

Original Article

# Female preference for male color is necessary and sufficient for assortative mating in 2 cichlid sister species

Oliver M. Selz,<sup>a,b</sup> Michele E.R. Pierotti,<sup>a,b</sup> Martine E. Maan,<sup>a,b,c</sup> Corinne Schmid,<sup>a,b</sup> and Ole Seehausen<sup>a,b</sup>

<sup>a</sup>Department of Fish Ecology and Evolution, EAWAG Swiss Federal Institute of Aquatic Science and Technology, Center for Ecology, Evolution and Biogeochemistry, Seestrasse 79, CH-6047 Kastanienbaum, Switzerland, <sup>b</sup>Aquatic Ecology and Evolution, Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, CH-3012 Bern, Switzerland and <sup>c</sup>Behavioural Biology Research Group, Center for Behaviour and Neurosciences, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

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A critical step for speciation in the face of gene flow is the origination of reproductive isolation. The evolution of assortative mating greatly facilitates this process. Assortative mating can be mediated by one or multiple cues across an array of sensory modalities. We here explore possible cues that may underlie female mate choice in a sympatric species pair of cichlid fish from Lake Victoria, *Pundamilia pundamilia* and *Pundamilia nyererei*. Previous studies identified species-specific female preferences for male coloration, but effects of other cues could not be ruled out. Therefore, we assessed female choice in a series of experiments in which we manipulated visual (color) and chemical cues. We show that the visibility of differences in nuptial hue (i.e., either blue or red) between males of the 2 species is necessary and sufficient for assortative mating by female mate choice. Such assortment mediated by a single cue may evolve relatively quickly, but could make reproductive isolation vulnerable to environmental changes. These findings confirm the important role of female mate choice for male nuptial hue in promoting the explosive speciation of African haplochromine cichlids.

**Key words:** assortative mating, cichlid, hue, mate choice, teleost fish.

## INTRODUCTION

Understanding how new species emerge and persist is a core question in evolutionary biology. In sexually reproducing species, nonallopatric speciation fundamentally requires overcoming recombination, which otherwise keeps breaking down favorable trait combinations required for divergent adaptation (Maynard Smith 1966; Mayr 1970; Felsenstein 1981; Kirkpatrick and Ravigné 2002). The evolution of assortative mating among individuals of diverging populations is one way by which reproductive isolation can emerge (Kondrashov and Shpak 1998; Gavrillets 2004) and can be critical for speciation with gene flow (Dieckmann and Doebeli 1999; Thibert-Plante and Hendry 2009, 2011; Servedio et al. 2011). Behavioral isolation can be promoted by divergent ecological and/or sexual selection, as proposed by theoretical models

(Turner and Burrows 1995; van Dooren et al. 1998; Higashi et al. 1999; Kondrashov and Kondrashov 1999; Via 2001; Doebeli and Dieckmann 2003; Débarre 2012; M'Gonigle et al. 2012) and supported empirically (Schluter 2009; Sobel et al. 2010; Maan and Seehausen 2011). The evolution of mate choice is one of the most crucial processes responsible for the generation and maintenance of biological diversity (Boake 2002).

Mate choice frequently involves multiple cues across multiple sensory modalities (Partan and Marler 1999, 2005; Candolin 2003; Hebets and Papaj 2005; Chenoweth and Blows 2006; Hohenlohe and Arnold 2010; Hebets 2011). Not all of these cues, and sexual preferences for them, need to diverge in order for reproductive isolation to evolve. Assortative mating may rely on divergence in a single cue (e.g., based solely on visual color patterns as in *Heliconius* butterflies, Jiggins et al. 2001, and in poison-dart frogs, Reynolds and Fitzpatrick 2007, or based on vibrational or acoustic signals as in treehopper insects, Rodriguez et al. 2006, and in North American crossbill birds, Snowberg and Benkman 2007, respectively) or involve multiple sensory modalities (e.g., visual and/or chemical cues in

Address correspondence to O.M. Selz. E-mail: [oliver.selz@eawag.ch](mailto:oliver.selz@eawag.ch).

pupfish, Kodric-Brown and Strecker 2001; Kodric-Brown and West 2014, and in three-spined stickleback fish, Rafferty and Boughman 2006, or visual, chemical, and tactile cues in plethodontid salamanders, Houk and Verrell 1993). The relationship between signal multimodality (i.e., signals received through more than 1 sensory channel) and the ease with which assortment evolves is not straightforward, as it depends on the phylogenetic time of emergence of these cues, relative to each other and relative to the stage of divergence, their underlying genetic architectures, and the additive or interactive effects of multiple sensory modes in signal assessment. Currently, a paucity of explicit theoretical modeling and empirical examination of multimodality in the context of assortative mating and speciation preclude a thorough understanding of its effects on the evolution and maintenance of reproductive isolation (Hohenlohe and Arnold 2010). Evaluating the role of sexual selection in speciation requires experiments that manipulate the number of signal modalities to understand the importance of individual cues in mate choice. In this study, we investigate the dimensionality of mate choice underlying reproductive isolation by identifying the cues that mediate assortative mating between sibling species of Lake Victoria cichlid fishes.

The haplochromine cichlid fish of the East African Lakes have become a model system in speciation and adaptive radiation research (Kocher 2004; Seehausen 2006). Male coloration is a target of sexual selection by female mating preferences both at the intra- and interspecific levels in many African cichlid species (Seehausen and van Alphen 1998; Coudridge and Alexander 2002; Jordan et al. 2003; Knight and Turner 2004; Maan et al. 2004; Pauers et al. 2004, 2010; Salzburger et al. 2006; Egger et al. 2008, 2010; Stelkens and Seehausen 2009; Pauers and McKinnon 2012; Maan and Sefc 2013; Selz et al. 2013; Tyers and Turner 2013), suggesting that female choice for male coloration might play an important role in the evolution of reproductive isolation and speciation of African cichlids (Dominey 1984; van Oppen et al. 1997; Seehausen and van Alphen 1999; Seehausen, Mayhew, et al. 1999; Wagner et al. 2012). Here, we focus on 2 populations of 2 closely related cichlid species from Lake Victoria, *Pundamilia pundamilia* and *Pundamilia nyererei*, which are at an incipient stage of speciation. To study reproductive isolation barriers that may have contributed to speciation, it is necessary to test species pairs at an incipient stage of the speciation process; when testing species that are completely reproductively isolated, one cannot distinguish reproductive barriers that contributed to species formation from those that arose after reproductive isolation is complete (Coyne and Orr 1989). The 2 species are similar in morphology and ecology as well as female coloration (Seehausen 2009), although differing strikingly in a secondary sexual trait, male nuptial hue. Males of both species have black vertical bars, but *P. pundamilia* males have blue-gray flanks, whereas *P. nyererei* males are bright yellow between the bars and crimson red above the lateral line. Intrinsic postzygotic barriers are unlikely to explain isolation of these species in sympatry, as fully viable and fertile interspecific hybrids can be obtained in the laboratory through several generations (van der Sluijs, van Dooren, Seehausen, et al. 2008). Therefore, restriction of gene flow between these species, as observed in nature (Seehausen et al. 2008), must be arising from prezygotic or extrinsic postzygotic mechanisms. Several experimental studies have addressed the role of female preference for male nuptial hue in the origin and maintenance of reproductive isolation in this species pair. Female mating preferences are significantly species assortative (Seehausen and van Alphen 1998; van der Sluijs, van Dooren, Hofker, et al. 2008) and they exert directional sexual selection for red coloration in *P. nyererei* (Maan et al. 2004, 2010).

Female mating preferences are heritable and oligogenic (Haesler and Seehausen 2005) and, as shown by Stelkens et al. (2008), they might exert disruptive selection on male nuptial hue when segregating in a hybrid population and thus facilitate sympatric speciation. Together, these findings suggest that female preferences for male color hue not only effectuate current reproductive isolation but may have contributed to the initial divergence of the 2 species as well (Maan and Seehausen 2010).

