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Developmental effects of visual environment on species-assortative mating preferences in Lake Victoria cichlid fish

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Pundamilia;
 sensory drive;
 speciation.

Abstract

Local adaptation can be a potent force in speciation, with environmental heterogeneity leading to niche specialization and population divergence. However, local adaptation often requires nonrandom mating to generate reproductive isolation. Population divergence in sensory properties can be particularly consequential in speciation, affecting both ecological adaptation and sexual communication. *Pundamilia pundamilia* and *Pundamilia nyererei* are two closely related African cichlid species that differ in male coloration, blue vs. red. They co-occur at rocky islands in southern Lake Victoria, but inhabit different depth ranges with different light environments. The species differ in colour vision properties, and females exert species-specific preferences for blue vs. red males. Here, we investigated the mechanistic link between colour vision and preference, which could provide a rapid route to reproductive isolation. We tested the behavioural components of this link by experimentally manipulating colour perception – we raised both species and their hybrids under light conditions mimicking shallow and deep habitats – and tested female preference for blue and red males under both conditions. We found that rearing light significantly affected female preference: shallow-reared females responded more strongly to *P. pundamilia* males and deep-reared females favoured *P. nyererei* males – implying that visual development causally affects mate choice. These results are consistent with sensory drive predictions, suggesting that the visual environment is key to behavioural isolation of these species. However, the observed plasticity could also make the species barrier vulnerable to environmental change: species-assortative preferences were weaker in females that were reared in the other species' light condition.

Introduction

The sensory drive hypothesis states that sensory systems, signals and communication behaviour co-evolve in concert with the sensory environment (Endler, 1992). In sexually reproducing species, this hypothesis predicts that the choosy sex will mate more often with individuals that they can more easily detect or

that elicit stronger sensory excitation and that preferences evolve for signals that are conspicuous in the local environment (Endler, 1992; Boughman, 2002). Modelling has shown that, even in the absence of geographical barriers, heterogeneous sensory environments may drive speciation when divergent selection acts on sensory systems used in mate choice (Kawata *et al.*, 2007). As such, natural selection on sensory systems (e.g. vision), in contexts other than mating (e.g. foraging), can result in correlated responses in mating preferences (Fuller & Noa, 2010). Aquatic systems have provided some of the strongest examples of sensory drive in visual communication (Reimchen,

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1989; Endler, 1992; McDonald *et al.*, 1995; Seehausen *et al.*, 1997, 2008; Boughman, 2001, 2002; Fuller, 2002; Boughman *et al.*, 2005; Fuller *et al.*, 2005; Maan *et al.*, 2006; Fuller & Noa, 2010; Morrongiello *et al.*, 2010), but a causal link between divergent visual adaptation and reproductive isolation has not been demonstrated. Of these many studies, only one has experimentally altered visual conditions during development to test whether environment-induced changes in visual system properties could affect mate choice: in killifish, Fuller & Noa (2010) observed no overall effect of developmental light conditions on female mating preferences for differently coloured males. However, interaction effects with individual genetic background and the light conditions during mate assessment suggested some influence of visual development on colour preference (Fuller & Noa, 2010). Thus, the relationship between visual system properties and visually mediated mate preferences deserves further study. Here, we experimentally manipulate the light environment to investigate how developmental plasticity in the visual system causally affects behavioural mate choice in *Pundamilia* cichlids from Lake Victoria, East Africa.

Pundamilia pundamilia (Seehausen *et al.*, 1998) and *Pundamilia nyererei* (Witte-Maas & Witte, 1985) are two closely related, rock-dwelling species of cichlid fish that co-occur at rocky islands in southern Lake Victoria (Seehausen, 1996). They are anatomically very similar and behave as biological species in clear waters but hybridize in more turbid waters (Seehausen *et al.*, 1997). Males of the two species are distinguished by their nuptial coloration; *P. pundamilia* males are blue/grey, whereas *P. nyererei* males are yellow with a crimson-red dorsum. Females of both species are yellow/grey in colour (Seehausen, 1996; van Alphen *et al.*, 2004). Although sympatric, the two species tend to have different depth distributions: *Pundamilia pundamilia* is found in shallower waters whereas *P. nyererei* extends to greater depths. High turbidity in Lake Victoria results in a shift of the light spectrum towards longer wavelengths with increasing depth and, as such, *P. nyererei* inhabits an environment largely devoid of short-wavelength light (Maan *et al.*, 2006; Seehausen *et al.*, 2008; Castillo Cajas *et al.*, 2012). Previous work has found female preferences for conspecific male nuptial colouration in both species (Seehausen & van Alphen, 1998; Haesler & Seehausen, 2005; Stelkens *et al.*, 2008; Selz *et al.*, 2014) as well as visual system differences: *P. nyererei* expresses higher levels of the long-wavelength-sensitive (LWS) visual pigment, (Carleton *et al.*, 2005) carries an LWS allele conferring a more red-shifted sensitivity, (Carleton *et al.*, 2005; Seehausen *et al.*, 2008) and shows greater behavioural sensitivity to long-wavelength light (Maan *et al.*, 2006) compared to *P. pundamilia*. These differences in visual system characteristics correspond to the differences in photic environment,

male coloration and female preference (Maan & Seehausen, 2010).

