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1 **Title**

2 **Relaxed trait covariance in interspecific cichlid hybrids predicts morphological diversity**
3 **in adaptive radiations**

4

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19

20 **Abstract**

21 The process of adaptive radiation involves multiple events of speciation in short
22 succession, associated with ecological diversification. Understanding this process requires
23 identifying the origins of heritable phenotypic variation that allows adaptive radiation to
24 progress. Hybridisation is one source of genetic and morphological variation that may spur
25 adaptive radiation. We experimentally explored the potential role of hybridisation in
26 facilitating the onset of adaptive radiation. We generated first- and second-generation hybrids
27 of four species of African cichlid fish, extant relatives of the putative ancestors of the
28 adaptive radiations of Lakes Victoria and Malawi. We compared patterns in hybrid
29 morphological variation with the variation in the lake radiations. We show that significant
30 fractions of the interspecific morphological variation and the major trajectories in
31 morphospace that characterize whole radiations can be generated in second-generation
32 hybrids. Furthermore we show that covariation among traits is relaxed in second-generation
33 hybrids, which may facilitate adaptive diversification. These results support the idea that

hybridisation can provide the heritable phenotypic diversity necessary to initiate adaptive radiation.

Introduction

Adaptive radiation involves multiple events of speciation in short succession, associated with ecological diversification and is often initiated when a population colonises new environments with a variety of available ecological niches (Simpson 1953; Schluter 2000; Losos 2010). Identifying the sources of heritable phenotypic variation required to initiate and sustain the process of adaptive radiation remains an outstanding challenge (Gavrilets and Losos 2009). Mutations and intraspecific recombination alone are often unlikely to produce and maintain sufficient levels of heritable phenotypic variation (Schlichting and Pigliucci 1998; Blows and Hofmann 2005; Orr and Unckless 2008; Hedrick 2013) to support the most rapid large radiations. Adaptive radiation is thus likely to require initially high levels of standing genetic variation in adaptive traits (Barrett and Schluter 2008). Interspecific hybridisation is one mechanism by which high levels of heritable phenotypic variation can be rapidly generated (Anderson and Stebbins 1954; Lewontin and Birch 1966; Arnold 1997; Seehausen 2004; Bell and Travis 2005; Hedrick 2013).

Hybridisation can affect patterns of genetic and phenotypic variation in two related ways that can facilitate and influence the process of adaptive radiation. Firstly, hybrids often display novel genotypes and phenotypes (Grant and Grant 1996; Bell and Travis 2005; Schwenk et al. 2008) that are intermediate to (Barton and Hewitt 1985; Mallet 2007; Rieseberg and Willis 2007) or outside of the range observed in both parental species combined (Slatkin and Lande 1994; Rieseberg et al. 1999; Stelkens and Seehausen 2009). Extreme traits arising through transgressive segregation increase the phenotypic variance upon which divergent natural selection can act, may facilitate the colonisation of new environments with novel selection pressures (Johnston 2004; Nolte et al. 2005; Gompert et al. 2006; Lucek et al. 2010; Nice et al. 2012; Pereira et al. 2013) and can increase the likelihood of ecological speciation (Mavárez et al. 2006; Larsen et al. 2010; Hermansen et al. 2011). Secondly, existing genetic and morphological covariance structures (Hallgrimsson et al. 2012) may be relaxed by hybridization, thereby reducing evolutionary constraint (Clausen and Heisey 1960; Grant and Grant 1994; Murren 2002; Ackermann et al. 2006) and increasing the likelihood of adaptive diversification (Parsons et al. 2011; Renaud 2009, 2012).

Hybridisation is particularly common when reproductively compatible species come together in novel or perturbed environments (Anderson 1948). Such conditions are likely to

arise from environmental events that bring together previously allopatric species (Anderson and Stebbins 1954). Hybridisation and adaptive radiation are thus favoured by similar environmental conditions, i.e. colonisation of novel environments, and a taxonomically broad evidence base suggests hybridisation may be a common feature of adaptive radiation (Barrier et al. 1999; Feder et al. 2003; Seehausen 2004; Herder et al. 2006; Grant & Grant 2008; Hudson et al. 2010; Joyce et al. 2011; Papadopulos et al. 2013).

This collective body of evidence underpins two complementary hypotheses for the role of hybridisation in adaptive radiation (Seehausen 2004). The 'hybrid swarm origin' hypothesis posits that hybridisation between distantly related colonising lineages can play an important role in initiating adaptive radiation, whilst the 'syngameon' hypothesis highlights the role of occasional hybridisation as adaptive radiation progresses. Both the hybrid swarm origin (Barrier et al. 1999; Feder et al. 2003; Seehausen et al. 2003; Joyce et al. 2011; Hudson et al. 2010; Genner and Turner 2012;) and syngameon hypotheses (Herder et al. 2006; Grant and Grant 2008; Dasmahapatra 2012; Nadeau et al. 2012; Papadopulos et al. 2013) are supported by observational and correlative genetic and phenotypic evidence. Whilst there is experimental evidence addressing hybrid speciation (Rieseberg et al. 1996; Fordyce et al. 2002; Greig et al. 2002; Mavárez et al. 2006; Melo et al. 2009; Selz et al. 'in review'), there remains little experimental evidence for the two potentially facilitating roles of hybridisation in adaptive radiation. Cooper et al. (2011) and Parsons et al. (2011) have experimentally addressed the role of the syngameon hypothesis for adaptive radiations but experimental work on the role of the hybrid swarm origin hypothesis is so far lacking.

Here we combine experimental and comparative methods to test predictions of the hybrid swarm origin hypothesis using African cichlid fish. The cichlid fish radiations of the three African great lakes (Tanganyika, Malawi and Victoria; hereafter LT, LM and LV, respectively) have produced famously specious and morphologically diverse and convergent endemic assemblages (Greenwood 1975; Kocher et al. 1993; Kocher 2004; Seehausen 2006; Young et al. 2009; Wagner et al. 2012). All three radiations originate from several distantly related lineages of riverine cichlids. There is evidence for hybridization between distantly related colonists early in the origins of radiations (Seehausen et al. 2003; Seehausen 2004; Joyce et al. 2011; Genner and Turner 2012; Loh et al. 2013), hybridization among radiation member species leading to hybrid speciation (Salzburger et al. 2002; Schliewen & Klee 2004; Schelly et al. 2006; Egger et al. 2007; Keller et al. 2012), and contemporary interspecific hybridization (Seehausen et al. 1997, 2008; Streelman et al. 2004; Konijnendijk et al. 2011; Egger et al. 2012). Diversification in the young LV (0.015-0.1 myr) and moderately young

LM (2-4 myr) radiations has been rapid and in situ, whereas the LT (8-16 myr) radiation is older and speciation was much slower (Genner et al. 2007; Day et al. 2008). We refer to the lake assemblages as 'radiations' for simplicity even though diversity in Lake Tanganyika has arisen in several distinct radiations in different lineages (Genner et al. 2007).

