



Differences among reciprocal hybrids of *Labeotropheus*

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Abstract Current evidence suggests that hybridization played a crucial role in the early evolution and diversification of the species flocks of cichlid fishes in the African Great Lakes. Nonetheless, evidence for hybridization in the extant cichlid fauna is scant, suggesting that hybridization is rare in the modern era, perhaps enforced by natural or sexual selection acting against F1 hybrids. Additionally, most experimental

studies of hybridization perform a hybrid cross in one direction, ignoring the reciprocal hybrid. In this study, we perform reciprocal crosses between sympatric congeners from Lake Malaŵi, *Labeotropheus fuelleborni* and *L. trewavasae*, in order to compare the body shape and coloration of males of both of these hybrids, as well as to examine how these hybrids fare during both inter- and intrasexual interactions. We found that *L. trewavasae*-sired hybrid males are intermediate to the parental species both morphologically and chromatically, while the reciprocal *L. fuelleborni*-sired hybrids are likely transgressive hybrids. Males of these transgressive hybrids also fare poorly during our mate choice experiments. While female *L. trewavasae* reject them as possible mates, male *L. trewavasae* do not make a distinction between them and conspecific males. Selection against transgressive F1 hybrids as observed in our crossing experiments may help explain why contemporary hybridization in Lake Malaŵi cichlids appears to be rare.

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Introduction

Until recently, hybridization was not viewed as a significant source of adaptive variation in animals. This is partially due to the long-standing view that

hybridization acts as a destructive force, leading to maladapted “dead ends,” but also due to the fact that there were very few convincing examples of adaptive introgression in animals (Mayr, 1963; Stelkens et al., 2009; Hedrick, 2013). Some of the best recent evidence for this phenomenon in vertebrates comes from the radiations of cichlid fishes in the African Great Lakes (e.g., Gante et al., 2016; Koblmüller et al., 2017; Meyer et al., 2017). These fishes represent the largest and most diverse radiations of vertebrate animals in the world, and have been rich study systems for evolutionary biologists, providing insights into speciation, adaptive radiation, and the role sexual selection can play in both of these processes (Barlow, 2000; Kornfield & Smith, 2000). Experiments involving controlled hybridization between species have provided evidence of morphological (e.g., McElroy & Kornfield, 1993; Albertson & Kocher, 2001; Stelkens et al., 2009), chromatic (e.g., Brzozowski et al., 2014; Albertson et al., 2014; Selz et al., 2014a), behavioral (Feller et al., 2020), and ecological (Selz & Seehausen, 2019) novelty. A study of hybrid novelty using species thought to be ancestral to the extant radiations in Lakes Tanganyika, Malaŵi, and Victoria found that inbred F2 individuals had a significantly increased range of morphological variation compared to that of both the parental and F1 generations (Selz et al., 2014b). This finding indicates that hybridization, far from constraining evolution, could be an important source of phenotypic novelty, especially under the ecological and environmental conditions in which these radiations occurred (e.g., Danley et al., 2012; Ivory et al., 2016; Santos-Santos et al., 2021). For example, after finding geological evidence of significant lake level fluctuations throughout the history of the Lake Malaŵi basin, Ivory et al. (2016) hypothesize that hybridization during times when the lake was shallow (“green lake” conditions) was an important source of phenotypic novelty that helped the cichlid flock radiate into the larger array of habitats and trophic niches available when lake levels subsequently rose (“blue lake” conditions), a scenario that has significant phylogenetic support (Genner & Turner, 2012, 2015).

While the above-listed studies powerfully demonstrate how hybridization could act to produce phenotypic variation during the early stages of adaptive radiation in the cichlids of the African Great Lakes, they do not directly address the fate of hybrids in the

extant cichlid communities of these lakes. Pauers et al. (2018) found that F1 hybrids of *Labeotropheus fuelleborni* and *L. trewavasae* were less efficient feeders than either of the parental species, leading to the expectation that such hybrids would not be successful in the wild. Additionally, many of these species are notoriously assortative with regards to mate choice, except under anthropogenically altered conditions (Seehausen et al., 1997; Egger et al., 2012), and evidence of hybridization in the wild is exceedingly rare (Stauffer et al., 1996; Smith et al., 2003). Furthermore, F1 hybrids are uncommon in the extant lacustrine radiations, and are unlikely to have much reproductive success (Crapon de Caprona, 1986; Ribbink, 1990).

The fate of hybrid cichlids during sexual selection has not often been studied. In the few available studies examining patterns of mate choice involving hybrids (e.g., Svensson et al., 2011, 2017; Selz et al., 2014a), only female choice, examined post hoc via molecular genetic assessment of parentage, was evaluated; assessments of female mate choice behavior, via receptive responses or numbers of visits (e.g., Pauers et al., 2010; Pauers & Grudnowski, 2022) have rarely been conducted (e.g., Haesler & Seehausen, 2005). While more involved and time-consuming to evaluate, direct observations of courtship and receptive behavior are important due to their potential additional insights on the degree of behavioral resistance to introgression (e.g., Barlow, 2000, 2002; Coyne & Orr, 2004). Additionally, studies of intrasexual aggression, comparing the responses of territorial males to conspecific and hybrid males, have not been conducted. Given the importance of territory acquisition to male fitness for many of these species (Ribbink, 1990; Barlow, 1993; Rometsch et al., 2021), exploring this would provide valuable insights into how hybrid males would fare in a natural population.

Also missing from current studies of hybridization in cichlids are comparisons of F1 offspring produced via reciprocal crosses between the parent species. In the relatively few studies in which reciprocal crosses were made, the resulting hybrid progeny were pooled in the subsequent analyses, thus obscuring their parentage (e.g., Husemann et al., 2017). Given the roles sexual conflict and chromosomal sex determination play in determining the phenotype (e.g., Roberts et al., 2009), it seems especially important to compare hybrid offspring from reciprocal crosses.

The genus *Labeotropheus* Ahl 1927 is a small genus of haplochromine cichlids endemic to Lake Malaŵi, the species of which have been crucial in studies of the role of hybridization in the evolution of phenotypic novelty this species flock (e.g., Albertson & Kocher, 2001; Albertson et al., 2014; Concannon & Albertson, 2015). Hybrids of *Labeotropheus fuelleborni*, in particular, have been used to elucidate both evolutionary development and adaptive introgression in numerous phenotypic traits. In the above-mentioned study of feeding performance in hybrid *Labeotropheus*, Pauers et al. (2018) produced experimental F1 hybrids of *Labeotropheus* by crossing male *L. trewavasae* with female *L. fuelleborni*, but did not produce reciprocal hybrids. These hybrids, although they had a distinct body shape, were most morphologically similar to the maternal species, *L. fuelleborni*, and the color pattern of the male hybrids was most similar to that of male *L. fuelleborni* (Pauers et al., 2018; Pauers, pers. obsv.). We thus wanted to conduct a comparative study of body shape and male coloration in the parental species and their reciprocal hybrids; we were especially curious whether such *L. fuelleborni*-sired hybrids would also be most phenotypically similar to their maternal species, or if they would display a different, perhaps transgressive, morphological and chromatic phenotype. Transgressive hybrids, those that have a phenotype outside of the combined range of their parental species, are of particular interest to evolutionary biologists, as they represent a potential source of adaptive novelty (Holzman & Hulsey, 2017; Husemann et al., 2017). Additionally, we were interested in how these hybrids would fare in behavioral contexts, specifically intra- and inter-sexual selection.