These patterns strongly implicate the involvement of divergent sensory drive. However, it remains to be established whether divergent visual adaptation directly causes divergent preferences for male colour signals. Such a mechanism would act quickly and could be one of the first documented cases of 'magic preferences' (Maan & Seehausen, 2012). To assess the causal link between variation in visual system properties and variation in mate preference, one must isolate and manipulate variation in visual perception. Specifically, manipulation of visual development should result in a change in female preference for differently coloured males: females reared in deep (red-shifted) light should have stronger preferences for red males. Manipulation of the light environment during mate assessment allows further exploration of the contribution of perceptual variation to mate preference. Specifically, deep-reared females may prefer red males under both deep and shallow test conditions (red is equally conspicuous in both light environments), but shallow-reared females may prefer blue males only when tested in shallow light (blue is an ineffective signal in the deep environment). Should light manipulations have no effect, then this would indicate that other factors, independent of colour perception, are more important in determining preference (e.g. genetic preference loci, social learning).

Here, we experimentally manipulated individual visual development and tested its consequences for female choice. We reared female fish under light conditions mimicking shallow and deep habitats of Lake Victoria and then tested preference for blue vs. red males under both light conditions. We found a small, but significant, effect of rearing light on female preference, supporting the causal involvement of visual development in behavioural reproductive isolation.

Materials and methods

Fish rearing & maintenance

F1 offspring of wild-caught *P. pundamilia* and *P. nyererei*, collected in 2010 at Python Islands (−2.6237, 32.8567) in the Mwanza Gulf of Lake Victoria (Fig. S1), were reared in light conditions mimicking those in shallow and deep waters at Python Islands. Fish were first transported to the Eawag Institute at Kastanienbaum, Switzerland; in September 2011, they were relocated to the University of Groningen, the Netherlands. Light conditions were the same for both locations – described in detail below. F1 families (hybrid and non-hybrid) were created opportunistically as reciprocal crosses, with 17 dams and 11 sires. Twenty crosses (five *P. nye* × *P. nye*; five *P. pun* × *P. pun*; four *P. nye* × *P. pun*; six *P. pun* × *P. nye*) resulted in a test population of 91 females from 19 families (two

P. nye × *P. nye* crosses were full-sibs; family details provided in Table S1). We included hybrids because their heterozygosity (particularly at loci influencing visual properties and mate preference) could allow us to more clearly observe an effect of our environmental manipulations, which may be obscured by strong genetic effects in the parental species. Indeed, F1 hybrid females have mate preferences that are weak and intermediate between the two parental species (Haesler & Seehausen, 2005). Hybridization occurs with low frequency at Python Islands (Seehausen *et al.*, 2008) and can be accomplished in the laboratory by housing females with heterospecific males. *Pundamilia* are maternal mouth brooders; to reduce the opportunity for imprinting (Verzijden & ten Cate, 2007), fertilized eggs were removed from brooding females approximately 6 days after spawning (mean ± SE: 6.3 ± 0.5 days post-fertilization; eggs hatch at about 5–6 dpf) and split evenly between light conditions. Upon reaching maturity, females ($n = 91$) were removed from family groups, PIT tagged (passive integrated transponders, from Biomark, Idaho, USA, and Dorset Identification, Aalten, the Netherlands) and housed together. Fish were maintained at 25 ± 1 °C on a 12L: 12D cycle and fed daily a mixture of commercial cichlid flakes and pellets and frozen food (artemia, krill, spirulina, black and red mosquito larvae). Stimulus males used in the preference trials (*P. pund.* = 9, *P. nye.* = 6) were also PIT tagged but were housed individually under standard aquarium lighting (white fluorescent), isolated by transparent, perforated partitions, to prevent aggression. This study was conducted under the approval of the Veterinary Office of Kanton Lucerne (01/10) and the Institutional Animal Care and Use Committee of the University of Groningen (DEC 6205B).

Experimental light conditions

Experimental light conditions were created to mimic the natural light environments of *P. pundamilia* and *P. nyererei* at Python Islands, Lake Victoria (Fig. S2). We measured down-welling irradiance (in $\mu\text{mol}/(\text{m}^2 \cdot \text{s})$) using a BLK-C-100 spectrophotometer and F-600-UV-VIS-SR optical fibre with CR2 cosine receptor (Stellar-Net, Tampa, FL, USA). Measurements were collected at 0.5 m increments to 5 m depth. In each measurement series, we took a minimum of two irradiance spectra at each depth and used the average for further analyses. We collected four independent measurement series (20/26 May and 4/5 June 2010, between 9:00 and 11:00 h). For each measurement series, we estimated the light environments experienced by *P. pundamilia* and *P. nyererei* by calculating a weighted average of the spectra at each depth, using the reported depth distribution of each species (at Python Islands – *P. pundamilia*: 1–3 m; *P. nyererei*: 1–5 m; Seehausen *et al.*, 2008).

The average of the four resulting species-specific light spectra was simulated in the laboratory (Fig. S2) by halogen light bulbs filtered with a green light filter (LEE #243, Andover, UK). Shallow conditions were blue – supplemented with Paulmann 88090 compact fluorescent 15W bulbs. Short-wavelength light was reduced in deep conditions by adding a yellow light filter (LEE #015). The resulting downwelling irradiance was measured using the same equipment as in the field. The light intensity differences between depth ranges in Lake Victoria are very small in relation to the variation that is due to changes in weather and sun angle (natural intensity levels can change rapidly – as much as 1000-fold in sun vs. cloud cover). As such, we did not attempt to mimic light intensity differences between environments (see Fig. S2).