We created first- and second-generation hybrids between: i) riverine species closely related to the putative ancestors of the LV and LM radiations, and ii) a riverine species and a generalist from the LM sand cichlid clade, both of which are implicated in giving rise to the young and rapidly diversifying rock-dwelling Mbuna clade of the LM radiation (Joyce et al. 2011; Loh et al. 2013). We compared patterns of morphological diversity among parental species, first- and second-generation hybrids, and representative species from the three great lake radiations. Previous work has shown that cichlid hybrids express a broader range of morphologies than their parental species (Parnell et al. 2008; Stelkens et al. 2009; Parsons et al. 2011) and reduced levels of integration between traits (Parsons et al. 2011), which are two predictions of the hybrid swarm origin hypothesis. Here, we test three further and increasingly refined predictions of this hypothesis: 1. hybrids display increased morphological variation and relaxed morphological covariance compared to parental species when projected into the morphospace of extant adaptive radiations, 2. the morphological covariance structures of extant radiations are more closely matched by interspecific hybrids than by any one ancestral species, and 3. the principal axes of hybrid morphospaces predict the principal axes observed in extant adaptive radiations better than do those of any one ancestral species.

Material and Methods

Parental species, first- and second-generation hybrids

We created parental type and hybrid lines using three riverine *Astatotilapia* species that are closely related to the radiations of Lakes Victoria and Malawi and considered archetypical for what the ancestors of these lake radiations would likely have looked like: *Astatotilapia calliptera* (Greenwood 1979), *A. tweddlei* (Greenwood 1979) and *A. burtoni* (Greenwood 1979). Additionally we used the lake dwelling cichlid *Protomelas taeniolatus* (Trewavas 1935). All three *Astatotilapia* species occur in lakes, rivers, streams and swamps in East and/or South Africa (Van Oijen et al. 1991; Skelton 1993; Kazembe 2006; Ntakimazi 2006; Konings 2007; Bills et al. 2010; Joyce et al. 2011). *A. burtoni* is found in Lake Tanganyika and surrounding rivers. *A. calliptera is* found in Lake Malawi and its catchment area, as well as in many rivers of southern East Africa from the Rovuma river and Lakes

Chilwa and Chiuta in its headwaters south to middle Mozambique. *A. tweddlei* is a member of the East African *A. bloyeti* species complex. The species complex is found in most coastal rivers from northern Tanzania down to the Rovuma river including Lakes Chilwa and Chiuta in its headwaters. *A. tweddlei* is confined to the Rovuma and its headwater lakes. We refer to the species as CAL for *A. calliptera*, TWE for *A. tweddlei*, BUR for *A. burtoni* and PRO for *P. taeniolatus*. Hybrid lines are named as: maternal species x paternal species - generation (e.g. CALxBUR-F2). The phylogenetic relationships between these and the great lake radiations are well established (Seehausen et al. 2003; Joyce et al. 2011; Loh et al. 2013). Both, *A. tweddlei* and *A. calliptera* are the nearest known living relatives of different lineages in the Lake Malawi radiation (Joyce et al. 2011). The riverine *Astatotilapia sp.* (including the three species used in this study) are the sister group to the whole Lake Victoria superflock and the Lake Malawi radiation, whereas *P. taeniolatus* is a basal member of the Lake Malawi radiation (Loh et al. 2013). The non-hybrid lines were laboratory populations founded by wild fish collected from Lake Malawi (CAL, PRO), Lake Chilwa (TWE) and rivers connected to Lake Tanganyika (BUR), and maintained in our laboratory (approximately five generations in captivity). We refer to these as the "parental lines" or "ancestral lines" depending on the context. The parents of the "hybrid lines" were taken from these populations.

We bred four types of hybrid F1 families (CALxPRO; CALxBUR; BURxTWE; CALxTWE) by holding 5-20 females of one species with one male of another. All F1 families derived from unique male-female combinations. F2 hybrid families of CALxPRO, CALxBUR and BURxTWE were obtained by breeding F1 sibs as above; F2 hybrids of CALxTWE were not obtained, because no mating events occurred among the F1 hybrids. Following spawning, fertilized eggs were removed from the female's mouth and transferred to an egg tumbler. After 15 days fry were moved to small aquaria (20 x 40 x 20 cm) for 15 days, then transferred to larger aquaria (50 x 40 x 30 cm) at a maximum density of 20 individuals. Families were raised in separate aquaria. Fish were fed a mixture of ground shrimp, peas and *Spirulina* powder two days a week, and with commercial cichlid flakes on other days. The water temperature (25 °C ± 2°C) and light:dark cycle (12:12 h) were the same for all aquaria.

All fish were digitally photographed after six months, near the age of sexual maturity. Pictures were taken of the left side of the live fish held in a transparent cuvette with a scale for subsequent size calibration. The total number of families and individuals present in each parental and hybrid line are given in Table 1.

Lake radiations

We collected digital photos from the left side (scale included) of preserved specimens from the three lake radiations (Young et al. 2009). The preserved cichlid specimens are representative species of each of the radiations in Lake Victoria, Lake Malawi and Lake Tanganyika that are stored at the Natural History Museum (London, U.K.), Africa Museum (Tervuren, Belgium), Naturalis Museum (Leiden, Netherlands) and the collections of O.S. The sample from LV included species now extinct due to eutrophication and invasive Nile perch (Witte et al. 1992; Seehausen et al. 1997). Most specimens were adult males. For the analyses we included 99 individuals from LV and LT and 97 individuals from LM. We included specimens from most genera and multiple specimens from polytypic genera to representatively sample the taxonomic and morphological diversity of each radiation (Young et al. 2009; Cooper et al. 2010). Each species is represented with one individual in the data set (Table S3).

