients in our study differ slightly from those found in previous studies

Table 3. The M matrix of eigenvectors and estimates of non linear selection (λ) acting on axes described by canonical rotation of the γ matrix and the p-values obtained with double regression method. Positive values of λ represent disruptive selection and negative values represent stabilizing selection. The response variable is the proportion of offspring sired by each male in the tank. Significant values are in bold.

M Vector	Male Traits								λ	p
	Male size	Gonopodium length	Orange %	Irid %	Sperm number	VCL	Behav. PC1	Behav. PC2		
m1	0.464	0.635	-0.556	-0.083	-0.057	-0.228	0.088	0.049	**0.152**	**0.050**
m2	0.03	-0.266	-0.090	0.307	-0.457	-0.243	0.153	0.732	**0.106**	**0.045**
m3	0.428	0.175	0.361	0.654	-0.239	0.127	-0.324	-0.223	0.058	0.804
m4	0.082	0.322	0.674	-0.126	0.303	-0.480	0.202	0.238	-0.006	0.783
m5	0.470	-0.091	0.198	-0.569	-0.116	0.394	-0.351	0.341	-0.025	0.566
m6	-0.507	0.511	0.185	-0.192	-0.622	0.143	-0.048	-0.020	**-0.081**	**0.001**
m7	0.212	0.031	0.130	0.047	-0.069	0.507	0.819	-0.047	-0.180	0.086
m8	0.266	-0.347	0.091	-0.305	-0.484	-0.459	0.163	-0.486	**-0.319**	**0.002**

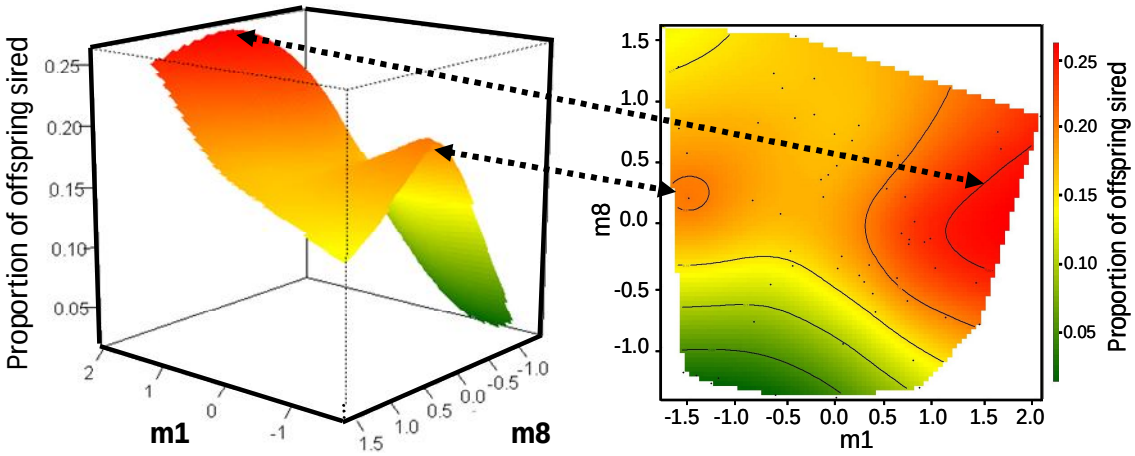

Figure 2. Fitness surface obtained with the thin-plate spline method and contour map of the surface. The surface describes the relationship between fitness and the m1 and m8 major vectors of selection. M1 is primary loaded by gonopodium length and orange coloration and is characterized by disruptive selection (positive λ), whereas on m8, loaded by velocity (and partially sperm number), selection appear to be stabilizing (negative λ).

(Head *et al.*, 2008). In particular, Head and coll. found a negative linear selection acting on sperm number and sneaky behaviour (negative value of β). These differences could also be due to small differences in the experimental design. However, the most likely explanation for these discrepancies is that fish used in the two experiments came from different populations, and there is evidence that female mating preferences can differ significantly across populations (Endler & Houde, 1995). A non-mutually exclusive explanation rests on the different sets of predictors used in the two studies. In fact, we included two measures of postcopulatory traits (sperm number at rest and velocity), whereas Head et al. only included sperm number after mating, which may also be influenced by recent mating rate.

Quadratic and correlational selection

Quadratic γ coefficients in our analysis did not reveal significant non linear selection acting on single traits. Some of these coefficients, however, were high in absolute value (male size=-0.408, gonopodium length=1.032, sperm number=-0.482, and sperm velocity=0.534. see Blows & Brooks, 2003; and Stinchcombe *et al.*, 2008 for a comparison) and their effect was maintained after canonical rotation of the matrix. Sperm velocity (VCL) was apparently subject to different selection regimes in the two analysis, but this difference was probably driven by the strong negative correlational selection acting on sperm velocity on the one hand, and sperm number and gonopodium length on the other (γ =-1.049, and γ =-1.440, respectively). Positive values of correlational coefficients indicate, in a vector space, that the sense of vector (=selection) is the same in the two traits and that traits are therefore selectively positively correlated, while the opposite occurs when coefficients are negative (Phillips & Arnold, 1989), resulting in contrasting selective pressure on the two traits (Walsh & Blows, 2009). Confirmation of the results, however, is nevertheless not simple and should be achieved after canonical rotation procedure, with which the effect of the correlational selection is integrated in the new matrix of m-vectors (Phillips & Arnold, 1989; Blows & Brooks, 2003).

Canonical rotation and fitness surface

Canonical rotation, produced results which were in large agreement with those of previous studies (Blows *et al.*, 2003), revealing disruptive selection on male ornaments (orange colouration). Furthermore, disruptive selection also involved gonopodium length and different

aspects of behaviour (courtship display rate and aggressive behaviour). More importantly, post copulatory traits (sperm number and sperm velocity) were characterized by stabilizing selection. This result is partially in contrast (but see below) with previous studies that showed a negative linear selection acting on sperm production (Head *et al.*, 2008).

Orange colouration is known to be an important determinant of male reproductive success in this species (Houde, 1997). Here we showed that the stronger selection vector acting on this trait is not linear and that it is constrained by an opposing selection vector acting on gonopodium length (another trait which morphology play a role in male attractiveness Brooks & Caithness, 1995; Evans *et al.*, 2011a). In our experiment individuals with higher reproductive success were characterized by opposite and extreme values of those two traits (also showed by correlational selection gradient 'γ'). This result can be explained considering the presence of different male mating tactics in this species. Males with larger orange spots may be favourite in cooperative mating by female preference for this trait. In contrast, less conspicuous males with a longer gonopodium may be more successful in forced copulations (Rosen & Tucker, 1961; Martin *et al.*, In press). Unfortunately, our experimental design did not allow to distinguish between the fitness associated to the two mating tactics, as the same male can easily switch between behaviours. Selection acting on male behaviour was also disruptive (even if weaker, m2). This vector indicates that successful males tendend to show low aggression rate and performed more displays or *viceversa* (PC2, see table 1). Loading factors associated with cooperative and coercive behaviour have opposite sign in PC2, in agree with the interpretation given for selection acting on m1.

Selection acting on postcopulatory traits, in contrast, was mainly stabilizing. The vector with the greatest λ absolute value (-0.319) was m8, indicating that selection surface of sperm velocity had a strong curvature, and the negative sign indicate that selection was stabilizing. Vector m6 was associated with sperm number and male body size. The sign of this vector was negative, but its value was relatively small, indicating that the curvature of the surface was not as pronounced as for other significant vectors.

Conclusions

Disruptive and correlational selection can contribute maintaining variability in sexually selected traits (see for example: Brodie, 1992 for snakes; and Smith, 1993 for birds). Our results suggest that this may be the case for the precopulatory traits studied in our fish population. On the contrary, variability should be reduced by directional and stabilizing selection (Walsh & Blows, 2009), as the one we observed on postcopulatory traits in this study. However, previous studies demonstrated a large variability for these traits (sperm number and velocity) in *P. reticulata* males (Gasparini & Pilastro, submitted; Evans, 2010; Evans, 2011). One possible explanation of this apparent contradiction relies in correlational selection acting on pair of traits. In fact, four of the significant gradients (see tab. 2) regard sperm traits and all of them are negative. This result suggests that correlational constraints are present between traits, and selection forces acting on those pairs of traits are counteracting. If this is the case, variability is maintained despite selection because effects on a trait will have a side-effect on the correlated trait (Walsh & Blows, 2009).

This is the first time, to our knowledge, in which multivariate selection analysis and fitness surface techniques are used in order to determine the shape and strength of selection acting simultaneously on pre- and postcopulatory traits. Our results suggest that selection in the guppy is far from being linear or directional and, more importantly, that the same trait (and combination of traits) can undergo different, contemporary selection pressures which are likely to largely reduce the potential for selection to reduce the genetic variability underlying these traits (Walsh & Blows, 2009). The expansion of this multivariate selection analysis to more

postcopulatory traits (e.g. sperm size or viability) could reveal other constraints. Future research should try to combine the use of this methodology with a quantitative genetic approach, in order to fully understand both selection forces acting on and genetic correlations/constrains between relevant sexually selected traits.

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Long-term costs of sperm production in the guppy

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Abstract

As predicted by sperm competition theory, if resources are limited, males of polyandrous species face a trade-off between pre- and postcopulatory traits. Despite the growing body of correlative and comparative evidence, fewer empirical works tested experimentally if an increased investment in postcopulatory traits results in a reduced investment in precopulatory traits. In the guppy, *Poecilia reticulata*, postcopulatory sexual selection appears to favour attractive males, whereas other evidence suggests that attractiveness is traded off with ejaculate quality. In this experiment we investigated the long term effect of an experimentally elevated mating rate on pre- and postcopulatory allocation. We manipulated the perceived mating opportunities of two groups of males reared for four months with unfamiliar (high mating rate group) or familiar (low mating rate group) females. Our results evidenced a phenotypic trade-off between ejaculate production (which was elevated) and courtship rate (which was reduced) in high-mating rate males.

Introduction

Lifetime fitness is maximized when resources are optimally allocated to the life history traits that concur into determining different fitness components. This is because, if resources are limited, an increased investment in one component (e.g. reproduction) will result in a reduced investment in other life-history traits (Roff, 1993). In polyandrous species, sexual selection continues after mating and males compete for both acquiring mates and for fertilizing their eggs (Parker, 1970). As predicted by sperm competition theory, trade-offs are therefore expected to arise between ejaculate and mate acquisition traits (Parker, 1998). It can safely be assumed that an increased investment in a pre- or postcopulatory sexually selected trait will result in an increased mating or fertilization success, respectively. However, it is more difficult to predict how the balance between pre- and postcopulatory investment will influence reproductive fitness, as this will be determined by the interaction between fitness functions and thresholds. If this is the case postcopulatory success of males can be constrained by precopulatory investment and vice versa, most likely leading to pre- and postcopulatory episodes of sexual selection balancing each other (Danielsson, 2001; Simmons & Emlen, 2006). Whereas there are many correlative and comparative evidences that sperm quantity or quality are traded off with other traits (see for example: Pitnick, 1996; Hosken, 2001; Froman *et al.*, 2002; Simmons & Roberts, 2005; Pitnick *et al.*, 2006; Rowe *et al.*, 2010; Klaus *et al.*, 2011; Lewis *et al.*, 2011), experimental studies on phenotypic trade-off are limited and reached opposite conclusions. Whereas an increased investment in precopulatory traits results in a reduced investment in postcopulatory traits in horned beetles (Simmons & Emlen, 2006), the opposite pattern has been found in a butterfly (Cordero, 2000). Understanding the complex interactions between pre- and postcopulatory sexual selection, uncovering possible trade-offs between traits involved in such mechanisms, is a key goal in evolutionary biology, as it can

explain the unexpected variability of sexually selected traits (Radwan, 2008).