Divergence in male coloration (blue [*P. pundamilia*] and red [*P. nyererei*]) and in female preference for color coincides with divergence in visual system properties in populations of the 2 species from clear water sites, such that *P. nyererei* has a red-shifted spectral sensitivity compared with *P. pundamilia* (Carleton et al. 2005; Maan et al. 2006; Seehausen et al. 2008). These differences in the visual system properties, that is, in visual pigment alleles, result from adaptation to different light regimes. The 2 species have narrowly parapatric depth ranges (Seehausen 1997), such that *P. nyererei* lives in deeper waters than *P. pundamilia* (Maan et al. 2006; Seehausen et al. 2008) and the light environment in deeper waters is red shifted (Castillo Cajas et al. 2012). This led to the suggestion that visual sensory drive might play an important role in the origin of reproductive isolation in this system (Maan et al. 2006; Seehausen et al. 2008; Maan and Seehausen 2010). In turbid water sites, where visual communication is impaired, there is a loss of observed genetic and phenotypic differentiation between the species (Seehausen et al. 1997; Konijnendijk et al. 2011) despite the presence of female preference variation (van der Sluijs et al. 2007). Also, when color differences are masked in laboratory experiments, there is a breakdown of assortative female preferences between populations of the 2 species (Seehausen and van Alphen 1998). These patterns strongly implicate visual cues as the major determinants of assortative mating. However, they need not be the only cue. Chemical signals are common in sexual communication in fish (Stacey 2003; Johansson and Jones 2007) and play a role in species-assortative mating in many different taxa, for example, in sticklebacks (McLennan 2004; Rafferty and Boughman 2006), gouramis (McKinnon and Liley 1987), swordtails (McLennan and Ryan 1997, 1999; Wong et al. 2005), and pupfish (Kodric-Brown and Strecker 2001; Kodric-Brown and West 2014). Two studies on Lake Malawi cichlid fish suggested that chemical cues might play a role in female assortative mating (Plenderleith et al. 2005; Blais et al. 2009). Also in Mozambique tilapia and in *Astatotilapia burtoni*, olfactory cues are emitted through increased urination during certain social contexts and used by one or both sexes to signal gravity or synchronize mating events (Almeida et al. 2005; Miranda et al. 2005; Maruska and Fernald 2012; Kidd, Dijkstra, et al. 2013; Kidd, O'Connell, et al. 2013). Recently, a study by Verzijden and ten Cate (2007) observed that species-specific mate preference in *P. pundamilia* and *P. nyererei* is affected and can be even inversed by sexual imprinting on maternal traits, perhaps through chemical cues. Thus, the sensory basis of haplochromine cichlid assortative mating might be multimodal.

Here, we examine the role of different mate choice cues that might underlie assortative mating by female choice between *P. pundamilia* and *P. nyererei*. In a 2-way mate choice design, we allowed females of both species to spawn with either conspecific or heterospecific males. We assessed female assortative mating, female spatial proximity to the 2 males, and male aggressive and courtship behavior, under 3 scenarios: 1) all possible cues such as behavioral, visual, or chemical cues were detectable to experimental females ("white light" treatment); 2) male color hue was masked, whereas other visual cues, for example, egg spots, behavior, and chemical

cues were retained ("green light" treatment); and 3) both male color hue was masked and chemical cues blocked, whereas any other visual cues were retained ("green light and no chemical communication" treatment).

## MATERIALS AND METHODS

### Model system

*Pundamilia pundamilia* (Seehausen et al. 1998) and *P. nyererei* (Witte-Maas and Witte 1985) are members of the *mbipi* group of rocky shore cichlids endemic to Lake Victoria in East Africa. The majority of haplochromines (including these 2 species) are maternal mouthbrooders and sexually dimorphic in a number of characters (e.g., size, color, and behavior) (Fryer and Iles 1972; Greenwood 1974). Female *P. pundamilia* and *P. nyererei* are very similar in morphology and color pattern, which is cryptically yellowish to brownish with dark vertical bars on the flanks (Figure 1). Males have black underparts and black vertical bars. The flanks of male *P. pundamilia* are blue-gray between the bars, with a bright metallic blue spinous dorsal fin and reddish soft dorsal, caudal, and anal fins (Figure 1). In contrast, males of *P. nyererei* are bright yellow between the bars on the lower flanks and bright crimson red above the lateral line. The entire dorsal fin is bright crimson and the caudal and anal fins are orange to red (Figure 1).

*Pundamilia pundamilia* and *P. nyererei* have been studied at several islands in the southern part of Lake Victoria (Seehausen et al. 2008). The 2 species are geographically fully sympatric, but within islands, they have narrowly parapatric depth ranges (Seehausen 1997), such that *P. nyererei* lives in deeper waters than *P. pundamilia* (Maan et al. 2006; Seehausen et al. 2008). They have been suggested to represent a natural "speciation transect" along the Mwanza Gulf and neighboring offshore islands in southern Lake Victoria (Seehausen 2009). Populations are distributed along a roughly north-south gradient of water turbidity: at the southern end of the transect, only 1 species occurs, which is intermediate in phenotype between *P. pundamilia* and *P. nyererei* and shows a unimodal distribution of male coloration and female preference. At the opposite (i.e., northern) end, 2 distinct, completely reproductively isolated species co-occur, which differ in male coloration and female preference. Between the 2 extremes, a gradient of levels of gene flow, paralleling trait and preference divergence, matches with the water turbidity gradient (Seehausen et al. 2008; Seehausen 2009).

In this study, we use laboratory-raised offspring from fish collected in 2001 and 2003 at Python Island and refer to these hereafter as PP for *P. pundamilia* and PN for *P. nyererei*. The waters at Python Island are of intermediate turbidity (Secchi reading ~100 cm; Castillo Cajas et al. 2012) and the 2 species hybridize occasionally; nevertheless, there is a strongly bimodal frequency distribution of male nuptial hue phenotypes (Seehausen et al. 1997, 2008; Dijkstra et al. 2007), visual pigment genotypes (Carleton et al. 2005; Seehausen et al. 2008), and female mating preference (Seehausen and van Alphen 1998; Haesler and Seehausen 2005; Seehausen et al. 2008; van der Sluijs, van Dooren, Hofker, et al. 2008). In contrast, the species are only weakly differentiated at neutral genetic loci (Seehausen et al. 2008). The populations of the 2 species at Python Island are at an incipient stage of speciation (Seehausen 2009). This makes the Python population appropriate for studying the initial divergence of the 2 species and thereby testing if the evolution of assortative mating relies on single or multiple cues and modalities.

### Housing

Fish were raised and maintained in monospecific groups consisting of both sexes in stock tanks that were part of a large recirculation system (25±2 °C; 12:12 h light:dark). Fish had no previous experience with heterospecifics. Fish were fed twice a week with a mixture of shrimps, peas, and spirulina powder and on other days with commercial cichlid pellets.