Morphological variance-covariance matrices

We used geometric morphometrics in tpsDig2 v.9.1 software (Rohlf 2006) to record the coordinates of 16 homologous landmarks and one homologous semi-landmark (Fig. 1). "Traditional" landmarks and semi-landmark can be used equally after Procrustes superimposition (Gunz and Mitteroecker 2013) and hence the 16 landmarks and the one semi-landmark were combined and treated equally in subsequent analysis (see below). We first included all individuals from the four parental, four F1 and three F2 hybrid lines and three lake radiations in one data set (Table 1). Using MorphoJ v.1.05a (Klingenberg 2011), the landmarks were geometrically scaled to a unit centroid size (= CS) and Procrustes superimposed, which controls for size but retains variation in shape (Rohlf and Slice 1990). For each fish, CS was then used as a measure of size (Zelditch et al 2004). For each observational group (N=14; 4 parental, 4 F1 and 3 F2 hybrid lines and 3 Lake radiations) we regressed the Procrustes coordinates on logCS to create size corrected residuals of the landmark coordinates (Klingenberg 2011). We then used the residuals to calculate the variance-covariance (VCV) and covariance (CV) matrix for each line (i.e. parental, F1 hybrid and F2 hybrid lines) and the three lake radiations. These matrices were used in subsequent analyses. Morphological (i.e. phenotypic) variance-covariance matrices (P matrices) reflect underlying patterns of genetic variance and covariance (G matrices) (Cheverud 1988; Roff 1995; Koots and Gibson 1996). Hence, we discuss morphological P matrices in the context of underlying patterns of heritable morphological variation.

To test whether families within lines differed in their morphological distribution, we calculated Procrustes distances (i.e. a multivariate measure of shape differences) between families within a line and between all lines (Klingenberg 2011). First we tested if the Procrustes distances between all lines and between one or several families within a line were significantly different based on a permutation test (N=10'000 permutations). Second we used a Kruskal-Wallis test with subsequent pairwise Mann-Whitney U tests to see if the Procrustes distances between lines are significantly larger than those found between families within each line and also if the Procrustes distances between families within lines differ between parental, F1 and F2 lines (Table S1).

Prediction 1. Hybridisation increases morphological variance and relaxes covariance structure compared to parental lines when projected into lake radiation morphospaces.

We used VCV matrices to first conduct principal component analyses (PCAs) for each lake radiation. We then projected the morphological variation of each parental and each hybrid line into the PCA of each lake radiation. This approach fixes the PCA axes of each lake radiation and then calculates from VCV matrices PC scores for each of the projected groups based on the fixed PCA axes of the lake radiation. We thereby test whether and to what extent the morphospace of individual parental and hybrid lines predicts patterns of interspecific diversity observed in each lake radiation. We measured the 95% confidence ellipse size on the two leading PC axes for the parental and hybrid lines (Fig. 2) to test the predictions that interspecific hybrids fill a larger (ellipse size) proportion of morphospace of lake radiations than individual species and display more relaxed covariance structure (ellipse structure, i.e. eccentricity).

The 95 % confidence ellipses were calculated and used for calculating the ellipse size for each hybrid line and each parental line and also for the combined range of each of the two parental lines that contributed to a hybrid line. As a measure to compare trait covariance among groups we used the morphological (P) variance/covariance matrix (Schluter 2000). Eccentricity (ε) reflects the shape of a variance/covariance matrix and is calculated as the ratio between the eigenvalues of the two leading eigenaxes (p_{max} and p_2). High eccentricity reflects strong covariance among morphological shape elements, whereas low values occur when ellipses are more circular ($\varepsilon \sim 1$) due to low covariance. We used one-way-ANOVA with Tukey post-hoc-test to evaluate whether the ellipse measures of morphological variation and eccentricity differ between the three groups (parental, F1 hybrid and F2 hybrid line) (Fig. 3). Furthermore we used one-way ANOVA with Tukey post-hoc-tests to determine if the F1 and

F2 hybrid lines displayed higher levels of morphological variation than the combined range of their respective parental lines.

Prediction 2: Comparing morphological variance-covariance structures

We used matrix correlation (R_m) to compare the structures of the variance-covariance (VCV) and covariance (CV - no diagonal elements) matrices of the hybrid and parental lines with those of the three lake radiations. We calculated R_m for all pairwise comparisons between experimental lines and radiations. Statistical significance was assessed using Mantel tests adapted for geometric morphometrics (Klingenberg et al. 2003), using 10,000 random permutations of the landmarks.

Because our sample sizes of the 11 lines and the three radiations varied (Table 1), we controlled for the potential effects of sampling error as follows. The maximum observable correlation between two matrices is not one, but a value, R_{max}, which corresponds to $(t_a t_b)^{0.5}$, where t_a and t_b represent the repeatability of matrices A and B respectively (Cheverud 1996). We calculated VCV (t_{vcv}) and CV (t_{cv}) matrix repeatability for each group following Maroig and Cheverud (2001). We then adjusted observed matrix correlations by dividing the observed matrix correlation (R_{obs}) by the maximum matrix correlation (R_{max}) as: $R_{adj} = R_{obs} / R_{max}$. Each group in the subsequent ANOVA (e.g. parental, F1 hybrid and F2 hybrid) contained the matrix correlation values that derived from comparing the VCV and CV matrices of each line with each of the three lake radiations. We used a one-way-ANOVA with Tukey post-hoc-tests to test if the adjusted pairwise matrix correlations calculated between each line and the three lake radiations differed between the groups (parental, F1 hybrid and F2 hybrid) (Fig. 4).

Prediction 3: Comparing trajectories of morphological diversification

We tested the prediction that the trajectories of morphological diversification observed in extant lake radiations are better predicted by hybrid populations than by individual ancestral species as follows. The VCV matrices of each line and each radiation were calculated independently to extract axes of variation in the global morphospace. For each line-specific PCA we retained the first four PC-axes, which each explained more than 5% of morphological variation (Table S2). We then compared the angles between the principal axes and multidimensional trajectories of each line (ancestral species, F1 hybrid and F2 hybrid line) with those of each lake radiation (LV, LM, LT), and among the lake radiations themselves. We used SpaceAngle6b (Sheets 2001; Zelditch et al. 2004) to compute the angle

between the first axes, 2-D planes, and higher dimensional spaces. First, we calculated the observed angle between two groups by re-sampling specimens from each group with replacement (500 bootstrapped replicates). Second, each group was randomly partitioned into two sub-samples and 4900 bootstrapped angles between them (within-group angles) were calculated. The angle between the two groups was considered significantly different if the angle between groups exceeded the 95% range of within-group angles of both groups (Table 2).

