

Title

Behavioural isolation may facilitate homoploid hybrid speciation in cichlid fish.

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Abstract

Hybrid speciation is constrained by the homogenizing effects of gene flow from the parental
 species. In the absence of postmating isolation due to structural changes in the genome, or
 temporal or spatial premating isolation, another form of reproductive isolation would be
 needed for homoploid hybrid speciation to occur. Here, we investigate the potential of
 behavioural mate choice to generate assortative mating among hybrids and parental species.
 We made three first-generation hybrid crosses between different species of African cichlid
 fish. In three-way mate choice experiments we allowed hybrid and non-hybrid females to
 mate with either hybrid or non-hybrid males. We found that hybrids generally mated non-
 randomly and that hybridization can lead to the expression of new combinations of traits and

preferences that behaviourally isolate hybrids from both parental species. Specifically, we find that the phenotypic distinctiveness of hybrids predicts the symmetry and extent of their reproductive isolation. Our data suggest that behavioural mate choice among hybrids may facilitate the establishment of isolated hybrid populations, even in proximity to one or both parental species.

Introduction

The role of hybridization in evolution is an intensively debated topic. Long considered an evolutionary dead end (Mayr 1942; Dobzhansky 1951), hybridization is now known to have diverse impacts on evolution (Arnold and Hodges 1995; Arnold 1997; Seehausen 2004; Mallet 2007; Abbott et al. 2013). Hybridization can cause the collapse of species into a hybrid swarm (Taylor et al. 2006) or facilitate adaptive introgression (Anderson 1949; Anderson and Stebbins 1954; Arnold 2006; Arnold and Martin 2009), allopolyploid hybrid speciation (Mallet 2007; Wood et al. 2009), homoploid hybrid speciation (Gross and Rieseberg 2005; Abbott et al. 2010) and adaptive radiation (Seehausen 2004, 2013). Since hybrids are usually produced in geographic proximity to one or both parental species, the development of some form of reproductive isolation is often required for a hybrid population to escape the homogenizing effect of gene flow from both parental species as first step towards establishing as a new species (Rieseberg 1997; Buerkle et al. 2000). The majority of documented cases of hybrid speciation involve intrinsic postmating reproductive isolation between the hybrid and the parental species (Mallet 2007). Two common causes of such isolation are chromosomal rearrangements (Grant 1949; Stebbins 1957) and allopolyploidy (Stebbins 1971; Rieseberg and Willis 2007), which are common in plants (Hegarty and Hiscock 2008; Woods et al. 2009). In animals, however, hybrid speciation involving polyploidy is less common (Mable 2004) than homoploid hybrid speciation (Mallet 2007). Homoploid hybrid speciation occurs when a genetically stable and fertile hybrid lineage emerges that acquires reproductive isolation from its parental species without changing ploidy level (Anderson 1949; Mallet 2007; Mavárez and Linares 2008; Abbott et al. 2010). Homoploid hybrid speciation relies on the build-up of barriers to gene flow between the hybrids and their parental species (Coyne and Orr 2004). In animals both prezygotic and postzygotic barriers have been shown to contribute to reproductive isolation between hybrids and their parental species with the former being more common than the latter (Mavárez and Linares 2008). Hence, in the

absence of intrinsic postmating isolation, i.e. chromosomal or genetic sterility barriers, homoploid hybrid speciation relies on reproductive isolation that is based on ecological or behavioural factors. Most of the described animal hybrid species differ ecologically from both parental species, by occupying spatially and/or temporally separated habitats (Nolte et al. 2005; Schwartz et al. 2005; Gompert et al. 2006; Larsen et al. 2010; Kunte et al. 2011; Nice et al. 2012). Consequently, the propensity of hybridization to generate genetic and evolutionary novelty, including hybrid species, is currently thought to be largely determined by the ecological context in which hybridization occurs (Mallet 2007; Arnold and Martin 2009). Existing theoretical models predict that homoploid hybrid speciation requires an available niche that is distinct from parental species' niches and strong ecological selection excluding the parental species from this niche (Buerkle et al. 2000; 2003; Duenez-Guzman et al. 2009). The result is geographical, temporal or ecologically mediated isolation of the hybrids from both parental species (Gross and Rieseberg 2005; Abbott et al. 2010).

In the absence of geographical, temporal or ecologically mediated isolation, behavioural mate choice through assortative mating can cause reproductive isolation. There are indeed cases of homoploid hybrid species that co-occur in sympatry with one (Mavárez et al. 2006; Schwander et al. 2008; Hermansen et al. 2011) or both parental species (Schliewen and Klee 2004; Keller et al. 2012). In half of the putative 25 cases of homoploid hybrid speciation in animals reviewed by Mavárez and Linares (2008), at least one parental species lived in sympatry with the hybrid species. Hence, behavioural factors must explain reproductive isolation. Two studies to date, namely in *Heliconius* butterflies (Mavárez et al. 2006; Melo et al. 2009) and *Pogonomyrmex* ants (Schwander et al. 2008), have shown that strong assortative mate choice contributes to reducing gene flow between incipient hybrid species and one of the parental species in the wild. The potential for such assortative mating depends on the mating traits evaluated by the choosing sex and its preferences for those traits. If mating traits and preferences for these are determined by several genes, hybridization may result in recombination among them. Thus, hybrids may display novel phenotypes (in traits and preferences) that are intermediate to, or similar to one of their parental species (Doherty and Gerhardt 1983; Gompert et al. 2006; Shaw et al. 2007; Magalhaes and Seehausen 2010; Raeymaekers et al. 2010), they may recombine features of both parents into new trait combinations or express transgressive variation in single traits, where phenotypic variation exceeds the trait range of both parental species combined (Bentley and Hoy 1972; Slatkin and Lande 1994; Mavárez et al. 2006; Olsson et al. 2006; Stelkens et al. 2009a; Cáceres et al. 2009). In first-generation hybrids, both additive effects and non-additive interactions

(epistasis and dominance) will contribute to the expression and to possible new combinations of such mating traits and preferences (Landry et al. 2007; Doyle et al. 2008; Michalak 2009). Non-additive genetic interactions can contribute to complementary gene action (Rieseberg et al. 1999), which could result in transgressive traits and preferences. Finally, for hybrid assortative mating to arise and contribute to reproductive isolation, such novel preferences in the hybrid must result in favouring the novel trait combination of the hybrid over those of the parental species.