Methods

Experimental animals

Wild caught adult specimens of *Labeotropheus fuelleborni* and *L. trewavasae* were obtained from a reputable Malaŵi-based exporter of cichlid fishes (Stuart M. Grant, Ltd.); both species were captured at the Chidunga Rocks in southwestern Lake Malaŵi. Twenty individuals of each species were obtained, ten of each sex. These adults were segregated by species and sex into four separate 160 l aquaria. Fishes were

fed to satiation daily with a mixture of spirulina flake food (e.g., Formula Two; Ocean Nutrition, Newark, CA, USA), cichlid pellets (e.g., Hikari Cichlid Excel; Kyorin Co., Ltd., Hyogo, Japan), and a variety of frozen foods (e.g., bloodworms, brine shrimp; various manufacturers). Water temperature was maintained at 25°C, and water quality was maintained using standard box filters and biweekly 50% water changes.

Intraspecific F1 individuals of both species were produced by moving randomly selected groups of adults (1–3 males with 2–5 females) into separate 160 l aquaria, which were maintained as described above. The breeding groups of fishes were fed to satiation daily with the assortment of foods provided to the fishes in the stock tanks. Mouthbrooding females were isolated in separate aquaria (80 l or 120 l) until their fry were released at about 21 days post-fertilization. Using this same breeding and rearing protocol, two different hybrids were produced via reciprocal crosses between *L. fuelleborni* and *L. trewavasae* by pairing 1–3 males of one species with 2–5 females of the other.

All F1 individuals, both the two intraspecific treatment groups (e.g., *L. fuelleborni* and *L. trewavasae*) and the interspecific hybrids (*L. fuelleborni*-sired hybrids and *L. trewavasae*-sired hybrids), were raised to adulthood under the same aquarium conditions under which the parental generation was maintained. Additionally, since all of these F1 individuals were fed the same diet, we were able to control for possible phenotypic plasticity in trophic anatomy and morphology (e.g., van Snick Grey & Stauffer, 2004). Once they were full grown and sexually mature, these F1 fishes were later used in the experiments described below.

For the morphometric and spectrophotometric analyses described below, we only used sexually mature males of each of the four treatment groups (*L. fuelleborni*, *L. trewavasae*, *L. fuelleborni*-sired hybrids, and *L. trewavasae*-sired hybrids). There is great sexual dimorphism in color pattern in these species, and given the role male color pattern plays in sexual selection in the *Labeotropheus*, we limited our analyses to the males (Pauers et al., 2004, 2008; Pauers & McKinnon, 2012). Additionally, the coloration of male cichlids from Lake Malaŵi tends to be more modular (i.e., consisting of several distinct biochemical, structural, or anatomical components, or modules; Klingenberg, 2009) and thus more

evolutionarily labile than that of female coloration, which is considered to be integrated (i.e., comprised of interconnected, covarying components; Klingenberg, 2009; Brzozowski et al., 2014; Albertson et al., 2014), and would be more likely to show variation from the parental species than female coloration. Since we limited the investigations of coloration to sexually mature males, we similarly limited our investigations of body shape to these same males, so that we would have congruent and complimentary data for each specimen in the analyses.

Many of our female *L. fuelleborni* had become reproductively senescent, and our attempts to obtain new *L. fuelleborni* stock, especially females, were hampered by a lack of *L. fuelleborni* from Chidunga Rocks in the U.S. aquarium trade, as well as a near cessation of imports of wild fishes from Lake Malawi due to shipping and supply chain issues caused by the COVID-19 pandemic. This limited our behavioral experiments to those involving *L. trewavasae* males and males resulting from the cross of a *L. fuelleborni* male and a *L. trewavasae* female.

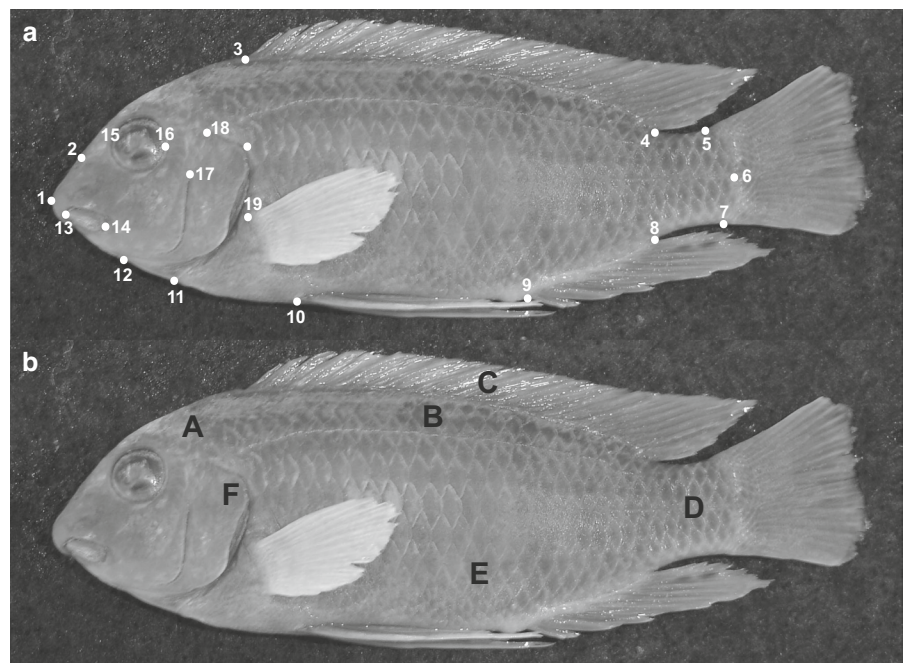
Geometric morphometrics

Sexually mature males of each of the four treatment groups were photographed with a digital camera

under fluorescent room lighting. The fishes were lightly anesthetized with MS-222 (approximately 25 mg/l), then removed from the MS-222 bath and placed on a black cloth such that the fish's left flank was facing up towards the camera; additionally, the fish's dorsal, anal, and pelvic fins were pinned in their erect positions. After the photographs were taken, the fish were transferred to a warm (28°C), aerated aquarium in which they were allowed to recover from the MS-222 before being returned to their stock aquaria. The photographs were imported into ImageJ, and 19 landmarks, following Pauers and McMillan (2015) and Pauers et al. (2018), were digitized from each specimen; see Fig. 1a for a picture and description of the landmarks.

