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# Disruptive sexual selection on male nuptial coloration in an experimental hybrid population of cichlid fish

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Theory suggests that genetic polymorphisms in female mating preferences may cause disruptive selection on male traits, facilitating phenotypic differentiation despite gene flow, as in reinforcement or other models of speciation with gene flow. Very little experimental data have been published to test the assumptions regarding the genetics of mate choice that such theory relies on. We generated a population segregating for female mating preferences and male colour dissociated from other species differences by breeding hybrids between species of the cichlid fish genus *Pundamilia*. We measured male mating success as a function of male colour. First, we demonstrate that non-hybrid females of both species use male nuptial coloration for choosing mates, but with inversed preferences. Second, we show that variation in female mating preferences in an $F_2$ hybrid population generates a quadratic fitness function for male coloration suggestive of disruptive selection: intermediate males obtained fewer matings than males at either extreme of the colour range. If the genetics of female mate choice in *Pundamilia* are representative for those in other species of Lake Victoria cichlid fish, it may help explain the origin and maintenance of phenotypic diversity despite some gene flow.

**Keywords:** disruptive sexual selection; female mating preference; hybridization; male coloration; *Pundamilia*; speciation

## 1. INTRODUCTION PART I: HYBRIDIZATION AND MATING PREFERENCES

Most zoologists throughout the twentieth century considered hybridization between animal species rare and of little relevance for the evolutionary process (Coyne & Orr 2004). Similarly, speciation in the face of gene flow was often relegated to the unimportant (Mayr 1963). These views changed in the last two decades with the advent of molecular genetic methods for tracking gene flow between species (Grant & Grant 1992; Arnold 1997; Barton 2001; Seehausen 2004). Hybridization between animal species is widespread, both in early stages of speciation (Grant *et al.* 2004) and between non-sister species (Salzburger *et al.* 2002; Schelly *et al.* 2006). Depending on circumstances, interspecific hybridization may have very different consequences. Where speciation is driven by divergent selection on few genes, even a low frequency of hybridization may slow down or prevent the differentiation of species across much of the genome (Wu 2001), and may permit cross-species transfer of globally favoured mutations (Anderson & Stebbins 1954; Grant 1963; Grant *et al.* 2004). However, if selection against hybrids is stronger than gene flow, low levels of gene flow may facilitate the evolution of assortative mating, a process referred to as reinforcement (Dobzhansky 1937; Servedio 2001, 2004). Alternatively, gene flow may lead to the coalescence of species into a hybrid swarm if effects of gene flow exceed those of selection (e.g. Ellstrand 1992; Rhymer & Simberloff 1996; Taylor *et al.* 2006). Finally, hybridization may facilitate the origin of new species with new adaptations arising through a recombination of existing genes (Schliewen & Klee 2004; Nolte *et al.* 2005) and may even facilitate bursts of adaptive radiation from hybrid swarms (Seehausen 2004). Theoretical considerations suggest that the evolutionary consequences of interspecific hybridization depend on the adaptive landscape

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experienced by the hybridizing populations (Buerkle *et al.* 2003; Seehausen *et al.* 2008). If species experience strong divergent or disruptive selection between environments, species may remain distinct despite hybridization, and additional species may arise from the recombination of existing variation if more than the two parental fitness peaks are available (Buerkle *et al.* 2003). Mate choice may play an important role in determining the likelihood and strength of divergent or disruptive selection that hybridizing species experience. If two hybridizing species possess open-ended but sign-inversed female preference functions, disruptive selection on male traits may facilitate retaining phenotypic cohesion despite gene flow (van der Sluijs *et al.* 2008). Such species may therefore maintain phenotypic differentiation under a wider range of environmental conditions (i.e. with weaker environmental selection against intermediates).

By this token, taxa that contain putative examples of selection-driven sympatric speciation may at the same time be candidates for hybrid speciation (Schliewen & Klee 2004). Models of sympatric speciation through mate choice make the assumption that heritable variation in female preferences within a single population exerts disruptive selection on male traits, such that males with intermediate trait value obtain fewer matings than males with high trait value of either sign (Turner & Burrows 1995; Higashi *et al.* 1999; Kondrashov & Kondrashov 1999; Takimoto *et al.* 2000; Arnegard & Kondrashov 2004; van Doorn *et al.* 2004). Yet, surprisingly few empirical tests of this assumption can be found in the literature. The observed high variability in pheromone composition of male *Colias* butterflies has been hypothesized to be due to disruptive sexual selection exerted by females of different colour morphs within populations (Sappington & Taylor 1990). In guppies and side-blotched lizards, within-population polymorphism in male sexual ornamentation may be promoted and maintained by disruptive female mating preferences on complex fitness surfaces with multiple peaks for male traits (Blows *et al.* 2003; Bleay & Sinervo 2007). Here, we present a test of disruptive sexual selection by female mate choice on variable male coloration in a laboratory population of hybrid males between the two fully sympatric cichlid fish sister species *Pundamilia pundamilia* and *Pundamilia nyererei*. Our experiment is complemented by a companion study on the same species, which focused on behaviours on the female side of the interaction during the mate choice process (van der Sluijs *et al.* 2008). Instead of measuring male mating success (MS) as we did here, their experiment investigated female mating preferences.