The guppy, *Poecilia reticulata*, is a polyandrous fish characterized by alternative male mating tactics (individual males can interchangeably adopt either a cooperative or a coercive mating tactic), high level sperm competition and a non-resource-based mating system. While some studies on guppies found that postcopulatory sexual selection favour attractive males (Matthews *et al.*, 1997; Evans *et al.*, 2003b; Pilastro *et al.*, 2004; Locatello *et al.*, 2006; Pilastro *et al.*, 2007), supporting the phenotype-linked fertility hypothesis, others suggest that sperm production have a negative selection coefficient (Head *et al.*, 2008), that attractiveness is traded for ejaculate quality (Evans, 2010), and that sperm characteristics are potentially genetically constrained (Evans, 2011). Moreover both pre- and postcopulatory traits show high variability in this species (Brooks & Endler, 2001; Evans, 2010; 2011). Although the large body of work exploring the interaction between sperm characteristic and other traits in this species (see above), and between mating rate a survival (Miller & Brooks, 2005), no manipulative experiments have been performed so far to evaluate if an investment in sperm production is traded-off with other reproductive investments. Here we investigated in male guppies the effect of an elevated mating a sperm investment and on traits involved in mate acquisition. As male guppies adjust sperm production in response to short-term changes in mating opportunities (i.e. within 1-3 days; Bozynski & Liley, 2003) and both males and females prefer to mate with unfamiliar individuals (Mariette *et al.*, 2010), we manipulate investment in sperm production by increasing male mating opportunities with unfamiliar females.

We predicted that males that increase their allocation to postcopulatory traits in response to an increased mating rate should show a reduced investment in traits associated with mating acquisition.

Methods

Fish Maintenance

Guppies used in the experiment were descendent of wild-caught fish from the lower part of Tacarigua River, Trinidad (see Gasparini *et al.*, 2010 for details in fish maintenance). We used 80 males between 6 and 8 months old collected from stock aquaria (sex ratio ~ 1:1) and randomly assigned to one of two experimental groups. A total of 198 gravid (=non virgin) females have been used for the treatment. Thirty gravid females have been used, after the treatment, for behavioural test. All females were collected from stock aquaria where the sex ratio was approximately 1:1. All fish were fed *ad libitum* twice a day before and during the experiment.

General Experimental Design

We divided the experimental males in two groups in which mating opportunities were manipulated. We performed 10 replicates per group. Each replicate consisted of 4 focal males and 6 females, placed in a 60l tanks, freely interacting for 16 weeks (average= 115 days, min= 110 days; max= 120 days). Male guppies significantly reduce their sexual activity after 5 days of interaction with the same female (Jordan & Brooks, 2010), in the High Mating Rate (HMR) tanks we changed females every two days, whereas in the Low Mating Rate (LMR) tanks females were changed every 10 ± 1 days. To equalize treatments, female in LMR treatment were manipulated in the same way as HMR females every two days, but not moved to another tank if not planned. During this period males' behaviour has been monitored up to 4 times.

Males dead during the treatment (12 in each treatment) were immediately replaced with new adult males collected from stocks and not considered for the subsequent analysis.

Before the treatment males that had been kept in physical isolation from females for 3 days were analysed for morphology (body size and colouration) and ejaculate characteristics (sperm number at rest and sperm velocity). At the end of the treatment males have been isolated in singular 1l tanks for 2 days. In this period males were physically but not visually isolated from 2 non familiar females. The third day of isolation males have been moved to a new tank for post treatment behavioural observations. At the end, test males were moved again in singular tanks

and the day after collected for ejaculate, morphology, and colour analysis.

Behavioural Trials

Male behaviour during the treatment

In the HMR group, male behaviour was recorded once in the day following the introduction of the new females. In the LMR group, male behaviour was recorded on the same day as above and for further three times during the following week (between days 4 and 10). This schedule has been repeated twice (SERIES1 and SERIES2): after approx. 5 weeks (average= 37 days, min=31 days, max=43 days), and after approx. 10 weeks (average= 72 days, min=63 days, max=81 days) from the beginning of the experiment. Each male was observed, in random order, for 5 min. We recorded the rate of courtship (no. of sigmoid displays), sneak attempts (no. of gonopodial thrust), and the time spent by the male following, courting or chasing a female (Head & Brooks, 2006). This last component of male behaviour represents the overall sexual activity of males and we will refer to it as "sexual activity". No copulations were observed during these observations. We were able to recognize males by their unique colour patterns.

Male behaviour after the treatment

At the end of the treatment, male sexual behaviour was measured in the same conditions for the two groups of males. Unfamiliar adult females (N=30) were allowed to settle in a tank for 12 hours. Each female was used twice, once with a HMR male and once with a LMR male (order was balanced between treatments). To evaluate the change in male behaviour during the time, each male was observed for 5 min after acclimatization (5 min) and other 5 min after an interval of 10 min. We registered: number of sigmoid display, number of gonopodial thrust and sexual activity (Head & Brooks, 2006).

Morphology and ornamentation

Body and colour spot size

Males were anesthetized with MS-222, put on a plastic ladder, and the excess of water was removed using blotting paper. A digital photograph of the fish placed along a ruler was then taken (Canon EOS 450D). Using ImageTool software (http://ddsdx.uthscsa.edu /dig/itdesc.html) we measured, from the digital photo of the left fish side, body area and standard length (from the snout to the base of the tail fin, SL). Total area of carotenoid and pteridine spots (hereafter "orange"), iridescent spots (blue, green, violet, hereafter "iridescent"), and melanistic black spots were also measured. Colour spot areas were subsequently standardized to male body area (%).

Colour spectral analysis

After the treatment, reflectance of orange and iridescent colouration was also measured. We performed all the measurements using a USB-2000+UV-VIS spectrometer (Ocean Optics, Inc., USA, range: 200-850nm) connected with a Deuterium-Halogen light source (DH-2000-BAL, Ocean Optics, Inc., USA. Range: 215-2000nm). We used a bifurcated 400 µm fibre-optic cable (Ocean Optics, Inc., USA) to illuminate the spots and measure their reflectance. The probe was covered with an opaque shield that allowed us to sample 1.5 mm diameter area. The cup maintained the sample at 4mm distance and removed the environmental light. Calibration was performed using a white standard (WS-1, Ocean Optics) before each male was measured. We measured one orange and one iridescent spot per male, repeating the measure 3 times for each spot. SpectraSuite software (OceanOptics) was used for data collection. We averaged the 3 spectral measurements of each spot (orange and iridescent separately) after discarding the data outside the UV-visible (300-700 nm wavelength) window (Young *et al.*, 2010). Spectral chroma ($[R_{max} - R_{min}]/R_{average}$; represents the spectral colour purity) and brightness ($R_{average300–700}$; represents the total amount of light coming from a unit area of surface) were calculated for each male spot and used in subsequent analysis (Montgomerie, 2006).

Ejaculate assays

After photography, sperm were stripped (Matthews *et al.*, 1997; Gasparini *et al.*, 2010)

for baseline sperm counts and sperm velocity. Briefly, each male was placed on a Petri dish under a dissection microscope with 0.5ml of physiological solution (0.9% NaCl). A gentle pressure was applied to the side of the abdomen to eject all sperm (packaged in bundles). Six sperm bundles were collected with a Drummond Micropipette and immediately used for computer assisted sperm analysis (Hamilton-Thorne CEROS). Sperm velocity assay was repeated twice on 3 bundles which were placed on a multi-well slide and activated with 150 mM KCl and 4 mg ml^{-1}BSA (Gasparini & Pilastro, 2011). We obtained three standard measures of sperm velocity: average path velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL). The mean of the two measures was used for each male. All other bundles were collected in a known volume of saline solution for sperm count. Sperm number was obtained using an *improved Neubauer haemocytometer* under a 400x magnification. The average of ten counts per male was used to estimate the number of sperm produced.

Results

Behavioural Trials

Male behaviour during the treatment

Pooling together the data obtained for each male during the treatment (min=1, max=8) there are no difference between the two groups (Student *t* test, p>0.34).

Treatment didn't affect differentially the two groups in the long time (GLMS: treatment: fixed factor; SERIES: fixed factor; experimental tank: random factor, nested in treatment; fish identity: random factor nested in tank and treatment; behaviours: dependent variables) as no significant interactions between treatment and SERIES were present (p>0.631). Males of both groups were more sexually active during the second SERIES but this difference was significant only for sexual activity (p=0.003).

Male behaviour after the treatment

Males' behaviour after the treatment has been analysed using a repeated measure GLM (tab. 1). Sexual activity and display rate presented a similar pattern and for both behaviours HMR males progressively reduced the frequency of their sexual behaviour during the 20 minutes with the female, while LMR males increased it (Fig. 1). In both groups, the rate of gonopodial thrusting increased during the observations.

Morphology and ornamentation

Body size and colour spot size

In both groups, males body size increased during the experiment (repeated measures GLM; 2 levels factor within subject= measure before and after treatment; factor between subjects= treatment). No significant differences were detected in the colour spots' area before and after the treatment and between groups (tab. 2), nor at the end of the experiment in colour spectral properties (Student *t* test, p>0.223).

Ejaculate traits

Sperm production and all parameters of sperm velocity (VAP, VSL, and weakly VCL) increased during the experiment but not differentially between treatments (repeated measures GLMs; 2 levels factor within subject= measure before and after treatment; factor between subjects= treatment) (Tab. 2).

Table 1. Male sexual behaviour after the treatment (repeated measures GLM). The Time factor represents the initial and final observation (5 minutes each), and indicate whether behaviour changed during the time. Time*treatment interactions represent different change in the two experimental groups. In bold significant components.

Behaviour	Factor	F	P
Sexual activity	Time	1.200	0.278
	Time*treatment	3.340	0.073
Display	Time	0.423	0.518
	Time*treatment	**4.332**	**0.042**
Gonopodial thrust	**Time**	**11.138**	**0.002**
	Time*treatment	1.238	0.271

Table 2. Differences between treatments in male size, colour area and sperm traits (number and velocity). Repeated measure GLMs. The Time factor represents the measures before and after the treatment, and indicates a difference between those 2 measures. Time*treatment interactions represent different change in the two experimental groups. In bold significant components.

Trait	Factor	F	p
Standard Length (mm)	**Time**	**213.819**	**<0.001**
	Time*treatment	0.015	0.902
Orange area %	Time	3.509	0.066
	Time*treatment	0.121	0.729
Black area %	Time	0.800	0.375
	Time*treatment	3.177	0.080
Iridescent area %	Time	1.644	0.205
	Time*treatment	0.000	0.993
Sperm Number ($x10^6$)	**Time**	**35.435**	**<0.001**
	Time*treatment	2.205	0.143
Sperm Velocity (VAP)	**Time**	**13.787**	**<0.001**
	Time*treatment	1.040	.312
Sperm Velocity (VSL)	**Time**	**17.862**	**<0.001**
	Time*treatment	1.866	.177
Sperm Velocity (VCL)	Time	3.402	.070
	Time*treatment	.542	.465

Ejaculate production and sexual behaviour

Difference in sperm production was marginally statistically significant after the treatment (T test: t=1.726, p=0.09) with HMR males producing in average more sperm (sperm number $x10^6$ in HMR= 14.85±2.41 SE; LMR=10.32±5.88 SE). We thus analysed the relationship between sperm production sexual behaviour after the treatment (average of display, gonopodial thrust and sexual activity performed in the two periods of observation). There is a general negative correlation between sexual behaviour and sperm production (all correlation are negative except between sperm number and gonopodial thrust in LMR group). Those correlation are stronger in HMR males where ejaculate production is significant negatively correlated with sexual activity (Pearson correlation=-0.508, p=0.006, N=28).