The morphometric analyses followed Pauers et al. (2018). Briefly, the (x, y) coordinates of each landmark for each specimen were subjected to a Generalized Procrustes Analysis in the program Coordgen8 (Sheets 2001) to reduce the effects of size, orientation, and position in the comparison of shape. Next, a multiple regression of shape on geometric centroid size was performed using the Standard6 software which eliminates shape differences due to size. These data were then subjected to a thin-plate spline procedure to generate geometric descriptors of shape variation called relative warps (RW); this procedure

Fig. 1 Landmarks for the geometric morphometric analyses, and locations from which spectral data were obtained. **a** Morphometric landmarks; the precise locations of these points are described in Pauers et al. (2018). **b** Spectral data locations: A: forehead; B: dorsum; C: dorsal fin; D: caudal peduncle; E: ventrum; F: operculum



was performed using the program TPSRelW (version 1.73; Rohlf, 2021). All deformation grids were generated with TPSRelW. We used Systat 10.0 (Systat Software, Inc.; Palo Alto, California, USA) to perform a MANOVA in which treatment group (e.g., *L. fuelleborni*, *L. trewavasae*, *L. fuelleborni*-sired hybrid offspring, and *L. trewavasae*-sired hybrid offspring) was used as the independent variable, and the RW scores were used as dependent variables; we then conducted Bonferroni-corrected pairwise tests among treatment groups for each RW. We also used Systat 10.0 to create bivariate plots of the RW scores.

Spectrophotometry

Adult males of all four treatment groups were placed individually in a weak solution of MS 222 (approximately 25 mg/l) and removed when the subject's respiration slowed and it lost equilibrium; anesthetized fishes were then transferred to an ice bath (Pauers et al., 2004). After removal from the ice bath, fish were placed on a piece of black cloth underneath a UV-transmitting quartz lens mounted on a macro tube, which directed light via a fiber-optic cable into an Oriel Instaspec IV CCD and illuminated the fish from its dorsal surface with a Newport 100 W ozone-free xenon lamp, a 385 nm LED flashlight, and a 15 W fluorescent blacklight. We obtained reflectance data from six spots on each fish (Fig. 1b); the recorded wavelengths were converted to actual reflectance measurements by dividing them by analogous measurements taken from a Spectralon white standard. After the measurements were made, the fish were transferred to a warm (28°C), aerated aquarium in which they were allowed to recover from the MS-222 before being returned to their stock aquaria.

Numerous measures of brightness, chroma, and hue were extracted from the reflectances using the pavo package (version 2.7.1) in R (Maia et al., 2019), and a principal components analysis (PCA) was performed on these chromatic variables in Systat 10.0 (Systat Software, Inc.; Palo Alto, California, USA). We then conducted an ANOVA in which treatment group was used as the independent variable and the principal components were dependent variables to determine whether the factor scores of our four treatment groups were statistically different; we additionally conducted Bonferroni-corrected pairwise

tests among treatment groups for each principal component.

Additionally, we were interested in the degree to which the color patterns of these treatment groups might be modular versus integrated; modular phenotypes consist of distinct, independent components; integrated phenotypes consist of interconnected, covarying components (e.g., Klingenberg, 2009). Male cichlids from Lake Malaŵi typically have coloration that is more modular, and thus more evolutionarily labile and responsive to sexual selection than the typically canalized and integrated color patterns of females (sensu Brzozowski et al., 2014; Albertson et al., 2014). We used the Principal Components Approach suggested by Brzozowski et al. (2014) and Albertson et al. (2014) to investigate the degree to which the color patterns of these fishes are integrated. We thus conducted principal components analyses separately for each treatment group using the same set of spectral characteristics as when all fishes were pooled, and examined the number of eigenvalues > 1, as well as the percent of variance explained by each principal component axis.

Sexual selection

We performed two experiments on sexual selection involving the two types of males available. The first of these experiments examined the aggressive reactions of male *L. trewavasae* to two opponents presented simultaneously, one male *L. trewavasae*, and one *L. fuelleborni*-sired hybrid male. Ten male *L. trewavasae* (total length: mean = 11.43 ± 0.63 cm) were the focal subjects, and ten unique pairs of opponents were created from five male *L. trewavasae* (total length: mean = 10.58 ± 0.47 cm) and five male *L. fuelleborni*-sired hybrids (total length: mean = 10.60 ± 0.28 cm). Following the methods of Pauers and Grudnowski (2020), the focal male *L. trewavasae* was placed in the central compartment of a three-part arena and allowed to acclimate for a period of 24–48 h; see Pauers and Grudnowski (2020) for a picture of the arena. On the day of the experiment, the two stimulus males were placed in two smaller Plexiglas aquaria that were suspended on opposite ends of the arena, and the fish were allowed to interact for ten minutes. The interactions were recorded on a digital video camera, and after the ten minute interaction period, the camera was stopped, and all individuals were removed

from the arena and measured for total length and had their eggspots counted before being returned to their respective cohort tanks.

We then reviewed the recording, counting the numbers of four aggressive behaviors (i.e., butts, bites, lateral displays, and frontal displays) the focal male directed to each opponent, as well as the sum of these four behaviors (“total aggression”). We specifically tested the hypothesis that the focal male *L. trewavasae* would direct more aggressive behaviors towards conspecific males, as found in Pauers and Grudnowski (2020). These data were checked for normality and analyzed using the appropriate (i.e., parametric versus nonparametric) statistical tests in Systat 10.0.

In the second experiment, we examined female mate choice in female *L. trewavasae* when offered a conspecific male and a *L. fuelleborni*-sired hybrid male, testing the hypothesis that females should direct more responses to conspecific males. The experimental arena consisted of a filtered, heated 132 L aquarium maintained at the same parameters as the cohort aquaria. Two semi-permanent, ultraviolet-transparent Plexiglas dividers were installed 30.5 cm from each side of the aquarium; these barriers demarcated the male territories, which had a ground area of 930.25 cm² and a volume of about 28.4 L. Each male was given a single brick (15.25 cm × 15.25 cm × 5 cm) which served as a territory and shelter; each brick was positioned such that a fish could hide behind or beside it and not be readily visible to the other fishes. Immediately in front of both clear dividers were removable opaque dividers, which allowed the fish to acclimate to the arena simultaneously without being in visual contact with one another. The centre portion of the arena, with a ground area of 1859.3 cm² and a volume of about 56.7 l, was the female’s territory; the female was also provided a small shelter in the form of a brick (15.25 cm × 15.25 cm × 5 cm) and an artificial plant.

A trio of fishes, a gravid female and two males, were randomly selected from their cohort aquaria and moved to the arena. Ten female *L. trewavasae* (total length: mean = 10.9 ± 0.20 cm) were the focal subjects, and ten unique pairs of opponents were created from five male *L. trewavasae* (total length: mean = 11.96 ± 0.66 cm; anal fin eggspots: mode = 3) and five male *L. fuelleborni*-sired hybrids (total length: mean = 10.94 ± 0.20 cm; anal fin eggspots:

mode = 3). The female was placed into the larger, central compartment and each male was placed separately into one of the smaller male territories. The fish were allowed to acclimate to the experimental arena for a period of 48 h. After the 48-h acclimation period, the opaque dividers were removed and the interactions between the female and both stimulus males were recorded using a video camera for 20 min with no humans present. After 20 min, the recording was stopped, and all individuals were removed from the arena and measured for total length and had their eggspots counted before being returned to their respective cohort tanks.

