A test of genetic association among male nuptial coloration, female mating preference, and male aggression bias within a polymorphic population of cichlid fish

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Abstract Both inter- and intrasexual selection have been implicated in the origin and maintenance of species-rich taxa with diverse sexual traits. Simultaneous disruptive selection by female mate choice and male-male competition can, in theory, lead to speciation without geographical isolation if both act on the same male trait. Female mate choice can generate discontinuities in gene flow, while male-male competition can generate negative frequency-dependent selection stabilizing the male trait polymorphism. Speciation may be facilitated when mating preference and/or aggression bias are physically linked to the trait they operate on. We tested for genetic associations among female mating preference, male aggression bias and male coloration in the Lake Victoria cichlid *Pundamilia*. We crossed females from a phenotypically variable population with males from both extreme ends of the phenotype distribution in the same population (blue or red). Male offspring of a red sire were significantly redder than males of a blue sire, indicating that intra-population variation in male coloration is heritable. We tested mating preferences of female offspring and aggression biases of male offspring using binary choice tests. There was no evidence for associations at the family level between female mating preferences and coloration of sires, but dam identity had a significant effect on female mate preference. Sons of the red sire directed significantly more aggression to red than blue males, whereas sons of the blue sire did not show any bias. There was a positive correlation among individuals between male aggression bias and body coloration, possibly due to pleiotropy or physical linkage, which could facilitate the maintenance of color polymorphism [Current Zoology 59 (2): 221–229, 2013].

Keywords Disruptive selection, Sexual selection, Speciation, *Pundamilia*, Linkage disequilibrium

Sexual selection theory predicts that mate choice can be a strong driver of sympatric speciation (Gavrilets et al., 2007; Kirkpatrick, 1982; Lande, 1981). Disruptive selection by female mate choice can facilitate speciation in sympatry in certain situations (Higashi et al., 1999; Payne and Krakauer, 1997) but to facilitate the emergence and persistence of trait polymorphisms within a population, speciation is more likely when disruptive selection is interacting with a negative frequency-dependent component of sexual selection on the same trait (van Doorn et al., 2004). Male-male competition can generate such negative frequency-dependent selection, because males often use the same cues that are used in mate choice (Mikami et al., 2004; van Doorn et al., 2004). Rare male phenotypes can have an advantage when males preferentially direct their aggression to males with similar coloration (Dijkstra et al., 2006; Seehausen and Schluter, 2004); hence, negative frequency-dependence may facilitate invasion of a novel male trait as well as coexistence of (incipient) species (reviewed in Dijkstra and Groothuis, 2011; Qvarnström et al., 2012; Seehausen and Schluter, 2004). Covariance among male aggression bias, coloration and female mate choice can emerge through genetic trait associa-
tions and thereby fuel sympatric speciation, while at the same time protecting against recombination by sexual reproduction (Servedio and Kopp, 2012; van Doorn et al., 2004).

An important question in models of speciation by sexual selection is how covariance evolves between mating preferences and secondary sexual traits. Such covariance can arise through genetic linkage, i.e. single gene pleiotropy, physical linkage, or non-random associations between alleles (Kronforst et al., 2006; Lande, 1981; Payne and Krakauer, 1997). Tight genetic linkage (or pleiotropy) promotes divergence and speciation because the effects of disruptive selection on one trait are transferred to the other trait, and traits that characterize incipient species are therefore less likely to be disassociated through recombination (Servedio and Kopp, 2012). One of the best examples of tight genetic linkage between traits that contribute to reproductive and ecological segregation comes from *Heliconius* butterflies. In this clade of mimetic butterfly species, there is evidence of tight genetic linkage and even pleiotropy between male mate preference and color pattern (Kronforst et al., 2006; Merrill et al., 2010). Non-random associations between alleles can further arise through assortative mating when there is large genetic variance in mating preferences (Lande, 1981).