The likelihood of behavioural isolating mechanisms to evolve in hybrids should be enhanced in taxa with strongly developed mate choice and with variation in phenotypic traits that can be recruited for mate choice (Crapon De Caprona 1986; Rosenthal 2013). African lake cichlid fish are known for their spectacular species diversity associated with diversity in coloration, behaviour and ecology (Kornfield and Smith 2000; Kocher 2004). Males in many of these species are conspicuously coloured, and colouration affects both inter- and intra-specific female mate choice (Couldridge and Alexander 2002; Seehausen and van Alphen 1998; Knight and Turner 2004; Maan et al. 2004; Pauers et al. 2004, 2010; Egger et al. 2008; 2010; Seehausen et al. 2008; Stelkens and Seehausen 2009a; Pauers and McKinnon 2012; Tyers and Turner 2013; reviewed in Maan & Sefc 2013). Hybridization is a common phenomenon in cichlids and cases of introgressive hybridization (Salzburger et al. 2002; Egger et al. 2012), homoploid hybrid speciation (Genner and Turner 2012; Keller et al. 2012) and signatures of past hybridization at the base of adaptive radiations (Seehausen et al. 2003; Joyce et al. 2005, 2011; Schwarzer et al. 2012; Loh et al. 2013) have been documented for cichlids.

Here, we use a series of mate choice experiments to investigate whether hybridization may generate novelty in traits and preferences possibly resulting in assortative mating among hybrid individuals. We explored mate choice in three F1 hybrid crosses between five different cichlid species with different pairwise genetic distances between them (i.e. divergence time) and different extents of differentiation in morphology and body colour. In a three-way mate choice experiment we allowed hybrid and non-hybrid females of each cross to mate with either hybrid or non-hybrid males. We predicted that the extent of behavioural reproductive isolation of F1 hybrids would increase with the genetic distance and the phenotypic dissimilarity between the parental species. This is because: 1. the opportunity for complementary gene action to occur in hybrids – which contributes to the expression of transgressive traits/preferences – increases with time since divergence from the last common ancestor (Stelkens et al. 2009b) and 2. the likelihood to obtain a novel intermediate

combination of traits and preferences should increase with the dissimilarity in traits recruited for mate choice in the parental species.

Material and Methods

Parental species and their F1 hybrids

We used five cichlid species to generate interspecific F1 hybrids: *Astatotilapia calliptera* (Greenwood 1979) a species found in Lake Malawi and surroundings waters and streams, *Protomelas taeniolatus* (Trewavas 1935) from Lake Malawi, *Pundamilia pundamilia* (Seehausen et al. 1998), *Pundamilia nyererei* (Witte-Maas and Witte 1985) and *Paralabidochromis* sp. “rockribensis” from Lake Victoria. All fish derived from laboratory populations bred and maintained at the Eawag Center of Ecology, Evolution and Biogeochemistry in Kastanienbaum, Switzerland. We will refer to these five species and their F1 hybrids as CAL for *Astatotilapia calliptera*, TAE for *Protomelas taeniolatus*, PUN for *P. pundamilia*, NYE for *P. nyererei*, ROC for *Pa. sp. “rockribensis”* and we use the same acronyms for the hybrid crosses, as follows: mother species x father species - generation, e.g CALxTAE F1.

We generated three F1 hybrid crosses between these five species. Two of the hybrid crosses represent intra-radiation crosses; PUNxROC and CALxTAE (both parents are from the same lake radiation, Victoria and Malawi respectively). The third hybrid cross is between a species from Lake Victoria and from Lake Malawi; CALxNYE. The species used to create the hybrid crosses varied in extent in morphology, body colouration and in their genetic relatedness (Fig. 1). We always paired females of a species, where males can be described as blue in general colour appearance (PUN, CAL), with a male of a species where males are red in general appearance (ROC, NYE, TAE). There was a parallel increase in genetic and colour distances between the parental species used to create the three F1 hybrid crosses, such that the parental species used to create the cross PUNxROC had the lowest genetic and colour distance, followed by the parental species of the cross CALxTAE and CALxNYE (Fig. 1.). Parental males of the cross PUNxROC also had the lowest morphological distance, followed by the parental males of the cross CALxNYE and CALxTAE (Fig. 1). Hence, we created crosses between parental species that always derived from a blue and red species, but with increasing distances in genetic relatedness, colour and morphology to test if genetic and/or

phenotypic distance of the parental species has an influence on the expression of assortative mating in hybrids.

All three F1 hybrid cross families (PUNxROC; CALxNYE; CALxTAE) were obtained by keeping between five and twenty females of one species together with one heterospecific male. Each of the F1 hybrid crosses is a full-sib family, i.e. each of the hybrid cross families derived from unique female-male pairings. None of the parental species females or males were used for creating more than one F1 hybrid cross family. We obtained 3, 8 and 2 full-sib F1 hybrid families of PUNxROC, CALxPNM and CALxTAE respectively. Since the species in this study are mouth brooders a female could be identified to have spawned by the presence of eggs in her mouth. Five days after a spawning the female was “stripped” by gently removing the fertilized eggs from her mouth, which were then raised in identical egg tumblers for 15 days. Fry were subsequently moved to small aquaria (20 x 40 x 20 cm) for 15 days, and then transferred into larger aquaria (50 x 40 x 30 cm) at a maximum density of 20 individuals per aquarium. Each family was raised in a separate aquarium. The aquaria were part of a large recirculation system, with water temperature at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and a 12:12 h light:dark cycle. Fish were fed twice a week with a mixture of ground shrimps and peas, enriched with spirulina powder, and on the other days with commercial cichlid pellets.

The three families (e.g. clutches) of PUNxROC were housed together in a single tank from approximately 1 year post-spawning, i.e. after the age of sexual maturity for these species. The eight families of CALxPNM were also housed together in a second single tank, approximately 1 year post-spawning. The two families of the F1 hybrid cross CALxTAE were kept in two separate tanks. Males and females of the parental species derived from single-species stock tanks. All the housing-tanks harboured densities of fish that allowed the fish to grow to full adult size. Males of the parental species and hybrid crosses used in the mate choice experiments were fully-grown adult fish with completely developed nuptial colours.

Description of mate choice setup

Two different mate choice setups were used in this study (see Table S1 for information about which setup was used for each female populations of the parental species and of the F1 hybrid crosses).