The video recordings were then reviewed, counting the number of times a female directly approached a male on the opposite side of the transparent barrier (Pauers & Grudnowski, 2022). The number of visits to a given male corresponds to the amount of time spent near that male, which is known to be indicative of male choice in the rock-dwelling cichlids of Lake Malawi (Coultridge & Alexander, 2001). These data were checked for normality and analyzed using the appropriate (i.e., parametric versus nonparametric) statistical tests in Systat 10.0.

Results

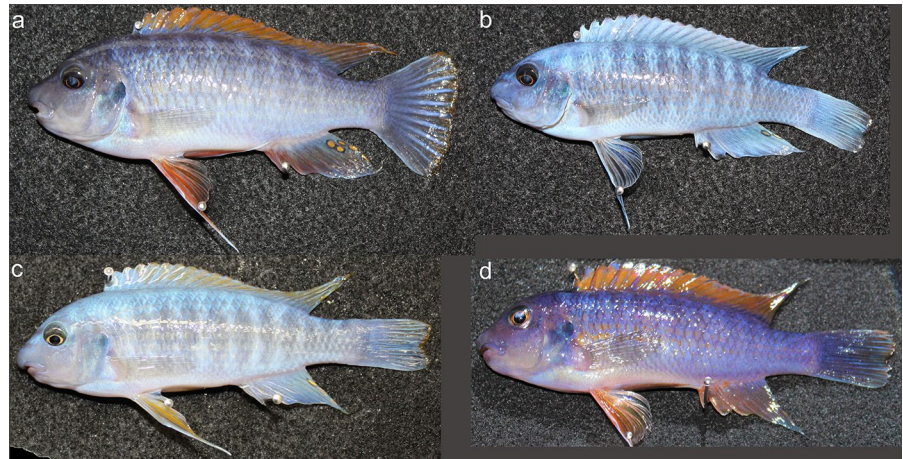
Experimental animals

Our experimental crosses produced 12 *L. trewavasae*-sired hybrid males and seven *L. fuelleborni*-sired hybrid males that survived to adulthood (Fig. 2). While many of the female *L. fuelleborni* were responsive to the male *L. trewavasae* and produced at least some hybrid offspring (e.g., Pauers et al., 2018), we noticed that the female *L. trewavasae* were resistant to male *L. fuelleborni*, and only two female *L. trewavasae* successfully spawned with a heterospecific mate (Pauers, pers. obsv.). We then selected 12 adult male *L. fuelleborni* and 12 adult male *L. trewavasae* from our cohort stocks for the morphometric and spectrophotometric analyses.

Geometric morphometrics

The relative warps analysis found significant differences in morphology among the four treatment groups of *Labeotropheus*. Relative Warp 1 explains

Fig. 2 Representative males of each treatment group. **a** *Labeotropheus fuelleborni*; **b** *L. trewavasae*; **c** *L. fuelleborni*-sired hybrid; **d** *L. trewavasae*-sired hybrid



25.07% of the variation in body shape and differentiates a deeper body and upturned neurocranium and snout, as seen in *L. fuelleborni* and the *L. fuelleborni*-sired hybrids, from a slender body with a straighter head and horizontal snout as seen in *L. trewavasae* and the *L. trewavasae*-sired hybrids. Relative Warp 2 explains 14.58% of the variation in shape among our specimens, and differentiates the prominently upturned neurocranium and snout of the *L. fuelleborni*-sired hybrids from the head profiles found in the other three treatment groups (Fig. 3).

In Fig. 3, it is interesting to note that *L. trewavasae*-sired hybrids have a body shape that is similar to that of *L. trewavasae*. Along both Relative Warps 1 and 2, these hybrids are significantly different from *L. fuelleborni*, though not from *L. trewavasae* (Table 1; Supplementary Table 1). The *L. fuelleborni*-sired hybrids, on the other hand, are significantly different from all of the other treatment groups of *Labeotropheus* along Relative Warp 1, but are only significantly different from the *L. trewavasae*-sired hybrids along Relative Warp 2 (Table 1; Supplementary Table 1).

Spectrophotometry

The average reflectances of each treatment group of *Labeotropheus* for each of the six patches measured are shown in Fig. 4. These reflectance curves illustrate the chromatic differences among the treatment groups, especially the *L. fuelleborni*-sired hybrids. These hybrids have strong ultraviolet reflectance in their caudal peduncle, operculum, and ventrum, with a peak at about 350 nm, but lack the additional peak

at 400 nm found in the other three treatment groups (Fig. 4). Interestingly, a somewhat inverse pattern is found in the dorsum and forehead reflectances, where the *L. fuelleborni*-sired hybrids lack a strong ultraviolet component to their coloration, especially in comparison to *L. trewavasae*, but have a strong peak at about 425 nm (Fig. 4). The reflectance of the dorsal fin distinguishes *L. trewavasae* from the other three treatment groups, with its unique broad peak throughout the ultraviolet and violet wavelengths, but lack of a subsequent and relatively less intense peak in the longer wavelengths as found in *L. fuelleborni* and both hybrids (Fig. 4).

The principal components analysis performed on all of the various measures of brightness, chroma, and hue reveals further distinctions among the four treatment groups. The loadings for the first two principal components are shown in Table 2. The first axis, Color PC 1, accounts for 19.35% of the variation in color, and discriminates between the high and narrow-peaked ultraviolet reflectance of the caudal peduncle and ventrum found on the *L. fuelleborni*-sired hybrids, as well as the more bimodal, ultraviolet and violet caudal peduncle and ventrum of the other three treatment groups (Fig. 5). The second principal component, Color PC 2, accounts for 16.97% of the variation in color, and distinguishes the unusual dorsum and operculum reflectances of the *L. fuelleborni*-sired hybrids from the other treatment groups (Fig. 5).

An ANOVA of the scores of Color PCs 1 and 2 for each treatment group indicates significant differences among the treatment groups for both (Table 3). The

