ABSTRACT

Genetic color polymorphisms are common in nature, and a major challenge for evolutionary biologists has been to understand how they are maintained despite the effects of directional selection. Recent studies suggest that frequency-dependent selection may explain the persistence of intraspecific variation in color. However, it remains to be determined whether frequency-dependent selection is the most predominant (or perhaps the only) mechanism to maintain such genetically-based polymorphisms.

Using a South American fish, *Poecilia parae*, my dissertation aims to elucidate the relative roles of natural and sexual selection in the maintenance of polymorphisms in natural populations. *Poecilia parae* males exhibit five distinct, Y-linked and co-occurring color morphs: (i) ‘immaculata’, the smallest and drab-colored males that resemble juvenile females; (ii) ‘parae’, the largest males that exhibit a striped tail and black vertical body bars that intensify during social interactions; and (iii) the blue, red, and yellow morphs that are of intermediate body size and display colorful body flanks. Field surveys indicate that the frequency of each morph remains relatively stable and consistent over multiple years.

Using a combination of observational and experimental studies, accompanied by techniques that aimed to characterize the visual ecology (e.g., water light transmission, visual sensitivity) of *P. parae*, I found that this striking color polymorphism is maintained by a complex balance between different components of natural and sexual selection. First, males of the five color morphs employ different behavioral mating strategies (hereafter alternative mating strategies, AMSs) to maximize their reproductive

success. These AMSs are also accompanied by differences in morphological traits, such as testes investment and sperm morphometry that complement the specific tactic. For instance, immaculata males are often categorized as an ‘unattractive’ male by females, which is correlated to its phenotypical appearance (smaller and drab). However, this morph is relatively abundant. I found that immaculata males specialize in ‘sneak’ copulations and have adaptations (i.e., larger testes and unique sperm morphology) that possibly confer a fertilization advantage during postcopulatory events, when competing with the sperm of more attractive males.

My studies also indicate that females have strong mating preferences for red and yellow males, with visual predators also favoring those males as prey. These findings suggest that the antagonistic interaction between pre-mating sexual selection favoring and predation acting against the red and yellow morphs may prevent them from eliminating other color morphs from the population. In fact, the red and yellow males were consistently found to be the rarest morphs across populations. Further, despite overall preference for red or yellow males, my analysis also detected female preferences for blue males. These results suggest that the interaction between female mating preferences and predation accompanied by variation in male reproductive strategies may allow for the maintenance of complex color polymorphism in natural populations.

Males of the different morphs further vary in their levels of aggressive behaviors. In a series of controlled lab experiments, I found that parae males gain successful matings by preventing other males from accessing females and/or modifying female mating preferences after test females witness successful agonistic interactions.

Finally, I found that the signaling environment of *P. parae* in nature is variable and that this contributes to temporal and spatial variation in how males are perceived by females. Although red males were typically the most conspicuous morph, blue males were sometimes more conspicuous than red males at several sampling sites. The results also revealed that an aquatic model predator is able to discriminate the same male color morphs that resulted also conspicuous for females. However, it is possible that males and females of *P. parae* are using parts of the background spectral that are least sensitive for predators.

In conclusion, my dissertation suggests that balancing selection defined by the interaction between various episodes of selection allows for the invasion of AMSs and thus the maintenance of the male color polymorphism in *P. parae*.

THE MAINTENANCE OF MALE COLOR POLYMORPHISM IN *POECILIA PARAE*

By

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CHAPTER I

PREFACE

Understanding the origin and maintenance of variation in natural populations has been a central problem in evolutionary biology for more than a century (Gray and McKinnon 2007; Roulin 2004). Of particular interest are the cases in which genetic variation is responsible for the dramatic differences in morphology (such as color) that are under genetic control (i.e. genetic color polymorphisms) and expressed by individuals of an interbreeding population (i.e., morph) (Ford 1965; Roulin 2004).

Genetic color polymorphisms are often linked to alternative mating strategies (AMSs), which are viewed as 'functional sets of behavioral patterns and/or morphologies' used with the purpose of acquiring mates (Shuster 2008). Hence, it is explicitly assumed that AMSs evolve when the mating success of males in a population becomes uneven (Gross 1996; Shuster and Wade 2003). Once the opportunity for strong selection in variable environments exists, it is expected that males should pursue alternative ways of securing matings or fertilization (Oliveira et al. 2008; Shuster and Wade 2003).

Fisher (1930) proposed that two morphs cannot exist in nature without a selective balance. That is, a polymorphism can be maintained if a morph gains temporal fitness benefits exploiting the disadvantages of the other but also suffering some fitness costs at some point in time so that the net benefit accrued by each morph become equal at equilibrium. This theoretical approach allowed Maynard-Smith (1982) to propose that morphs can reach the estimated equal net benefit by effects of frequency-dependent selection, where a morph will experience increase reproductive success when rare (negative) but not when it becomes abundant (positive). So far, only three biological systems offer solid evidence for this prediction: side-blotched lizard (*Uta stansburiana*);

Californian marine isopod (*Paracerceis sculpta*); swordtail (*Xiphophorus nigrensis*) (Ryan et al. 1992; Shuster and Wade 1991; Sinervo and Lively 1996), with only the side-blotched lizard representing a color polymorphism. It remains to be seen whether frequency dependent selection is the only or predominant way to maintain polymorphisms in nature.

The aim of my dissertation is to elucidate the selective forces that allow for the maintenance of polymorphisms within populations of a single species. My research investigated how natural and sexual selection interact to maintain a striking sex-limited and Y-linked color polymorphism in *Poecilia parae*, a live-bearing fish from South America. The rationale behind my work is as follows: whereas it is increasingly accepted that the existence of color variation between individuals of an interbreeding population are under directional sexual selection, very little is known about how different color males that are characterized by two or more discrete alternative mating strategies resulting from a genetic polymorphism are maintained in the population. Only a handful of species fits this category and from which it has been inferred that the average fitness of each strategy should be equal. At least, there is a very limited body of empirical research indicating that when fitness is unequal and there are not evolutionary mechanisms maintaining multiple strategies, selection should cause the extinction of the strategy with the lower fitness (see above). Although my work does not focus on the measuring the fitness of each alternative strategy, I provide strong evidence of the possible mechanisms that allow for the maintenance of the five different types of males in *P. parae*.

There are currently several mechanisms proposed to explain the maintenance of color polymorphisms (neutral polymorphism, disruptive selection, frequency-dependent selection, Heterosis, immigration-selection balance, mutation-selection balance). A number of reviews (Ayala and Campbell 1974; Galeotti et al. 2003; Gray and McKinnon 2007; Hedrick 1986; Roulin 2004; Sinervo and Calsbeek 2006) have already described the relevance of each mechanism offering theoretical and empirical support. Consequently, I briefly describe those mechanisms that may help explaining the maintenance of the color polymorphism in *P. parae*. It is important to mention that in most cases, the maintenance of color polymorphisms of a single species can be explained by several other mechanisms. For example, although it has been well established that guppy (*Poecilia reticulata*) color patterns evolve as a compromise between conspicuousness and attractiveness to females and crypsis to predators (Endler 1978; Endler 1980; Endler 1991), the maintenance of the color polymorphism is explained by negative frequency-dependent mating success and predation, disruptive correlational selection, sensory bias, and divergent selection coupled with gene flow, which in turn indicates the magnitude of complexity of the polymorphism. Unfortunately to this point we still know little about the contribution of each mechanism and how they might interact (Gray and McKinnon 2006).

When AMSs are maintained in a balance by different selective mechanisms no particular AMS can predominate (Sinervo and Calsbeek 2006). Frequency-dependent selection, where each behavioral phenotype depends upon its frequency in the population, is often the primary form of selection evoked to explain the evolutionary stability of color polymorphisms (Sinervo and Calsbeek 2006). This is because in several color polymorphic species, there is a consistent trend for a ‘rare morph’ advantage in terms of predation (Bond and Kamil 2006; Olendorf et al. 2006) and mating success (Bleay and

Sinervo 2007; Farr 1977; Hughes et al. 1999) and even in relation to sexual conflict (Svensson and Abbott 2005) and social dominance (Dijkstra et al. 2005).

A first step to determine the effects of frequency-dependent selection should be by following the variation of morph frequencies between populations and years. For instance, the three AMSs in the side-blotched lizard (*Uta stansburiana*) cycles among years (4-5 years) from a high frequency of orange, to yellow, then blue (Sinervo and Lively 1996; Zamudio and Sinervo 2000). Similarly, there are clear fluctuations in the frequency of female morphs in the trimorphic blue-tailed damselfly (*Ischnura elegans*) between populations and across years (Svensson and Abbot 2005). In both populations, the genetic cycles in morph is driven by frequency-dependent selection favoring rare morphs in any given year. Although I have not directly addressed the relative importance of frequency-dependent selection in *P. parae*, my data suggests no significant changes in the frequencies of AMSs between populations for at least the last 50 years (see Chapter III).

Alternative to frequency-dependent selection, when colour polymorphism is sex limited and linked to sexual function, the evolution and maintenance of such variation may be explained by the interaction between sexual and natural selection (Endler 1992; Houde and Endler 1990; Kingston et al. 2003; Pierotti et al. 2009; Seehausen and Schluter 2004). Females often prefer males with bright and colorful signals because these signals are honest indicators of male quality (Andersson 1994; Stein and Uy 2006). However, female mating decisions may favour AMSs depending on a number of morph-related or environmental factors (Barlow 1973; Alonzo and Sinervo 2001; Brooks and Endler 2001; Kingston et al. 2003; Rios-Cardenas et al. 2007). For instance, under high risks of predation, bright and colorful male guppies (*P. reticulata*) are less preferred by females than drab males (Gong & Gibson 1996; Houde 1997), suggesting that susceptibility to visual predators offset the advantages of mating with conspicuous males. Such interaction between female mating preferences and susceptibility to predation may in turn allow new AMSs to invade and persist within populations (Bond and Kamil 2006; Gamble et al. 2003).

Finally, a recent stream of empirical research has examined the role of heterogeneous environments in the maintenance of color polymorphisms (reviewed in Boughman 2002; but see also Fuller et al. 2005; Gray et al. 2008), especially since color perception (by mates and predators) depends on segments of the habitat in which sexual and natural selection occurs. Indeed, the perception of color signals depends on the light under which the signal is perceived, the background against which the signal is viewed, the medium through which the signal travels, and the sensory capabilities of the receiver (Endler 1991). Therefore, variation in any of these components can select for divergent phenotypes. For instance, several studies have documented strong relationships between the visual background and male color patterns suggesting that color traits often show higher contrast and conspicuousness (e.g. sticklebacks: Boughman 2001; bluefin killifish: Fuller 2002; golden collared manakins: Uy and Endler 2004) and conspicuousness favoring different color male morphs at different segments of the habitat correlates with increase reproductive success (Gray et al. 2008).

Males of the South American poeciliid *Poecilia parae* exhibit five distinct morphs (Figure 1, Chapter II): (i) immaculata, the smallest and drab-colored males that resemble juvenile females; (ii) parae, the largest males that exhibit a striped tail and black vertical body bars that intensify during social interactions; and (iii) the blue, red, and yellow morphs that are of intermediate body size and display colorful body flanks (Bourne et al. 2003; Lindholm et al. 2004). Males and females mate promiscuously, with males providing no resources during mating (Liley 1966). *P. parae* breeds year-round (Bourne et al. 2003; Liley 1966), creating an opportunity for intense competition among males to identify and monopolize sexually receptive females. Females can store sperm for up to three months and produce a succession of litters from a single insemination (Hurtado-Gonzales, unpublished). Gestation is 26 ± 3 days and number of young produced per brood varies from 3 – 31 and depends on the size and age of the mother (Watson-Rodney 2005).

The distinct variation in male color in *P. parae* is one of the most striking polymorphisms found in vertebrates. These visually divergent phenotypes are straightforward to score, and many of the factors influencing their mating success can be readily measure. Previous studies in *P. parae* have elucidated three outcomes relative to this work: (1) Liley's (1966) study have documented the rarity and difficulties in finding the red and yellow males in their natural habitats since 1960's; (2) Bourne et al. (2003) reported that *P. parae* females show strong preferences for mating with carotenoid-based red and yellow males, with immaculata males being never preferred and that such preferences correspond to their mating success; and, (3) Lindholm et al. (2004) show that all male color morphs interbreed and the color phenotype of the sons is inherited from the father (Y-linked color polymorphism).

My dissertation is divided in four additional chapters. Chapters II - IV have been already published and chapter V is being prepared for submission.

In Chapter II, I document the occurrence of alternative mating strategies in *P. parae*. For this purpose, I quantified (i) the relative mating success of each color morph by using behavioral trials, and (ii) the relative testes investment and sperm morphometry to look for evidence of whether competition for mates extends to post-copulatory events. My results suggest that males are exploiting different components of sexual selection (i.e., pre-copulatory and post-copulatory), with colorful males obtaining in greater proportion mating success from courtships rather than from sneak copulations (i.e., parae, blue, red and yellow). The least attractive immaculata males circumvents female mating preferences by using a sneaker strategy accompanied by morphological adaptations (i.e., larger testes size and relatively longer sperm), which may translate to fertilization advantages during episodes of sperm competition when facing bigger and more attractive competitors (i.e., parae, blue, red and yellow).

In Chapter III, my aim was to explore the relative roles of natural and sexual selection in favoring the maintenance of rare color morphs in nature. Previous studies examining female mating preferences in *P. parae* have clearly demonstrated that red and yellow males are the most preferred males (Bourne et al. 2003; Lindholm et al. 2004). If increased mating success leads to increased reproductive success, then red and yellow males should be consistently the most abundant morphs. However, based long-term field surveys, red and yellow males have been consistently rare since the 1960s (Liley, 1966; Lindholm et al., 2004; see Chapter III in this dissertation). A possibility is that red and yellow males persist in low frequencies because the strong female mating preference for these males is offset by elevated risks to visual predators (e.g. guppies; Endler, 1980). Consequently, I explored (i) the possibility that individual females are differing in their level of mate preferences and (ii) the susceptibility of color male morphs to predation risk. My results provide evidenced that the interaction between variable female mating preferences and selective predation can explain the frequencies of both carotenoid-based morphs (red and yellow). I also found that some females indeed prefer parae and blue males, indicating that individual female mating preferences may partially explain their maintenance.

In Chapter IV, I determined how overt male aggression can influence male mating success and facilitate the persistence of the color polymorphism in *P. parae*. If indeed, particular morphs specialize in male-male aggression, I predicted that agonistic interactions (i) may limit the mating opportunities of the attractive (i.e., red and/or yellow) morphs, (ii) have the potential to influence female mating preferences, and (iii) enhance the mating success of aggressive males. Results consistent with these predictions would suggest that the opportunity to exploit male-male competition may facilitate the maintenance of genetically-based polymorphisms. The main results to emerge from chapter IV is that parae and yellow males have evolved the use of overt aggressions to enhance their mating success by monopolizing access to females and by modifying female preferences after wining agonistic encounters

Finally, in chapter V with the use of physiological models that simulate how organisms receive and sense signals, I tested whether spatial heterogeneity of the visual environment contributes to the maintenance of the color polymorphism in *P. parae*. Specifically, I determined whether there was a role for variable visual environment in dictating the level of conspicuousness of color males as perceived by conspecifics and predators. I found that the most attractive males are the most conspicuous morphs (i.e., red and yellow), within and between populations. However, there is an important intersite variation (within populations) in which the visual background seems to favor the conspicuousness of blue males. In terms of sexual selection, the results of chapter V suggest a potential role for variable environment, which in turn, should favor individual variation in female mating preferences and male-male competition. In terms of natural selection, there is an agreement in the discrimination of conspicuous males by females and predators. That is predators can contrast attractive males as well as females but not as intense in terms of chromatic contrast, which could suggest that *P. parae* tune their signals to their visual background which for predators may not be that sensitive in terms of spectral properties.

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CHAPTER II

ALTERNATIVE MATING STRATEGIES MAY FAVOR THE PERSISTENCE OF A GENETICALLY-BASED COLOR POLYMORPHISM IN A PENTAMORPHIC FISH

Hurtado-Gonzales, J. L. and J. Albert. C. Uy. 2009. Alternative mating strategies may favour the persistence of a genetically based colour polymorphism in a pentamorphic fish. *Animal Behaviour* 77: 1187–1194

ABSTRACT

Males of the pentamorphic fish, *Poecilia parae*, show a striking colour polymorphism that ranges from drab coloration resembling females to brilliant black and red stripes. We tested the hypothesis that the observed polymorphism may persist because each morph uses a unique but equally successful mating strategy. We quantified the mating behavior, testes investment and sperm morphometrics of each morph. We found that the smallest morph (immaculata), which resembles drab females, used a ‘sneaker’ tactic (i.e. forgoing courtship and mostly ‘stealing’ copulations) as a mating strategy. Immaculata males also had larger testes, and produced larger ejaculates and sperm with longer flagella, which should provide advantages during sperm competition. The largest morph (parae) and the coloured blue, red and yellow morphs used a combination of courtship and sneaker tactics. However, they achieved greater copulation success as a result of courtships. The testes investment of parae and yellow morphs were similar but larger than those of the blue and red males. Our results suggest that male morphs of the pentamorphic *Poecilia parae* use unique mating strategies that exploit different aspects of pre- and postcopulatory sexual selection, which, in turn, may help maintain their relatively stable frequencies in the wild.

INTRODUCTION

Sexual selection has given rise to a broad array of male traits related to intense competition for mates (Andersson 1994). These include weapons that allow males to monopolize access to females or display traits that are attractive to choosy females (Andersson 1994). However, males that cannot monopolize or attract females may evolve alternative behaviours that allow them to circumvent male–male competition, female choice or both (Gross 1996; Brockmann 2001; Shuster & Wade 2003). As in the case of genetically polymorphic species, these alternative mating behaviours are commonly accompanied by morphological, physiological and life history differences that make it possible to categorize individuals as alternative morphs and their mating behaviours as alternative mating strategies (AMSs, *sensu* Gross 1996).

Species that are known to display genetically based polymorphisms and in which the polymorphism is associated with AMSs are few (e.g. pigmy swordtail: Zimmerer & Kallman 1989; Californian marine isopod: Shuster & Wade 1991; *Urosaurus ornatus* lizards: Thompson et al. 1993; the ruff: Lank et al. 1995; side-blotched lizard: Sinervo & Lively 1996; Zamudio & Sinervo 2000). In these systems, the use of AMSs should result in a stable equilibrium in which each mating strategy can increase in frequency at a certain point in time but not drive the others to extinction (Sinervo & Lively 1996; Ryan et al. 1992; Zamudio & Sinervo 2000).

A recent mechanism proposed to explain the coexistence of AMSs associated with polymorphism in male coloration posits that alternative morphs exploit a variable sensory environment, resulting in habitat-specific selection pressures and the persistence of multiple color forms (e.g., Fuller et al. 2005; Chunco et al. 2007; Uy & Stein 2007; Gray et al. 2008). That is, colour perception by females and predators is depends on the environment where predation and mating interactions occur (‘environment-contingent sexual selection’; Gray & McKinnon 2007). Thus, genetic colour polymorphisms may be maintained by frequency-dependent selection acting within populations (e.g. via predation and/or mating success) and/or environmental heterogeneity that influences signal perception (e.g. Endler 1992; Boughman 2002).

The live-bearing fish *Poecilia parae* has five distinct Y-linked morphs (Liley 1966; Lindholm et al. 2004): the drab-coloured (immaculata), the coloured-tail (parae), and the blue, red, and yellow morphs (see Fig.1). Previous experiments indicated that females (which are dull in coloration) have a strong preference for carotenoid-coloured males (red and yellow), which translated into greater copulation success for the preferred morphs (Bourne et al. 2003). If no other selective forces (e.g. predation; male-male competition) are acting on *P. parae* populations, strong female preferences for carotenoid-based morphs should result in the fixation of red and yellow phenotypes in their natural habitat. However, long-term field surveys indicated that the less preferred morphs are actually more abundant (immaculata: 44%; parae: 42%; blue: 9%; yellow: 4%; red: 1%), and that the frequencies of the five alternative male morphs are constant across years (i.e. 5 years of survey; see Lindholm et al. 2004; J. L. Hurtado-Gonzales & J. A. C. Uy, unpublished data). As a first step to elucidate a possible explanation for the observed high frequency of the less preferred morphs and the overall persistence of all five morphs, our aim for this study was to determine whether male morphs of the *P. parae* have evolved alternative mating strategies.

Males of *P. parae* use two well-defined mating tactics: courtship displays and sneak copulations (Liley 1966). During courtships, males change the intensity of their body coloration, and display their body flanks and fins to females during a set of short movements repeated several times in quick succession. These courtship displays result in successful copulations if females are sexually receptive (Liley 1966). Sneak copulations, in contrast, do not involve courtships and do not require female cooperation. To achieve a sneak copulation, a male places himself outside of a female's range of vision by approaching her from behind. Then, the male places himself directly below the female's anal region and inserts his gonopodium into the female's genital pore (Liley 1966). This form of behaviour, commonly found in poeciliids, can circumvent female precopulatory mate choice and reduce competition among males (e.g. Houde 1997). Following courtships and sneak copulations that result in successful sperm transfer, males suddenly jump away from the female, spiraling upwards and performing two or three body jerks (Liley 1966; Houde 1997; Pilastro et al. 2002); hence, successful matings in *P. parae* can also be easily quantified.

Parker (1990) showed that, in theory, males specializing in sneaking behaviour face high rates of sperm competition, whereas dominant or attractive males face less intense sperm competition. As a consequence, males that specialize in sneak copulations (i.e. 'sneakers') are predicted to invest in traits or behaviours that increase their competitive ability in sperm competition (e.g. larger testes, larger ejaculate size). This prediction has been widely supported in a variety of taxa (reviewed in Wedell et al. 2002), but it has been rarely explored in genetically based polymorphic species (but see Shuster 1987; Jukema & Piersma 2006). Considering the behavioural mating differences between morphs of *P. parae*, which are accompanied by high rates of promiscuity, we predicted that the smallest morph, immaculata, which lacks conspicuous coloration and resembles a juvenile female, would specialize in sneak copulations. In contrast, we predicted that the coloured (blue, red and yellow) and parae morphs may invest in courtships to advertise their body colour and size to females, respectively. Finally, we predicted that the observed differences in mating behaviours would be accompanied by differential investment in sperm production and sperm morphometry, favouring morphs that have a mating disadvantage or that specialize in sneak copulations.

METHODS

Experimental Fish

Wild-caught male and female *P. parae* (Fig. 1) used in these experiments were collected from clear water ditches (0.3–1.5 m) in Guyana during June–August 2006, March 2007 and May–July 2008 from two populations: west (6º41’ N, 58°12’W) and east (6º47.2’N, 58° 09’W) of the Demerara River. To quantify male mating behaviours, we used individuals only from the west population. However, to determine testes investment and their respective spermatic correlates, we used individuals from west and east populations. To avoid misidentifying a juvenile of the four coloured morphs as an immaculata male, we used only immaculata males that were at least 19 mm in total length, the minimum size at which the four coloured morphs begin expressing their colours (Liley 1966; J. L.

Hurtado-Gonzales & J. A. C. Uy, unpublished data). Live individuals were maintained in our laboratories in Georgetown (Guyana) and Syracuse University (U.S.A.) on a diet of commercial flake food and live brine shrimp (12:12 h light:dark cycle, 28° ± 1°C).

Quantifying Male Mating Behaviours

We captured adult males and females in source ditches from the west population. All individuals were kept in communal tanks for 3 days following capture to reduce stress. Males were, then sorted and housed by morph type. From the pool of sorted morphs, 15 males from each morph (N = 75) were individually isolated for 5 days to increase male sexual responses and to reduce male–male interaction effects. Pregnant females were isolated individually until their fry emerged. We used postpartum females since females are more sexually receptive following the end of the pregnancy cycle (Liley 1966; Houde 1997). Trials in which males and females were unresponsive after 10 min of observations were excluded from our analyses, and were not used again. In total, we obtained mating behaviour from 60 males (12 immaculata, 12 parae, 12 blue, 13 red and 11 yellow).

Dimensions of experimental tanks were 32 x 22 x 24 cm (L x W x H). The tanks were filled with treated tap water (14 cm in depth) and a thin layer of gravel, and illuminated with a full spectrum light (70 cm above the tank). The tanks were covered on three sides with brown, craft paper, with the uncovered side facing a SCL-810 Hi8 Samsung camcorder. Trials were conducted from 0600 to 1200 hours and from 1500 to 1800 hours, which are times when *P. parae* are most active (J. L. Hurtado-Gonzales, personal observations). Before each trial, a male and a female were placed in the test tank, which had a removable barrier that prevented physical and visual contact. Fish were allowed to acclimate for 10 min. At the start of the experiment, the opaque barrier was lifted, allowing the test female and male to interact. After trials ended, each individual was anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester) and a lateral digital picture of each individual was taken. We measured the total length (tip of the upper jaw to tip of caudal fin) for each individual from the digital images using Sigmascan Pro®v5.0.0 (San Jose, CA, U.S.A.). From the videos, we recorded the number of (1) courtship displays, (2) copulations as a result of courtships, (3) gonopodial thrusts (hereafter sneak copulations) and (4) failed sneak copulations (hereafter sneak attempts). Behaviours performed by each male were quantified, and then standardized by the length of the experiments (i.e. behaviour per 10 min) and converted to proportions. Classes of mating behaviours were based on detailed ethograms of P. parae compiled by Liley (1966).

Determining Testes Investment

To obtain a natural measure of testes investment, 288 males (west: N = 168; east: N = 120) were caught from local ditches and were immediately euthanized in a water bath containing MS-222. We measured male total length (see Methods above), and removed their gonads. Gonads and soma were dried in a conventional laboratory oven at 80°C for 48 h, and weighed to the nearest 0.001 g (ATI Cahn C-35, Cahn Instruments, Boston, MA, U.S.A.).

Quantifying and Characterizing Sperm Numbers and Sperm Morphometry

Males of each morph (immaculata: N = 10 west, N = 7 east; parae: N = 10 west, N = 10 east; blue: N = 10 west, N = 10 east; red: N = 7 west, N = 6 east; yellow: N = 10 west, N = 5 east) were isolated from communal tanks for 5 days to ensure and standardize sperm replenishment. Males were then stripped for sperm to quantify the number of sperm and size of ejaculate following standard method used in guppies (*P. reticulata*; Kuckuck & Greven 1997). Likewise, to characterize and compare sperm morphology, we followed methods developed for guppies (Matthews et al. 1997). Briefly, after stripping a male, all recovered sperm packages were initially diluted in 100 ml of physiological solution (NaCl 0.9%) or until we obtained a proper counting concentration needed for a Neubauer chamber haemocytometer (ca. 100). The mean number of sperm produced by each morph was quantified by taking the average of sperm count from five counts, then multiplying it by the sample dilution factor and the initial volume. From the same sample, we placed 7 ml of sperm dilution on a microscope slide, covered it with a cover slip and observed it at 1000_ magnification. We obtained digital images of individual sperm with a Dage CCD72 camera mounted on an Olympus BX60 microscope, allowing us to measure the lengths of the head, midpiece, flagellum, and total spermatozoa to the nearest 0.01 mm using NIH freeware (http://rsb.info.nih.gov/nih-image). We measured 20 spermatozoa per male (N = 1700 spermatozoa for 85 males/5 morphs).

Statistical Analyses

Statistical analyses were performed using SPSS 15.0 for windows (SPSS, Chicago, IL, U.S.A.). All data were tested for normality. Behavioural data were analyzed using MANCOVA (covariates: total body length of males and females; fixed factors: morph type (immaculata, parae, blue, red and yellow); dependent variables: number of courtship displays, copulations as a result of courtship displays, sneak copulations and sneak attempts). Proportions were arcsine transformed. When the MANCOVA was significant, we performed univariate tests for each dependent variable. Moreover, to determine differences between morphs, we used Tukey multiple comparison tests. For testes investment among morphs within and between populations, we used two approaches: (1) the gonadosomatic index (GSI = testes mass/body mass), which assumes proportional increase of testes size with sperm demand (de Vlaming et al. 1982; Stockley et al. 1997) and (2) the testes allometry model (TAM), which has been used to quantify testes variation in fish that have conditional mating strategies (Tomkins & Simmons 2002). We analyzed GSI with a nonparametric factorial design (Shirley 1987), since the GSI data did not meet the assumptions of parametric tests. For the TAM, we log-transformed testes and soma mass, and constructed a general linear model (GLM; covariate: log soma mass (LSM); fixed factor: morph type and population (west and east); dependent variable: log testes mass (LTM)). The model included full factorial interactions.

We analyzed sperm morphometry with MANOVA to compare the difference in variance between populations and between morphs, and their respective interactions. All descriptive values are reported in their original units (mm or mg), and expressed as means ± SE. Significant differences between morphs were also determined using Tukey multiple comparison tests.

RESULTS

Male Mating Behaviours

After controlling for individual length (Table 1), we found that the five distinct morphs differ in their mating behaviours (MANCOVA: $F_{16, 153.39} = 2.71$, $P = 0.0008$). Male morphs vary in the number of courtship displays initiated ($F_{4, 53} = 5.16$, $P = 0.001$). The Tukey test indicates that parae, blue and yellow morphs initiate more courtship displays to females than the immaculata and red morphs (all $P < 0.05$; Fig.2a). A significant difference was also found in the proportions of sneak attempts ($F_{4, 53} = 3.41$, $P = 0.01$), where the immaculata males performed significantly more attempt sneak copulations than any other morph, and the blue morphs the least (all $P < 0.05$; Fig.1a). Considering all the successful copulations achieved as a result of elaborated courtships between parae, blue, red, and yellow morphs, we found no statistical difference ($F_{4, 53} = 1.78$, $P = 0.\ 15$). However, all larger morphs achieved greater number of copulations as a result of elaborate courtships than did immaculata males, which obtained none. Likewise, there was a difference in the proportions of successful sneak copulations ($F_{4, 53} = 6.05$, $P = 0.004$), from which immaculata males achieved greater success than the other four morphs (all $P < 0.01$; Fig. 2b).

Intermorph Body Length Differences Within and Between Populations

The total body length of male morphs differed within (ANOVA, $F_{4, 278} = 25.55$, $P < 0.001$; Fig. S2, EMS) and between the east and west populations (ANOVA: $F_{1, 278} =$

14.55, $P < 0.001$). Males of the west population were longer in body length. Within populations, immaculata was the shortest morph ($P < 0.01$), whereas parae was the longest morph ($P = 0.004$). We did not find any difference in the total body length between the blue, red, and yellow morphs (all $P > 0.05$), which were intermediate in body length to the immaculata and parae morphs.

Testes Investment

Testes mass (mg) differed among morphs (ANOVA: $F_{4, 283} = 4.19$, $P < 0.01$). Testes of parae and yellow males and of blue and red males were 90% and 70%, respectively, of the *immaculata* absolute testes mass (Fig. 4). We found no difference in GSI between populations ($\chi_1^2 = 0.6$, n = 2; $P = 0.44$), and no interaction between population and morph type ($\chi_4^2 = 6.47$, n = 5; $P = 0.17$). After pooling the GSI data from both populations, we found that the five male morphs varied in their GSI (Kruskal-Wallis, $H_{4, 288} = 62.27$, $P < 0.001$), with the immaculata morph exhibiting the largest GSI investment ($P < 0.001$), and blue and red morphs the least ($P < 0.001$).

Population had no effect on LTM (population: $F_{1, 268} = 0.15$, $P = 0.69$), and there was no interaction between testes size among morphs and population (population x morph type: $F_{4, 268} = 1.05$, $P = 0.38$; population x LSM): $F_{1, 268} = 0.29$, $P = 0.59$; population x morph types x LSM: $F_{4, 268} = 1.19$, $P = 0.32$). Thus, we combined data from both populations for subsequent analyses. The full model confirmed that there was an effect of LSM and morph type on LTM, but the interactions were not significant (Table 2). The reduced model showed a strong association between morph type and testes investment, which followed a linear relationship with LSM: immaculata ($r^2 = 0.26$, $P < 0.001$); parae ($r^2 = 0.11$; $P = 0.002$); blue ($r^2 = 0.35$; $P < 0.001$); red ($r^2 = 0.42$; $P < 0.001$); and yellow ($r^2 = 0.23$; $P = 0.001$) (Fig. 5). Coincident with the GSI results, the LGM of immaculata males was higher than the other four morphs ($P < 0.001$). Parae and yellow males had larger testes investment than blue and red males ($P = 0.01$).

