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ORIGINAL ARTICLE

Genetic interactions controlling sex and color establish the potential for sexual conflict in Lake Malawi cichlid fishes

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Sex-determining systems may evolve rapidly and contribute to lineage diversification. In fact, recent work has suggested an integral role of sex chromosome evolution in models of speciation. We use quantitative trait loci analysis of restriction site-associated DNA -tag single nucleotide polymorphisms to identify multiple loci responsible for sex determination and reproductively adaptive color phenotypes in Lake Malawi cichlids. We detect a complex epistatic sex system consisting of a major female heterogametic ZW locus on chromosome 5, two separate male heterogametic XY loci on chromosome 7, and two additional interacting loci on chromosomes 3 and 20. Our data support the known chromosomal linkage between orange blotch color and ZW, as well as novel genetic associations between male blue nuptial color and two sex determining regions (an XY and ZW locus). These results provide further empirical evidence for a complex antagonistic sex–color system in this species flock and suggest a possible role for, and effect of, polygenic sex-determining systems in rapid evolutionary diversification.
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INTRODUCTION

One of the most fundamental and surprisingly diverse processes in the life history of an organism is the determination of sex. An array of environmental and genetic controls has been discovered across divergent taxa, yet in the majority of cases the sex-determining loci remain unidentified. Although sex-determining systems are frequently associated with a sex-specific heteromorphic chromosome pair (that is, male XY or female ZW), more cryptic and complex systems are widespread (Ezaz *et al.*, 2006). For example, among teleost fishes one finds both simple and polygenic sex determination, yet no general patterns exist and even the master sex-determining loci may vary within genera (Matsuda *et al.*, 2002; Nanda *et al.*, 2002; Mank *et al.*, 2006; Yoshida *et al.*, 2011). The maintenance of functional diversity in such an integral genetic and developmental system sets sex determination apart from other more highly conserved pathways (Wilkins, 1995; Carroll, 2000).

The importance of reproductive isolation in speciation has led to interest in the role of intersexual conflict, and sex-determining systems, in this process (Kitano *et al.*, 2009; Qvarnstrom and Bailey, 2009; Meiklejohn and Tao, 2010). Sexually antagonistic selection is known to produce intersexual conflict (Rice, 1992, 1998; Chapman, 2006) and possible resolution of such conflict involves the evolution of sex-determining loci linked to alleles favoring male- or female-specific traits (Kocher, 2004; Streelman *et al.*, 2007). Lande *et al.* (2001) incorporated color-based sexual selection and sexual conflict into speciation models, with cichlid fishes in mind. In this conception, a dominant female sex determiner (W) invades an XY male heterogametic system, reinforced by a W-linked novel color and unlinked loci for mate preference. Divergence is achieved in sympatry by assortative mating through a complex interplay of new sex systems, repressors/modifiers of sex determination and linked color patterns.

Kocher (2004) envisions a complementary scenario in which new sex-determining systems and associated sex-specific color patterns are favored during different stages of population growth, after founder events due to changes in lake levels. Together, these models suggest (i) the evolution of new sex-determining systems linked to male- or female-specific traits (that is, color patterns) and (ii) the possibility of a 'speciation engine' (Kocher, 2004) as assortative mating by novel color patterns resolves sexual antagonism. In theory, it is possible that new and old sex-determining loci might remain polymorphic within and between populations, providing standing variation for interacting sex-determining systems (Wilkins, 1995; van Doorn and Kirkpatrick, 2007, 2010). The evolutionary dynamics of sex systems as a result of sexually antagonistic selection has been suggested as important for the high speciation rates of some fishes (Seehausen *et al.*, 1999a; Lande *et al.*, 2001; Kocher, 2004; Ross *et al.*, 2009), including the cichlids of Lake Malawi (LM), East Africa.