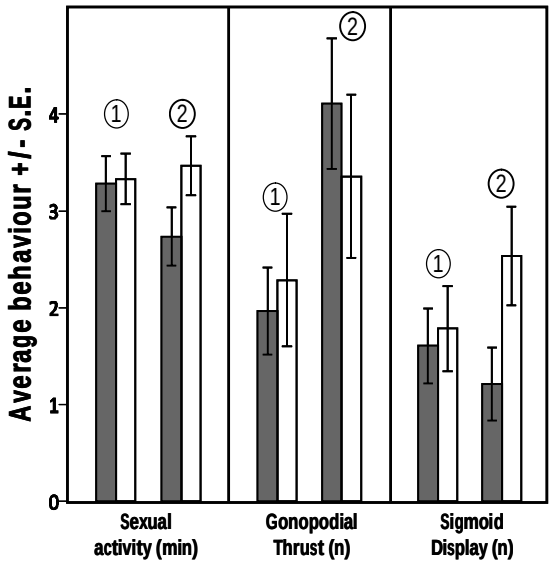

Figure 1. Sexual behaviour after the treatment. For each behaviour we presented mean values and standard errors. On the left behaviour measured during the firs five minutes (1) and in the right after ten minutes (2). In gray HMR group and in white LMR group.

Discussion

Results on male morphology and ejaculate investment suggest that male condition improved during the two months of treatment and/or that males invested progressively more in reproduction, as both pre- and postcopulatory traits increased their expression. Improvement in male condition my depend on less intense competition for food due to low population density in the experimental tanks as compared to stock tanks in which fish were maintained before entering into the treatment (Reznick *et al.*, 2001; Arendt & Reznick, 2005).

Orange and iridescent spot areas increased in both groups (with marginal significance in orange colouration) probably as an effect of larger body size, or because experimental males improved their condition during the treatment. No difference between groups have been detected in colouration (spot dimension and colour intensity), however, suggesting that changes in mating opportunities did not affect, at least in our experiment, investment in ornamentation.

Ejaculate investment in the long period increased significantly in both groups (both sperm number

and sperm velocity), and this increase was larger in HMR males, although the difference was only marginally significant between treatments (tab. 2).

A detailed analysis of behaviour after the treatment revealed a difference in mating effort in the two treatment groups. Sexual activity and number of sexual display performed by males show an opposite pattern during the post-treatment behavioural observations. Whereas LMR males showed e sustained sexual activity and even increased their mating effort during time, HMR males showed an oppositely pattern. In a similar experimental design, Jordan and Brooks (2010) found that in the short-medium period (21 days) males housed with familiar females dedicated more time to foraging than males housed with novel females. This situation could lead to energetic costs, which could explain the observed reduction in courtship rate. Sperm production in HMR males after the treatment was negatively correlated with male sexual activity. Reduced endurance of HMR males may therefore be caused by previous increased investment in sperm production. Number of gonopodial thrust was not different between treatments. This behaviour is typically less energetic costly to males than courtship display (Houde, 1997) suggesting that the difference in other behaviour is due to a reduced amount of resources available for males. Ejaculate investment is known to be energetically costly in many species (Olsson *et al.*, 1997; Snook, 2005; Parker & Pizzari, 2010) and our results confirm that guppy males cannot increase both mating effort and sperm production. More importantly, we found evidence that sperm production is phenotypically traded off with precopulatory mating effort potentially leading to fitness costs and selection consequences for males (Kotiaho, 2001).

This is the first case, to our knowledge, in which the effects of experimental increase in sperm production are tested in a vertebrate. Our result add to a growing body of evidence that a increasing postcopulatory effort is traded off with precopulatory traits, supporting one of the prediction of the sperm competition theory and potentially explaining maintenance of variability in postcopulatory sexually selected traits.

Immunocompetence, condition-dependence and sexually selected traits in the guppy

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Abstract

Condition dependence is a fundamental prediction of the "genic capture hypothesis". In polyandrous species, genetic and phenotypic variability underlying sexually selected traits is usually large, despite the strong sexual selection. The expectation that the expression of sexual traits is associated with condition comes from the prediction that only high quality males are able to afford the cost of possessing such exaggerated traits. Moreover, many loci are expected to affect condition, and hence condition should provide a broad target for intrinsic (e.g. mutations) and extrinsic (e.g. parasites, stress) factors that counter the depletion of genetic variance through selection. We examined condition dependence of sexually selected traits in male guppies (*Poecilia reticulata*), a species in which pre- and postcopulatory success strongly influences male reproductive fitness and that exhibits relatively high levels of additive genetic variation. In this polyandrous fish condition dependence has been demonstrated for behavioural and ornamental traits, but rarely tested in postcopulatory traits. We compared the expression of behavioural, ornamental, and ejaculate traits of one group of males in which condition was manipulated by eliciting an immunological challenge (LPS Injection) with those of a control group. Immune experimental manipulation had only effect on postcopulatory trait whereas male colours were not affected. However, both groups of males showed a reduced condition after the treatment, suggesting that injection per se had a strong effect on males.

Introduction

To explain the unexpected high variability (Tomkins *et al.*, 2004; Walsh & Blows, 2009) in sexually selected traits, Rowe and Houle (1996) proposed that male traits under directional sexual selection become costly and hence condition-dependent. As mutations affecting male condition will have concomitant effects on condition-dependent traits and because condition is determined by a large proportion of the genome, any intrinsic (e.g. mutation) and extrinsic (e.g. parasites, nutritional stress, ecc.) could have a disproportionally larger effect on sexually selected traits as compared to traits subject to weaker or stabilizing selection. Iin addition, given this large 'genic' target, mutations will arise as rapidly as selection removes them, thus maintaining variability in condition-dependent sexual traits despite persistent directional selection (Tomkins *et al.*, 2004). This is the so called 'genic capture hypothesis'.

Condition dependence in precopulatory sexually selected traits has now been well recognized in many taxa (see for example: David *et al.*, 2000; Kotiaho, 2001; Bonduriansky & Rowe, 2005; Birkhead *et al.*, 2006; Kemp & Rutowski, 2007;

Punzalan *et al.*, 2008; Rashed & Polak, 2010; Albo *et al.*, 2011). Yet, most sexually reproducing species are polygamous and females mate with two or more males within a single reproductive episode (polyandry). As a consequence, sperm competition (Parker, 1970) and cryptic female choice (Eberhard, 1996) will also affect male fitness outcome. Postcopulatory traits typically exhibit moderate to very high levels of genetic variation (Simmons & Kotiaho, 2002; Moore *et al.*, 2004; Birkhead *et al.*, 2005; Simmons & Moore, 2009; Snook *et al.*, 2010; Evans, 2011). In contrast to the large body of evidence that precopulatory traits are condition dependent, postcopulatory traits have been relatively little studied from this point of view (Gage & Cook, 1994; Simmons & Kotiaho, 2002; Schulte-Hostedde *et al.*, 2005; Vermeulen *et al.*, 2008; Perry & Rowe, 2010). In particular, only a handful of studies, conducted on invertebrates, have tested how pre- and postcopulatory sexually selected traits simultaneously respond to manipulations of male condition (Simmons & Kotiaho, 2002; Knell & Simmons, 2010; Lewis *et al.*, 2011). This comparison is interesting, however, as it can indirectly indicate the relative strength of sexual selection on the two types of traits, assuming that stronger selection should lead to a stronger correlation between condition and trait expression.

Our model species is the guppy, *Poecilia reticulata*, a tropical fresh-water fish with a polyandrous, non-resource based mating system and internal fertilization. Guppies are sexually dimorphic, with males showing a noticeable, variable colouration composed by orange (carotenoid and pteridine based), iridescent (structural colour), and black (melanin based) spots (Houde, 1997). When females are receptive (3-5 days after parturition) males can mate cooperatively. In this case females usually prefer more colourful males and males with higher courtship rate (Endler & Houde, 1995). During the rest of the cycle (approximately 3-4 weeks) females are usually unreceptive and males can only attempt mating through forced copulations, termed gonopodial thrusts (Houde, 1997). As females actively mate with 2-3 males during their receptive period, and are forcibly inseminated with high frequency during the whole reproductive cycle (Pilastro & Bisazza, 1999; Evans *et al.*, 2003a)) males face high level of sperm competition (Evans & Pilastro, 2011). Male postcopulatory success has been shown to be determined by sperm number, velocity and viability (Boschetto *et al.*, 2011, Fitzpatrick, J. L. & Evans, J. P., unpublished data). Both precopulatory display traits and postcopulatory sperm straits are therefore subject to directional sexual selection in this species (Endler & Houde, 1995; Pilastro *et al.*, 2002; Evans *et al.*, 2003b; Pilastro *et al.*, 2004; Locatello *et al.*, 2006; Pilastro *et al.*, 2007; Boschetto *et al.*, 2011). Despite this, high genetic variability has been revealed both for size of the males' colour spots and courtship behaviour (Brooks & Endler, 2001; Evans, 2010) and for sperm viability and sperm velocity (Gasparini, 2009; Evans, 2011). Here we tested in *P. reticulata* the prediction of genic capture hypothesis' that sexually selected traits are condition-dependent. We analysed condition-dependence on both pre- and post-copulatory sexually selected traits. Ornaments and behaviour are known to be condition-dependent in this species (Grether, 2000; van Oosterhout *et al.*, 2003; Karino & Haijima, 2004; Kolluru & Grether, 2005; Kolluru *et al.*, 2009), but this prediction has rarely been tested for postcopulatory traits (but see Zajitschek & Brooks, 2010). As condition-dependence of a trait could be set to a threshold because of trade-offs with other sexually selected traits (Demary & Lewis, 2007; Klaus *et al.*, 2011; Lewis *et al.*, 2011), we studied the condition-dependence on the overall set of pre- and postcopulatory sexually selected traits. In our experiment, we considered in detail two elements affecting male precopulatory success (sexual behaviour and ornamentation) and two sperm characteristics (number and velocity). Moreover we obtained an estimate of the overall condition of males after treatment by measuring male's ability to escape a simulated predator. To evaluate the condition-dependence of these traits we analysed their expression after manipulating male condition.

Different techniques have been used in the guppy in order to modify extrinsic male condition (Cotton *et al.*, 2004); one of those is by inducing a parasite infection (see for example Kolluru *et al.*, 2009). We used a slightly different approach, consisted in injecting a solution of bacterial lipopolysaccharides (LPS). This antigen results effective in eliciting an immune response in other fish species (M.B. Rasotto, pers. comm.) and is commonly used in evolutionary biology experiments. Our technique has the advantage to induce an inflammatory response that is followed by the production of specific antibodies, without simultaneously introducing a metabolically active, replicating pathogen. The manipulation of condition should therefore be consistent between individuals. We compared expression of sexually selected traits in males injected with LPS solution (n=20) with males injected with saline solution (n=20). Condition-dependent traits expression was expected to be reduced in LPS injected males.

