Female mating preference functions predict sexual selection against hybrids between sibling species of cichlid fish

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The evolutionary outcome of interspecific hybridization, i.e. collapse of species into a hybrid swarm, persistence or even divergence with reinforcement, depends on the balance between gene flow and selection against hybrids. If female mating preferences are open-ended but sign-inversed between species, they can theoretically be a source of such selection. Cichlid fish in African lakes have sustained high rates of speciation despite evidence for widespread hybridization, and sexual selection by female choice has been proposed as important in the origin and maintenance of species boundaries. However, it had never been tested whether hybridizing species have open-ended preference rules. Here we report the first experimental test using *Pundamilia pundamilia*, *Pundamilia nyererei* and their hybrids in three-way choice experiments. Hybrid males are phenotypically intermediate. Wild-caught females of both species have strong preferences for conspecific over heterospecific males. Their responses to F_1 hybrid males are intermediate, but more similar to responses to conspecifics in one species and more similar to responses to heterospecifics in the other. We suggest that their mate choice mechanism may predispose haplochromine cichlids to maintain and perhaps undergo phenotypic diversification despite hybridization, and that species differences in female preference functions may predict the potential for adaptive trait transfer between hybridizing species.

Keywords: female mating preference; sexual selection; hybrid attractiveness; speciation; cichlid fish; hybridization

1. INTRODUCTION

The evolutionary outcome of interspecific hybridization, i.e. the likelihood of species collapsing into a hybrid swarm, as opposed to persisting or even diverging further, depends on the balance between gene flow and selection against hybrids (Turelli *et al.* 2001). Where sympatric with the parental species, hybrids may suffer from ecological intermediacy, but additionally or alternatively, female mating preferences can be a source of selection against hybrids. The case for this seems particularly strong if (i) hybrid males are phenotypically intermediate, (ii) preference rules are open-ended in both species but (iii) sign-inversed between them and (iv) the sign of the preference has a simple Mendelian basis. Open-ended preference functions describe female responsiveness that is the strongest at one end of the male trait axis and the weakest at the other end.

Cichlid fish in African lakes are a rapidly radiated group of vertebrates with sustained high rates of species origination despite fairly widespread evidence for hybridization (Seehausen 2006). In fact, some of the best examples for sympatric speciation (Schliewen *et al.* 1994), phenotypic cohesion in the face of gene flow (Samonte *et al.* 2007; Seehausen in press) and hybrid speciation (Salzburger *et al.* 2002; Schliewen & Klee 2004) are African cichlids. Sign-inversed preferences for male nuptial coloration between species have been demonstrated at least for one group of colourful Lake Victoria cichlids, the genus *Pundamilia* (Seehausen & van Alphen 1998; Stelkens *et al.* 2008).

In many theoretical and simulation models of sympatric speciation, intermediate phenotypes are at a fitness disadvantage caused by a lower mating success relative to that of phenotypes at either end of the phenotypic scale (Higashi *et al.* 1999; Takimoto *et al.*

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2000; Lande *et al.* 2001; van Doorn *et al.* 2004). Similarly, even though reinforcement models often assume that when species come into secondary contact, hybrids have reduced viability or fertility (Liou & Price 1994; Kirkpatrick & Servedio 1999; Servedio 2001), reinforcement can occur even if only sexual selection is directly acting against hybrids (Kawata & Yoshimura 2000). Disruptive sexual selection may be important in the maintenance of species boundaries (Jiggins *et al.* 2001; Kirkpatrick & Ravigne 2002). Population divergence in mating preferences does often evolve faster than genic incompatibilities (Coyne & Orr 1989). Disruptive sexual selection between species upon secondary contact has been shown in three-spined sticklebacks (Vamosi & Schluter 1999) and *Heliconius* butterflies (McMillan *et al.* 1997; Mallet *et al.* 1998; Naisbit *et al.* 2001).