Experimental set-up

Mate preference trials, based on procedures previously employed in *Pundamilia* (Seehausen & van Alphen, 1998; Maan *et al.*, 2004; Haesler & Seehausen, 2005) took place from May 2012 to September 2014. Prior to testing, one F1 *P. pundamilia* and one F1 *P. nyererei* male was introduced on either end of female housing tanks, behind transparent, perforated partitions. This was done to ensure that all females had interacted with males of both species before preference assessment, and to induce female receptivity. After 1 week, males were replaced with different individuals (and sides switched) and again allowed 1 week of interaction. Following this period, females were only exposed to males during experimental trials. Preceding the experimental period for a batch of test females, females were habituated to the experimental tank by allowing them, as a group (10–15 females), to explore the tank for 1–2 h (without stimulus males and under the light conditions in which they were reared).

At least 12 h before trials, one male of each species (F1, nonhybrid) was isolated behind a transparent partition on opposite ends of the test tank, containing an air filter and shelter (PVC tube and stone). An area was designated at 20 cm in front of each stimulus male as an ‘interaction zone’ for behaviour scoring (see below). Water temperature was kept at 25 ± 1 °C, water quality was maintained via 3 continuously running filters (one per compartment, described below), and the bottom of the tank was covered with fine gravel. Experimental tanks were illuminated with the same light configuration as described above, with each female tested repeatedly in both shallow and deep light conditions (filters were switched and blue light turned on/off depending on test condition).

We used two different experimental set-ups. Set-up #1 used a tank 120 cm × 40 cm × 41 cm with solid, transparent partitions, preventing chemical exchange between the fish. Males were confined to an area

24 cm × 40 cm × 32 cm and were visually isolated from one another by opaque dividers in the centre portion of tank (with large holes through which females could pass, Fig. 1a). The region between opaque dividers was intended as an area of refuge for females and was further supplemented with a PVC tube. In total, 205 trials (with 72 females) were conducted in set-up #1.

Success rate in this set-up was 64%, with low courtship intensity of males and/or low responsiveness of females in the remainder of trials (for trial success criteria see below). To increase fish activity and responsiveness, preference tests were modified into set-up #2 (Fig. 1b). As chemical communication may enhance cichlid reproductive behaviour (Caprona & Crapon, 1980; Kidd *et al.*, 2013) but does not affect female mate preference in *Pundamilia* (Selz *et al.*, 2014) see also results), set-up #2 allowed for chemical exchange between the fish. We used a larger tank, 150 cm × 50 cm × 51 cm, with transparent partitions with small holes. Males were confined to an area of 30 cm × 50 cm × 38 cm, with no visual barrier between them. Female refuge was provided by a PVC tube and two plastic plants in the centre portion of the tank. 76 trials were completed in set-up #2 (using 47 females), with a modest but statistically significant increase in success rate (77%; $\chi^2 = 4.77$, $df = 1$, $P = 0.029$). Of these 47 females, 28 had also been tested in set-up #1 (under both light conditions but with different stimulus males); 19 were tested solely in set-up #2. Thus, we tested 91 females in total, with 19

tested solely in set-up #2, 44 tested solely in set-up #1, and 28 tested in both.

Mate preference trials

For each trial, a gravid female was introduced into the centre portion of the tank and allowed to interact with the males. Female gravidity was determined visually prior to testing by the presence of a swollen abdomen and scored on a scale 1–5 (Seehausen & van Alphen, 1998). Only females scoring 3+ were used in preference tests. Males were scored for courtship behaviour – lateral display and quiver (McElroy & Kornfield, 1990), the first two behaviours in the sequence of the haplochromine courtship ritual (Seehausen, 1996) – whereas female fish were scored for their responses to each male courtship event (positive or negative). Following previous studies in *Pundamilia* (Seehausen & van Alphen, 1998; Maan *et al.*, 2004; Haesler & Seehausen, 2005), positive female response was classified by an observable interest in male behaviour – moving towards males and/or remaining engaged in interaction (i.e. still trying to gain access to the male through the plastic partition following male courtship). Negative responses were classified as a general disinterest – moving away and/or not responding to male behaviour. Females ($n = 91$; see Table S1 for family counts reared in each light condition) were tested repeatedly (median = 3, min/max = 1/7), under both shallow and deep light conditions (only 15 fish were not tested in both conditions, see Table S2) with different combinations of stimulus males (*P. pun.* = 9, *P. nye.* = 6; combined into 29 different stimulus pairs; stimulus pair usage: median = 9, min/max = 3/24). Males in a stimulus pair were matched as much as possible for standard length (SL), differing by an average of 9% (*P. pundamilia* males are typically larger, mean SL: *P. pun.* = 86.49 ± 3.31 mm; *P. nye.* = 83.43 ± 2.29 mm). Trials were scored directly using JWatcher (v1.0, 2000–2012, Blumstein, Daniel, and Evans) and video-recorded (a portion of trials were scored from videos). Behavioural scoring started when females entered a male interaction zone and was paused when females left this zone, until a total of 20 min of interaction time (combined across the two zones) was reached. Trials were considered successful if 20 min of interaction time was recorded within one hour and each male had performed at least three quiver displays.