We tested principal axes and higher multidimensional trajectories: i) Pmax = PC1 only, ii) the first two PC axis combined (PC1-2), iii) including all PC axes explaining >5% of variation (PC1-4). As the number of PC axes explaining >5% of variation varied between groups, we used the number of PC axes of the group with the fewest PC axes explaining more than 5% variance for all the other groups too. This was four PC axes. Hence, by excluding axes explaining less than 5% of the variance we focused on testing similarity between the principal trajectories of morphological diversification.

Statistics

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.

Results

Normality assumptions were satisfied (assessed by Shapiro-Wilk tests and Q-Q-plots) for all data sets with one exception for which non-parametric test were used.

The Procrustes distances between families within the parental, F1 and F2 hybrids lines were small and none were significant in the CALxTWE F1, CALxBUR F1, TWExBUR F1, and BURxTWE F2 lines. Also in the other lines in six cases one and in two cases more than one of the Procrustes distances between families was marginally significant. Procrustes distances between lines were on average larger than those between families within a line and all were significantly different (P<0.001). The Procrustes distances between families within parental, F1 and F2 hybrid lines were significantly smaller than those between all lines (parental, F1 and F2 hybrid lines) (Kruskal-Wallis, H=27.17, P<0.001; see Table S1). We acknowledge that data sets with small number of families per lines should be treated with some caution due

306 to possible family effects. In our data set the Procrustes distances between parental, F1 and F2
307 hybrid lines did not differ and hence any possible family effects would have to be small
308 (Table S1).
309
310 *Prediction 1. Hybridisation increases morphological variance and relaxes covariance*
311 *structure*
312 When projected into the morphospace of the three radiations, hybrid lines accounted
313 for more of the total diversity observed in extant radiations than individual parental lines
314 (mean ellipse size and standard deviation: parental lines = 0.16±0.05, F1 hybrids = 0.18±0.08,
315 F2 hybrids = 0.27±0.14; ANOVA, $F_{2,30}$=3.63, P=0.039; Fig. 2 & 3). Tukey post-hoc tests on
316 ellipse sizes revealed that the only significant difference was between F2 and parental lines
317 (F1 versus F2 P=0.110; F1 versus parental P=0.840; F2 versus parental P=0.032).
318 Across all lines, the ellipse sizes of F1 and F2 hybrids were not significantly larger
319 than the ellipses of their combined parental species (parental line = 0.20±0.05, F1 =
320 0.18±0.08, F2 = 0.27±0.14; ANOVA, $F_{2,30}$=2.22, P=0.13). However, this was due to the small
321 morphological variation found both in F1 and F2 hybrid lines of CALxPRO, which were
322 consistently smaller than those of the other hybrid lines. When excluding the combined
323 parental PRO and CAL ellipse sizes and ellipse sizes of the F1 and F2 hybrid CALxPRO
324 lines, the F2 hybrid lines had significantly larger ellipse sizes than their parental species
325 combined (parental line = 0.21±0.05, F1 = 0.20±0.06, F2 = 0.34±0.10; ANOVA, $F_{2,21}$=7.17,
326 P=0.005; post hoc: P=0.006) and the F1 hybrids (post hoc: P=0.005).
327 The parental lines had significantly more eccentric ellipses (mean and standard
328 deviation: ε = 1.81±0.33) than F1 hybrids (ε = 1.53±0.20) and F2 hybrids (ε = 1.27±0.18)
329 (ANOVA, $F_{2,30}$=11.73, P<.001; post-hoc: F1 hybrids vs. parental species P=0.044; F2
330 hybrids vs. parental species P<.001) (Fig. 2 & 3). The F1 hybrids showed a tendency to have
331 more eccentric ellipses than the F2 hybrids (P=0.056).
332
333 *Prediction 2: Comparing morphological variance-covariance structures*
334 Repeatability was high for both VCV (range: 0.88-0.98; mean±sd: 0.95±0.03) and CV
335 matrices (0.86-0.97; 0.94±0.03). All pairwise VCV and CV matrix correlations were
336 significant (Mantel test, all P<0.001). The highest correlations were between the lake
337 radiations, reflecting their parallel diversification into sets of convergent phenotypes (VCV:
338 0.88±0.05; VC: 0.84±0.06). The correlations with the three lake radiations were higher for F2
339 (VCV: 0.71±0.08; CV: 0.60±0.11) and F1 hybrids (VCV: 0.68±0.08; CV: 0.57±0.10) than for

parental species (VCV: 0.64±0.07; CV: 0.50±0.09) (Fig. 4). These differences were almost significant for the CV matrices (one way ANOVA: $F_{2,30}$=2.9, P=0.071; post-hoc: parents vs. F1 P=0.264, parents vs. F2 P=0.062, F1 vs. F2 P=0.723), whereas the trend was somewhat weaker for the VCV matrices ($F_{2,30}$=2.52, P=0.123).

Prediction 3: Comparing trajectories of morphological diversification

The leading axis of morphological variation (PC1) as well as the two- and four-dimensional shape space (PC1-2, PC1-4) did not differ between the three lake radiations, suggesting diversification has accumulated along common trajectories through the global morphospace (Table 2).

In comparisons with the three lake radiations, hybrid lines had more similar diversification trajectories than parental lines, particularly along the principal axes. The parental lines had similar trajectories as the lake radiations in 6 of 12 comparisons (50%) for the principal axes, for 4 of 12 comparisons (33%) for the 2-D plane, and for 6 of 12 comparisons (50%) for the 4-D space (Table 2). For the same comparisons, the F1 hybrids had similar trajectories in 9 of 12 (75%), in 4 of 12 (33%), and 4 of 12 (33%) cases, respectively (Table 2). The F2 hybrids were similar to the lake radiations in 7 of 9 (78%), 4 of 9 (44%), and 5 of 9 (56%) comparisons (Table 2).