The LM cichlid flock consists of hundreds of species that have arisen in the past 500 000 to 1 000 000 years, presumably the fastest and the most extensive vertebrate adaptive radiation known (Won *et al.*, 2005; Seehausen, 2006). The rapidity of this radiation, extreme endemism (Parnell and Streelman, 2011) and continued low levels of gene flow between species (Mims *et al.*, 2010) has led to a phenomenon wherein genetic incompatibilities between lineages are considerably outpaced by speciation (Stelkens *et al.*, 2010). The integrity of species is maintained by pre-zygotic mechanisms—assortative mating based on visual (Seehausen *et al.*, 1997, 2008; van Oppen *et al.*, 1998; Knight and Turner, 2004; Maan *et al.*, 2004; Carleton, 2009), auditory (Amorim *et al.*, 2004, 2008; Simoes *et al.*, 2008; Smith and van Staaden, 2009; Danley *et al.*, 2011) and olfactory cues (Jordan *et al.*, 2003; Plenderleith *et al.*, 2005; Cole and Stacey, 2006) that sometimes breakdown. Interspecific and intergeneric

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hybridization has been well documented within all of the African cichlid species flocks, including LM (Ruber et al., 2001; Salzburger et al., 2002; Smith and Kornfield, 2002; Smith et al., 2003; Streelman et al., 2004; Gerlai, 2007; Mims et al., 2010). Hybridization among such young species often produces viable, fertile offspring and maintains standing genetic and phenotypic variation (that is, the 'hybrid swarm' hypothesis, Seehausen, 2004; Albertson and Kocher, 2005; Genner and Turner, 2005; Loh et al., 2008; Parnell et al., 2008). In fact, researchers exploit this situation, where species behave genetically like populations, by designing interspecific (or intergeneric) crosses to identify links between genotype and phenotype (Streelman et al., 2003, Albertson et al., 2005; Parnell et al., 2012).

Using this strategy, Streelman, Albertson and Kocher mapped the genetic basis of orange-blotch (OB) color to linkage group 5 in an intergeneric cross between Labeotropheus fuelleborni and Metriaclima zebra, two members of LM's rock-dwelling lineage of species (Streelman et al., 2003). They showed that OB was presumably linked to a female heterogametic sex determiner (W), while a male heterogametic locus (Y) was observed on a different chromosome (Albertson, 2002). The segregation of (i) OB on linkage group 5 within multiple species of rock-dwelling Malawi cichlids (Roberts et al., 2009), as well as (ii) both sex-determining systems within species of the genus Metriaclima (Ser et al., 2010) has been confirmed using selective screening of color- and/or sex-associated markers. The study by Ser et al. (2010) is particularly notable here because it demonstrated that some Metriaclima species segregate both XY and ZW sex systems and also speculated that other sex determiners must be present in the genomes of LM rock-dwelling cichlids.

The goal of our study is twofold. First, we aim to identify additional loci contributing to sex determination and color pattern in LM rock-dwelling cichlids and to ask whether these, as well as previously identified loci, segregate in unstudied rock-dwelling species. Second, we develop an experimental design powered to quantify, at a genome-wide scale, the genetic contributions and interactions among loci with effects on sex and color. Using quantitative trait loci (QTL) analysis of restriction site-associated DNA (RAD)-tag single nucleotide polymorphisms (SNPs) in a new F_2 hybrid cross, we identified five genomic regions (and epistatic interactions) contributing to sex determination in LM rock-dwelling cichlids. We confirm the association between a major ZW locus and OB color and highlight novel linkage between male nuptial color and both the ZW plus a male-heterogametic XY system. Our results are bolstered by consistency with previous work in different LM rock-dwelling species and contribute to an overall understanding of the interplay between sex and color genetic systems in this young species assemblage.