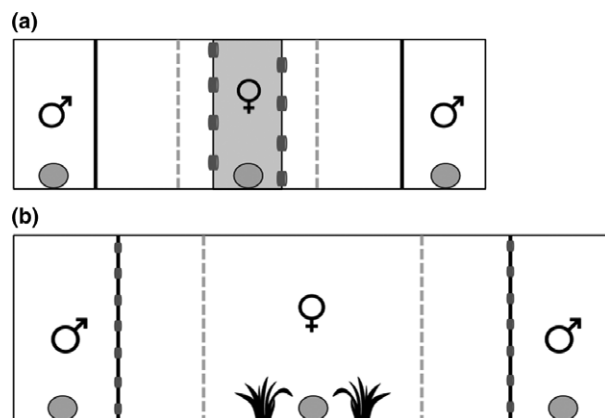


Fig. 1 Experimental set-ups – Dashed lines indicate the ‘interaction zone’ of females with each stimulus male, and solid lines represent barriers between fish. All fish were provided a PVC tube for shelter and each compartment (female and male) had a continuously running filter. Aquaria drawn to scale. (a) Set-up #1: opaque dividers with large holes for female passage provided an area of refuge. Males were chemically isolated from females. (b) Set-up #2: opaque dividers were removed; plastic plants were added. Barriers between males and females had small holes allowing chemical exchange.

Behaviour Scoring

Female preference

Female positive and negative responses to each male courtship behaviour was totalled for each trial and female preference scores were calculated as the difference in the proportions of positive responses to male courtship between the two males (Seehausen & van

Alphen, 1998; Maan *et al.*, 2004; Haesler & Seehausen, 2005). For example, lateral display (LD) based preference was calculated as follows:

$$\text{Preference LD} = \frac{(\text{Positive to } P. \text{ nye LD})}{(\text{Total } P. \text{ nye LD})} - \frac{(\text{Positive to } P. \text{ pun LD})}{(\text{Total } P. \text{ pun LD})}$$

The result is a measure of preference ranging from -1 to 1 , with positive scores indicating a preference for *P. nyererei* males and negative scores indicating a preference for *P. pundamilia* males. Quiver (Q)-based preference was calculated in an analogous manner.

For both *P. pundamilia* and *P. nyererei* females (not hybrids) we also calculated species-assortative preference, by rescaling all scores such that positive scores indicate preference for conspecifics and negative scores indicate preference for heterospecifics.

Fish activity

We examined overall female activity, irrespective of male behaviour (using the number of times females moved from one section of the tank to another), and overall female responsiveness to male courtship (i.e. the overall proportion of positive responses to the courtship of both stimulus males). Male activity was assessed as the total number of courtship displays.

Statistical analysis

Linear mixed models

We tested for influences on female preference with linear mixed modelling using the *lmer* function in the *lme4* package (Bates *et al.*, 2013) in R (v3.2.2; R Development Core Team). Included in full models were the fixed effects (and interactions) of rearing light condition (shallow vs. deep), testing light condition (shallow vs. deep), female genetic background (*P. pun.*, *P. nye.* or hybrid), and set-up (#1 vs. #2). Random effects included female identity, male identity, parental identity, and observer identity to account for: (i) the repeated testing of females, (ii) individual differences among stimulus males, (iii) repeated usage of stimulus males, (iv) shared parentage among test females (Table S1), (v) multiple scorers of behaviour. The optimal random effect structure of models was determined by parametric bootstrapping using the *PBmodcomp* – *pbkrtest* package (Halekoh & Højsgaard, 2014) and the significance of fixed effect parameters was determined by likelihood ratio tests via *drop1* in the *MASS* package (Ripley *et al.*, 2015). Minimum adequate statistical models (MAM) were selected using statistical significance (Crawley, 2002; Nakagawa & Cuthill, 2007). We then used the *KRmodcomp* – *pbkrtest* package (Halekoh & Højsgaard, 2014) to test the MAM against a model lacking the significant parameter(s), which allowed us

to obtain the estimated effect size of fixed effect parameters under the Kenward-Roger (KR) approximation (Kenward & Roger, 1997, 2009). We used the same approach to evaluate the extent of species-assortative preference. In addition to considering all trials together, we also analysed the data separately for each set-up and species group. All analyses followed the procedures detailed above and, in the case of more than two categories per fixed effect parameter (i.e. female species group); we used post hoc Tukey (*glht* – *multcomp* package (Hothorn *et al.*, 2008)) to obtain parameter estimates. The significance of parameter intercepts was checked using *lsmeans* in package *lmerTest* (Kuznetsova *et al.*, 2013).

Repeatability

The repeatability of individual female preference was estimated as: $R_A = (MS_A - MS_W) / [MS_A + (n_0 - 1) * MS_W]$, using *rpt.aov* in package *rptR* (Nakagawa & Schielzeth, 2010). R_A is the ANOVA-based repeatability estimate, MS_A is the mean between-individual sum of squares, and MS_W is the mean within-individual (residual) sum of squares. The correction term n_0 (equation 5 in Nakagawa & Schielzeth, 2010) is lower than the average sample size if sample sizes vary among individuals (as is the case for this study; repeated testing varied among individual females, detailed in Table S2). This downward correction accounts for the overestimation of variance in smaller groups (Nakagawa & Schielzeth, 2010). Both overall and species-specific repeatability were examined, as well as repeatability among test light conditions.

Results

Female preference is affected by rearing light

Rearing light had a significant effect on female preference for both male courtship behaviours, with shallow-reared females responding more positively to *P. pundamilia* males than deep-reared females: LD ($F_{1,70.48} = 4.61$, $P = 0.035$; Fig. 2a) and quiver ($F_{1,69.81} = 4.26$, $P = 0.042$; Fig. 2b). Shallow-reared females preferred *P. pundamilia* males (intercept significantly different from zero for LD: -0.0476 , $P = 0.05$, 95% CI $[-0.09, 0.00]$ but not for Q: -0.0451 , $P = 0.1$, 95% CI $[-0.10, 0.01]$); deep-reared females exerted no preference (intercepts not different from zero; LD: $P = 0.6$; Q: $P = 0.3$). Effects of test light (LD: $P = 0.17$; Q: $P = 0.40$) and female species (LD: $P = 0.76$; Q: $P = 0.87$) were nonsignificant, as were any interactions among the parameters. There was no effect of stimulus male size ($P > 0.7$). Analyses for each female species group separately yielded qualitatively similar results ($P > 0.1$ for all parameters, species-specific preference estimates for rearing and test light combinations are provided in Table S3).