Ejaculate Volume and Sperm Morphometry

We found no differences in ejaculate volume (µl) between morphs across populations (population x morph type: $F_{4, 75} = 0.33$, $P = 0.86$; population x total length: $F_{1, 75} = 0.05$, $P = 0.82$; population x morph types x total length: $F_{4, 75} = 0.33$, $P = 0.86$). After removing non-significant effects and interactions, we found that morphs varied in sperm numbers ($F_{4, 79} = 12.32$, $P < 0.01$; Fig. 6), with the immaculata morph producing more sperm per ejaculate (mean log ejaculate = 9.96 ± 0.14) compared to all other morphs ($P < 0.01$). No difference was detected in the amount of sperm released in ejaculates by parae (8.04 ± 0.13), blue (8.64 ± 0.13), red (9.20 ± 0.14), and yellow (8.85 ± 0.13; all $P > 0.05$) males. However, we found a positive relationship between body size (as a covariate) and ejaculate size, suggesting that variation within morphs also exists ($P < 0.001$).

The head, midpiece and flagellum, and total sperm lengths did not differ between the west and east populations (MANOVA: $F_{3, 73} = 0.62$, $P = 0.60$). After pooling data across populations, we found that morphs did not differ in sperm head length, but differed in midpiece, flagellum, and sperm total length (Table 3). Immaculata males had the shortest midpiece ($P < 0.03$) but the longest flagellum ($P < 0.01$) compared to the other morphs. Males of the yellow morph had longer flagella compared to parae, blue and red morphs ($P = 0.03$). The longest sperm was produced by the immaculata morph; however, sperm length of the immaculata morph did not differ from that of yellow males. No relationship was found among head, midpiece, flagellum or total sperm length across morphs ($P > 0.10$).

DISCUSSION

Our results suggest that male morphs of the pentamorphic *P. parae* have evolved unique alternative mating strategies (AMSs) to exploit and/or circumvent female mate choice. Our behavioural experiments show that drab, immaculata males typically do not court females and instead gain successful copulations exclusively through a ‘sneaker’ tactic (Fig. 2). In contrast, the largest morph (parae) obtained a greater number of successful copulations after initiating courtships rather than by sneak copulation attempts. Furthermore, additional experiments indicate that parae males are typically dominant over the other morphs during male–male competition (J. L. Hurtado-Gonzales & J. A. C. Uy, unpublished data). This morph may therefore specialize in monopolizing access to females through courtship displays and by outcompeting other males through agonistic behaviours. The colorful morphs (blue, red and yellow) use a combination of sneaker and courtship tactics (e.g. mixed mating strategy). However, blue males gained more successful copulations after initiated courtships than through sneak attempts. Red males generally performed fewer courtships before obtaining copulations, suggesting that red males may simply rely on their body coloration instead of elaborate courtship displays in attracting females. Finally, yellow males were more successful in obtaining matings when they used the sneaker tactic than were the blue and red morphs (Fig. 2b). In summary, results from these behavioural experiments suggest that the three colorful morphs used a strategy that was intermediate to the mating strategy used by the smallest immaculata males (i.e. sneaker tactic) and by the largest parae males (i.e. courtship tactic). Behavioural differences in AMSs based on courtship and sneaker behaviours have also been reported in males of the pygmy swordtail, *Xiphophorus nigrensis* (Zimmerer & Kallman 1989) and northern mountain swordtail, *Xiphophorus nezahualcoyotl* (Morris et al. 2008). In these swordtails, larger males exclusively perform elaborate courtships. Smaller males, in contrast, either initiate courtship displays in the absence of large males or sneak copulation attempts when large males are present (Zimmerer & Kallman 1989).

If, indeed, the use of these different behavioural strategies is adaptive in *P. parae*, the strategies should be accompanied by morphological traits that enhance their success. For instance, because immaculata males are primarily defined as ‘sneakers’, they should face a greater risk of sperm competition and thus evolve traits that will enhance their competitive abilities during sperm competition (Parker 1990). We found evidence supporting this prediction, with the immaculata morph having larger relative testes and producing larger ejaculates and sperm with longer flagella (Fig. 5, Table 3). It is expected that investing in larger ejaculates will result in more sperm successfully fertilizing female eggs during sperm competition (Gage et al. 2004). In addition, sperm with longer flagella may have greater mobility and swim faster, enhancing their ability to gain access to unfertilized eggs (e.g. Katz & Drobnis 1990). Colorful and attractive males, in contrast, are expected to face lower risks of sperm competition and should thus invest in traits or behaviours that maximize their ability to acquire matings. Indeed, we found that colorful males frequently initiated courtship displays to attract females; however, we also found differences in testes investment between colour morphs, with yellow males investing more than the red and blue morphs. In fact, we found a trend suggesting that yellow males are more successful than the other colour morphs during sneak copulation attempts. Together, these observations suggest additional complexity to the possible AMSs used by the colorful morphs.

The link between the colour polymorphism and AMSs suggests a mechanism for the persistence of the five morphs and the high frequency of drab immaculata males, which is the least preferred morph by females (Bourne et al. 2003; J. L. Hurtado-Gonzales, D. Baldassare & J. A. C. Uy, unpublished data). Because immaculata males mostly used the 'sneaker' mating strategy and invested more in spermatogenetic tissue, our results suggest that their high frequency in the wild may be due to advantages during postmating sexual selection (e.g. sperm competition or cryptic female choice). That is, immaculata males may be circumventing female choice through sneak copulations, and/or transfer more and potentially faster sperm. Because of this, immaculata males may have a higher reproductive success than the colorful and larger male morphs despite being at a disadvantage in precopulatory sexual selection. In contrast, colorful males may persist in the population by exploiting both precopulatory female choice and sneak copulation attempts but are kept at lower frequencies because of their disadvantage in postmating sexual selection.

An alternative (although not mutually exclusive) possibility is that the high frequency of immaculata males and the rarity of attractive males may be due to

differential predation risks between morphs. Immaculata males may be less conspicuous and thus less susceptible to predation than their colorful and larger counterparts.
For instance, in guppies, female mate choice favors conspicuous coloration (larger and brighter orange or red spots), while predation disfavors these spots (Endler 1983). Red and yellow males, on the other hand, may suffer high levels of predation but still may be maintained in the population because of female preference for rare morphs (e.g. Hughes et al. 1999; Eakley & Houde 2004).

A final alternative for the persistence of the colour polymorphism in *P. parae* may be that females change their preferences depending on the presence of predators (e.g. Houde 1997) or varying visual environments, which results in variation in female mating preferences and resulting selection for variable males (see Henson & Warner 1997; but see Morris et al. 2003; Rios-Cardenas et al. 2007). For instance, it is possible that the effectiveness of male courtship displays is shaped by the visual environment, which changes with the time of day and where courtships occur (Endler 1993). In this scenario, parae and blue morphs may be more conspicuous in open areas (e.g. increased levels of blue light), whereas red and yellow males may be more conspicuous in shaded areas (e.g. increased levels of long wavelength of light). Likewise, if colorful males are more conspicuous to predators than are immaculata males, then females may prefer to mate with drab males in habitats where predators are abundant (e.g. Endler & Houde 1995).

Although we cannot definitively provide evidence to explain the persistence of the five colour morphs, our results showing that the genetically based polymorphism in *P. parae* is linked to alternative mating strategies are consistent with the hypothesis that pre- and postcopulatory sexual selection favoring different mating strategies can mediate the persistence of colour polymorphism in the wild. We are currently testing whether sneaker males indeed have postmating advantages and/or are least susceptible to predators, and whether these mechanisms can help explain their consistently high frequency in the wild.

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TABLES

Table 1.

Total lengths of male morphs (MTL) and females (FTL) and alternative mating behaviors, of *P. parae*. All measurements are given as mean ± SE. Mating behaviors are expressed in number of behaviors/10min-trial period.

MORPHS	**N**	**MTL (mm)**	**FTL (mm)**	**ATTEMPTS**	**SNEAKS**	**COURTHSIP**
Immaculata	12	20.39 ± 0.11	29.84 ± 0.78	5.59 ± 1.17	0.82 ± 0.14	0.74 ± 0.40
Parae	12	26.17 ± 0.31	35.67 ± 1.54	5.17 ± 0.48	0.08 ± 0.04	4.81 ± 1.13
Blue	12	22.64 ± 0.22	36.85 ± 1.43	2.19 ± 0.78	0.22 ± 0.09	5.17 ± 0.56
Red	13	23.13 ± 0.38	30.46 ± 2.26	2.11 ± 0.55	0.22 ± 0.07	1.51 ± 0.51
Yellow	11	24.56 ± 0.19	32.17 ± 0.66	3.73 ± 0.91	0.35 ± 0.13	4.65 ± 0.60

Table 2. Results from the ANCOVA model (full model and reduced model) of log testes mass on the pentamorphic *P. parae*, using log soma mass as the covariate. The significance level was set to 0.01

	FULL MODEL				**REDUCED MODEL**			
SOURCE	**df**	**Mean SS**	***F***	***p***	**df**	**Mean SS**	***F***	***p***
Model	9	0.37	12.07	<0.001	5	0.64	21.24	<0.001
Morph type	4	0.02	0.81	0.52	4	0.44	14.69	<0.001
Log soma mass	1	2.27	75.06	<0.001	1	2.46	81.77	<0.001
Morph type * Log soma mass	4	0.02	0.73	0.58				
Error	278	0.03			282	0.03		

Table 3. The mean ± SE of spermatozoon components measurements of *P. parae* across morph types ($n = 85$). Measurement units are expressed in micrometers (µm). P values in MANOVA were calculated with 4, 80 degrees of freedom

	MORPH TYPES					**MANOVA**	
SPERM COMPONENTS	***Immaculata***	***Parae***	**Blue**	**Red**	**Yellow**	$F_{4,80}$	P
Head length	7.08 ± 0.09	7.06 ± 0.08	7.21 ± 0.08	7.21 ± 0.10	6.97 ± 0.09	1.33	0.267
Midpiece	5.75 ± 0.18	6.51 ± 0.16	6.76 ± 0.16	6.57 ± 0.20	6.84 ± 0.19	6.12	<0.01
Flagellum length	55.36 ± 0.69	51.33 ± 0.64	51.97 ± 0.64	52.06 ± 0.79	54.93 ± 0.73	7.40	<0.01
Total sperm length	68.19 ± 0.68	64.89 ± 0.63	65.94 ± 0.63	65.83 ± 0.78	68.74 ± 0.72	5.93	<0.01

FIGURE CAPTIONS

Figure 1. Female and the five male morphs of *Poecilia parae*.

Figure 2. Proportion of (a) elaborated courtships and sneak attempts, and (b) copulations as a result of courtships and sneak attempts. Data based on individual 10-minute trials of observations for the five *P. parae* color morphs. Lower case or upper case letters above the bars indicate significant differences between morphs ($P < 0.05$) as a result of Tukey's multiple comparisons within each mating behavior and within each form of mating success. Note that none of the courtships resulted in successful copulations for immaculata males.

Figure 3**.** Mean (± standard error) body length of males of the pentamorphic *P. parae* from the west and east populations. Samples sizes are shown within each bar. Different letters denote significant differences between morphs within and between populations. *$P < 0.05$).

Figure 4**.** Mean (± SE) testes mass of immaculata ($N = 80$); parae: ($N = 79$); blue: ($N = 58$); red ($N = 26$); yellow ($N = 45$) *P. parae* males.

Figure 5. Log testes mass regressed on log soma mass for immaculata males [$n=80$; $\log(\text{testes mass})=0.11*\log(\text{soma mass})-1.86$] compared with (a) parae morph [$n=79$; $\log(\text{testes mass})=-0.61*\log(\text{soma mass})-1.68$]; (b) blue [$n=58$; log(testes mass)=-

0.12*log(soma mass)–2.27]; (c) red [n=26; log(testes mass)=-0.37*log(soma mass)-1.77]; and (d) yellow [n=45; log(testes mass)=-0.11*log(soma mass)-1.71].

Figure 6. Log of number of sperm stripped at rest regressed on total length for *P. parae* immaculata males ($r = 0.54$, $n = 17$, $P = 0.03$), compared to (a) parae ($r = 0.70$, $n = 20$, $P < 0.01$) and blue ($r = 0.59$, $n = 20$, $P < 0.01$) males and (b) red ($r = 0.72$, $n = 13$, $P < 0.01$); and yellow ($r = 0.83$, $n = 15$, $P < 0.01$) males.

FIGURES

Figure 1

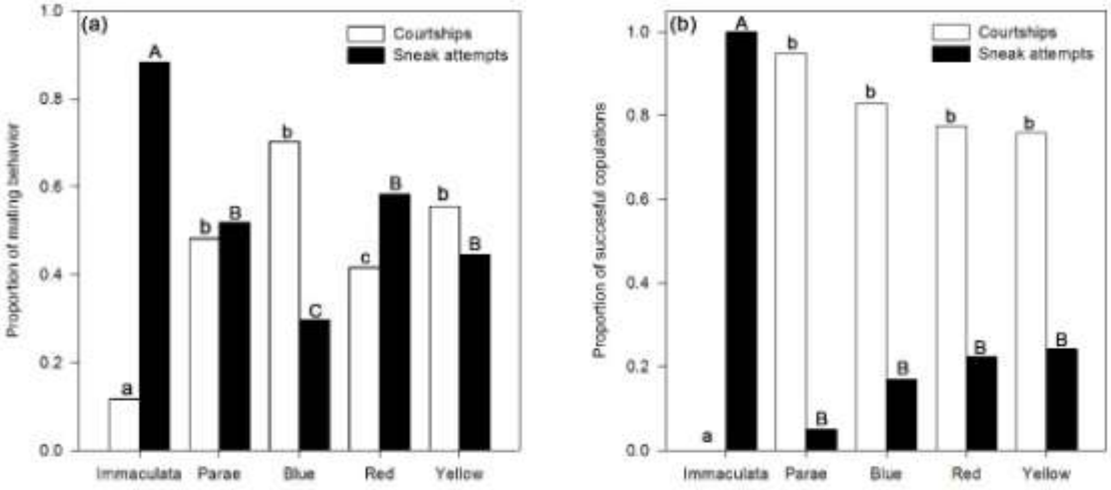

(a)
Courtships
Sneak attempts
Proportion of mating behavior
1.0
0.8
0.6
0.4
0.2
0.0
a
A
b
B
b
C
c
B
b
B
Immaculata
Parae
Blue
Red
Yellow
(b)
Courtships
Sneak attempts
Proportion of successful copulations
1.0
0.8
0.6
0.4
0.2
0.0
a
A
b
B
b
B
b
B
b
B
Immaculata
Parae
Blue
Red
Yellow

Figure 2

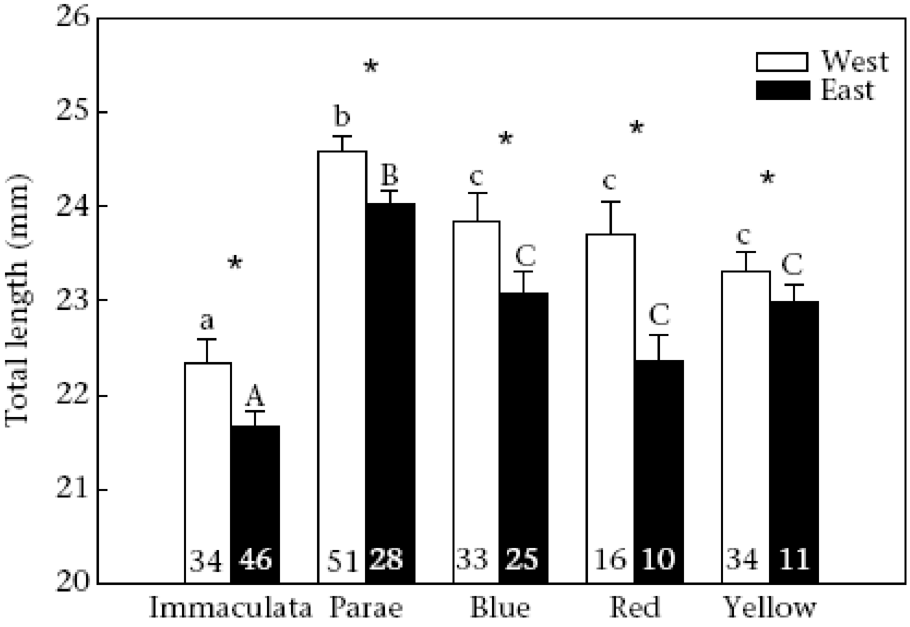
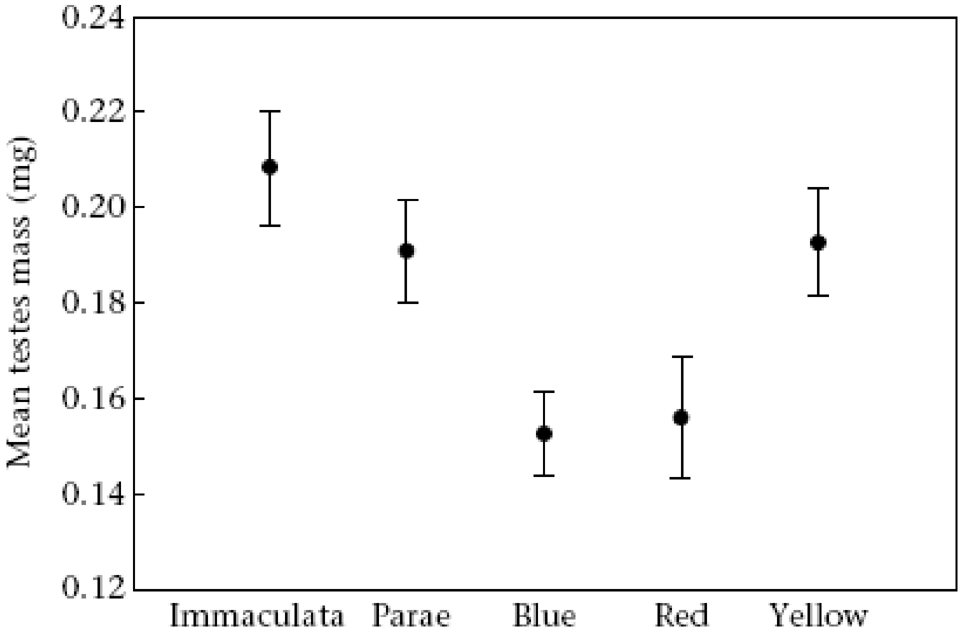
Total length (mm)
26
25
24
23
22
21
20
West
East
Immaculata
Parae
Blue
Red
Yellow
a
A
b
B
c
C
c
C
c
C
*
*
*
*
*
34
46
51
28
33
25
16
10
34
11

Figure 3



Mean testes mass (mg)
0.24
0.22
0.20
0.18
0.16
0.14
0.12
Immaculata
Parae
Blue
Red
Yellow

Figure 4

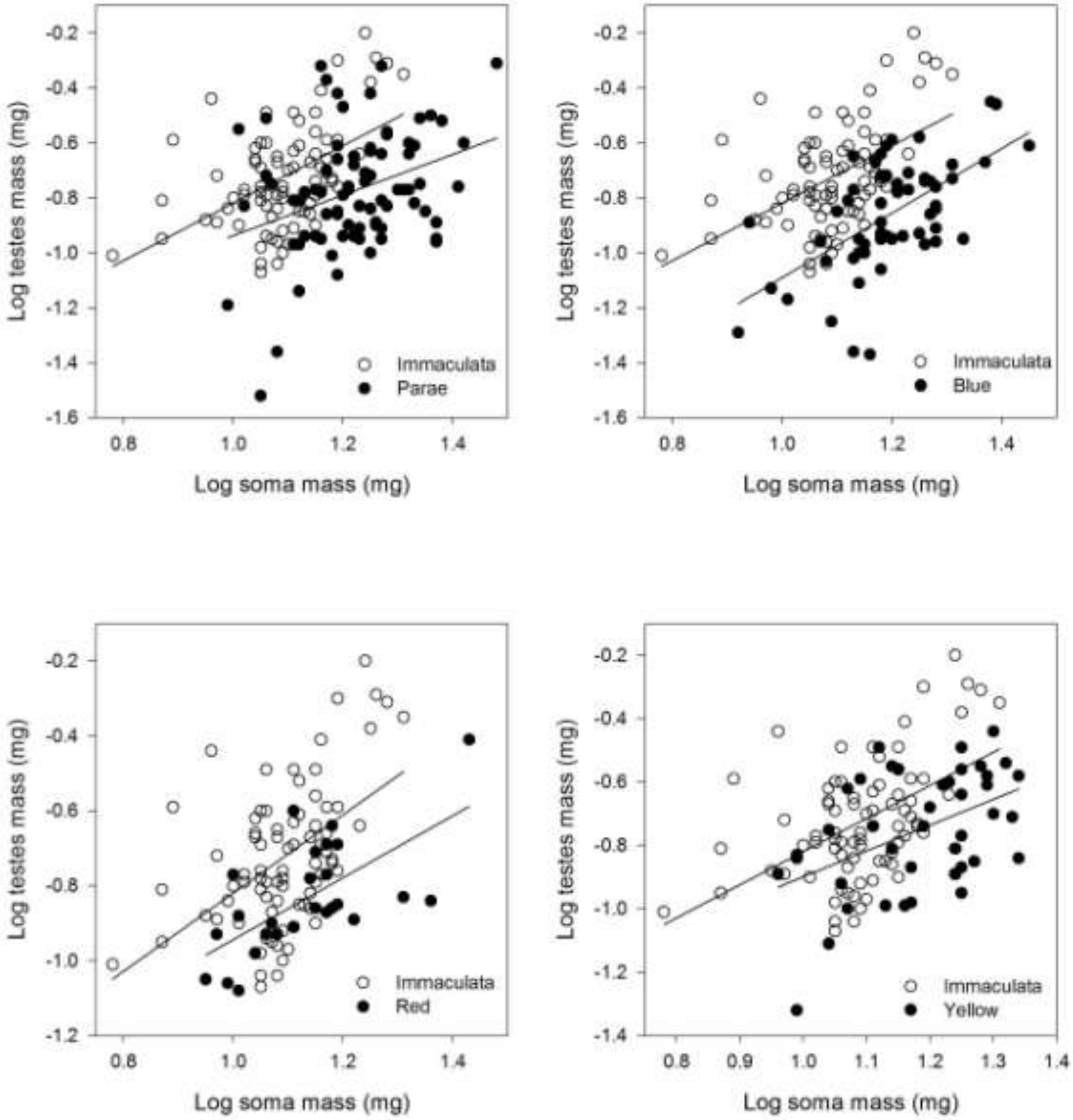
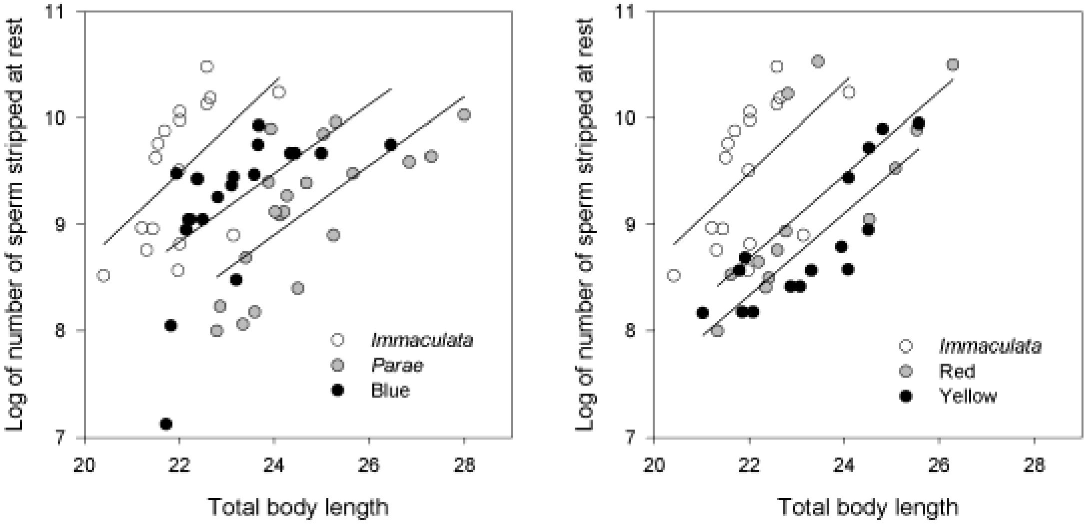

Log testes mass (mg)
Log soma mass (mg)
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
0.8
0.9
1.0
1.1
1.2
1.3
1.4
○ Immaculata
● Parae
○ Immaculata
● Blue
○ Immaculata
● Red
○ Immaculata
● Yellow

Figure 5



Log of number of sperm stripped at rest
11
10
9
8
7
20
22
24
26
28
Total body length
Immaculata
Parae
Blue
Log of number of sperm stripped at rest
11
10
9
8
7
20
22
24
26
28
Total body length
Immaculata
Red
Yellow

Figure 6

CHAPTER III

INTERACTION BETWEEN FEMALE MATING PREFERENCES AND PREDATION MAY EXPLAIN THE MAINTENANCE OF RARE MALES IN THE PENTAMORPHIC FISH *POECILIA PARAE*

Hurtado-Gonzales, J. L., Baldassarre D. T., and Uy, J. Albert. C. 2010. Interaction between female mating preferences and predation may explain the maintenance of rare males in the pentamorphic fish *Poecilia parae. Journal of Evolutionary Biology* 23, 1293-1301

ABSTRACT

Variation in mating preferences coupled with selective predation may allow for the maintenance of alternative mating strategies. Males of the South American live-bearing fish *Poecilia parae* fall in one of five discrete morphs: red, yellow, blue, stripe-colored tail (parae) and female mimic (immaculata). Field surveys indicate that the red and yellow morphs are the rarest, and that their rarity is consistent across years. We explored the role of variable female mating preference and selective predation by visual predators in explaining the rarity of red and yellow males, and more generally, the maintenance of this extreme color polymorphism. We presented wild-caught *P. parae* females and *Aequidens tetramerus*, the most common cichlid predator, with the five male color morphs in separate trials to determine mating and prey preferences, respectively. We found that a large proportion of females shared a strong preference for the rare carotenoid-based red and yellow males, but a distinct group also preferred the blue and parae morphs. The cichlid predator strongly preferred red and yellow males as prey. Together, these results suggest that the interaction between pre-mating sexual selection favoring and predation acting against the red and yellow morphs may explain their rarity in the wild. The trade-off between sexual and natural selection, accompanied by variation in female mating preferences, may therefore facilitate the maintenance of the striking color polymorphism in *P. parae*.

INTRODUCTION

Color polymorphism, genetically-based discrete color patterns expressed by interbreeding individuals within a population, is widespread in nature (Ford, 1965; Oxford & Gillespie, 1996; Roulin, 2004). In theory, color polymorphism can be maintained by different mechanisms such as disruptive selection, mutation-selection balance, heterosis, and balancing selection (Ford, 1965; Levins, 1968; Endler, 1986; Roulin, 2004; Gray & McKinnon, 2007). However, when color polymorphism is sex-limited and linked to sexual function, the evolution and maintenance of such variation may be explained by the interaction between sexual and natural selection (Houde & Endler, 1990; Endler, 1992; Sinervo *et al.*, 2001; Kingston *et al.*, 2003; Seehausen & Schluter, 2004; Pierotti *et al.*, 2009).

Whenever competition for mates is intense, males could evolve variation in behavioral, morphological, physiological and life history strategies to maximize reproductive success (Gross, 1996; Shuster & Wade, 2003; Oliveira *et al.*, 2008). If this variation is genetically-based, they are referred to as alternative mating strategies (Gross, 1996), and their occurrence has been documented in several species (Zimmerer & Kallman, 1989; Shuster & Wade, 1991; Thompson *et al.*, 1993; Lank *et al.*, 1995; Sinervo & Lively, 1996; Hurtado-Gonzales & Uy, 2009). Frequency-dependent selection is often invoked to explain the stable coexistence of alternative strategies (Gross, 1996), but far less attention has been paid to the interaction between female mating preferences (but see Brooks & Endler, 2001a; Morris *et al.*, 2003; Rios-Cardenas *et al.*, 2007) and risk of predation (Bond & Kamil, 2006; Endler 1986; Merilaita & Lind, 2005).

Females often prefer males with bright and colorful signals because these signals are honest indicators of male quality (Andersson, 1994; Stein & Uy, 2006). However, female mating decisions may favor alternative strategies depending on a number of morph-related or environmental factors (Barlow, 1973; Alonzo & Sinervo, 2001; Brooks & Endler 2001a; Kingston *et al*., 2003; Rios-Cardenas *et al*., 2007). For instance, under high risks of predation, bright and colorful male guppies (*Poecilia reticulata*) are less preferred by females than drab males (Gong & Gibson, 1996; Houde, 1997), suggesting that susceptibility to visual predators offset the advantages of mating with conspicuous males. Such interaction between female mating preferences and susceptibility to predation may in turn allow alternative mating strategies to invade and persist within populations (Bond & Kamil, 2006; Gamble *et al*., 2003).

Males of the live-bearing fish *Poecilia parae* display one of the most prodigious examples of intraspecific color polymorphisms (Liley, 1966; Bourne *et al*., 2003; Lindholm *et al*., 2004; Hurtado-Gonzales & Uy, 2009). Males in this species occur in five discrete, Y-linked color morphs (Fig. 1 in Hurtado-Gonzales & Uy, 2009): the drab-colored immaculata (i.e., female mimic), the stripe-colored tail parae, the structural-colored blue and the carotenoid-based red and yellow morphs. Each of the five color morphs employs alternative mating strategies that ranges from males exploiting female mate choice to males winning access to females via male-male competition (i.e., red, blue, yellow, and parae) to males mimicking juvenile females and using a sneaker tactic to gain forced copulations (i.e., immaculata) (Bourne *et al*., 2003; Hurtado-Gonzales & Uy, 2009). Previous studies have shown that females of *P. parae* strongly prefer the carotenoid-based red and yellow morphs (Bourne *et al*., 2003; Lindholm *et al*., 2004), leading to the expectation that red and yellow males should be the most abundant males in the population. However, our field surveys from 2006 through 2009 (this study) along with published data from 2002-2005 (Lindholm *et al*., 2004; Watson-Rodney, 2005), indicate that the red and yellow morphs are consistently the least abundant males. With such a precopulatory mating advantage, why are red and yellow males consistently rare?

In this study, we conducted a series of controlled experiments and extensive field surveys to test whether individual variation in female mating preferences and trade-offs between female mating preference and selective predation can help explain the maintenance of the color polymorphism, as well as the persistence of red and yellow males at low frequencies. First, we re-examined female mate choice because previous studies did not control for intrasexual interactions (Bourne *et al*., 2003; Lindholm *et al*., 2004), which can influence the outcome of female choice (Radesater & Halldorsdottir, 1993; Nilsson & Nilsson, 2000; Shackleton *et al*., 2005). We then explored the role of visual predators in keeping attractive males rare by 1) comparing the frequency of the most common predator (*Aequidens tetramerus*: Cichlidae) with the frequency of each color morph from our field surveys, and 2) conducting laboratory experiments to determine if predators preferentially approach and attack certain color morphs.

METHODS

Collection and husbandry

Our collection sites are located on the east (6˚ 47. 2' N, 58˚ 09' W) and west (6˚ 41' N, 58˚ 12' W) sides of the Demerara River, Guyana. Individuals of *Poecilia parae, P. reticulata* and the cichlid predator *Aequidens tetramerus* were collected in 2007 and 2008. Pregnant females of *P. parae* were individually housed to monitor their breeding status, and used for our mate choice experiments within four days after parturition. Cichlid predators were individually housed to reduce stress and aggression among them, while morphs of *P. parae* (immaculata, parae, blue, red and yellow) and males of *P. reticulata* were housed in communal tanks. All fish were maintained at 12L: 12D light cycle, at 28 ± 1˚C, and fed with commercial flake food and live brine shrimp (for poeciliids) or commercial pellets (for cichlids).

Morph frequencies and predator relative abundance

To estimate morph frequencies in the wild, we combined published and unpublished surveys of morph abundances from the east and west populations as follows: data from counts done by Lindholm *et al*. (2004) for east and west populations (January-February 2002), Watson-Rodney (2005) for west population (May-August 2003-2004), and data collected by JLHG (this study) for east and west populations (January-March, May-August, December 2006-2009). We sampled transects in the east (n = 15 sites, mean ± SD: 25.8 ± 2.18 m length, 0.76 ± 0.073 m width, 0.93 ± 0.045 m depth) and west (n = 15 sites, mean ± SD: 29.97 ± 2.97 m length, 0.96 ± 0.058 m width, 0.88 ± 0.056 m depth) populations from 2006 to 2009. These transects were used only for survey purposes. To capture individuals, we blocked segments in each of our sampling transects with fish nets, re-sampling each transect for three consecutive times or until no additional *P.*

parae were caught. All captured individuals were placed in containers with treated water and constant aeration and immediately sorted and counted. Since male morphs of *P. parae* resemble each other during their juvenile stage, we only used mature males that displayed fully developed coloration (i.e., for parae, blue, red, and yellow morphs) and/or with total body length of $\geq$ 18 mm (for immaculata). At this body length, all colored morphs express adult characteristics (Watson-Rodney, 2005; Hurtado-Gonzales & Uy, 2009).