## 2. INTRODUCTION PART II: THE STUDY SYSTEM

The Lake Victoria cichlid fish genus *Pundamilia* has increasingly become a model system in evolutionary ecology (Seehausen 2008). At some islands in the lake, bluish, yellow and reddish males all inhabit the same microhabitat and occupy the same trophic niche (Seehausen 2008). Here, male colour phenotypes and female mating preferences have a unimodal frequency distribution, and no genetic differentiation between the colour types could be detected at 11 neutral marker loci. At other islands, two genetically differentiated species (one with blue and the other with red males) coexist, have distinct ecologies and hybridize at some but not other islands. The main cause of variation in this progress towards speciation seems to be variation in the aquatic light regime that facilitates disruptive selection on male coloration in clear but not turbid water (Seehausen *et al.* 1997; O. Seehausen *et al.* 2008, unpublished). Populations from turbid waters phenotypically resemble laboratory populations generated by hybridization between the two species from clear waters. Hybrids between *P. nyererei* and *P. pundamilia* do not suffer from any intrinsic fitness disadvantage (van der Sluijs *et al.* 2008*a*).

Geographical (Seehausen & van Alphen 1999; Seehausen & Schluter 2004) and ecological distribution patterns (Seehausen 1997), field observations of phenotype frequency distributions in sympatric populations (Seehausen 1997) and behavioural experiments in the laboratory (Seehausen & van Alphen 1998; Haesler & Seehausen 2005) are consistent with the hypothesis that divergent female mating preferences for different male coloration are the main factor that restricts gene flow between the sibling species. Intrasexual selection, acting on the same components of male nuptial coloration (Dijkstra *et al.* 2005, 2006), may simultaneously stabilize the phenotype frequencies through negative frequency-dependent fitness advantages in male–male competition (Seehausen & Schluter 2004; Dijkstra *et al.* 2006; P. D. Dijkstra, C. Hemelrijk, O. Seehausen & T. Groothuis 2008, unpublished). Hence, female preferences and male aggression biases are two intrinsic sources of disruptive selection on the same phenotype trait. Theoretical modelling suggests that sympatric speciation by sexual selection may be possible under such conditions (van Doorn *et al.* 2004). It is clear that hybridization between the *Pundamilia* species occurs, that gene flow is common at some islands and that phenotypic differentiation can be maintained despite this gene flow, potentially making *Pundamilia* an excellent candidate for studying the 'porous genome' phenomenon associated with selection-driven speciation (Wu 2001).

No experimental test of disruptive selection on the *Pundamilia* phenotypes or any other cichlid phenotypes existed to date. We produced first and second generation hybrids between the blue species *Pundamilia pundamilia* and the red species *P. nyererei,* both from Python islands (Mwanza Gulf, Lake Victoria), and used them to experimentally test for disruptive sexual selection. The main difference between the species at Python islands lies in the coloration of flanks, dorsum and dorsal fin of the males. *Pundamilia pundamilia* males have blue-grey bodies and a bright metallic blue dorsal fin (see figure 3*c*, far left); *P. nyererei* males are yellow on the flanks with a bright crimson red dorsum and dorsal fin (see figure 3*c*, far right). Females of both species are cryptically yellow-brownish in coloration. For a detailed description of the biology of the two species see Seehausen (1996) and for the evolutionary ecology see Seehausen (2008). The same parental lines and $F_1$ hybrids that we used here were previously employed in an experiment investigating the genetic

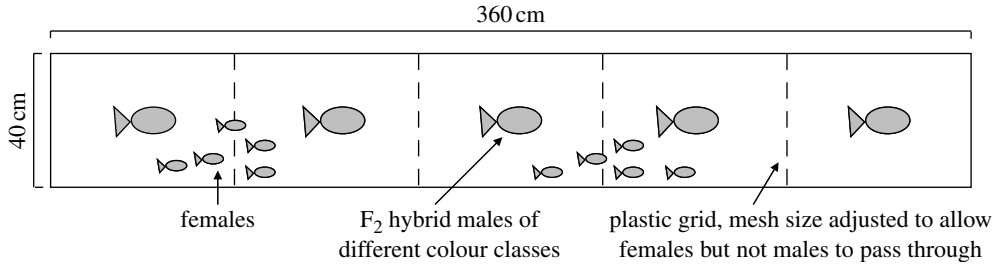


Figure 1. Schematic of the experimental aquarium. Males were constrained to their compartments and could interact visually but not physically with adjacent males. Female groups numbered at least 10 individuals. We used *P. nyererei* and *P. pundamilia* females in experiment 1, $F_1$ hybrid females in experiment 2 and $F_2$ hybrid females in experiment 3.

architecture of species differences in mating preferences (Haesler & Seehausen 2005). This latter study found that $F_1$ hybrid females had no preferences between red and blue males, but that female preference segregated in the $F_2$ hybrid generation. $F_1$ hybrid males have intermediate, predominantly yellow colour. The majority of $F_2$ hybrid males are also of intermediate colour, but some $F_2$ males with red coloration (see figure 3*c*, second from right), reminiscent of *P. nyererei*, and entirely blue coloration (see figure 3*c*, second from left), resembling *P. pundamilia*, are regularly obtained. In $F_2$ hybrid males, the genes determining nuptial colour are expected to segregate independently of other possible species differences, except for tight linkage or pleiotropy.

In the first experiment, we asked whether the mating preferences of females of the red and blue sister species are indeed based on red versus blue male nuptial coloration, once male coloration and other species differences are decoupled. In experiments 2 and 3, we investigated whether segregation of female mating preference genes can facilitate disruptive selection on male nuptial coloration in a population with variable, but mainly intermediate (yellowish) male coloration. We made three predictions. (i) When competing for males in an assemblage of non-hybrid females of the two species, reddish hybrid males will have higher MS than bluish hybrid males with female *P. nyererei*. Conversely, bluish hybrid males will have higher MS than reddish hybrid males with *P. pundamilia* females. (ii) When competing for mates in a population of $F_1$ hybrid females, MS will not be predicted by male colour because the $F_1$ hybrid generation consists only of heterozygous hybrid females. (iii) When competing for mates in a population of $F_2$ hybrid females, the more red and the more blue hybrid males will have higher MS than that of intermediate yellowish coloration.