Disruptive sexual selection has been proposed to be important in the speciation of the endemic cichlid fish of Lakes Victoria and Malawi (Van Oppen *et al.* 1998; Seehausen & van Alphen 1999; Maan *et al.* 2006). However, the prerequisite of open-ended preference rules that are sign-inversed between the species, surprisingly, had never been tested in any cichlid fish. To test this, intermediate male phenotypes have to be produced by hybridizing species. Here we report the first of such experiments in which we tested whether the sympatric, closely related and hybridizing species *Pundamilia pundamilia* (Seehausen *et al.* 1998) and *Pundamilia nyererei* (Witte-Maas & Witte 1985) do have such preference rules. Interspecific hybrids can be obtained readily by giving females access only to heterospecific males and are fully viable and fertile (van der Sluijs *et al.* 2008b). An earlier study already showed that females prefer conspecific males under white light but not under colour-masking light (Seehausen & van Alphen 1998). In a companion study, we tested whether these preferences do exert disruptive selection on male coloration, when coloration is dissociated from other differences between the species (Stelkens *et al.* 2008). Here we use that same species pair to ask whether (i) hybrid male phenotypes are intermediate and (ii) the female preferences are open-ended, two prerequisites for selection against hybrids.

2. MATERIAL AND METHODS

(a) Description of the species
Males of *P. pundamilia* are blue-grey whereas that of *P. nyererei* have a bright red dorsum with yellow flanks. Furthermore, *P. pundamilia* have four to six broad vertical black bars and *P. nyererei* have six to eight narrower vertical bars on the flanks (Seehausen 1996). The *P. pundamilia* and *P. nyererei* females have a slightly different coloration, being grey-brownish and grey-yellowish, respectively, and can be distinguished only with difficulty.

(b) Fish collection and breeding
All fishes for breeding were collected at Python islands in the southern part of Lake Victoria. The mean visibility, measured as Secchi disc readings between 1991 and 2003, is 98 ± 12 cm at Python islands. Although the two species hybridize occasionally at this location, there is a strongly bimodal frequency distribution of variation in male nuptial colour phenotypes (Seehausen 1997, in press; Dijkstra *et al.* 2007).
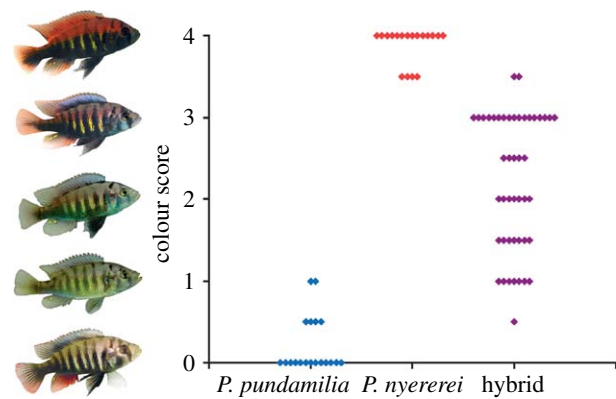

Figure 1. Male colour scores for each male type. The three different male types, *P. pundamilia* ($n = 19$), *P. nyererei* ($n = 18$) and hybrid males ($n = 46$), are shown on the *x*-axis. The males were assigned to five different categories based on total body coloration, shown on the *y*-axis. Overlapping data points were offset by a small arbitrary amount for display purposes only.

P. pundamilia and *P. nyererei* inhabit the rocky shores between 0 and 6 m water depth, but at Python island the *P. pundamilia* males keep territories in shallower water and the *P. nyererei* males are more abundant in deeper water (Seehausen 1997, in press).