The relative abundances of aquatic predators were quantified using transects conducted during the summer of 2007 in the east and west populations. We only recorded the presence of *Rivulus stagnalis* (Rivulidae), *Apistogramma steindachneri, A. tetramerus* (Cichlidae), and *Hoplias malabaricus* (Erythrinidae). With the exception of *R. stagnalis* and *A. tetramerus*, the two other predators were only caught occasionally. *Rivulus stagnalis* feed mostly on fry, while *A. tetramerus* feed on adult poeciliids (Watson-Rodney, 2005). We, therefore, only tested for an association between the relative abundances of *A. tetramerus* and the five color morphs.

General experimental protocol

Experimental tank (Fig. 1) dimensions were 50 x 45 x 28 cm (L x W x H) filled to a depth of 14 cm with water for female choice experiments (but up to 20 cm for the predator experiments) and divided across its width by a glass partition that split the aquarium into two sections. The shorter section contained six compartments of approximately 8 x 7 x 20 cm each. The sides of these compartments were blocked with an opaque non-colored thin mesh, to prevent adjacent fish from establishing visual contact. Tanks were supplied with a thin layer of gravel, illuminated with full spectrum light (70 cm above the tank), and were covered on four sides with brown, kraft paper. Before trials, stimuli individuals selected from the stock populations were anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester), and photographed using a digital camera. As a metric reference, we include a 100 mm ruler in each photo. The total length (tip of the upper jaw to tip of caudal fin) for each individual was obtained from the digital images using Sigmascan Pro®v5.0.0 (San Jose, CA, USA). With the exception of test predators that were deprived of food for 24 h prior to testing, test females and stimuli in both the female choice and predator-prey preference experiments were fed to satiation before experiments started. Immediately after, stimuli individuals were randomly assigned to one of the six compartments by a third party who did not participate in scoring the trials. To prevent visual contact between the test and stimuli individuals during acclimation time (i.e., 20 min period), a removable barrier was fitted, which was then lifted at the start of each trial. Trials were run for 20 min, and were continuously recorded with a Samsung camcorder (SCL-810 Hi8) on a tripod positioned about 50 cm above the tank.

From the video tapes, we scored active association time in seconds (females facing morphs, and moving left and right smoothly and repeatedly) as an index of female mating preferences. We excluded periods of time in which females remained stationary facing a male compartment. Laboratory experiments in other fish species have confirmed that this index is an effective assay of female mating preferences (Godin & Dugatkin, 1996; Kodric-Brown & Nicoletto, 1996; Houde, 1997; Kingston *et al.*, 2003; Morris *et al.*, 2003; Cummings & Mollaghan, 2006). For predators, time spent near the stimulus (sec) was used as a measure of predator discrimination or preference for a particular prey type. We recorded the time in which *A. tetramerus*, once habituated to the artificial conditions of the tank, moved slowly to approach and inspect a male and then bump softly into the glass to attack. Several studies in poeciliids and other fish taxa confirm that this type of experimental approach effectively measures prey preferences by visual predators (Rosenthal *et al.*, 2001; Godin & McDonough, 2003).

Experiment 1: female mate choice

Female mate choice trials were run in 2007 (east: n = 32 females; mean ± SE: 29.11 ± 0.7mm total length, west: n = 18; 28.83 ± 0.65 mm) and 2008 (east: n = 34; 29.91 ± 0.21 mm, west: n = 32; 29.13 ± 0.11 mm). Since females may preferentially associate with other fish for schooling (Liley, 1966; Houde, 1997), we included a female as a part of the experimental stimuli to control for schooling behavior. Thus, the stimuli consisted of the five male morphs (immaculata, parae, blue, red, and yellow) and a female. With the exception of immaculata males, which are usually 15% smaller than the other morphs (Hurtado-Gonzales & Uy, 2009), males used in female preference trials were similar in total body length (i.e., within 7 %). To estimate repeatability of female preferences, trials were repeated after 24 hours using the same set of males and females (see statistical analyses for further details). Repeatability (Lessells & Boag, 1987; Boake, 1989) quantifies the proportion of phenotypic variation among individuals, which can also be an indication of the upper limit of heritable variation for female preference (Bakker & Pomiankowski, 1995; Brooks & Endler, 2001b).

Experiment 2: predator prey preference

Experiments to assay predator preference for *P. parae* morphs were run in 2007 (east: n = 16 cichlids; mean ± SE: 164.1 ± 3.38 mm total length, west: n = 16; 138.3 ± 2.86 mm) and 2008 (east: n = 32; 164.7 ± 2.08 mm, west: n = 32; 150.7 ± 2.66 mm). Each predator test included the five male morphs (immaculata, parae, blue, red and yellow) and a male of a sympatric prey species, the guppy *P. reticulata*. Guppies are the most common prey in the wild, and so predators may have formed a search image for this prey. The inclusion of guppies therefore represents a control for the most common prey model for the cichlid predator. We did not estimate repeatability for predator preferences. Thus, each predator was tested only once.

Statistical Analyses

Total association time (sec) that each female or predator spent during each trial with a potential mate or prey was converted to proportions (Fig. S1 and S2). Since the analysis of proportions is subject to a constant-sum constraint (i.e., equal to 1 or 100%), the nature of the data is non-independent (Aitchison, 1986). Thus, any attempt in analyzing proportions using standard parametric or nonparametric multivariate statistical approaches can lead to erroneous conclusions (Jackson, 1997). Hence, we analyzed our data using compositional log-ratio techniques, following the methods of compositional data analysis (Aitchison, 1986; Buccianti *et al.*, 2006; Pierotti *et al.*, 2009).

We used the package CoDaPack (http://ima.udg.edu/Recerca/EIO/inici_cat.html), which runs as an Excel ® add-Ins. CoDaPack implements the basic methods of compositional data based on log-ratios (Thió-Henestrosa & Martín-Fernández, 2006). In brief, zero values in our data (i.e., no interactions between test subject and stimuli) were replaced by using the "random zero replacement" command in CoDaPack. This is an important step since zero values would preclude the log-ratio transformations. Random zero replacement value can be assigned by default or manually. We set the value as 0.003, which did not affect the total compositional values expressed by either females or predators. Second, we transformed our data to the isometric-log-ratios (ilr) (Egozcue *et al*., 2003) to represent a compositional value as a real vector. As a result, the transformed data is said to fluctuate (i.e., the closure of the data is opened) (Buccianti *et al*., 2006). We determined whether there was any statistical difference for individual female preferences for potential mates depending on population (east and west) and year (2007 and 2008) by running a multivariate analysis of variance (MANOVA). Population and year did not factor in the model (both main effect and interaction; all $P > 0.191$), and so we pooled all female preference data (n = 116 females). We estimated repeatabilities of female preference (Lessells & Boag, 1987; Boake, 1989) for all the females tested and considering the groups defined by the cluster analysis. For this purpose, we used the equation developed by Pierotti *et al*. (2009) to calculate repeatability index in multivariate data [detail explanation for the applicability of modified estimator of repeatability index see pg. 2387-2388, appendix B, Pierotti *et al*. (2009)].

To determine if predators differ in their preference for a potential prey, we run an analysis of variance (ANOVA). We used population (east and west) and years (2007-2008) as factors, and the ilr-transformed predator preference values as the dependent variable. Again, population and year did not factor in the model (all $P > 0.182$), and so we pooled all the predator preference data (n = 96). Our aim was to test for differences in the geometric means of preferences expressed by females and predators for potential mates and prey, respectively. Due to the nature of the ilr transformations, it is difficult to infer the direction of the preference (i.e., which males are the most preferred). However, if the tests are non-significant, it may represent a general consensus for predators to display the same preferences as females do as mating preferences. Therefore, a cluster analysis is a robust way to explore the possibility that females and predators share similar preferences for particular morphs. The ilr-transformed data of individual females and predators were clustered using mahalanobis distance (Aitchison, 1986; Pierotti *et al.*, 2009) following the Ward clustering algorithm (Ward, 1963). We obtained two clusters representing female mating preferences and predator prey preferences. Cluster validation indices are presented in Table 1S (supporting electronic materials).

The distribution of vectors of female preferences, according to the cluster groups, was graphed with package "compositions" (http://www.stat.boogaart.de/compositions) (van den Boogaart & Tolosana-Delgado, 2008) within the statistical package "R" (http://www.cran.R-project.org, R development Core Team Ver. 2.92 as in 2009). With "compositions" we were able to visualize tables of multiple ternary diagrams, in which compositional geometric mean vectors were plotted (Fig. S3 and S4). The interpretation of these diagrams is as follows. Two components (i.e., the two base angles) in any particular plot correspond to a pairwise combination of the five morphs reflecting female mating or predator prey preferences towards each color morph (I = immaculata, P = parae, B = blue, R = red, and Y = yellow). The vertex angle represents the geometric means of the remaining components (i.e., the other morphs). The location of the mean, if biased towards either base angle, represents the level of preference. For all other statistical analyses, unless indicated, we used the software SYSTAT 12 for Windows®.

RESULTS

Color morph and predator relative abundances

The sex ratio (female: male) of *P. parae* was nearly 1:1 for the east (1.07: 0.93) and west (1.03: 0.97) populations. Surveys across years (Fig. 2) indicate that the immaculata and parae morphs are consistently the most abundant color morphs, with the blue males typically being the third most abundant morph. The red and yellow are the least abundant morphs within and between populations, with the red morph being the rarest in the west population and the yellow in the east population (Fig. 2).

The relative abundance of the most common cichlid predator *A. tetramerus* was 2.74 ± 0.73 individuals per sampling transect. The relative abundance of *A. tetramerus* negatively correlated with the relative abundance of the red morph (r = - 0.55, *P* = 0.03). A similar negative trend was seen between *A. tetramerus* and yellow morph abundances (r = - 0.48, *P* = 0.06). No significant correlations was found between the abundances of *A. tetramerus* with that of females (r = - 0.15, *P* = 0.58), immaculata (r = - 0.27, *P* = 0.33), parae (r = - 0.13, *P* = 0.63) and blue (r = - 0.24, *P* = 0.37) morphs.

Female mate choice

Female association time with each morph was highly variable, ranging from 4 to 506 sec. In all the behavioral trials, females spent more time with each of the five males than with control females (geometric mean = 6.9 % of total time), indicating that females associated with males for reasons other than schooling. The MANOVA indicated that females vary in their preferences for potential mates (Wilks λ= 0.757, $F_{8, 1148}$ = 21.462, $P < 0.001$). The homogeneity of the log-ratio variances and covariances was also statistically significant, indicating differences among groups in female mating preferences (Box's M test = 234.74, $F_{12, 2450515}$ = 19.384, $P < 0.001$). The resulting cluster of individual female mating preferences identified three major groups (Fig 3A): females with a preference for (1) both carotenoid red and yellow males, (2) red males only, and (3) parae, blue, red, and yellow males (Fig. 3A). Our analysis detected a weak preference for females to associate with immaculata males and control females. Repeatability of female mating preferences was moderately high for all the clustering groups (group 1 = 0.36, group 2 = 0.29, and group 3 = 0.21).

Predator prey preference

The time that the cichlid predator *A. tetramerus* spent with specific *P. parae* morphs ranged from 2 to 336 sec. We found that the amount of time *A. tetramerus* spent associated with the different *P. parae* morphs and a congener, the guppy (*P. reticulata*), differed significantly (ANOVA: $F_{5, 475}$= 27.70, $P < 0.001$). The resulting cluster of predator prey preferences identified three major groups (Fig 3B): predator preference for (1) the yellow and guppy males, (2) red males only, and (3) red and yellow males. In general, the cichlid predators preferred fish with carotenoid-based coloration.

DISCUSSION

Our experiments demonstrate variation among *Poecilia parae* females in their mating preferences for each color morph. The compositional clustering analysis indicated that a large group of females preferred the rare carotenoid-based red and yellow males, but another group shared preferences for blue and parae males. These distinct female mating preferences were also highly repeatable, indicating consistency in pre-mating behavior of individual females. These results indicate that variable female mating preferences and the consistent variation among females in the strength of their preferences may partially explain the persistence of the four colorful male morphs (red, yellow, blue, and parae). However, the compositional analysis detected only a weak female mating preference for the drab immaculata males (Supporting Information Material, Fig. S3), suggesting that mating preference does not keep this morph type in high frequency. Alternatively, our previous work indicates that immaculata males have larger relative testes and primarily use a sneaker mating strategy (Hurtado-Gonzales & Uy, 2009). That is, the immaculata morph foregoes courtship and attempts force copulations with females, suggesting that their high frequency in the population may be a result of their effectiveness in post-copulatory sexual selection. Together, these results suggest that variable female mating preferences and alternative mating strategies by males may allow for the persistence of the five *P. parae* color morphs.

Although females varied in their mating preferences, the majority preferred red and yellow males, and this is consistent with previous studies (Bourne *et al*., 2003; Lindholm *et al*., 2004). If increased mating success leads to increased reproductive success, then red and yellow males should be consistently the most abundant morphs in the wild. However, based on our and previously published surveys, rarity of red and yellow males has been consistent and stable since the 1960's (Liley, 1966; Lindholm et al., 2004; Hurtado-Gonzales & Uy, 2009). One possibility is that red and yellow males persist in low frequencies because the strong female mating preference for these males is offset by elevated risks to visual predators (e.g., guppies; Endler, 1980). Indeed, our field surveys indicate a negative association between the abundances of the most common cichlid predator *A. tetramerus* and the abundances of the red and yellow color morphs. Predator abundance, however, does not predict the frequency of immaculata, parae, and blue morphs. More directly, our predator experiments showed that *A. tetramerus* preferentially approached red and yellow *P. parae* males. The compositional cluster analysis further identified a group with a preference for the common co-occuring prey, the guppy *P. reticulata* (Fig S4). In our study site, *P. parae* is sympatric with three other poeciliids (*P. reticulata*, *P. picta*, and *P. vivipara*). Males of *P. reticulata* and *P. picta* also display carotenoid-based color patches, and *P. reticulata* is the most abundant poeciliid. Therefore, cichlid predators may prefer red and yellow males because predators have developed a general search image for carotenoid color patches or share similar sensory biases as the majority of female *P. parae* (Reznick & Endler, 1982; Rosenthal *et al.*, 2001; Godin & McDonough, 2003).

Frequency-dependent selection tends to be the most common mechanism for the maintenance of color polymorphisms (Gray & Mckinnon, 2007); nevertheless, *P. parae* seems to follow different evolutionary dynamics. While a major visual predator may be affecting the relative abundances of red and yellow males, there is a strong female preference for these rare morphs. The positive and negative selective pressures on these males may therefore create a balance favoring the maintenance of red and yellow morphs in natural conditions. In addition, rarity of the most preferred morphs via predation may allow for the invasion and maintenance of alternative mating strategies (e.g., Bond & Kamil, 2006). This tradeoff between natural and sexual selection can then be accompanied by variation in female mating preference to further facilitate the persistence of alternative strategies (see also Rios-Cardenas et al. 2007).

The main result for frequency-dependent selection is that the relative abundances of morphs should fluctuate overtime (e.g., Gray & Mckinnon, 2006). In the case of side-blotched lizard, for instance, there is clear evidence for the fluctuation of the frequency of morphs across years, with a clear advantage for rare phenotypes (e.g., Sinervo & Lively, 1996). In contrast, the abundances of the five morphs of *P. parae* are relatively stable, with no evidence of cycles in relative abundances of all five morphs (Liley, 1966; Lindholm et al., 2004; this study). The same frequency patterns are also found across the geographical distribution of this species (Lindholm *et al*., 2004; Hurtado-Gonzales, unpublished). Hence, frequency dependent selection is unlikely to explain the persistence of the five color morphs in the wild.

Although our study provides evidence that the interaction between variable female mating preferences and selective predation can explain the frequencies of both carotenoid-based morphs (red and yellow) and that of the immaculata males, the persistence of the blue and parae morphs need further explanation. Our results suggest that some females indeed prefer parae and blue males, and this may partially explain their maintenance. The parae morph is typically larger than other males and often more aggressive (Hurtado-Gonzales & Uy, 2009; Hurtado-Gonzales & Uy, unpublished). It is, therefore, possible that parae males primarily rely on overt male-male aggression in monopolizing females and securing matings, resulting in high abundances in the wild. Blue males have unique color patches that reflect in the ultraviolet and short wavelengths. As the ditches, where *P. parae* are found show variation in ambient light (Hurtado-Gonzales & Uy, unpublished), these males may persist in the wild by exploiting microhabitats rich in short wavelength light, thereby increasing their conspicuousness and attractiveness to females. Our ongoing field observations and experiments are exploring these possibilities and should provide a more complete explanation for the maintenance of the striking color polymorphism in *P. parae*.

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FIGURE CAPTIONS

Fig. 1 Experimental tank design

Fig. 2 Frequencies of *P. parae* color morphs for the a) west and b) east populations across years. Total number of individuals sampled for each year is given above the bars [2002, Lindholm et al. (2004); 2004, Watson-Rodney (2005); and 2006-2009, this study)].

Fig. 3 Clustering of geometric means of individual (a) female mating preferences and (b) predator prey preferences using the Ward hierarchical clustering algorithm based on Aitchison distance (log-mahalanobis distance) between the variables. Results are presented in dendograms.

FIGURES

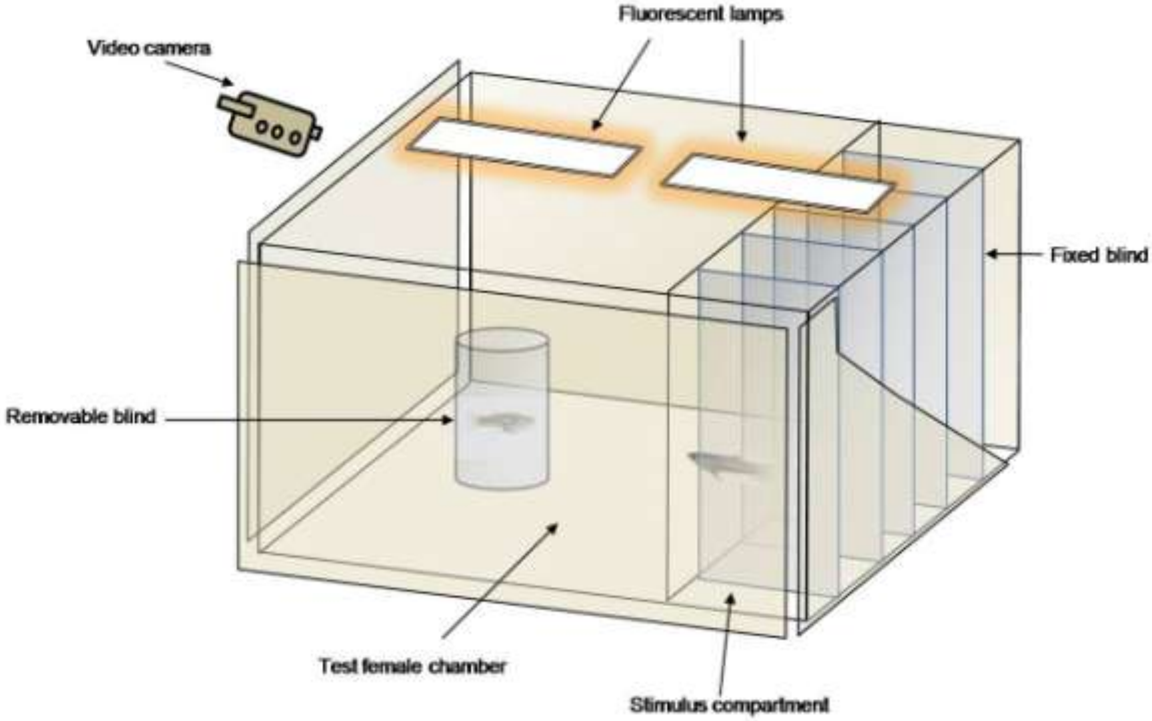
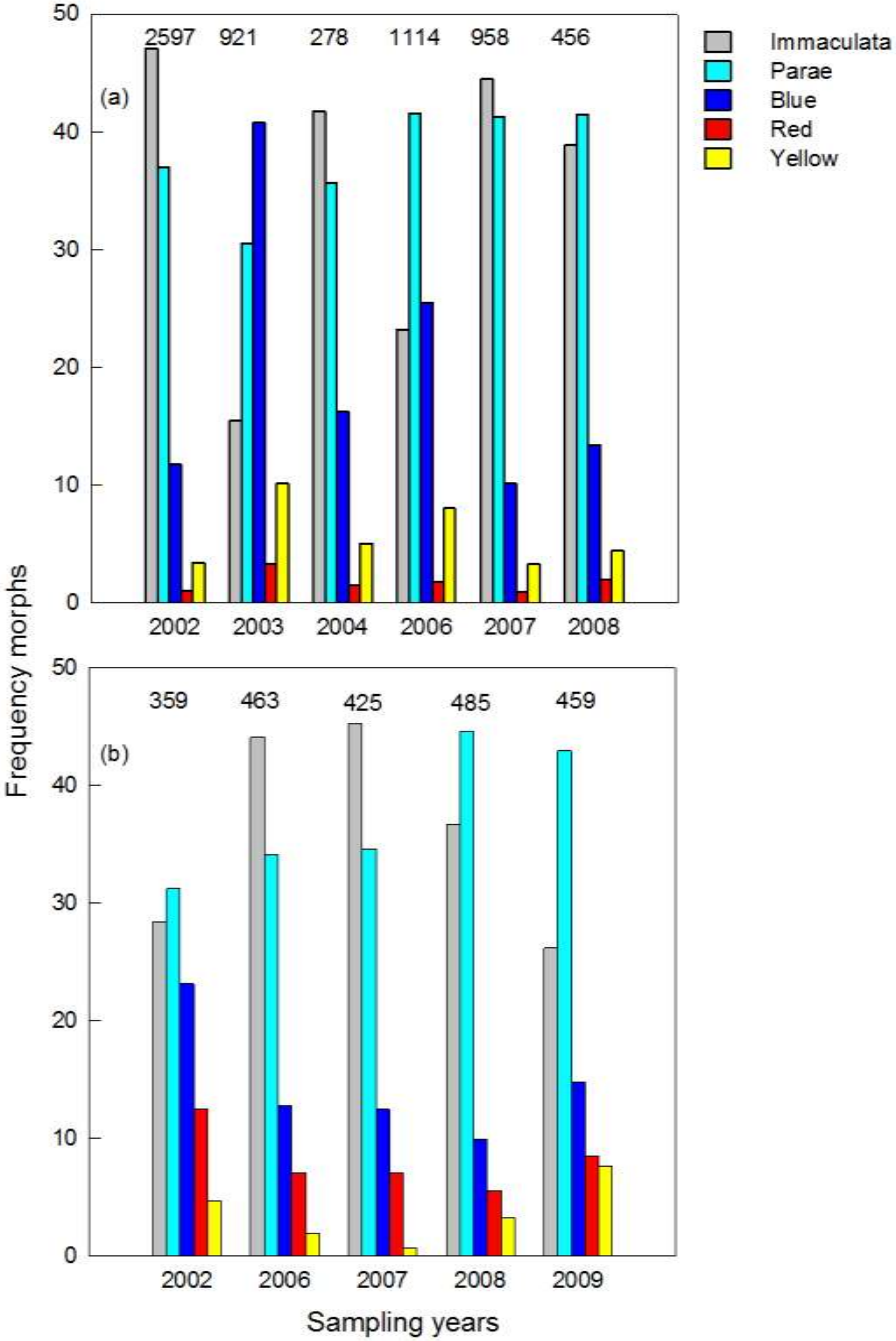


Figure 1



(a)
(b)
2597
921
278
1114
958
456
359
463
425
485
459
Immaculata
Parae
Blue
Red
Yellow
Frequency morphs
Sampling years
0
10
20
30
40
50
2002
2003
2004
2006
2007
2008
2009

Figure 2

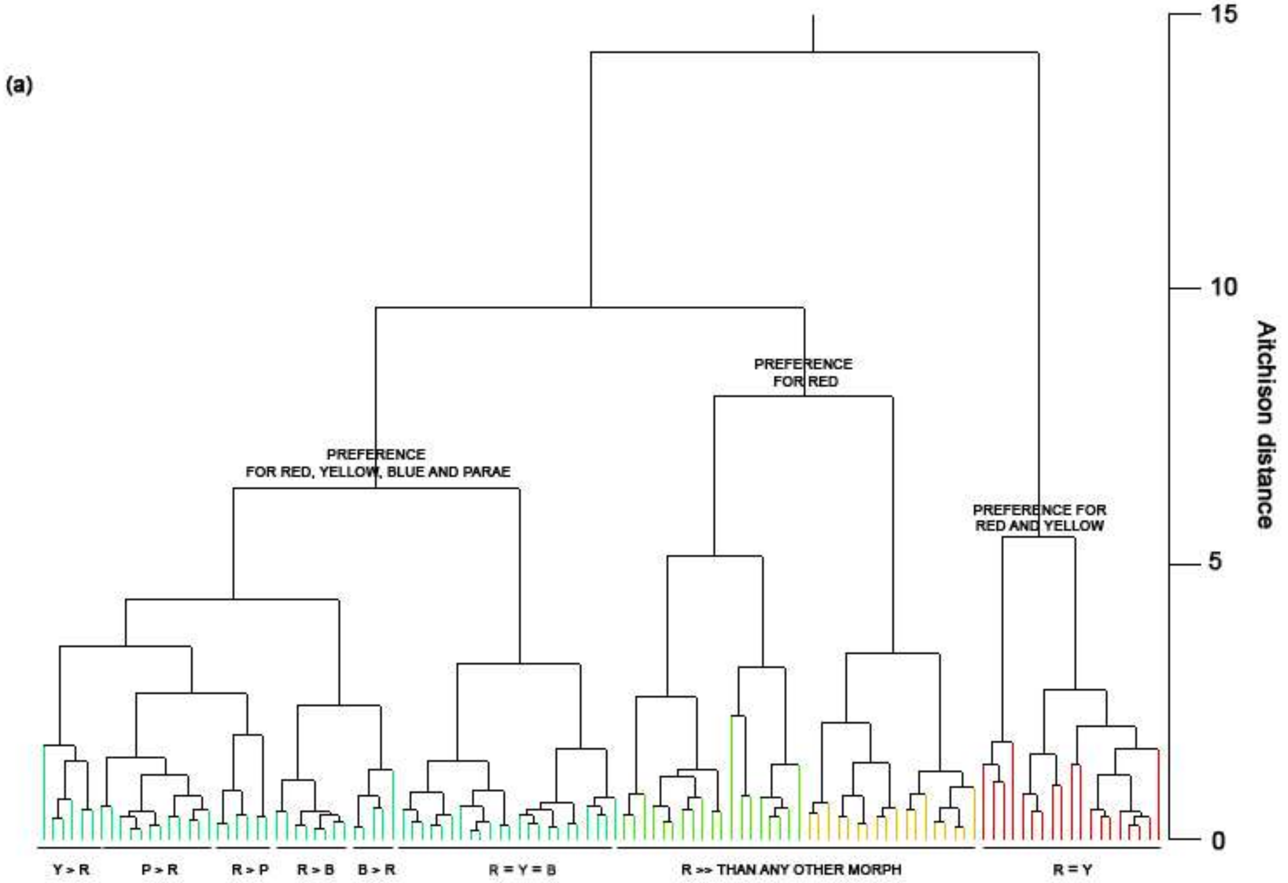

(a)
PREFERENCE FOR RED, YELLOW, BLUE AND PARAE
PREFERENCE FOR RED
PREFERENCE FOR RED AND YELLOW
15
10
5
0
Aitchison distance
Y > R
P > R
R > P
R > B
B > R
R = Y = B
R >> THAN ANY OTHER MORPH
R = Y

Figure 3a

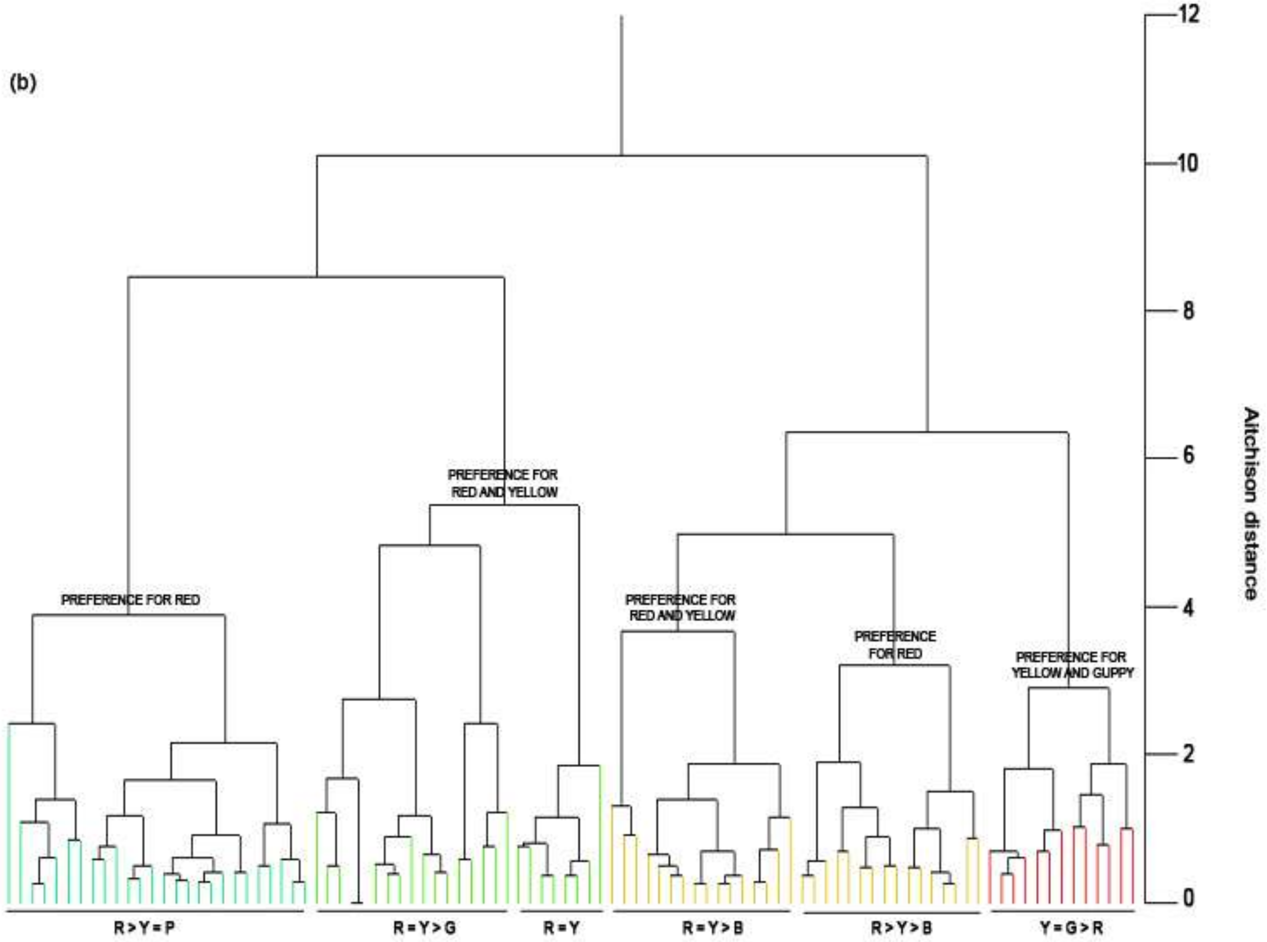

(b)
PREFERENCE FOR RED
PREFERENCE FOR RED AND YELLOW
PREFERENCE FOR RED AND YELLOW
PREFERENCE FOR RED
PREFERENCE FOR YELLOW AND GUPPY
R>Y=P
R=Y>G
R=Y
R=Y>B
R>Y>B
Y=G>R
12
10
8
6
4
2
0
Aitchison distance

Figure 3b

SUPPORTING ELECTRONIC MATERIALS

Table S1. Cluster validity algorithms for female mating and predator preferences. RMSSTD: Root-mean-square standard deviation of new formed cluster, CHF: Calinski-Harabasz method, PTS: Pseudo T-square, DB: Davies-Boldin's Index for each hierarchy. D: Dunn's cluster separation.