Divergent sexual selection likely played an important role in the rapid radiation of cichlid fish species from the African Great Lakes Victoria and Malawi (Dominey, 1984; Maan et al., 2004; Seehausen et al., 1999; Seehausen et al., 1997; van Oppen et al., 1998). The haplochromine mating system, with exclusive female parental care in the form of mouthbrooding, is conducive to strong sexual selection. Experimental evidence shows that female mate choice based on male nuptial coloration can maintain species boundaries (Kidd et al., 2006; Seehausen and van Alphen, 1998; van der Sluijs et al., 2008b). Females of closely related Lake Victoria cichlid species prefer males of their own species under white light but choose randomly when color differences are masked by monochromatic light (Seehausen and van Alphen, 1998). Similarly, in some species of Lake Malawi cichlids, females prefer conspecific males when females only have a visual cue (Kidd et al., 2006). There is further evidence for directional sexual selection on male coloration by female mating preferences, where females prefer brightly colored over dull colored males of their own species (Maan et al., 2004). Furthermore, male nuptial coloration of closely related cichlid species is often extremely diverse, even though morphology and ecology are strikingly similar (Genner et al., 1999; Seehausen and Schluter, 2004).

Mating success results from the interaction between female mate choice and male-male competition (Wong and Candolin, 2005) and haplochromine cichlids further experience intrasexual selection on male color by male-male competition for mating territories (Pauers et al., 2008; Seehausen and Schluter, 2004). Males aggressively defend their territories against neighboring males and other intruders and use nuptial coloration in intrasexual aggression signaling. Intrasexual competition is an important component of sexual selection, because males require a territory to gain access to matings and territory size is an important factor affecting female choice (Dijkstra et al., 2008b; Gerlai, 2007; Maan et al., 2004).

Cichlid fish within the genus *Pundamilia* are a good example of species that are under directional and disruptive sexual selection. These species vary in their degree of genetic and phenotypic differentiation for blue and red color polymorphism (Seehausen, 2009). In Southern Lake Victoria, most clear water sites are predominated by the bimodal male phenotype consisting of two, genetically and phenotypically differentiated, sibling species with distinct male phenotypes: *P. pundamilia* males have blue-grey body coloration (Seehausen et al., 1998), whereas *P. nyererei* have a bright red dorsum with yellow flanks (Witte-Maas and Witte, 1985). Females of both species are cryptically colored and difficult to distinguish. While most populations of *P. pundamilia* and *P. nyererei* inhabit Lake Victoria sympatrically, turbidity of certain sites within the lake locally prevents or has prevented assortative mating in the past; hence at these turbid water sites male phenotype distribution is unimodal and females have varying mate preferences (Dijkstra et al., 2007; Seehausen, 1997; Seehausen et al., 1997; van der Sluijs et al., 2008a).

Several studies have demonstrated preference-trait covariance in genetic crosses between sister species, such as in *Heliconius* butterflies (Kronforst et al., 2006), field crickets (Gray and Cade, 2000), and *Drosophila* (Blows, 1999). In *Pundamilia* cichlids, female mate preferences are mainly based on male nuptial coloration (Seehausen and van Alphen, 1998) and are genetically different between sister species (Haesler and Seehausen, 2005; van der Sluijs et al., 2008b). In this study, we tested for covariation among female mating preference, male aggression bias and male nuptial coloration in *Pundamilia*. We examined this in a *Pundamilia* popula-
tion with broad but unimodal distributions of male phenotypes and female preferences (van der Sluijs et al., 2008a). We selected a panmictic population because any covariation between preferences and other traits within this population is most likely due to tight genetic linkage (or pleiotropy) rather than linkage disequilibrium, which would have been lost in a panmictic population. Such tight genetic linkage would facilitate divergence and speciation in the face of gene-flow.