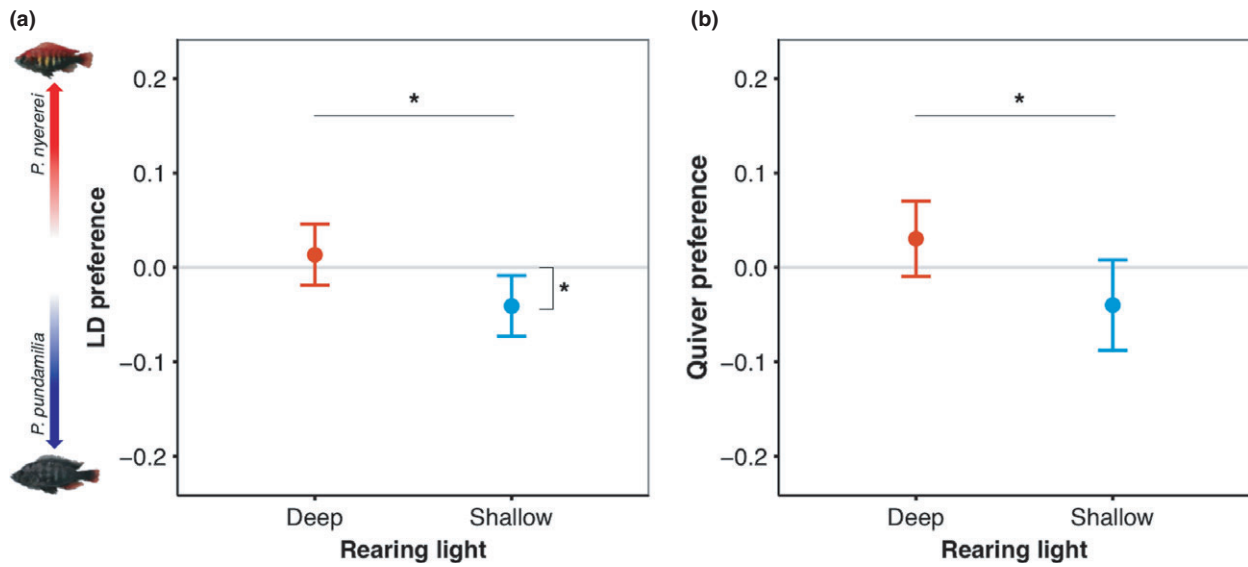


Fig. 2 Effect of rearing light conditions on female preference – Females from different rearing light conditions showed significantly different mate preferences, measured as differential response to either male lateral display (a) or male quiver (b). Females reared in shallow light conditions displayed more positive responses to *Pundamilia pundamilia* male courtship. *Indicates $P < 0.05$, error bars represent 95% C.I.

Species-assortative preference

To explore the extent of species-assortative preference, we reran all models excluding hybrid females on rescaled female preference scores. We found a significant interaction between rearing light and female species for both LD ($P = 0.035$, Fig. 3a) and quiver ($P = 0.009$, Fig. 3b), indicating that both species are

more responsive to conspecific males when reared under light conditions natural to each species (i.e. *P. nyererei* females reared in deep and *P. pundamilia* females reared in shallow), compared to their sisters raised in unnatural light conditions. Tests of the preference intercepts revealed that shallow-reared *P. pundamilia* females preferred conspecific males (intercept differed significantly from zero for LD: 0.082, $P = 0.03$,

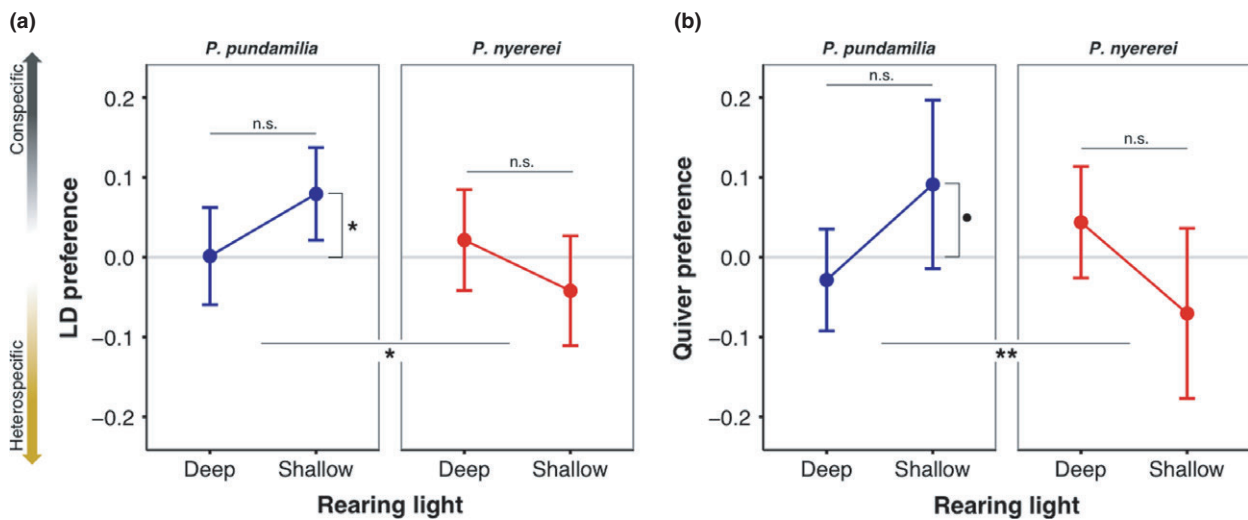


Fig. 3 Species-specific effects of rearing light on preference – Significant interaction between rearing light and female species on both LD-based (a) and Q-based (b) preference for conspecific males: rearing light conditions affected female preference in opposite directions in *Pundamilia pundamilia* and *Pundamilia nyererei*. **Indicates $P < 0.01$, *indicates $P < 0.05$, •indicates $P < 0.1$, error bars represent 95% C.I.