		RMSSTD	CHF	PTS	DB	D
Female mating Preferences	4	0.993	18.666	12.865	2.288	0.1
	5	0.891	21.897	14.399	1.824	0.206
Predator prey Preferences	5	0.815	13.511	8.896	1.763	0.136
	6	0.981	13.967	7.481	1.874	0.107

Fig. S1 Box plots showing distribution of raw data for female preferences (proportion of association time) by population and year. Range (whiskers), 25% and 75% percentiles (lower and upper limit of the box), median (black line), mean (red line), and outliers (black cross) are shown.

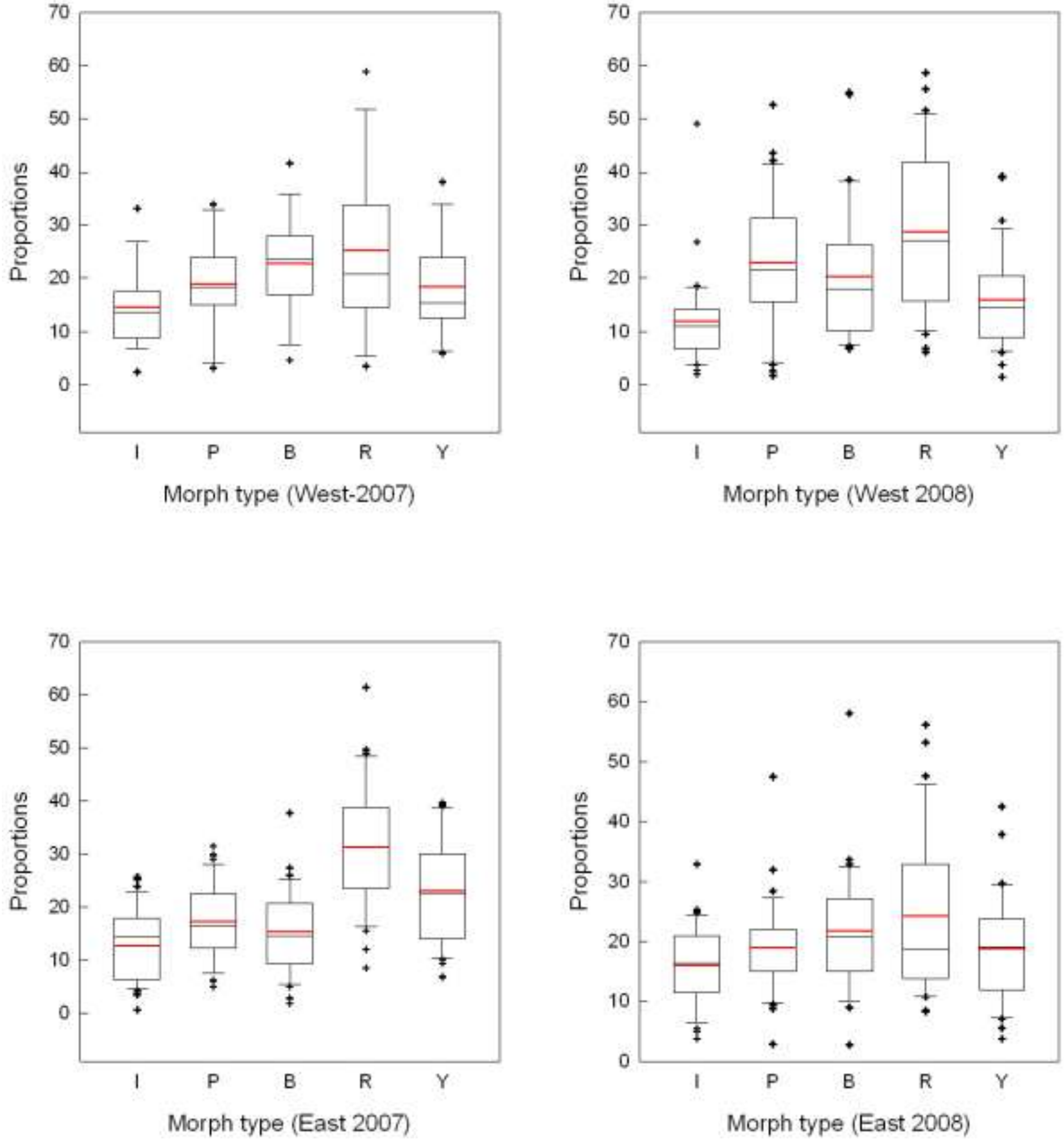
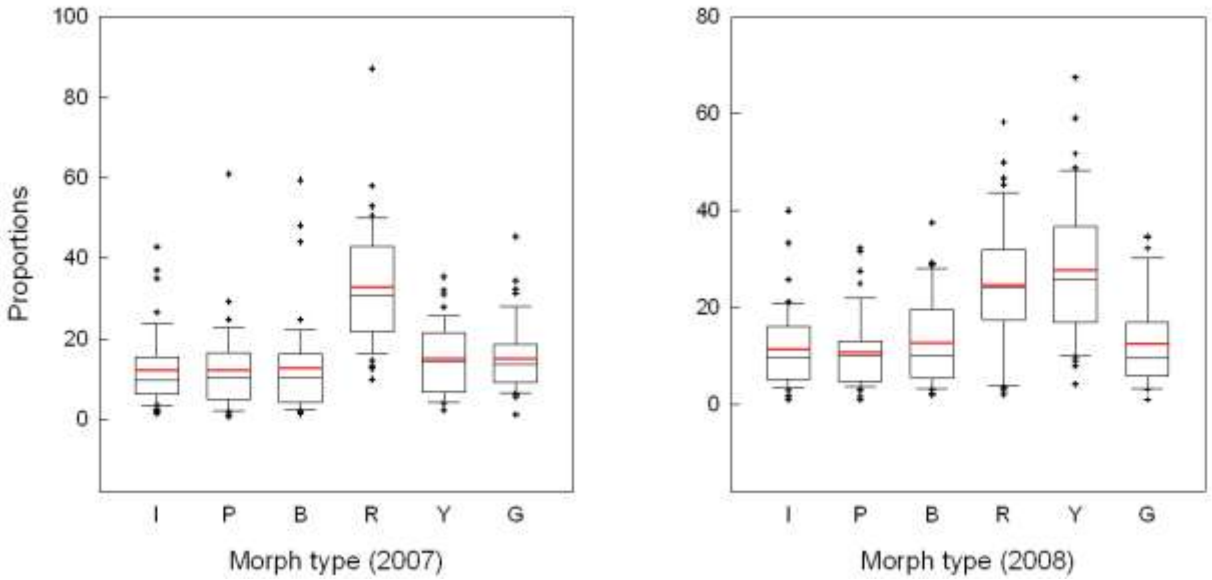


Fig. S2: Box plots showing distribution of raw data for predator preferences (proportion of association time) by population and year. Range (whiskers), 25% and 75% percentiles (lower and upper limit of the box), median (black line), mean (red line), and outliers (black cross) are shown.



Appendix S1. Interpretation of matrices of ternary diagrams.

A ternary diagram is used to identify trends and relationships between groups of samples. The matrices presented in this supplementary section were obtained by using Aitchison geometric means from individuals grouped following the cluster analysis. Hence, the matrices helped to reinforce the grouping criteria as appeared in the dendograms. There is a more precise way to evaluate these relationships and can be found in Aitchison (1986) and Buccianti *et al*., (2006). In here, we offer a way for a quick and basic interpretation based on the location of the geometric means. For this purpose refer to Figure S3a, where we concluded that the mating preferences for Red are equal to that of Yellow (Red = Yellow). Figures are expandable.

A ternary diagram has three apexes. In this section, the top apex of all diagrams are represented by an asterisk (*) containing all others morphs or components of the sample according to the individual female preference. The left apex represents the first morph to be compared with a second morph, whose initials are found on the left and right apexes, respectively. Diagonally, there is the initial of each morph subject to comparison and that divides the diagrams in two segments: upper and lower. The upper and lower segments of the diagram contain the same information. The lower segment presents the information in reversed order. For instance, in the upper part the comparison is between female preferences for I (immaculata) and P (parae), in the lower part it reverses and becomes the comparison between P and I. Both the location of the geometric and confidence regions are reversed but the interpretation is the same. The decisions are based on the location of the geometric mean (black open dot) enclosed by the confidence regions (red lines). In Fig S3a, when I is compared to P and others (*), the diagram suggest a weak preference for both I and P (the mean is greater than 50% for *, 30% favoring I and 20% for P). Then, to inspect I against B (blue), the distribution of the geometric mean is located around * (50%), I (15%), and B (35%). Considering I with R (red) and I with Y (yellow), there is a tendency for the geometric mean to group towards the apexes corresponding to both R (~ 70%) and Y (~ 60%), respectively. In the case of P with B level of preferences are quite similar suggesting * 40%, P 30% and B 30% suggesting again a weak preference when these two morphs are compared with the others. The other possible comparisons are between P and R and P and Y, which still favor the preferences for R and Y. Identical trends are found for the comparisons of preferences for B with R and B with Y. However, the location of the mean between R and Y suggests an equal proportion in the preferences (R 40% and Y 40% and 20% for *). Noticed that in all possible comparisons the level of preferences for I tends to be lower than that the preferences displayed for the other morphs. The same interpretation applies for Fig. S4.

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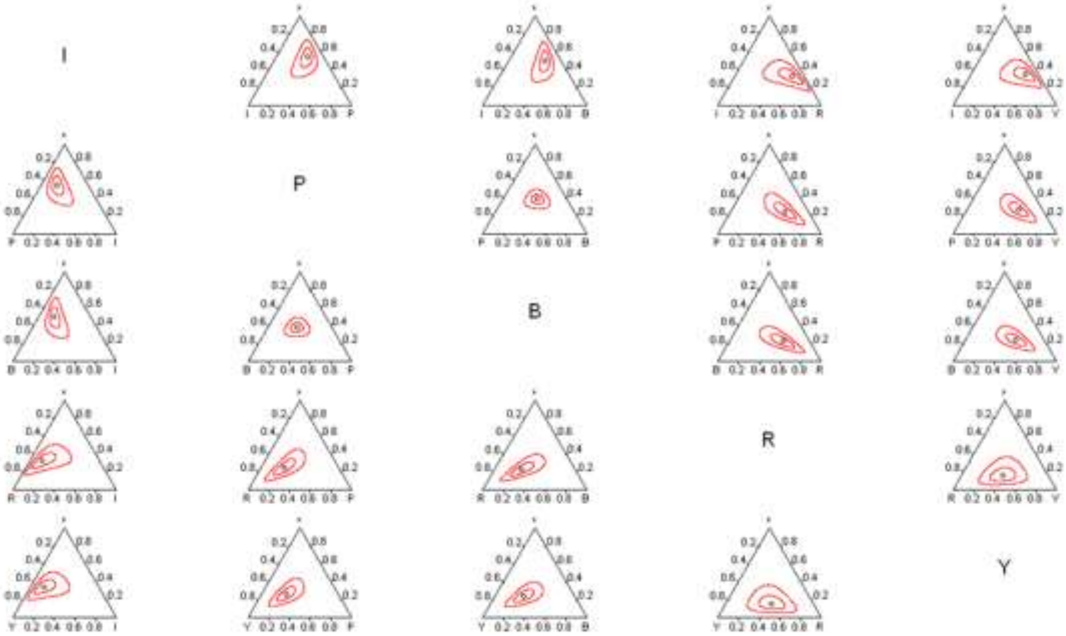
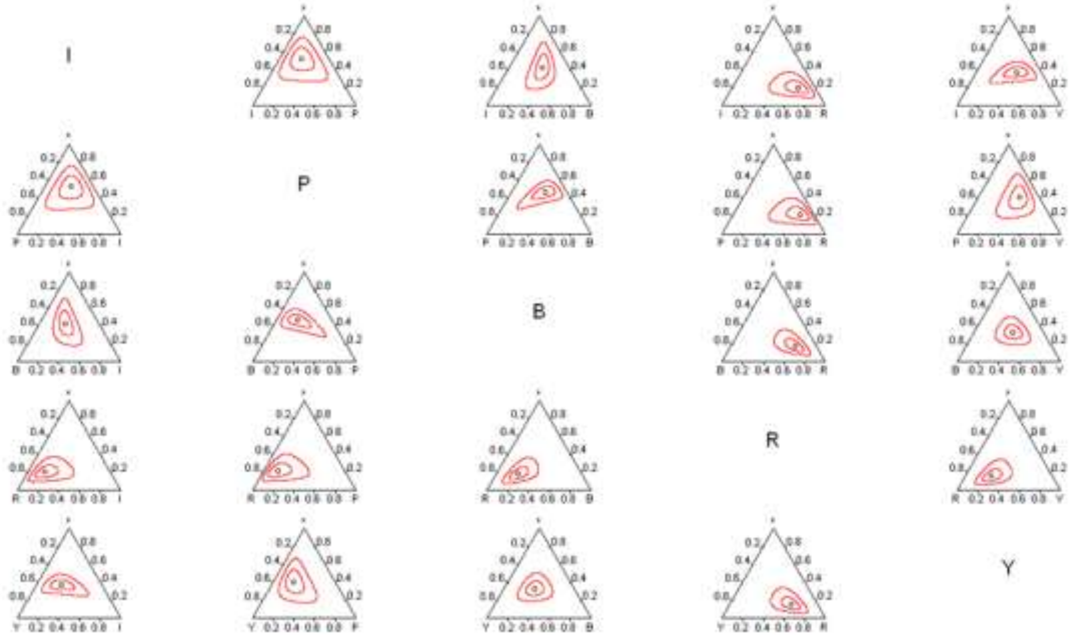
Fig. S3 Matrix of ternary diagrams of the geometric mean (black open dot) and confidence regions (red lines) of a five sub compositions of individual female mating preferences (N = 116) for color males (I= immaculata, P= parae, B= blue, R= Red, Y= yellow). The Matrix of preferences represent the sub-groups as identified by the compositional clustering for (a) RED = YELLOW (N = 19 females); (b) RED >> THAN ANY OTHER MORPH (N = 37 females); (c) RED= YELLOW=BLUE (N = 23 females); (d) BLUE > RED (N = 5 females); (e) RED > BLUE (N = 8 females); (f) RED > PARAE (N = 6 females); (g) PARAE > RED (N = 12 females); and, (h) YELLOW > RED (N = 6 females). Although treatment females were part of the analysis they were not considered graphically due to their low contribution.

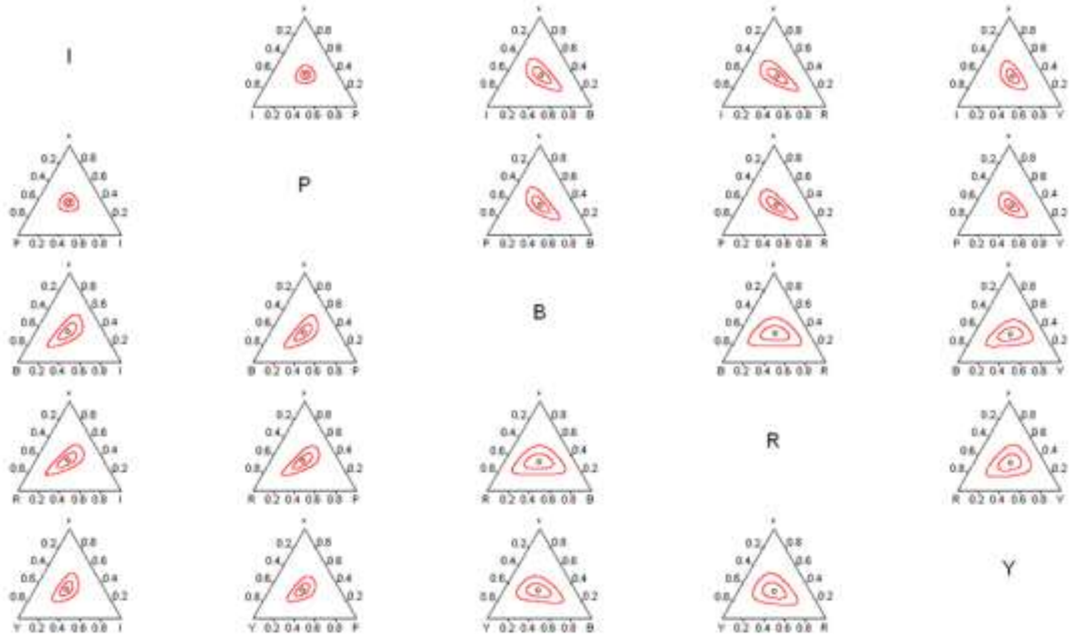
(a) RED = YELLOW



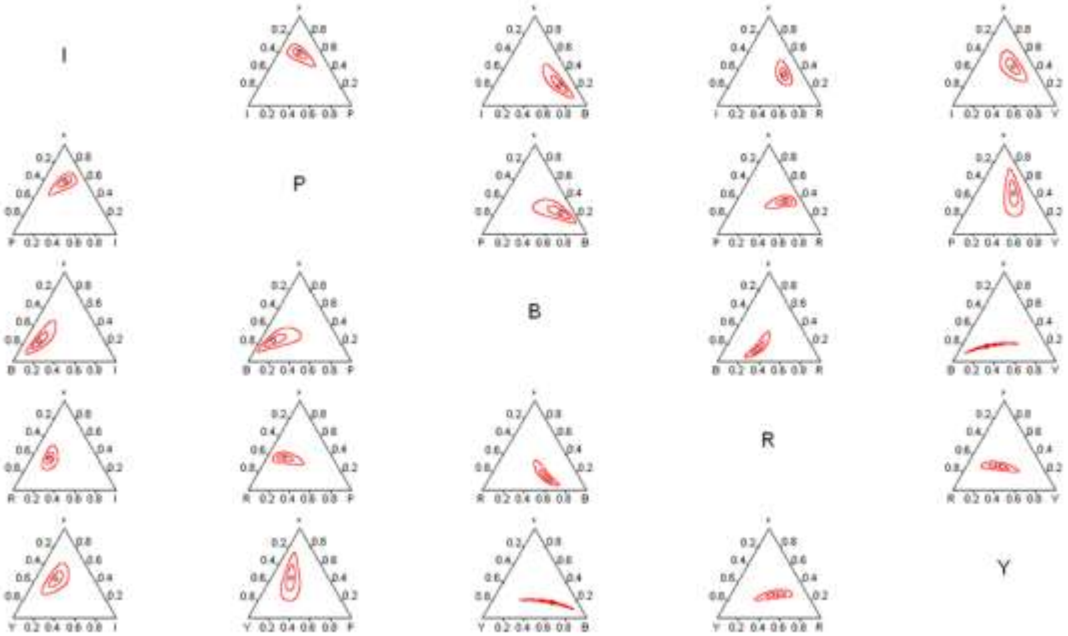
(b) RED >> THAN ANY OTHER MORPH



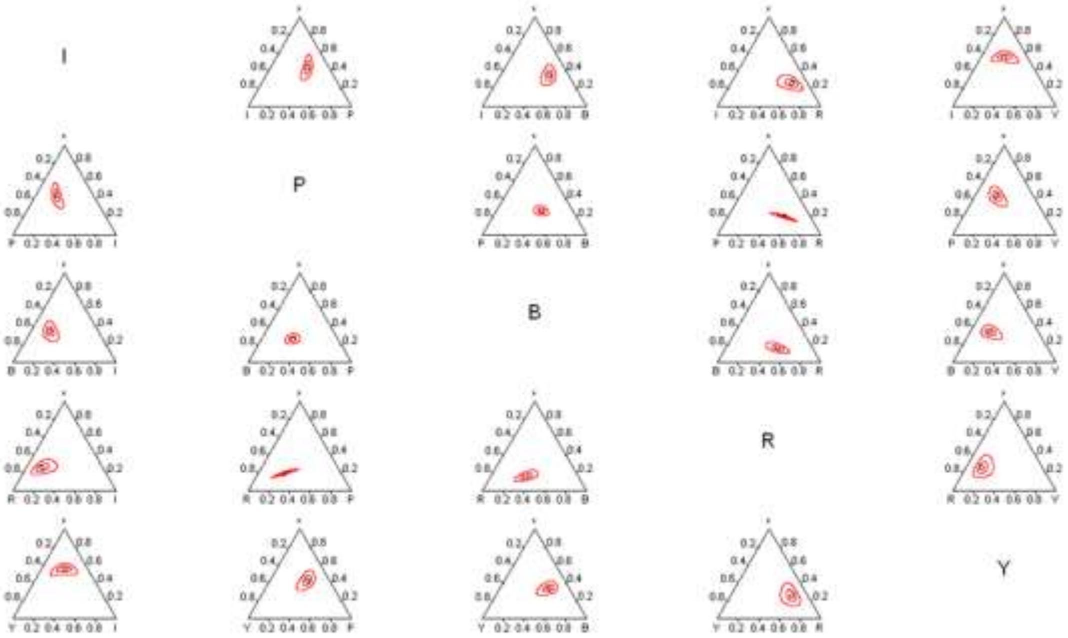
(c) RED = YELLOW = BLUE



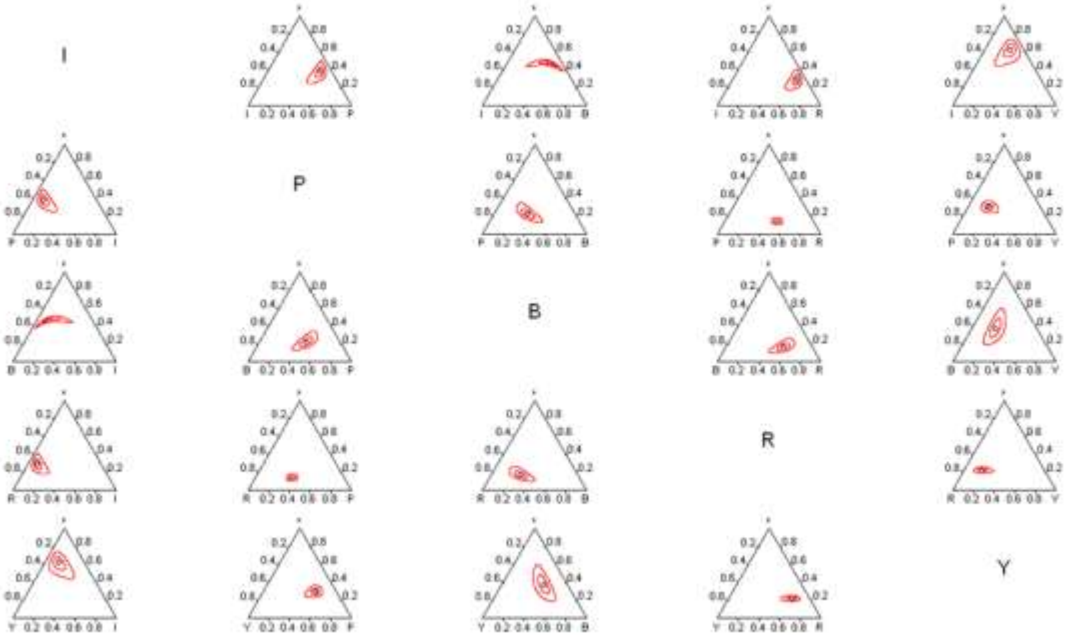
(d) BLUE > RED



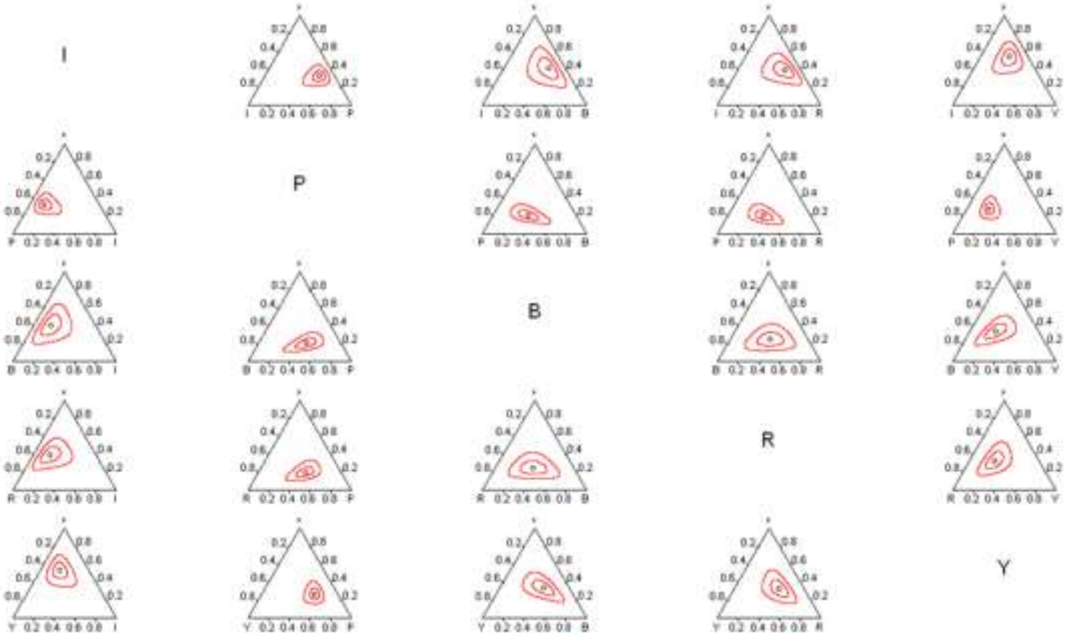
(e) RED > BLUE



(f) RED > PARAE



(g) PARAE > RED



(h) YELLOW > RED

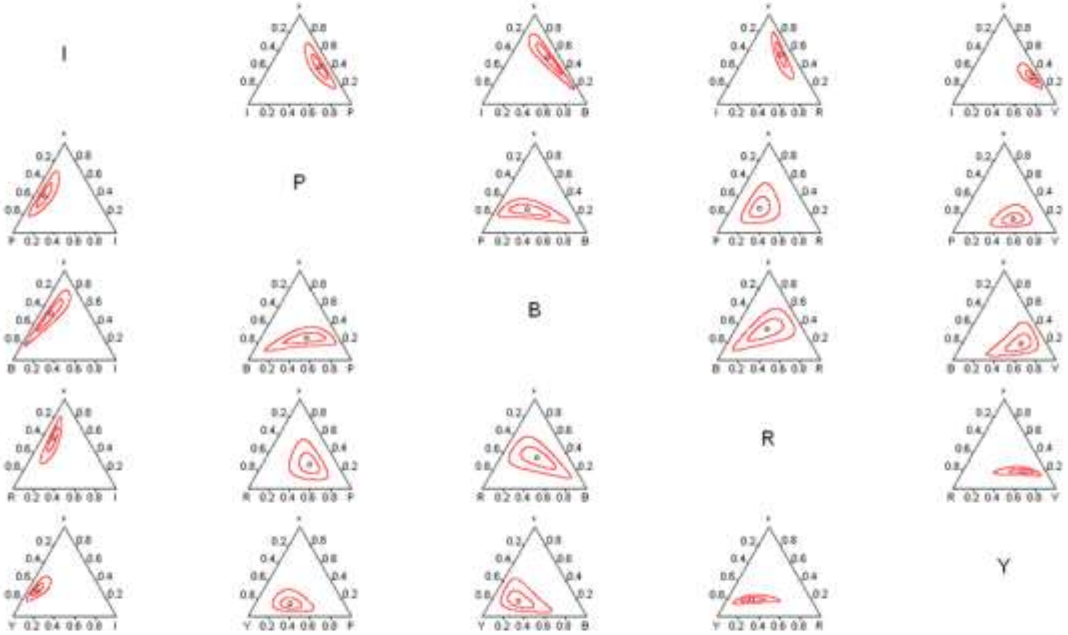
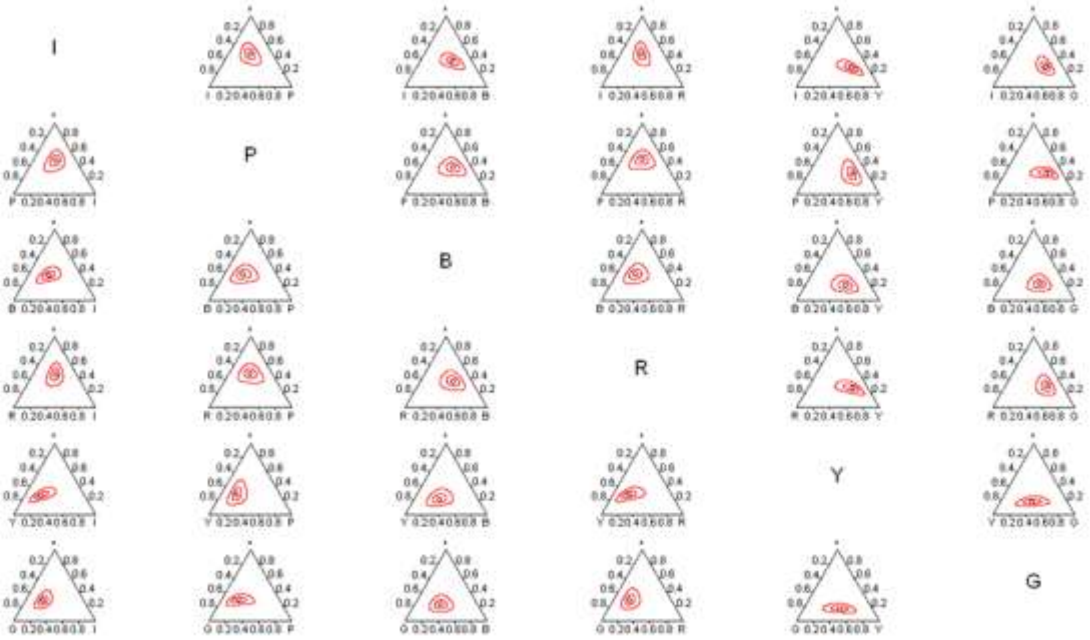
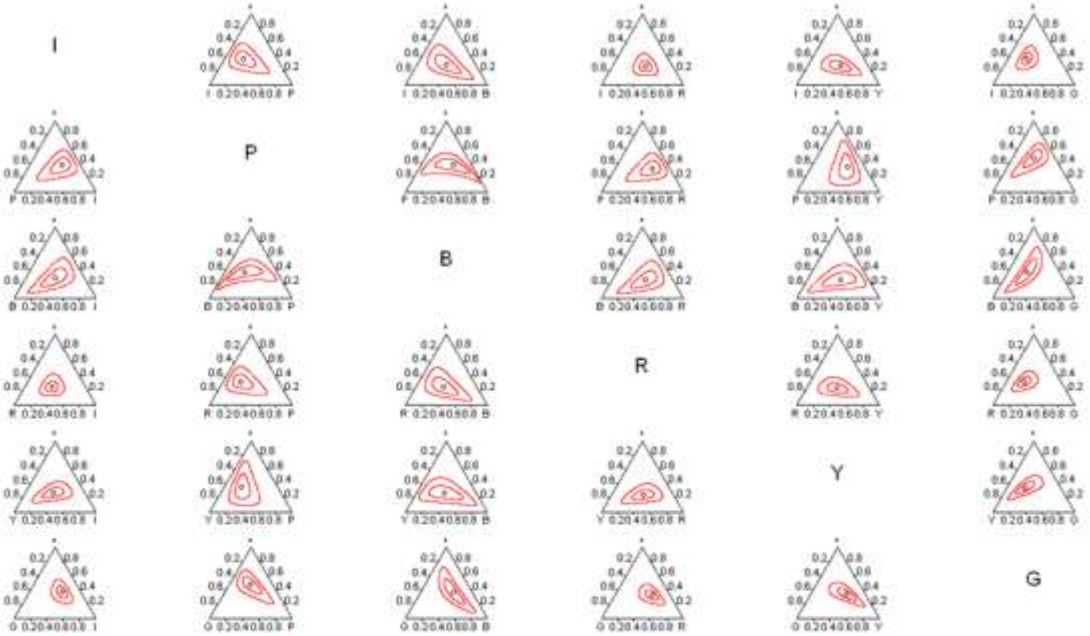


Fig. S4 Matrix of ternary diagrams of the geometric mean (black open dot) and confidence regions (red lines) of a six sub compositions of individual predator prey preferences (N = 96 predators) for color morphs (I= immaculata, P= parae, B= blue, Y= yellow) and male guppy (G). Matrix of preferences for (a) YELLOW = GUPPY > RED (N = 13 predators); (b) RED > YELLOW > BLUE (N = 16 predators); (c) RED = YELLOW > BLUE (N = 8 predators) (d) RED = YELLOW (N = 8 predators); (e) RED = YELLOW > GUPPY (N = 17 predators), (f) RED > YELLOW = PARAE (N = 26 predators).