Discussion

Theoretically, hybridisation may facilitate adaptive radiation by increasing levels of heritable phenotypic diversity, relaxing genetic constraint, and generating novel trait combinations that provide new trajectories along which diversity can accumulate in response to divergent natural selection. There is empirical evidence demonstrating hybridisation can increase diversity (e.g. Grant and Grant 1994; Albertson and Kocher 2005; Stelkens and Seehausen 2009) and relax constraint (e.g. Parsons et al. 2011; Renaud et al. 2009, 2012), and hybridisation appears common in adaptive radiations (Seehausen 2004; Abbott et al. 2013). To date, however, direct evidence for hybridisation's role in initiating and sustaining diversification in natural adaptive radiations is limited. By combining experimental and comparative methods using putative ancestors and extant radiations of African cichlids, our results provide new support for refined predictions of the 'hybrid swarm origin' hypothesis of adaptive radiation (Seehausen 2004, 2013). Compared to parental lines, hybrid lines display: increased diversity and relaxed constraint when projected into the morphospace of the extant

radiations, have morphological variance-covariance and covariance structures more similar to extant radiations, and have trajectories of diversification that more closely match those of extant radiations in the global morphospace.

Both F1 and F2 hybrids occupied greater volumes of the extant radiation morphospaces compared to their parent species. The difference was pronounced for F2 hybrid lines, which sometimes occupied a significantly greater volume of morphospace than that of both their parental species combined. These observations are consistent with previous work demonstrating that hybridisation can increase morphological diversity in African cichlids (Albertson and Kocher 2005; Stelkens et al. 2009; Cooper et al. 2011; Parsons et al. 2011), and that the magnitude of the effect increases with divergence time between parental species (Stelkens and Seehausen 2009; Stelkens et al. 2009), but only until to a point where hybrid breakdown will occur (Edmands 1999). Such transgressive segregation likely occurs through complimentary gene action, is an important source of additive genetic variation, and is expected to manifest more in F2 than F1 hybrids (Rieseberg et al. 1999; Stelkens and Seehausen 2009). Our results advance this body of work by demonstrating that these general patterns hold when parental and hybrid lines are projected into the morphospace of extant adaptive radiations.

Our ellipse analysis revealed that hybrid lines displayed lower eccentricity than parental lines when projected into the morphospace of the extant radiations. Our first-generation hybrid crosses and particularly our second-generation hybrid crosses showed a significant reduction in covariance among traits when compared to the parental species. These findings suggest hybridisation relaxes genetic constraint, creates new morphological combinations and may thus facilitate phenotypic diversification in response to novel forms of directional and divergent natural selection. Relaxation of the G and P matrices may be particularly important during the early stages of adaptive radiation, when phenotypes are likely to be subjected first to relaxation of previously experienced selection in the ecological release phase (Yoder et al. 2010), followed by complex, multidimensional forms of diversifying selection in directions not previously experienced by these populations (Gavrilets 2004; Ito and Dieckmann 2007). By relaxing constraint, hybridization may first facilitate the expansion to new areas in morphospace in response to ecological release, and second adaptive diversification in response to new diversifying selection in such environments. Hybrid populations may thus be able to evolve along a wider variety of morphological trajectories and respond more quickly to novel selection regimes (Grant and Grant 1994; Deng et al. 1999; Young et al. 2010; Hallgrimson et al. 2012; Villmoare 2013).

Our experimental hybrid lines were not only more diverse with lower eccentricity when projected onto the lake radiations morphospace. Their VCV and CV matrices were more similar to those observed in the extant radiations. This pattern was particularly strong in comparisons with the youngest LV radiation, a point upon which we elaborate below. This pattern was supported by the analysis of trajectories in the global morphospace. Compared to parental lines, hybrid lines were more similar to the extant radiations. The first axes of hybrid line morphological diversity were similar to those of extant radiations more often than were those of parental species. For F2 hybrids, the first axes were consistently similar to those of the LV and LM radiations. Across all dimensions, the morphological trajectories of hybrids were more similar to the youngest LV radiation than to the older radiations.

Our results compliment and extend those of two previous studies that combined experimental and comparative approaches to explore the role of hybridisation in African cichlid adaptive radiation. Cooper et al. (2011) and Parsons et al. (2011) studied patterns of cranial shape variation in second-generation hybrids of LM cichlids and, similar to our results, they found the primary axes of morphological diversity in hybrids matched those of the wider LM radiation. These studies, however, created hybrids between radiation member species taken from within the extant LM radiation, whereas we created hybrids using three putative ancestors and one basal member of the LM radiation. Thus, whereas the previous results speak to the role of hybridisation in the course of adaptive radiation (the 'syngameon' component of the hybrid swarm hypothesis), our design provides the first experimental test for the role of hybridisation in initiating adaptive radiation (e.g. the 'origin' component of the hybrid swarm hypothesis; Seehausen 2004). These studies together provide support for both components of the hybrid swarm hypothesis, and suggest that hybridisation across a range of phylogenetic and temporal contexts may create genetic and phenotypic architectures that manifest more broadly in adaptive radiations.

Unequivocal experimental evidence that hybridisation facilitates niche shifts and promotes adaptive diversification in African cichlids is lacking (Genner and Turner 2012). While relevant experiments are tractable in principle, the approach of comparing patterns of diversity within 'ancestral hybrids' and extant radiations provides valuable insights. Our results reveal that F2 hybrids occupy a significantly larger fraction of the radiations morphospace than their parental species, and that the principal axes of diversity in morphospace amongst hybrids more closely match those observed amongst species of extant radiations. Thus, the novel morphologies and trajectories of 'ancestral hybrids' match those that have arisen in the expanded morphospaces of adaptive radiation (Fig. S1). To the degree

that the morphological diversity observed in extant radiations is adaptive, hybrid phenotypes thus 'predict' the occurrence of niche shifts associated with speciation during adaptive radiation. One likely scenario by which this could occur is if some hybrid genotypes gain a fitness advantage through occupying novel, previously vacant niches and subsequently become new incipient species whilst morphologically diverging in response to novel selection pressures (Seehausen 2004; Mallet 2007). The plausibility of such a scenario is supported by previous work demonstrating that under certain conditions even distantly related cichlids readily hybridise and produce fertile offspring. Stelkens et al. (2009) found that hybrid crosses between cichlid species that had diverged for at least 3, perhaps up to 7 million years were viable and fertile. Furthermore, their crosses included two used in this study (CALxPRO, CALxBUR), which have divergence times similar to the hypothesized multiple ancestors of several large cichlid radiations (LV, Seehausen et al. 2003; LM, Joyce et al. 2011; Loh et al. 2013; Lake paleo-Makgadikgadi, Joyce et al. 2005).