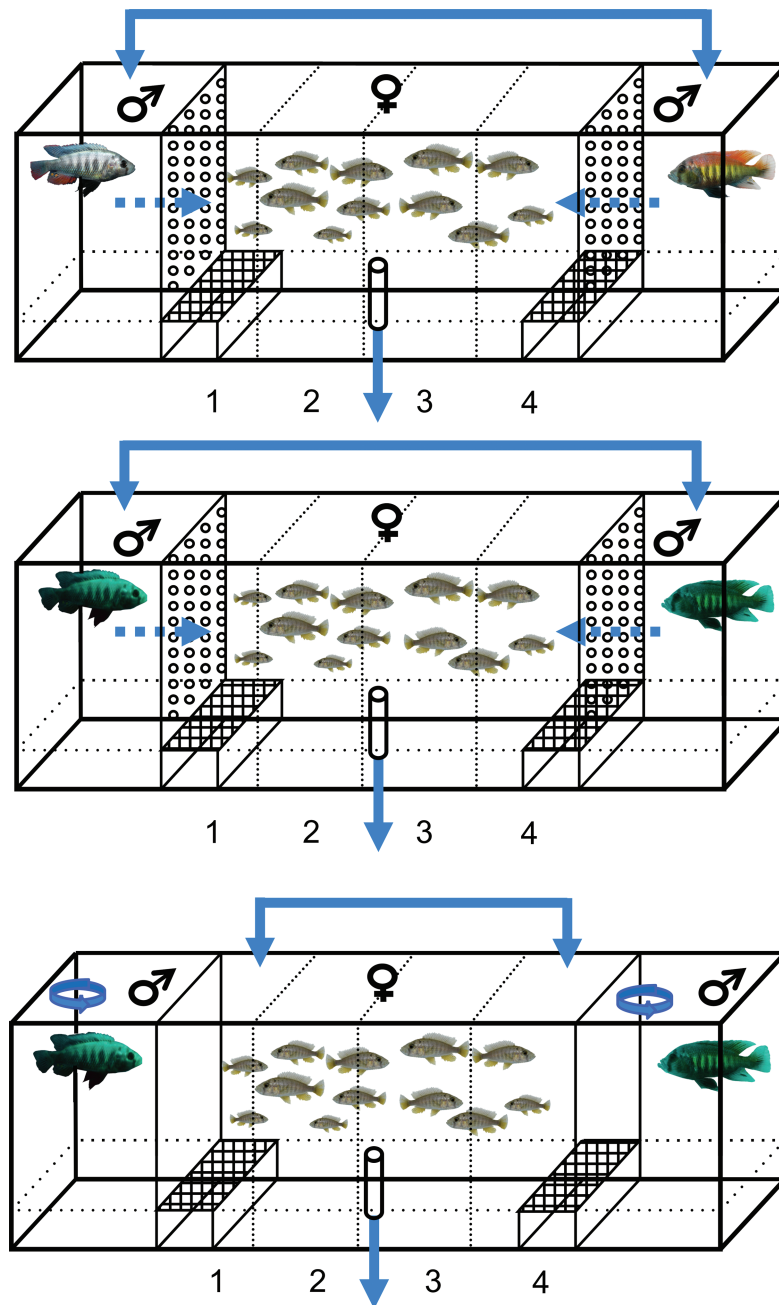
### Experimental setup

Experiments were conducted in 1 room containing 5 identical tanks (40×40×196 cm) that were divided into 2 smaller male compartments (40×40×40 cm) adjacent to a central female compartment (40×40×116 cm) (Figure 1). Each aquarium was equipped with its own box filter with continuous water circulation; twice a week, one-fourth of the aquarium water was exchanged. The water outlet was situated in the middle of the central compartment; 2 water inlets were in the male compartments. The direction of the water flow was tested for each of the aquariums separately with color-dyed water; the water flowed from both male compartments toward the central female compartment. This setup was used for the "white light" treatment and "green light" treatment (see below). In the "green light and no chemical communication" treatment, the 2 water inlets were in the female compartment and each male compartment had its own internal filters. Compartments were separated by transparent acrylic partitions, with or without perforations (see below). On both ends of the central compartment, directly in front of the partitions, egg collectors (Kidd et al. 2006) were installed (for a detailed description, see below). The collectors were constructed of transparent acrylic, 30 cm wide and covered by transparent plastic mesh with lattice spacing large enough to allow eggs to fall through during spawning. All compartments had their floor covered with sand to the height of the egg collectors. Each male compartment was equal in size and contained equally sized PVC tubes placed at the same spot in each male compartment as refuges and to ensure territoriality. The occupation of a territory is important in mate choice in cichlids. Most haplochromine cichlids initiate courtship to females at the surrounding of their territory and lead them subsequently to their territory for spawning, which can consist of a rocky surface or crevice or a sand bower (Seehausen 1996; Genner et al. 2008). Occupying a territory is vital for male mating success by gaining access to spawnings (Parker and Kornfield 1996; Maan et al. 2004). Also, the quality of the occupied territory, (for example, size and location) further influences the mating success of males (Stauffer et al. 2005; Gerlai 2007; Dijkstra et al. 2008; Genner et al. 2008).

### Treatments

The complete experiment included 3 treatments, which differed in light condition and the availability of chemical communication. The 3 experimental treatments were conducted sequentially using the same basic setup and aquaria. To allow for chemical communication between the sexes ("white light" treatment and "green light" treatment, see below), the males' partitions were perforated, with 1 hole (diameter approx. 1 cm) every 2 cm<sup>2</sup>. Replacing these with unperforated partitions in the "green light and no chemical communication" treatment prevented chemical communication.

Illumination was provided by broad-spectrum 58W fluorescent light tubes (Dennerle: TROCAL-de luxe African Lake). To inhibit female perception of species differences in male nuptial hue ("green light" treatment and "green light and no chemical communication"



**Figure 1**

Schematic representation of the experimental tank used in the “white light” treatment, the “green light” treatment, and the “green light and no chemical communication” treatment (top to bottom). The sexes of both species are shown as they appear under broad-spectrum light conditions (“white light” treatment) and the males of both species are shown as they appear under monochromatic light conditions (“green light” treatment and “green light and no chemical communication” treatment). Horizontal dashed lines indicate the level to which the floor was covered with sand. Vertical dashed lines represent the 4 sectors (numbered from 1 to 4) of the central compartment used to score the spatial distribution of females. Blue arrows indicate water flow (for details, see Materials and Methods).

treatment), the tubes were covered with light filters (as in [Dijkstra et al. 2005](#)). One layer of yellow optical filter (Lee 010, Medium Yellow) and 2 layers of blue optical filter (Lee 172, Lagoon Blue) resulted in a green light spectrum. The light filter restricted the spectrum of light to a narrow wavelength band with peak intensities at 491 and 543 nm.

Females of both species were first tested in the “white light” treatment (male hue differences among other visual cues were visible and chemical cues were available), which ran between May and

September 2008; subsequently, females of both species were tested in the “green light” treatment (male hue differences were masked, whereas other visual cues and chemical communication were available), which ran between October 2008 and June 2009; and finally PP females were tested in the “green light and no chemical communication” treatment (both male hue was masked and chemical cues were blocked), which ran from August 2009 until May 2010. Only PP females were exposed to the “green light and no chemical communication” treatment because the results of the “green light”



treatment suggested that the PP females retained some preferences for conspecifics while PN females were mating at random.

For each treatment, females were introduced into the aquaria for acclimatization 1 week prior to the introduction of males. Each experimental tank contained a monospecific group of 4–12 females (the mean number of PN females present in both treatments was 12; the mean number of PP females present in the “white light” treatment and the “green light” treatment was 10 and in the “green light and no chemical communication” treatment, it was 8). Males in each pair (see below) were randomly assigned to the left and right compartment on first introduction and subsequently the position of the species was alternated (left or right) each time a new pair was introduced. After each spawning event (i.e., at least 15 eggs present in the egg collector), the male pair and the female that spawned were replaced (with 1 day in-between) with individuals not previously used in the same treatment. The nonspawning females remained in the tank. Females had to lay a certain amount of eggs to be identified as having spawned with the below-mentioned criteria. In most cases, where less than 15 eggs were present in the egg collector, females could not reliably be identified to have spawned and hence the replicates continued. We acknowledge that this may have resulted in mate choice copying (Pruett-Jones 1992; Dugatkin 1996) within a replicate. However, we rarely found eggs on the next day after a spawning of less than 15 eggs the day before. In the few cases, where this happened, we added the eggs from the next day to the batch of eggs from the previous day. Also, only in a few of the replicates that continued and hence contained several batches, that is, several spawnings, was the sign of mate choice the same, that is, either being always assortative, disassortative, or random, and hence the possibility of mate choice copying existed. From a total of 16 replicates in PN females in the “white light” treatment, 6 replicates contained multiple spawnings and in none was the sign of mate choice the same between spawnings. For PN females in the “green light” treatment, 10 out of 19 replicates contained multiple spawnings and in 2 of these was the sign in mate choice the same (each once assortative and disassortative). In PP females in the “white light” treatment, 6 out of a total of 15 replicates were multiple-batch replicates and of these, two showed the same sign in mate choice (twice assortative). In the “green light” treatment, out of a total of 18 replicates, 9 were multiple-batch replicates and 3 of these showed the same sign in mate choice (all assortative). In the “green light and no chemical communication” treatment, out of a total of 14 replicates, 9 contained multiple batches and 1 replicate showed the same sign in mate choice (assortative). In the majority of replicates, the sign of mate choice was not the same and thus spawnings were treated as independent data points in the analysis. Females that died were replaced with individuals not previously used in the same treatment. A female that had spawned was identified either by the presence of eggs in its mouth or by the outline of the ventral region (which is concave after spawning) and the urogenital papilla (which swells just before oviposition and remains swollen for up to 24 h afterward). In 2 cases, 2 females spawned at the same time, once in a PN group and once in a PP group (i.e., eggs were found in the egg collector and 2 females had swollen papilla); hence, both females were removed and replaced. In both cases, a batch of eggs was found in both collectors; each batch of eggs was counted as independent spawning and thus both females were counted as independent data points. Because we are interested in mate choice at the population level and did not attempt to assign individual-based female mating preferences (Wagner 1998;

Chenoweth and Blows 2006), these multiple batches of eggs were included in the analysis.

Female size (standard length, to the nearest 0.1 mm) and weight (to the nearest 0.01 g) were measured after spawning; there were no significant differences in weight and size between treatments (PN:  $N = 32$ , [U test]—size: “white light” treatment =  $65.76 \pm 6.01$  mm [standard deviation, SD]/“green light” treatment =  $67.08 \pm 8.34$  mm;  $U = 122$ ,  $P = 0.850$ ; weight: “white light” treatment =  $8.88 \pm 2.31$  g/“green light” treatment =  $9.94 \pm 3.57$  g;  $U = 116$ ,  $P = 0.680$  and PP:  $N = 39$  [Kruskal–Wallis test]—size: “white light” treatment =  $66.66 \pm 3.58$  mm/“green light” treatment =  $67.77 \pm 7.61$  mm/“green light and no chemical communication” treatment =  $69.99 \pm 6.77$  mm;  $H = 1.88$ ,  $P = 0.391$ ; weight: “white light” treatment =  $10.61 \pm 2.35$  g/“green light” treatment =  $11.45 \pm 4.65$  g/“green light and no chemical communication” treatment =  $11.55 \pm 4.01$  g;  $H = 0.570$ ,  $P = 0.751$ ).