MATERIALS AND METHODS
Production of F_2 to map sex and color in new species
We chose two LM cichlid species from the rock-dwelling (mbuna) clade, Cynotilapia afra and Pseudotropheus elongatus, in which sex determination has not been studied previously. These species co-occur at several sites across the lake and differ in a number of phenotypes of interest, including craniofacial morphology, coloration, tooth shape and density and gut length. Fishes collected from LM were acquired from Old World Exotics fish suppliers (Miami, FL) and several C. afra males were placed in a 189-L aquarium with P. elongatus females at a ratio of 1:2. Within a few weeks, the dominant male (and subsequent sire) had established an obvious hierarchy over his conspecifics and had fertilized the eggs of the dam. Fin clips (25 mm²) were taken from both the dam and sire for RAD-tag library preparation and the pair was kept in a breeding tank with dither fish to diffuse male aggression. Offspring were taken from the dam after hatching, grown up in net pens and moved to

consecutively larger aquaria as they grew. This pair produced several F_1 broods, which were maintained and intercrossed ($F_1 \times F_1$) in 189-L aquaria for 2 years during which time they produced over 40 broods totaling some 600 F_2 offspring (mean brood size = 15). Each F_2 family was isolated and numbered by brood sequence. A total of 397 F_2 hybrids were euthanized (tricaine methane sulfonate, MS-222, in accordance with established Georgia Institute of Technology IACUC protocols) and processed for DNA samples and a suite of morphological traits (for example, below; Parnell et al., 2012). When sampled, individuals were at least 75 mm total length (approximately 1-year old).

Color assignment and sex determination
Following euthanasia, hybrid individuals were photographed in a full-body lateral view for color assignment. The C. afra sire (Mara yellow type) had yellow body color with black bars (for example, Figure 1a). The P. elongatus dam exhibited the yellow–orange 'O' color, putatively the homozygous morph of OB that lacks black blotches (Figure 1i; Lande et al., 2001). All F_1 hybrid individuals were OB. We used binary scoring to map the genetic basis of OB (Supplementary Figure S1); F_2 individuals were assigned to two color classes, 'OB' where 'O' is also scored OB (1) or 'not OB' (0). We observed a blue color among male F_2 that was not seen in either of the parental individuals (see Figure 1) but is a known nuptial phenotype common in these and other LM species (Ribbink et al., 1983; Svensson et al., 2011). To map the genetic basis of blue color, we assigned F_2 individuals to two categories, 'blue' (1) or 'not blue' (0). Note that with this assignment scheme, it is possible for fish to be both OB and blue (Figure 1f; Supplementary Figure S1) and in fact this phenotype is observed in males of P. elongatus. Fish were dissected and sex was determined from gross morphology (all fish were sexually mature) and assigned binary coding as male (1) or female (0).

DNA extraction, RAD library preparation and Illumina Sequencing
Malawi cichlids have highly similar genomes and share polymorphism across population, species and major lineage boundaries; for instance, the nucleotide diversity observed across survey-sequenced genomes from five diverse representatives of the species flock was less than that among laboratory strains of zebrafish (Loh et al., 2008). Therefore, we sought a strategy to identify informative markers for mapping before the genotyping phase of our study. Genomic DNA was purified from sire and dam fin tissue using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) and RAD-tag libraries were prepared and sequenced from each as described elsewhere (Parnell et al., 2012). The RAD libraries for each parent were assembled, aligned and alternately fixed SNPs were identified and selected for genotyping in the intercross population (F_2).

SNP genotyping in F_2 individuals
A total of 382 F_2 individuals as well as sire and dam were genotyped at 384 SNP markers by the Emory Biomarker Service Center (Emory University). The Illumina (San Diego, CA, USA) BeadArray genotyping platform was coupled with the GoldenGate assay (Illumina) in which oligonucleotide pool assays are designed specifically to discriminate between alleles at a SNP (Oliphant et al., 2002; Fan et al., 2003). Marker genotypes were examined to evaluate accuracy of initial sequencing during SNP discovery and 12 loci were removed because they did not segregate between the parents. Another 12 markers that contained one heterozygous parental genotype were removed, as well as seven others with low genotyping success in either parental or F_2 individuals. This left a fully informative set of 353 SNP markers, with nearly complete genotypic data across the F_2 population (0.6% missing data).