To test for covariance among female preference, male nuptial coloration and male aggression bias, our approach was twofold: We first tested for preference-trait covariation by crossing randomly-selected females with either blue or red colored males from the same panmictic population. Female offspring of blue and red colored sires were then tested in mate choice trials with red and blue male stimuli (stimulus males originated from a clear water site). A positive genetic association between female mating preference and male coloration would become apparent if female offspring of red sires prefer the red over the blue species and vice versa. Second, coloration as well as aggression biases of male offspring were measured. Color differences between blue and red *Pundamilia* species are heritable (Seehausen, 2009; van der Sluijs et al., 2008b) but whether or not variation in color within a single population is heritable remained to be determined. We tested this by evaluating whether male offspring of a redder sire differ significantly in coloration from male offspring of a bluer sire. Additionally, if male coloration and male aggression bias are genetically linked, male offspring of a bluer sire should direct aggression towards blue males but males of a redder sire should direct aggression towards red males.

At the individual level, males should direct more aggression towards individuals that are similar to themselves. By contrast, an absence of associations among mating preference, aggression bias and male coloration could be due either to a lack of genetic linkage or to breakdown of weak linkage disequilibrium by laboratory crossings, but would make physical linkage very unlikely.

1 Materials and Methods

1.1 Fish

Males and females were collected at a turbid water location with a Secchi disk reading of 50±7 cm, Luanso Island in the southern part of Lake Victoria (Carleton et al., 2005), Tanzania in May 2001 and shipped to the University of Hull, United Kingdom. The most colorful males, one red and one blue, were selected visually from among 20 males and crossed to several females of the same population (Fig. 1). At maturity, female offspring were shipped to the University of Leiden, the Netherlands, for female mate choice trials and male offspring were shipped to the University of Groningen, the Netherlands, for male aggression bias trials and photography for color scoring. At all locations, fish were kept in aquaria at a temperature of 24 – 26 °C and 12 : 12h light:dark cycle. The fish were fed daily with a mixture of fresh shrimp and peas, or dry commercial cichlid pellets / flakes. For female mate choice trials, we used wild caught *P. pundamilia* (blue) and *P. nyererei* (red) stimulus males, which were collected near three islands of a small island group (Python Islands) in February 2003. Stimulus males for the male aggression bias trials were first generation lab-bred offspring of wild

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**Fig. 1  Experimental design**

Red males (top) and blue males (bottom) were allowed to mate with arbitrarily selected females from the same population. Differences in body coloration of the male offspring was determined and mating preference measured in female offspring. Male offspring were further tested for aggression bias.
caught *P. pundamilia* and *P. nyererei*. Both species were collected at Python Islands and Kissenda Island, in May 2001 and February 2003. At these islands *P. pundamilia* and *P. nyererei* are phenotypically differentiated and largely assortatively mating incipient species (Seehausen et al., 1997).

### 1.2 Crossing scheme

We obtained nine clutches of eight different dams, of which four were sired by the blue male and five by the red male. One clutch consisted of females only, whereas another consisted of males only. In total, we obtained 48 females; 18 females from of the blue sire and 30 females of the red sire. We further obtained 33 male offspring, ten males of the blue sire, and 23 males of the red sire. One male of the red sire died before we could test its aggression bias. All clutches were raised in full-sib groups. Colors are only expressed after sexual maturity, making it unlikely that individuals imprinted on sibling visual phenotypes.

### 1.3 Inheritance of male coloration

Photographs of 33 males were taken under standardized light conditions to test for color differences between males of the blue and the red sire. These males were assigned to a color category on a 0–4 point color scale, ranging from blue (0) to largely red (4) (van der Sluijs et al., 2008a) by two independent observers IS and PD. There was a general agreement between observers (Pearson correlation = 0.751, \( P < 0.001, n = 33 \)); hence the average score of the two observers was used for further analysis. To test for differences in coloration between males of the blue and the red sire, we fitted the data to linear mixed-effect models (LMER) in R (version 2.4.0 Ihaka and Gentleman, 1996) with Poisson distribution and log link function. We included clutch number as a random effect and determined the effects of sire and PD. There was a general agreement between observers because they died prior to the end of the experiment. In total, 284 successful trials were conducted.

All males were weighed (±0.1 g) and measured for standard length (±0.01 mm). Four male pairs (eight different males) were matched by standard length with a maximum difference of 10% and two male pairs were randomly assembled from the first four pairs which resulted in a maximum difference of 16%. Males of each pair had a fixed position in the tank in order to have each male species equally often in the right and left compartment of the experimental tank. Mate choice trials were done blind with respect to the color of the sire of the test female.