Males in a pair were size matched (to less than 8% of the mean size of the 2 males in a pair) because body size has been shown to be important for mate choice in cichlids (McKaye 1986; Hert 1991; Gerlai 2007). Male standard length and weight were measured before introduction to the tank and after replacement, and the means used for further analysis. Size and weight differences were measured such that the standard length or weight of the larger or heavier male was subtracted from the standard length or weight of the smaller or lighter male. In PN female tanks, the average length and weight of all males and the difference between males within a male pair in the “white light” treatment ( $N_{\text{male pair}} = 16$ ) was  $87.19$  (average length)  $\pm 1.88$  mm (SD) and  $23.07$  (average weight)  $\pm 2.50$  g and in the “green light” treatment ( $N_{\text{male pair}} = 19$ ) it was  $86.70 \pm 2.27$  mm and  $21.17 \pm 2.13$  g. Comparable differences in length and weight between males in the PP female tanks were observed for the different treatments: “white light” treatment ( $N_{\text{male pair}} = 15$ )  $87.95 \pm 2.1$  mm and  $23.81 \pm 4$  g, “green light” treatment ( $N_{\text{male pair}} = 18$ )  $86.73 \pm 2.07$  mm and  $20.78 \pm 2.16$  g, and “green light and no chemical communication” treatment ( $N_{\text{male pair}} = 14$ )  $80.33 \pm 2.52$  mm and  $15.92 \pm 1.44$  g.

After a spawning, fish were photographed and injected with a 12-mm passive integrated transponder tag for individual identification (Biomark, Idaho). In males, the number of vertical bars and the number of egg spots on the anal fin were counted to assess the possible effect of variation in these traits on female preference (see below). Due to practical constraints, measurements for some individuals could not be taken (weight and standard length of 6 out of 77 females and of 2 male pairs out of 84 pairs; bar and spot counts of 10 male pairs out of 82 pairs). Altogether a total of  $N = 54$  *P. nyererei* males and  $N = 49$  *P. pundamilia* males were used. The number of unique male pairs used in tanks with PP females was 15, 20, and 14 in the “white light” treatment, the “green light” treatment, and the “green light and no chemical communication” treatment, respectively, and 16 and 19 in the “white light” treatment and the “green light” treatment, respectively, in tanks with PN females.

Three males died during the experiment. In these cases, the remaining male was removed and a new male pair was introduced. None of the males in the “white light” treatment were used in more than 1 male pair. In the “green light” treatment and the “green light and no chemical communication” treatment, males were used in up to 2 different pairs, but only for testing females of the species that the male had not previously been tested with. The reused males were paired to form novel, unique male combinations, such that reused males were never paired with the same heterospecific male twice.

## Behavioral observations

We scored female spatial positioning as an indirect measure of female preference as follows. The central compartment was visually divided into 4 equally sized sectors resulting in 2 central sectors and 2 sectors each adjacent to one of the male compartments (Figure 1). During 20-min observation trials, the number of females in each sector was noted every 2 min and then averaged over the trial. The total number of trials (days) for PP females was 28, 21, and 24 in the “white light” treatment, the “green light” treatment, and the “green light and no chemical communication” treatment, respectively, and for PN females, 27 and 18 in the “white light” treatment and the “green light” treatment, respectively.

Behavioral elements of male courtship in *Pundamilia* were recorded because differences in courtship intensity can influence female preference (Endler 1995; Barbosa and Magurran 2006). Recorded elements of male behavior were “approach” (male approaching a female that is less than 15 cm away from the male compartment), “lateral display” (the male displays its body and fins by posing sideways in front of the female with erected fins), “quiver” (a high-frequency shaking movement), and “lead swim” (swim toward the spawning pit—generally, the male shelter—with rapid tail beats) (McElroy and Kornfield 1990; Seehausen 1996). Additionally, rare aggressive behaviors were also recorded including “aggressive lateral display” and “attack” behavior (biting and butting) at the acrylic barriers directed clearly to a female. Observations were conducted during 20 min for each tank, with 10 min for each male. The total number of observational trials (days) in tanks with PP females was 42, 21, and 23 in the “white light” treatment, the “green light” treatment, and the “green light and no chemical communication” treatment, respectively, and in tanks with PN females was 42 and 14 in the “white light” treatment and the “green light” treatment, respectively. For each male, the frequencies of behavioral elements were expressed as a proportion of the total of both males in the pair; for example, for lateral displays:  $LD_{PN\text{ male}} = LD_{PN\text{ male}} / (LD_{PN\text{ male}} + LD_{PP\text{ male}})$ .

## Mate choice

Female choice was scored based on actual spawning events by using the “egg collector” experimental design described in Kidd et al. (2006) (see also Nelson 1995; Plenderleith et al. 2005; Blais et al. 2009; Stelkens and Seehausen 2009; Tyers and Turner 2013). The egg collector allows the observer, without having witnessed the actual spawning event, to assign eggs to each male. The eggs were usually found in 1 batch on the floor of either of the 2 egg collectors. Occasionally, both collectors contained eggs and/or females still carried eggs in the mouth. When a female spawned with both males, a chi-square test was conducted on the number of eggs spawned with each male. If significant, the male with the larger number of eggs was considered to be the sire. If not significant, it was regarded as a split spawning and both males were assigned as sires. When females carried eggs in their mouth, the eggs were collected by gently opening the mouth of the female to let the eggs fall out. These eggs were added to the number of eggs found in either collector, whereby the calculation for the extrapolation was based on the ratio of the number of eggs found in the collectors. This happened 2 times in PP females and 5 times in PN females across all 3 treatments. The classification of spawnings into disassortative and assortative spawnings or split-decision spawnings is solely to count the number of different spawning categories. The statistical test for measuring assortativeness or the lack thereof was based on

the actual number of eggs found or the ratio between the numbers of eggs in the 2 collectors from each spawning (i.e., each spawning event was the unit of replication; see below).

## Data analysis

### Behavior

When normality assumptions were satisfied (Shapiro–Wilk test), parametric statistics were used to analyze the data, otherwise non-parametric tests were applied. Statistical analyses were done using the statistical software packages R v. 2.13.0 (R development Core Team 2013) and PAST v. 2.03 (Hammer et al. 2001). All tests are 2 tailed.

Differences in male behavior, between species and toward female species, were analyzed with Wilcoxon signed-rank tests. The spatial positioning of females per sector was tested by a Friedman test, and as a post hoc test, the Wilcoxon signed-rank test was used. *P* values that derived from the post hoc test were corrected for multiple comparisons with the Holm–Sidak method (Abdi 2010). Additionally, the spatial positioning of females, pooled for sectors 1 and 2 and 3 and 4, was tested with an unpaired Student’s *t*-test. Finally, to test if the average spatial positioning of females per sector differed between treatments, we used a chi-square test.

### Mate choice

To quantify the degree of assortative mating of females of either species, we used the following:

- an Assortative Mating Index (AMI) calculated for each spawning, using  $AMI = (E_{con}) / (E_{total})$ , where  $E_{con}$  is the number of eggs spawned with the conspecific male and  $E_{total}$  is the total number of eggs spawned. The AMI can range from 0 to 1, with a value of 0 indicating a preference for heterospecifics, a value of 1 indicating a preference for conspecifics, and a value of 0.5 indicating no preference. The AMI was tested against 0.5 (no preference) using a *U* test with spawning as unit of replication.
- the numbers of eggs spawned toward the conspecific and heterospecific males, compared using a Wilcoxon signed-rank tests with spawning as unit of replication.

Differences in AMI and clutch size between treatments were tested with Kolmogorov–Smirnov tests and *U* tests, respectively.