Methods

Fish Maintenance

Guppies used in the experiment were descendent of wild-caught fish from the lower part of Tacarigua River, Trinidad. Fish are reared in standard condition in 65l and 125l stock aquaria where the sex ratio is approximately 1:1. (see Gasparini *et al.*, 2010 for details in fish maintenance). We used 40 males between 6 and 8 months old collected from stock aquaria and randomly assigned to one of two experimental groups. A total of 20 non virgin mature females have been used after the treatment for behavioural tests. All females were collected from stock aquaria where the sex ratio was approximately 1:1. All fish were fed *ad libitum* before and during the experiment.

Experimental Design

Experiment consisted in a three step procedure (fig. 1): 1) traits of each male (size, colouration and ejaculate traits) were measured before the treatment; 2) males were then injected, and 3) after three (sexual and anti-predator behaviour) and four days (ejaculate traits and ornamentation) males were measured again. Methodology used before and after the treatment to measure traits was the same. Measure of sperm traits was performed after 4 days of isolation during which males were reared in standard condition in 1 litre tank physically but not visually isolated from females (during this period males are able to completely replenish sperm reserves, Bozynski & Liley, 2003; Gasparini *et al.*, 2009). In order to avoid possible difference in sperm stores due to recent copulations in the stock tank, before the beginning of the experiment all males were stripped and then isolated. Males used for the experiment have been divided in two groups which undergone exactly the same treatment except for the solution used for injection. In the treatment group (hereafter LPS group) males were injected with 1,25 µl of a solution composed by LPS (lipopolysaccharides) and saline solution (0.9% NaCl; concentration of LPS was 10 mg/kg of fish body mass. As male

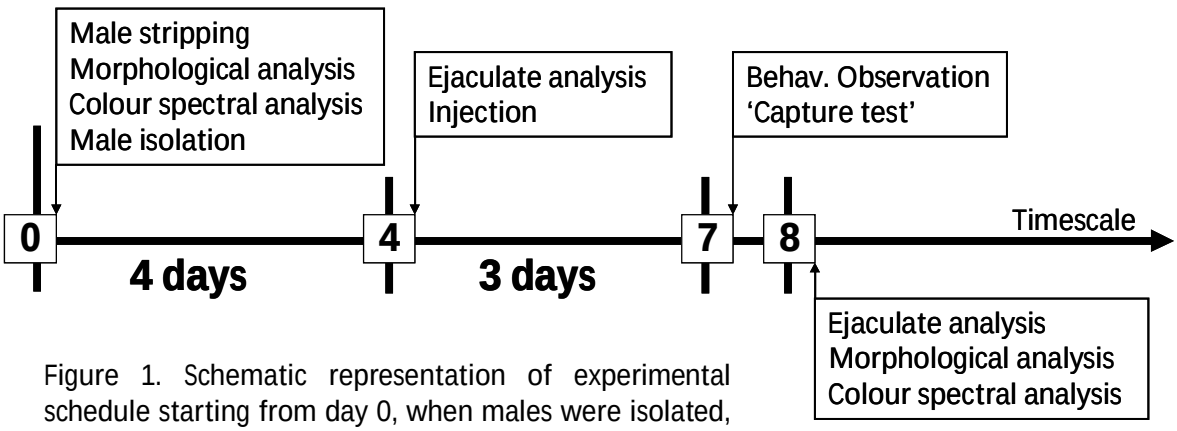

Figure 1. Schematic representation of experimental schedule starting from day 0, when males were isolated, to day 8, when males were released.

guppies weight is approximately 0.1 g the final amount of LPS injected in each male was 1 µg). LPS is a component of bacterial membrane (*E. coli* lipopolysaccharides serotype O55:B5, Sigma Chemical) which elicit an immunological response in other fishes (M.B. Rasotto, pers. comm.). Control group males were injected with the same amount of physiological saline solution (hereafter PHYSIO group). Injection was performed with the commercial insulin syringes with a 0.26 mm needle diameter. A screw plunger was used to ensure the injection of a precise amount of solution. Different syringes were used for injecting males of the two groups. Before each injection the needle was sterilized. Briefly, each male was placed on a polystyrene support covered with medical sterile tape. Injection was then performed in the dorsal part of the left side of the fish, near the caudal peduncle. After this procedure fish were isolated again in singular tanks. Treatment (from isolation to last measure) lasted 8 days. Males have been anesthetized with MS-222 (on day '0', '4' and '8'). All males except two (one in each group) survived the treatment. Final sample size was therefore 38 (19+19).

Male size and Ornamentation

On day '0' and day '8', anesthetized males were photographed near a ruler (Canon 450D camera, equipped with Canon EF*S* 60mm MACRO lens and circular flashlight). Pictures have been analysed using UTHSCSA ImageTool software (http://ddsdx.uthscsa.edu /dig/itdesc.html). Standard length, body area and colour spot area have been measured on the left side of each male. Spots were assigned to tree different categories: orange (carotenoids and pteridin, orange and yellow spots), back (melanin, dark spots) and iridescent (structural, green-white, blue-violet spots). After photography males were gently placed on a dark plastic support and spectral characteristic of a single orange spot were measured tree times. We used a S2000 spectrometer equipped with an ADC1000-USB (Ocean Optics, Inc., USA, range: 350-1000nm) connected with a Halogen light source (HL-2000-FHSA-LL, Ocean Optics, Inc., USA. Range: 360-1700nm). Spots were illuminated and their reflectance measured with a bifurcated 400 µm fibre-optic cable (Ocean Optics, Inc., USA). The distal end of the fibre probe was fitted with an opaque, black plastic tube that allowed us to sample 1,5 mm Ø area, to maintain the probe end at the constant distance of 4 mm from the fish surface, and to exclude ambient light. Before measuring each male we calibrated the spectrometer using a white standard (WS-1, Ocean Optics). Data were digitalized with SpectraSuite software (OceanOptics). Raw spectra consisted in an array of data points (≈2000) representing the reflectance in the 360-1000 nm wavelength interval (one measure every 0.325 nm). We considered only the visible range (400-700 nm) and then the three spectral measure of each spot were averaged. Yellow chroma, calculated as the proportion of reflectance occurring between 550 and 625 nm over the total reflectance ($R_{550–625}/R_{400–700}$), represents the colour purity in the region of the spectrum corresponding to carotenoids and was used in subsequent analysis as an index of spot colour quality (Montgomerie, 2006).

Ejaculate assays

After being photographed, males were stripped for baseline sperm counts and sperm velocity analysis (Matthews *et al.*, 1997; Gasparini *et al.*, 2010). Males were placed on a Petri dish under a dissection microscope with 0.5ml of physiological solution and a gentle pressure was applied to the side of the abdomen. Released sperm bundles (sperm packages) were collected with a Drummond Micropipette. Computer assisted sperm analysis (Hamilton-Thorne CEROS) was performed was performed twice using 3 bundles each placed on a multi-well slide and activated with 150 mM KCl and 4 mg ml^{-1}BSA (Gasparini & Pilastro, 2011). Three standard measures of sperm velocity were obtained: average path velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL). The mean of the two measures was used for each male. All other bundles were collected in a known volume of saline solution for sperm counting using an *improved Neubauer*

haemocytometer under a 400x magnification. The average of ten counts per male was used to estimate the size of sperm reserves at rest.

Sexual and anti-predator behaviour

On day 7th, males were removed from their isolation tank and placed in a 20l observation tank where 2 non virgin adult females were allowed to settle for 24 hours. Observations took place in the morning (between 08.00 and 12.00) and consisted in observing the male for 10 min (after 5 min of acclimatization) recording the number of sigmoid display (stereotyped courting behaviour during which the male bends and vibrate his body in front of the female), forced mating attempts ('gonopodial thrusts' during which the male quickly approach the female from behind and tries to insert his gonopodium into female's gonopore), and physical contacts performed by the males towards the females by nipping her genital area (see Houde, 1997 for a detailed description of behaviours). The total time the male spent following, chasing or courting one female was recorded and used as an index of sexual activity (modified from Head & Brooks, 2006). After behavioural trials, males were tested in a simulated predator-evasion trial. This test consisted in recording the time needed to capture the fish with a hand fishnet using a standardized procedure. This measure is commonly used as an index of fish ability to escape predators and gives an indication of male general condition (adapted to adults from Evans & Magurran, 2000). For example, inbred guppies show a significantly reduced capture time as compared to their outbred counterpart and the measure is highly repeatable (C. Gasparini & A. Pilastro, unpubl. obs.).

Statistical analysis

Male ornamentation, size and ejaculate traits are all compared between the two groups using univariate repeated measure general linear models (GLM). The two level factors represent

Table 1. Differences between treatments in precopulatory traits (proportion of colour spot area on total body area and orange chroma) and postcopulatory traits (sperm number and velocity). Repeated measures GLM. The *Time* factor represents the measures before and after the treatment, and indicates a difference between those 2 measures. Significant *Time*treatment* interaction indicates the trait changed differently in the two experimental groups. In bold significant effects. Mean trait values (±S.D.) before and after the treatment are given on the right columns.

Trait	Factor	F	p	LPS		PHYSIO	
				before	after	before	after
Orange %	**Time**	**19.037**	**<0.001**	9.22±3.15	7.31±2.3	8.15±3.4	6.69±2.54
	Time*treatment	0.255	0.617				
Black %	**Time**	**6.169**	**0.019**	3.11±1.57	2.72±1.5	2.88±1.14	2.19±1.3
	Time*treatment	0.416	0.524				
Iridescent %	Time	1.025	0.320	6.57±2.81	7.59±2.52	8.02±2.30	8.46±3.38
	Time*treatment	0.034	0.856				
Orange Chroma	**Time**	**19.232**	**<0.001**	0.31±0.02	0.29±0.02	0.31±0.02	0.29±0.02
	Time*treatment	0.432	0.515				
Sperm Number (log.transformed)	**Time**	**10.987**	**0.002**	6.63±0.48	6.22±0.61	6.72±0.34	6.65±0.32
	Time*treatment	**5.224**	**0.028**				
VAP (µm/s)	Time	0.467	0.449	88.13±11.46	81.17±12.42	87.58±10.56	91.57±9.03
	Time*treatment	**6.376**	**0.016**				
VSL (µm/s)	Time	1.068	0.308	81.17±10.10	72.77±3.09	79.99±10.16	83.78±9.00
	Time*treatment	**7.483**	**0.010**				
VCL (µm/s)	Time	0.350	0.558	115.81±10.65	111.84±12.37	116.17±8.23	118.14±8.85
	Time*treatment	**3.055**	**0.089**				

the change in the traits before and after the treatment. Treatment has been used in the model as factor 'between subjects'. Colour spot size, colour index, sperm number and sperm velocity were dependent variables. Male sexual and anti-predator behaviours were measured after the treatment and compared between groups with Student T tests. All analyses were performed with PASW 18.

Results

Effect of LPS on male ornaments

Males used in the experiment did not differ in body size between groups (Student t test; standard length: p=0.713; body area: p=0.978, n=38). Both LPS and saline-solution injections had an effect on fish traits (tab. 1). Melanic (size) and orange (size and chroma) but not iridescent spots were reduced after treatment. This change was not statistically different between treatment groups (no interaction between factors, see tab. 2).