Response ratio is defined as the number of positive responses of the female to the total number of courtship displays of the male. To test for differences in female mating preference between females of the blue sire and females of the red sire, we fitted binomial generalized linear models (GLM) and linear mixed-effect models (LMER) with logit link functions in R (version 2.4.0 Ihaka and Gentleman, 1996). We fitted models which included sire, body weight, and standard length of the stimulus males as covariates. Models further included each trial as a covariate (GLM), or random effect (LMER), to correct for potential differences in courtship
frequency between males in one trial and pseudoreplication. We used the lme4 library version 0.9975-10 (Bates and Sarkar, 2006) for LMER. We checked models for overdispersion and adjusted statistics by switching to F-statistics and a quasi-likelihood approach when necessary. F-tests or Chi-square tests were performed on stepwise removal of variables. Covariates were excluded from the models if they had no significant effect ($P > 0.05$) on female preference. LMER models were further used to test for differences in courtship activity of males of the two species, in which male courtship activity was the response variable, male species was the explanatory variable and male identity added as a random effect variable.

1.5 Male aggression test

32 males were tested for aggression bias using simulated-intruder-choice tests (Dijkstra et al., 2007). A test aquarium consisted of a larger experimental compartment ($55 \times 35 \times 39$ cm, $l \times w \times h$) with a PVC tube as potential refuge for the test male, and a smaller compartment ($5 \times 35 \times 39$ cm) for a dither fish. The dither fish was a small male of *P. pundamilia* that stimulated territoriality of the test male. The dither fish was separated from the test male by a transparent partition. At the start of a trial, one blue and one red stimulus male, each enclosed in a watertight Plexiglas cylinder (cross section 13 cm), were simultaneously introduced to the compartment of the test male with a distance of 20 cm between each other. Immediately upon introduction of the stimulus males, aggressive behavior was scored for a period of five minutes.

Scoring was done blind with respect to the color of the sire of a given test male. Male aggressive behavior consists of display behavior in which a male approaches the intruder (frontal display) and shows the lateral side of the body with stretched fins (lateral display), and attack behavior in which males attempt to bite or butt (Baerends and Baerends-Van Roon, 1950). In these trials, physical contact was impossible because stimulus males were in Plexiglas cylinders. 13 different stimulus pairs (26 different males) were matched by standard length with a maximum difference of 5%. Males were successfully tested between one and eight times, and four times on average. Aggression bias was defined as the ratio of aggressive behaviors by a test male to the red stimulus male, divided by the total number of aggressive behaviors by the same test male to both stimulus males. Binomial LMER models with logit link functions were fitted to the data. An interaction term of male identity and clutch number was included in the models as random effect variable. The size and significance of the effects of dam, sire, color of test male, and placement of the stimulus males in the tank (blue male on the left and red male right, or the reverse) on aggression biases were tested by stepwise removal of each variable and chi-square tests on the models.

2 Results

2.1 Inheritance of male coloration

The average color score of the male offspring of the blue and red sires is plotted in Fig. 2. The minimum model to predict the color of the males retained clutch number as a random effect (variance clutch effect = $5e-10$, variance residual = $2.24e-5$) and identity of the sire as fixed effect ($\chi^2 = 5.68$, $df = 1$, $P = 0.017$). The identity of the dam had no effect on color of the males ($\chi^2 = 6.98$, $df = 6$, $P = 0.323$). Color scores of males of the red sire were significantly higher (i.e. redder) than those of males of the blue sire [estimate ($SE$) = 0.99 (0.48), $P = 0.037$].

2.2 Female mating preference

The average female response ratios to males of the blue and red species are presented for each clutch (Fig. 3; clutch number 9 consisted of males only). In GLM, only trial number had a significant effect on female response ratio ($F_{282, 285} = 2.11, P < 0.001$), and the remaining covariates did not influence the response ratio ($P > 0.05$). If we assume that random effects of trial number are normally distributed, we can use LMER with trial number as random effect ($SD$ random effect trial number = 0.337, $SD$ residual = 0.581) to estimate the size of the effects of sire and dam on female response ratios.