The three lake radiations that we compared with our experimental hybrids differ widely in age (LV, 0.015-0.2 myr; LM, 2-4 myr; LT, 8-16 myr) (Genner et al. 2007), offering a rare temporal insight into patterns of morphological diversification during adaptive radiation. Our results provide a new context for previous work (Young et al. 2009; Cooper et al. 2010) that showed that morphological diversity accumulated rapidly, that levels of extant total diversity are non-linearly age-ordered, and that despite differences in colonization history, phylogenetic context and ecological conditions, the three radiations are diversifying along similar morphological trajectories (Young et al. 2009; Cooper at al. 2010). Our results are consistent with the idea that hybridisation contributes to the early bursts of diversification observed in cichlid radiations. The patterns of diversity in the second-generation hybrids most closely resembled those of the youngest LV radiation, to a lesser extent those of LM, and were least similar to those of the oldest LT radiation. Combined with molecular evidence implicating an initial and on-going role of hybridisation in the two younger radiations (Seehausen et al. 2003; Streelman et al. 2004; Joyce et al. 2011; Genner and Turner 2012; Keller et al. 2013; Loh et al. 2013), this body of work suggests hybridisation may play a key role during the initial stages of diversification, while the role of mutation in providing heritable variation may increase through time.

Support for the 'hybrid swarm origin' hypothesis (Seehausen 2004) can come from three complementary types of evidence. First, there should be evidence for hybridisation that predates the radiation. Second, there should be evidence that the patterns of morphological diversity observed in extant radiations are derived principally from hybridisation between

divergent lineages rather than de-novo mutations. Third, there should be evidence that the morphological diversity among species that originated through hybridisation is adaptive. Molecular evidence from LV and the Mbuna radiation of LM is consistent with the first prediction (Seehausen et al. 2003; Joyce et al. 2011; Loh et al. 2013). This study provides support for the second prediction by showing that experimental hybridisation between putative ancestor species creates patterns of morphological diversity that predict those observed in extant radiations. To the degree that extant patterns of between-species diversity in these radiations are adaptive, our results also support the third prediction, though the definitive test will require multigenerational experiments subjecting parental and hybrid lines to ecologically relevant divergent natural selection. Such experiments are not easily feasible with cichlids. However, we suggest that combining experimental hybridisation with comparative analyses of morphological diversity and genomic analyses of the underlying genetic changes as well as their phylogenetic histories will be the way to go in exploring the role of hybridisation in adaptive radiation.

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Table 1. Number of families and individuals in each parental and hybrid line and each lake radiation. For the three lake radiations 99 species of each Lake Victoria and Lake Tanganyika and 97 species from Lake Malawi were used (one individual per species).

Parental line	**N families (N total number of individuals (bold): N individuals per family)**
A.burtoni (BUR)	4 (**46**: 8 / 11 / 13 / 14)
A.calliptera (CAL)	3 (**68**: 16 / 19 / 33)
P.taeniolatus (PRO)	3 (**56**: 11 / 22 / 23)
A.tweddlei (TWE)	2 (**29**: 8 / 21)

Hybrid line		**N families (N total (bold): N individuals per family)**	
Female parent	**Male parent**	**F1 Hybrid**	**F2 Hybrid**
A.calliptera (CAL)	*P.taeniolatus* (PRO)	2 (**64**: 22 / 42)	2 (**93**: 21 / 72)
A.calliptera (CAL)	*A.burtoni* (BUR)	4 (**45**: 2 / 6 /18 / 19)	3 (**62**: 9 / 15 / 38)
A.burtoni (BUR)	*A.tweddlei* (TWE)	3 (**45**: 5 / 16 / 24)	4 (**69**: 1 / 2 / 26 / 40)
A.tweddlei (TWE)	*A.burtoni* (BUR)	2 (**54**: 24 / 30)	2 (**29**: 1 / 28)
A.calliptera (CAL)	*A.tweddlei* (TWE)	2 (**21**: 6 / 15)	

Table 2. Comparing trajectories of morphological diversification. Principal component axes derived from line-specific PCAs were used to compare the angles between the axes of each of the parental, F1 hybrid and F2 hybrid lines to each of the lake radiations and among lake radiations. The orientation in shape space (angle in degrees between the two groups compared, based on PC1, PC1-2 and PC1-4) is considered significantly different ($P < 0.05$, in bold) if the observed bootstrapped (500 times) angle between groups exceeds both within-group bootstrapped (4900 times) angles between sub-samples of each group. See Table 1 for abbreviations.