Note that predictions (ii) and (iii) hold only if preference-heterozygous hybrid females mate randomly between red, blue and yellow males, as opposed to having a preference for intermediate (yellow) males, i.e. if the effects of blue- and red-preference alleles cancel out in hybrids. Prediction (iii) then arises because in the $F_2$ generation female preference alleles for red and blue segregate (Haesler & Seehausen 2005). While most $F_2$ hybrid females carry similar proportions of the different preference alleles and are expected to mate at random just like $F_1$ hybrid females, segregation of preference genes also produces females carrying unequal numbers of the preference alleles with different sign, favouring either red or blue males and hence exhibiting mating preferences.

Our data are consistent with the three predictions and provide evidence for the following: (i) male nuptial coloration is an important determinant of male MS in *P. pundamilia* and *P. nyererei* and (ii) segregation of female preference genes can exert disruptive sexual selection on male nuptial coloration in a population of males with variable coloration.

## 3. MATERIAL AND METHODS

Most non-hybrid females used in this experiment (21 *P. nyererei* and 9 *P. pundamilia*) were laboratory bred, deriving from fishes collected at Python islands (Mwanza Gulf, Lake Victoria, Tanzania) in 1992, and maintained first at the University of Leiden, The Netherlands, later at the Universities of Southampton and Hull, UK. The remaining non-hybrid females were wild-caught fishes, taken from the same location at Python islands in 2003 (six *P. nyererei* and five *P. pundamilia*). All hybrid males and females were bred from the 1992 laboratory lines between 2000 and 2002. Fishes were reared in stock tanks in a large recirculation system. Hybrids and non-hybrids had never encountered each other prior to the experiments. Fishes were fed twice a day, once with dry food and once with a blend of shrimps, peas and *Spirulina* powder. Water temperature was maintained between 24 and 26°C. Aquaria were illuminated on a 12 D : 12 L regime using full-spectrum fluorescence tubes including UV light. Fishes were individually PIT (Passive Integrated Transponder) tagged and fin clipped by A.M.S (UK home office license; project number PPL60/3295) one month before being introduced into the experimental tank.

Experiments were carried out in a $360 \times 75 \times 40$ cm aquarium that was divided into five equal size compartments, each containing one hybrid male (figure 1). A 'partial partitioning' design (Hert 1989; Turner *et al.* 2001) was used, allowing for full interaction between males and females and spawning. Compartments were separated by plastic grids with adjustable mesh size, allowing the females, but not the larger males, to pass through the grid. Each compartment contained a small cave made from three bricks, providing shelter for the male and motivating it to establish a territory. Light conditions, temperature and food supply were identical to those in the stock tanks. Food was distributed in equal quantities among the five compartments.

Experiments were conducted in three series between April 2003 and June 2004 in the following order: $F_2$ hybrid males competing for matings with non-hybrid females to test prediction (i); $F_2$ hybrid males competing for $F_1$ hybrid females to test prediction (ii); and $F_2$ hybrid males competing for $F_2$ hybrid females to test prediction (iii).

Scoring colour phenotypes on a six-point scale (Dijkstra *et al.* 2007) was done independently by two investigators (R.B.S. and O.S.) and the mean was used. For experiment 1, we assigned hybrid males to two colour classes, assigning scale points 0, 1 and 2 to 'bluish' ($n=14$), and 3 and 4 to 'reddish' ($n=16$). In each replicate, either three bluish and two reddish males or two bluish and three reddish males were used. For experiments 2 and 3, hybrid males were assigned to three colour classes: scale point 0, 'blue' ($n=6$); scale points 1 and 2, 'intermediate' ($n=17$); and scale points 3 and 4, 'reddish' ($n=13$; note that the very red phenotype, representing scale point 5, is not regularly recovered in $F_2$ families). In each replicate for experiments 2 and 3, three intermediate, one blue and one reddish male were present.

Male brightness was scored on a three-point scale (bright, intermediate and dull) from digital photos that were taken of all males before and during mating trials. Brightness varies with the motivation of males and is a combined measure of contrast, saturation and reflectivity. Colour class on the other hand can be assigned independently of motivation. In total, 45 $F_2$ hybrid males from 13 different families were used.

Females were introduced into the experimental tank prior to the males in groups of at least 10 individuals and were allowed to acclimatize and explore all five compartments. Subsequently, five males were randomly allocated to the compartments. Once one female had spawned, we concluded the replicate and removed the males. Males were weighed on a pan balance to the nearest 0.01 g and their standard length was measured. Condition factor for each male was calculated as $100\times$weight (g)/length $(\mathrm{cm})^{2.76}$ (Bolger & Connolly 1989). We used these measurements to control for the effects of size and condition on male MS. Brooding females were left in the tank for one week to avoid premature release of the eggs before these contained sufficient DNA for molecular paternity assignment. After a week, the brooding female was removed and the eggs were collected by gently opening the mouth of the female to let the eggs fall out. The eggs, a fin clip of the mother and fin clips of all five potential fathers were preserved in 95% ethanol. After each spawning, one of the five males, chosen at random, was replaced by another male of the same colour class and all males were again randomly allocated to compartments. In this way, we ensured that each male had a different set of competitors for every replicate spawning event, and that each female chose from a different set of males (the few cases where more than one female spawned in the same replicate were treated as pseudoreplicates in the analysis, see below). Brothers from the same family were never used in the same replicate to avoid misassignment of paternity. Likewise, females and males used in our experiments never came from the same family.