![Fig. 2](image_url)  
Fig. 2  Color scores of male offspring of blue and red sires (color scores ranged from 0 = blue to 4 = red; open dots)  
Filled dots represent the average color score. Overlapping data points were offset by a small arbitrary amount for display purposes only.
Sire identity did not have any significant effect on the response ratio of the females \( (\chi^2 = 0.205, df = 1, P = 0.651) \) but dam identity was significant \( (\chi^2 = 83.06, df = 6, P < 0.001) \). Male courtship activity of stimulus males differed significantly between species \( (\chi^2 = 24.09, df = 1, P < 0.001) \). Males of the blue species (average number of quivers = 14.83 ± 1.11 SE) courted more frequently than males of the red species (average number of quivers = 13.32 ± 1.11). In the models, we corrected for this difference by taking the number of positive responses of the female to the total number of courtship behaviors and by including trial number as covariate or random effect.

### 2.3 Male aggression bias

The left / right placement of the stimulus males in the tank \( (\chi^2 = 1.736, df = 1, P = 0.188) \) and the identity of the dam \( (\chi^2 = 8.393, df = 6, P = 0.211) \) had no significant effect on the aggression bias and were excluded from further analyses. An interaction term between male identity and clutch number was included as random effect variable, but was not significant (variance random effect = 0.471, variance residual = 0.686). There was a trend for the identity of the sire to affect male aggression biases \( (\chi^2 = 4.837, df = 2, P = 0.089) \).

Males of the blue sire showed no aggression bias [estimate \( (SE) = -0.392 (0.253) \), \( z = -1.553, P = 0.120 \), back transformed estimate = 0.597], whereas males of the red sire had a significant aggression bias for red males [estimate \( (SE) = -0.597 (0.288) \), \( z = -2.075, P = 0.038 \), back transformed estimate = 0.645]. Furthermore, own color of the test male had a significantly positive effect on aggression bias \( [\chi^2 = 264.31, df = 1, P < 0.001, \text{estimate } (SE) = 0.272 (0.171)] \). The latter result indicates that males with a higher red score have a higher aggression bias towards males of the red species. The own color effect remained significant when the clutches of the blue sire \( (\chi^2 = 50.83, df = 1, P < 0.001) \) were considered separately from the clutches of the red sire \( (\chi^2 = 214.58, df = 1, P < 0.001) \). The average aggression biases and coloration of male offspring of the blue and red sire are shown in Fig. 4.

### 3 Discussion

We tested for genetic associations among three phenotypic traits implicated in speciation within the Lake Victoria cichlid genus *Pundamilia*: male nuptial coloration, female mating preference for male nuptial coloration, and male aggression bias based on nuptial coloration. Genetic associations among these traits could potentially facilitate speciation without geographical isolation (Kirkpatrick, 1982; Lande, 1981; van Doorn et al., 2004). More specifically, tight genetic linkage (or pleiotropy) of traits that contribute to reproductive and ecological segregation promotes divergence and speciation in the face of gene flow, because traits characterizing incipient species are less likely to be disassociated through recombination (Servedio and Kopp, 2012). Studying the covariance between these three traits in...
laboratory crosses of different phenotypes collected from a variable wild population revealed that within-population variation in male coloration is heritable. This finding is consistent with earlier studies on *P. pundamilia* and *P. nyererei* populations which showed that interpopulation and interspecific differences in male nuptial coloration are heritable (Seehausen, 2009; Seehausen et al., 1997; van der Sluijs et al., 2010; van der Sluijs et al., 2008b).