### Hue-independent visual cues

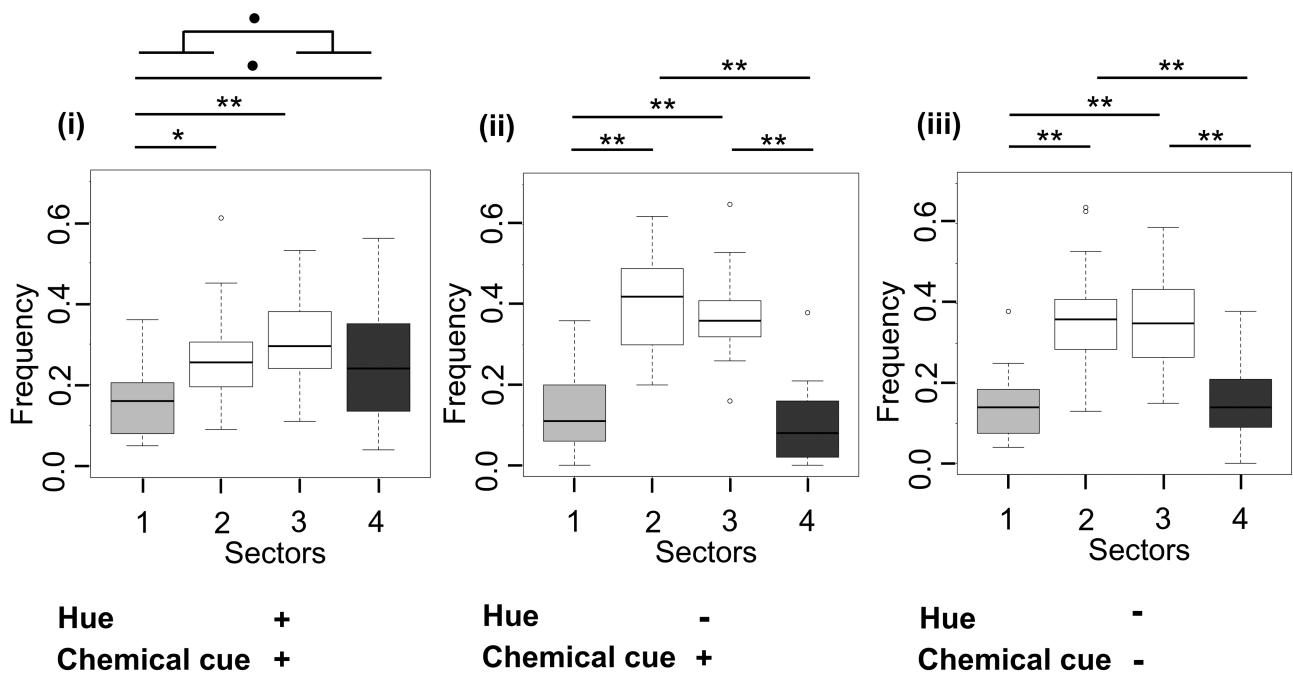
Differences in weight, size, number of vertical bars, and egg spots between males in a pair were tested with Wilcoxon signed-rank tests. To test for an effect of these traits on female mating preference or clutch size, we used Kendall’s tau ( $\tau$ ) rank correlations.

## RESULTS

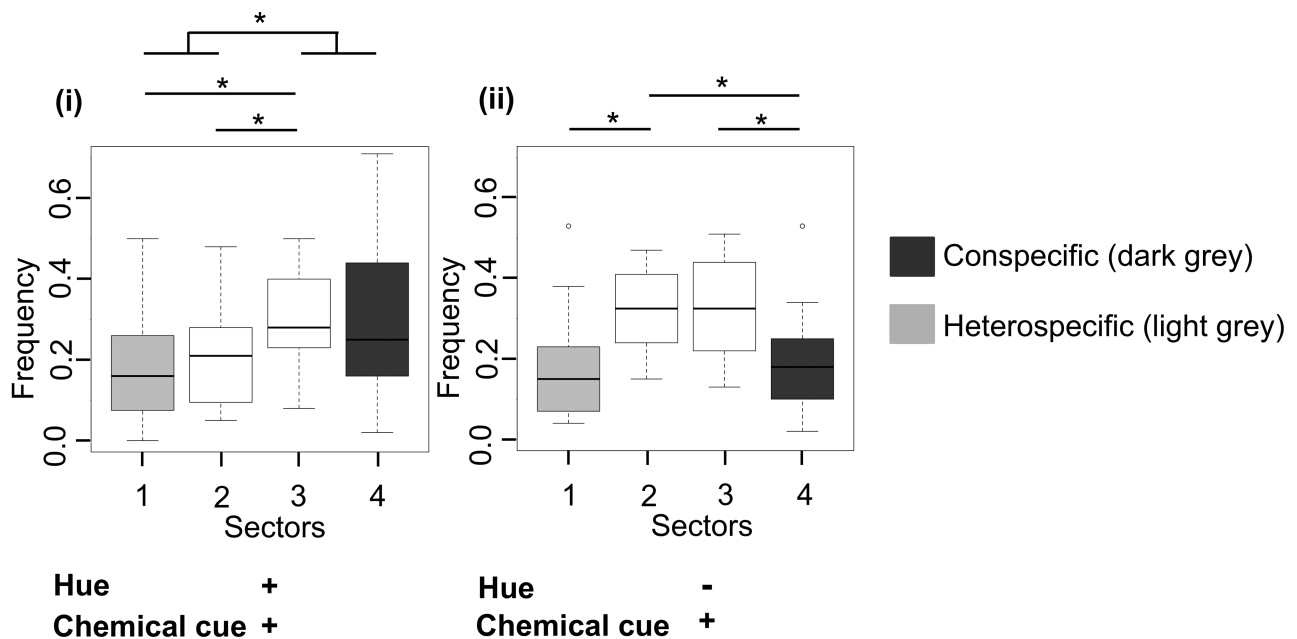
### Behavior

In the “white light” treatment, females of both species showed a skewed spatial distribution toward the compartment with the conspecific male (Figure 2A,B). Both PP and PN females showed a significant preference to be more often in the vicinity of the conspecific male compartment (Table 1). Once the hue differences between males were masked (“green light” treatment), females of both species no longer showed skewed distributions but rather aggregated in the middle of the female compartment and showed no preference for either conspecific or heterospecific

**(A)**  
***P.pundamilia***



**(B)**  
***P.nynererei***



**Figure 2**

Female spatial distribution during 20-min observations. Panels show the spatial distribution of PP (A) and PN (B) females in the “white light” treatment (i), the “green light” treatment (ii), and the “green light and no chemical communication” treatment (iii). The spatially skewed distribution of females of both species toward the conspecific male compartment compared with the heterospecific male compartment in the “white light” treatment was lost once hue differences between males were masked in the “green light” treatment and the “green light and no chemical communication” treatment.  $P < 0.1$ ,  $*P < 0.05$ , and  $**P < 0.001$ . Box plots show medians, first and third quartile, confidence intervals (whiskers), and outliers (asterisks).

**Table 1****Test statistics of the spatial positioning per sector of both female populations in each treatment**

Treatment	Female population	Friedman test with sequential Bonferroni correction	Sector						t-Test	
			1 versus 2	1 versus 3	1 versus 4	2 versus 3	2 versus 4	3 versus 4	Sector 1 + 2 versus 3 + 4	
“White light”	PP	$\chi^2 = 23.24, P < 0.001$	$P = 0.001$	$P < 0.001$	$P = 0.054$	$P = 0.125$	$P = 0.770$	$P = 0.318$	$t = 1.77, P = 0.081$	
“White light”	PN	$\chi^2 = 23.97, P = 0.018$	$P = 0.400$	$P = 0.033$	$P = 0.300$	$P = 0.032$	$P = 0.479$	$P = 0.778$	$t = 2.45, P = 0.026$	
“Green light”	PP	$\chi^2 = 41.77, P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.346$	$P = 0.601$	$P < 0.001$	$P < 0.001$	$t = -0.05, P = 0.962$	
“Green light”	PN	$\chi^2 = 11.62, P = 0.008$	$P = 0.020$	$P = 0.100$	$P = 0.900$	$P = 0.900$	$P = 0.046$	$P = 0.046$	$t = -0.45, P = 0.660$	
“Green light and no chemical communication”	PP	$\chi^2 = 40.33, P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.852$	$P = 0.808$	$P < 0.001$	$P < 0.001$	$t = -0.05, P = 0.935$	

Friedman test and Wilcoxon signed-rank post hoc test were used to compare sectors. *P* values were corrected for multiple comparisons with the Holm–Sidak method (Abdi 2010). Pooled sectors 1 and 2 and 3 and 4 were tested with an unpaired Student's *t*-test.

males (Table 1). The blocking of chemical cues resulted in a similar distribution of the PP females toward the center of the female compartment (Table 1). The average spatial positioning of females of both species per sector significantly differed between the “white light” and “green light” treatments (PP:  $\chi^2 = 25.17, P < 0.001$ ; PN:  $\chi^2 = 10.24, P = 0.017$ ). In the “green light and no chemical communication” treatment, the average spatial positioning of PP females was not significantly different from that in the “green light” treatment ( $\chi^2 = 2.15, P = 0.542$ ), but it was significantly different from that in the “white light” treatment ( $\chi^2 = 8.94, P = 0.030$ ).

Overall, males of the 2 species showed similar levels of courtship and aggressive behavior, directed toward females of either species (most  $P > 0.1$ ; Figure 3A,B). However, in the “white light” treatment with PP females, PP males approached and presented lateral display to the females more often than did PN males (AP:  $z = 2.33, P = 0.020$ ; LD:  $z = 2.02, P = 0.044$ ) and in the “green light” treatment, PP males had a tendency to approach PN females more often than did PN males (AP:  $z = 0.190, P = 0.058$ ).