Genetic linkage map construction
A genetic linkage map was produced with SNP marker genotype data using JoinMap 3.0 (Dyazma, Wageningen Netherlands) software (van Ooijen and Voorrips, 2001), as described (Parnell et al., 2012). The map was created using Kosambi's mapping function, a logarithm of the odds (LOD) threshold of 1.0, a recombination threshold of 0.4, a jump threshold of 5.0 and a ripple function with no fixed order of loci. A LOD threshold of 4.0 was used to join 344 loci in 22 linkage groups with a total map size of 1082 cM and average marker distance 3.67 ± 1.71 cM. To facilitate comparison to other genetic maps in Malawi (Streelman et al., 2003; Albertson et al., 2005) as well as tilapiine
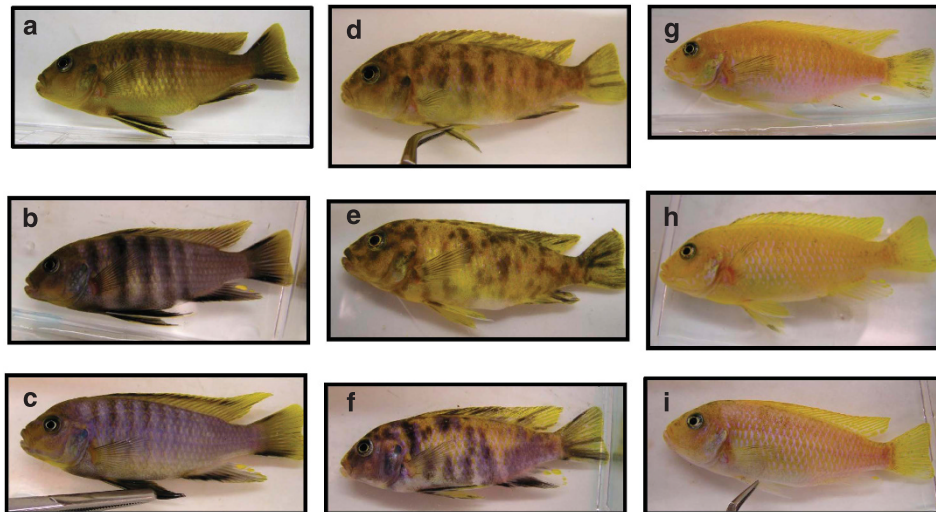
Figure 1 Representative color phenotypes of F_2 hybrid individuals. (**a**) Representation of the sire color pattern, *Cynotilapia afra* (although this is a F_2 individual pictured) and (**i**) displays the dam, *Pseudotropheus elongatus* ('O' phenotype = homozygous WW) phenotype. (**b**), (**c**) and (**f**) show individuals scored as 'blue' with (**b**) and (**c**) being blue-barred and (**f**) as blue-blotched ('blue-OB'). Individuals (**d**), (**e**) and (**g**–**i**) represent the 'OB' phenotype with (**g**–**i**) displaying the 'O' color (homozygous WW), which does not occur in males. (**d**) and (**e**) are OB individuals representative of the phenotype of all F_1 individuals produced.

cichlids (Lee *et al.*, 2005), our linkage group names represent consensus cichlid chromosomes.