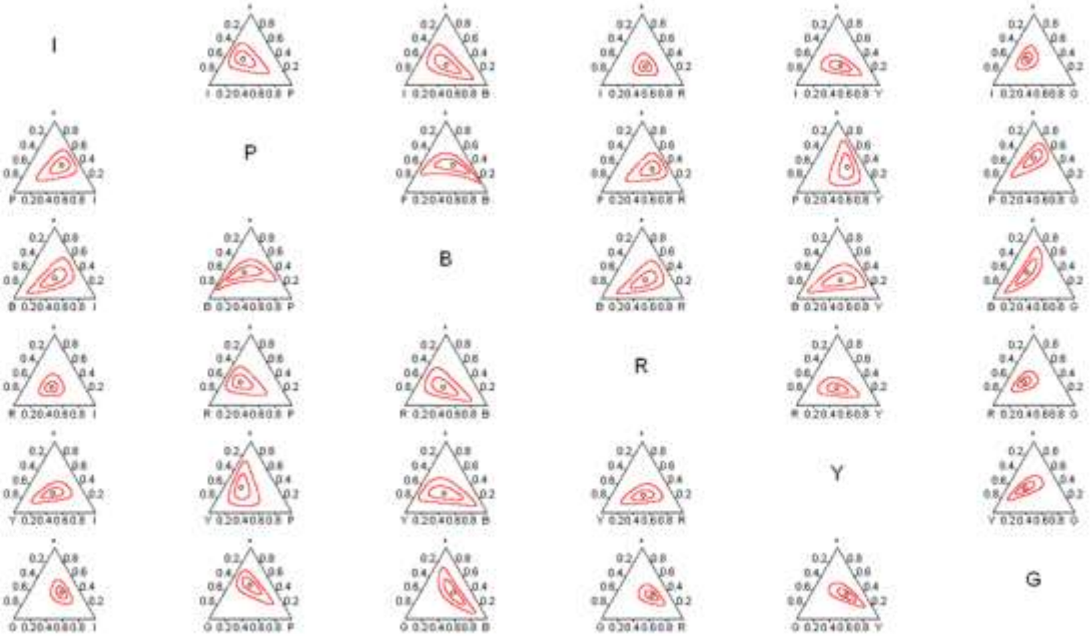
(a) YELLOW = GUPPY > RED



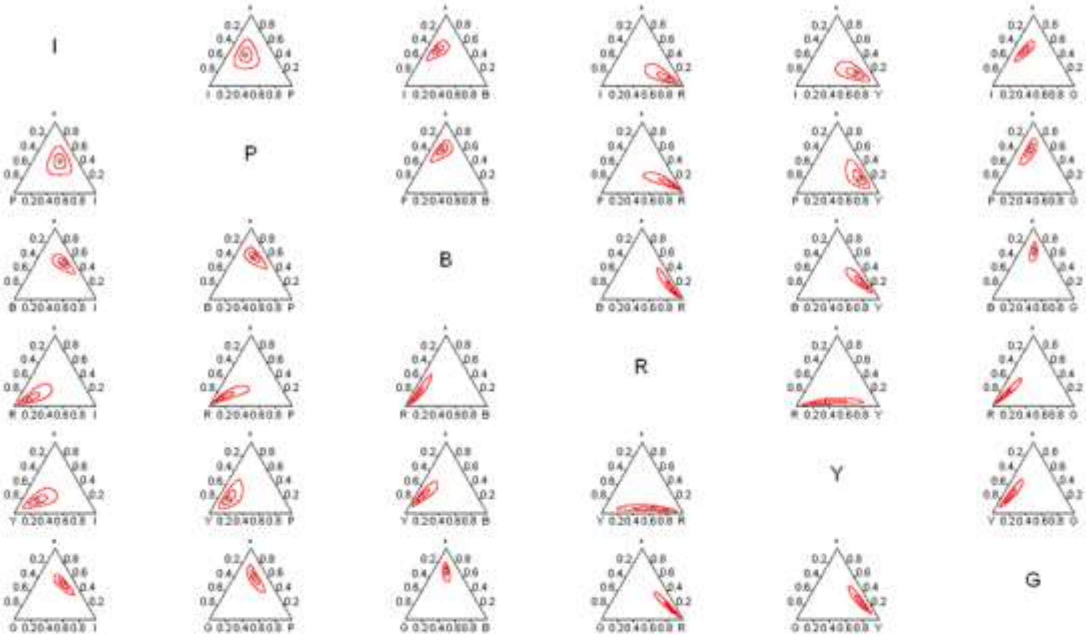
(b) RED > YELLOW > BLUE



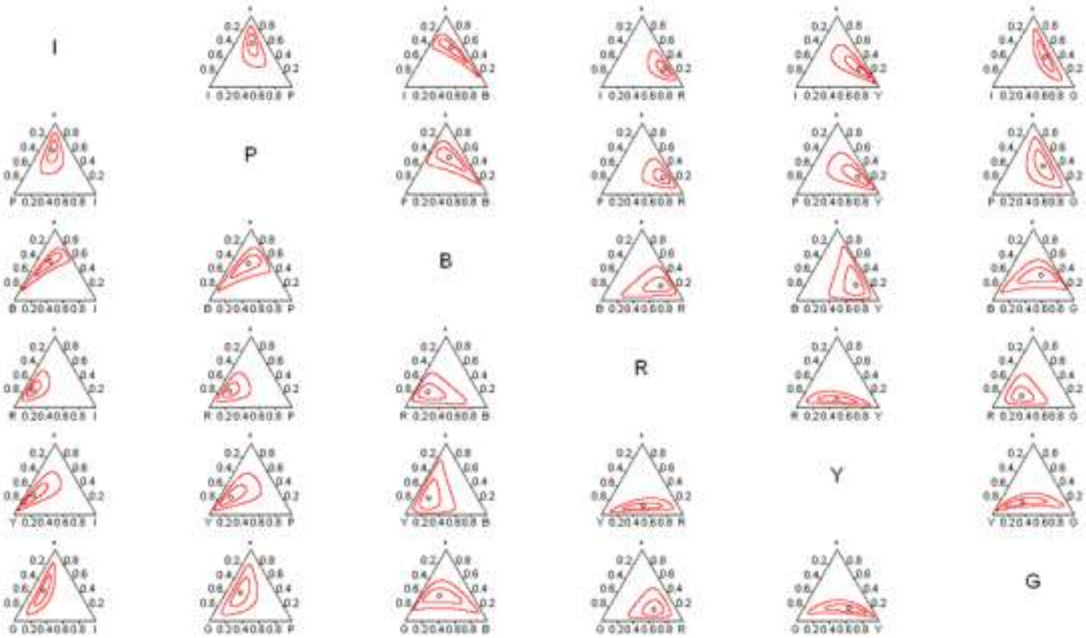
(c) RED = YELLOW > BLUE



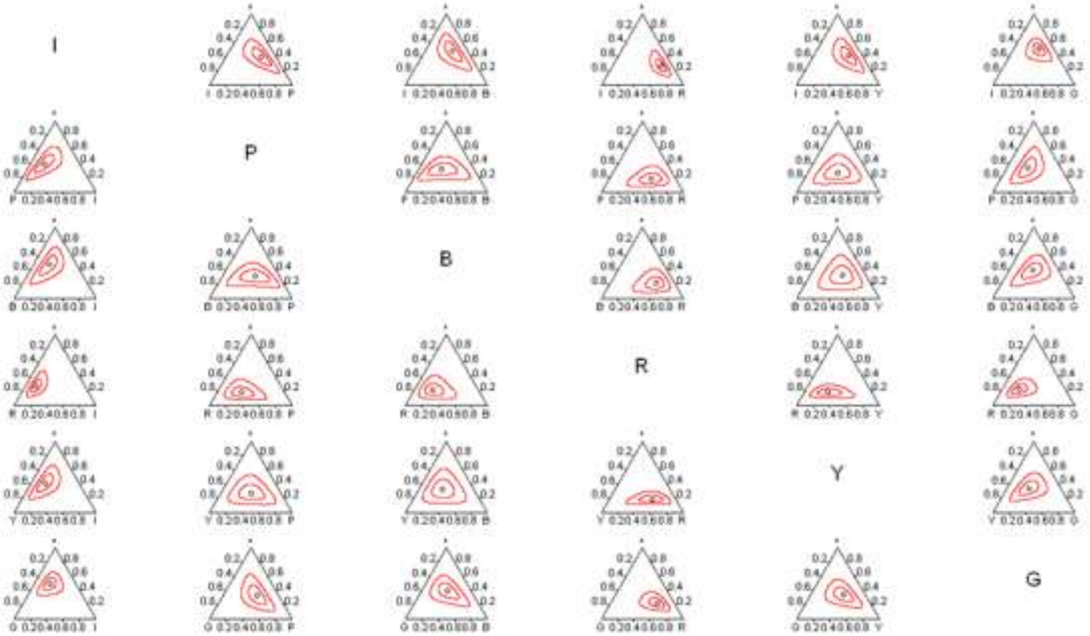
(d) RED = YELLOW



(e) RED = YELLOW > GUPPY



(f) RED > YELLOW = PARAE



CHAPTER IV

INTRASEXUAL COMPETITION FACILITATES THE EVOLUTION OF ALTERNATIVE MATING STRATEGIES IN A COLOUR POLYMORPHIC FISH

Hurtado-Gonzales, J. L. & J. A. C. Uy. 2010. Intrasexual competition facilitates the evolution of alternative mating strategies in a colour polymorphic fish. BMC Evolutionary Biology 10:391.

ABSTRACT

Intense competition for access to females can lead to males exploiting different components of sexual selection, and result in the evolution of alternative mating strategies (AMSs). Males of *Poecilia parae,* a colour polymorphic fish, exhibit five distinct phenotypes: drab-coloured (immaculata), striped (parae), structural-coloured (blue) and carotenoid-based red and yellow morphs. Previous work indicates that immaculata males employ a sneaker strategy, whereas the red and yellow morphs exploit female preferences for carotenoid-based colours. Mating strategies favouring the maintenance of the other morphs remain to be determined. Here, we report the role of agonistic male-male interactions in influencing female mating preferences and male mating success, and in facilitating the evolution of AMSs. Our study reveals variation in aggressiveness among *P. parae* morphs during indirect and direct interactions with sexually receptive females. Two morphs, parae and yellow, use aggression to enhance their mating success (i.e., number of copulations) by 1) directly monopolizing access to females, and 2) modifying female preferences after winning agonistic encounters. Conversely, we found that the success of the drab-coloured immaculata morph, which specializes in a sneak copulation strategy, relies in its ability to circumvent both male aggression and female choice when facing all but yellow males. Strong directional selection is expected to deplete genetic variation, yet many species show striking genetically-based polymorphisms. Most studies evoke frequency dependent selection to explain the persistence of such variation. Consistent with a growing body of evidence, our findings suggest that a complex form of balancing selection may alternatively explain the evolution and maintenance of AMSs in a colour polymorphic fish. In particular, this

study demonstrates that intrasexual competition results in phenotypically distinct males exhibiting clear differences in their levels of aggression to exclude potential sexual rivals. By being dominant, the more aggressive males are able to circumvent female mating preferences for attractive males, whereas another male type incorporates subordinate behaviours that allow them to circumvent male aggression and female mating preferences. Together, these and previous results indicate that exploiting different aspects of social interactions may allow males to evolve distinct mating strategies and thus the long term maintenance of polymorphisms within populations.

BACKGROUND

Intense sexual selection can lead to competing males evolving alternative ways to obtain fertilizations, thereby enhancing their reproductive success [1]. In genetically-based polymorphic species, alternative mating strategies (AMSs) are characterized by distinct behavioural and morphological traits that help males (hereafter morphs) ameliorate their mating disadvantages when facing superior competitors [2-4]. For example, in some polymorphic lizards [5,6], birds [7-9], fish [10,11] and marine isopods [12], large and/or colourful males are aggressive and defend breeding territories to exclude competitors. As a consequence, subordinate males have evolved AMSs, such as sneak copulations that are accompanied by adaptations to sperm competition [1] to possibly circumvent overt aggression. AMSs are expected to be maintained as long as the resulting average fitness of one strategy equals that of the others co-occurring in the population [4], with frequency-dependent selection favouring rare over common phenotypes [2, 4,13].

Sexual selection theory predicts that strong mating preferences for males with elaborate ornaments that reflect their quality or dominance should deplete genetic variation in these traits [14-17]. There are, however, cases in which males as a result of high variance in mating success and thus strong sexual selection exhibit striking, genetically-based polymorphism in display traits (e.g., [10,18-20]). Further, several studies in a broad range of taxa ([5, 7-10,12,21-30] see also table 12.2 in [4]) consistently suggest that such variation in male phenotypes (e.g., extreme differences in body lengths, behaviours, physiology) is adaptive and correlates with asymmetric social dominance relationships defining AMSs. A mechanism by which variation in male phenotypes is maintained is through frequency dependent selection for AMSs [2, 4, 12,13]. For instance, in the side-blotched lizard (*Uta stansburiana*), males have evolved AMSs (orange-throated: aggressive and territorial; yellow-throated: sneaker; blue-throated: mate guarding) and the relative fitness of each strategy fluctuates depending on the frequency of the competing strategies from one generation to the next [5]. Under frequency-dependent selection, the rare strategy always has an advantage [2,31]. The same mechanism is often invoked to explain polymorphisms that are not shaped by sexual selection [32-36].

Although not necessarily independent of frequency dependent selection, a less explored mechanism for the maintenance of polymorphisms involves complex, balancing selection in which different aspects of sexual and natural selection select for unique phenotypes (e.g., [13]). For instance, female mating preferences may favour colourful males, while agonistic male-male competition may favor large males [10]. This lack of synergism between the two aspects of sexual selection may allow for the invasion of AMSs and thus promote the maintenance of polymorphisms [10,18-20,22].

In many species, males can use overt aggression as a tactic to circumvent female choice [37-39]. For instance, dominant males can prevent attractive males from gaining access to females [10,40-43]. However, male aggression can also facilitate female choice if winners of male contests signal their quality (e.g., vigour, tenancy of better territories) to females [37-39]. Further, in many taxa, the success of mating attempts ultimately relies on female consent [44], and thus, females may still exercise choice despite overt male aggression [41,42]. This potential for conflict and synergism between intrasexual competition and intersexual mate choice can result in opportunities for certain males to exploit different components of sexual selection [3], facilitating the evolution and maintenance of AMSs [4,44,45]. Here, we explore how overt male aggression can influence male mating success and facilitate the persistence of colour polymorphism in the pentamorphic fish *Poecilia parae*.

The South American poeciliid *P. parae* exhibit five Y-linked, discrete colour morphs [46,47]. These morphs include: (i) immaculata, the smallest and drab-coloured males that resemble juvenile females; (ii) parae, the largest males that exhibit a striped tail and black vertical body bars that intensify during social interactions; and (iii) the blue, red, and yellow males that are of intermediate body size and display colourful body flanks [46,48]. Males and females mate promiscuously, with males providing no resources during mating [48]. *Poecilia parae* breeds year-round [48,49], suggesting an opportunity for intense competition among males to identify and monopolize sexually receptive females. In *P. parae*, the carotenoid-based red and yellow morphs are strongly preferred by the majority of females as mates, and the smaller immaculata males are the least attractive males [46,49,50]. However, because the immaculata morph specializes in sneak copulations with apparent adaptations for sperm competition [51], such a mating strategy would be successful only if sneaker males can circumvent pre-copulatory female choice, intrasexual aggression, or both (e.g.,[4,5,7]. It remains to be seen, however, whether immaculata males can indeed circumvent male aggression and female choice and consequently gain successful matings. The persistence of the other two morphs (*i.e.*, parae and blue) may be the result of their competitive abilities in open mixed groups [46, 49].

In this study, we experimentally test for the role of male-male competition in the evolution and maintenance of AMSs. If indeed, particular morphs specialize in male-male aggression, we predict that agonistic interactions (i) may limit the mating opportunities of the attractive (i.e., red and/or yellow) morphs, (ii) have the potential to influence female mating preferences, and (iii) enhance the mating success of aggressive males. Results consistent with these predictions would suggest that the opportunity to exploit male-male competition may facilitate the maintenance of genetically-based polymorphisms.

RESULTS

(a) *Female mate choice and male dominance*

On average, females spent 30.81 ± 15.67 % of the time in the region close to the test males. In the experiment where males were not allowed to interact (pre-male competition), females spent more time with the parae, blue, red and yellow than with the drab immaculata males (Figure 1a). After observing male-male interactions (post-male competition), females switched their mating preferences when parae males were winners of interactions against blue (paired t_{14}= $P < 0.01$), red (paired t_{14}= $P < 0.01$), and yellow males (paired t_{14}= $P = 0.1$; Figure 1b). Similarly, aggression displayed towards the blue males modified female choice favouring red (paired t_{14}= $P < 0.01$) and yellow (paired t_{14}= $P < 0.01$) males (Figure 1c). We found no statistical differences between the mate choice scores of red and yellow males (paired t_{14}= $P = 0.23$) for the pre-male and post-male competition experiments (Figure 1d).

On average, parae males dominated blue, red and yellow males (binomial tests, all $P < 0.01$). Immaculata males never initiated aggressive interactions during the staged contests against other males, and they were attacked most frequently by yellow males (Mean ± SE: 12 ± 3.41 aggressive behaviours 10 min^{-1}) and in less proportion by parae males (2.7 ± 0.83 aggressive behaviours 10 min^{-1}). Blue males typically lost to red (binomial test, $P = 0.01$; $n = 15$) and yellow (binomial test, $P < 0.01$; $n = 15$) males. Yellow males were dominant in 60% of their encounters with red males (binomial test, $P = 0.30$; $n = 15$).

In addition, the five morphs differed in the rate of received (Kruskal-Wallis test: $H_{4, 260}$: 37. 55, $P < 0.001$) and initiated (Kruskal-Wallis test: $H_{4, 260}$: 74. 03, $P < 0.001$) aggressive behaviours (Figure 2a). Overall, the immaculata and parae morphs were less likely to be attacked when compared to the blue, red, and yellow males (all comparisons, $P < 0.001$). In contrast, parae males initiated more aggressive behaviours than the immaculata, blue and red morphs (all comparisons, $P < 0.003$). Parae and yellow males did not differ significantly in rate of initiating aggression ($P = 0.09$).

(b) *Competition for access to females and mating success*

The total number of aggressive behaviours was fewer when males were allowed to directly interact with females than when females were separated from males and only observed male-male interactions (direct: Mean ± SE: 7.12 ± 1.07 aggressions 10 min^{-1}; $n = 220$; indirect: 25.85 ± 2.91 aggressions 10 min^{-1}; $n = 220$; Mann-Whitney $U = 147$, $P < 0.001$). Morphs differed in the number of aggressive behaviours received (Kruskal-Wallis test: $H_{4, 260}$: 35. 09, $P < 0.001$) and initiated (Kruskal-Wallis test: $H_{4, 260}$: 56. 74, $P <$

0.001; Figure 2b) during the open aquaria experiment. Parae and yellow males received fewer attacks ($P < 0.01$), but, along with red males, initiated the greater proportion of aggressive behaviours ($P < 0.02$). Compared to parae, blue, and red males, yellow males initiated more aggressive behaviours (Mean ± SE: 23.7 ± 4.18; n= 10; 2 % sparring, 53% chasing, and 45% attacks) against immaculata males. In contrast, parae males initiated the least number of aggressive behaviours towards immaculata males (Mean ± SE: 1 ± 0.52 aggressive behaviours 10 min^{-1}), yet directed more attacks to blue (Mean ± SE: 24.13 ± 7.59; n= 15) and red (14.6 ± 4.46; n= 15) males.

The number of copulations (Mean ± SE: 0.56 ± 0.07 10 min^{-1}) gained as a result of individuals being able to fend off their competitors differed significantly among morphs (Kruskal-Wallis test: $H_{4, 260}$: 28.31, $P < 0.001$; Figure 3). Parae males experienced an increase in mating success when competing against blue (Wilcoxon matched paired test, z = 2.69, n= 15, P = 0.007) and red (Wilcoxon matched paired test, z = 2.2, n = 15, P = 0.02; Figure 3) males, but not with yellow males (Wilcoxon matched paired test, z = 1.53, n = 15, P = 0.13).

Yellow males obtained significantly more copulations after winning against blue males (Wilcoxon matched paired test, z = 2.67, n= 15, $P < 0.01$) and marginally significant after winning against immaculata males (Wilcoxon matched paired test, z = 1.83, n = 10, P = 0.06). Moreover, 11.67 % of copulations obtained by yellow males were the result of aggressive interactions (*i.e.*, sparring) with females and sneak copulations.

DISCUSSION

Our results suggest that certain *P. parae* morphs have evolved the use of overt aggression as an AMS. Several lines of evidence support this interpretation. First, males of the parae and yellow morphs consistently dominated their competitors during staged contests by excluding rival males from gaining access to females. Second, when females and competing males were allowed to freely interact, parae and yellow males gained a substantial proportion of matings by directly monopolizing females and limiting the mating opportunities of the blue and red morphs, explicitly showing that aggression indeed results in increased mating success for dominant males. Finally, females observing male-male interactions modified their mate choice favouring dominant over subordinate males for contests that involved males of the parae, red, and yellow morphs. These results suggest that the opportunity for intense intrasexual competition can facilitate the evolution and maintenance of AMSs when females display strong preferences for attractive but not necessarily dominant males (see also [10,40,43]).

During the staged contests, immaculata males, the smallest and the least preferred males by females, were typically submissive. In our study, the mating success of immaculata males relied on the strategy of appearing like juvenile females to avoid harassment when approaching receptive females and in taking advantage of their smaller body size to sneak copulations. The juvenile female-mimic strategy was mostly effective when immaculata males were competing with the parae, blue, and red males. These behavioural patterns suggest that the immaculata morph has evolved a strategy to female mating preferences for colourful males [46,49,50]. When male-male competition is excluded, a large proportion of females strongly preferred red and yellow males; however, some females showed consistent preference for parae and/or blue males [50]. In the experiments where females were physically separated from males with a clear barrier, red and yellow males did not differ in their ability to attract females, even after the test females observed yellow males dominate over red males. However, in the experiments where males and females could physically interact, yellow males were dominant over red males and were capable of restricting access of red males to females. This difference in aggression translated in greater association time with females for yellow males. Surprisingly, however, the observed preference did not differ in their realized mating success (i.e., number of copulations in 10 min^{-1}), suggesting that even though aggressive yellow males can restrict the access of red males to females, female preference for red males was able to counteract male aggression. That is, in cases in which red males were subordinate, females still managed to circumvent the attempts of dominant yellow males to monopolize matings and mated with the attractive red males. Similar female mating behaviours have also been reported in guppies (*P. reticulata*), wherein paternity in multiply sired broods was biased towards subordinate males ([40], but see also [54]).

How then does intrasexual competition contribute to the maintenance of the striking colour polymorphism in *P. parae*? Because sexual selection operates at distinct stages (e.g., pre- and post mating intersexual choice, pre- and post mating intrasexual competition), males can evolve unique strategies that specialize in one or few stages of sexual selection (reviewed in [3]). For instance, females may have strong mating preferences for particular males, but the most dominant and aggressive males can exclude attractive males from gaining access to females [37-39]. Other AMSs can circumvent both female choice and male aggression by mimicking females and adopting a sneaker strategy (e.g., yellow sneaker in side-blotched lizard; beta and gamma males in marine isopods; satellite and faeders in the ruff [5,52,53]). Furthermore, males exploiting post copulatory sexual selection could also evolve traits that enhance their competitive abilities during sperm competition (e.g., allocating ejaculates containing faster sperm; see [3]) or post-copulatory female choice (e.g., [55]).

Indeed, several studies have demonstrated that under intense episodes of sexual selection, males experiencing continuous mating disadvantages should evolve strategies that exploit different components of sexual selection (e.g., the ruff, [7]; side-blotched lizard: [56]; pigmy swordtail [10]; Gouldian finch, [8]; marine isopods, [53]). In these examples, frequency-dependent selection is the most plausible explanation in facilitating the co-existence of the AMSs [2,4]. In *P. parae*, however, frequency-dependent selection cannot fully explain the persistence of the five morphs, as their frequency in nature is consistent over time ([46,48]; see Fig. 2. in [50]). Field surveys from 2002 to 2009 indicate that immaculata and parae males are the most abundant, followed by blue males, with red and yellow males being rarest [50]. In this case, different forms of balancing selection may help explain the persistence of the five morphs in *P. parae.* For instance, females show a strong mating preference for the red and yellow males [46,49,50], and so these males should be most abundant. Red and yellow males, however, are rare, and this may be due to strong predator (i.e., cichlids) preference for prey with carotenoid colour patches [50]. Therefore, the interaction between female mating preference and selective predation may provide opportunities for less attractive males to evolve AMSs, and invade and persist in the population. Our study suggests that the parae (and also yellow) morph use intrasexual aggression to monopolize females and obtain matings. However, in this and many other systems, female cooperation is needed to achieve successful matings (e.g.

[44]), and so, while parae males are able to monopolize access to females, female mate choice may still counteract the effects of male aggression, and prevent parae and yellow males from driving the other morphs to extinction. The drab, female-mimic immaculata morph represents an alternative strategy of circumventing both female mating preferences and male-male competition, as well as being unattractive to visual predators (see also [46]). This form of balancing selection in which female mate choice and intrasexual competition, combined with predator preferences for red and yellow males, may ultimately provide opportunities for multiple mating strategies to evolve and persist in the population.

Lastly, although blue males gained some matings as a result of aggressive interactions and variable female mating preferences [50], the factors that allow for the persistence of this morph remains unclear. Recent studies suggest a strong role for environmental heterogeneity in favouring the maintenance of colour polymorphisms [57-59]. For instance, as shown in the pentamorphic Sulawesi fish, *Telmatherina sarasinorum* [59], female mating preferences may vary as a response to spatial and/or temporal fluctuations in the visual environment [57]. Preliminary surveys of *P. parae* habitat indicate that the visual habitat is variable, with some areas being rich in short wavelength light. This suggests the intriguing possibility that, in their natural settings, blue males may be able to exploit these microhabitats to appear more conspicuous and thus more attractive to females (Hurtado-Gonzales & Uy; in prep). The role of sexual selection under environmental heterogeneity may explain the persistence of the blue *P. parae* morph.

CONCLUSIONS

Frequency dependent selection is typically evoked to explain the evolution and maintenance of genetically-based polymorphisms. In these scenarios, rare morphs have a selective advantage over common morphs, resulting in cyclical fluctuation of morph frequencies. Alternatively and somewhat independent of frequency dependent selection, complex balancing selection may facilitate the evolution of polymorphisms by allowing the persistence of alternative mating strategies. In our work, the antagonistic interaction between various components of sexual selection, in addition to natural selection by predators, allows for the invasion of AMSs and thus the maintenance of the striking polymorphism in *P. parae*.

METHODS

Female mating preferences and male aggression were assessed using wild caught fish from the west coast of the Demerara River (6˚ 41’ N, 58˚ 12’ W), Republic of Guyana. Males were sorted by morph type (n = 40 immaculata; n = 55 parae; n = 55 blue; n = 38 red; and n = 41 yellow) and housed in separate aquaria with non-experimental females at equal sex ratio. Fish were maintained in 20 gal aquaria with treated water at 27 ± 1°C, on a 12:12 h light: dark cycle, and fed daily with live brine shrimp and Tetra-Min® (Melle, Germany) flakes two times per day. All experiments were carried out in Georgetown, Guyana. Before starting each trial, all fish were fed to satiation.

Test females (n = 130) were periodically captured, individually housed in small 250 ml plastic containers to monitor their breeding status, and used within four days after parturition. We selected experimental females with standard lengths ≥ 20 mm (Mean ± SD: 26.48 ± 2.98 mm, Intervals 20.06 – 31.44 mm, n = 130). Considering the high levels of promiscuity in *P. parae* and that females breed year round [48, 49], these females have likely bred at least once. Thus, we assumed that the selected standard length (≥ 20 mm) would be an indication that test females have been exposed to all five morphs under natural conditions. Another advantage in using post-partum females compared to naïve virgin females is that females mating preferences is shaped by previous experience of male phenotypes [60]; consequently, experienced females are more likely to posses better discrimination capabilities than naïve females [60,61]. Finally, previous mate choice experiments in *P. parae* likewise used experienced females [46,59,50]. These studies show that experienced females are responsive towards all males. Hence, for consistency, we followed the same criteria.

Identification of morphs

Although the five *P. parae* morphs are easily identified by their patterns of coloration as adults, juvenile males resemble each other [46,47]. We used two methods to insure that the classification of males used for the experiments were accurate. First, we only used males with developed gonopodial hoods, which is a good indicator of sexually mature males in poeciliids [61-63]. Second, based on our observations of the development of fry in our laboratory, individuals express their distinct color patterns and have well differentiated gonopodium when they attain the body length of 13.9 - 14.5 mm (51–90 days in development; unpublished data). Similarly, Lindholm *et al*. [34] found that males reach sexual maturity when they attain a body length of 8.5 – 11.5 mm (n = 22) under laboratory conditions. Under experimental breeding conditions, growth of *P. parae* fry is slower and coloration is less intense compared to wild caught individuals, and so for the wild-caught males used in our study, we opted a more conservative, minimum standard length of 16 mm to reduce the possibility of choosing immature individuals. Having identified sexually-mature males, we then classified them into one of the five *P. parae* morphs.

Matching body size for experimentation

Each test male was isolated two days before facing their assigned competitor and a corresponding female. Prior to each experiment, isolated test individuals were anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester) and photographed using a camera (Canon EOS Rebel XTi 400D digital, Japan). Each picture included a ruler as a metric reference. From each digital image, we measured the total length (tip of the upper jaw to tip of caudal fin) and standard length (tip of the upper jaw to the base of the caudal peduncle) of each fish. Body lengths were obtained by using Sigma Scan Pro® v 5.0.0 (San Jose, CA, USA). With the exception of the small immaculata morph, test males were matched in length as much as possible (Table A1 in Additional file 1). Due to the fewer number of red and yellow males during our collections (see above), some individuals were used twice.

(a) *Female mating preferences and male dominance*

We assessed female mating preferences before (‘pre-male competition’) and after (‘post-male competition’) females observed male-male interactions. Each test female participated in a total of four trials with the same males in the following sequence: pre-competition mate choice → females observe male-male interactions (indirect) → post-competition mate choice → females and males directly interact (detailed below).

In the pre-male competition experiment, a random test female was presented with a dichotomous choice of males of two different morphs. Each female was only tested with one pair of males (in the sequence outlined above). All trials were staged in experimental 1.8 gal tanks (Figure A1, Additional file 1) partitioned in two zones by a transparent glass. The larger zone (60% of the total length of the tank) was used as the female test chamber. The smaller zone was further partitioned in two equal-sized compartments using a removable opaque plexiglass. The tank was supplied with a thin layer of gravel, filled to a depth of 14 cm with water, illuminated with a full spectrum light (70 cm above the tank), and covered on three sides with brown, kraft paper. The two small compartments were occupied by two randomly assigned males that were able to see the test female but not each other. The test female was placed in a removable opaque compartment situated in the centre of the female’s test chamber. Males and female were acclimatized for 20 min before the start of each experiment. At the start of the experiment, the female compartment was lifted, and a Samsung Hi-8 SCL 860 camcorder positioned 30 cm away from the uncovered side of the tank recorded female mate choice for 10 min. Recordings started as soon as the test female approached the first male. From the video recording, we measured the amount of time (in seconds) that a female spent within a body length distance to the glass separating her from the males, and actively moving left and right while facing one of the two males. Periods in which a female remained within one body length of a particular male but did not reflect active inspection (i.e., not facing the male) were excluded. In *P. parae*, and poeciliids and other fishes, association time is a good index of female mating preferences [10,11,41-43,46,48-50,61,64,65].

Before running the post-male competition mate choice experiment, we transferred the three individuals (i.e., the two males and female) to a new 1.8 gal tank (Figure A1b), which was divided in two equal-sized sections by a clear glass. One section was occupied by the female and the other by the two competing males. The two males and female were allowed to acclimate for 10 min in individual, opaque removable compartments. At the start of the experiment, the partition separating the males was lifted, and we videotaped male-male interaction for 10 minutes, starting with the first male-male interaction. The test female was allowed to observe the two males through a transparent glass, with no physical contact. From the videotapes, we quantified all aggressive behaviours, but focused on three aggressive behaviours that occurred frequently: sparring, chasing, and attacks (see Table A2 for detailed definitions in Additional file 1). A male was declared dominant after the other male stopped approaching or interacting with the test female or opposing male, or assumed a headstand position whenever the dominant male approached (Table A2).