Effect of LPS on sperm traits

Post copulatory traits were strongly affected by LPS treatment (tab. 1). Sperm number decreased in both experimental groups but this decrement was stronger in LPS group males. Sperm velocity parameters change significantly after the treatment but, while in LPS group males sperm velocity weakly decreased, in PHYSIO group males the velocity increased (tab. 1). The difference between treatments was significant except for VCL (p=0.089).

Effect of LPS on sexual and anti-predator behaviour

No males mated during the behavioural trials and only one control male performed sigmoid displays. We therefore excluded this behaviour from subsequent analysis. Treatment didn't affect males in any of the other behaviours observed (Tab. 2). Even after removing one outlier control male whose capture time was 10 longer than the average, results of the capture test showed that LPS males were captured more rapidly than their control counterparts (Student t test, $t = 2.494$; p=0.018 after log-transformation, fig. 2).

Discussion

Condition can be defined in several ways. According to Rowe and Houle (1996) condition is the pool from which resources are allocated. Condition has the property that higher values confer greater fitness, and it can have genetic and environmental components (Iwasa & Pomiankowski, 1999). Practically, experimental manipulation of condition can be more difficult, and different approaches have been used. In order to estimate condition-dependence of sexually selected traits is essential to have a measure of the condition itself or to measure a trait that it is close to fitness (Cotton *et al.*, 2004). Here we evaluated males' condition using their anti-predator behaviour, measured through a "capture test". This is a reliable method to estimate anti-predator behaviour (Evans & Magurran, 2000) and has shown to be highly repeatable in our lab (unpublished data). Clearly, the ability of escaping a predator can have great fitness consequences in natural conditions. In our experiment males injected with LPS solution were captured in average easier than control group males (fig. 2) supporting the expectation that treatment had an effect on male condition.

Table 2. Difference between experimental groups in sexual behaviour. Mean values of groups are compared using a Student T test. Average values (± standard deviation) for each group are given.

Behaviour	T	p	df	LPS	PHYSIO
Gonopodial Thrust (n)	-1.254	0.218	36	0.58±1.87	1.63±3.15
Physical contacts (n)	-0.585	0.562	36	0.89±2.31	1.26±1.48
Sexual interest (sec.)	-0.797	0.431	36	77.7±95.92	105.66±119.02

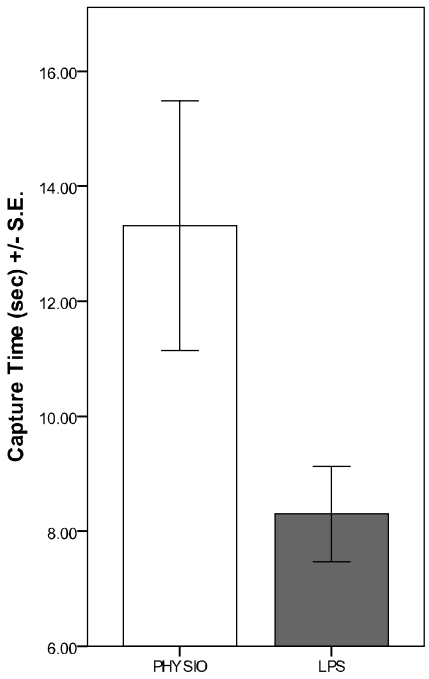

Figure 2. Difference between groups in the 'Capture time' test. Male in the LPS group where caught more easily (Student T test, $p<0.05$)

Thus, we have a clear indication that LPS reduce male condition. Our intent, however, was to compare condition-dependence across sexually selected traits, with particular focus on differences between pre- and postcopulatory traits.

Our study showed that eliciting an immunological response in male guppies has a significant effect on male condition, as indicated by the shorter capture time of LPS males as compared to controls. Moreover, LPS males showed a reduced investment in postcopulatory traits as compared with males injected with saline solution. In contrast, precopulatory traits were reduced in all males, but not differently between treatments.

Ornaments in guppies are very important as their expression is associated with the probability to mate with females (Rios-Cardenas & Morris, 2011). Surprisingly male colouration was not affected by LPS more than by saline solution. In both groups, males show a decline in orange and black spot size but this decrement was not different between the groups. This result was unexpected as carotenoids, which are not synthesised by animals (Goodwin, 1984), are important components of the immunological cascade and have an antioxidant role which contrasts the effect of free radicals produced during inflammatory response. For this reason in guppies, as in other species, investment of carotenoids in colouration is traded-off with investment in immunocompetence (Olson & Owens, 1998; Blount *et al.*, 2003; McGraw & Ardia, 2003; Grether *et al.*, 2004; Baeta *et al.*, 2008). As expected, more colourful guppies show better resistance to parasites (Kolluru *et al.*, 2006) and infected guppies showed reduced orange colouration (Houde & Torio, 1992), suggesting that males have the capability to plastically allocate their carotenoid pool from colouration to the immune function. In our experiment no positive correlations have been observed between orange colouration (neither area nor chroma) and other measured male traits. Males that were more orange before the treatment were not more active during behavioural tests nor performed better in the capture test.

The lack of differences between groups, both for orange and black coloration, may be due to a stronger effect of injection *per se* in comparison with the effect of different solutions injected. This explanation, however, seems unlikely as LPS treatment strongly affected postcopulatory traits. Alternatively this result can be due to a threshold in condition-dependence of orange and black ornamentation. In both groups, in fact, we observed a significant reduction in these traits, suggesting that colouration may respond strongly to any initial reduction in condition. A third group of unmanipulated males would have been necessary to better understand the effect of manipulation per se.

In contrast, we found no effect of LPS on iridescent spots (see for examplePeters *et al.*, 2011). Structural colours are generally more stable than pigment colours and the treatment may have been too short or made to late in development to affect this colouration (check: Fox, 1976; McGraw *et al.*, 2002; Kemp & Rutowski, 2007). In fact, studies involving

inbreeding stress or food limitation in early developmental stages, before or during moult show to have an effect on iridescent colouration (see for examples: Hill, 2006; Kemp & Rutowski, 2007 for insects; and Griggio *et al.*, 2011 for birds).

Males treated with LPS solution performed, in average, less gonopodial thrusts, physical contacts and were less active (see table 2). The differences between groups, however, were not statistically significant. Males of both groups did not perform sigmoid display during trials. This result was unexpected as courtship behaviour in normally performed in similar experiments. This behaviour is more energetically costly than sneaky attempts (Houde, 1997) and the absence of sexual display in both groups may be explained, as for an orange ad black colouration, by an effect of manipulation per se on condition. This result agrees with previous studies revealing that this component of male sexual behaviour is costly and a may be very sensitive to a reduction of male condition (van Oosterhout *et al.*, 2003; Kolluru & Grether, 2005; Kolluru *et al.*, 2009; Head *et al.*, 2010). Furthermore, in these behavioural tests we used sexually unreceptive females, which elicit courtship behaviour from males at a lower rate as compared to receptive (virgin or post-partum) females.

In the guppy postcopulatory sexual selection plays a key role in determining male reproductive fitness (Boschetto *et al.*, 2011; Evans & Pilastro, 2011). Sperm production is likely to be costly (Olsson *et al.*, 1997; Parker & Pizzari, 2010) and Zajitschek and colleagues (2010) suggested that some ejaculate traits exhibit condition dependence. In accord with this prediction, we observed a significant reduction in ejaculate traits (quality and number). Sperm production and velocity were negatively affected by LPS injection. In both groups sperm number after the treatment decreased significantly, but this decrement was as twice as more pronounced in the LPS group (average sperm decrement in sperm number: LPS=3.20 × 10^6 ± 0.84 S.E.; PHYSIO=1.36 × 10^6 ± 0.83 S.E.). Effect of LPS on sperm velocity was even stronger: whereas in LPS group sperm velocity decreased after the treatment, in the PHYSIO group this parameter slightly increased (see table 1). Our results agree with previous work on other species reporting evidence for condition dependence in sperm production (Gage & Cook, 1994; Simmons & Kotiaho, 2002; Perry & Rowe, 2010) and velocity (Losdat *et al.*, 2011). Immune activation produces large amounts of reactive species and it has been suggested that it induces oxidative stress (Swindle & Metcalfe, 2007; Sorci & Faivre, 2009). Sperm are highly susceptible to oxidative stress (Aitken, 1999; Tremellen, 2008; Helfenstein *et al.*, 2010), and this susceptibility can explain why in our experimental groups we found a significant effect of LPS particularly in postcopulatory traits.

Conclusions

In conclusion, we demonstrated that immunity system activation has a short term effect on male condition (anti-predator behaviour) and that this manipulation influences both precopulatory and postcopulatory sexually selected traits, although differently between trait types. In particular, we found a stronger effect of LPS on sperm traits. In contrast, precopulatory traits were reduced in both groups when expression before and after manipulation was compared. This raises the possibility that manipulation per se may have affected the expression of precopulatory traits. Unfortunately, we do not have an unmanipulated control group and the effect of injection per se on male condition cannot be ascertained. Irrespective of the effect of manipulation on condition, and possibly on postcopulatory traits, the observed effect of LPS on sperm traits strongly suggest that ejaculate traits are probably particularly affected by immunological stresses.

Our findings support previous studies revealing that precopulatory traits exhibit condition dependence in this species (Grether, 2000; van Oosterhout *et al.*, 2003; Karino & Haijima, 2004; Kolluru & Grether, 2005; Kolluru *et al.*, 2009), but also show that LPS injection results in

a concomitant decline of traits under postcopulatory sexual selection.

These results add to a growing number of studies that test critical components of the genic capture hypothesis (Rowe & Houle, 1996; Tomkins *et al.*, 2004), thus potentially explaining the high level of variability observed in both pre- and post-sexually selected traits.

Key challenges for future work include carrying out more targeted investigations of condition dependence (e.g. during different developmental stages), identifying the genetic basis of condition, and exploring the reproductive fitness implications of such manipulations (see Tomkins *et al.*, 2004).

Condition-dependent expression of pre- and postcopulatory sexually selected traits in guppies

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Abstract

Sexually selected traits typically exhibit high variability, despite the expectation that persistent selection should erode such variation. According to the genic capture hypothesis (GCH), such variation arises because these traits depend on condition, which in turn presents a large mutational target due to its dependency on genes at many loci. In this paper we test critical components of the GCH in the guppy *Poecilia reticulata,* a freshwater fish in which traits subject to both pre- and postcopulatory selection exhibit high levels of phenotypic and additive genetic variation. Specifically, we examine how both pre- and postcopulatory sexually selected traits simultaneously respond to manipulations of male condition by comparing behavioural, ornamental, and ejaculate traits between males fed different diets (*ad libitum* vs restricted food). We found that males fed restricted diets exhibited reductions in courtship, orange spot pigmentation and sperm viability. These results demonstrate that the expression of behavioural, ornamental and ejaculate traits are sensitive to food stress, and support critical components of the GCH by revealing condition dependence in the expression of these highly variable pre- and postcopulatory traits.

Introduction

Sexually selected traits typically exhibit high phenotypic and genetic variation, despite the expectation that strong directional selection should progressively erode such variability (Tomkins *et al.*, 2004). To explain how this variation can be maintained, Rowe and Houle (1996) proposed the 'genic capture hypothesis', which predicts that male traits under directional sexual selection should become costly and condition-dependent, and that condition depends on many life-history traits. This prediction therefore implies that the expression of sexually selected traits is determined by a large proportion of the genome, and consequently that mutations affecting male condition will have concomitant effects on these traits. Given this large mutational target, mutations will arise as rapidly as selection removes them, thus maintaining variability in condition-dependent sexual traits despite persistent directional selection (Tomkins *et al.*, 2004).