Our results further showed that mating preferences of females of the blue sire did not differ from those of the red sire; hence no evidence for genetic associations between female preference and male coloration became apparent. Dam identity had a significant effect on mate preference of the daughters. The lack of strong linkage disequilibrium could be due to random mating in a large fraction of the population that lives in very turbid water, restricting assortative mating opportunities (van der Sluijs et al., 2008a), which is further supported by a lack of genetic differentiation between blue and red phenotypes in these turbid water locations (Seehausen, 2009). The absence of strong linkage disequilibrium does not exclude the possibility that preference and color are in weak linkage disequilibrium. However, our sample sizes do not allow for a precise determination of the level of linkage disequilibrium and a larger number of families is needed for estimation. Linkage disequilibrium decays by 50% per generation between unlinked loci when random mating occurs (Bakker and Pomiankowski, 1995; Hawthorne and Via, 2001); hence maintenance of weak linkage disequilibrium seems unlikely.

Two lines of evidence support a genetic association between male color and aggression bias: (1) Males of the redder sire showed an aggression bias towards red males, whereas males of the bluer sire did not have an aggression bias (i.e. they were equally aggressive towards both colors); (2) Male aggression biases were positively associated with own body coloration with redder males biasing more aggression towards red males. This ‘own color effect’ was significant when combining all males as well as when considering males from the red and the blue sire separately. Collectively, this suggests that there is a genetic association between male color and aggression bias, potentially due to pleiotropy or physical linkage that could facilitate the maintenance of own-type aggression biases in the face of gene-flow.

Aggression biases or mating preferences in our experiment may also have been influenced by learning from other individuals. Imprinting on the mother’s phenotype is unlikely to have affected aggression bias, because females of the variable population are phenotypically very similar. Moreover, a cross-fostering experiment in *Pundamilia* using individuals of a clear water population showed that female mating preferences can be strongly affected by sexual imprinting on the mother’s phenotype (Verzijden and ten Cate, 2007), but the authors did not detect any effect of imprinting on the development of aggression biases in males (Verzijden et al., 2009). By contrast, *Pundamilia* males, including those from a panmictic population, that were only exposed to blue males developed no aggression bias, whereas exposure to both blue and red phenotypes during the adult phase led to a blue bias (Dijkstra et al., 2008a). In the current study, learning was less likely to influence aggression biases, first because the possibility to learn from the color of siblings was limited as completely red males were lacking in all sib groups (Fig. 2), and second because males of the red sire were exposed to a wider range of color phenotypes (both blue and more reddish sibs similar to Dijkstra et al., 2008a) compared to males of the blue sire who were mainly exposed to blue siblings (see Fig. 2). This would predict that males of the red sire should bias aggression towards blue, yet we found that males of the red sires in fact biased aggression towards red.

We found no evidence for genetic associations between male nuptial coloration and female mating preference. It thus appears that in the initial stages of speciation, strong disruptive selection is required for the
buildup of linkage disequilibrium between these traits. However, the genetic association between male nuptial coloration and male aggression bias maintains aggression bias of redder males towards red males even in the face of gene flow, which in theory could generate negative frequency dependent selection. Sustained frequency dependent selection could facilitate speciation by favoring the invasion of rare-male phenotypes and maintenance of male color variation prior and during the course of speciation.

The limited genetic covariance in traits that contribute to reproductive isolation suggests that strong disruptive selection is required to initiate and complete speciation if it was to happen in sympatry. Possible factors contributing to the sympatric maintenance of color variation in *Pundamilia* are environmental heterogeneity in ambient light conditions (Seehausen et al., 2008) and color-specific physiological trade-offs between sexual signaling and health maintenance (Dijkstra et al., 2011). These findings are consistent with the idea that speciation occurred by a combination of sexual and ecological selection (Maan and Seehausen, 2011; Weissing et al., 2011). These findings are consistent with the idea that speciation occurred by a combination of sexual and ecological selection (Maan and Seehausen, 2011; Weissing et al., 2011).

In summary, we found no evidence for genetic associations between male nuptial coloration and female mating preference, but there is evidence for genetic associations between male nuptial coloration and male aggression bias. The latter could facilitate invasion of rare-male phenotypes and maintenance of male color variation prior to speciation that could potentially pave the way for speciation, most likely aided by divergent ecological selection.

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