For the post-male competition experiment, both males and female were immediately returned to the first experimental tank, with the competing males assigned to the opposite compartment from the pre-male competition experiment. We quantified female mating preferences as in the pre-male competition experiment outlined above.

(b) *Competition for access to females and mating success*

To determine how male aggression directly affects copulation success, we allowed the same set of males and females to directly interact using an open-aquarium design. Males and the test female were transferred to individual compartments placed in the centre of a new 1.8 gal tank (Figure A1c in Additional file 1) and allowed to acclimate for 10 min. Individuals were released simultaneously, and were allowed to interact for 10 minutes, starting from the time of first interaction between males or a male and a female. All trials were video recorded, and we quantified all aggressive male-male interactions, aggressive male-female interactions, and number of successful copulations (as defined in [51]) that occurred during the 10 min trial).

Since other studies have shown that odours and pheromones influence mate choice and recognition in poeciliids (e.g., [66-68]), to reduce these effects, we used several aquaria, which allowed us to change the water between experiments.

(c) *Analyses and statistics*

Association time from pre- and post-male competition experiments were transformed to proportions for standardization purposes. We calculated preference for a particular male as the difference between the proportions of time spent between the two males. For instance, with M_A and M_B representing total proportion of time spent with male A or B, respectively, positive values of this index ($M_A – M_B$) would indicate a preference for male A and negative values a preference for male B. To determine whether there was a switch in female mating preferences after observing male-male interactions, we subtracted the post-male competition preference score from the pre-male competition preference score [i.e., $(M_A - M_B)_{\text{2nd experiment}} - (M_A - M_B)_{\text{1st experiment}}$]. We determined if females switched or enhanced their mate preferences between trials using a paired t-test. Preference scores were arcsine transformed to meet the assumptions of parametric tests [69].

We used two assays to measure the level of dominance of males of one particular morph over the other. First, we used the number of times individuals won against an opponent. For the dyadic contests over females, the outcomes (i.e., win or loss) were analysed with two-tailed exact binomial tests. Second, we also quantified the number of aggressive interactions between competitors, totalling then grouping the three types of the most frequent-observed aggressive behaviours [sparring (3%), chasings (21%), and attacks (71%)] into two categories: aggressions received and initiated. Both categories (aggressions received and initiated) were analysed using Kruskal-Wallis non-parametric ANOVAs. To determine which morphs were attacked less and attacked more, we ran multiple comparisons of mean ranks. We also performed Kruskal-Wallis non-parametric ANOVAs and multiple comparisons of mean ranks to analyse aggressions received and initiated during the open aquarium experiments. To compare whether there were differences in the number of attacks performed by males during direct and indirect interactions with females, we used a Mann-Whitney test. Finally, the difference in number of gained or lost copulations to the competitors was analysed with Wilcoxon matched pair tests. All data sets were tested for normality and analysed with STATISTICA$^{©}$ ver. 7. StatSoft, Inc, 2007 OK, U.S.A.

AUTHORS' CONTRIBUTIONS

JLHG conceived the study, carried out the experiments, analysed the results and drafted the manuscript in partial fulfilment of a doctoral degree at Syracuse University (USA). JACU contributed to the study design, supervised the study, edited and revised the manuscript critically. Both authors have read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1 Mean (± standard error) female mate choice scores during pre- (empty bars) and post- male competition (filled bars) trials for immaculata, parae, blue and red morphs. Positive results indicate female preference for pictured males (i.e., Male A) while negative results indicate preference for the assigned opponents (i.e., Male B, or males in X-axis).

Figure 2 Mean (± standard error) proportions of received (empty bars) and initiated (filled bars) aggressive behaviours 10 min^{-1} for each morph when (a) females were separated but were able to observe male-male interactions, and (b) females freely interacted with competing males in open aquaria. Bars with different letters above are significantly different (see text).

Figure 3 Mean (± standard error) rates (10 min^{-1}) of gained (empty bars) and lost (filled bar) copulations by immaculata, parae, blue and red morphs (in pictures) against assigned opponents in the experiment where females could physically interact with competing males.

FIGURES

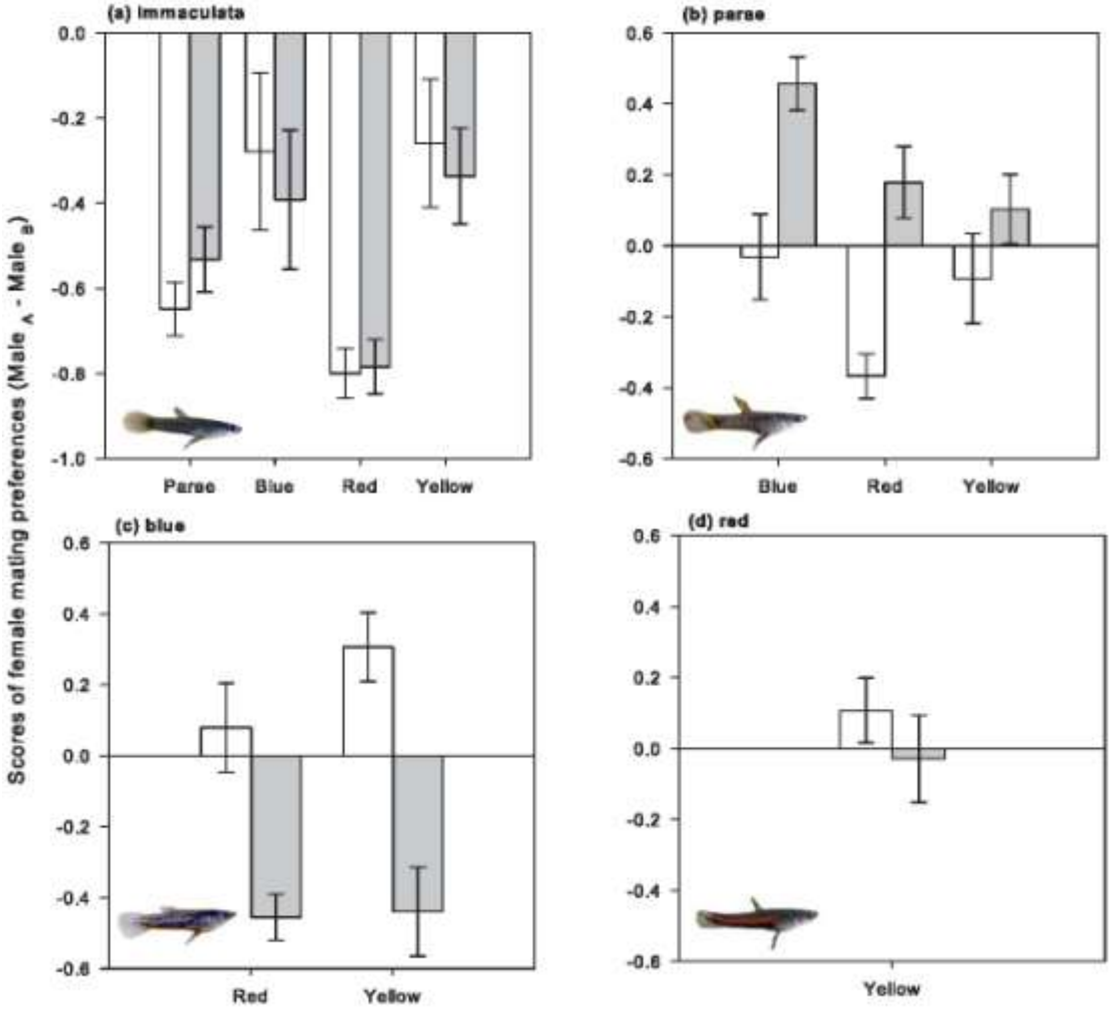
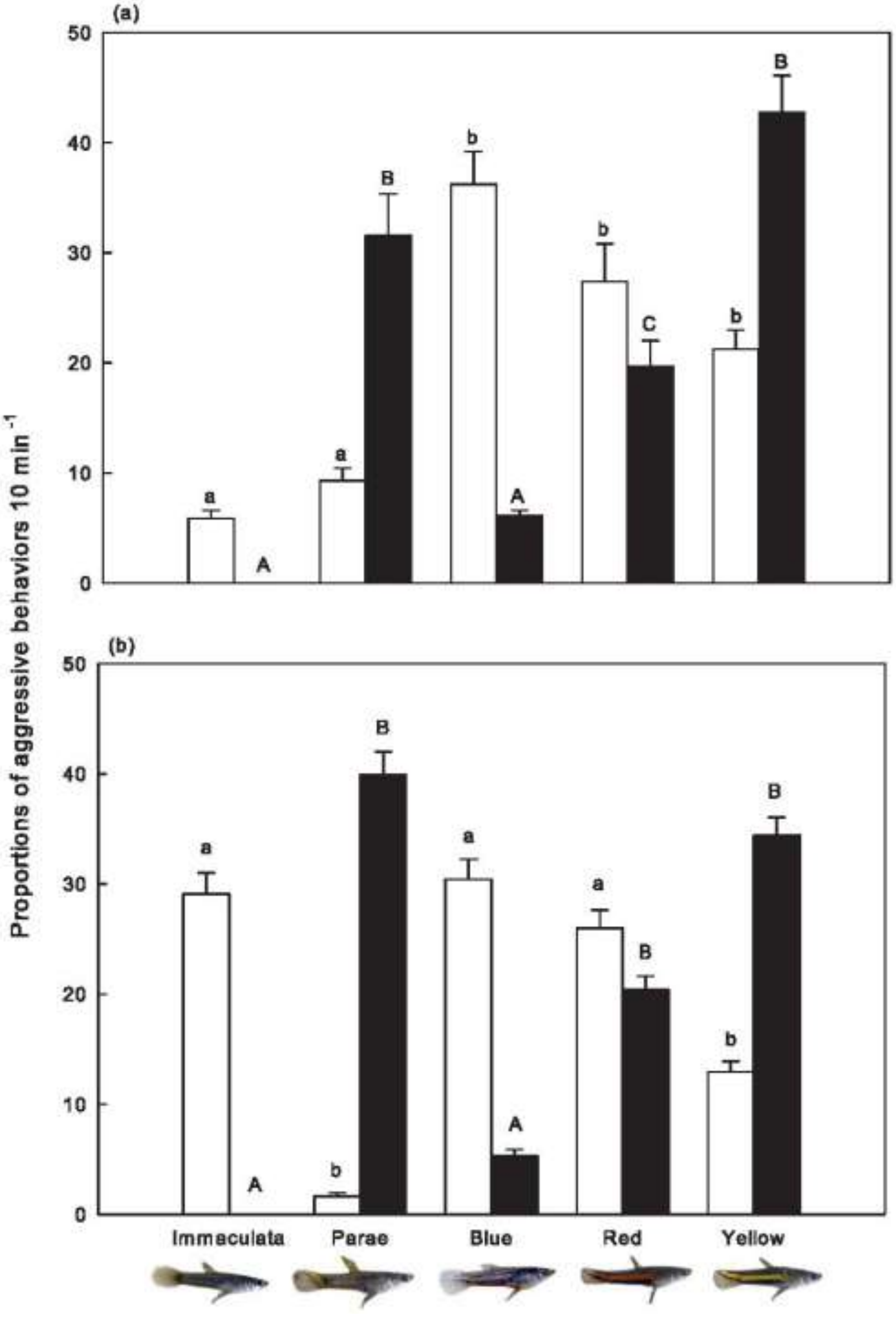


Figure 1



(a)
(b)
Proportions of aggressive behaviors 10 min $^{-1}$
50
40
30
20
10
0
a
A
a
B
b
A
b
C
b
B
a
A
b
B
a
A
a
B
b
B
Immeculata
Parae
Blue
Red
Yellow

Figure 2

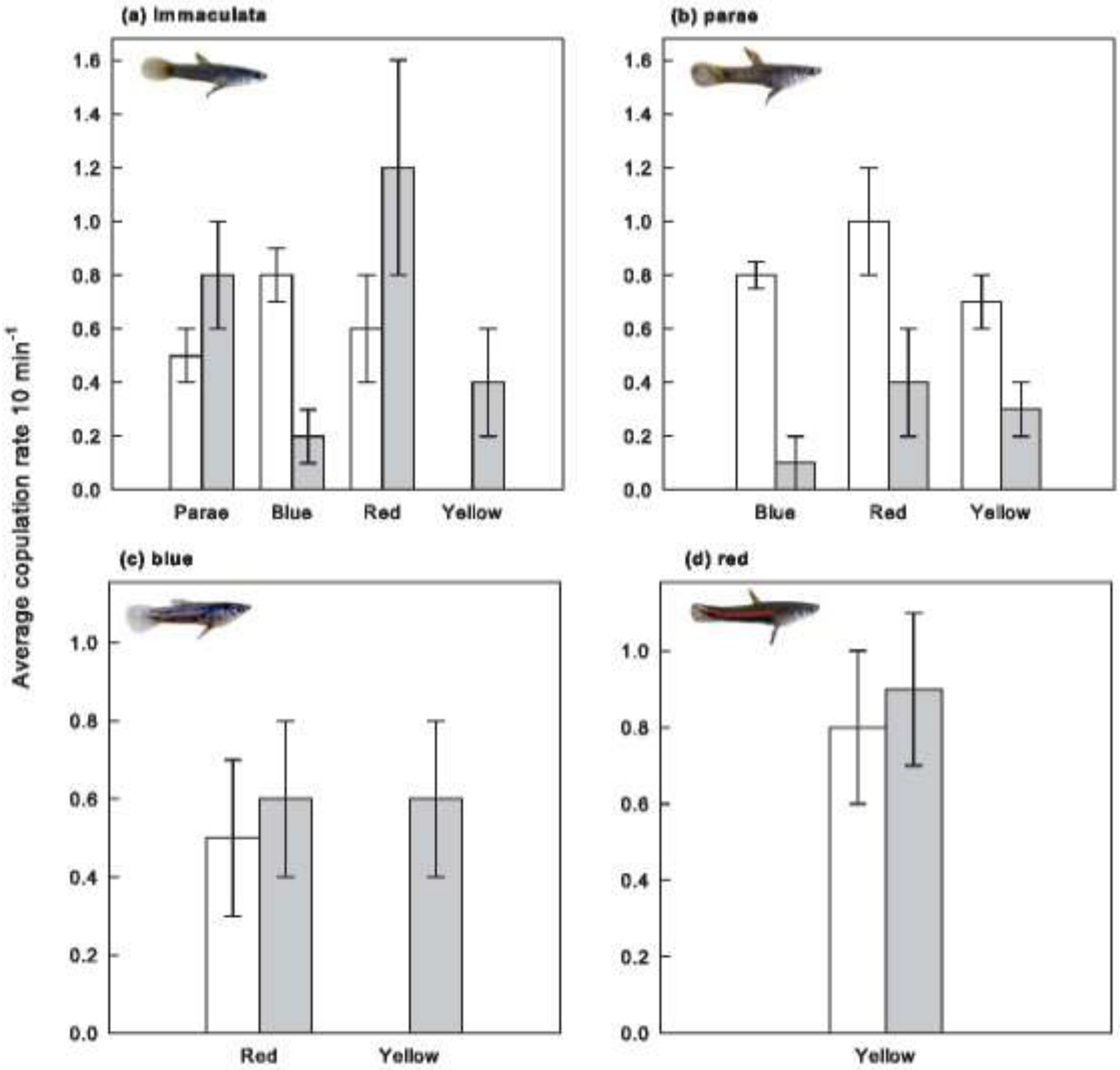

(a) immaculata
(b) parae
(c) blue
(d) red
Average copulation rate 10 min⁻¹
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Parae
Blue
Red
Yellow

Figure 3

SUPPORTING ELECTRONIC MATERIALS

Table A1. Standard body length (mm) of *Poecilia parae* males that were used in male-male competition trials. Columns to the left provide the descriptive statistics (Mean ± SD, minimum and maximum) for males from one morph, while adjacent columns to the right provide the descriptive statistics for the corresponding males used from a second morph **(**I = immaculata, P = parae, B = blue, R = red, Y= yellow). Sample sizes for each set of trials are provided (*n)*. The final two columns to the right provide the mean difference (± SD) between competing males (M_A-M_B). Negative values indicate that male 2 (M_B) was larger on average than male 1 (M_A).

M_A	Mean	SD	Min	Max	M_B	Mean	SD	Min	Max	n	M_A - M_B	SD
I	16.831	0.395	16.168	17.401	P	19.272	0.658	18.35	20.38	10	-2.440	0.296
	16.684	0.42	16.033	17.387	B	17.133	0.273	16.625	17.401	10	-0.449	0.19
	16.782	0.482	16.059	17.48	R	18.607	0.151	18.267	18.813	10	-1.825	0.35
	16.82	0.553	16.066	17.455	Y	18.143	0.254	17.739	18.568	10	-1.322	0.331
P	18.951	0.891	16.833	19.876	B	18.864	0.92	17.56	20.161	15	0.087	0.364
	19.717	0.465	19.075	20.342	R	19.879	0.467	19.442	20.9	15	0.161	0.196
	19.396	1.042	16.457	20.257	Y	19.731	0.377	19.334	20.735	15	-0.335	0.868
B	19.928	0.685	19.038	20.972	R	19.685	0.662	18.877	20.9	15	0.242	0.209
	19.24	0.737	18.437	20.083	Y	19.308	0.629	18.705	20.735	15	-0.068	0.447
R	18.924	0.409	18.267	19.418	Y	18.78	0.627	17.739	19.869	15	0.144	0.326

Table A2. Aggressive behaviours observed during male-male interactions in *Poecilia parae*. Modified from the terminology and description Liley (1966) offered for *P. reticulata* (the guppy).

Sparring: This behaviour is mostly initiated by a single male (challenger). The challenger will approach to the other male presenting one of its flanks with the dorsal and ventral fins fully extended and the body slightly arched towards the side facing the flank of the opponent. The body of the challenger will start quivering as an invitation to initiate the sparring. If the opponent accepts the challenge, it will immediately adopt an anti-parallel or parallel position to that of the challenger. During this period, the body and fin colorations become intense. Once both males are placed in their position, they will keep their body quivering and performing circular motions and moving upwards. During circling, both males would keep their initial posture. This behaviour can last between 2 – 45 seconds and can be repeated several times if none of the males are willing to retreat.

Tail slapping: This behaviour follows sparring and can be initiated by either the challenger or the opponent. The slap would be either directed towards the flank or head region of the opponent. If dominance has not yet been determined, males will continue tail slapping for short periods of time (2 - 4 seconds), and males will re-group in sparring position. However, if one of the males retreats, the tail slapping will be followed by an attack.

Chasing: the dominant male will go behind the loser male performing consecutive attacks. Chasing usually has an average of three attacks and/or chase and ends with male’s complete submission by head standing.

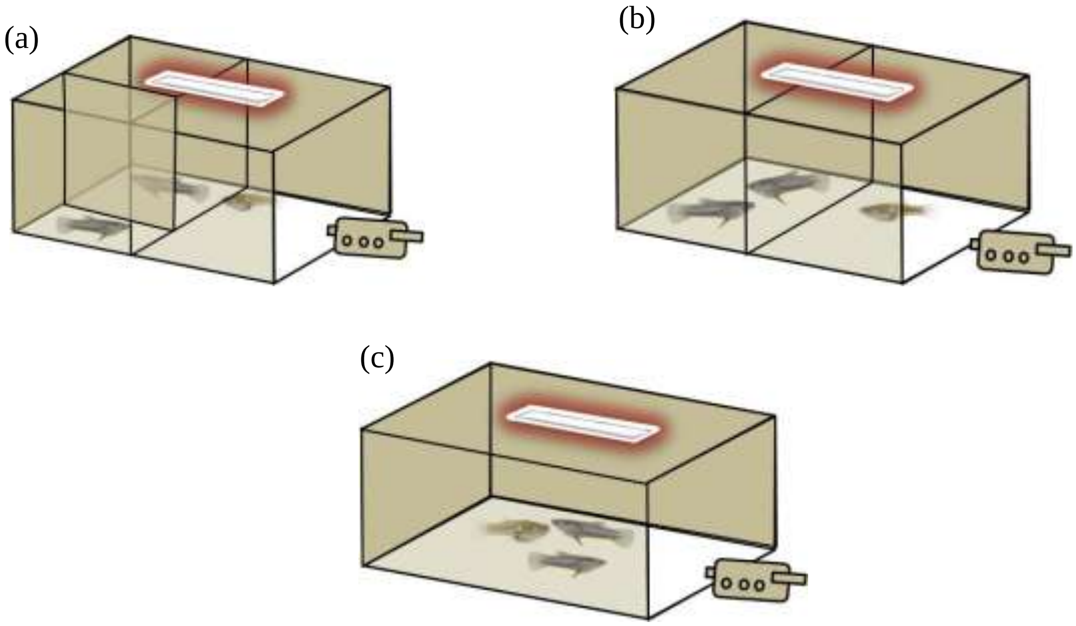
Attack: attacks are comprised by nibbles directed towards the flank of the other individual. However, the winner can nibble and keep attacking until the opponents flee, or can give a warning nibble and will resume their behaviour (e.g., eating or courting).

Escape: the male losing the male-male interaction usually flee away of the dominant male. If the male decides to remain close to the dominant male, attacks can continue and followed by chasing.

Headstands: to stop chase or attacks, the subordinate male will tilt his body vertically forming and angle $> 45°$ to the substrate.

Blocking: this behaviour is commonly observed after a male assumes the dominant role. The male will block access of other males to the female by not allowing other males to approach to the females. The dominant male will use his body to intercept the approach of the other males.

Figure A1. Experimental tanks. (a) Tank use for the female choice trials. The glass separating both males is a fixed non transparent barrier. (b) Tank use to determine aggressive behaviours between males, with a female observing the interactions through a transparent glass barrier. (c) Tank use to determine aggressive behaviours between males with the direct interaction of a female.



CHAPTER V

VARIATION IN THE VISUAL ENVIRONMENT MEDIATES THE MAINTENANCE OF COLOR POLYMORPHISM IN A POECILIID FISH

Hurtado-Gonzales, J. L., Loew, E. R., Uy, J. Albert C. *In Prep*. Variation in the visual environment mediates the maintenance of color polymorphism in a poeciliid fish.

ABSTRACT

Variation in the visual habitat may alter the appearance of male color patterns that are perceived by females, resulting in variation in mating preferences and therefore allowing the maintenance of alternative phenotypes in natural populations. Here, I tested this prediction by combining measurements of the visual environment, fish coloration and the sensitivity of visual photopigments of the South American color polymorphic *Poecilia parae* and that of their common cichlid predator, *Aequidens tetramerus*. As measured by the perceived differences between color patches and natural backgrounds, I found an important variation in the perception of conspicuousness of color male morphs by females as an effect of intersite variation in visual background. The results also revealed that cichlids are able to discriminate the same male color morphs that were also conspicuous for females. However, it is possible that males and females of *P. parae* are using parts of the spectral background that are least sensitive for predators. These results suggest a mechanism to explain the observed variation in female mating preferences, and in the maintenance of blue morphs in wild populations of *P.parae*.

INTRODUCTION

The expression of exaggerated courtship traits often evolve under the conflicting forces of sexual and natural selection (Andersson 1994; Endler 1980; Zuk and Kolluru 1998). For instance, individuals displaying elaborate signals that are attractive to the opposite sex are also preferentially targeted by predators or other natural enemies (Rosenthal et al. 2001; Zuk and Kolluru 1998; Hurtado-Gonzales et al. 2010). Under such scenarios, the tradeoff in attractiveness to females and susceptibility to predators can result in reduction in trait elaboration (Endler 1980) or alternative ways of communicating signals only detected by conspecifics (Banks 2001; Cummings et al. 2003).

The perception of signals depends on the physical properties of the habitat (e.g., ambient light and background, medium of light dispersion) and the sensory physiology of the receivers assessing the signals or cues (Banks 2001; Endler 1993; Endler 1992; Endler 1991; Endler and Mielke 2005; Fleishman and Persons 2001). Therefore, variation in any of these components of the signaling environment may influence the tradeoff between attractiveness and susceptibility to predation, as well as promote the evolution of alternative courtship colorations that correspond with the variable environment (Endler 1980; Levine and McNichol 1979; Chunco et al. 2007). Several studies provide support for this hypothesis, showing that variation in the photic environment [manakins: (Uy and Endler 2004; Uy and Stein 2007); African cichlids: (Dalton et al. 2010; Maan et al. 2006; Seehausen et al. 1999); bluefin Killifish: (Fuller 2002); pentamorphic Sulawesi fish: (Gray et al. 2008)] and sensory physiology [African cichlids: (Carleton et al. 2005); sticklebacks: (Boughman 2001); bluefin killifish: (Fuller et al. 2003); Guppy: (Hoffmann et al. 2007); passerine and avian predators: (Håstad et al. 2005); Crab spider: (Théry and

Casas 2002)] can select for signals that are similarly or differently exploited by predators and conspecifics and could play an important role in favoring the persistence of color polymorphisms (e.g., Bond 2007; Roulin 2004)

Although several studies in aquatic systems have established an association between visual physiology and the photic environment (Archer and Lythgoe 1990; Carleton et al. 2005; Endler 1992; Lythgoe 1979; McDonald and Hawryshyn 1995), only a handful of these have modeled the simultaneous effects of variation in visual environment and physiology on the outcome of female mate choice and predation (Boughman 2001; Endler 1991; Fuller et al. 2005; Fuller and Travis 2004; Kemp et al., 2008; Kemp et al. 2009; McDonald and Hawryshyn 1995). In the blue killifish (*Lucania goodei*), for instance, male coloration has evolved to match female visual physiology despite extreme variation in photic environments across populations (Fuller and Travis 2004). Similarly, in the threespine stickleback (*Gasterosteus aculeatus*), variable light environments have selected for distinct spectral tuning, and males in turn, have evolved traits to match both the visual environment and visual physiology of females (Boughman 2001). More recently, it has been reported that male color polymorphism in *Telmatherina sarasinorum* may be maintained by variation in the signaling environment resulting in variable, microhabitat-specific sexual selection on each morph (Gray et al. 2008). Altogether, these studies indicate the simultaneous importance of the variable visual habitats and receiver physiology in mediating the conflicting pressures of natural and sexual selection, which in turn, may lead to the origin and maintenance of distinct phenotypes.

Males of the live-bearing fish *Poecilia parae* exhibit five Y-linked discrete color morphs (Lindholm et al. 2004): (i) immaculata, the smallest and drab-coloured males that resemble juvenile females; (ii) parae, the largest males that exhibit a striped tail and black vertical body bars that intensify during social interactions; and (iii) the blue, red, and yellow males that are of intermediate body size and display colorful body flanks (Lindholm et al. 2004; Liley 1966). Previous field and laboratory studies suggest that the male-specific color polymorphism in *P. parae* may be maintained by a complex balance between the opposing effects of sexual and natural selection. First, controlled laboratory experiments indicate that variation in female mating preferences for specific morphs, with a majority of tested females preferring the carotenoid-based red and yellow morphs (Hurtado-Gonzales et al. 2010; Bourne et al. 2003; Lindholm et al. 2004). However, strong female preference for red and yellow males were also shared by their most common cichlid predator, offsetting the mating advantage of red and yellow morphs and thus keeping them relative rare in natural populations (Hurtado-Gonzales et al. 2010). Further, we have proposed that the outcome of the interaction between strong female mating preferences and predators for ‘rare’ males may have offered unique opportunities for less successful males to evolve alternative mating strategies (Hurtado-Gonzales and Uy 2010). We have documented, for instance, that the parae morph is quite effective in excluding sex competitors due to their agonistic behaviors that are always directed to blue, red, and yellow males but not to the drab immaculata morph (Hurtado-Gonzales and Uy 2010). In addition, the least preferred immaculata morph efficiently circumvents both female mate choice and male-male competition by relying on their similar appearance to juvenile females to steal copulations (Hurtado-Gonzales and Uy 2010; Hurtado-Gonzales and Uy 2009). No studies has yet to incorporate sensory data of the pentamorphic *P. parae* and that of their most common cichlid predator to explore how the perception of male color patches used during courtships may vary within a single species under different natural backgrounds. Variation in visual habitats and sensory parameters may, in turn, allow for the persistence of multiple color morphs within a single species

Here, I explore how (i) variation in the intensity of predation within and between populations, (ii) heterogeneity of the visual environment, (iii) variability in female visual perception (Endler 1991; Levine and McNichol 1979), and (iv) the interaction of these factors may lead to variation in female mating preferences, which, in turn, may allow for the persistence of multiple and discrete male mating strategies within a single species.

METHODS

My study sites were located on the east (6º 47.2' N, 58º 09' W, n = 15 sites) and west (6º 41' N, 58º 12' W, n = 15 sites) sides of the Demerara River, Republic of Guyana (see Hurtado-Gonzales et al. 2010 for characterization of sampling sites). Hereafter, both sampling areas will be referred to as the east and west populations. During January-March 2006 and May-August 2007, I recorded the relative abundance of females and the five *P. parae* male color morphs, and that of their common predator, *Aequidens tetramerus.* Fish were collected using dipnets and sampling occurred until individuals of *P. parae* and/or *A. tetramerus* were no longer present in subsequent samplings.

In January 2010, I visited the same sampling sites to collect males and females of *P. parae* which were transported to our laboratory in Syracuse University. Fish were maintained in 20 gal aquaria with treated water at 27 ± 1°C, on a 12:12 h light: dark cycle, and fed daily with live brine shrimp and Tetra-Min® (Melle, Germany) flakes two times per day until used for microspectrophotometry (MSP) analysis.

1. Reflectance measurements

Immediately after capture (January 2010), females and males sorted by morph type were housed in separate aquaria. Spectral reflectance of five randomly drawn individuals of each morph and five females were measured within 4 h of capture. I focused on the color patches present on the flanks of the body region because these patches are conspicuous, used during courtships and correlate with mating preferences (e.g., Liley 1966; Lindholm et al. 2004). I did not measure color patches on the caudal and dorsal fins because of the difficulties in gathering repeatable spectrometry readings of these small and translucent patches.

The color patches were illuminated and scanned at a 45° of their flank with an Ocean Optics USB2000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA) and a pulse xenon flash light source (Ocean Optics PX-2; as in Uy and Endler 2004). To allow for comparison across scans, each reflectance measure was standardized with a spectrally flat 97% reflecting spectralon white standard (Labsphere; North Sutton, NH, USA), and a dark current reading. Scans (Figure 1) were taken from a *ca*. 1-mm-diameter circle across 300–700 nm spectral range.

2. Characterization of the Visual Habitat

I collected spectral irradiance (Figure 2a) and background radiance scans (i.e., ambient light; Figure 2b) in the 30 different sites [east (n = 15) and west (n = 15) populations] previously sampled and where *P. parae* is common. Measurements were taken early in the morning (6:00 – 8:00 h) when *P. parae* was most socially active (Liley 1966). I used a submersible Planar Irradiance collector (Hydro-Optics, Biology, and Instrumentation Laboratories, WA, USA) attached to an Ocean Optics USB2000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA) to collect ambient light and background measures.

At each site, irradiance was collected horizontally (i.e., downwelling) every 5 cm to a depth of 20 cm because *P. parae* inhabits shallow waters and most social and foraging activities occur within this depth (Liley 1966). Background radiance was measured by modifying the submersible irradiance probe with a black opaque cap of 40 mm in length and 4 mm in diameter so that only light from a small solid angle can reach the detector surface. Radiance scans were taken every 15° angle until completing a semicircle, starting at an approximate location where an individual of *P. parae* was observed or likely to interact with females. The aim of these radiance scans was to measure the average visual background against which an individual would be perceived by a conspecific or predator. To allow for direct comparison among scans, the spectrophotometer was calibrated with a standard light source (LiCor 1800-02; see Endler and Théry 1996).

Brightness was determined by summing the irradiance or radiance spectrum from 300 – 700 nm (n = 15 sites in the east and n = 15 sites in the west populations). I then used a principal component analysis (PCA) to derive variables that represent the color properties of ambient light and visual background. To do so, I first standardized each spectrum for ‘brightness’. That is, the raw spectrum was summed and then divided at each wavelength by the summed spectrum (see Endler 1990). The standardization of the data, thus, would factor out total ‘brightness’ so that we could compare the shape of the spectra and calculate PC factors that specifically represent spectral properties independent of brightness.