Round tank setup:

We ran a full contact, partial partition mate choice design in a large circular tank (10 meter in outer circumference, 0.8 meter in width and 0.8 meter in height) in which six males (two males of each parental species and two F1 hybrid males) held non-overlapping territories of equal size and quality (1 meter in diameter, 0.8 meter in height). Each male was enclosed in a large net cage with mesh sizes that permitted the smaller females to enter and leave, but retained males inside their territories. Each male compartment contained a PVC tube as a refuge to allow territoriality. The male's positions in the tank were randomized. For some replicates (see below), only 3 males instead of 6 were introduced, i.e. one male of each parental species and one hybrid male, due to limiting numbers of males (for details see Table S1).

Rectangular tank setup:

The round tank setup could only be used when all males were larger than the tested females. This was not the case for all replicates (see Table S1) with the CAL and TAE female populations and for one out of ten replicates with the CALxTAE female population in the CAL/TAE/CALxTAE mate choice experiments. Also for a few replicates (3 out of 7) with the ROC and for a few replicates (2 out of 6) with the PUN female populations the round tank setup could not be used. In these cases we used a full contact mate choice design in a large rectangular aquarium (7 meter in length, 0.75 meter in depth, 0.35 meter in height) where males and females could freely interact. Three equally sized PVC tubes were placed in an equal distance to one another and to both ends of the aquarium. The tubes acted as a refuge and to allow territoriality. No difference in assortative mating was observed between the two different setups used for the female mate choice experiments, suggesting the setups do not influence female mate choice. Female populations of PUN and ROC, which were tested in either setup more than once, showed in both setups positive assortative mating for conspecific males (see results below and Table S1).

In both setups, females were introduced into the aquaria for acclimatisation one week prior to the introduction of males. The two experimental tanks (round tank and rectangular tank) contained a single population consisting of females of one of the parental species or of one of the F1 hybrid crosses. Before introduction into the tank, male size (standard length to the nearest 0.1 mm) and male weight (to the nearest 0.1 g) were measured. After each replicate (see below) these measures were taken again and the means were used for further

analyses. An experimental replicate continued until a female had spawned. The tanks were inspected daily for spawnings. As soon as at least one female had spawned, all males were removed from the tank, were photographed (Canon 60D), injected with a 12 mm PIT-tag for individual identification (Biomark, Idaho) and a piece of fin-tissue for DNA was taken. Five days after the spawning event all females that spawned were removed from the tank and a new set of males was introduced. A unique combination of males was established and introduced into each replicate with one exception; in the PUN female mate choice experiment the same male combination was used twice, i.e. in two replicates. Hence, one or several females could spawn in a replicate, which consisted of a newly introduced and unique (with one exception) combination of males from the F1 hybrid cross and the respective parental species. The number of males used in each female population mate choice experiment and the number of unique combinations of males are given in Table S2. The females that spawned were “stripped”, a piece of tissue for DNA was taken and they returned to the stock tank. The eggs were transferred to and raised in identical egg tumblers until day fourteen post-spawning. Subsequently all the fry were sacrificed with MS222 and preserved in 95% ethanol.

Parentage assignment

DNA was extracted from fins of the parents and fry using 150 µl Chelex® 100 (BioRad) in 5% concentration (weight/volume) diluted in ultra-pure water and adding 10 µl buffer solution and 15 µl Proteinase K. Microsatellite amplification was performed using a PCR microsatellite multiplex containing 5µl QuantiTect Multiplex PCR Kit (Qiagen), 3µl of DNase free water, 1µl of primer mix (pPun05, pPun07, pPun17, pPun21, pPun32 (Taylor et al. 2002)) and 1µl of template DNA per reaction. PCR conditions consisted of 35 cycles of 30 seconds at 94°C, 90 seconds at 57°C, 90 seconds at 72°C, followed by a final elongation of 30 min at 72°C and subsequently kept at 4°C. The PCR product was diluted with 50µl DNase free water and 1µl of this product was added to 24µl of de-ionized formamide (SLS, CEQ; Beckman Coulter) and 0.3µl of 400-bp DNA size standard (Beckman Coulter) per reaction. Denaturated fragments were run on an automated DNA sequencer (CEQ 8000; Beckman Coulter). On average a total of 9 ± 4 (s.d.), i.e. $33\% \pm 22$ (s.d.) (Table S1), of the fry per clutch were genotyped at the above-mentioned microsatellite loci, as well as all mothers and potential fathers. We repeated the PCR amplification and genotyping deliberately for all

parents at least once. Genotypes were visualized using the program GeneMarker[®] (SoftGenetics) and scored manually. The assignment of offspring and parents was done using a parental-exclusion program running in Visual Basic for Excel[®] (Windows Inc.) (VITASSIGN V8-5.1; Vandeputte et al. 2006). We allowed for up to two mismatches to assign a sire (Vandeputte et al. 2006; McLean et al. 2008).

Analysis of genetic, morphological and colour distances between parental species and hybrids

We took estimates of genetic distances between parental species from Stelkens et al. (2009a), based on mitochondrial D-loop sequences (Fig. 1).

We quantified estimates of differentiation in morphology and coloration between males from the hybrid crosses and their respective parental species (Fig. 1), i.e. traits that are possibly recruited by females for mate choice. To quantify differentiation in coloration between parental species and F1 hybrid crosses we visually scored features related to colouration of males in breeding dress (Salzburger et al. 2006; Stelkens and Seehausen 2009a; Egger et al. 2012) at 19 homologous points on the body (Fig. S1A; numbers mark the 19 colour points): 1) upper part of mouth, 2) head, 3) third dorsal fin ray, 4) middle dorsal fin ray between point 3 and 5, 5) third last dorsal fin ray, 6) inner rim of dorsal fin, 7) outer rim of dorsal fin, 8) upper caudal fin, 9) lower caudal fin, 10) outer rim of caudal fin, 11) outer rim of anal fin, 12) outer part of anal fin, 13) inner part of anal fin, 14) body colour above anal fin, 15) rear end of back along the lateral line, 16) body colour behind pectoral fin, 17) cheek, 18) presence or absence of egg spots and their distribution on the anal fin (in one line or scattered) and 19) presence or absence of vertical bars or horizontal stripes (Salzburger et al. 2006). At every point, colour was scored as either being translucent, white, grey, black, blue, green, yellow, orange or red (Stelkens and Seehausen 2009a). The 19 colour scores were collected of between 6 to 15 males per species or F1 hybrid cross. The qualitative score was converted into a distance metric by using the “simple matching coefficient” (SMC) (Digby and Kempton 1987; Stelkens and Seehausen 2009a), which divides the number of matching characters by the total number of characters in individual pairwise comparisons, and results in a estimate of overall colour difference between groups. To account for within-group variation the colour distances were corrected by subtracting the average intra-population distance from

the average inter-population distance. Factorial correspondence analysis for categorical data was used to visually depict the two main axes of variation in colouration.