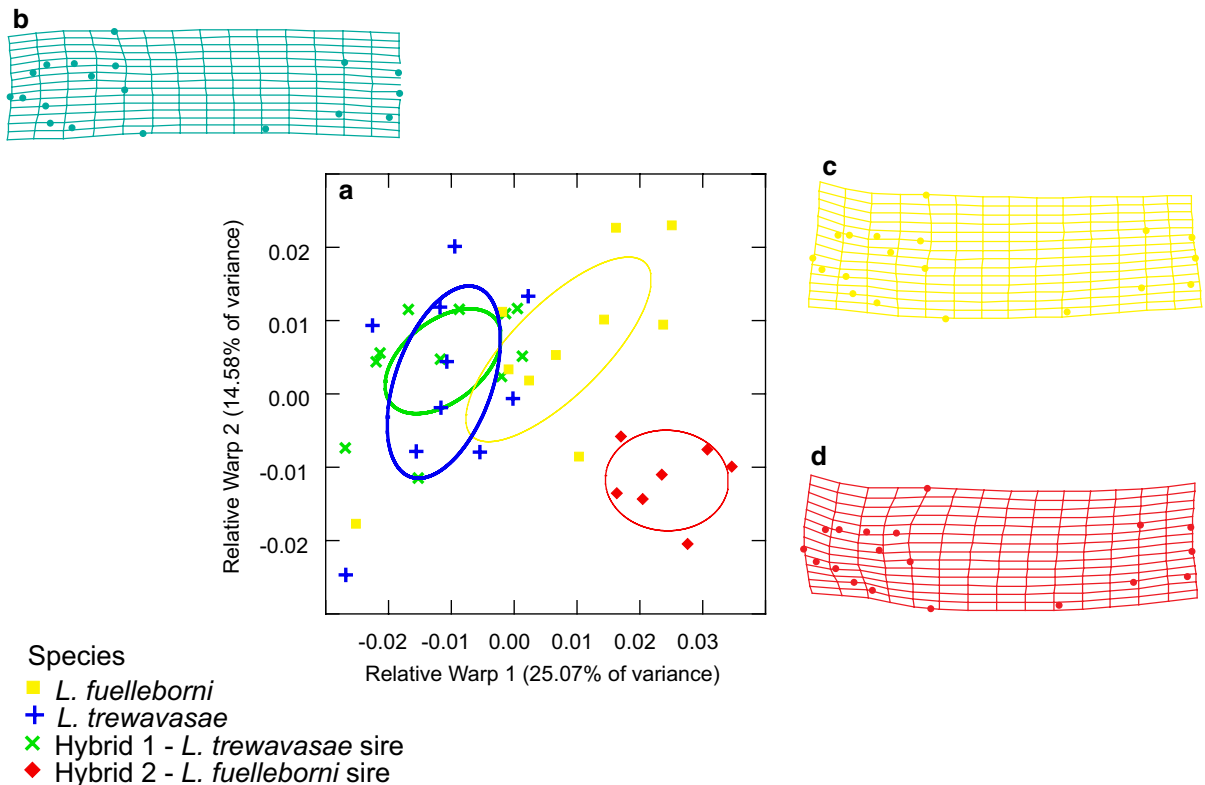


Fig. 3 **a** Relative warp plot for the geometric morphometric data. Relative Warp 1 accounts for 25.07% of the variation in body shape among the four treatment groups, and distinguishes the deeper-bodied *L. fuelleborni* and *L. fuelleborni*-sired hybrids from the slender *L. trewavasae* and *L. trewavasae*-sired hybrids. Relative Warp 2 accounts for 14.58% of the variation, and distinguishes the more drastically upturned neu-

rocranium of the *L. fuelleborni*-sired hybrids from the other three treatment groups. The ellipses are 95% confidence ellipses around the group centroid. **b** Deformation grid for both *L. trewavasae* and *L. trewavasae*-sired hybrids. **c** Deformation grid for *L. fuelleborni*. **d** Deformation grid for *L. fuelleborni*-sired hybrids. All deformation grids exaggerated 5X

Table 1 MANOVA on relative warps 1–5; Wilks' $\lambda=0.40$; $F_{15,83}=12.196$; $P\leq 0.001$

Variable	Sum of squares	df	Mean square	<i>F</i>	<i>P</i>
RW 1	0.007	3	0.002	20.829	≤ 0.001
Error	0.004	34	0.000		
RW 2	0.002	3	0.001	4.718	≤ 0.01
Error	0.004	34	0.000		
RW 3	0.002	3	0.001	7.131	≤ 0.001
Error	0.004	34	0.000		
RW 4	0.001	3	0.000	5.908	≤ 0.01
Error	0.004	34	0.000		
RW 5	0.000	3	0.000	0.039	0.989
Error	0.004	34	0.000		

Relative Warps 6–34 were ns; data not shown. Results of pairwise comparisons of treatment groups can be found in Supplementary Table 1

L. fuelleborni-sired hybrids are significantly different from the other three groups along both Color PC 1 and 2 (Table 3). The *L. trewavasae*-sired hybrids are significantly different from *L. trewavasae* along both, but not from *L. fuelleborni* (Table 3).

Exploring modularity and integration in the pigmentation of the four treatment groups using the approach of Brzozowski et al. (2014) and Albertson et al. (2014) revealed that, unlike the other three treatment groups, the pigmentation of the *L. fuelleborni*-sired hybrid males is consistent with an integrated color pattern. For both parental species, nine eigenvalues were > 1 , with the first axis accounting for 29.81% of the variation in *L. fuelleborni*, and 30.20% in *L. trewavasae*. The *L. trewavasae*-sired hybrids had 10 eigenvalues > 1 , and the first principal component axis explained 29.73% of the observed variation.