Because my radiance and irradiance readings were done early in the morning, at the beginning of each measurement, we additionally recorded tree cover indicated as (0 = none; 10 – 50 % = low, and more than 50% = high; as in Fuller 2002). Then, we measured the water depth (cm) of each sampled portion of the stream.

3. Microspectrophotometry

To characterize the spectral sensitivity of *P. parae* cones, seven adult males [immaculata (*n* = 2), parae (*n* = 2), blue (*n* = 2) and yellow (*n* =1) morphs] and 10 adult females were used for microspectrophotometry (MSP) analyses at Cornell University, Ithaca, New York. MSP readings were collected following methods described in Loew (1994). In brief, each fish was housed in complete darkness for 24 hours prior to the MSP analyses. Each fish was euthanized with MS-222 (3-aminobenzoic acid ethyl ester) and the eyes enucleated under dim red light (Safelight N° 2, 15 W bulb, Kodak$^{©}$, USA). All subsequent preparations were done using infrared light (> 800 nm, Safelight N° 11, Kodak$^{©}$, USA) using image converters. The eyes were hemisected and the retinas removed under buffer solution (cold Ca/Mg-free Ringer’s solution pH 7.4 supplemented with 5 % sucrose). Retinas were then separated from the retinal pigment epithelium and macerated using razor blade fragments and tungsten needles (Loew 1994, Loew et al. 2002). A small sample of the treated retina was transferred to a cover slip and placed under a second cover slip edged with silicone grease. The MSP settings and functioning used in this study is described in Loew (1994) and Loew et al. (2002). The 1.5 μm^2 rectangular measuring aperture was produced by demagnification using a Leitz (Oberkochen, Germany) 180X quartz mirror objective. A Zeiss 100X Ultrafluar (0.85 NA) collected the transmitted light and focused it onto the photomultiplier photocathode. Retinal cells were selected individually. Determination of double cones was based on intact pairs of examined cells only. MSP scans were collected at 1 nm intervals scanning from 750 to 350 nm, and back from 350 to 750 nm (Loew 1994). Comparisons of both scans are commonly used as good indicators of over-filtering and bleaching.

The $\lambda_{\max}$ (the wavelength at maximum absorbance for a template-derived visual pigment best fitting the experimental data) was determined by using a template fitting (as in Fuller et al. 2004). The process of determination of $\lambda_{\max}$ involves the following steps: (i) smooth the data, (ii) determine the peak absorbance ($X_{\max}$), (iii) normalize the absorbance curve, (iv) fit the templates, (v) calculate the standard deviation (SD) of $\lambda_{\max}$, and (vi) compare with the actual data and choose the best fit (Loew 1994).

Pre-selected spectra were smoothed prior to normalization with digital filter routines, ‘Smooft’ (Press, 1989). For instance, a smoothed spectrum was overlaid on the raw data and visually compared for over-filtering or for spurious data points that had shifted the apparent maximum. If shifts were perceived, then the unsmoothed data were used. The peak absorbance ($X_{\max}$) used in the normalization represented the calculated maximum of the best fit Gaussian to the data points 20 nm either side of the estimated-by-eye absorbance maximum of the alpha band. Using *Xmax*, the data were then normalized using standard methods (Mansfield 1985; MacNichol 1986). Finally, normalized data were best-fitted using the A_1 templates (Lipetz and Cronin 1988). Since MSP wavelength error is ± 1 nm, all visual pigments are reported to the nearest integer. Absorbance spectra from 30-40% of cells measured were retained for analysis since the information from other cells was of insufficient quality for template fitting. Hence, inspected cells are greater than the sample sizes I report in this study.

4. Quantitative models of perception

To analyze the perception of color signals, I used two different approaches. First, I estimated chromatic contrast based on the Euclidian distance between a color patch and the visual background plotted in tetrahedron space. Hence, I used the model developed by Chittka (1992) as implemented in Avicol ver. 5.0 (Gomez, 2011). This color space model uses data on: (i) the reflectance spectra of a color patch (Figure 2), (ii) reflectance spectrum of the visual background (derived by subtracting radiance from irradiance), (iii) irradiance spectrum of the ambient light illuminating the color patch, and (iv) the spectral sensitivity of the photoreceptors from *P. parae* (Figure 3a) and the cichlid predator (Figure 3b) visual systems to estimate the difference in photon capture of cones between an object and its background. Due to the absence of data characterizing the visual spectra sensitivity of the cichlid *A. tetramerus*, I used the available published information from *A. pulcher*, a congener and major predator of adult guppies in Trinidadian streams (Endler, 1980). Thus, to model spectral sensitivity, I used templates fitted to MSP data in *P. parae* (as explained in results) and *A. pulcher* (Kröger et al. 1999) cone peak sensitivities estimate (SWS = 453 nm; MWS = 530 nm; LWS = 570 nm). Absorbance functions were calculated using the Govardovskii's pigment absorbance template equations (Govardovskii et al. 2000) considering that *P. parae* and the cichlid predator possess A1 (rhodopsin; this study) and A2 (porphyropsin; Wagner and Douglas 1989) visual pigments, respectively. To my normalized visual pigments absorbance, I also incorporated normalized spectra data from lens transmittance estimated from *P. reticulata* [the guppy; T_{50} = 315 nm; (Thorpe et al. 1993)], and for *A. tetramerus* [T_{50} = 432 nm; (Douglas and McGuigan 1989; Kirsch et al. 1989; Muntz 1973)]. The lens transmission (T_{50}) normalized curves were built using equation 7 in Endler and Mielke (2003) by setting the maximum reflectance to 1 (Figure 1A additional supporting file 1).

Considering that receptor noise limits the discrimination of signals from its background or other color patches (Vorobyev and Osorio 1998, Vorobyev et al. 2001), I used a second approach that allowed me to make a more realistic comparison between conspecifics and perception of color signals by the predator. Although the physiological model of Vorobyev et al. (2001) depends on several assumptions (as described for this study below), it determines the difference threshold or 'just noticeable difference' (JNDs) that would allow an organism to distinguish between objects. A JND is the minimum amount by which the intensity of a stimulus must be changed to allow an organism to detect an object from the background. Hence, JNDs with values ≤ 1 would indicate that two objects are indistinguishable, while higher JNDs values indicate that two colors are correspondingly distinguishable. The Avicol ver. 5.0 (Gomez 2011) freeware program incorporates the Vorobyev et al. (2001) model requiring the same information for the Chittka's model (1992) as mentioned above, but adding (i) information about the ratios of cones between classes from *P. parae* and *A. tetramerus* and (ii) estimation of the Weber fraction for LWS cones in *P. parae* and predator. The Weber fraction represents the amount of units that would allow an individual to perceive two colors of the same intensity as different from each other. The estimation of visual cone densities were derived from published data of *Poecilia reticulata* retina based on the relative encounter rates of different cone classes inspected during retina preparations (Archer and Lythgoe 1990). This resulted in a cone density of 1: 2: 2: 4 (VSWS, SWS, MWS and LWS, respectively). For the cichlid predator, I used the relative densities of photoreceptor estimated for the South American cichlid, *Amphilophus longimanus*, inhabiting clear freshwater streams (cone density = 1: 2.6: 3.4; SWS, MWS and LWS, respectively; Levine et al. 1979; Levine and McNichol 1979). Finally, I assumed a conservative Weber fraction of 0.05 (at threshold) for the LWS in fish and that a JND < 1 indicates that individuals are unable to discriminate two color patches or objects. Overall results were similar even after adjusting the Weber values between 0.04, and 0.01).

Although the models used in this study are not designed especially for fish vision, their applicability comprises a majority of taxa that range from insects, including anurans to mammals [bees (Hempel de Ibarra et al. 2001), crustaceans (Cummings 2007), fishes (Marshall & Vorobyev; Lettieri et al. 2009), anurans (Siddiqi et al. 2004), birds (Vorobyev et al. 1998), and primates (Osorio et al. 2004)].

5. Statistical Analyses

All data were checked for normality, and all statistical analyses were implemented using STATISTICA v. 7.0.0. StatSoft, Inc. (Tulsa, OK, U.S.A).

To test whether perceived conspicuousness differed between morphs, I compared the chromatic contrast values of the five morphs as perceived by conspecifics and predators, using nested ANOVAs. ‘Population’ (east and west), sites (sampling sites within-populations) and ‘morphs’ (males and females of *P. parae*) were used as fixed factors/independent variables. The dependent variables were represented by the values of chromaticity as obtained by Chittka’s quantitative model (see section 4) and were designated as perception by ‘conspecifics’ or ‘predators’. ‘Morphs’ were nested within ‘sites’, which in turn, was within ‘populations’. I used Fisher’s LSD method for *post hoc* comparisons to determine differences in the perceived conspicuousness of *P. parae* males and females.

Using the jnds values, I then tested whether color contrast values of morphs and females of *P. parae* as discriminated by conspecifics differ from the contrast values perceived by predators. Hence, I conducted a paired t-test analysis. For this purpose, I considered all male color morphs and females of *P. parae* as ‘conspecifics’ and *Aequidens* spp cichlid as “predator”. Comparisons were run for populations, east and west.

I used a one-way ANOVAs to compare and test for differences in brightness and spectral properties (i.e., hue and chroma represented by PC scores) of ambient light and visual background between populations.

Finally, to determine which variables best explained or predicted the variation in the relative abundance of color morphs, I performed a backwards stepwise multiple regression analysis with morph abundance as the dependent variable and populations (n = 2; dummy variable), tree cover (dummy variable), water depth (cm), brightness and PC scores (i.e., hue and chroma) of the visual environment (i.e., irradiance and radiance; as specified above and in results) and the relative abundance of predators.

RESULTS

1. Ambient light and visual background

Unstandardized irradiance and radiance spectra characterizing the visual habitat of *P. parae* and that of its most abundant predators are summarized in Figure 2. The PCA for the standardized irradiance resulted in three PC scores accounting for 95.69 % of the total variation between sites in hue and chroma (PC1= 38.08%; PC2 = 29.37%; PC3 = 28.24). Component loadings show that PC1 was positively associated with the UV wavelength spectrum, PC2 positively associated with middle wavelengths (500-540 nm, green), and PC3 was positively associated with short wavelengths (420-460 nm, violet- blue; Figure 4a). I did not detect any statistically difference on overall brightness between populations (Table 1).

The PCA for the standardized radiance extracted four PC scores accounting for 96.71 % of the total variation in hue and chroma (PC1= 35.72%; PC2 = 32.22%; PC3 = 18.06; PC4 = 10.71). Component loadings of the radiance analysis indicates that PC1 correlates positively with UV wavelengths, PC2 correlates positively with medium wavelength (540-580nm, green-yellow), and PC3 correlates positively with short wavelengths (peak at 480 nm, blue). PC4 explained some of the existent variation in long wavelengths (660-680 nm, orange-red; Figure 4b), which was absent in the PCA of the standardized ambient light data. An ANOVA indicated that the east and west populations showed significant variation in the spectral properties of the background related to UV and short wavelengths only (PCs 1 and 3; Table 1). Finally, there was a significant difference in brightness, with west population showing greater total intensity in irradiance and radiance compared to the east population (Figure 2; Table 1).

2. *Microspectrophotometry*

The MSP analysis suggests that *P. parae* possess spectral sensitivities that best fit the A1 visual pigment templates. I discovered the presence of four cone classes and individual rod cells. Rod cells had an average λ_{max} of 503 ± 1.2 nm (mean ± SD, with 6 ± 3.6 cells/individual; n = 17 individuals; λ_{min} = 499 nm; λ_{max} = 505 nm). Two single cones were discovered: one with very short λ_{max} of 356 ± 1.4 nm (mean ± SD, with 2 ± 0.9 cells/individual; n = 11 individuals; λ_{min} = 354 nm; λ_{max} = 357 nm, “UV”), and another short wave sensitivity with an average λ_{max} of 407 ± 1.6 nm (mean ± SD, with 4 ± 1.7 cells/individual; n = 17 individuals; λ_{min} = 404 nm; λ_{max} = 410 nm; “V”). The other cones are composed of paired cones (i.e., two fused cones) with one cone more sensitive to shorter wavelengths than the other (double cones) or both cones expressing the same wavelength sensitivity (twin cones). The three visual pigments representing the long-wavelengths and that are the most dominant had an average λ_{max} of 456 ± 1 nm (mean ± SD, with 3.5 ± 1.4 cells/individual; n = 17 individuals; λ_{min} = 454 nm; λ_{max} = 459 nm), 526 ± 0.8 nm (mean ± SD, with 3.9 ± 1.4 cells/individual; n = 17 individuals; λ_{min} = 525 nm; λ_{max} = 528 nm), and 543 ± 1 nm (mean ± SD, with 3.4 ± 1.2 cells/individual; n = 17 individuals; λ_{min} = 542 nm; λ_{max} = 546 nm). Although the analysis did not suggest sex or morph differences in cone sensitivities, I found that some individuals expressed 526/533 or 553/553 double cones, and both types of double cones were never present in the retina of the same individual.

3. *Chromatic contrasts as perceived by conspecifics and predators*

The analysis suggests significant population differences in the levels of chromatic contrast of *P. parae* color patterns as perceived by conspecifics (nested ANOVA: $F_{1, 75}$ = 19.9, $P < 0.001$). Further, the nested ANOVA indicated an important sites[population] (nested ANOVA: $F_{14, 75}$ = 18.87, $P < 0.001$) and morph[site] (nested ANOVA: $F_{75, 75}$ = 2.46, $P < 0.001$) effect in perceived chromatic contrasts (Figure 5a-b; Figure 1B additional supporting file 1). Fisher LSD post-hoc comparisons suggest that the red males are overall the most conspicuous (all $P < 0.04$) in both populations (i.e., east and west), with no significant differences among immaculata, parae, blue and yellow males (all $P > 0.07$).

The chromatic contrasts of *P. parae* as perceived by the common cichlid predator did not differ between the east and west populations (nested ANOVA: $F_{1, 75}$ = 3.17, $P = 0.08$) or sites nested in populations (nested ANOVA: $F_{14, 75}$ = 1.37, $P = 0.19$) and morph nested in sites (nested ANOVA: $F_{75, 75}$ = 0.68, $P = 0.95$). Hence, I reanalyzed the data by pooling sampling sites from the east and west populations. This analysis indicated a significant differences among sites in chromatic contrast as detected by predators (nested ANOVA: $F_{14, 75} = 3.87$, $P < 0.01$; Figure 5c-d; Figure 1C additional supporting file 1). Although predators seem to coincide with the perception of females after contrasting morphs using different visual backgrounds at each site, the analysis did not capture any significant differences in morphs[sites] (nested ANOVA: $F_{75, 90} = 0.63$, $P = 0.98$).

Analyses of perceived conspicuousness using Vorobyev's et al.'s (2001) physiological model provided similar patterns to analyses using Chittka's (1992) (Figure 1B additional supporting file 1). The distribution of jnds values indicates that red is the most detectable morph (east: 45.34 ± 4.25 jnds, n = 15 sites; west: 34.89 ± 5.83 jnds, n = 15 sites) by females. The second most detectable morph varies across populations. Yellow males were more conspicuous (33.42 ± 3.17 jnds, n = 15 sites) in the east whereas blue males were more conspicuous (27.94 ± 2.41 jnds, n = 15 sites) in the west population. Figure 6 shows differences in the perception of the two most noticeable males comparing east and west populations and as perceived by females considering their jnds values. Note, however, that although red males were typically most conspicuous, blue males were sometimes more conspicuous than red males at several sampling sites (Fig. 6)

With respect to predators, the Vorobyev's model indicates that the most discernable males in the east were the red (9.55 ± 0.91 jnds) and yellow morphs (8.86 ± 0.92 jnds). Immaculata, parae, blue, and females show very low discrimination values (< 5 jnds; Figure 1C additional supporting file 1). Along the sampling sites in the west population, the blue, red and yellow males show higher jnds values (> 6 jnds) than immaculata, parae and females (< 5 jnds). Figure 6c-d shows differences in the perception of the three most noticeable male color patterns comparing east and west populations and as perceived by predators based on jnds values.

A further analysis of the noticeable differences (i.e., jnds) among morphs suggest that color patches that are used in mate choice and male-male competition are more conspicuous to conspecifics than to predators, and the pattern is consistent for comparisons made between the east ($t_{178} = 18.65$, $P < 0.001$) and west ($t_{178} = 13.39$, $P < 0.001$) populations (Figure 7).

The stepwise regression analysis indicates that the relative abundances of male color morphs and females of *P. parae* are predicted by the properties of the photic environment (Table 2). The abundance of the three colorful males (blue, red and yellow) is influenced by the UV-blue light available from the ambient light (Table 2). Blue and yellow males were most abundant in habitats with low levels of UV light. Conversely, red males were abundant in habitats with high levels of UV light (Figure 8). Both red and yellow males were also more common in brighter sites with high overall intensity (Table 2).

The regression model suggests that the relative abundance of the immaculata, parae and females is positively associated with the amount of medium to long wavelengths light in the background. Finally, the stepwise analysis showed that only the abundances of yellow males and females are affected by the presence of predators (Table 2).

DISCUSSION

Perception of color signals by conspecifics

My results suggest that variation in signaling environment translated to variation in conspicuousness of male color morphs. Although red males were predominantly the most conspicuous morph, blue males were more conspicuous than red males in some habitats within the west population. Likewise, the relatively high contrast values of the parae and the drab immaculata males and females (Figure 1B-C additional supporting file 1) suggest that the conspicuousness of both morphs is related to the inherent properties of their color patterns containing light patches (i.e., highly reflective unsaturated colors such as silver/gray) adjacent to body areas with low reflectance and chroma (i.e., dark gray). This type of color patterns is conspicuous in nearly all light conditions (Endler 1992).

Interactions between males and females have shown the existence of variable individual mating preferences in *P. parae*. That is, a great proportion of tested females found red and yellow males as the most attractive males whereas others found blue and parae males to be most attractive (Hurtado-Gonzales et al. 2010; but see also Bourne et al. 2003 and Lindholm et al. 2004). These results are in agreement with the current findings suggesting that variation in individual female mating preferences may be the result of increase male conspicuousness under different segments of their habitat. Hence, these findings provide a mechanism supporting the observed variation in female mating preferences (Hurtado-Gonzales et al. 2010), and in particular, a role for the visual environment contributing to the maintenance of the blue morph in the population.

Traits that are often most attractive and conspicuous to females are also detectable by same sex competitors, with important outcomes during male-male interactions that may affect the mating success of conspicuous males (Qvarnström and Forsgren 1998; Wong and Candolin 2005). Here I present evidence that all color patterns are readily discriminated against any natural background by competitors and this is a relevant outcome given that intrasexual competition in *P. parae* also dictates the mating success of dominant males over attractive males (Hurtado-Gonzales and Uy 2010).

Perception of color signals by predators

A major prediction in the theory of sexual selection is that in the context of mate choice and predation, females and predators often coincide in their attraction for conspicuous signals (Andersson 1996; Boughman 2001; Uy and Endler 2004; Cummings et al. 2003; Rosenthal et al. 2001). Predators however should have different spectral sensitivities compared to their prey. That is, natural selection should favor color patterns that are most conspicuous to conspecifics and less conspicuous to predators (Endler 1980). The first set of results suggests that predator and conspecific perception of color morphs is similar, but there is a trend in which predators are able to discriminate red, yellow and blue males better compared to parae and immaculata males and females (Figure 1B and 1C Additional supporting file 1). This differential perception of conspicuousness may translate in higher risk of predation depending on the background against which a particular male color morph is viewed. The second set of analyses suggests that *P. parae* color patches are always more conspicuous to conspecifics than to predators. Both color based-models (i.e., Chittka’s and Vorobyev’s) agree in that the perception of predators coincides with that of females, in which the most conspicuous morphs resulted also the most conspicuous prey for predators. Nevertheless, as previously suggested in other taxa (e.g., birds: Håstad et al. 2005), males of *P. parae* bear color patches that are significantly more conspicuous to conspecifics than to their most common aquatic predator. In sum, these patterns suggest a ‘secret channel’ or a way for colorful males to reduce the costs of bearing colorful traits.

Visual Habitat

In this study, the analysis with regard to habitat light intensity and spectral quality across sites between the east and west populations consistently suggest that differences in perception of male color morph are mediated mainly by the visual background. Variation in UV light can predict the relative abundance of male color morphs (i.e., blue, red and yellow) across sites and also between populations, presumably because these males appear more attractive to females and thus experience higher mating success. In contrast, the most abundant males (i.e., immaculata and parae) were affected by the spectral properties of the radiance background favoring medium wavelengths over shorter and longer wavelengths, and were positively associated with deeper streams, which may contribute to faster scattering of shorter wavelengths. These findings are consistent with studies that document linear relationship between the visual background and the predominant color patterns, in which color traits often show higher chromatic contrast values and experience increased conspicuousness [sticklebacks *Gasterosteus aculeatus* (Boughman 2001), bluefin killifish *Lucania goodei* (Fuller 2002), golden-collared manakin *Macanus vitellinus* (Uy and Endler 2004)].

Finally, the relative abundance of predators characterizing each sampled habitat was found to be only associated with the relative abundances of yellow males and females with no significant effects on any other male color morph. Although the relationship did not significantly predicted the abundance of the other color males (i.e., red and blue), beta coefficients of the stepwise regression model indicated a negative trend where color male morphs tend to be more abundant in habitats with lower numbers of predators.

Visual pigments

My study incorporates the measurement of photoreceptor sensitivities of *P.parae* established with the use of microspectrophotometry (see section 2, in results). Within the limitations of the MSP approach, I identified all the opsin proteins being expressed in recordable amounts. However, the existence of other opsin proteins at very low levels that are co-expressed in some cells is still possible. The results draw some important parallels suggesting that the visual pigments of *P. parae* appear to be as complex as the one documented for the closely related and also color polymorphic guppy (*P. reticulata*) (Archer and Lythgoe, 1990, Weadick and Chang, 2007, Hoffmann et al., 2007, Ward et al., 2008) within the long wavelength sensitive cones. *P. parae* inhabits shallow clear waters and it is also found syntopically with guppies. In contrast to most temperate freshwater species both have visual pigments that are likely pure rhodopsin. This is supported by my study that indicates that all inspected photoreceptors were best fit by a vitamin A1-based pigment template. Second, my results suggest the presence of UV, blue/violet, green and variable red/orange sensitive cone opsin proteins. The long wavelength sensitive cones represent an important polymorphic class (between 526 and

553 nm) that identifies three different peaks of absorption at 533, 543 and 553 nm. Similarly as in guppies (Archer and Lythgoe, 1990), I did not find any evidence suggesting that cone polymorphism is sex-linked in *P. parae*, despite the fact of the extreme color polymorphism exhibited by males. However, the MSP data suggests that some individuals of *P. parae* can express at least one different LWS described either as 526/533 nm or 553/553 nm paired cones, but both are never present in the same individual.

A future study will aim to explore how the evidenced polymorphism in cone sensitivity in the long wavelength affects the conspicuousness of conspecifics. At this point, this study has only evidenced color discrimination using the peaks of absorbance of the recorded visual pigments.

Are the color-based models reliable in the prediction of morph conspicuousness?

Variation in the perception of conspecifics due to differences in visual backgrounds is consistent with previous findings that uncovered variation in female mating preferences in *P. parae*. However, based on morph frequencies recorded since 2002 (Hurtado-Gonzales et al. 2010; but see also Liley 1966, for some older records), there is an indication that for the most attractive red and yellow males higher conspicuousness may not necessarily translate into increase reproductive success. I have proposed that because carotenoid-based colorations represent conspicuous visual signals for a major cichlid predator, the relative abundance of the red and yellow males may be, to a certain extent, conditioned by the effects of natural selection in the form of predation (Hurtado-

Gonzales et al. 2010). That is, while females contribute to the mating success of red and yellow males, predators may play an important role in keeping carotenoid-based males rare. In this study, there is evidence that the cichlid predator is able to discriminate carotenoid-based color morphs compared to all others and there is also evidence that both carotenoid-based morphs experience higher risk of predation compared to all other male color morphs (Hurtado-Gonzales et al. 2010).

This balance between natural and sexual selection (by maintaining attractive males rare) may have opened new possibilities for other alternative mating strategies to have a sufficient selective advantage to be maintained in the population. The drab immaculata male that resembles the coloration and appearance of juvenile females has been found consistently to be the least attractive morph by females (Bourne et al. 2003; Lindholm et al. 2003; Hurtado-Gonzales et al. 2010). Although in this study, immaculata males have high contrast values when compared to different natural backgrounds, they are unlikely to be chosen during pre-copulatory events and even unable to win during direct confrontation against other type of males (Hurtado-Gonzales and Uy 2010). However, immaculata males can successfully circumvent both female mate choice and male-male competition by taking advantage of their female-like appearance and by using a sneak copulation strategy (Hurtado-Gonzales and Uy 2009; Hurtado-Gonzales and Uy 2010). The sneaker strategy of immaculata males is also accompanied by a relatively larger investment in testes size, making them perhaps more competitive in sperm competition (Hurtado-Gonzales and Uy 2009). In contrast, the parae morph is likely to take advantage of their increase conspicuousness and gain some mating success by means of elaborated courtships. Nonetheless, I have shown that the parae morph enhances their mating success by 1) circumventing female mating preferences by preventing other males from gaining access to females and 2) modifying female preferences after a successful aggressive interaction with other competitors are witnessed by receptive females (Hurtado-Gonzales and Uy 2010).

A relevant outcome of this study is that blue males may persist in the population because females vary in their mating preferences. That is, in habitats in which blue males are more conspicuous than red and yellow males, the blue morph should experience overall higher mating success (Hurtado-Gonzales et al. 2010; Hurtado-Gonzales and Uy 2009). Males that are conspicuous are more attractive to females in *Poecilia reticulata* (Houde 1997), for instance.

In summary, the present study considers the photic conditions when *P. parae* and their most common predator, the cichlid *A. tetramerus* are active and color vision is utilized. The results for color comparisons at the species level indicate that females of *P. parae* are able to perceive the red male as the most conspicuous among males. According to my initial prediction, variable visual habitat seems to shape the dynamics of male color conspicuousness to the point that in some places blue males are able to gain increase chromatic contrast. This observation suggests that different phenotypes may be favored by the same sexual selective process under different lighting conditions (Gamble et al., 2003). That is, female mate choice likely favors the maintenance of the most conspicuous morphs, in habitats where a color morph (i.e., red, yellow and blue) appears conspicuous. This, along with previous work on different species adds to the growing evidence suggesting a strong role for environmental heterogeneity in favoring the maintenance of color polymorphisms in other fish species (e.g., Boughman 2001; Fuller 2004; Gray et al. 2008; Lettieri et al. 2009).

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TABLES

Table 1. Results of one-way ANOVAs for the comparisons of the PCA scores from the habitat light measurements of *P. parae*.

Variable	**d.f.**	**F**	***P***	**Mean (PC scores) comparisons**
Brightness				
Irradiance	1, 28	4.56	0.04	west > east
Radiance	1,28	3.43	0.07	west > east
Very short wavelengths				
UV				
Irradiance	1, 28	1.10	0.30	east > west
Radiance	1,28	5.39	0.02	east > west
Short wavelengths				
Violet and Blue				
Irradiance	1, 28	0.79	0.38	east > west
Radiance	1,28	8.04	0.01	east > west
Medium wavelengths				
green-yellow				
Irradiance	1, 28	2.83	0.10	east > west
Radiance	1,28	0.63	0.44	east > west
Long wavelengths				
orange-red				
Radiance	1, 28	3.65	0.06	east > west

Table 2. Summarized statistics for model validity and model variables as discerned by the multiple regression stepwise criteria for each of the color morphs and females of *P*. *parae*. (+) or (-) signs of the standardized *β* coefficients. Additional variables refers to the variables that although no significant remained in the final model. Variance explained refers to values of the adjusted R^2.

Morph/female	**Variables significant in initial model**	**Variables significant in final model**	**Additional variables in final model**	**Variance explained (%)**	**Model**	***P***
Immaculata	None	Depth (+) Radiance MW (+) Radiance LW (-)	Brightness Radiance (+) Radiance SW (-)	28	$F_{5,24} = 3.28$	0.021
Parae	Radiance MW Radiance LW	Radiance MW (+) Radiance LW (-)	Radiance UV(-)	42	$F_{3,26} = 7.88$	0.001
Blue	None	Radiance UV (-) Radiance SW (-) Irradiance UV (-)	None	53	$F_{3,26} = 11.93$	<0.001
Red	Cover (-) Radiance LW (-) Brightness Irradiance (+)	Cover (-) Radiance SW (+) Radiance LW (-) Brightness Irradiance (+) Irradiance UV (+) Irradiance SW (-)	None	43	$F_{6,23} = 4.73$	0.003
Yellow	Irradiance UV	Brightness Irradiance (+) Irradiance UV (-) Irradiance SW (+) Abundance Predators (-)	Radiance SW (-) Radiance LW (-)	49	$F_{6,23} = 5.71$	0.001
Female	Abundance Predators (-) Radiance SW (+)	Brightness Radiance (+) Radiance SW (+) Abundance Predators (-)	Irradiance UV (-)	35	$F_{4,25} = 4.92$	0.005

FIGURES LEGENDS

Figure 1. Mean reflectance of males (n = 5, for each male type) and females (n = 5).

Figure 2. Mean (± 1 SE) ambient light (a) spectra and (b) background characterizing the habitats in the east (filled circles) and west (open circles) populations where *P. parae* and their predators are found.

Figure 3. Normalized absorbance spectra of cone visual pigments of *P. parae* (A) and *A. pulcher* (B). Cones are VSWS= very short-wavelength sensitive, SWS= short-wavelength sensitive, MSW= medium-wavelength sensitive, and LWS= long-wavelength sensitive.

Figure 4. Component loadings from PCA of standardized (a) irradiance and (b) radiance.

Figure 5. Conspicuousness of *P. parae* assayed by the perceived difference in colour (chromatic contrast) against natural backgrounds at different sampling sites. In the upper panels, contrasts as perceived by conspecifics in the east (a) and west (b) populations. In the lower panels, contrasts as perceived by predators in the east (c) and west (d) populations. Symbols are connected by lines to facilitate better visualization.

Figure 6. Differences in females (upper panels) and predator (lower panels) perception of the two most conspicuous males in the east and west populations. Colors represent the corresponding morph type, with the upper portion of the circle representing the most

conspicuous male color morph. (Red for red, yellow for yellow, blue for blue, and cyan for parae morphs).

Figure 7. Mean (± 1SE) differences in average discriminability (Δs, which represents JNDs values) between color patches of all five *P.parae* morphs as viewed by conspecifics and by predators. Data are presented as box plots indicating their medians and quartiles. Extreme data points are represented by the 5th and 95th percentiles. Prey = *P. parae*; and predators = *Aequidens* spp; E = east; W = west.

Figure 8. Relationship between the abundances of the three most colorful males of *P. parae* and abundances of UV light in the visual background. Each data point represents a sampled site.