Males and females of both species were collected in February 2003. All fishes were shipped to Leiden, The Netherlands, and kept in aquaria at $24 \pm 1°C$ and 12 L : 12 D. They were fed a blend of fresh shrimps and peas 3 days a week and dry commercial cichlid pellets the other days. Females were allowed to mate with conspecific or heterospecific males and the male offspring was used in three-way female choice trials. Heterospecific crosses were made in two directions, hence with *P. nyererei* mother and *P. pundamilia* father and vice versa. In September 2005, we collected females of both species from the same island for the preference tests. Females were collected as adults, thus of an unknown age; however, female mate choice is highly repeatable and does not change through its reproductive life (O. Svensson *et al.* 2005–2008, unpublished data). Standard length was measured with digital callipers (± 0.01 mm), fishes were weighed (± 0.1 g) and photographed for colour analysis.

(c) Male colour analysis and number of bars
Photographs were taken of 19 *P. pundamilia*, 18 *P. nyererei* and 46 hybrid males (26 male offspring of a *P. nyererei* female and a *P. pundamilia* male, and 20 male offspring of a *P. pundamilia* female and a *P. nyererei* male). The males were placed in a photo-cuvette and photographed with a Sony digital camera (DSC-F707). A Kodak colour strip (no. Q13, Eastman Kodak, Rochester, NY) was attached to the front glass to calibrate the photos in Photoshop v. 6.0 (Adobe Systems, Inc.). Males were assigned to a five-point (0–4) colour scale based on total body coloration (adjusted from Dijkstra *et al.* 2007; van der Sluijs *et al.* 2008b; Seehausen in press): 0, blue; 1, yellow flank but no red, spiny part of dorsal fin blue; 2, yellow flank with some red along the upper lateral line, spiny dorsal fin blue; 3, yellow flank with a partially red dorsum upwards from the upper lateral line, but a grey body crest and largely blue spiny dorsal fin; and 4, yellow flank with a completely red dorsum between the upper lateral line and the body crest, red spiny dorsal fin (figure 1). We also counted the number of vertical bars of the males between the pectoral fin and the caudal end of the dorsal fin from photos. The parental populations at Python islands differ significantly in this trait (Seehausen 1997).
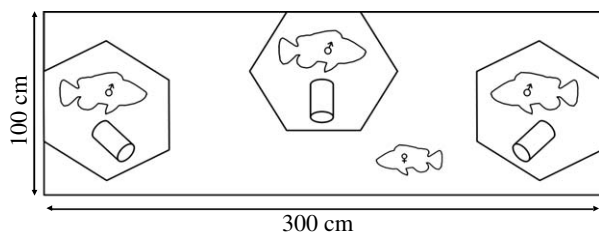
Figure 2. Plan view of the experimental tank with the males in Perspex enclosures. The test female can choose to interact with the males.

Significance of differences between male types was tested with Mann–Whitney U test for non-normal data in SPSS v. 12.0.1 (SPPS, Inc.).

(d) Experimental set-up of female mate preference tests

One week before the onset of the trials, fishes were put into individual isolation tanks. Males had visible contact with one neighbouring male to stimulate territoriality and only females had female neighbours. Wild-caught females were given a three-way choice between one *P. pundamilia*, one *P. nyererei* and one hybrid male. Each trio was used to test one *P. nyererei* female and one *P. pundamilia* female. We used 11 *P. nyererei* females each of which was tested between one and three times and 13 *P. pundamilia* females each of which was tested one or two times with different male trios. In total, we used 20 different male trios. In half of them, the males were matched for standard length with less than 12% difference and the other half assembled randomly. Ten different *P. nyererei* males, 11 different *P. pundamilia* males and 15 different hybrid males (eight male offspring of a *P. pundamilia* female and *P. nyererei* male and seven male offspring of the reciprocal cross) were used. Males were placed in six-sided watertight Perspex enclosures with a width of 50 cm. Olfactory communication between males and female was excluded. The three enclosures were in a tank of $300 \times 100 \times 60$ cm ($l \times w \times h$) with equal distances between them (figure 2). Within a trio, males were kept in the same position in the tank (left, right or middle) in the trials with both female species. The different male types were equally often in each position. A polyvinyl chloride tube was placed in each enclosure, which males adopted as the centre of their territory. Males were allowed to acclimatize in the enclosures overnight. A trial was started by releasing a female in the middle of the tank. Courtship behaviours of the males and response of the female was scored during 20 min using OBSERVER v. 3 event recording software (Noldus).