To measure differentiation in morphology between males of the parental species and the F1 hybrid crosses (Nolte et al. 2005; Stelkens et al. 2009; Larsen et al. 2010) we used geometric morphometrics in tpsDig2 v.9.1 software (Rohlf 2006) based on 16 homologous landmarks and one homologous semi-landmark (Fig. S1B). “Traditional” homologous landmarks and semi-landmark can be used equally after Procrustes superimposition (Crispo and Chapman 2011; Gunz and Mitteroecker 2013) and hence the 16 homologous landmarks and the one semi-landmark were combined and treated equally in subsequent analysis (see below). All photographs were size-calibrated using a scale on the photo. Using MorphoJ v.1.05a (Klingenberg 2011) the landmarks of all specimens were geometrically scaled to a unit centroid size (= CS) and superimposed with Procrustes superimposition, which controls for size but retains variation in shape (Rohlf and Slice 1990). Thereafter CS was used as the measure of size for each specimen (Zelditch et al 2004). We combined the males of each F1 hybrid cross and their respective parental species into three separate datasets. These three datasets were separately Procrustes superimposed and then the Procrustes coordinates were regressed against centroid size to remove potential allometric size effects. Variance-covariance matrices were calculated from the residuals and used in a principal component analysis (PCA) with each one of the three F1 hybrid crosses and the respective parental species. The first two principal component axes were plotted to visually depict the two main axes of variation in morphology. Procrustes distances were calculated between F1 hybrid crosses and the respective parental species and between the parental species. This method calculates the Procrustes distance of each individual to the multivariate mean of each group (species or F1 hybrid cross) and results in a geometric estimate of overall shape differences between groups (Clabaut et al. 2007). The following 17 landmarks were used to quantify differentiation in morphology of males (Fig. S1B; numbers correspond to the numbers depicting landmarks on figure 1): 1) anterior tip of maxilla, 2) junction of head and dorsal scales, 3) anterior insertion point of dorsal fin, 4) posterior insertion point of dorsal fin, 5) dorsal junction of caudal fin and caudal peduncle, 6) ventral junction of caudal fin and caudal peduncle, 7) posterior insertion point of anal fin, 8) anterior insertion of anal fin, 9) anterior/dorsal insertion of pelvic fin, 10) anterior/ ventral insertion of pectoral fin, 11) dorsal insertion of pectoral fin, 12) posterior extreme of operculum (mostly the opercular blotch), 13) ventral-posterior extreme of preoperculum, 14) center of the eye, 15) anterior reach of the

eye, 16) anterior reach of the premaxillary groove, and 17) a semi-landmark to depict the curvature of the head; a line is drawn between the landmarks 1 and 2 and at the middle of this line a second line is drawn 90° degrees to the first. The landmark is then placed where the second line crosses the outline of the head (Crispo and Chapman 2011).

Data analysis

Statistical analyses were done using MorphoJ v.1.05a (Klingenberg 2011), the statistical software R v. 2.13.0 (R Development Core Team 2011) and PAST v. 2.03 (Hammer et al. 2001). All statistical tests are two-tailed.

We used a Binomial test to test whether females of each parental species and hybrid cross significantly preferred to mate with conspecific males over either heterospecific male. Differences in the weight and size between males of the F1 hybrid crosses and the parental species were tested for each mate choice experiment with a Kruskal-Wallis test, and if significant, we additionally performed a pairwise Mann Whitney U-test with Bonferroni correction in order to correct for the number of comparisons. To quantify the degree of assortative mating between a hybrid cross and either parental species, a “hybrid assortative mating index” (HAMI) was calculated: $HAMI = S_{\text{hybrid cross}} / S_{\text{total}}$, where $S_{\text{hybrid cross}}$ is the number of times a hybrid male was a sire and S_{total} is total number of sires in a pair-wise comparison between the hybrid cross and either parental species. The HAMI can range from 0 to 1, with a value of zero indicating a preference for males of a parental species, a value of one indicating a preference for males of the hybrid cross and a value of 0.5 indicating no preference. The relationship between the degree of assortative mating in hybrids and the morphological or colour distances between hybrid crosses and their parental species was calculated with an ordinary least squares regression.

Results

Females of all parental species mated assortatively, mating (with one exception) only with conspecifics and not with heterospecifics nor with hybrids (Binomial test all: $P < 0.03$; Fig. 2A, C, D, F, I). CAL females showed the same pattern of positive assortative mating, but one out of 6 females spawned with a CALxTAE hybrid male (Binomial test: $P = 0.220$, Fig.

2G). Females of two of the hybrid crosses showed asymmetric assortative mating. Females of PUNxROC (Fig. 2B) and CALxNYE (Fig. 2E) mated with males of one of the parental species and with hybrid males (Binomial test both: $P>0.15$), but never with males of the other parental species (Binomial test both: $P<0.03$). Females of the third hybrid cross, CALxRPO (Fig. 2H), showed symmetrical assortative mating, mating significantly more often with hybrid males than with males of either parental species (Binomial test both: $P<0.03$).

Principal component analysis on morphology and factorial correspondence analysis on colour revealed that the hybrid crosses and the respective parental species overlapped to various extents in morphology and colouration. The first two principal components on morphology explained between 39.1% and 73.6% cumulatively of the morphological variation in each of the three data sets, which each contained one of the hybrid crosses and both parental species (Fig. 3A, B, C). The first two factorial components of colour explained between 71.4% and 94 % cumulatively of colour variation for each of the three data sets (Fig. 3D, E, F). Weakest morphological differentiation was found between the males of the Lake Victoria species ROC, PUN and their hybrids PUNxROC. These showed strong overlap in morphology along both morphological axes and the morphological distance between the mean of the parental species (Procrustes distance = 0.03) and between those of the hybrid cross and the parental species were small and similar (both Procrustes distances = 0.02, Fig. 1A,B,C). In factorial correspondence analysis based on colour there was a strong overlap between males of PUNxROC and PUN, which were the two male groups the PUNxROC hybrid females exclusively mated with (Fig. 3D). ROC males were completely non-overlapping with the former two and PUNxROC hybrid females never mated with these. Also, in colour distances the PUNxROC and PUN males showed only small dissimilarity in colour (single matching coefficient = 0.1) when compared to the dissimilarity in colour found between PUNxROC and ROC (0.57) and between ROC and PUN (0.80, Fig. 1A,B,C).