The majority of studies testing for condition-dependence have been confined to male precopulatory sexually selected traits (see for example: David *et al.*, 2000; Kotiaho *et al.*,

2001; Bonduriansky & Rowe, 2005; Birkhead *et al.*, 2006; Boughman, 2007; Kemp & Rutowski, 2007; Punzalan *et al.*, 2008; Rashed & Polak, 2010; Albo *et al.*, 2011 and Cotton *et al.*, 2004, for a review on methodology). Yet in most sexually reproducing species, females mate with two or more males within a single reproductive episode (polyandry) and sexual selection continues after mating in the form of sperm competition, where ejaculates from different males compete for fertilizations (Parker, 1970), and cryptic female choice, where females influence the outcome of this competition (Thornhill, 1983; Eberhard, 1996). As with precopulatory sexually selected traits, those subject to postcopulatory sexual selection typically exhibit moderate to very high levels of genetic variation (Simmons & Kotiaho, 2002; Moore *et al.*, 2004; Birkhead *et al.*, 2005; reviewed by Evans & Simmons, 2008; Simmons & Moore, 2009; Snook *et al.*, 2010; Evans, 2011). However, there is considerably less empirical evidence that condition dependence contributes towards the maintenance of such variation in these traits (Gage & Cook, 1994; Simmons & Kotiaho, 2002; Schulte-Hostedde *et al.*, 2005; Vermeulen *et al.*, 2008; Perry & Rowe, 2010) and only a handful of studies, conducted on invertebrates, have tested how pre- and postcopulatory sexually selected traits simultaneously respond to manipulations of male condition (Simmons & Kotiaho, 2002; Knell & Simmons, 2010; Lewis *et al.*, 2011).

In this manuscript we experimentally test for condition dependence in pre- and postcopulatory sexually selected traits in the guppy, *Poecilia reticulata*, a tropical freshwater fish with a polyandrous, non-resource based mating system and internal fertilization. Guppies are sexually dimorphic, with males exhibiting complex colour patterns composed of orange (carotenoid and pteridine based), iridescent (structural), and black (melanin based) spots (Houde, 1997). During their brief period of sexual receptivity (within a few days of producing a brood), females will mate cooperatively with males, preferentially choosing individuals with high levels of courtship and orange pigmentation over less vigorous and drabber males (Endler & Houde, 1995). Outside these (*ca.* monthly) brood cycles females are sexually unreceptive to male courtship and copulations can only be achieved through the use of forced mating attempts, termed gonopodial thrusts (Houde, 1997). As females are highly polyandrous, sperm competition is intense in this species (Evans & Pilastro, 2011). The outcome of sperm competition is influenced by relative differences in sperm number and sperm velocity between competing males (Boschetto *et al.*, 2011), while recent (unpublished) evidence confirms that sperm viability is the primary determinant of competitive fertilization success when artificial insemination is used to compete ejaculates from rival males (Fitzpatrick, J. L. & Evans, J. P.; unpublished data). Both pre- and postcopulatory traits therefore appear to be subject to directional sexual selection in this species (Endler & Houde, 1995; Pilastro *et al.*, 2002; Evans *et al.*, 2003b; Pilastro *et al.*, 2004; Locatello *et al.*, 2006; Pilastro *et al.*, 2007; Boschetto *et al.*, 2011). Despite this, quantitative genetic studies reveal that both pre- and postcopulatory sexually selected traits exhibit relatively high levels of additive genetic variation. In particular, the size of the males' colour spots and courtship behaviour have high coefficients of additive genetic variance (Brooks & Endler, 2001; Evans, 2010), while recent work on postcopulatory traits reveals that traits such as sperm viability and sperm velocity exhibit moderate to high levels of genetic variation (Evans, 2011).

In our study we focused on condition dependence in both pre- and postcopulatory sexually selected traits in guppies. In this species, some behavioural and ornamental sexually selected traits have been shown to exhibit condition dependence (Grether, 2000; van Oosterhout *et al.*, 2003; Karino & Haijima, 2004; Kolluru & Grether, 2005; Kolluru *et al.*, 2009), but the possibility that postcopulatory traits also reflect male condition has received less attention(but see Zajitschek & Brooks, 2010). Despite this, several studies have reported that pre- and postcopulatory sexually selected traits can be phenoptyically correlated in guppies

(e.g. Matthews *et al.*, 1997; Evans & Magurran, 2001; Locatello *et al.*, 2006; Pitcher *et al.*, 2007; Gasparini et al. unpiblished), and results from a recent quantitative genetic study are consistent with the idea that for some pre- and postcopulatory traits, notably the area of iridescence and sperm velocity, investment in sexual ornamentation comes at a cost of investment in ejaculate quality (Evans, 2010). Our current study uses an experimental approach to explore how the interplay between pre- and postcopulatory traits is affected by male condition. We focus on two precopulatory (sexual behaviour and colour ornamentation, including both total area of colour spots and their spectral characteristics) and four postcopulatory (the number, velocity, viability and morphology of sperm) traits. We tested for condition dependence in these traits by comparing their expression between groups of guppies fed different diets. In the first group males were fed *ad libitum* while in the second group males were fed a restricted diet (limited food quantity). We then tested the prediction that the expression of condition-dependent traits (both pre- and postcopulatory) will be reduced in the food limited group.

Materials and Methods

Focal population and experimental diets

All fish were descendents of wild-caught guppies originating from the Alligator Creek River, Queensland, Australia. Stock populations were maintained at 26°C with 12:12 h light-dark cycle and fed *Artemia salina* nauplii daily. Males used for the experiment were taken at random from these mixed sex stock aquaria and placed individually into 2l tanks from which they had visual access to two adult females. Opaque screens placed between adjacent tanks prevented visual interactions among males. Experimental males (n=90) were assigned at random to one of two experimental diet groups: *ad libitum* (n=45) and food restricted (n=45). Males assigned to the *ad libitum* group were fed *ca.* 150 fresh *Artemia* nauplii twice daily, six days per week. We chose this amount following preliminary trials which confirmed that fish fed this quantity of *Artemia* foraged constantly for eight to 10 minutes but rarely finished all nauplii during this time. Fish assigned to the restricted food group were fed one third of the amount of the *ad libitum* group (*ca.* 50 *Artemia* nauplii) twice daily, six days per week (Grether *et al.*, 2008; Kolluru *et al.*, 2009). These fish invariably finished the nauplii within five minutes. 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 males within each group throughout the feeding trials. The diet trials lasted approximately one month (33.81 days ± 1.31 SD) and did not differ significantly in duration between treatments (Student t-test: t_{88}=-0.402, P=0.689).

Behavioural observations

After the diet treatments, the sexual behaviour of males was observed between 08:30 and 14:30, which corresponds with the peak of sexual activity in this species (Houde, 1997). For these trials we used eight replicate observation tanks (28.5 x 14.5 x 19 cm, filled to 15 cm), each containing aquarium gravel and lit by overhead fluorescent tubes (Philips Lifemax TL-D 36W/840 Cool White). In each trial a non-virgin female from a mixed sex (stock) aquarium was placed in the tank and allowed to settle overnight. Females were approximately matched for size (by eye) across trials and used only once. The following day, one male was placed in the aquarium and observations began after five minutes or when the male began to actively court (i.e. performing courtship displays) or chase (i.e. performing thrusts or showing sexual interest in) the female. For each 15 minute trial, we recorded male mating behaviour as the number of sigmoid displays (males arch their body in a characteristic s-shaped posture and quiver), forced mating attempts (termed 'gonopodial thrusts', where males approach females from behind and attempt to force copulations without prior courtship or female solicitation), and the time spent by the male courting or chasing the female (a measure of the male's overall sexual interest in the female, hereafter 'sexual interest').

After each trial males were returned to their individual tanks under the same diet treatment for a further week before being used for the body size, colour pattern and sperm analyses. This period of isolation ensured that males would have fully replenished their sperm supplies prior to sperm counts and sperm analyses (Bozynski & Liley, 2003; Gasparini *et al.*, 2009).

Male body size and area of colour patterns

Male body size and colour ornaments were measured 40.73 days (± 1.35 SD) following the feeding trials, and this time period did not differ significantly between treatments (Student *t*-test; t_{87}=0.021, *P*=0.983). Males were euthanized in iced water and their body surface gently dried with blotting paper. Each male was then photographed under standard lighting conditions (two 13W fluorescent bench lamps) against a measurement scale on a white background using a digital camera (Canon EOS 400D). From these photographs, we used *ImageJ* software (http://rsbweb.nih.gov/ij/) to measure body size and the total area of the coloured spots. Measurements, made on the left side of each male's body, included body area (including caudal fin but excluding dorsal fin), and standard length (from the snout to the base of the caudal fin, hereafter SL). We also measured the total area of orange and yellow carotenoid and pteridine spots (hereafter "orange"), structural iridescent spots (blue, green, violet, hereafter "iridescent"), and melanic black spots.

Spectral analysis of colour patterns

Immediately after the photography, we measured the spectral reflectance of the iridescent and orange colour patterns. We performed all the measurements in a dark room using a USB-4000 spectrometer (Ocean Optics, Inc., USA) and a miniature Deuterium Tungsten light source (Analytical Instrument Systems, Inc., NJ; range 200-1700nm). Illumination was provided at 45° to the left side of the fish with a 600 µm fibre optic cable. The detector probe (Ø = 400 µm) was situated orthogonally to the illuminating probe and focused with a UV/VIS collimating lens (range: 200-2000nm) to sample a 1mm Ø in the illuminated area. Detector and light source were placed 16mm and 3mm, respectively from the fish's body. Each fish was placed on a sloped, black Perspex block to ensure that the body was presented flat to the detector and light source. The block was positioned on a vertically adjustable photography stand so that the distance between the fish and illumination/detection probes could be finely controlled. Calibration was performed relative to a dark and white standard (WS-1, Ocean Optics) before each male was measured. One orange and one iridescent spot per male were measured three times each (Kemp *et al.*, 2008), selecting different positions close to the centre of the largest spot in each case. Measurements were performed quickly (within approx. 4 minutes of the death of the individual) to minimise any fading in colour pattern reflectance (Gray *et al.*, 2011) and spectral data were collected using *SpectraSuite* software (OceanOptics).

We considered data in the UV-visible wavelength range (300-700 nm) and discarded data outside this interval (Young *et al.*, 2010). We then reduced each spectrum (from the ≈ 3,600 data points collected) by averaging over 5nm intervals to obtain 81 measures of reflectance for each sample. For each male we averaged the three spectral measurements for each spot (for orange and iridescent patches separately). To compare the spectral characteristics of orange and iridescent spots, we used principal component analysis (PCA) (Cuthill *et al.*, 1999). Principal component 1 typically accounts for a large proportion (>90%) of the variability observed in the PCA and usually represents mean spectral reflectance (correlated with brightness), while subsequent PCs represent the shape of the reflectance spectrum (which is correlated with hue, chroma, and saturation; see Cuthill *et al.*, 1999). PCAs were performed for orange and iridescent colour patches separately, using all males in the analyses. We only considered principal components with eigenvalues greater than one and obtained two PCs for orange spots (OR-PC_{1-2}) and four PCs for iridescent spots (IR-PC_{1-4}).