Courtship behaviour (illustrated in Seehausen 1996) usually starts with an approach of the male and is followed by a lateral display, in which it shows its flank and stretches the fins. Subsequently, the male will start to shake his body, which is called quiver (Q) and will try to lead the female to the spawning site, where it starts circling, after which spawning and fertilization of the eggs may take place. In our trials, spawning did not occur because the fishes were separated by the Perspex enclosure. In successful trials, all males had courted at least once and the female responded positively, by approaching to at least one male. Unsuccessful trials were repeated with the same fish after a pause of at least 1 day. In total, we conducted 85 trials to obtain 40 that were successful by these standards.

(e) Data analysis of female mate preference

Female mate preference is defined as the number of positive responses of the female over the total number of quivers

displayed by the male. Female mate preferences were estimated using the lme4 library, v. 0.9975-10 (Bates & Sarkar 2006) for binomial generalized linear mixed models with logit link function in R software, v. 2.4.0 (Ihaka & Gentleman 1996; Maan *et al.* 2004; van der Sluijs *et al.* 2008*a*). An interaction term between identity and species of the females was built into the model as a random effect to remove pseudoreplication resulting from multiple use of the same female. Furthermore, we included standard length, weight or condition factor of the males (calculated as $100 \times$ weight (g)/standard length (cm)$^{2.76}$ after Bolger & Connolly 1989) as covariates. The interaction between female species and male type (*P. pundamilia*, *P. nyererei* or hybrid) was included in the models to estimate the preference of females of the two species for the three different male types. We fitted models that included each trial performed with one female as a fixed effect, to correct for differences in courtship frequency of the males in one trial. Minimum adequate models were built by stepwise removal of model terms using χ^2 test. Model terms were removed from the models if removal had no significant effect on model fit. We checked models for overdispersion and adjusted statistics by switching to *F*-statistics and a quasi-likelihood approach (McCullagh & Nelder 1989) when there was significant overdispersion. Female preference for the different male types was compared by means of simultaneous confidence intervals (CIs) with the multcomp library, v. 0.991-7 (Hothorn *et al.* 2006). These are adjusted for the fact that several pairs of groups are compared simultaneously. CIs for difference in female mating preference for conspecific, heterospecific and hybrid males were estimated using the same library, to test symmetry in female mating preferences of the two species.

Differences in courtship frequency between the three types of males were also tested with linear mixed-effect models but with Poisson distribution and log link function. Male quiver was the response variable, male type the explanatory variable and male identity a random effect. Courtship frequency of the three male types was compared by means of simultaneous CIs.

3. RESULTS

(a) Male colour analysis and number of bars

Data on male colour scores and number of bars were not normally distributed; therefore, we used Mann–Whitney U tests to compare the different male types (table 1 for averages, standard errors and all test results). There was no significant difference ($p = 0.909$) between colour scores of hybrid males from *P. pundamilia* mother, and a *P. nyererei* father, and those of the reciprocal cross. Therefore, we pooled these datasets. The *P. nyererei* males had significantly higher colour scores, i.e. more red and yellow, than the *P. pundamilia* males. Hybrids were intermediate in coloration and were significantly different from both parental species. The variance in coloration in hybrids was larger than that in the parental species (figure 1).

The number of bars differed significantly between the parental species and, as expected, *P. nyererei* males had the highest number of bars. Here, we could not pool the data from the two directions of the cross since these were different. Hybrids were intermediate in the number of bars although hybrids of *P. nyererei* mother and *P. pundamilia* father did not differ significantly from *P. pundamilia* males.