Slightly stronger morphological differentiation was found between males of the two parental species CAL and NYE (0.04). CALxNYE hybrid males overlapped more and showed lower morphological distance against males of NYE (0.02) than against males of CAL (0.04) (Fig. 3B). Similarly, only males of CALxNYE and NYE overlapped in colour (Fig. 3E) and these were again the two male groups with which the CALxNYE hybrid females exclusively mated (Fig. 3E). CAL males did not overlap with the former two male groups and never achieved a mating with CALxNYE hybrid females. Based on colour distances males of CALxNYE and NYE had much smaller dissimilarities in colour (0.38)

when compared to those found between CALxNYE and CAL (0.98) and between CAL and NYE (1.37).

The largest morphological distance, and no overlap in morphology, was found between males of CALxTAE, CAL and TAE (Fig. 3C). CALxTAE males were distinctly separated from both parental species along the first major morphological axis and intermediate to both parents along the second major axes. The morphological distances between CALxTAE males and males of either parental species (both 0.07) were even slightly larger than those between CAL and TAE males (0.05). Likewise, CALxTAE, TAE and CAL males showed no overlap in colour (Fig. 3F) and hybrid females of CALxTAE mated much more often with hybrid males than with males of either parental species. CALxTAE males were separated from both parental species along the second major colour axis and were intermediate to both parents along the first axes. The dissimilarities in colour between CALxTAE and CAL males were smaller (0.62) than those between CALxTAE and TAE males (1.04) and between the parental species CAL and TAE (1.05).

A significant positive relationship was found between the degree of hybrid assortative mating and the colour distance between the hybrid crosses and their respective parental species ($r(6)=0.81$, $P=0.050$), but not for the morphological distance between hybrids and parents ($r(6)=0.440$, $P=0.382$).

The average weight and size of parental species males and hybrid males used in the mate choice experiments are given in Table S3 and significant differences in male weight and size in Table S4. Except for the mate choice experiments with the female populations PUN, ROC, PUNxROC and CAL (tested with males of CALxTAE, CAL and TAE) the average male weight of the parental species and of the hybrid crosses differed in at least one pairwise comparison (see Table S4 for direction of differences). Also, except for the mate choice experiment with the ROC female populations the average size of the parental species and the hybrid crosses differed (Table S4).

Discussion

Non-allopatric homoploid hybrid speciation can in principle occur when reciprocal behavioural reproductive isolation exists between the parental species and the hybrid population. Here, we investigated experimentally whether assortative mating can arise instantaneously as a result of the recombinant origin of novel mating traits. We crossed cichlid fish with varying pairwise genetic, morphological and colour distances and studied

behavioural reproductive isolation between hybrids and parents in the lab. We have shown that hybridization can lead to the expression of new combination of traits and preferences that behaviourally isolate F1 hybrids completely from one or nearly complete from both parental species. Specifically, the extent of phenotypic distinctiveness in colour of hybrids and both parental species was associated with the extent of behavioural reproductive isolation between hybrids and parental species. This suggests that hybrids could become reproductively isolated from their parental species, which might facilitate the initial establishment of hybrid populations in the absence of any spatial or temporal isolation. Females of all parental species showed strong and symmetrical behavioural isolation, from hybrids and heterospecifics. Females of the hybrid cross CALxTAE also showed symmetrical isolation, mating much more often with hybrid males than with males of either parental species. Females of the other two hybrid crosses, PUNxROC and CALxNYE, showed asymmetrical isolation, mating not only with hybrid males but also with males of the parental species that most resembled the hybrid colour phenotype.

The asymmetrical and symmetrical assortative mating found among the three hybrid crosses corresponds to the observed phenotypic dissimilarity in colour between hybrid males and those of the parental species. In two hybrid crosses the females mated only with males of the parental species that overlapped in multivariate colour-space with the hybrid males. In the third hybrid cross (CALxTAE) the males showed extreme transgressive features both in colour and morphology (Fig. 3C, F). The hybrids lied outside the range of both parental species in multivariate colour- and morphological space, and the hybrid females mated significantly more often with the hybrid males than with males of either parental species. Hence, in the hybrid cross CALxTAE the traits and most likely the preferences too are strongly transgressive resulting in preferences that favour novel trait combinations of hybrids over those of the parental species, which leads to strong assortative mating among hybrid individuals. Neither genetic distance between parental species (Stelkens and Seehausen 2009b), nor the dissimilarity of phenotypic traits between parental species predicts the observed patterns of asymmetrical and symmetrical assortative mating in our hybrids. We acknowledge that these findings should be treated with caution due to the lack of replication. Yet, the observed phenotypic dissimilarity in colour, but not morphology, between hybrid males and those of the parental species significantly predicts the degree of assortative mating in hybrids, which is consistent with the well-established importance of nuptial coloration in cichlid mate choice (Maan & Sefc 2013). We show that hybridization can produce novelty in morpho- and colour-space and in preference leading to asymmetrical and symmetrical

assortative mating in hybrids based on the phenotypic dissimilarity in colour between hybrids and their parental species. The observed hybrid trait variation resulting in different patterns of assortative mating in hybrids will affect the direction and strength of gene flow between parental species and hybrids. This could possibly result in hybrid speciation or adaptive introgression (Abbott et al. 2013).