QTL mapping of sex and color

The linkage map was used to determine genomic locations for sex, OB and blue color in the F_2 population using the R/qtl package (Broman and Sen, 2009). We used an iterative approach by scanning for single QTL with standard and composite interval mapping, followed by two-dimensional scans to (i) identify QTL by QTL interactions and (ii) detect additional QTL. Finally, using results of the previous steps we built multiple QTL models incorporating QTL interactions and covariates. In the multiple QTL model process, we used a forward–backward selection algorithm to add and remove QTL based on overall model effects and the effects of single QTL as they were removed from the model. Genotype–phenotype associations are scored using the LOD, which represents the $\log_{10}$ likelihood ratio comparing the hypothesis of a QTL at a marker location to the null hypothesis of no QTL (LOD = $(n/2)\log_{10}$ (RSS_0/ RSS_1); RSS = residual sum of squares, Broman and Sen, 2009). The variance in a phenotype is assigned to each significant QTL (or covariate) and reported as percentage variance explained (PVE) in the analysis output. The total variance accounted for by QTL is a proxy for the heritability of a trait and is calculated as $1-10^{-(2/n)LOD}$ (Broman and Sen, 2009). Significance thresholds for LOD scores were estimated using 1000 permutations of phenotypes relative to genotypes to build a distribution of maximum genome-wide LOD scores. From this distribution, the 95th percentile LOD score was calculated to serve as a threshold for significant QTL associations (Broman and Sen, 2009). Due to the discrete nature of our data set, we used the Haley–Knott regression mapping method and binary phenotype modeling.

RESULTS

Sex

Our dataset for the analysis of sex included 353 individuals, with complete phenotype data exhibiting a sex ratio of 58:42 female to male. Two QTL of major effect were detected on chromosomes 5 and 7 during the single-locus scan for sex (Figure 2). Simultaneously scanning for two QTL plus interactions supported these loci and indicated a second locus on chromosome 7 exhibiting epistasis, with the major effect locus on chromosome 5. Using the results of the single and double scans, a multiple QTL model was constructed and
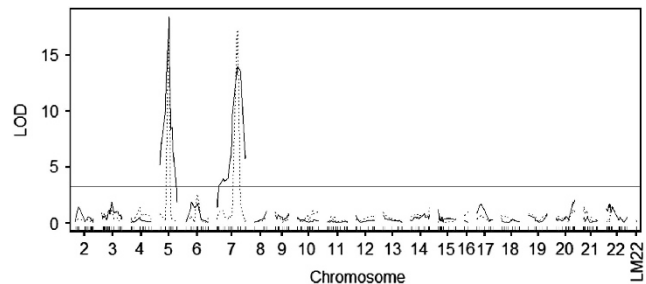
Figure 2 Single QTL scans for loci affecting sex. The solid line indicates standard interval mapping and dashed line indicates composite interval mapping. The horizontal axis denotes chromosome number with hash marks representing marker positions; vertical axis is log-likelihood score (LOD) of marker association with phenotype. The horizontal bar indicates significant LOD score (3.5; $P<0.05$).

tested in multiple iterations, producing a significant final model (LOD = 53.36). The full model incorporated five loci, three epistatic effects and accounted for over 50% of variance in sex (PVE = 51.5; see Table 1).

In the multiple QTL model, the largest effect was seen at position 26.3 cM on chromosome 5 (5@26.3), explaining an estimated 23.5% of the phenotypic variance and matching the precise position indicated in single and double QTL scans. This region of chromosome 5 has previously been suggested as the location of a ZW sex-determining system in which the female allele (W) is dominant (Streelman *et al.*, 2003; Ser *et al.*, 2010). The QTL behaves in a roughly similar fashion here (Figure 3 and Table 2). Individuals with WW genotypes were always female, regardless of genotypes at other loci, while sex ratios (fraction of females) for ZW and ZZ genotypes were 0.54 and 0.43, respectively. The second large-effect locus (7@67.2) accounted for 10.83% of the variance (LOD = 14.88) in sex (Table 1), and its action and position suggests that it represents an XY system (Ser *et al.*, 2010). The major ZW locus is semi-dominant

in sex determination to this XY and all other loci; two W alleles at the ZW locus trump any combination of alleles elsewhere in the genome. However, the sex of ZW and ZZ individuals depends strongly upon genotypes at 7@67.2, as well as those at other loci (Figure 3 and below).