95% CI [0.007, 0.157] but not quite for Q: 0.087, $P = 0.07$, 95% CI [-0.008, 0.183]; Fig. 3), whereas deep-reared *P. pundamilia* did not (LD: $P = 0.93$; Q: $P = 0.46$). For *P. nyererei*, neither deep-reared nor shallow-reared females had a significant preference ($P > 0.23$ in all cases). Tukey post hoc showed no significant differences between-individual species/treatment combinations ($P > 0.11$ in all comparisons).

When pooling both species and re-categorizing rearing light (deep vs. shallow) as natural or unnatural for each species, rearing environment significantly affected the extent of species-assortative preference for quiver ($F_{1,36.30} = 5.19$, $P = 0.028$; nonsignificant for LD: $F_{1,37.00} = 2.75$, $P = 0.105$, Fig. 4). Differences between test light conditions (LD: $P = 0.12$; Q: $P = 0.97$) and female species (LD: $P = 0.50$; Q: $P = 0.82$) were nonsignificant. Tests of the natural rearing environment intercept revealed marginal nonsignificance for both LD ($P = 0.08$) and quiver ($P = 0.07$), indicating a nonsignificant overall tendency for species-assortative preference when females were reared under light conditions that mimicked their natural environments, but not when they were reared in unnatural conditions ($P > 0.21$).

Repeatability of female preference

Repeatability of female preference was overall low ($R_{LD} = 0.103$; $R_Q = 0.07$). *Pundamilia pundamilia* and hybrid females showed somewhat higher repeatability ($R_{LD} = 0.153$; $R_Q = 0.145$; and $R_{LD} = 0.176$; $R_Q = 0.061$, respectively) than *P. nyererei* ($R_{LD} = -0.059$;

$R_Q = 0.013$). Low repeatability was not due to testing females in two different light conditions and two different set-ups: test light conditions did not influence preference, and we found low repeatability also within each test light condition and within each set-up (see Table S4).

Set-up effects

There was no difference in female preference between set-ups (fixed effect of set-up was nonsignificant in all preference models: $P > 0.28$). However, females in set-up #2 were more active ($F_{1,65.73} = 17.54$, $P < 0.001$) and displayed more positive responses to male LD ($F_{1,177.26} = 4.46$, $P = 0.035$; similar trend for quivers: $F_{1,129.34} = 3.24$, $P = 0.074$). For stimulus males, we found an increase in LD frequency ($F_{1,229.83} = 15.97$, $P < 0.001$) but not quiver frequency ($P = 0.41$). Separate analyses of female preference in set-up #1 and #2 revealed qualitatively similar results to those presented above (see Table S5), confirming that set-up influenced fish activity but had no effect on preference scores.

Effect of test light on fish activity

Test light had no effect on overall female responsiveness to male courtship; females were slightly more responsive to LD in deep test light ($F_{1,164.32} = 2.57$, $P = 0.11$), but there was no difference between light conditions for quiver ($P = 0.74$). Test light also had no effect on female activity ($P = 0.18$). Female species groups did not differ in their responsiveness to male LD