Cichlids are known for their diversity in colour (Kocher 2004; Seehausen 2006) and male colouration has been shown to influence female mate choice both at the intra- and interspecific level (Seehausen and van Alphen 1998; Knight and Turner 2004; Maan et al. 2004; Pauers et al. 2004, 2010; Egger et al. 2008; Stelkens and Seehausen 2009a). It has been suggested to play a role in the evolution of reproductive isolation (Dominey 1984; Seehausen and van Alphen 1999; Seehausen et al. 1999; Wagner et al. 2012). Specifically, several studies on populations of different cichlid species from Lake Malawi and Tanganyika and on different species from Lake Mweru have shown that colour pattern dissimilarity predicts the strength of assortative mating observed between the populations or species (Salzburger et al. 2006; Stelkens and Seehausen 2009a; Egger et al. 2010; Tyers and Turner 2013). Moreover cichlid fish from Lake Malawi and Victoria, the two lakes where our parental species originate from, have very diverse visual systems (Carleton et al. 2005; Jordan et al. 2006; Hofmann et al. 2009). In Lake Victoria cichlids the variation in and sensitivity of the visual sensory repertoires has been shown to correlate with male colouration (Miyagi et al. 2012) and it has been suggested that visual sensory drive might play an important role in the origin of reproductive isolation in Lake Victoria cichlids (Maan et al. 2006; Seehausen et al. 2008). Recent work by Sandkam et al. (2012) has shown that the Amazon molly (*Poecilia formosa*), a “frozen” F1 hybrid species that reproduces asexually through gynogenesis (Vrijenhoek 1979), expresses novel visual repertoires inherited from both parental species. These novel visual repertoires influence the behavioural response to colour stimuli in a manner similar or intermediate to that of both parental species (Sandkam et al. 2012). Such novel hybrid-sensory repertoire results when two species with differently tuned sensory systems form an F1 hybrid, which will have an expanded sensory repertoire that is the sum of the variance between both parental species (Sandkam et al. 2012). Similar expansions of the sensory repertoire might be important in our experimental hybrids for which differences in the sensory repertoire between species is known (Hofmann et al. 2009). Future work on visual systems of parental species and hybrids of cichlids coupled with behavioural tests using colour stimuli may elucidate whether the hybrid-sensory expansion hypothesis could also apply to this group.

Only one other study system in animals, to our knowledge, has shown similarly strong symmetrical and almost complete behavioural isolation between hybrids and both parental species based on colour traits (Mavárez et al 2006; Melo et al. 2009). In *Heliconius* butterflies Melo et al. (2009) showed that a novel wing colour pattern generated in the lab in backcross hybrids, phenotypically resembles the colour pattern of a naturally occurring hybrid species and resulted in positive assortative mating among experimental hybrids. Both experimentally reconstructed backcross hybrids and a naturally occurring hybrid species preferred the colour pattern of the backcross hybrid over that of either parental species, and both parental species showed positive assortative preferences in two-way mate choice experiments. Based on the experiments by Melo et al. (2009) and previous studies in *Heliconius* butterflies (Crane 1955; Naisbit et al. 2001; Konforst et al. 2006) these authors concluded that colour is a critical cue for genetically inherited mate preference. The most likely explanation for the assortative mating observed in our hybrid crosses is that they are also genetically inherited. We raised the hybrids such that imprinting of fry on the foster mother was impossible, and horizontal imprinting on siblings does not affect adult mate choice in cichlids (Verzijden et al. 2008). Two other studies using two-way mate choice experiments show that asymmetrical and symmetrical patterns of assortative hybrid mate choice can be underlain by traits other than coloration and relate to the dissimilarity of these traits between hybrids and parental species. Symmetrical assortative mating has been shown in reciprocal F1 hybrid crosses of *Anastrepha* fruit flies, where F1 hybrid females of both cross types preferred to mate with hybrid males of both cross types rather than with either of the parental males (Segura et al. 2011). The hybrid males of both cross types produced novel and very similar blends of pheromones (Cáceres et al. 2009) and the authors concluded that assortative mating of hybrid females was based on these novel pheromone traits (Segura et al. 2011). Asymmetrical assortative mating has been observed in reciprocal F1 hybrid crosses of *Hyla* tree frogs, where the mating calls of F1 hybrid males of both cross types were indistinguishable and resembled partially the calls of one of the parental species, but were distinctive to that of the other parental species (Doherty and Gerhardt 1983). F1 hybrid females of both cross types showed no preference for the calls of both reciprocal F1 hybrid males to the calls of one of the parental species, which had similar calls to that of the F1 hybrid, but they preferably chose both of the reciprocal F1 hybrids to the other parental species (Doherty and Gerhardt 1983). Thus, in each of these three studies the direction of assortative mating was found to depend on the dissimilarity of the mate choice trait between hybrids and parental species.

Our mate choice experiments suggest that interspecific hybridization in Haplochromine cichlids could lead to hybrids showing positive assortative mating and together with the observed positive assortative mating among the parental species results in behavioural reproductive isolation between hybrids and both parental species. Assuming multiple hybridization events between two parental species (Rosenthal 2013) resulting in repeated production of hybrid individuals, the chance of finding hybrid mates increases and a multiple generation hybrid population could establish from several independent interspecific matings (Bateson 2002). Yet, a major additional obstacle of homoploid hybrid speciation is for the hybrid population to escape competition from its parental species (e.g. Rieseberg 1997; Buerkle et al. 2000; Coyne and Orr 2004; Mallet 2007). When behavioural reproductive isolation between the hybrids and the parental species is present, ecological niche partitioning should follow in order to allow non-allopatric homoploid hybrid speciation. We suggest that incorporating mechanisms causing positive assortative mating in hybrids into current models of homoploid hybrid speciation will considerably increase the range of conditions under which interspecific hybridization can contribute to the evolution of new species. Such speciation may be particularly important in young but extremely species-rich radiations such as the cichlid fish of Lake Victoria.

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Figure 1. Representative photos of males from the hybrid crosses and their parental species and the genetic, morphological and colour distances between hybrids crosses and their parental species. The parental species that can be described as blue in general appearance are at the left side and those that are red in general appearance are to the right. In the middle column are the F1 hybrids that derived from crossing the two parental species. From top left to bottom right: A) *Pundamilia pundamilia* (PUN), B) F1 hybrid PUNxROC, C) *Paralabidochromis* sp. “rockkribensis” (ROC), D) *Astatotilapia calliptera* (CAL), E) F1 hybrid CALxNYE, F) *P. nyererei* (NYE), G) *A. calliptera* (CAL), H) F1 hybrid CALxTAE and I) *Protomelas taeniolatus* (TAE). Given are the genetic distance (GD) between parental species (straight line connecting parental species) and morphological (Procrustes distance = PD) and colour (single matching coefficient) distances (CD) between parental species (dashed line) and between each F1 hybrid cross and the respective parental species (dashed line with arrows).