The mean number of replicates per male was $3.3\pm2.6$ with non-hybrid females, $3.6\pm1.6$ with $F_1$ hybrid females and $5.7\pm3.4$ with $F_2$ hybrid females. Even though most males were used in several replicate spawning trials, we did not pseudoreplicate measures of male MS: we either calculated for each male the mean MS from all its repeat uses, obtaining one data point per male for all further analyses, or included 'male identity' as a factor in logistic regression analyses of MS (see below). Once removed from the experimental tank, males were returned to stock tanks and given a week before they were used again. Replicates lasted from 2 hours to 10 days depending on how soon a female spawned. The five males in each replicate were videotaped in random order for collection of behavioural data. Although males had no physical contact, we recorded aggressive behaviour directed to adjacent males visible through the plastic grids. Courtship and aggression intensities were quantified using Noldus Observer Video Pro v. 5.0 software to analyse the video tapes. Seven relevant elements of male courtship and aggression behaviour were scored: *butting*; *frontal display*; *lateral display*; *quiver*; *lead swim*; *presenting egg dummies*; and *circling* (Baerends & Baerends-van Roon 1950). Behavioural data were recorded without knowledge of a male's MS and quantified as counts of events during 15 min recording time.

### (a) *Paternity assignment*

A total of 63 broods were obtained. Twenty per cent of the eggs of each clutch, but not less than five eggs (when the clutch contained fewer than 25 eggs), the mother and all five potential fathers of each clutch were genotyped at five microsatellite loci: Ppun5; Ppun7; Ppun17; Ppun21; and Ppun32, developed from a *P. nyererei*×*P. pundamilia* hybrid (Taylor *et al.* 2002). As clutch size ranged from 5 to 75 eggs, the number of eggs genotyped varied from 5 to 15 eggs per clutch. With a minimum of five eggs per clutch, the probability that an allele at a given locus was missed is $0.5^5$ per parent. DNA was extracted using the HotSHOT method (Truett *et al.* 2000). Microsatellite loci were amplified using Cy5- and Cy5.5-labelled forward primers. Each reaction contained 1 µl DNA template, 5 pmoles forward and reverse primers, 0.2 mM dNTPs and 0.25 units Taq polymerase and 1–2 mM $MgCl_2$. Annealing temperatures were as described in Taylor *et al.* (2002). Successful amplification was confirmed on a 1.5% agarose gel before genotyping individuals on a Beckman Coulter CEQ 8000 capillary sequencer. Microsatellites were scored with the CEQ 8000 Series Genetic Analysis System.

### (b) *Calculating mean mating success for each male*

Mean mating success (MMS) of each individual male was calculated from the results of the paternity assignment using the equation

$$\mathrm{MMS}=\frac{\sum_{i=1}^{N}\frac{S_i}{S_{i\_\mathrm{total}}}}{N}, \tag{3.1}$$

where $S_i$ is the number of spawnings of the individual male in replicate (i); $S_{i\_\mathrm{total}}$ is the total number of spawnings of all males in replicate (i); and $N$ is the total number of replicates in which the given male was used. In experiment 1, MMS of each male with *P. nyererei* and with *P. pundamilia* females was calculated separately considering spawnings with either only *P. pundamilia* or only *P. nyererei* females in $S_i$ and $S_{i\_\mathrm{total}}$. Usually only one female spawned per replicate, but in a few cases two or three spawned before the replicate could be terminated. The term in the numerator of equation (3.1) allowed us to use this information without either pseudoreplicating or arbitrarily excluding spawnings. MMS potentially ranges from 0 (a male never mated) to 1 (if a male was the only one that spawned in each replicate in which it was used). One clutch was sired by two different males (the only case of multiple paternity among 63 clutches) and we attributed to each male half of the clutch.

### (c) *Analysing male mating success*

To test the prediction (i) that reddish hybrid males have higher MS than bluish hybrid males with female *P. nyererei*, whereas the opposite would be true with female *P. pundamilia*, we compared the MMS of bluish and reddish males with females of each species using unpaired *t*-tests allowing for unequal variance. We also compared for each bluish male and

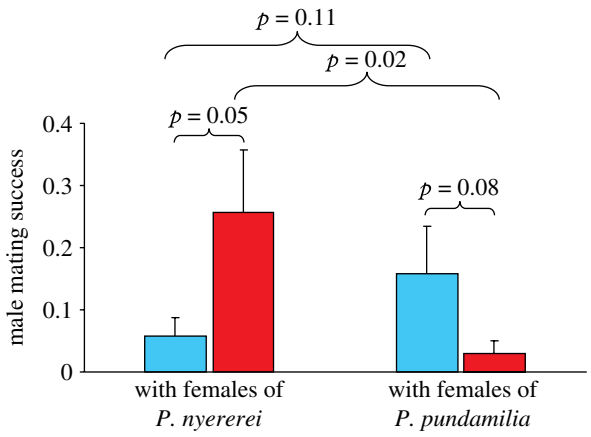


Figure 2. Mean individual mating success (MMS) of reddish (red bars) and bluish (blue bars) $F_2$ hybrid males with the females of *P. nyererei* or *P. pundamilia*. Error bars represent 1 s.e. Values of $p$ are from $t$-tests comparing MMS of red and blue hybrid males with either *P. pundamilia* or *P. nyererei* females separately. Fisher's exact combined probability test is significant for both possible ways of grouping the results. Male sample sizes are $n=14$ for bluish males and $n=16$ for reddish males. Female sample sizes are $n=14$ for *P. nyererei* and $n=11$ for *P. pundamilia* females.

each reddish male its MMS with *P. pundamilia* and with *P. nyererei* females using paired $t$-tests.