Ejaculate assays

Sperm collection

Following the spectrometry, sperm were collected (Matthews *et al.*, 1997) for ejaculate assays (baseline sperm counts, and measures of sperm velocity, morphology, and viability) largely following the methods of Evans (2009). Briefly, males were placed on a glass slide under a dissecting microscope and 40 µl of extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.49mM MgCl2, 0.41mM MgSO4, 10 mM Tris, pH 7.5) was added to the base of gonopodium. Pressure was gently applied to the male's abdomen to eject all sperm. From the ejaculate, eight spermatozeugmata (sperm bundles) were extracted for the sperm viability assay (see below). The remaining bundles were activated with 40 µl 150 mM KCl solution in 2 mg/l bovine serum albumin. Computer assisted sperm analysis (CASA) of motility (see below) was immediately performed using two replicate measures for each male, each comprising three sperm bundles (approx. $8x10^4$ sperm cells). Sperm bundles not used for CASA and the sperm viability assay were collected in a known volume of saline solution and 1% formalin (to prevent sperm degradation). Samples were then stored at 4°C until counted.

Computer-assisted sperm analysis

For each ejaculate sample, two sub-samples (each containing three spermatozeugmata) were tested to estimate the within-sample (i.e. within-male) repeatability in sperm velocity measures. In both cases, the freshly activated samples were placed in a separate well of a 12-cell multitest slide (MP Biomedicals, Aurora, OH, USA) coated with a 1% polyvinyl alcohol to prevent sperm from sticking to the glass slide (Wilson-Leedy & Ingermann, 2007). Each sample was then analysed using the CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) for three standard measures of sperm velocity: 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. The threshold values for defining static cells were predetermined at 24.9µm/s for VAP and VCL, and15µm/s for VSL (Evans, 2009). An average of 84.12±77.69 SD sperm tracks per sample were used to estimate sperm velocity. Repeatability (Lessells & Boag, 1987) within samples was high for all parameters (VAP: r=0.93±0.015 SE; VSL: r=0.93±0.015 SE; VCL: r=0.92±0.018 SE) and therefore the mean of the two measures was used for each measure. As sperm velocity parameters were positively correlated with each other (Pearson>0.54, $p<0.001$), we used just VAP in subsequent analysis of sperm swimming velocity (Evans, 2009).

Sperm viability assays

A live/dead sperm viability kit (Invitrogen, Molecular Probes) was used to estimate the proportion of live sperm per stripped ejaculate. Live sperm are labelled green (with the membrane-permanent nucleic acid stain SYBR-14) and dead or damaged sperm are labelled red (with propidium iodide, a membrane-non-permanent stain). After staining, the samples were observed under a fluorescence microscope (see Evans, 2009 for detailed protocol) and the proportion of live and dead spermatozoa were assessed from 200 sperm cells per male.

Sperm number and morphology

Sperm counts were performed using an improved Neubauer hemocytometer under 400x magnification (Leica DM1000 microscope) after vortexing each sample for 10 seconds. The average of five counts per male was used to estimate the total number of sperm in each stripped ejaculate (Gasparini *et al.*, 2010). Sperm counts were corrected to allow for sperm that had been removed from each sample for the CASA and viability assays (see Evans, 2009).

Photographs of each male's sperm were obtained under 1000x magnification (Leica DM1000 microscope) using a digital camera (Leica DFC320). Where possible, 20 undamaged spermatozoa were analysed per male (mean

number of sperm cells analysed per male=19.58±2.66 SD; range 6–20). *ImageJ* software was used to measure the length of the head, midpiece and flagellum (Gasparini *et al.*, 2010).

Statistical analysis

All males (n=89) survived until the end of the experiment except one, which died after the behavioural trial so that we were unable to collect sperm and morphology traits for this individual. Univariate General Linear Models (GLMs) were used to test for an effect of diet treatment (fixed factor) on body area, the total area of orange, black and iridescent spots and the number of sperm in the males' ejaculates (all dependent variables in separate models; Table 1). As these traits are typically linked to variation in male body size (Evans *et al.*, 2011b) we controlled for the effect of this parameter by entering standard length as a covariate in these models (Table 1). We first confirmed that there was no interaction between standard length and diet treatment (P values all > 0.2) prior to removing this term from our final models (following Engqvist, 2005). Multivariate GLMs (Cuthill *et al.*, 1999) were used to test for an effect of diet treatment (fixed factor) on the spectral characteristics of orange and iridescent spots by entering the principal components (OR-PC_{1-2} and IR-PC_{1-4} in separate tests) as dependent variables in the models. The remaining behavioural and sperm traits were analysed using either Student *t*-tests (parametric data) or Mann-Whitney U-tests (for non-parametric data), depending on whether the data were normally distributed.

Results

Food limitation treatment had a significant effect on male body area (Table 1), confirming that fish fed *ad libitum* had fuller body shapes (i.e. were fatter) than those on restricted diets. The different dietary treatments had no effect on male standard length over the course of the (one-month) experiment (Student *t*-test: t_{87}=0.753, P=0.453).

Effect of diet on precopulatory male traits

We found that food-limited males performed fewer sexual (sigmoid) displays and exhibited a reduction in sexual interest during the behavioural trials. By contrast, we detected no significant difference in the number of gonopodial thrust attempts between the groups (Table 1).

Our analysis revealed a highly significant effect of food treatment on the total area of orange spots. Specifically, we found a significant reduction in the area of orange spots in males fed a restricted diet compared to their well-fed counterparts (Table 1). By contrast, we found no significant effect of treatment on the total area of iridescent or black spots (Table 1). Interestingly, despite the effect of diet treatment on the area of (orange) colour spots, our multivariate analysis of spot spectral characteristics (described by the PCs) revealed no overall differences in mean percentage reflectance (brightness), hue or saturation between the two experimental groups (Table 1).

Effect of diet on postcopulatory traits

Our analysis revealed a significant effect of diet treatment on sperm viability; males fed a restricted diet produced ejaculates comprising a significantly lower proportion of live sperm than those fed the *ad libitum* diet (Table 1). By contrast, we found no significant effect of diet treatment on sperm velocity, components of sperm length or the number of sperm within stripped ejaculates (Table 1). When we excluded males that did not produce sperm (*n*=4 in the *ad libitum* group and *n*=6 in the restricted group), our results remained substantially unchanged (Univariate General Linear Model. Fixed factor: diet treatment, dependent variable: sperm number ($x10^6$), covariate: SL. No interaction between treatment and SL has been detected. Fixed factor, F=0.367, p=0.546; covariate, F=0.231, p=0.632).

Table 1. Effect of food limitation (diet treatment) on male body size and the mean (±*SE*) values of different pre- and postcopulatory traits. Results of the statistical tests used are given, along with their associated p-values. Body length (SL) was included as a covariate in the univariate GLMs and had a significant effect on several male traits (significant values are shown in italic).

		Restricted		**Libitum**					
	Trait	*Mean±SE*	*N*	*Mean±SE*	*N*	*Model*	**Test statistic**	***P***	***SL***
Body size	*Body area (mm^2)*	60.68±1.02	44	64.07±0.85	45	Univariate GLM	*9.598*	*0.003*	*<0.001*
Pre	*Sexual interest (min)*	3.71±0.57		9.64±0.63		Student *t*-test	*7.01*	*<0.001*	
	Gonopodial thrust (n)	3.16±0.72	45	4.42±0.89	45	Mann-Whitney	880.5	0.278	
	Sigmoid Display (n)	6.29±1.31		21.18±1.72		Student *t*-test	*6.884*	*<0.001*	
	Orange area (mm^2)	4.84±0.31		6.86±0.36			*17.735*	*<0.001*	*<0.001*
	Black area (mm^2)	2.23±0.15	44	2.44±0.15	45	Univariate GLM	0.886	0.349	0.614
	Iridescent area (mm^2)	7.34±0.37		7.16±0.37			0.602	0.44	*<0.001*
	Orange PC1	-0.13±0.13		0.13±0.17			0.853	0.43	
	Orange PC2	-0.05±0.13		0.05±0.17					
	Iridescence PC1	0.11±0.18	44	-0.11±0.12	45	Multivariate GLM			
	Iridescence PC2	-0.05±0.16		0.05±0.14			1.204	0.315	
	Iridescence PC3	-0.1±0.16		0.09±0.14					
	Iridescence PC4	-0.17±0.17		0.17±0.13					
Post	VAP (µm/s)	88±2.66	38	92.16±2.77	40	Student *t*-test	1.079	0.284	
	Sperm Head (µm)	3.72±0.02		3.75±0.02			0.74	0.461	
	Sperm Midpiece (µm)	3.02±0.04	36	3.04±0.06	41	Student *t*-test	0.252	0.802	
	Sperm Flagellum (µm)	46.16±0.16		45.99±0.15			-0.774	0.441	
	Sperm Number ($x10^{-6}$)	3.29±0.49	44	3.88±0.44	45	Univariate GLM	0.749	0.389	0.809
	Sperm Viability	0.54±0.02	38	0.62±0.02	41	Student *t*-test	*3.706*	*<0.001*	

Discussion

Our results reveal that short-term dietary manipulations can have dramatic effects on the expression of pre- and postcopulatory sexually selected traits. We found that males assigned to the food-restricted group exhibited reductions in both behavioural and morphological components of their precopulatory sexual displays, including courtship rate, sexual interest and the area of orange pigmentation. These reductions in precopulatory trait expression were accompanied by concomitant reductions in sperm viability, confirming that at least one component of ejaculate quality also exhibits condition dependence. Previous work on the population used in our study suggests that reductions in all of these traits are likely to have important consequences for male reproductive fitness. As in other guppy populations (Rios-Cardenas & Morris, 2011), the area of orange pigmentation and intensity of courtship displays are important determinants of male sexual attractiveness (Nicoletto, 1993; Brooks & Endler, 2001), while recent findings from controlled (artificial) insemination trials reveal that sperm viability is the primary determinant of competitive fertilization success (Fitzpatrick, J. L. & Evans, J. P.; unpublished data). Our finding that both traits are influenced by diet therefore leads us to conclude that male condition will be an important factor underlying the relative success of individuals during pre- and postcopulatory episodes of sexual selection.

Condition dependence in precopulatory traits

We observed a striking (three-fold) reduction in courtship rate in the restricted diet group, supporting the previous assertion that this component of male sexual behaviour is costly and therefore a potentially important signal of male condition for females during mate choice (van Oosterhout *et al.*, 2003; Kolluru & Grether, 2005; Kolluru *et al.*, 2009; Head *et al.*, 2010). However, in contrast with most of these studies (but see Kolluru *et al.*, 2009) we also found that male sexual motivation (our measure of 'sexual interest') was influenced by diet, suggesting that in our focal population courtship effort also functions as a reliable indicator of male quality (as observed in snakes: Shine & Mason, 2005; and in spiders: Shamble *et al.*, 2009). Interestingly, we found that the number of forced mating attempts (gonopodial thrusts) was unaffected by food limitation. This latter finding indicates that males incur relatively low costs during gonopodial thrusts (Houde, 1997), but also suggests that they do not compensate for the reduction of mating displays by increasing their reliance on alternative mating tactics under food stress. Thus, despite the negative (genetic) correlation between courtship and sneaking reported previously for this population (Evans, 2010), we find no evidence that this trade-off is further mediated by environmental effects.