	Comparison		PC1 max. angle = 90		PC1-2 max. angle = 127.28		PC1-4 max. angle = 180	
Generation	Group 1	Group 2	Observed Angle Between Groups	Within-Group Angles Group1/ Group 2	Observed Angle Between Groups	Within-Group Angles Group1/ Group 2	Observed Angle Between Groups	Within-Group Angles Group1/ Group 2
	LV	**LM**	45.55	65.40 / 81.67	42.01	68.65 / 46.44	55.01	89.60 / 43.42
	LV	**LT**	39.7	66.45 / 21.91	60.71	68.04 / 84.57	80.92	89.56 / 89.43
	LM	**LT**	26.3	81 / 21.82	35.33	44.35 / 84.70	88.16	43.18 / 89.40
	BURxBUR	**LV**	68.25	51.59 / 77.68	**90.95**	55.76 / 80.72	**98.97**	96.31 / 91.42
	CALxCAL	**LV**	60.46	57.72 / 72.77	**74.72**	41.55 / 74.45	91.98	93.71 / 90.30
	PROxPRO	**LV**	**88.54**	61.23 / 77.06	79.15	90.42 / 80.23	94.22	100.41 / 91.02
	TWExTWE	**LV**	**84.4**	68.26 / 82.57	**89.7**	69.90 / 84.51	**114.31**	105.70 / 93.46
F1	**CALxPRO**	**LV**	54.06	44.89 / 75	89.24	89.59 / 76.52	99.09	100.16 / 90.62
F1	**CALxBUR**	**LV**	63.89	35.51 / 77.72	67.67	84.36 / 80.86	**97.13**	90.36 / 91.43
F1	**BURxTWE**	**LV**	37.56	71.87 / 65.63	64.86	51.43 / 68.32	**93.84**	72.61 / 89.04
F1	**CALxTWE**	**LV**	66.41	87.41 / 84.79	66.07	88.63 / 87.47	96.52	106.58 / 96.05
F2	**CALxPRO**	**LV**	50.92	86.23 / 67.19	62.53	89.05 / 71.59	88.51	94.71 / 89.45
F2	**CALxBUR**	**LV**	59.85	46.07 / 74.80	66.22	52.28 / 76.37	77.72	93.87 / 90.76
F2	**BURxTWE**	**LV**	46.78	43.40 / 66.01	57.28	51.03 / 69.80	**97.5**	93.52 / 89.93
	BURxBUR	**LM**	**88.44**	51.22 / 85.23	**96.3**	55.58 / 68.60	92.33	95.78 / 66.22
	CALxCAL	**LM**	58.44	60.53 / 84.03	**92.32**	42.15 / 53.11	87.09	93.34 / 51.71
	PROxPRO	**LM**	67.42	60.48 / 84.48	76.77	90.30 / 60.74	97.64	100.93 / 60.89
	TWExTWE	**LM**	49.45	68.64 / 86.20	**83.15**	69.50 / 82.70	101.37	105.30 / 82.32
F1	**CALxPRO**	**LM**	62.32	45.51 / 84.13	**93.52**	90 / 54.40	**113.15**	99.70 / 53.03
F1	**CALxBUR**	**LM**	**87.55**	35.45 / 84.80	**91.67**	84.06 / 72.87	87.63	89.92 / 69.11
F1	**BURxTWE**	**LM**	46.92	71.70 / 81.64	**92.78**	51.09 / 45.26	**88.9**	70.37 / 44.13
F1	**CALxTWE**	**LM**	75.26	87.32 / 86.77	**90.72**	87.82 / 86.49	97.66	105.93 / 90.11
F2	**CALxPRO**	**LM**	60.78	86.42 / 82.27	87.79	88.93 / 47.21	90.41	94.29 / 44.61
F2	**CALxBUR**	**LM**	83.26	44.31 / 83.72	**89.33**	52.65 / 56.59	82.09	94.16 / 54.85

F2	**BURxTWE**	**LM**	60.24	44.02 / 81.45	**79.85**	50.97 / 45.87	**102.77**	93.19 / 43.93
	BURxBUR	**LT**	**81.19**	51.60 / 28.87	**101.35**	55.93 / 86.19	**106.11**	95.59 / 91.81
	CALxCAL	**LT**	**69.23**	56.59 / 24.62	83.03	41.10 / 85.57	**103.92**	93.84 / 90.26
	PROxPRO	**LT**	**85.76**	59.01 / 26.42	81.14	90.44 / 86.41	**118.74**	100.10 / 91.03
	TWExTWE	**LT**	56.75	67.26 / 35.84	87.47	71 / 87.53	**122.62**	104.70 / 94.35
F1	**CALxPRO**	**LT**	**74.27**	45.25 / 25.06	**98.7**	89.77 / 85.78	**120.24**	100.02 / 90.80
F1	**CALxBUR**	**LT**	**88.37**	34.55 / 29.18	**87.05**	83.85 / 86.82	**91.95**	90.06 / 91.85
F1	**BURxTWE**	**LT**	53.71	71.07 / 21.87	**85.63**	51.23 / 83.82	86.41	71.26 / 89.54
F1	**CALxTWE**	**LT**	82.66	87.50 / 42.55	**102.33**	89.03 / 88.83	**107.74**	106.58 / 98.48
F2	**CALxPRO**	**LT**	56.74	85.64 / 22.44	**97.72**	89.16 / 84.59	92.82	94.79 / 89.26
F2	**CALxBUR**	**LT**	**82.82**	43.21 / 25.50	**100.35**	52.28 / 85.55	**94.43**	93.79 / 90.72
F2	**BURxTWE**	**LT**	**62.9**	42.93 / 21.63	**97.07**	51.28 / 84.72	**107.78**	93.54 / 89.10

Figure 1. Location of landmarks used in morphometric analysis. F2 hybrid individual between the two riverine cichlid species *Astatotilapia burtoni* and *A. tweddlei*. Numbers mark the 17 landmarks used for geometric morphometric analysis in this study: 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).

Figure 2. Patterns of morphological variation and eccentricity of parental and hybrids lines when projected into the morphospace of the three Lake radiations. Principal component plots (PCA) with the first two components from the morphospace of each Lake radiation (black dots). The morphological variation of each parental lines and the respective F1 (light blue) and F2 (dark blue) hybrid lines (Panel A: CALxPRO; Panel B: only F1 CALxTWE; Panel C: BURxCAL; Panel D: BURxTWE) are projected in the morphospace of each Lake radiation (black dots from left to right in each panel: LV, LM and LT). Circles represent the 95% confidence ellipses. F1 and F2 hybrid lines on average showed larger morphological variation (ellipse size) than did the parental lines and the ellipses of both the F1 and F2 hybrid lines were less eccentric than those of the parental lines. See Table 1 for abbreviations.