To test the predictions (ii) and (iii), i.e. testing for differences in MMS among three $F_2$ hybrid male phenotype classes, we used one-way ANOVAs. The shape of the relationship between MMS and male colour, indicating the form of selection, was assessed by fitting linear and quadratic regressions. A significant fit of a linear regression term suggests directional selection, whereas a significant fit of a quadratic regression suggests disruptive selection. Before analysis, all data were Box–Cox transformed to obtain normality.

To assess the effect of male nuptial colour class on MS, relative to the effects of other male traits, we performed multiple regressions of MMS against male colour, size, weight and condition factor, as well as logistic regressions of MS against male colour, brightness, behaviour and male identity as covariates. For the latter, we had to use male MS data of each replicate separately rather than a male's MMS because male brightness and behaviour varied across replicates, hence averages of these variables calculated across repeat-use replicates is not meaningful. The corresponding response variable MS is therefore a binary variable that was scored as '1' if a male sired a clutch and as '0' if not. The variable male identity controlled for individual male effects in these logistic regressions. The regressions were calculated for each experiment separately as well as for the data from all experiments pooled. The variable 'colour' used for model fitting here corresponds to the two (experiment 1) and three (experiments 2 and 3) colour classes that the males were assigned to (see above). For the regression pooling of the data of all the three experiments, we reassigned males used in experiment 1 to the three classes used in experiments 2 and 3. Minimum adequate models were calculated, applying likelihood-ratio tests with backward stepwise removal of variables with $p>0.10$. We used Wald-$\chi^2$ tests to test for the effect size of each variable in the models. If the Wald test was not significant, the variable was removed from the model. To obtain a single compound measure of behavioural activity, we applied a principal component analysis (PCA) on the correlation matrix of the seven courtship and aggression frequencies and used PC1 in all subsequent analyses of MS.

To control for position effects of males in the experimental tank, we used one-way ANOVA on male MS. To test for unintentional associations between the different male traits and male position in experimental tank, we used $\chi^2$-tests. All analyses were done in JMP Professional v. 6.0.0 (SAS Institute), except the quadratic regression fitting that was done in R (Ihaka & Gentleman 1996).

## 4. RESULTS

### (a) *No position effect*

Male MS did not depend on the position of the male in the experimental tank in any of the three experiments (one-way ANOVAs, experiment 1: $F_{4,85}=0.64$, $p=0.63$; experiment 2: $F_{4,50}=0.38$, $p=0.82$; and experiment 3: $F_{4,97}=0.33$, $p=0.86$), and no other male trait was associated with male position in any of the experiments (for brevity only test results for pooled data of all experiments shown; colour class: $\chi^2_8=6.83$, $p=0.55$; brightness: $\chi^2_8=4.73$, $p=0.79$; behaviour: $F_{4,192}=0.54$, $p=0.71$; weight: $F_{4,232}=1.16$, $p=0.33$; size: $F_{4,232}=1.52$, $p=0.2$; condition factor: $F_{4,232}=1.36$, $p=0.25$).

### (b) *Mating success of $F_2$ hybrid males with non-hybrid females*

To measure the MS for 30 different $F_2$ hybrid males, we obtained clutches from 14 different *P. nyererei* females, choosing from 11 different sets of five $F_2$ hybrid males, and from 11 different *P. pundamilia* females, choosing from 9 different sets of five $F_2$ hybrid males. Differences in the number of spawnings per female type (i.e. *P. pundamilia*, *P. nyererei*, $F_1$ hybrid and $F_2$ hybrid females) are due to the temporal irregularity of spawning events and the varying numbers of available females per type.

When competing for *P. nyererei* females, reddish $F_2$ hybrid males had significantly higher MS than bluish males (unpaired $t$-tests, $n_1=16$, $n_2=14$, $t=1.68$, $p$ (one tailed) $=0.05$; power ($\alpha=0.05$)$=0.39$), whereas when competing for *P. pundamilia* females bluish $F_2$ hybrid males tended to have higher MS than reddish $F_2$ hybrid males ($t=-1.45$, $p$ (one tailed)$=0.08$; power ($\alpha=0.05$)$=0.27$). Hybrid males that resembled the male nuptial coloration of one species had significantly higher MS with females of that species than did those hybrid males that resembled the heterospecific males (Fisher's exact combined probability test: $F_4=11.04$, $p=0.02$; figure 2).

Reddish $F_2$ hybrid males had significantly higher MS with females of *P. nyererei* than with females of *P. pundamilia* (paired $t$-tests, $n=14$, $t=-2.17$, $p$ (one tailed)$=0.02$), whereas a trend in the opposite direction was observed for bluish $F_2$ hybrid males ($n=16$, $t=1.28$, $p$ (one tailed)$=0.11$). Hybrid males had significantly higher MS with females of the species whose male nuptial coloration they resembled than with the females of the other species (Fisher's exact combined probability test: $F_4=11.83$, $p=0.02$; figure 2).