Table 1. Average colour score, the number of males and the number of bars of males of the parental species and the hybrids. The s.e.m. are given in brackets. The scores of the males of hybrid crossings of different parents are given separately. The first mentioned is the maternal species. Mann–Whitney *U* tests for differences in colour scores and in number of bars between male types, with *n*, *z* and *p* values.

male type		*n*	colour score (s.e.)		*n* bars (s.e.)
P. pundamilia		19	0.21 (0.08)		5.11 (0.15)
P. nyererei		18	3.89 (0.05)		6.36 (0.11)
hybrid		46	2.23 (0.12)		5.61 (0.12)
P. nyererei×*P. pundamilia*		26	2.21 (0.17)		5.35 (0.17)
P. pundamilia×*P. nyererei*		20	2.25 (0.18)		5.95 (0.11)
colour male type 1	colour male type 2	*n₁*	*n₂*	*z*	*p*
P. pundamilia	*P. nyererei*	19	18	−5.48	0.000[a]
P. nye×*pun*	*P. pun*×*nye*	26	20	−0.11	0.909
P. pundamilia	hybrid	19	46	−6.24	0.000[a]
P. nyererei	hybrid	18	46	−6.23	0.000[a]
bars male type 1	bars male type 2	*n₁*	*n₂*	*z*	*p*
P. pundamilia	*P. nyererei*	19	18	−4.61	0.000[a]
P. pundamilia	*P. nye*×*pun*	19	26	−1.14	0.254
P. pundamilia	*P. pun*×*nye*	19	20	−3.78	0.000[a]
P. nyererei	*P. nye*×*pun*	18	26	−3.88	0.000[a]
P. nyererei	*P. pun*×*nye*	18	20	−2.41	0.016[a]
P. nye×*pun*	*P. pun*×*nye*	26	20	−2.51	0.012[a]

[a] Difference is significant.

(b) *Female mate preference*

The average female response ratio of the raw data is presented for each species with 95% CIs in figure 3. The random effect of the interaction of female identity and female species was very small (7.26×10^{-10}). Therefore, we switched to binomial generalized linear models that included trial effect parameters correcting for pseudoreplication. An additional consideration was that binomial mixed models allow only approximate inference, based on PQL or Laplace approximations, and using such models for inference can be riskier than fitting a model with fixed effects only.

Female preferences were influenced by the significant interaction between female species and male type ($F_{2,77} = 9.00$, $p < 0.001$). Female preferences were not influenced by the standard length, weight or condition factor of the males ($p > 0.05$).

Pundamilia pundamilia females showed the highest mate preference for *P. pundamilia* males, followed by hybrid males, and the lowest for *P. nyererei* males. The difference between the preferences to *P. pundamilia* and *P. nyererei* males was significant, but there was no difference between the responses to hybrid and conspecific males. *P. nyererei* females showed the highest mate preference for *P. nyererei* males and similarly low preferences to both *P. pundamilia* and hybrid males. The difference between the responses to *P. nyererei* and both *P. pundamilia* and hybrid males was significant, but there was no difference between responses to hybrid and heterospecific males (see table 2a for parameter estimates and simultaneous CIs). Despite large standard errors on the parameter estimates, the differences between them were significant in several cases. That is then due to a positive covariance between these estimates in the observed Fisher information matrix. This means that among all models with parameter values similar to
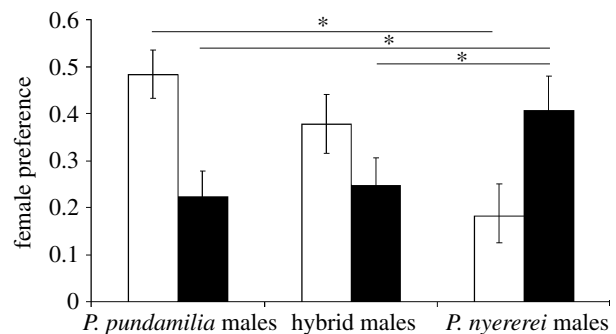

Figure 3. Female mate preferences for *P. pundamilia*, hybrid and *P. nyererei* males with 95% CI. Female mate preference is the average response ratio of the raw data, which is the proportion of positive responses of the female over the total number of quivers. The open bars represent *P. pundamilia* females and filled bars represent *P. nyererei* females. Significant differences are indicated with an asterisk.

the estimates in table 2a, the models that have pairwise differences between groups similar to the ones reported in table 2a consistently have the largest likelihood.