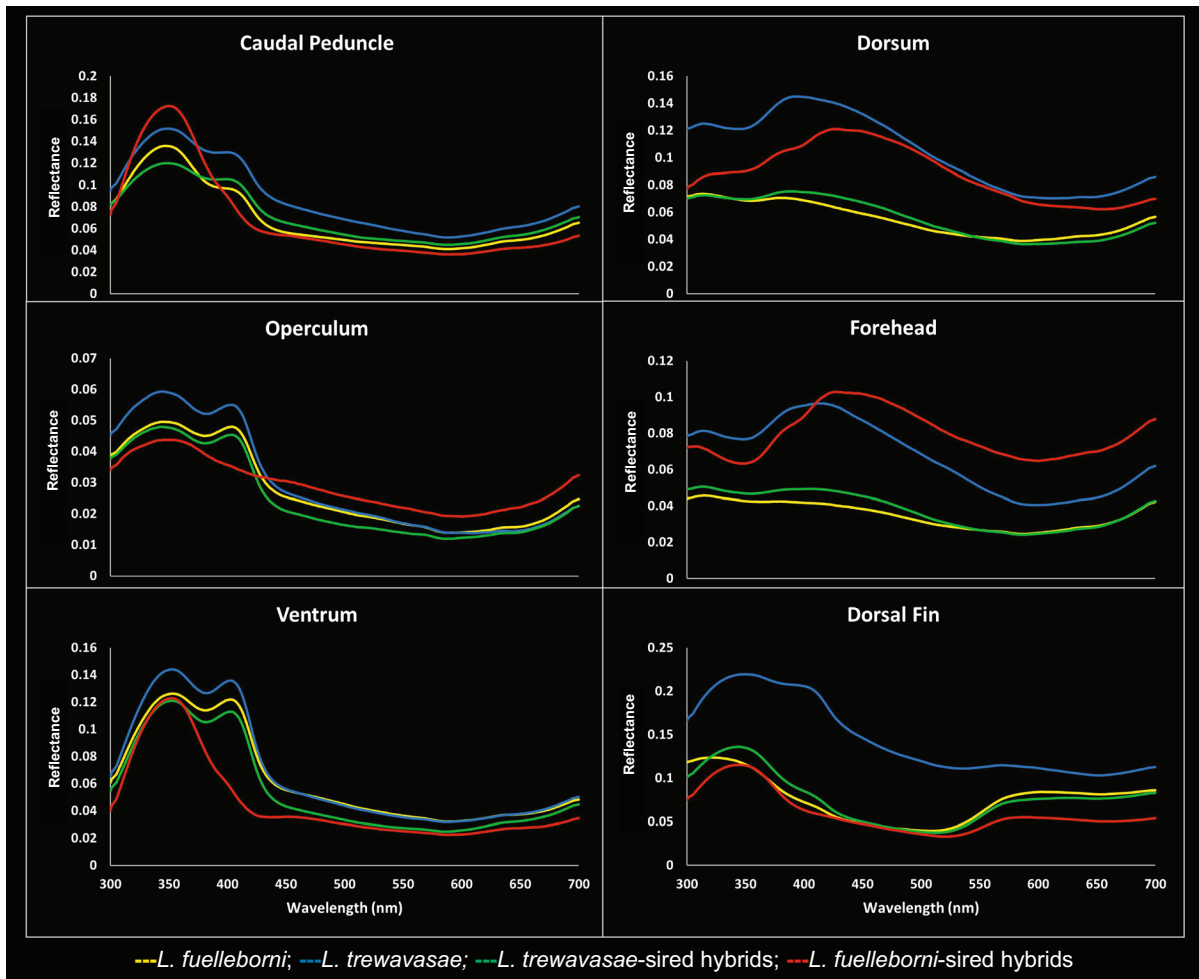


Fig. 4 Reflectances obtained at the six body locations for all four treatment groups. The titles of each plot are the body region from which the reflectances were measured. Each curve is the average of all the members of a given treatment

group; error bars are omitted for clarity. Yellow: *L. fuelleborni* ($n=12$); blue: *L. trewavasae* ($n=12$); green: *L. trewavasae*-sired hybrids ($n=12$); red: *L. fuelleborni*-sired hybrids ($n=7$)

Finally, the *L. fuelleborni*-sired hybrids had six eigenvalues > 1 , and the first axis accounted for 36.88% of the variation in the color of these fishes. The relatively lower number of eigenvalues with values > 1 , and the greater percentage of variation explained by the first principal component, are both indicative of integration (Table 4).

Male–male aggression

All 10 focal male *L. trewavasae* responded to the stimulus males, yielding usable data for these analyses. The data were not normally distributed, so

Wilcoxon signed rank tests were used to test for pairwise differences in the aggression directed to the two different opponents. The focal males did not discriminate between *L. trewavasae* and *L. fuelleborni*-sired hybrid opponents, directing approximately equal numbers of aggressive behaviors to either opponent. None of the differences were significant, neither the total sum of all aggressive behaviors (total aggression: $Z = -1.020$, $P = 0.308$; Fig. 6a), nor the number of individual aggressive behaviors (lateral displays: $Z = -0.803$, $P = 0.407$; frontal displays: $Z = -1.219$, $P = 0.223$; bites: $Z = -0.840$, $P = 0.401$; butts: $Z = -0.867$, $P = 0.386$).

Table 2 Principal component loadings from spectral variables; note that all variables were entered in the same analysis, but are separated here by body region for clarity and simplicity

Variable	Caudal Peduncle		Dorsum		Dorsal Fin	
	PC 1	PC 2	PC 1	PC 2	PC 1	PC 2
Total brightness	0.642	0.463	0.411	0.667	0.523	0.059
Mean brightness	0.642	0.463	0.411	0.667	0.523	0.059
Intensity	0.138	0.656	0.439	0.651	0.467	− 0.004
Ultraviolet chroma	− 0.491	0.404	0.324	− 0.723	0.102	− 0.064
Violet chroma	− 0.441	0.385	0.372	− 0.670	0.192	− 0.009
Blue chroma	0.677	− 0.245	0.162	0.674	0.286	0.406
Green chroma	0.374	− 0.369	− 0.346	0.569	− 0.260	− 0.110
Yellow chroma	0.289	− 0.398	− 0.484	0.298	− 0.304	− 0.226
Red chroma	0.161	− 0.407	− 0.387	− 0.272	− 0.279	− 0.314
Spectral saturation	− 0.418	0.397	0.317	− 0.154	− 0.057	− 0.322
Contrast	− 0.043	0.622	0.473	0.576	0.221	− 0.091
Peak λ	0.004	0.016	− 0.107	0.672	− 0.223	0.166
λ of maximum negative slope	0.285	− 0.193	− 0.361	0.163	− 0.364	0.215
λ of median reflectance	0.193	− 0.059	0.375	− 0.189	0.066	− 0.064
	Forehead		Opercular Tab		Ventrum	
	PC 1	PC 2	PC 1	PC 2	PC 1	PC 2
Total brightness	0.266	0.724	0.522	0.603	0.743	0.312
Mean brightness	0.266	0.724	0.522	0.603	0.743	0.312
Intensity	0.353	0.678	0.703	0.376	0.630	0.447
Ultraviolet chroma	0.544	− 0.579	0.452	− 0.646	− 0.609	0.000
Violet chroma	0.631	− 0.498	0.517	− 0.615	− 0.504	− 0.149
Blue chroma	0.320	0.537	0.093	0.388	0.686	− 0.336
Green chroma	− 0.552	0.516	− 0.489	0.594	0.461	0.287
Yellow chroma	− 0.806	0.300	− 0.495	0.541	0.425	0.289
Red chroma	− 0.800	− 0.129	− 0.564	0.393	0.096	0.230
Spectral saturation	0.444	− 0.309	0.402	− 0.560	− 0.600	0.036
Contrast	0.511	0.519	0.764	0.166	0.379	0.367
Peak λ	− 0.423	0.332	− 0.017	0.179	0.319	0.054
λ of maximum negative slope	− 0.113	− 0.020	− 0.540	0.410	− 0.270	− 0.024
λ of median reflectance	− 0.403	− 0.192	− 0.433	0.186	0.038	− 0.052

Female mate choice

Of the 10 attempted trials, nine yielded usable data; in one case, a male was able to get around the permanent transparent divider and was found in direct contact with the female during the acclimation period. In the nine trials that provided usable data, the data were normally distributed, so parametric analyses were used to analyze the data.

With respect to the males, despite the difference in mean length between *L. trewavasae* and *L.*

fuelleborni-sired hybrid males noted above, the difference in length between a pair of males did not influence the number of responses directed to either opponent ($R^2=0.124$; $F_{1,7}=0.989$; $P=0.353$). While the *L. fuelleborni*-sired hybrid males tended to perform more displays than *L. trewavasae* males (hybrids: 9.0 ± 3.7 displays; *L. trewavasae*: 3.6 ± 1.1 displays), this difference was not significant ($t_8=1.623$, $P=0.143$). The great difference in the average number of displays performed was due to a single hybrid male who performed 34 displays, and, when this