We also analysed the spectral properties of male colour ornaments, which for some traits (notably orange) are known to be important in influencing mate choice in guppies (Endler & Houde, 1995; Brooks & Endler, 2001). Despite our finding that the size of orange spots was influenced by diet, we found no evidence that the spectral properties of these pigments was affected by treatment. This result was unanticipated in the light of previous work on guppies reporting significant condition dependence in the spectral properties of orange spots, specifically their chroma (saturation) and brightness (Grether, 2000). One possible explanation for our finding is that pigment deposition is not uniformly reduced in the orange spots, but instead pigmentation fades from the periphery of each colour spot. If so, a reduction in carotenoids available for coloration may reduce the visible area of the orange spots without changing the chromatic characteristics in the centre of the spot (but see: Grether, 2000; Karino & Haijima, 2004). This would allow males to maintain some aspects of signal quality (e.g. spot colour and saturation) while reducing the expression of potentially less important cues (i.e. spot size). Another explanation for the discrepancy between this study and previous work (Grether, 2000) is that there is population variation in the relationship between carotenoid intake and pigment spectral characteristics; for example, changes in dietary carotenoid

concentration may have a larger effect on orange chroma in some populations compared with others (Grether, 2000). Alternatively, skin shrinkage associated with the body area reduction observed in food-limited males may have compensated for any reduced concentration of carotenoids in the pigmented area, causing a reduction of spot size but balancing the reduction in the quantity of carotenoids available for male ornamentation. Alternatively, carotenoids may not have been limited in our diets, despite the reduction of total food available in the restricted diet group. Finally, other pigments that contribute to orange coloration, such as pteridins, that are synthetised by the male, may have compensated for a reduced availability of carotenoids (but see Grether *et al.*, 2001). Clearly there is a need to carry out experiments that combine manipulations of carotenoid content with the analysis of pigment to test these ideas further.

Although we report that the size of orange spots exhibits condition dependence (a finding also reported for guppies by Grether, 2000), we found no evidence that the area of melanin (black) or iridescent pigmentation was affected by the diet manipulations. The latter finding for iridescence is consistent with recent work on blue tits (Peters *et al.*, 2011) and may be explained because structural colours are generally more stable than pigmented colour spots except where condition is manipulated during key developmental stages of ontogeny (Fox, 1976; McGraw *et al.*, 2002; Kemp & Rutowski, 2007). Indeed, studies involving stress in early developmental stages have revealed condition dependence in both melanic and iridescent colouration (for example see: Kemp & Rutowski, 2007 for insects; and Griggio *et al.*, 2009 for birds). An interesting direction for future work is to explore condition dependence in these traits over a greater period encompassing key phases of growth and development.

Condition dependence in postcopulatory traits

Female guppies are highly promiscuous and postcopulatory sexual selection plays a key role in determining male reproductive fitness in this species (Boschetto *et al.*, 2011; Evans & Pilastro, 2011). Sperm production is generally known to be costly (Olsson *et al.*, 1997; Parker & Pizzari, 2010) and previous inbreeding studies on guppies suggest that some ejaculate traits exhibit condition dependence (Zajitschek & Brooks, 2010). We therefore expected to observe a significant reduction in a range of ejaculate traits (quality and number) in food-limited males. Our findings for sperm viability were consistent with this expectation; we observed almost a 10% reduction in the proportion of live sperm in stripped ejaculates from food-restricted males compared to their well-fed counterparts. As we note above, this finding is likely to have important fitness implications in guppies, as our recent work has shown that sperm viability is an important determinant of competitive fertilization success in the population used in our analysis (Fitzpatrick, J. L. & Evans, J. P.; unpublished data).

Interestingly, we found no evidence that sperm traits other than viability were significantly affected by diet treatment, although both sperm number (stripped ejaculate size) and sperm velocity (average path velocity) exhibited downward trends in the food-restricted group (see Table 1). Furthermore, we detected no observable effect of diet treatment on any of the observed sperm length measures, despite recent evidence from other taxa reporting remarkable levels of within-individual plasticity in sperm length (Crean & Marshall, 2008; Immler *et al.*, 2010). In some respects, therefore, our results contrast with previous work on other species reporting evidence for condition dependence in sperm numbers and length (Gage & Cook, 1994; Simmons & Kotiaho, 2002; Perry & Rowe, 2010). To our knowledge, however, our study is the first to experimentally test for condition dependence in sperm velocity through resource manipulation. As our treatments would have spanned the entire spermatogenesis cycle (approx. one month, Billard & Escaffre, 1969), our results suggest that investment in sperm number, size and velocity is not sensitive to food availability, at least during the time frame used

in this experiment. However, as we argue above for iridescent structural colours (see discussion above), the lack of any observed effect of diet on these traits may arise because our feeding trials did not span key developmental phases during ontogeny. However, in the case of sperm production, this argument seems unlikely as there is abundant evidence from guppies and other species that males can rapidly adjust their sperm allocation according to current mating opportunity (Wedell *et al.*, 2002; Bozynski & Liley, 2003; Rudolfsen *et al.*, 2006; Gasparini *et al.*, 2009) . An alternative explanation is that, when resources are limited, males allocate strategically to postcopulatory traits at the expenses of body maintenance and precopulatory traits. This may be due to the fact that sperm competition is so extreme in this species (Evans & Pilastro, 2011), that any reduction in investment to postcopulatory traits would have a disproportionately high effect on fitness.

Conclusions

In conclusion, we show that diet manipulations influence the expression of both precopulatory (sexual behaviour and ornamentation) and postcopulatory sexually selected traits (sperm viability). These results support the assertion from previous studies that precopulatory traits exhibit condition dependence in this species (Grether, 2000; van Oosterhout *et al.*, 2003; Karino & Haijima, 2004; Kolluru & Grether, 2005; Kolluru *et al.*, 2009) but also show that traits under postcopulatory sexual selection can exhibit concomitant declines in response to dietary stress. Our findings therefore suggest that male condition will simultaneously influence mate acquisition and competitive fertilization success, although we still lack direct experimental evidence that male reproductive fitness is compromised by such manipulations of condition. Importantly, our results add to a growing number of studies that test critical components of the genic capture hypothesis (Rowe & Houle, 1996; Tomkins *et al.*, 2004), thus potentially explaining the high level of variability observed in sexually selected traits. Key challenges for future work include carrying out more targeted investigations of condition dependence (e.g. during different developmental stages or through specific manipulations of diet composition), exploring the fitness implications of such manipulations, and identifying the genetic basis of condition (see Tomkins *et al.*, 2004). We anticipate that such investigations will greatly improve our understanding of the importance of condition dependence in sexual selection and its evolutionary implications.

RIASSUNTO

La selezione sessuale, descritta da Darwin nella sua opera *"The Descent of Man and Selection in Relation to Sex"*(1871) è una delle forze trainanti in natura e, nella maggior parte degli esseri viventi, determina fortemente la loro evoluzione. Negli ultimi tre decenni, la ricerca scientifica nell'ambito della selezione sessuale ha visto una rapida crescita, e numerosissimi lavori sia teorici che sperimentali hanno chiarito numerosi aspetti della selezione sessuale sia pre- che postcopulatoria. Nonostante ciò, la coesistenza di due fondamentali condizioni in natura fa si che esista ancora un paradosso evolutivo irrisolto: nella maggior parte dei caratteri si osserva una grande variabilità genetica nonostante la presenza di una forte selezione, sia naturale che sessuale. Poiché la selezione dovrebbe esaurire la variabilità genetica, queste due condizioni (variabilità da una parte e selezione dall'altra) sono in diretto conflitto. Poiché questa situazione riguarda la maggior parte dei tratti che formano il fenotipo di un organismo, o almeno tutti quei caratteri sotto una qualche forma di selezione, è facilmente immaginabile come questo problema abbia attirato l'attenzione di moltissimi ricercatori.

Durante il mio dottorato di ricerca ho mi sono dedicato allo studio di una parte di questo problema, concentrandomi sui caratteri maschili selezionati sessualmente. Questo campo è stato ampliamente studiato ma la maggior parte degli sforzi fatti per comprendere questa contraddizione è stata compiuta esclusivamente in un contesto di selezione precopulatoria, ed in particolare per quanto riguarda un suo caso particolare, quello del paradosso del lek.

Tuttavia, nel caso in cui le femmine di una specie siano sessualmente promiscue (situazione quasi totalmente diffusa nel regno animale), ci si aspetta la presenza di una selezione direzionale per i caratteri maschili legati alla competizione spermatica. Ed infatti quello che si osserva è che le caratteristiche dell'eiaculato hanno un ruolo importante nel determinare il successo riproduttivo maschile. La selezione su questi tratti è quindi forte ma, come per i caratteri precopulatori, numerose evidenze sperimentali dimostrano la presenza di un'elevata variabilità sia genetica che fenotipica in caratteri soggetti a selezione postcopulatoria.

Molte ipotesi sono state formulate per spiegare il mantenimento della variabilità genetica nei tratti selezionati sessualmente. In particolare, durante il mio dottorato ho testato le previsioni di tre di queste principali teorie, applicabili sia ai tratti pre- che postcopulatori. Per prima cosa ho verificato la presenza di selezione non lineare disruptiva e correlazionale prendendo in considerazione un ampio set di caratteri maschili. In secondo luogo, ho verificato che esistano dei trade-off di tipo energetico tra i tratti pre- e postcopulatori, come proposto nella 'teoria della competizione spermatica' di Parker. Infine, ho testato una delle condizioni fondamentali della teoria della 'cattura genica' proposta da Rowe e Houle (1996) e cioè che i tratti selezionati sessualmente siano condizione-dipendenti.

Durante il dottorato ho svolto quattro esperimenti principali utilizzando come specie modello *Poecilia reticulata*, comunemente chiamata guppy. Questo piccolo pesce tropicale d'acqua dolce è particolarmente adatto per i miei scopi. I maschi presentano infatti caratteristiche soggette sia a selezione precopulatoria (ornamenti, dimensioni e comportamento sessuale) che a selezione postcopulatoria (numero, velocità e vitalità degli spermatozoi) ed inoltre si osserva in questi caratteri un'elevata varianza genetica additiva. Appare quindi evidente la presenza della contraddizione prima descritta. Con il primo esperimento (primo articolo) ho

descritto, per la prima volta, la selezione non lineare che agisce sull'insieme dei tratti sia pre- che postcopulatori. Ho poi misurato il costo a lungo termine imposto dalla produzione di spermi (secondo articolo) con l'obiettivo di determinare i trade-off presenti tra i tratti pre- e postcopulatori. Negli ultimi due articoli (terzo e quarto) ho testato l'ipotesi di condizione-dipendenza in un ampio set di tratti selezionati sessualmente.

I miei risultati suggeriscono che in questa specie la selezione non lineare può essere più importante di quanto stimato in precedenza e, in particolare, che la selezione disruptiva e correlazionale possono contribuire a mantenere il polimorfismo osservato nei tratti selezionati sessualmente. Inoltre l'investimento a livello postcopulatorio nell'eiaculato presenta per i maschi di Poecilia un costo in termini di successo precopulatorio (possibilità di accoppiarsi), in accordo con la teoria della competizione spermatica. Infine, i tratti sessualmente selezionati, sia pre- che postcopulatori, mostrano una forte dipendenza dalla condizione del maschio, confermando così uno degli assunti dell' ipotesi della cattura genica.

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