These results suggest an asymmetry in response to conspecific over hybrid males of females of the two species, but when the responses of all females to conspecific males were compared by means of simultaneous CIs with the response to hybrid males, we found that there was no difference. The same is true for the response to heterospecific and hybrid males. Females of the two species differed in their response to conspecific and heterospecific males, with females of the blue species better able to distinguish between these male types (table 2b).

Male type had a significant effect on the number of quivers ($\chi^2_2 = 7.38$, $p = 0.03$). Male identity was included as random effect (s.d. effect = 0.25, s.d.

Table 2*a*. Parameter estimates and standard errors of minimum GLM for female mating preference (upper panel) and standard errors. In the lower panel, we show simultaneous CIs (95%) for mating preference differences of the two female species between the three male types. All estimates are relative to the average response in the first trial and parameter estimates for trial effects are omitted (37 effects).

female species	male type	female mating preference (s.e.)
P. pundamilia	*P. pundamilia*	0.15 (0.84)
P. pundamilia	hybrid	−0.21 (0.81)
P. pundamilia	*P. nyererei*	−1.16 (0.84)
P. nyererei	*P. pundamilia*	0.20 (0.74)
P. nyererei	hybrid	0.30 (0.74)
P. nyererei	*P. nyererei*	1.08 (0.78)

female species	male type 1	male type 2	female mating preference difference	lower	upper
P. pundamilia	*P. pundamilia*	*P. nyererei*	1.31	0.29	2.34[a]
P. pundamilia	*P. pundamilia*	hybrid	0.36	−0.42	1.15
P. pundamilia	*P. nyererei*	hybrid	−0.95	−1.99	0.10
P. nyererei	*P. pundamilia*	*P. nyererei*	−0.88	−1.60	−0.16[a]
P. nyererei	*P. pundamilia*	hybrid	−0.10	−0.83	0.62
P. nyererei	*P. nyererei*	hybrid	0.78	0.04	1.15[a]

[a] Difference is significant; CI does not include zero.

Table 2*b*. Differences between the mating preferences of females of both species pooled calculated by simultaneous CIs between the three male types to test symmetry in mate preferences (95%).

male type 1	male type 2	female mating preference difference	lower	upper
conspecific	heterospecific	2.19	0.90	3.49[a]
conspecific	hybrid	−0.41	−1.56	0.75
heterospecific	hybrid	−0.84	−2.16	0.48

[a] Difference is significant; CI does not include zero.

residual $= 0.50$). *P. pundamilia* males courted on average more frequently ($Q = 14.09$, s.e. $= 0.16$) than *P. nyererei* males ($Q = 7.24$, s.e. $= 0.17$). Courtship frequencies of hybrid males were intermediate and not significantly different from males of either parent species ($Q = 10.29$, s.e. $= 0.14$).

4. DISCUSSION

Hybridization is common among species of the cichlid fish and other young adaptive radiations. Direct female mating preferences for different male phenotypes, both with perhaps relatively simple genetic architectures, have been suggested to play an important role in the origin and maintenance of cichlid species (Kocher 2004). Sign-inversed preferences for male nuptial coloration characterize sympatric and hybridizing species of *Pundamilia* in Lake Victoria (Seehausen & van Alphen 1998; Stelkens *et al.* 2008), and the species difference in preference is caused by few genes with large effects (Haesler & Seehausen 2005). Here we have tested two additional conditions for female mating preferences to maintain species differences despite hybridization. We have shown that (i) hybrid males in *Pundamilia* are intermediate between the species in phenotype and courtship frequency (in contrast with, for example, a reduced courtship intensity in *Drosophila* hybrids, described in Noor 1997) and (ii) females of both species appear to have open-ended preference