## Mate choice

When all cues were available (“white light” treatment), females of both species significantly preferred to mate with conspecific males (Figure 4). Females of both species laid a significantly larger number of eggs with conspecific males when hue was visible (Figure 4) (PP:  $z = 3.02, P = 0.003$ ; PN:  $z = 2.971, P = 0.002$ ). Analysis at the level of the AMI yielded similar results. PP females mated assortatively in 19 out of 25 spawnings; in 4 instances, eggs were laid with the heterospecific male and 2 times a split spawning occurred ( $AMI_{PP} = 0.78, U = 125, P < 0.001$ ). Mating in PN females was species assortative in 14 out of 23 spawnings; 3 times the heterospecific male was chosen and 6 times the females spawned with both males ( $AMI_{PN} = 0.78, U = 80.5, P < 0.001$ ).

In the “green light” treatment, neither female species discriminated between conspecific and heterospecific males based on the number of eggs spawned (PP:  $z = 1.466, P = 0.140$ ; PN:  $z = 0.74, P = 0.460$ ) nor as measured by the AMI. PP females spawned with the conspecific male in 30 out of 47 spawnings; 15 times they mated with the heterospecific male and twice with both males ( $AMI_{PP} = 0.62, U = 869.5, P = 0.053$ ). PN females mated with the conspecific male in 20 out of 46 spawnings; 17 times they spawned with the heterospecific male and 9 times a split spawning occurred ( $AMI_{PN} = 0.53, U = 989, P = 0.56$ ).

Because PP females, but not PN females, still showed weak indications for assortative mating in the “green light” treatment, we exposed PP females to a third treatment in which neither male

nuptial hue was visible nor chemical communication available. In this “green light and no chemical communication” treatment, PP females did not discriminate between conspecific and heterospecific males. Females laid similar numbers of eggs with either male (PP:  $z = 0.81, P = 0.420$ ) and chose the conspecific male in 15 out of 43 spawnings; 12 times they spawned with the heterospecific male and 16 times a split spawning occurred ( $AMI_{PP} = 0.51, U = 903, P = 0.844$ ).

The decrease in AMI from the “white light” treatment to the “green light” treatment was significant in PN females ( $D = 0.348, P = 0.049$ ) but not in PP females ( $D = 0.223, P = 0.392$ ). Furthermore, in PP females, the decrease in AMI from the “green light” treatment to the “green light and no chemical communication” treatment was not significant ( $D = 0.270, P = 0.076$ ), but the decrease from the “white light” treatment to the “green light and no chemical communication” treatment was significant ( $D = 0.394, P = 0.015$ ).

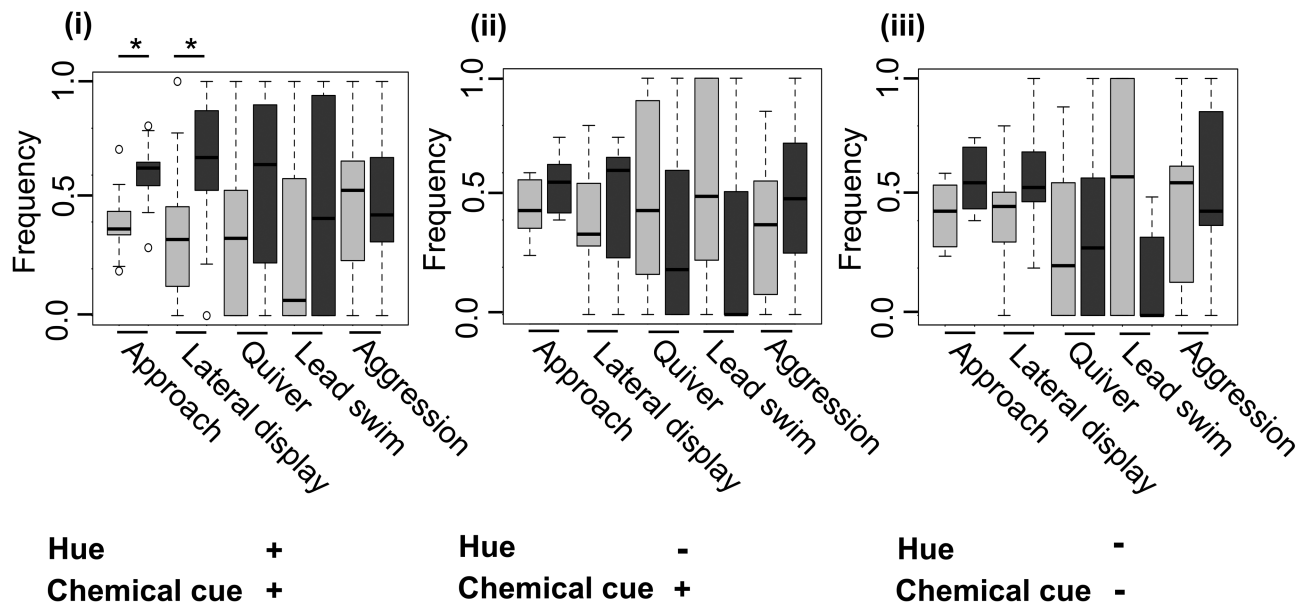
We observed a significant drop in clutch size in both female species when males' color hue in the “white light” treatment (PP:  $34.9 \pm 32.2$ ; PN:  $28.6 \pm 29.6$ ) was concealed in the “green light” treatment (PP:  $14.5 \pm 21.4$ ; PN:  $14 \pm 19.2$  and PP:  $U = 529.5, P = 0.027$ ; PN:  $U = 662.5, P = 0.019$ ). In the “green light and no chemical communication” treatment ( $11.7 \pm 12.4$ ), PP females laid clutches that were comparable to those in the “green light” treatment ( $U = 1548, P = 0.944$ ) but significantly smaller than those in the “white light” treatment ( $U = 572, P = 0.009$ ).

## Hue-independent visual cues

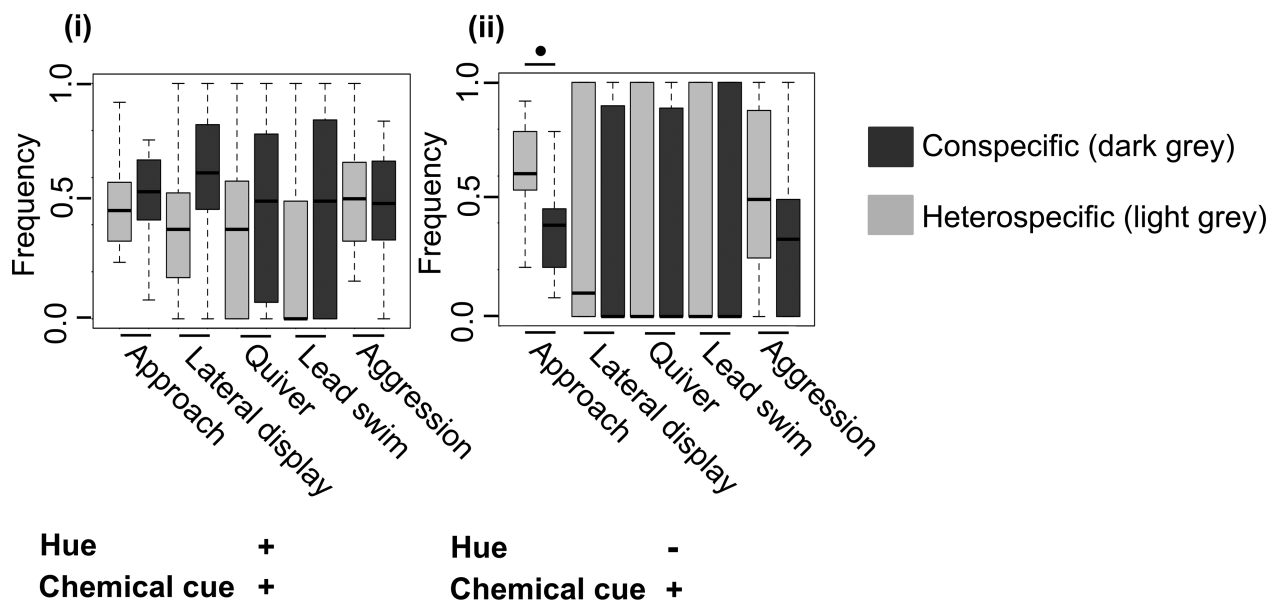
Some species-specific color pattern differences (Seehausen 1997) were not removed by masking color cues by green light. Therefore, we tested whether these differences could restore assortative mating in the “green light” treatment and the “green light and no chemical communication” treatment in which male hue was not visible. We found no support for this effect although the males of the 2 species did systematically differ in a few characters. PN males had more vertical bars (mean number of vertical bars over all treatments and standard error: PN =  $6.1 \pm 0.10$ ) than did PP males (PP =  $5.3 \pm 0.09$ ) in all treatments of both female species (all  $P < 0.05$ ) with one exception; in the “green light” treatment with PP females, there was a trend for PN males to have a higher number of vertical bars ( $z = 1.82, P = 0.070$ ). PN and PP males did not differ in the number of egg spots in all but one treatment (all  $P > 0.250$ ); in the “green light and no chemical communication” treatment of the PP females, PN males had significantly more egg spots than PP males (PN =  $6.1 \pm 0.10$ , PP =  $5.3 \pm 0.09$ ;  $z = 2.05, P = 0.041$ ). These differences in vertical bars and egg spots did not