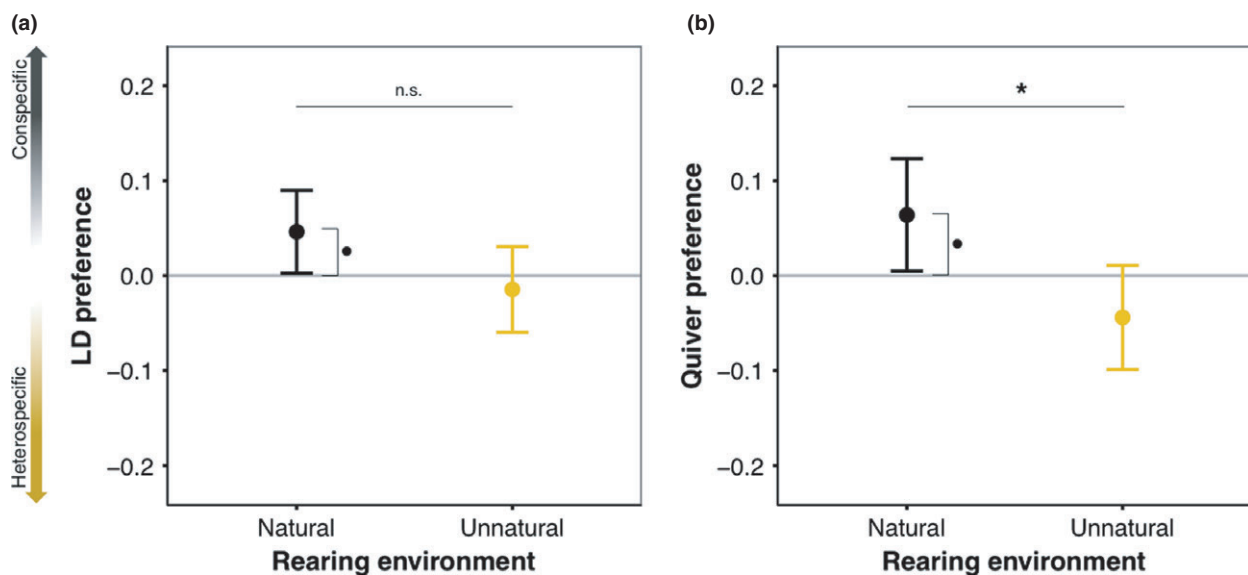


Fig. 4 Rearing environment influences species-assortative preference – Comparison of species-assortative preference between females reared in light environments ‘natural’ vs. ‘unnatural’ (to their own species) showed that (a) LD-based preference did not differ, but (b) quiver-based preference for conspecific males was stronger in females reared in natural light conditions. *Indicates $P < 0.05$, *indicates $P < 0.1$, and error bars represent 95% C.I.

under different test light conditions (Tukey post hoc: $P > 0.3$ in all cases), but in shallow test light, *P. pundamilia* females responded more to male quivers than *P. nyererei* females did ($z = 2.90$, $P = 0.04$). There was no difference in the deep test light condition ($P > 0.9$). Hybrid females were generally more active than *P. pundamilia* and *P. nyererei* (switched sides more, $P = 0.01$), whereas *P. pundamilia* and *P. nyererei* females did not differ in activity ($P > 0.9$).

Total male courtship activity did not differ between shallow and deep test light (LD: $P = 0.60$; Q: $P = 0.18$). *Pundamilia pundamilia* males showed higher courtship frequency in deep light (LD: $F_{1,220.14} = 3.44$, $P = 0.064$; Q: $F_{1,212.68} = 4.41$, $P = 0.036$), but *P. nyererei* courtship did not differ between light conditions (LD: $P = 0.21$; Q: $P = 0.46$).

Discussion

The sensory drive hypothesis predicts that population divergence in sensory properties can affect both ecological fitness and sexual communication. *Pundamilia pundamilia* and *P. nyererei* differ in colour vision, correlated with the different light environments of their natural habitats, and females display divergent preferences for conspecific male coloration. Here, we tested the causal link between visual development and preference by rearing both species and their hybrids in light conditions mimicking distinct habitats in Lake Victoria and assessing female mate preference. We found small but significant effects of the rearing light treatments: shallow- and deep-reared females had significantly different preferences, and shallow-reared females significantly preferred *P. pundamilia* males. When reared in conditions unnatural to their own species (i.e. *P. pundamilia* in deep light conditions and *P. nyererei* in shallow light conditions), females showed weaker species-assortative preferences. Preference scores were not affected by the availability of chemical communication, nor the light conditions during mate preference assessment, suggesting additional factors beyond visual perception.

Effect of rearing light

Pundamilia pundamilia and *P. nyererei* are naturally depth-segregated at multiple locations in southern Lake Victoria (Seehausen *et al.*, 2008). Both within and between locations, visual system characteristics, female preferences and male coloration co-vary with variation in the visual environment (Carleton *et al.*, 2005; Seehausen *et al.*, 2008; Maan *et al.*, 2010; Castillo Cajas *et al.*, 2012). By rearing each species (and their hybrids) under light conditions mimicking this variation, we have shown that the visual environment during development significantly influences female preference. We propose two possible explanations for this result.

First, haplochromine cichlids exhibit plasticity in visual properties: the expression levels of retinal pigments can be influenced by light (Van der Meer, 1993; Hofmann *et al.*, 2010; Smith *et al.*, 2012). Thus, the shallow rearing environment may have induced an up-regulation of blue-sensitive pigment, generating increased sensitivity to the blue coloration of *P. pundamilia* males. Conversely, up-regulation of red-sensitive pigment in the deep rearing environment may have induced greater sensitivity to the red coloration of *P. nyererei* males. Previous studies in fish have reported behavioural effects of manipulating the visual environment during development, with changes in opsin expression accounting for at least some of the variation (Fuller & Noa, 2010; Fuller *et al.*, 2010; Smith *et al.*, 2012). However, the relationship between opsin expression and behaviour is not straightforward. In killifish, for example, female preference for differently coloured males was found to result from complex interactions between genetic background, rearing environment and testing environment (Fuller & Noa, 2010). Analyses of light-induced effects on pigment expression in *Pundamilia* are underway, but the observations presented here do indicate some involvement of perceptual variation: the effect of rearing condition seems more pronounced in the shallow test environment (see Fig. S4). However, if female preference was completely determined by variation in visual perception, we should have seen a much stronger interaction between rearing light and test light. For example, the preference of shallow-reared females for blue males should be weaker under deep testing conditions, where the blue coloration of *P. pundamilia* is not conspicuous (see Fig. S3). Thus, our results indicate that other factors are involved (discussed in more detail below).

A second explanation concerns potential effects of female exposure to male phenotypes prior to testing. All fish were reared in family groups (until sexual maturity), and females were visually presented with males of both species prior to testing. Thus, females gained experience with both male phenotypes, in the light conditions they were reared in. Given that light conditions influence the radiance of male colours, females in the two different light conditions gained different experience with male phenotypes. Although filial imprinting seems unimportant in the development of haplochromine mate preference (Verzijden *et al.*, 2008), we cannot rule out that, for example, deep-reared females are less likely to develop preferences for blue males because they never perceived this phenotype prior to being tested. Individually housing experimental fish and controlling prior exposure to male colour would be required to explore these effects.