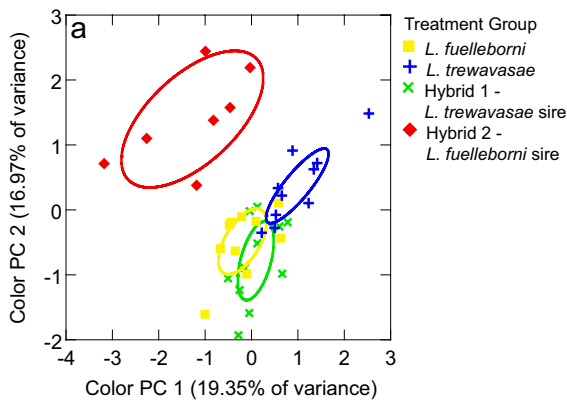


Fig. 5 Plot of color principal component 1 (Color PC 1) versus color principal component 2 (Color PC 2); ellipses are 95% confidence around the group centroid. Color PC 1 explains 19.35% of the variation in coloration among the treatment groups, and largely encompasses the strong ultraviolet components of the caudal peduncle and ventrum; see Table 2 for factor loadings. Color PC 2 accounts for 16.97% of the variation, and differentiates a very reflective dorsum, as found in *L. trewavasae* and the *L. fuelleborni*-sired hybrids at the positive end, versus an operculum that reflects strongly in the violet wavelengths, as found in *L. fuelleborni*, *L. trewavasae*, and *L. trewavasae*-sired hybrids, at the negative end (c.f. Table 2)

individual is excluded from the dataset, the average number of displays performed by *L. fuelleborni*-sired hybrid males falls to 5.8 ± 1.9 . Finally, the number of displays performed by one male was not related to the number of displays performed by his opponent ($R^2 = 0.179$; $F_{1,7} = 1.521$; $P = 0.257$).

Female receptive behavior towards the two different males was consistent. Female *L. trewavasae* directed significantly more receptive responses to *L. trewavasae* males than they did to *L. fuelleborni*-sired hybrid males ($t_8 = -6.415$, $P \leq 0.001$; Fig. 6b).

Discussion

Male hybrids of *Labeotropheus fuelleborni* and *L. trewavasae* from the Chidunga Rocks in southwestern Lake Malaŵi produced from reciprocal crosses differ in body shape and coloration. The hybrids resulting from a cross of a male *L. trewavasae* and a female *L. fuelleborni* have a body shape intermediate to the parental species, and a coloration most similar to that of the males of the maternal species; this agrees with the findings of Pauers et al. (2018). Conversely, the

hybrids that are produced with a male *L. fuelleborni* and a female *L. trewavasae* present a combination of morphological and color features that are unique and different to that of either parental species, as well as that of the reciprocal hybrid. As such, while the *L. trewavasae*-sired hybrids are phenotypically intermediate to their parental species, the *L. fuelleborni*-sired hybrids have a novel combination of morphometry and pigmentation, and are consistent with what would be expected in a transgressive hybrid.

Pauers et al. (2018) found that the body shape of F1 hybrids created by crossing female *L. fuelleborni* and male *L. trewavasae* contributed to a reduced foraging efficiency. In this study, hybrids produced by *L. trewavasae* sires and *L. fuelleborni* dams had an upturned and expanded neurocranium not found in either of the parental species that contributed a slower growth rate in laboratory conditions (Pauers et al., 2018). Given how consequential body shape is for foraging and maneuvering in the aquatic environment (Higham et al., 2015), especially for the algae-grazing cichlids of Lake Malaŵi (Stauffer & Posner, 2006; Rupp and Hulsey, 2014), the inability for these hybrids to thrive under these conditions is not surprising. It would thus be especially enlightening to see how the *L. fuelleborni*-sired hybrids fare in a similar experiment.

Brzozowski et al. (2014) and Albertson et al. (2014), using their innovative approach to the analysis of color patterns of the cichlids of Lake Malaŵi, found that males have less integrated, more modular color patterns. Such color patterns, consisting of separate, distinct units of pigmentation, would allow them to evolve rapidly, especially in response to sexually selection (Brzozowski et al., 2014; Albertson et al., 2014). In their analyses, they used the number of eigenvalues > 1 , as well as the amount of variance explained by each principal component, to determine the degree to which color patterns were modular; a large number of eigenvalues > 1 and a lower amount of variance explained by each principal component indicated a greater degree of modularity, versus integration, in a color pattern. Our results of the principal components analysis of the spectral data are consistent with this pattern. Male *L. fuelleborni*, *L. trewavasae*, and *L. trewavasae*-sired hybrids, while differing somewhat in their spectral characteristics, nonetheless have modular color patterns, as demonstrated by the relatively large number of eigenvalues > 1 , and the

Table 3 Analysis of variance for color principal components

(A) Color PC 1 ($n=38$; multiple $R^2=0.584$)					
Variable	Sum of squares	df	Mean square	F	P
Treatment Group	21.619	3	7.206	15.929	≤ 0.001
Error	15.381	34	0.452		
Pairwise comparisons					
	<i>L. fuelleborni</i>	Hybrid 1 (<i>Lt</i> Sire)	Hybrid 2 (<i>Lf</i> Sire)		
Hybrid 1 (<i>Lt</i> Sire)	– 0.288 ^{ns}				
Hybrid 2 (<i>Lf</i> Sire)	1.080*	– 1.368***			
<i>L. trewavasae</i>	1.183**	0.895*	2.263***		
(B) Color PC 2 ($n=38$; multiple $R^2=0.653$)					
Variable	Sum of squares	df	Mean square	F	P
Treatment Group	24.144	3	8.048	21.285	≤ 0.001
Error	12.856	34	0.378		
Pairwise comparisons					
	<i>L. fuelleborni</i>	Hybrid 1 (<i>Lt</i> Sire)	Hybrid 2 (<i>Lf</i> Sire)		
Hybrid 1 (<i>Lt</i> Sire)	0.297 ^{ns}				
Hybrid 2 (<i>Lf</i> Sire)	– 1.883***	2.180***			
<i>L. trewavasae</i>	0.856*	1.153***	– 1.027**		

ns not significant

* $P=0.05$; ** $P=0.01$; *** $P=0.001$

lower amount of variance explained by each principal component axis. Conversely, the *L. fuelleborni*-

rock-dwelling cichlids of Lake Malaŵi, and especially the *Labeotropheus*, have long been known

Table 4 Number of eigenvalues and percentage of variance explained by each principal component for the principal component analyses performed on the spectral data for each treatment group

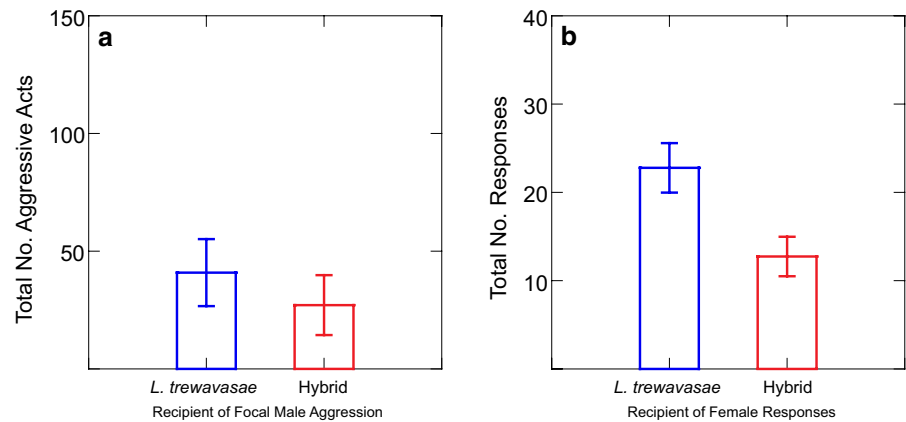
Treatment group	Number of eigenvalues > 1	Percent variance explained by each axis									
		1	2	3	4	5	6	7	8	9	10
<i>L. fuelleborni</i>	9	29.81	16.12	13.90	11.30	8.78	6.84	5.44	4.19	3.62	–
<i>L. trewavasae</i>	9	30.20	17.86	14.31	11.74	8.62	6.71	4.37	3.93	2.26	–
<i>L. fuelleborni</i> -sired hybrids	6	36.88	18.51	17.40	12.74	9.83	4.64	–	–	–	–
<i>L. trewavasae</i> -sired hybrids	10	29.73	18.25	12.44	10.29	9.72	5.79	5.13	4.00	3.18	1.46

sired hybrid males had a spectrally unique color pattern, one that was rich in ultraviolet wavelengths, but lacking violet and blue wavelengths, at several of the body regions we sampled. Additionally, for this treatment group, there were fewer eigenvalues > 1, and a greater percentage of variance was explained by each principal component.