**(A)**  
***P. pundamilia***



**(B)**  
***P. nyererei***

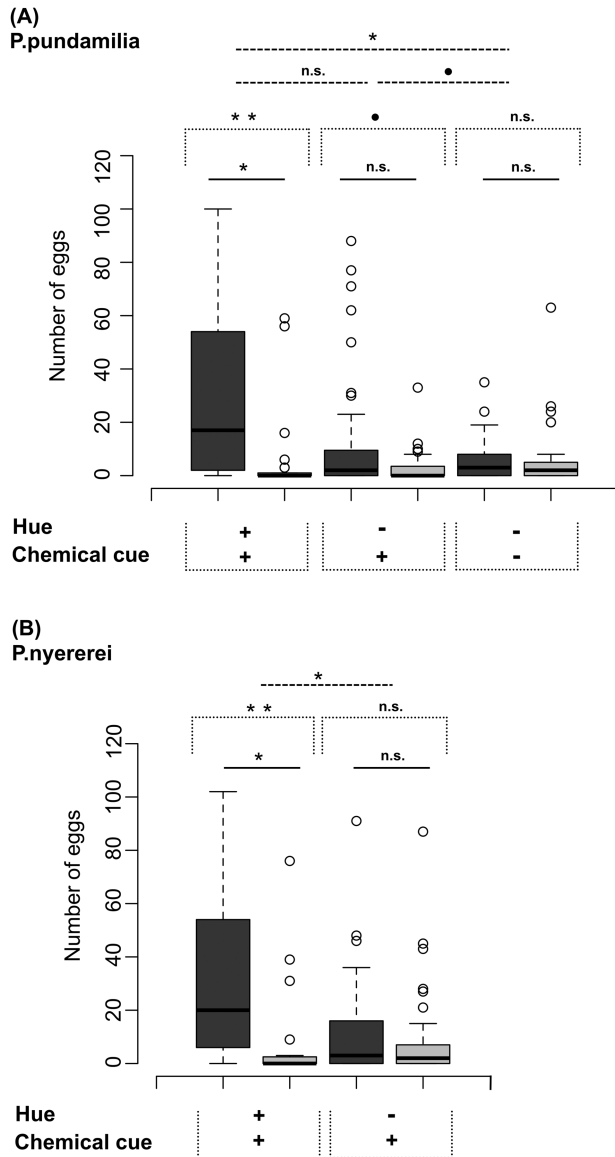


**Figure 3**

Male behavior during 20-min observations. Courtship and aggressive behaviors of PP (A) and PN (B) males in the “white light” treatment (i), the “green light” treatment (ii), and the “green light and no chemical communication” treatment (iii). Females of both species received more courtship from conspecific males in the “white light” treatment. See Materials and Methods for a detailed description of male behavior.  $P < 0.1$ ,  $*P < 0.05$ . Box plots show medians, first and third quartile, confidence intervals (whiskers), and outliers (asterisks).

increase assortative mating (all  $P > 0.1$ ), with 2 exceptions. In the “white light” treatment, PN females mated more strongly species assortative, and tended to spawn larger clutches, when the conspecific male had a higher number of vertical bars than the heterospecific male (median difference in vertical bar number and range: 0.5, from -1 to 3; AMI:  $z = 2.07$ ,  $P = 0.039$ ,  $\tau = 0.41$ ; clutch

size:  $z = 1.95$ ,  $P = 0.052$ ,  $\tau = 0.35$ ). However, PP females mated significantly less assortatively in the “green light” treatment when the conspecific male had a fewer number of vertical bars than the heterospecific male (-1, from -2 to 0; AMI:  $z = 2.09$ ,  $P = 0.039$ ,  $\tau = 0.31$ ). Thus, species differences in vertical bars and egg spots did not rescue assortative mating when other cues were unavailable.



**Figure 4**  
The number of eggs laid by (A) PP and (B) PN females with the conspecific or heterospecific male for all treatments. PP and PN females mated species-assortative in the “white light” treatment but not in the “green light” treatment and the “green light and no chemical communication” treatment. Solid lines above the box plots indicate Wilcoxon signed-rank test results for the difference in egg number spawned with conspecific or heterospecific males. Dotted lines above box plots indicate *U* test results for the deviation in the AMI from random mating in each treatment and dashed lines above the dotted lines indicate Kolmogorov–Smirnov test results for the difference in the AMI between treatments. \* $P < 0.1$ , \*\* $P < 0.05$ , \*\*\* $P < 0.001$ , and n.s. = not significant. Box plots show medians, first and third quartile, confidence intervals (whiskers), and outliers.

## DISCUSSION

Speciation with gene flow frequently requires assortative mating, which may be mediated by various sexual signals and cues. Multimodality of signaling might strengthen assortative mating once it is established but delay or hamper its initial evolution (Schluter and Price 1993; Iwasa and Pomiankowski 1994). A single

mating cue, particularly when oligogenic (as is the case in *Pundamilia* where both female color preference, Haesler and Seehausen 2005, and male nuptial hue, Magalhaes and Seehausen 2010, are controlled by few loci), might provide a more efficient transmission of divergent ecological selection to nonrandom mating and thereby facilitate speciation with gene flow (Dieckmann and Doebeli 1999; Kondrashov and Kondrashov 1999; Gourbiere 2004). Hence, determining the traits that underlie assortative mating furthers our understanding of how reproductive isolation emerges during speciation and how it is maintained.

Our experimental results show that a single mating cue, namely male nuptial hue, is sufficient for interspecific female mate choice between the populations of the sympatric sister species *P. nyererei* and *P. pundamilia* that are at an incipient stage of speciation. Females of both species mated species assortative when the color difference between males was visible. This assortativeness broke down after color differences between the males were masked despite the availability of other possible species-recognition signals. We also found that the female spatial behavior data correspond well with the female mate choice data. The skewed spatial distribution of females toward the compartment that holds the conspecific male and the elevated frequency of courtship interactions between conspecific males and females were both lost once color differences were unavailable. These results experimentally confirm that male nuptial hue is necessary and sufficient to mediate premating reproductive isolation between *P. nyererei* and *P. pundamilia*.

The species divergence in male coloration and female preference in *Pundamilia* correlates with divergent underwater light regimes and divergent visual sensitivities (Seehausen et al. 2008). *Pundamilia nyererei* inhabits deeper waters with more red-shifted light conditions and has a more red-shifted retinal visual pigment composition than *P. pundamilia* (Seehausen et al. 2008). Moreover, the extent of differentiation in visual sensitivity predicts the strength of reproductive isolation in this species pair. This led to the suggestion for a role of divergent sensory drive in the evolution of reproductive isolation between the 2 species (Maan et al. 2006; Seehausen et al. 2008). Our results support this hypothesis by showing that no additional cue is needed to initiate and maintain species-assortative mating.

In a study by Seehausen and van Alphen (1998), investigating the same *Pundamilia* populations, female behavioral mating preference was species-assortative under broad-spectrum light conditions and broke down under monochromatic light conditions. Despite the fact that this study allowed for restricted chemical communication between the sexes (unidirectional water overflow from male to female compartments, such that any chemical cues could not be emitted by the males specifically to the females during courtship interaction), had a small sample size (4 gravid females per species and treatment), and used a behavioral measure of female preference, that is, female response to male courtship, rather than actual mating, the results are remarkably consistent with our study. Also, in our study, another behavioral measure of female preference, namely the proximity to a male, showed that when color differences were visible, female groups of both species showed a strong spatial bias toward the conspecific male compartments, corresponding to the species-assortative mating of both female species. Once color differences were masked, female groups of both species lost this spatial bias and also mated randomly. Together, the findings from Seehausen and van Alphen (1998) and from this study suggest that male nuptial hue determines both female behavioral preference and female mate choice, in both species.