An unlinked QTL identified on chromosome 20 (20@28.1) exhibited significant effects in the model (LOD = 11.44, PVE = 8.14)

Table 1 Results of multiple QTL mapping (MQM) for factors controlling sex

	df	LOD	PVE	P-value (χ^2)
Full model	25	53.36	51.46	<0.000001

Drop one QTL at a time ANOVA table:

	df	LOD	PVE	P-value (χ^2)	Sig.
5@26.3 (ZW)	6	29.15	23.50	<0.000001	***
7@67.2 (XY)	6	14.88	10.83	<0.000001	***
20@28.1 (X′Y′)	12	11.44	8.14	<0.000001	***
3@27.0 (Z′W′)	6	6.30	4.32	0.000006	***
7@44.2 (X″Y″)	6	5.80	3.97	0.000164	***
brood	3	3.75	2.53	0.0006	***
20@28.1:7@67.2	4	6.10	4.18	0.000012	***
3@27.0:20@28.1	4	5.24	3.57	0.000075	***
5@26.3:7@44.2	4	4.09	2.77	0.000842	***
20@28.1:brood	2	1.86	1.24	0.013884	*

Abbreviations: ANOVA, analysis of variance; LOD, logarithm of the odds; PVE, percentage variance explained; QTL, quantitative trait loci.
The log-likelihood score (LOD), PVE, and the χ^2 P-value for the full model are indicated in the top portion. The lower portion of the table represents the results of dropping a factor from the model one at a time. Putative QTL are indicated as chromosome@position in centimorgans (cM), 'brood' represents inclusion of family as a cofactor and epistatic interactions are indicated by factor:factor notation. Significant results are indicated as *P<0.05, **P<0.005 and ***P<0.0005.

and epistasis with XY (Table 1). This locus behaves similarly to the putative XY (thus is labeled X′Y′) and interacts with a fourth QTL on chromosome 3 (3@27.0) that subsequently behaves like a second ZW (thus labeled Z′W′). A fifth QTL (7@44.2) was detected 23 cM proximal to the putative XY (7@67.2). Genotypes here are similar to those at the distal XY site due to linkage; however, they differ in 37% of F_2 individuals. The behavior of this locus in sex determination appears to be as a complementary effector to the XY, functioning as another male determiner (thus labeled X″Y″). Epistatic interactions had modest variance effects and relatively low LOD scores, yet they are integral to the strength of the overall model as well as the explanation of the effects of haplotype on sex (Figure 3). A significant effect of F_2 family ('brood') was detected in this analysis. This is most likely due to skewed sex ratios in a handful of families (4 skewed female, 1 skewed male) as well as a single family that was entirely male. Parentage (individual identity of mother or father) of F_2 fish had no effect on sex when held as a covariate.
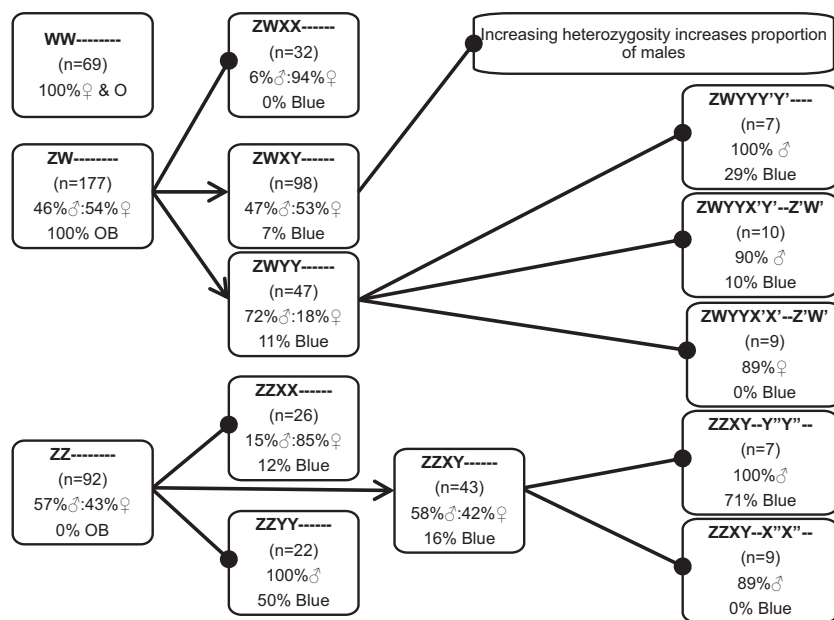
Color