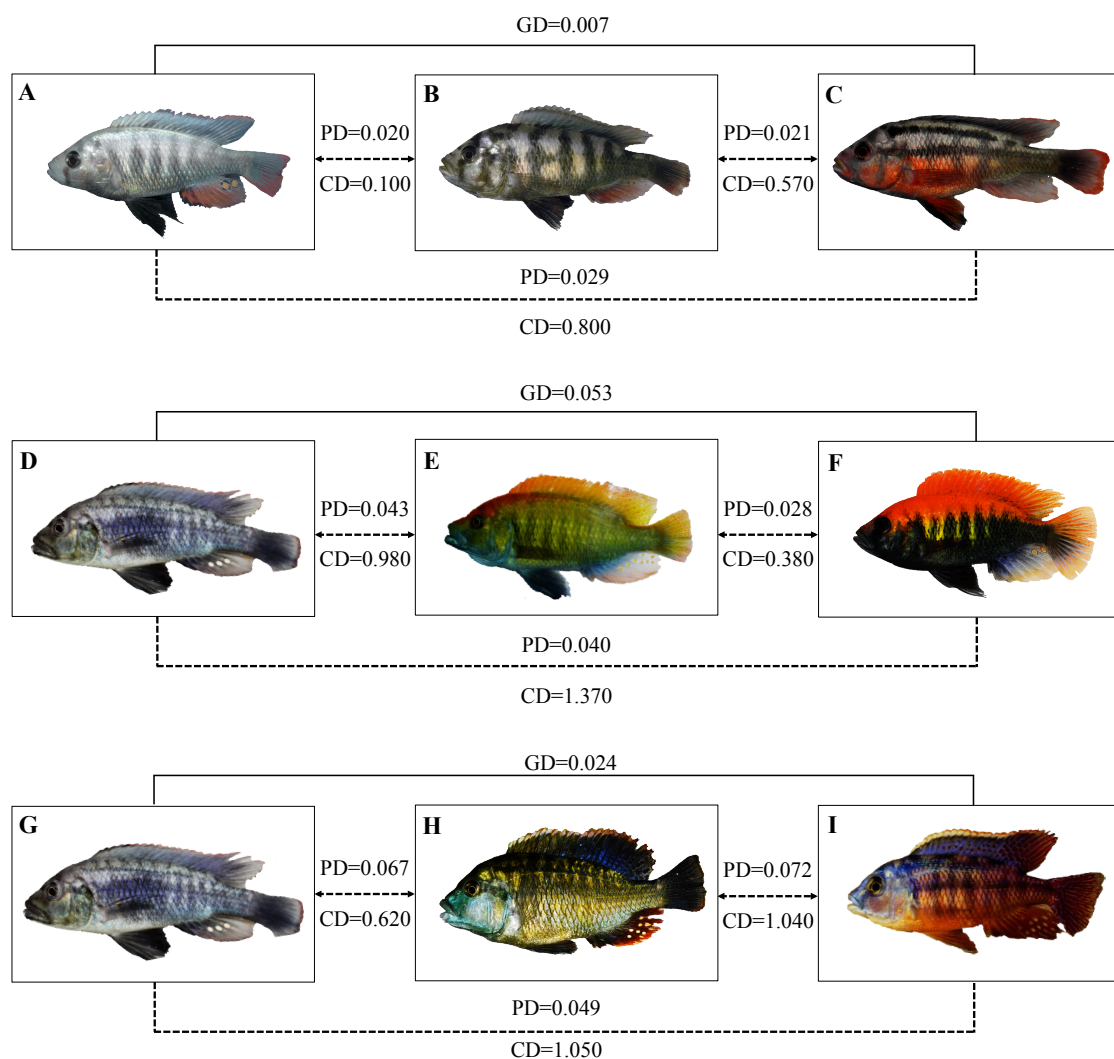


Figure 2. Number of spawning events where a female of one of the parental species or of one of the F1 hybrid crosses mated with a parental or F1 hybrid male. Females of all parental species mated assortatively, mating (with one exception) only with conspecifics and neither with heterospecific nor with hybrids (Binomial test all: $P<0.03$) (A, C, D, F, I). CAL females showed the same pattern of positive assortative mating, but one out of 6 females spawned with a CALxTAE male (Binomial test: $P=0.220$) (G). Females of two of the hybrid crosses showed asymmetric assortative mating, mating with males of one of the parental species and with hybrid males (Binomial test both: $P>0.15$) but never with males of the other parental species (Binomial test both: $P<0.03$) (B & E). Females of the third hybrid cross showed symmetrical assortative mating, mating much more often with F1 hybrid males than with males of either parental species (Binomial test both: $P<0.03$) (H).