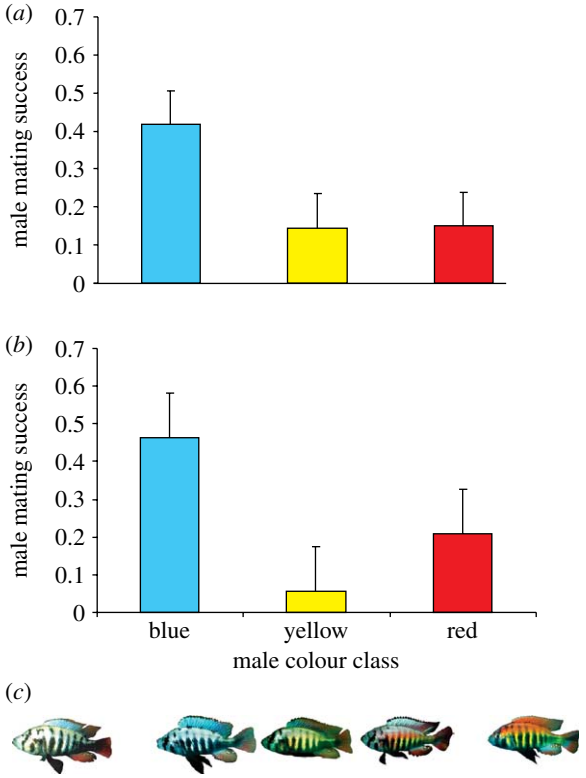


Figure 3. Mean individual mating success (MMS) of $F_2$ hybrid males (colour of bar indicates three male colour classes: blue, yellow (intermediate), reddish) with (*a*) $F_1$ hybrid females and (*b*) $F_2$ hybrid females. Error bars represent 1 s.e. (*a*) Male sample sizes are $n=2$ for blue, $n=8$ for yellow and $n=7$ for reddish males. Female sample size is $n=14$. (*b*) Male sample sizes are $n=5$ for blue, $n=11$ for yellow and $n=6$ for reddish males. Female sample size is $n=24$. (*c*) Three $F_2$ hybrid males representative for the three colour classes (the three phenotypes in the middle) and a *P. pundamilia* male (far left) and a *P. nyererei* male (far right) for comparison.

### (c) *Mating success of $F_2$ hybrid males with $F_1$ hybrid females*

To measure the MS for 17 different $F_2$ hybrid males, we obtained 14 clutches from 14 different $F_1$ hybrid females with 12 different sets of each $F_2$ hybrid males. No significant effect of male nuptial colour class on MS was detected (one-way ANOVA with male colour class as three-level factor (blue, intermediate and reddish): $F_{2,14}=1.50$, $p$ (two tailed)$=0.26$, power ($\alpha=0.05$)$=0.265$; figure 3*a*).

### (d) *Mating success of $F_2$ hybrid males with $F_2$ hybrid females*

To measure the MS for 22 different $F_2$ hybrid males, we obtained 24 clutches from 24 different $F_2$ hybrid females with 18 different sets of five $F_2$ hybrid males. Male nuptial colour class had a significant effect on MS (one-way ANOVA with colour class as three-level factor (blue, intermediate and reddish): $F_{2,19}=3.92$, $p$ (two tailed)$=0.038$, power ($\alpha=0.05$)$=0.633$, figure 3*b*). Tukey–Kramer *post hoc* comparisons revealed that MS differed significantly between blue (highest) and intermediate (lowest) hybrid males. The MS of reddish hybrid males was intermediate between those of blue males and males of intermediate coloration.

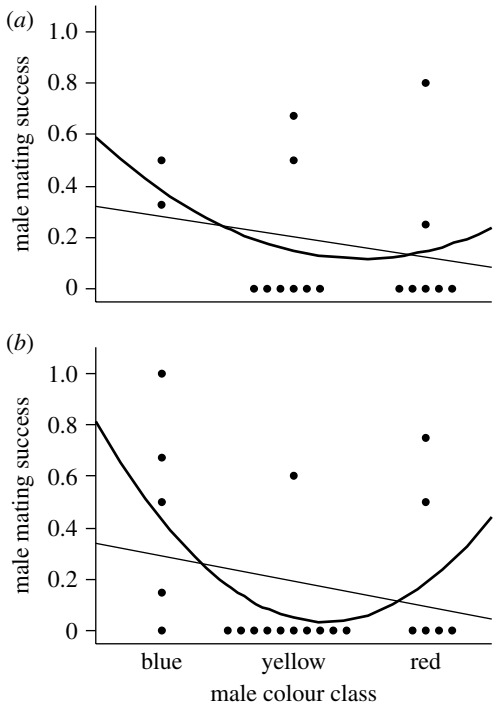


Figure 4. Linear (thin line) and quadratic (thick line) regression fit of male mating success on male colour class. (*a*) With $F_1$ hybrid females, linear fit: $R^2=0.09$, $p=0.25$; quadratic fit: $R^2=0.18$, $p=0.26$. Sample sizes are $n=2$ for blue, $n=8$ for yellow (intermediate) and $n=7$ for red males. (*b*) With $F_2$ hybrid females, linear fit: $R^2=0.07$, $p=0.24$; quadratic fit: $R^2=0.29$, $p=0.038$. Sample sizes are $n=5$ for blue, $n=11$ for yellow and $n=6$ for red males. Overlying data points are shown offset on the $x$-axis for illustrative purpose only.