Regardless of the underlying mechanism, our results show that the environment during development can directly affect female preference. We found that when reared in light conditions unnatural to their own

species, females had weaker species-assortative preferences than females reared in natural light conditions. This suggests that female preference and, by extension, reproductive isolation are linked to heterogeneity in the local environment, which has implications for natural systems. Any change in local conditions, through, for example, environmental change or migration, could influence sexual preferences and the extent of reproductive isolation (Fisher *et al.*, 2006; Frederick & Jayasena, 2011; Senior *et al.*, 2014).

Weak species-assortative preference

Even when females were reared in a light environment natural to their own species, we found only weak species-assortative preferences. This is surprising, given that several earlier studies have reported female preferences for conspecific male colour in both *P. pundamilia* and *P. nyererei* (Seehausen & van Alphen, 1998; Haesler & Seehausen, 2005; Selz *et al.*, 2014). This may be explained by our experimental light conditions – the spectra of our light treatments, while mimicking natural conditions, differ dramatically from the standard aquarium lighting used in previous studies (Fig. S2). Yet, one would still expect species-assortative preferences in the shallow light condition with broad-spectrum lighting, which should be somewhat comparable to the fluorescent white light used in previous studies. We provide two additional explanations for the weak species-assortative preferences we observed: lack of maternal imprinting and selective disappearance.

In contrast to filial imprinting, maternal imprinting has been shown to be an important determiner of female preference in Lake Victoria cichlids, including *P. pundamilia* and *P. nyererei* (Verzijden & ten Cate, 2007; Verzijden *et al.*, 2008). Maternal care of offspring (females mouth brood for about 3 weeks, followed by a brief period of guarding) may allow female imprinting on mothers' phenotype, thus driving assortative preferences. In contrast to previous studies of preference (Seehausen & van Alphen, 1998; Haesler & Seehausen, 2005; Dijkstra *et al.*, 2008; van der Sluijs *et al.*, 2008), the opportunity for maternal imprinting was greatly reduced in our females. Fry were removed from brooding females at 5–6 dpf and housed in family groups until testing. A recent study also removed fry early in the brooding period (Selz *et al.*, 2014) but these authors examined the number of spawning events rather than behavioural preference. Possibly, imprinting on maternal phenotype has stronger effects on behavioural measures of preference and does not interfere with actual mating decisions.

A second potential explanation concerns the selective survival of each species in the experimental light treatments. We found that when reared in 'unnatural' light conditions, both *P. pundamilia* and *P. nyererei* survive at a lower rate (~40% reduction at 12 months) than their

'naturally' reared counterparts (Maan *et al.*, in press). Within species, survival may be nonrandom, with surviving females differing from nonsurviving females in properties that also affect preference. For example, *P. pundamilia* females that survive in deep conditions may have more *P. nyererei*-like visual properties, which could affect preferences. At Python Islands, *P. pundamilia* and *P. nyererei* are strongly differentiated at the LWS locus, but there are a small percentage of mismatched allele profiles in each species (Seehausen *et al.*, 2008). As all fish used in this experiment were derived from wild-caught individuals, it is reasonable to expect some degree of genetic variation in the visual systems of our laboratory population. This variation may have led to a nonrandom test population of females, selected by genotype-dependent survival. Similarly, perhaps those individuals that do survive in 'unnatural' light conditions are more plastic, allowing them to cope with sub-optimal conditions (Ghalambor *et al.*, 2007; Crispo, 2008). This could have generated a population of test females that do not exhibit strong mate preferences, thus potentially explaining weak conspecific preferences and low repeatability. Previous work in *Pundamilia* reported higher female preference repeatability (pooled over two series: $R = 0.59$, (Haesler & Seehausen, 2005)), but these scores were for females reared and tested under white light. Our use of fish reared in manipulated light conditions likely contributed to the low repeatability values reported in this study.

Set-up effect

We used two experimental set-ups: set-up #1 isolated the effects of male coloration and visual perception by preventing chemical communication, whereas set-up #2 allowed chemical exchange. Chemical communication in fish is important (Liley, 1982) and can affect mating behaviour in cichlids (Caprona & Crapon, 1980; Kidd *et al.*, 2013). Consistent with this, we found higher trial success rates and higher levels of fish activity in set-up #2. Although we cannot attribute these changes solely to chemical communication, because set-ups also differed in tank size and partitions, we can conclude that female preferences were not affected by the availability of chemical cues in set-up #2. This is in line with Selz *et al.* (2014), who demonstrated that chemical communication alone does not generate species-assortative mating in *Pundamilia*. Thus, the results presented here support the notion that visual signals are key in preference determination in *Pundamilia*.

Conclusion

Our results provide behavioural support for divergent sensory drive in *P. pundamilia* and *P. nyererei*. We have shown that light conditions during development

significantly influence female preference; even to the extent that species assortment is affected. These results highlight the importance of environmental heterogeneity in maintaining species isolation, if only in the initial stages of speciation. Future work will be aimed at establishing the underlying mechanisms, such as phenotypic plasticity in visual development and/or the influence of early experience with male colour phenotypes.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Python Islands, Lake Victoria.

Figure S2 Natural and experimental light conditions.

Figure S3 Males in manipulated light treatments.

Figure S4 Interaction between rearing light and test light.

Table S1 Test female families.

Table S2 Female testing scheme.

Table S3 Species-specific preference estimates.

Table S4 Repeatability of female preference.

Table S5 Preference estimates.

Table S6 Set-up repeatability.

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