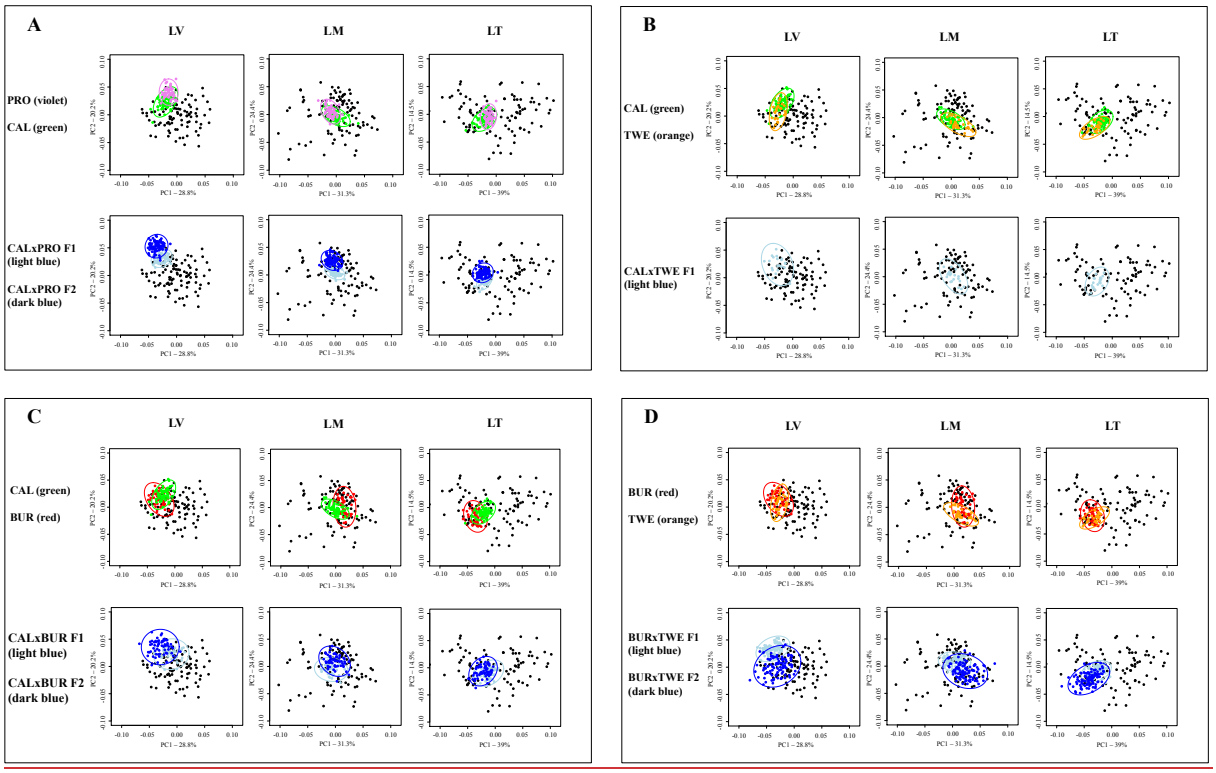

Figure 3. Ellipse sizes and eccentricities derived from the projection of each parental, F1 or F2 hybrid line into the morphospace of each Lake radiation. The ellipse sizes and eccentricities of the parental (in black; from left to right: BUR, CAL, PRO, TWE) and hybrid lines (F1 in dark grey; from left to right: CALxTWE F1, CALxPRO F1, CALxBUR F1, BURxTWE F1, and F2 in mild grey; from left to right: CALxPRO F2, CALxBUR F2, BURxTWE F2) when projected into the morphospace of each lake radiation (from left to right: LV, LM, LT). The upper row shows the ellipse sizes of each group (parental, F1 hybrid and F2 hybrid line) and the middle row shows the eccentricity for the ellipse of each group. When projecting the parental and hybrid lines into the morphospace of each lake radiation the F1 and F2 hybrid lines on average showed larger morphological variation (ellipse size) than did the parental lines and the ellipses of both the F1 and F2 hybrid lines were less eccentric than those of the parental lines. See Table 1 for abbreviations.

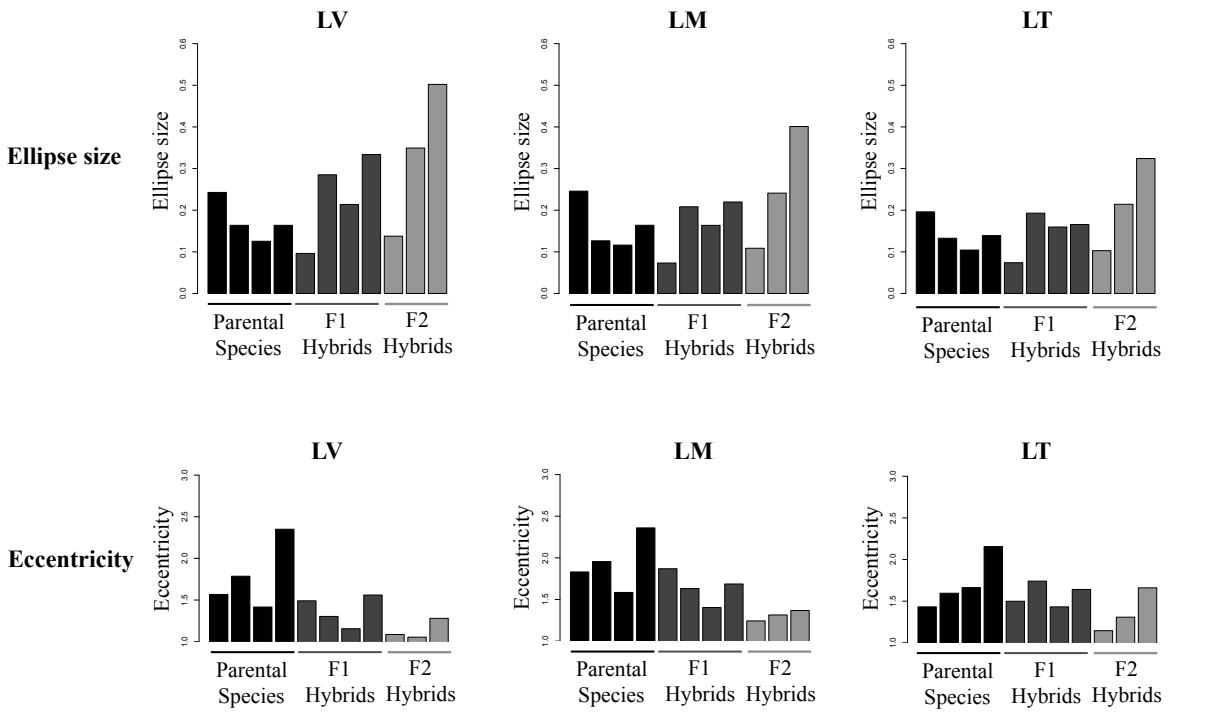

Figure 4. Variance-covariance and covariance matrix correlations between parental and hybrid lines and the lake radiations. Matrix correlations (from Mantel tests) for comparisons of the parental lines (in black; from left to right: BUR, CAL, PRO, TWE), F1 hybrid (in dark grey; from left to right: CALxTWE F1, CALxPRO F1, CALxBUR F1, BURxTWE F1) and F2 hybrid lines (in mild grey; from left to right: CALxPRO F2, CALxBUR F2, BURxTWE F2)) with each lake radiation (from left to right: LV, LM, LT) and among the lake radiations (in light grey). A gradual increase of the correlations from parental to F1 hybrid lines, F2 hybrid lines and lake radiations was observed both for variance-covariance matrices (upper row) and covariance matrices (lower row). The correlations with the three lake radiations were higher for F2 than for parents and based on the CV matrices showed a trend to differ between the two groups.