Color phenotypes were available for 340 F_2 individuals. We mapped two color traits, orange blotch (OB) and blue (Figure 1 and

Table 2 Association between sex and genotype at the putative ZW locus (chromosome 5 at 26.3 cM)

	ZZ	ZW	WW
Male	57% (n = 52)	46% (n = 82)	0
Female	43% (n = 40)	54% (n = 95)	100% (n = 69)

All homozygous 'W' individuals are female, while heterozygotes were slightly skewed towards females and homozygous 'Z' individuals were slightly skewed towards males, due to additional loci and their interactions.

Figure 3 Patterns in sex and color phenotypes based on associated haplotypes at loci identified in multiple QTL models (see text). Locus order in the figure is as follows: ZW→XY→X′Y′→X″Y″→Z′W′ (see Table 1). Locus positions are: ZW = 5@26.3, XY = 7@67.2, X′Y′ = 20@28.1, X″Y″ = 7@44.2, Z′W′ = 3@27.0. Arrowheads indicate that haplotype patterns are further resolved at the next step while filled circles indicate the final level of resolution. Dashes within a haplotype represent locus genotypes that do not show a clear effect on the phenotype.

Table 3 Results of multiple QTL mapping (MQM) for factors controlling the orange blotch (OB) color pattern

	df	LOD	PVE	P-value (χ^2)
Full model	5	311.82	98.54	<0.000001

Drop one QTL at a time ANOVA table:

	df	LOD	PVE	P-value (χ^2)	Sig.
5@26.3	4	308.58	94.24	<0.000001	***
sex	3	0.36	0.01	0.655	
5@26.3:sex	2	0.12	0.00	0.761	

Abbreviations: ANOVA, analysis of variance; LOD, logarithm of the odds; PVE, percentage variance explained; QTL, quantitative trait loci.
The log-likelihood score (LOD), PVE and the χ^2 P-value for the full model are indicated in the top portion of the table. The lower portion of the table represents the results of dropping a factor from the model one at a time. Putative QTL are indicated as chromosome@position in centimorgans (cM), 'sex' represents inclusion of sex as a cofactor and epistatic interactions are indicated by factor:factor notation. Significant results are indicated as *$P<0.05$, **$P<0.005$ and ***$P<0.0005$.

Table 4 Results of multiple QTL mapping (MQM) for factors controlling blue color

	df	LOD	PVE	P-value (χ^2)
Full model	31	63.25	57.54	<0.000001

Drop one QTL at a time ANOVA table:

	df	LOD	PVE	P-value (χ^2)	Sig.
sex	9	28.22	19.77	<0.000001	***
7@44.2	10	13.76	8.70	<0.000001	***
LM22@0.8	4	13.53	8.54	<0.000001	***
5@27.9	6	10.08	6.21	<0.000001	***
14@42.1	4	9.85	6.06	<0.000001	***
6@43.8	4	8.79	5.37	<0.000001	***
15@15.7	4	7.29	4.41	<0.000001	***
18@39.8	6	5.77	3.45	<0.000001	***
sex:LM22@0.8	2	8.41	5.13	<0.000001	***
5@27.9:7@44.2	4	8.05	4.89	<0.000001	***
sex:14@42.1	2	6.38	3.83	<0.000001	***
sex:6@43.8	2	5.78	3.46	<0.000001	***
7@44.2:18@39.8	4	4.11	2.43	0.001	**
sex:15@15.7	2	4.07	2.40	<0.000001	***

Abbreviations: ANOVA, analysis of variance; LOD, logarithm of the odds; PVE, percentage variance explained; QTL, quantitative trait loci.
The log-likelihood score (LOD), PVE and the X^2 P-value for the full model are indicated in the top portion of the table. The lower portion of the table represents the results of dropping a factor from the model one at a time. Putative QTL are indicated as chromosome@position in centimorgans (cM), 'sex' represents inclusion of sex as a cofactor and epistatic interactions are indicated by factor:factor notation. Significant results are indicated as *$P<0.05$, **$P<0.005$ and ***$P<0.0005$.