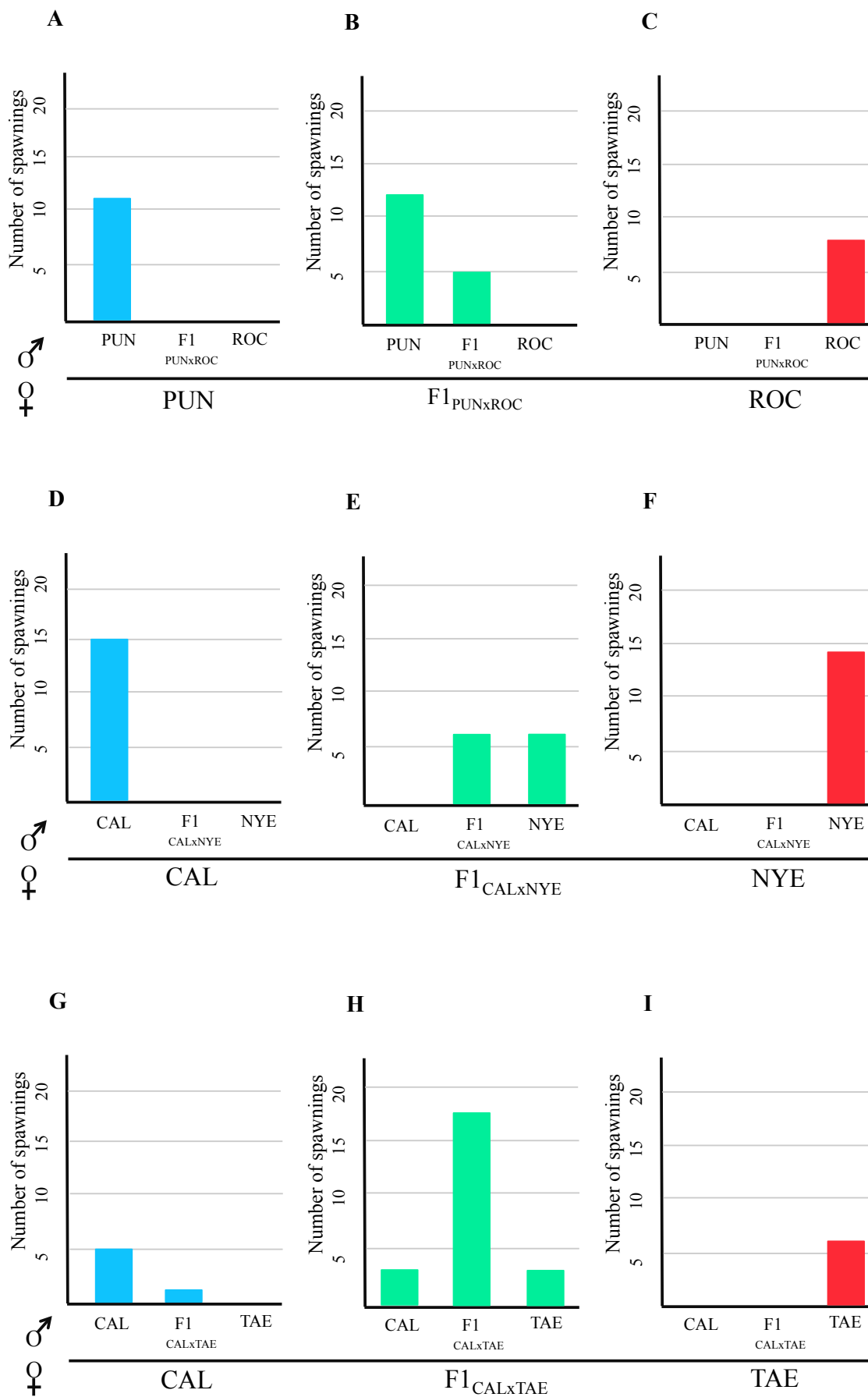


Figure 3. Plots from Principal Component Analysis based on 17 homologous landmarks (A-C) and from Factorial Correspondence analysis on 19 colour metrics (D-F) for the three mate choice experiments. A colour code under the plots depicts the two parental species (red and blue) and the F1 hybrid cross (green) for each of the mate choice experiments. Percentage of variation in morphology and colour explained by the first two principal component and factorial correspondent axes, respectively, is given within the plots. Circles represent the 95% confidence interval ellipses for each parental species and hybrid cross.

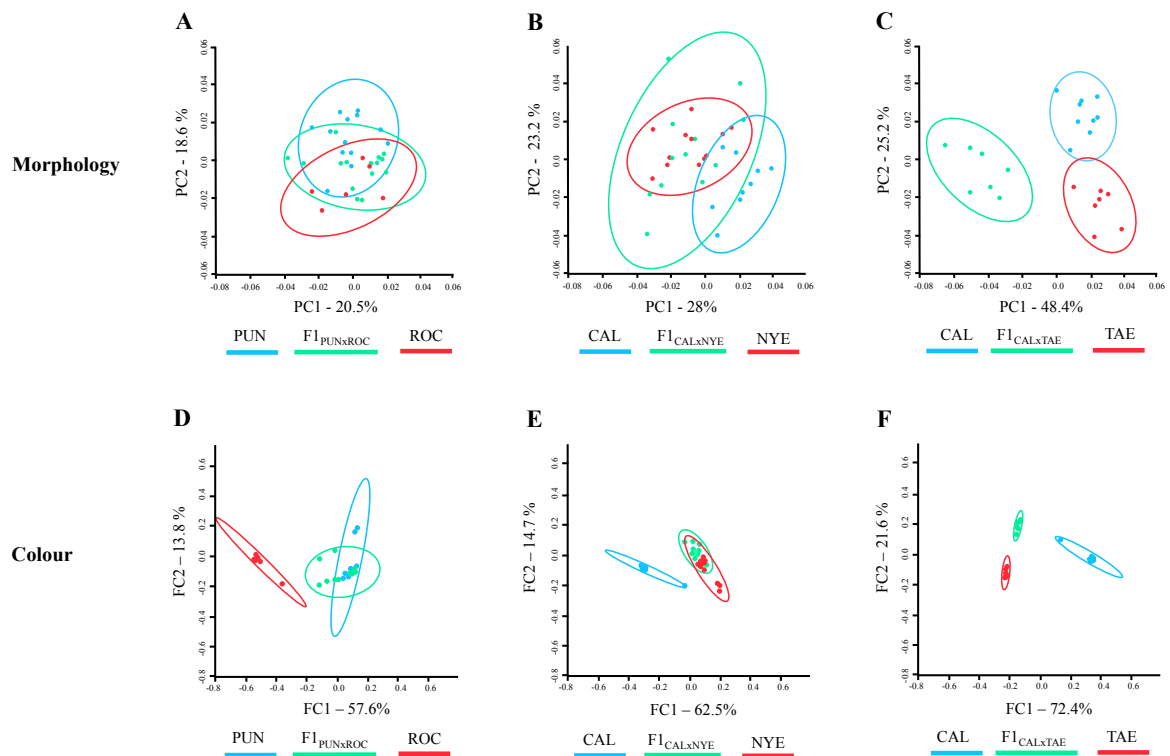


Figure 4. Testing colour (circle) and morphological (triangle) distance as predictor of the degree of assortative mating among hybrids (represented by the Hybrid Assortative Mating Index (HAMI)). The degree of hybrid assortative mating was not predicted by the dissimilarity in morphology between hybrids and parents ($r(6)=0.440$, $P=0.382$), but significantly increased with the colour distance between the hybrid crosses and their parental species (linear regression line shown; $r(6)=0.81$, $P=0.050$). Colour distance (simple matching coefficients) were calculated from male coloration scores at 19 points along the body and morphological distance (Procrustes distance) were calculated from 17 homologous landmarks (See figure S1A-B and material and methods for a detailed description).