### (e) *The shape of the relationship between colour and mating success of hybrid males*

Neither a linear ($R^2=0.09$, $p=0.25$) nor a quadratic ($R^2=0.18$, $p=0.26$) regression term provided significant fit for the relationship between MS and male nuptial colour using data from experiment 2 with $F_1$ hybrid females (figure 4). By contrast, a quadratic regression term provided a significant fit using data from experiment 3 with $F_2$ hybrid females ($R^2=0.29$, $p=0.038$), whereas a linear regression term did not ($R^2=0.07$, $p=0.24$; figure 4). We hence have to reject the null hypothesis of uniform MS among males of different coloration when competing for matings in a population of $F_2$, but not in a population of $F_1$ hybrid females. The relationship between male colour and MS when competing for matings in a population of $F_2$ hybrid females is consistent with disruptive selection on male colour.

### (f) *Effects of male size, brightness, behaviour and colour on hybrid male mating success*

Although male size is important for female mate choice in many fishes (Rogers & Barlow 1991; Reynolds & Gross 1992), neither size, weight nor condition factor of males explained significantly any of the variation in MS of the $F_2$ hybrid males in any of the three experiments (with non-hybrid females: $R^2=0.08$, $p=0.57$; with $F_1$ hybrid females: $R^2=0.11$, $p=0.67$; and with $F_2$ females: $R^2=0.09$, $p=0.62$).

The minimum adequate model to explain variance in MS with *P. nyererei* females (experiment 1; colour as

two-level factor) contained male brightness and colour (model: $R^2=0.41$, $p<0.01$; brightness: $p=0.001$; colour: $p=0.024$). The minimum adequate model to explain variance in MS with *P. pundamilia* females contained colour, brightness and behaviour (model: $R^2=0.58$, $p<0.01$; colour: $p=0.023$; brightness: $p=0.063$; behaviour: $p=0.07$). Male identity was not retained in either case. Similar results were obtained to explain variance in MS with $F_2$ hybrid females (experiment 3; colour as three-level factor), where the variance was best predicted by male behaviour and colour (model: $R^2=0.44$, $p<0.01$; behaviour: $p<0.001$; colour: $p=0.006$). Male brightness and identity were not retained in the minimum adequate model. By contrast, variance in MS with $F_1$ hybrid females (experiment 2; colour as three-level factor) was explained by neither male colour nor brightness. Instead, male behaviour alone provided the best fit (model: $R^2=0.12$, $p=0.07$; behaviour: $p=0.072$). Male identity was not retained in this model either.

When data from all the three experiments were pooled, the minimum adequate model explaining variance in MS contained colour, brightness and behaviour (model: $R^2=0.33$, $p<0.01$; colour: $p=0.007$; brightness: $p=0.004$; behaviour: $p=0.002$; colour as three-level factor). Male identity again explained no significant part of the variance. This means that the differences in male MS between experiments 2 and 3 were not explained by male identity.

Finally, we tested for associations of male colour with weight, size, condition factor, brightness and behaviour. The only significant association was with behaviour in experiment 1 (red males were more active: $\chi^2_2=6.97$, $p=0.03$) but not in the other experiments. When data of all experiments were pooled, behaviour was not associated with male colour class ($\chi^2_2=3.58$, $p=0.17$). There was no significant association of colour class with any of the other variables in any of the three experiments when testing each dataset separately. The same was true when data from all the three experiments were pooled (weight: $\chi^2_2=4.6$, $p=0.1$; size: $\chi^2_2=3.68$, $p=0.16$; condition factor: $\chi^2_2=0.25$, $p=0.88$; brightness: $\chi^2_2=8.61$, $p=0.07$).

## 5. DISCUSSION

Discrete heritable variation in female mating preferences for alternative forms of a male trait can cause divergent and, if heterozygous females mate randomly, disruptive selection on the male trait. Heritable variation of this type may arise in a population by mutation, introgressive hybridization or complete admixture of previously distinct populations or species. In many cases, such variation may be lost from a population, but it may be maintained by negative frequency-dependent sexual selection (Sinervo 2001; Seehausen & Schluter 2004) or condition-dependent variation in mating preference functions (Bleay & Sinervo 2007). Depending on the starting conditions, i.e. the initial extent of linkage disequilibrium with male traits, however, such preference polymorphisms may also facilitate reinforcement (e.g. Servedio 2001, 2004) and parapatric or sympatric speciation (e.g. Higashi *et al.* 1999; Takimoto *et al.* 2000). Even though quite thoroughly investigated theoretically, very little experimental data have been published to test the feasibility of disruptive selection by female mate choice. Cichlid fish of the east African Great Lakes are suitable objects for such tests because divergent mating preferences have been demonstrated between species in laboratory experiments (Knight *et al.* 1998; Seehausen & van Alphen 1998; Couldridge & Alexander 2002; Kidd *et al.* 2006) and in the wild (Holzberg 1978; Seehausen *et al.* 1998). Even though there is evidence from a Lake Malawi cichlid species pair that female choice may be based on olfactory cues (Plenderleith *et al.* 2005), in most of the experimental studies demonstrating divergent female mating preferences olfactory cues were excluded (Seehausen & van Alphen 1998; Couldridge & Alexander 2002; Jordan *et al.* 2003; Kidd *et al.* 2006; van der Sluijs *et al.* 2008). This may be taken as support for the hypothesis that divergent female mate choice between species is often based on male nuptial coloration. However, other possible male courtship cues, such as sound, were not excluded and could potentially confound the interpretation. The hypothesis that divergent female mate choice is based on male nuptial coloration had, prior to our study, been tested only indirectly by manipulating ambient light (Seehausen & van Alphen 1998). The experiment that we have reported here, in which females of both species chose among hybrid males of variable colour, represents a more direct test. Effectively, our approach using $F_2$ hybrid males separates the colour of the males from other traits that may differ between the species, except for pleiotropy or tight linkage. Also in contrast to previous studies, we assessed male MS by allowing the fishes to spawn followed by paternity tests to assign MS to males. Hence, we were able to count actual fertilization rates rather than giving only estimates for the popularity of males.