rules at least over the male trait range tested, responding most strongly to conspecific males and least strongly to heterospecific males, two prerequisites for female mate choice to potentially cause selection against hybrids. These results are consistent with the hypothesis that the mate choice mechanism in Lake Victoria cichlid fish facilitates the maintenance of phenotypic differences in the face of hybridization and gene flow, i.e. the porous genome model of speciation (Wu 2001). In another paper (Stelkens *et al.* 2008), we do indeed find evidence that segregation of the female mating preferences exerts disruptive selection on male coloration in a laboratory hybrid population too.

If the outcome of our experiment predicts the situation in nature, hybridization would in clear water environments perhaps be relatively rare since interspecific female mating preferences tend to maintain species boundaries. However, once hybridization has occurred, *P. pundamilia* females seem to discriminate less against hybrid males than *P. nyererei* females. Consequently, *P. nyererei* genes might introgress into *P. pundamilia* perhaps more readily than vice versa. Even if both hybridizing species had open-ended preference functions with inversed sign, and assortative mate choice was symmetric between the species, the direction of gene flow between species could be affected by interspecific differences in shape and slope of the preference function.

2876 I. van der Sluijs *et al.* *Mate preferences in hybridizing cichlids*

Here we tested only prerequisites for disruptive sexual selection on male nuptial coloration. It is probable that natural selection acts on *Pundamilia* male coloration too. Conspicuous nuptial coloration is important to attract females in these cichlids (Maan *et al.* 2004), but being conspicuous can be costly in terms of an increased predation risk. Conspicuousness depends on the light environment, the contrast with the natural background, and the visual system of the predator (Endler 1978). Male nuptial coloration may evolve to optimize the trade-offs between mate attraction and predator avoidance (Endler 1983). Work on other systems has shown that hybrid male coloration may be a poor match to the environment. Therefore, hybrid males may suffer from higher predation and predation could be an additional source of disruptive selection (Mallet & Barton 1989; Naisbit *et al.* 2001; Godin & McDonough 2003; Stuart-Fox *et al.* 2003). While we cannot rule this out at present for *Pundamilia*, we have no indication that this might be so. Hybrid males may match the yellowish background light in turbid waters better than either red or blue non-hybrid males. However, it is hard to see that blue and red males would match the background in clear water better than yellowish males would.

Territories of males of the red species tend to be in deeper water than those of the blue species. This depth segregation decreases with water turbidity and is completely lost at islands with highly turbid waters (Seehausen 1996, in press). Maan *et al.* (2006) found that females of the blue and red species of a population from a clear water island had significant differences in their behavioural sensitivities to red and blue light coinciding with the body coloration of conspecific males. This could imply that in early stages of speciation, the visual system adapts to the light environment at different water depths, leading to divergence in female mating preferences for the male nuptial colours that best match their visual sensitivity. Alternatively, the difference in visual sensitivity can also be a secondary adaptation to a different visual environment. There is also molecular evidence for differences in the visual system between these two species. Individuals of the blue and red species differed in the relative expression levels of three opsin genes in directions that corresponded with the difference in male body coloration. Moreover, the red and blue species differed in their main LWS (long wavelength-sensitive cone pigment) allele, the red species possessing an LWS allele that was red shifted by several nanometres relative to the allele of the blue species (Carleton *et al.* 2005).

Interspecific differences in the shape of the preference function could be an important predictor of the potential for adaptive trait transfer between hybridizing species. In the future, our predictions of asymmetrical introgression could be tested with molecular and population genetic tools. It would perhaps predict larger molecular and adaptive variation in *P. pundamilia* due to introgression of *P. nyererei* genes. Future work should also address effects of intermediate coloration on fitness components other than attractiveness to females.

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