Proximity tests have been used as an indirect measure of female preference and mate choice in many studies on fish (e.g., stickleback: McLennan 2004; Rafferty and Boughman 2006/gudgeon and minnows: Gozlan et al. 2014/swordtails: Ryan and Wagner 1987; Basolo 1990; McLennan and Ryan 1999/rainbow fish: Young et al. 2010/mosquito fish: Kahn et al. 2010/darters: Williams and Mendelson 2013/sand goby: Forsgren 1992/pupfish: Kodric-Brown and Strecker 2001; Kodric-Brown and West 2014/Convict cichlids: Dechaume-Moncharmont et al. 2011, 2013/Lake Malawi cichlids: Mellor et al. 2011, 2012/West-African river cichlid: Thünken et al. 2012). However, such proximity measures may reveal associations between the sexes that are not embedded in a mating context (Barlow 2002). For example, Kidd, O'Connell, et al. (2013) could show in the cichlid fish *A. burtoni* that intraspecific female preference for males depends on their reproductive status, such that the association time with a male only reflects actual mating decisions at the day of spawning but not before. Several other studies in fish have investigated if the time a female spends with a male is a reliable predictor of a subsequent mating decision. A concordance between an individual's association time and its mating decision has been found in Malawian cichlids (Coultridge and Alexander 2001), West-African river cichlids (Thünken et al. 2007), and Central American convict cichlids (Santangelo 2005; Dechaume-Moncharmont et al. 2011) but not in sailfin mollies (Gabor 1999), killifish (Aspbury and Basolo 2002), and Lake Tanganyika cichlids (Egger et al. 2008). Hence, incorporating female preference—measured as the response of females to male courtship behavior— or actual mating decisions for the species under investigation is desirable to predict that association time is a reliable indicator of female mating intentions. Although this has not been conducted in Lake Victoria cichlids, evidence that female preference (Seehausen and van Alphen 1998) and female mate choice for conspecifics and female association time with conspecifics, measured as spatial bias (both in this study), all break down once color (hue) of males is absent is a strong indication that the observed spatial bias in our study predicts to some extent female preference and mate choice, respectively.

Two studies on Lake Malawi cichlid fish suggested that chemical cues might play a role in female assortative mating (Plenderleith et al. 2005; Blais et al. 2009). In our experimental “green light” treatment, where hue was absent but chemical cues available, we observed a few instances where females of *P. pundamilia* laid large clutches with conspecific males. We did not observe this when chemical communication was also blocked in the “green light and no chemical communication” treatment. This might suggest that *P. pundamilia* females may in some instances rely on a species-specific cue other than male nuptial color, and this may well be a chemical cue. Alternatively, sound of courting males as an acoustic cue could have also contributed to these few larger clutches (see below). However, independent of the exact cue involved, this sensory modality is insufficient for restoring the mating preference for conspecific males in the majority of females or spawning events.

Males of African cichlids—when in proximity to females—may also produce sound during courtship as part of their reproductive repertoire (Amorim et al. 2004). The low-frequency sounds that the courting males in our experiment may have produced could be audible through both perforated and unperforated acrylic partitions (Maruska et al. 2012) but possibly be affected in all 3 treatments by the external noise produced by the filters and especially by the internal filter noise in the “green light and no chemical communication” treatment (see Engelking et al. 2010; Slabbekoorn et al.

2010). These sounds can differ between species (Lobel 1998, 2001; Amorim et al. 2004, 2008; Danley et al. 2011) including the 2 species in this study (Verzijden et al. 2010). Yet, the evidence that species differences in sound can influence assortative mating is weak (Amorim et al. 2004). Sound influences intraspecific female preference in 2 African cichlid fish species (*P. nyererei*: Verzijden et al. 2010; Estramil et al. 2013; *A. burtoni*: Maruska et al. 2012), but no study in cichlids has tested its effect on interspecific female preferences. Notably, when males of *P. nyererei* were not visible, females did not show any preference for compartments with conspecific sound over those with white noise or no sound (Estramil et al. 2013). This indicates a dominant role for visual cues in courtship interactions. Thus, even though low-frequency sounds have been shown to affect courtship interactions, it seems unlikely that they provide a sufficient basis for interspecific mate choice in *Pundamilia*.

Besides male color (hue), chemical and acoustic cues, species-specific variation in other quantitative male traits such as body size, weight, and the numbers of vertical bars or egg spots could contribute to mate recognition in both *Pundamilia* species. Yet, none of these cues could explain interspecific mate choice in our experiments although males of the 2 species systematically differed in the number of vertical bars and once also in the number of egg spots, and the availability of these cues was not affected by our treatments. Moreover, the breeding color of a fish does not only contain the element of color (hue) but also of pattern that results from multiple colors and melanization (Williams and Mendelson 2013). The differences in pattern between the 2 *Pundamilia* species could have served as a species-recognition signal because these elements were also not affected by our treatments, but they seemed to not convey any such information. Specific manipulations of other signals than color (hue), for example, chemical, acoustic, or color pattern signals, between individuals of the 2 populations used in this study will be necessary to test whether these sensory modalities, although not required for assortative mating, might play a supporting role in mate discrimination (e.g., egg spots: Hert 1989, 1991; Coultridge and Alexander 2001; Coultridge 2002; Theis et al. 2012).

Cichlids display complex behaviors, especially during courtship and spawning (Baerends and Baerends-van Roon 1950; Fryer and Iles 1972; Dominey 1984) and differences in courtship behavior elements (behavioral innovations) or in the transition from one element to another could be species specific and serve as a species-recognition signal. However, male courtship behavior in haplochromines is rather stereotypic and conserved among species (McElroy and Kornfield 1990). The same sequences of behavioral interactions between the sexes are common to the rock-dwelling cichlids from all 3 African great lakes and they are thought not to be associated with divergence among populations or species and thus not to be central to mate recognition (McElroy and Kornfield 1990; Seehausen 1996). Previous studies on the sister species *P. pundamilia* and *P. nyererei*—including our study—did not observe any differences in male courtship behavior (Seehausen and van Alphen 1998; Maan et al. 2004, 2010; Haesler and Seehausen 2005; van der Sluijs et al. 2007; Dijkstra et al. 2008; van der Sluijs, van Dooren, Hofker, et al. 2008), but differences in male courtship intensity have been observed (Seehausen and van Alphen 1998; van der Sluijs, van Dooren, Hofker, et al. 2008; this study). Males of several fish species discriminate between conspecific and heterospecific females based on visual and/or chemical cues and may show stronger courtship behavior toward conspecific females (Knight and Turner 1999; Seehausen, van Alphen, et al. 1999; McLennan 2004; Pierotti and Seehausen 2007; Pierotti et al. 2009; Pauers et al. 2010; Pauers and

McKinnon 2012; Zoppoth et al. 2013), which in turn could influence the behavior of females (Forsgren 1997). Differences in courtship intensity may influence female choice because it may enhance the perception of other male traits, such as color or size (Endler 1995; Barbosa and Magurran 2006). In our experiment, male courtship bias toward conspecific females was weakened once color was masked in tanks with *P. pundamilia* females and even reversed in tanks with *P. nyererei* females. Male behavior in *P. pundamilia* female tanks did not change further when chemical communication was additionally blocked. This indicates that chemical cues may not be involved in male discrimination of conspecific and heterospecific females. Seehausen and van Alphen (1998), using the same species pair as in our study, showed that the slight differences between the species in female morphology and coloration had no effect on male courtship, suggesting that males do also not visually discriminate between females. Instead, the stronger courtship behavior of males toward conspecific females in the “white light” treatment of this study could have been a consequence of female preference, as reflected by female positional bias, because female responsiveness tends to elicit male courtship (Collins 1994; Houde 1997; Maan et al. 2004; Takahashi et al. 2008).

We conclude that this study, together with previous studies on the *Pundamilia* system, suggests that female preference for conspecific males is based primarily on visual signals, and the contribution of other sensory modalities seems to be minor. Female mate choice based solely on male nuptial color reduces gene flow between *P. pundamilia* and *P. nyererei* and may have played a crucial role in the evolution of reproductive isolation. On the one hand, the phenomenon that a single mate choice cue is sufficient to guide female species-assortative mating may have facilitated speciation with gene flow in this system. On the other hand, reliance on visual cues alone could make the coexistence of *Pundamilia* species vulnerable to disruption of this communication channel. Indeed, natural populations of this species pair exhibit different levels of gene flow, correlated with variation in water transparency and thereby the opportunity for visual communication (Seehausen et al. 1997, 2008; Konijnendijk et al. 2011). Thus, identifying the dimensionality of traits that mediate assortative mating not only contributes to understanding speciation but may also help to understand the fate of species in changing environments (van der Sluijs et al. 2011).

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