Consistent with the hypothesis that divergent mating preferences are based on nuptial coloration, the more red hybrid males in our experiments had higher MS with females of *P. nyererei* (a species with red males) than the more blue hybrid males, whereas the latter had higher MS with females of *P. pundamilia* (a species with blue males). We found no other male trait (i.e. weight, size, condition factor, brightness, behaviour) to be associated with the blue versus red nuptial coloration. Hence, the differential MS of hybrid males with females of the two species can most likely be attributed to variation in red or blue male coloration, or in an unknown trait pleiotropic or tightly linked to male nuptial coloration.

Female mating preferences for red versus blue males are heritable and probably oligofactorial. Heterozygous females mate at random (Haesler & Seehausen 2005). Complementary to our study, van der Sluijs *et al.* (2008) did the first comparison of female preference functions between *P. pundamilia* and *P. nyererei*, showing preference functions in both species that were open ended over the range of male phenotypes tested, and sign inversed between the species. If the absence of preferences for red or blue males observed in $F_1$ hybrid females by Haesler & Seehausen (2005) is because preference alleles for red and blue cancel out to make a no preference, as opposed to a yellow

preference, segregation of female mating preferences in the $F_2$ generates some females with preference for red, some with preference for blue and a majority of females that mate at random. In a population in which female preferences segregate, more extremely coloured males are then predicted to have higher MS than males of intermediate colour. By contrast, when living in a population of $F_1$ hybrid females, MS should be unrelated to colour because preference-heterozygous females have no mating preferences for blue, red or yellow males. Consistent with these predictions, we found a significant effect of male coloration on male MS in a population of $F_2$, but not in a population of $F_1$ hybrid females. In a population of $F_2$ hybrid females, males of more extreme coloration (reddish, blue) had higher MS than intermediately coloured (yellowish) males. Male colour, brightness and behaviour were strong predictors of male MS with *P. pundamilia*, *P. nyererei* and $F_2$ hybrid females alike. Male colour explained more of the variance in hybrid male MS with both non-hybrid female types than any other male variable, in agreement with other experiments that investigated non-hybrid females (Haesler & Seehausen 2005, van der Sluijs *et al.* 2008). The frequency distribution of the strength of preference for red versus blue in our laboratory-bred $F_2$ hybrid population had a single mode on no preference (Haesler & Seehausen 2005). The data we report here provide first experimental evidence that such a unimodal distribution of female preference variation in a fully admixed population can exert disruptive selection on male nuptial coloration.

Our results are relevant to scenarios of both secondary contact between species with hybridization and sympatric speciation. Theoretically, if mate choice exerts disruptive sexual selection, it might facilitate sympatric speciation (Turner & Burrows 1995; Payne & Krakauer 1997; Higashi *et al.* 1999). However, the mechanism is inherently unstable (Arnegard & Kondrashov 2004; Kirkpatrick & Nuismer 2004) unless a mechanism is in place that stabilizes trait polymorphisms during the process (van Doorn *et al.* 2004). Our experimental data are consistent with this inherent instability: males at one tail of the hybrid male phenotype distribution (blue) had higher MS than males at the other tail of the distribution. Also, in another study, blue males tended to have an overall higher MS that the authors attributed to a slightly larger body size and display rates of blue males (Seehausen & van Alphen 1998). Even though the latter does not apply to our results (neither male size nor behaviour was correlated with male coloration with one exception in experiment 1 where red males were more active in courtship than blue males), our data may imply that blue males attract, overall, more matings than red males. In the absence of any compensation, our experimental population, if allowed to evolve, would probably become fixed for blue male coloration. However, there is evidence that intrasexual selection on the same male nuptial colour traits may stabilize colour polymorphisms in *Pundamilia*. Red males dominate blue males in dyadic interactions (Dijkstra *et al.* 2005) and both phenotypes have own-type biases in aggression (Dijkstra *et al.* 2006; P. D. Dijkstra, C. Hemelrijk, O. Seehausen & T. Groothuis 2008, unpublished). Theoretical modelling suggests that sympatric speciation by sexual selection can be stable if disruptive intra- and intersexual selection operate on the same male trait (van Doorn *et al.* 2004). Without wanting to make any inference about the geographical mode of speciation, our demonstration that segregating female preferences can exert disruptive selection on male coloration, together with the work on male aggression behaviour, suggest that the interaction between intra- and intersexual selection may help explain the frequent sympatric maintenance despite gene flow of red and blue sister species pairs of cichlid fish in Lake Victoria (Seehausen *et al.* 1997).

In our experiment, we were interested in asking does the segregation of female mating preferences facilitate disruptive selection on the male trait. The validity of our test is independent of whether the genetic diversity in our experimental population reflects that in wild populations. However, populations of *Pundamilia* containing phenotypic variation similar to that in our experimental population can be found at several places in Lake Victoria (Seehausen 1997; van der Sluijs *et al.* 2008*b*), mainly where high water turbidity constrains mate choice. Our experimental results suggest that the potential for disruptive selection by female choice may be retained in such populations as long as the preference polymorphisms persist. If the genetics of mate choice in *Pundamilia* were representative for other species of Lake Victoria cichlids, it may help explain why these fishes evolved and retain substantial phenotypic differentiation despite gene flow.

This research adhered to the Association for the Study of Animal Behaviour/Animal Behavior Society Guidelines for the use of Animals in Research, local legislation, and institutional code of conduct.

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