FIGURES

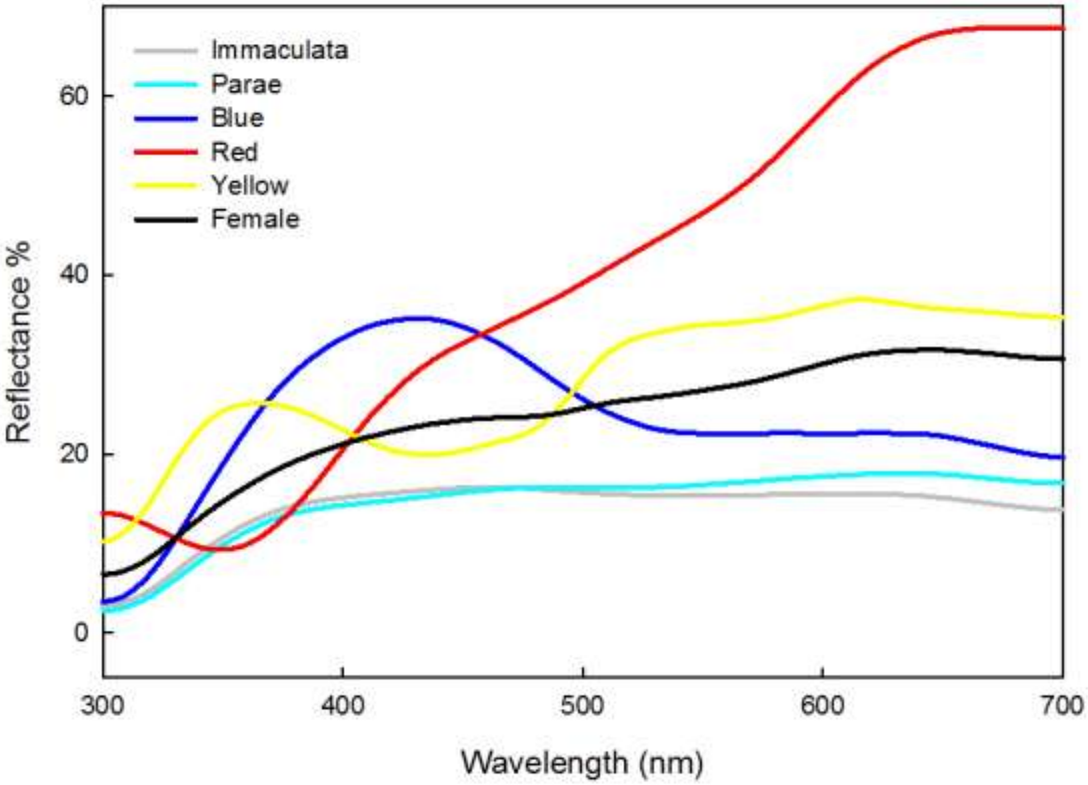


Figure 1

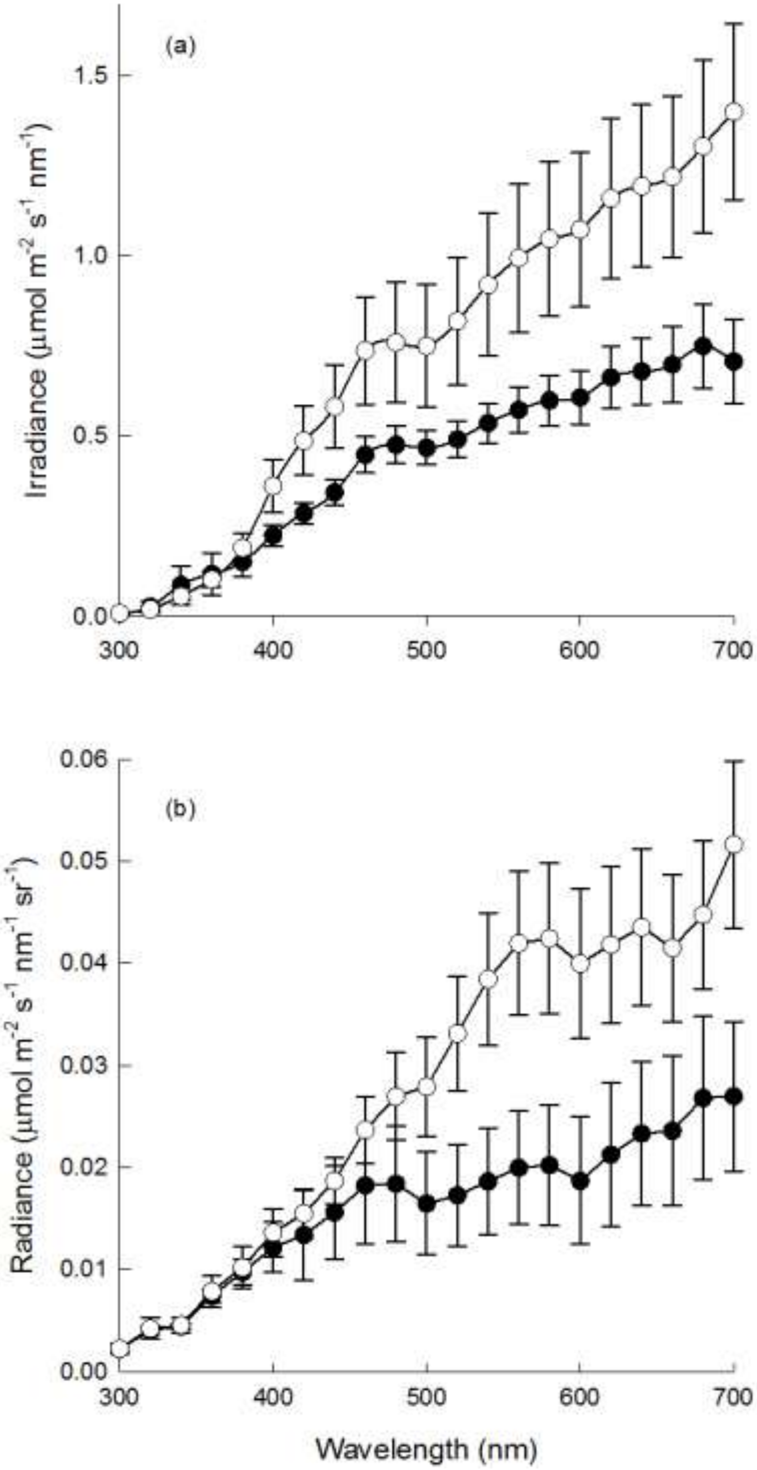
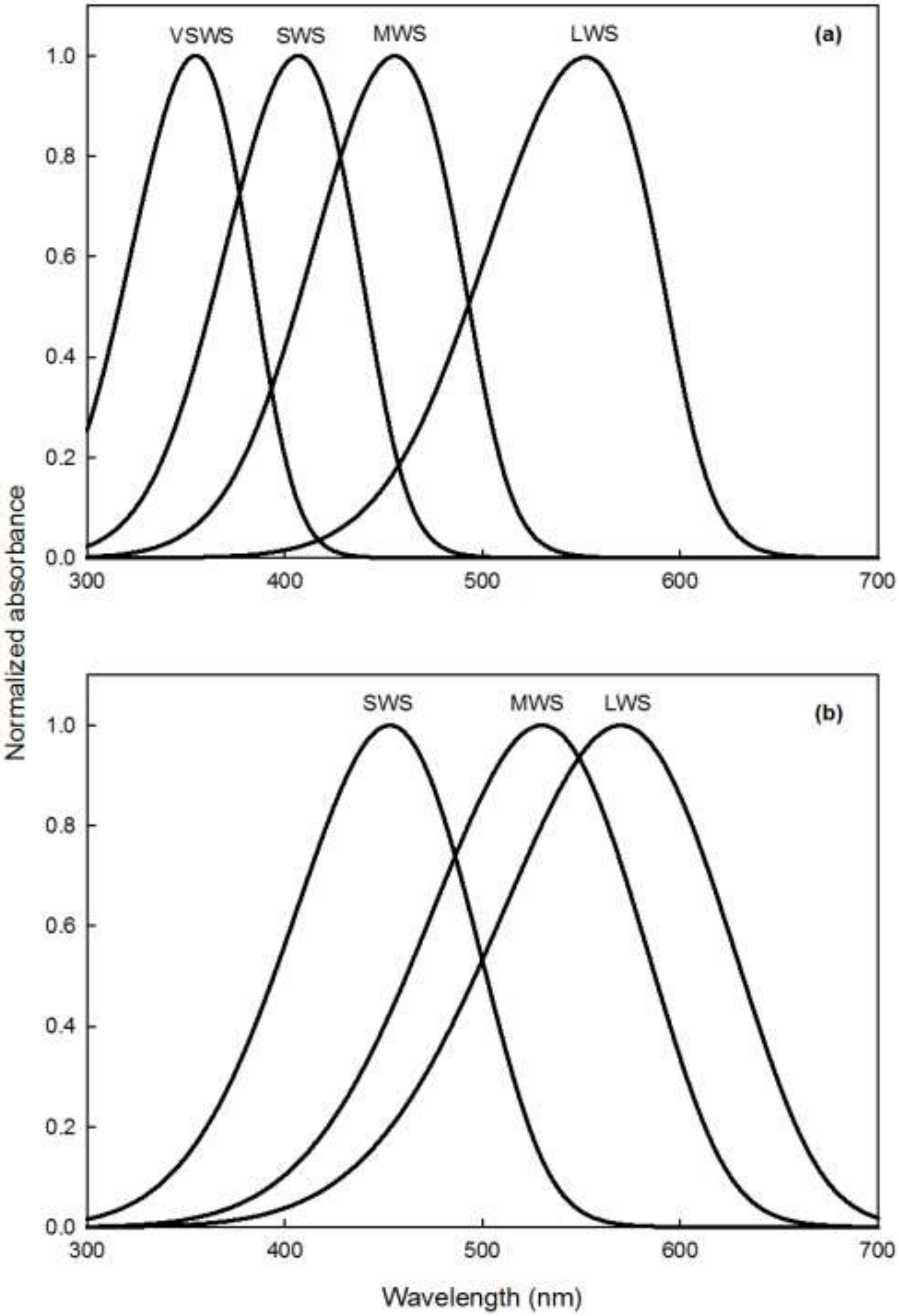

(a)
Irradiance (μmol m^{-2} s^{-1} nm^{-1})
0.0
0.5
1.0
1.5
300
400
500
600
700
(b)
Radiance (μmol m^{-2} s^{-1} nm^{-1} sr^{-1})
0.00
0.01
0.02
0.03
0.04
0.05
0.06
300
400
500
600
700
Wavelength (nm)

Figure 2



VSWS
SWS
MWS
LWS
(a)
(b)
Normalized absorbance
1.0
0.8
0.6
0.4
0.2
0.0
300
400
500
600
700
Wavelength (nm)

Figure 3

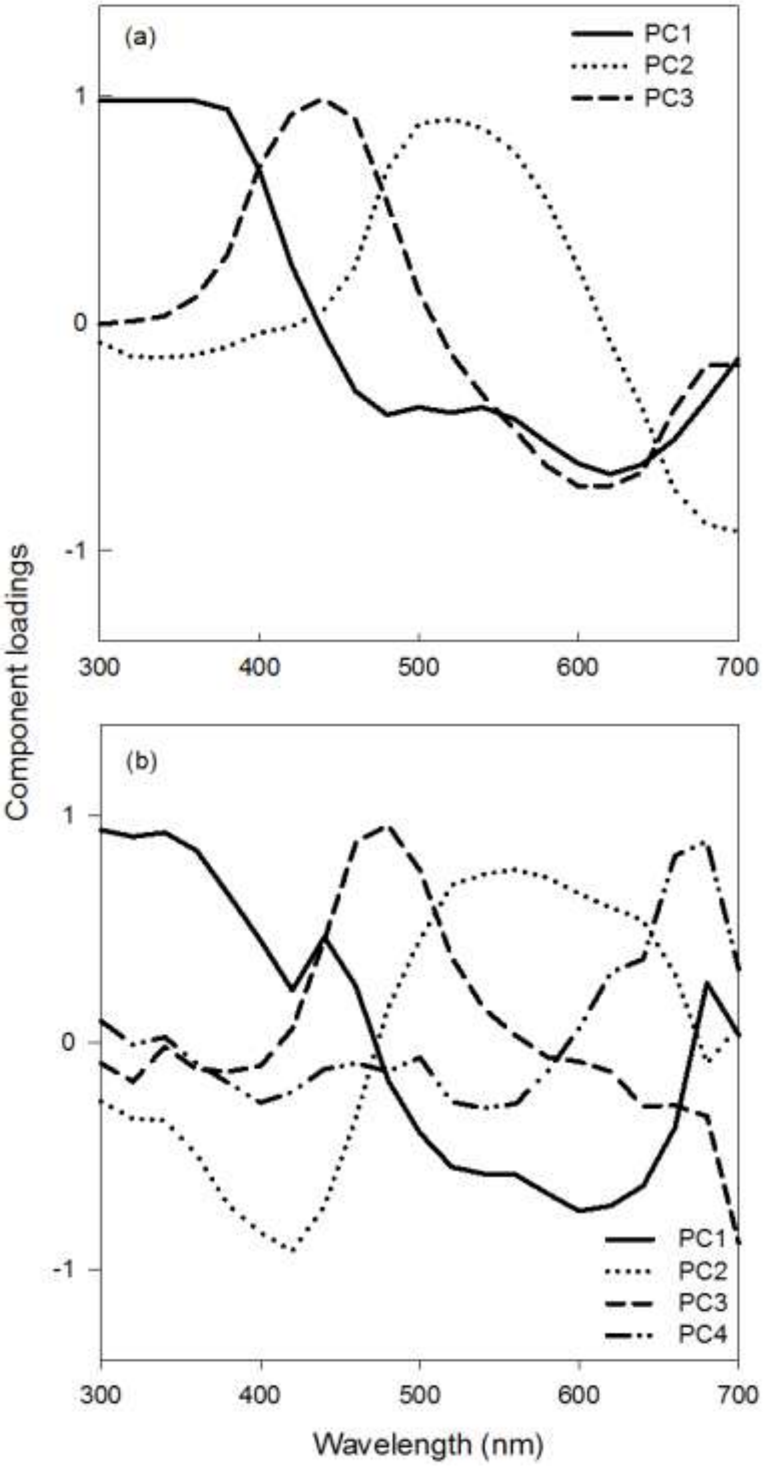

(a)
PC1
PC2
PC3
1
0
-1
300
400
500
600
700
Component loadings
(b)
1
0
-1
PC1
PC2
PC3
PC4
300
400
500
600
700
Wavelength (nm)

Figure 4

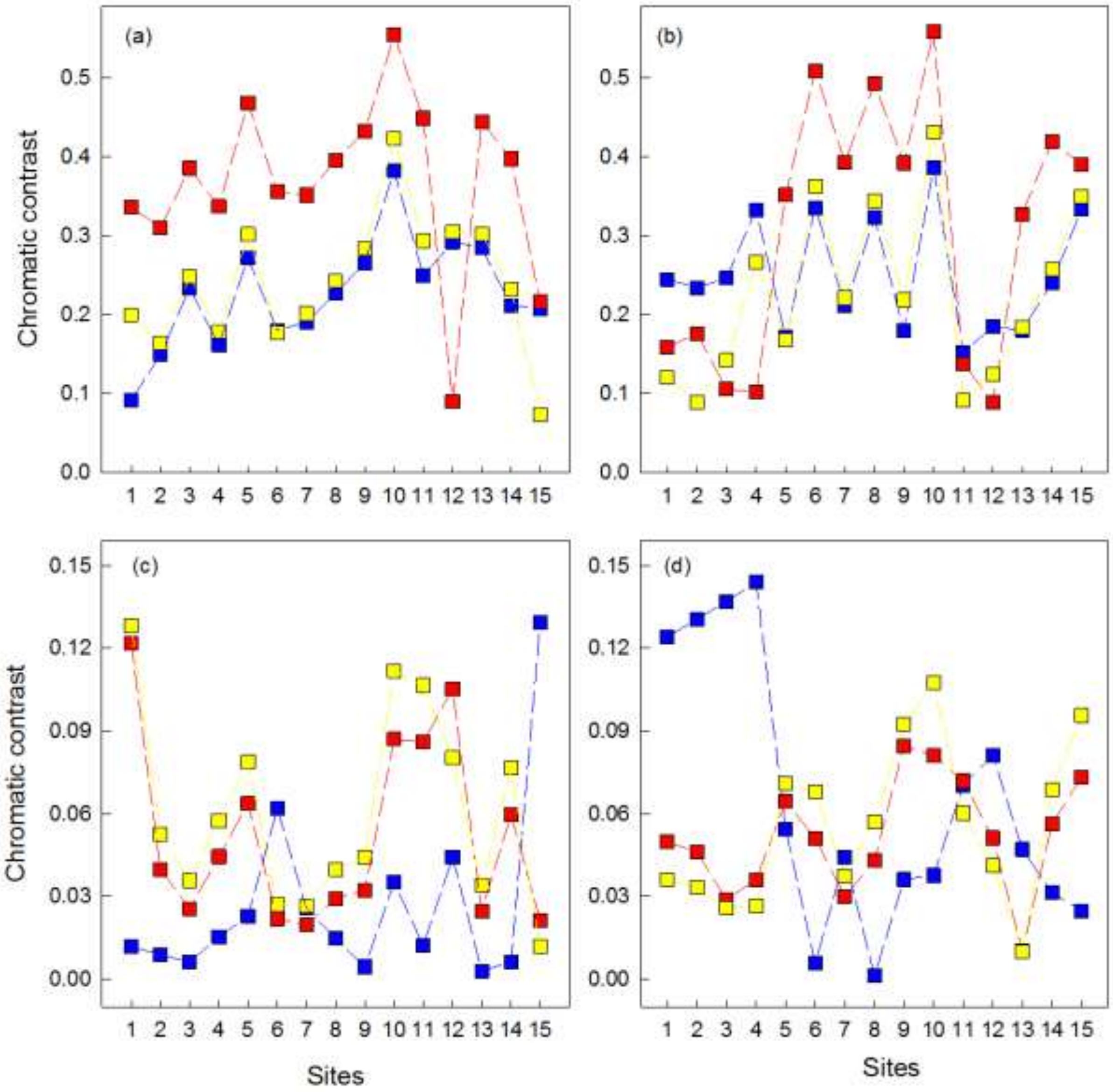

(a)
(b)
(c)
(d)
Chromatic contrast
0.0
0.1
0.2
0.3
0.4
0.5
0.00
0.03
0.06
0.09
0.12
0.15
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Sites

Figure 5

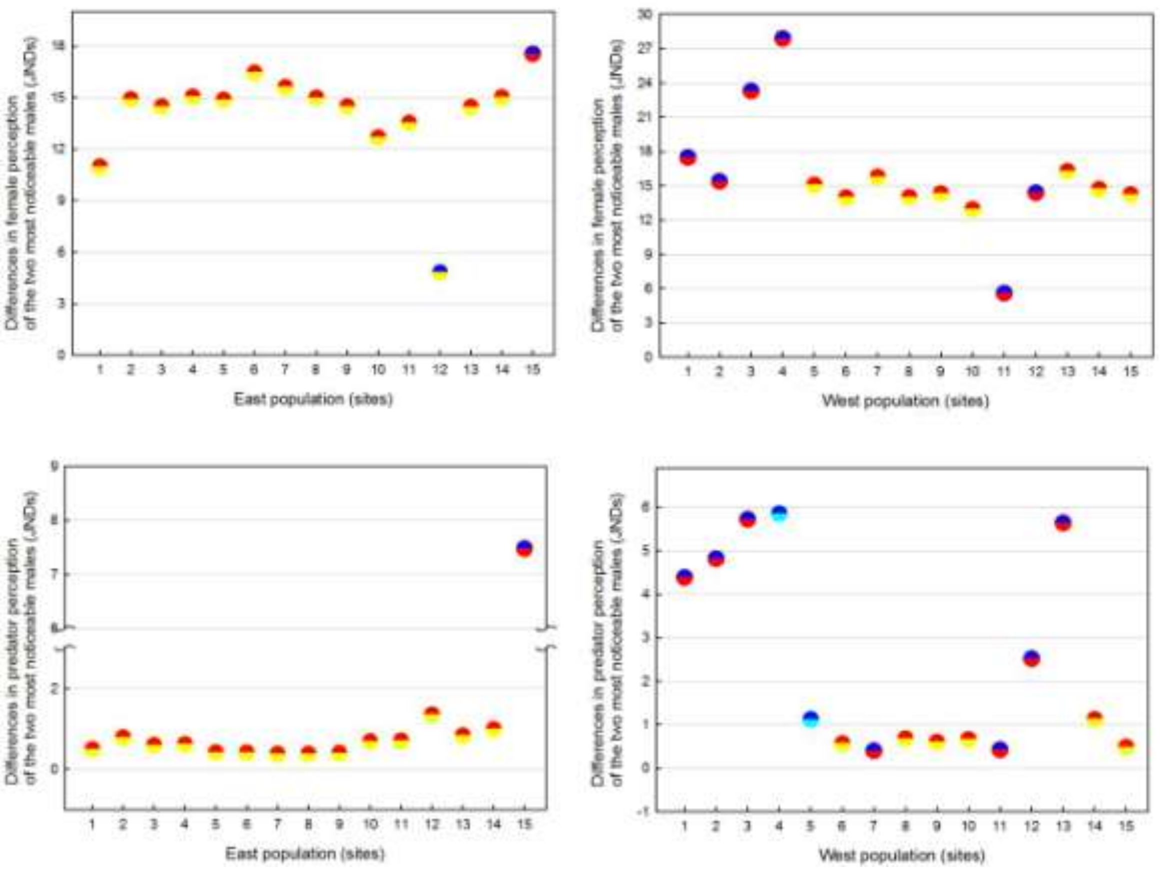
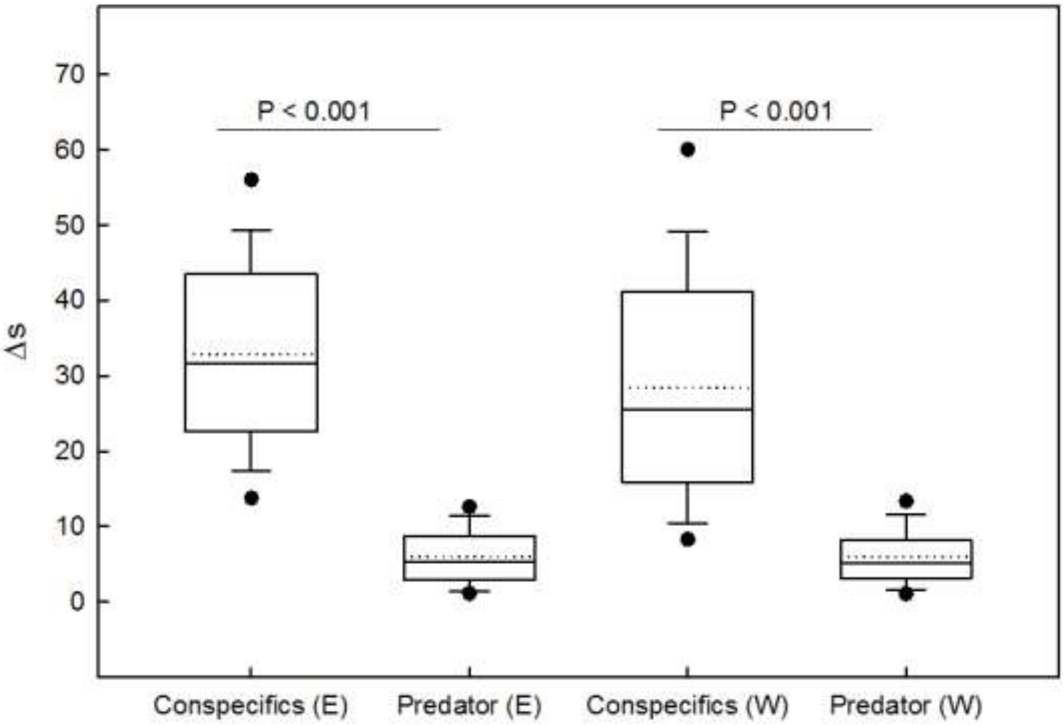

Differences in female perception of the two most noticeable males (JNDs)
0
3
6
9
12
15
18
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
East population (sites)
Differences in female perception of the two most noticeable males (JNDs)
0
3
6
9
12
15
18
21
24
27
30
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
West population (sites)
Differences in predator perception of the two most noticeable males (JNDs)
0
2
6
7
8
9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
East population (sites)
Differences in predator perception of the two most noticeable males (JNDs)
-1
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
West population (sites)

Figure 6



70
60
50
40
30
20
10
0
Δs
P < 0.001
P < 0.001
Conspecifics (E)
Predator (E)
Conspecifics (W)
Predator (W)

Figure 7

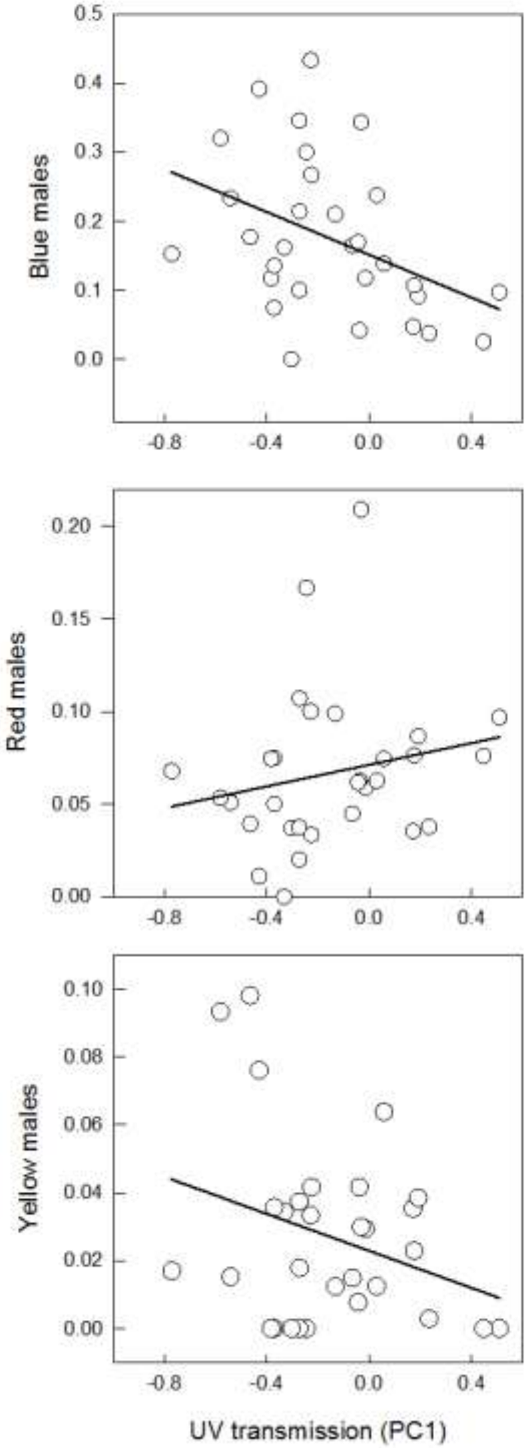

Blue males
Red males
Yellow males
UV transmission (PC1)
0.5
0.4
0.3
0.2
0.1
0.0
0.20
0.15
0.10
0.05
0.00
0.10
0.08
0.06
0.04
0.02
0.00
-0.8
-0.4
0.0
0.4

Figure 8

SUPPORTING ELECTRONIC MATERIALS

Additional supporting file 1

Figure 1C. Normalized lens transmission spectra of *P. reticulata* (dotted line) and *A. pulcher* (filled line).

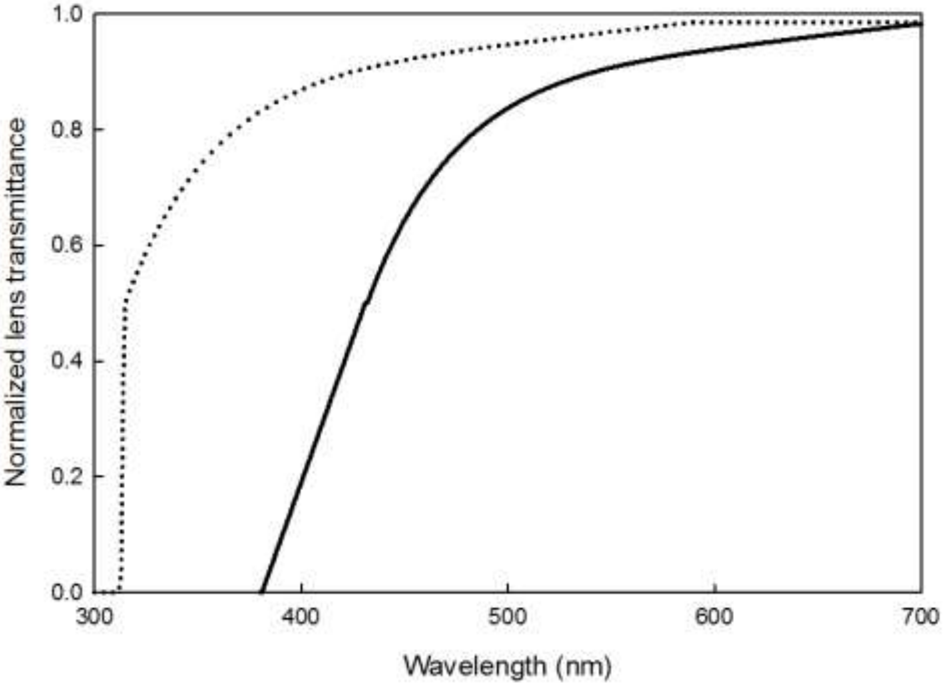


Figure 1B. Box plots showing distribution of chromatic contrast values for conspecifics (upper panel) and predators (lower panel) as estimated by Chittka's model (1992) for the east (a and c) and for the west (b and d) populations, respectively. Range (whiskers), 25% and 75% percentiles (lower and upper limit of the box), median (black line), mean (dotted line), and outliers (black dots) are shown. (I = immaculata; P = parae; B = blue; R = red; Y = yellow and F = female)

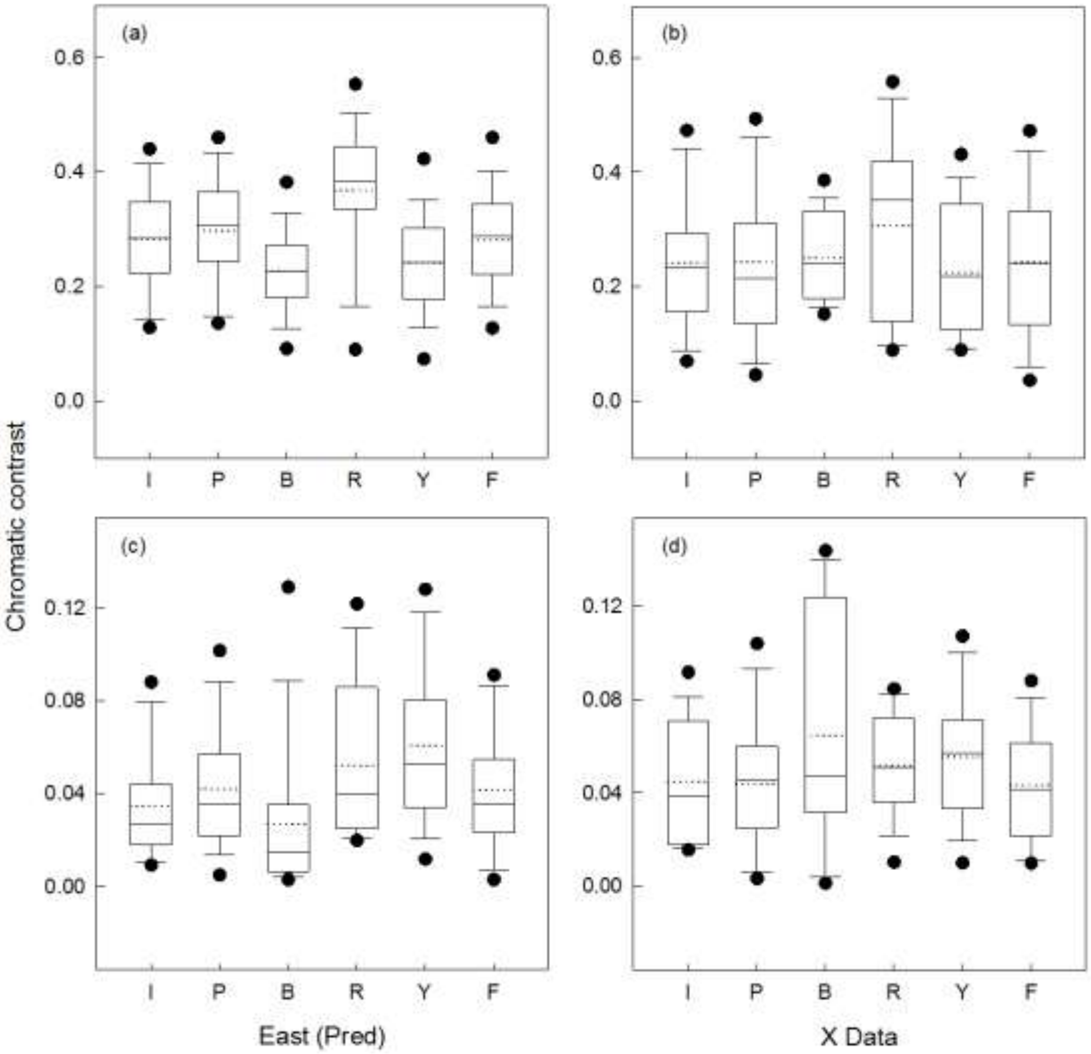


Figure 1C. Box plots showing distribution of chromatic contrast values (jnds) conspecifics (upper panel) and predators (lower panel) as estimated by Vorobyev's et al. model (2001) for the east (a and c) and for the west (b and d) populations, respectively. Range (whiskers), 25% and 75% percentiles (lower and upper limit of the box), median (black line), mean (dotted line), and outliers (black dots) are shown. (I = immaculata; P = parae; B = blue; R = red; Y = yellow and F = female).