It is not surprising, then, that these transgressive hybrid males would not be recognized by female *L. trewavasae* as a potential mate. Female

to select their mates visually, especially on the basis of male nuptial color (Jordan et al., 2003; Knight & Turner, 2004; Pauers et al., 2004; Pauers & Grudnowski, 2022). The body shape of these *L. fuelleborni*-sired hybrids, and especially their unusual coloration, makes them visually distinct from *L. trewavasae* males, at least to a human observer, although the spectral distribution of their color pattern also suggests that these differences should be

Fig. 6 Responses of male and female *L. trewavasae* to conspecific and *L. fuelleborni*-sired males. **a** Male *L. trewavasae* directed slightly more aggressive acts to conspecific opponents, but this difference is not statistically significant. **b** Female *L. trewavasae* were much more responsive to male *L. trewavasae* than they were to male *L. fuelleborni*-sired hybrids



visible to female *L. trewavasae* (Carleton & Kocher, 2001; Pauers et al., 2004).

The results of the male-male aggression experiments are less clearly interpreted. Male rock-dwelling cichlids from Lake Malaŵi use the color patterns of their opponents to ascertain the greatest threat to their ability to attract a mate (i.e., males colored most similarly to themselves) and modulate their aggression accordingly (Pauers et al., 2008; Pauers & McKinnon, 2012; Pauers & Grudnowski, 2020). Given the differences in coloration between *L. trewavasae* and *L. fuelleborni*-sired males, it is reasonable to expect that focal male *L. trewavasae* would direct more aggressive behaviors to *L. trewavasae* opponents, but this was not the case. The focal male *L. trewavasae* in these experiments directed equal numbers of aggressive behaviors to both opponents. It could be possible that the spectral characteristics of the color pattern of *L. fuelleborni*-sired hybrids are just similar enough to those of *L. trewavasae* that they do not meet some inherent threshold of species recognition (Crapon de Caprona, 1986; Ribbink, 1991), assuming that males identify their opponents using visual cues (Pauers et al., 2008, Pauers & McKinnon, 2012). The results from this experiment suggest that, in a natural and unaltered habitat, the *L. fuelleborni*-sired hybrid males would be treated as a conspecific by males of both parental species, and would thus have no advantage over male *L. trewavasae* during intrasexual aggressive encounters. To the best of our knowledge, this is the first time that the performance of male hybrids of Lake Malawi cichlids in intrasexual aggressive encounters has been tested.

Taken together, the results of our behavioral experiments strongly suggest that these transgressive *L. fuelleborni*-sired hybrids are not likely to be successful competitors or mates in a natural population in an undisturbed habitat (but see Seehausen et al., 1997). Young rock-dwelling cichlids in Lake Malaŵi typically do not migrate more than a few meters from where they are released, and are thus most likely to join the community frequented by their mothers (Ribbink et al., 1983; Ribbink, 1991). Since the *L. fuelleborni*-sired hybrid males used in our experiments were carried by female *L. trewavasae*, in Lake Malaŵi they would come to sexual maturity surrounded by *L. trewavasae*; our results suggest that these hybrids would struggle in sexually selected interactions with adults of their parental species.

Given the lack of *L. fuelleborni* females and *L. trewavasae*-sired hybrid males in our experiments, results of our behavioral studies provide an incomplete picture of the way hybrid males might interact in a natural hybrid zone of *Labeotropheus*. Nonetheless, these results, coupled with the morphological and spectrophotometric data, allow us to propose further investigations to determine whether or not these findings are broadly applicable. An obvious place to start would be to perform reciprocal experiments in which female *L. fuelleborni* are offered a choice between *L. trewavasae*-sired hybrid males and male *L. fuelleborni*. Since the *L. trewavasae*-sired hybrid males are very similar in appearance to *L. fuelleborni*, both morphologically and chromatically, it might be possible that these hybrids would receive attention from female *L. fuelleborni*. With regards to male-male aggression, the similarity, at

least to a human observer, between *L. trewavasae*-sired and *L. fuelleborni* males suggests that focal male *L. fuelleborni* should direct equal amounts of aggression towards conspecific and *L. trewavasae*-sired opponents. Finally, it would be extremely informative to examine the mate choice preferences of both *L. fuelleborni*- and *L. trewavasae*-sired females. If these females prefer non-hybrid, parental males, that could explain the presence and persistence of gene flow among the cichlids of Lake Malaŵi (Mims et al., 2010; Selz et al., 2014a, b; Hulsey et al., 2017). On the other hand, if hybrid females prefer hybrid males, this could lend credence to the hypothesis that hybridization is an important source of phenotypically divergent species in Lake Malaŵi (Feller et al., 2020).

Although morphological and behavioral evidence from the wild is exceedingly rare, molecular genetics reveals that hybridization has undoubtedly occurred during the evolution of the species flock of cichlids endemic to Lake Malaŵi (e.g., Mims et al., 2010; Loh et al., 2013; Meier et al., 2017; Malinsky et al., 2018; Svartal et al., 2020). Our results indicate that hybridization does indeed generate phenotypic novelty in the *Labeotropheus*, but that the emergence of novelty depends upon the parentage of the hybrids, since one direction produces intermediate hybrids, and the other produces novel combinations of traits. Perhaps most importantly, our experiments suggest that one reason for the rarity of hybrids in modern day Lake Malaŵi could be the fact that transgressive males were neither preferred by females of their maternal species, nor did they receive less aggression from males of the maternal species.

In the vast majority of studies involving hybridization in cichlids from Lake Malaŵi, experimental crosses were made in only a single direction (e.g., McElroy & Kornfield, 1993; Albertson & Kocher, 2001; Brzozowski et al., 2014; Pauers et al., 2018). In the relatively few studies in which reciprocal crosses were made, the resulting hybrid progeny were pooled in the subsequent analyses, thus obscuring their parentage (e.g., Husemann et al., 2017). Given the results presented in this paper, as well as the roles sexual conflict and chromosomal sex determination play in determining the chromatic phenotype of male cichlids from Lake Malaŵi (Roberts et al., 2009), it is important for future studies to compare hybrid offspring from reciprocal crosses.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests None of the authors have any competing interests to report.

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