Supplementary Figure S1), in separate analyses. Approximately 75% of F_2 individuals were OB (or 'O'). Notably, 100% of F_1 individuals exhibited the OB phenotype. Previous work has indicated a single large-effect QTL for the OB phenotype on cichlid chromosome 5, explained by alleles at the *pax7* locus (Streelman *et al.*, 2003; Roberts *et al.*, 2009). This QTL was detected in each step of our analysis and found to account for over 90% of the phenotypic variance in OB color pattern (Table 3, Supplementary Figure S2). OB (5@26.3) is mapped coincident with the inferred ZW sex-determining locus (above). The presence of a single W allele is sufficient to express OB in all individuals, while homozygous WW individuals exhibit the 'O' phenotype.

The blue phenotype was an emergent trait in this cross between a homozygous OB ('O') dam and a black-barred yellow sire (see Figure 1). 'Blue' individuals included those with blue/orange blotches (approximating a blue OB pattern; a male phenotype in *P. elongatus*) as well as fish with black bars on blue background (similar to 'BB' or 'blue-barred' in Streelman *et al.* (2003); see Supplementary Figure S1). Ten percent of F_2 exhibited blue color with the majority being 'BB' type. Our analysis revealed several factors associated with this phenotype (full model LOD = 63.25; Table 4, Supplementary Figure S3). The largest factor contributing to blue was the covariate sex (only males are blue; PVE = 19.77) while the largest PVE (8.70) for a putative QTL was at position 44.2 on chromosome 7 (Table 4). There were no effects of F_2 family ('brood') or parentage on this trait. We detected a handful of loci of moderate effect on several chromosomes, as well as epistatic interactions between QTL and sex (Table 4). The QTL for blue color on chromosome 7 (position 44.2) is coincident with an XY sex determiner (X″Y″ above). A second locus of particular interest for the blue phenotype is found on chromosome 5, 1.6 cM away from OB/ZW. Just as the OB polymorphism has been linked to sex-determining loci in Malawi cichlids (Streelman *et al.*, 2003; Roberts *et al.*, 2009; Ser *et al.*, 2010), here we observe a similar phenomenon for blue color.

DISCUSSION
Diverse sex systems in diverse cichlids
Sex-determining loci are important in evolution because they may (i) resolve sexual conflict between traits favored by selection in males versus females (for example, color patterns), (ii) evolve rapidly and

contribute to reproductive isolation and/or (iii) act as genomic attractors for genes controlling phenotypes subject to natural selection. This is particularly true in lineages where multiple, recently evolved sex determiners segregate as polymorphism, and sex chromosomes are in the early stages of evolution (Streelman *et al.*, 2007). LM rock-dwelling cichlids are ideal for investigating sex determination due to the existence of multiple sex systems not only between but also within species (Albertson, 2002; Streelman *et al.*, 2003; Ser *et al.*, 2010). The Malawi radiation has been so rapid that biological species continue to exchange genes by hybridization (for example, Mims *et al.* (2010)) and major intrinsic post-zygotic incompatibilities separate only the most divergent lineages (Stelkens *et al.*, 2010).

Using QTL analysis of RAD-tag SNPs, we have identified and characterized the (additive and epistatic) effects of five sex-determining regions in a single hybrid cross between LM rock-dwelling cichlid species. We mapped the genetic basis of two color phenotypes with putatively conflicting roles in male versus female fitness (see below; Holzberg, 1978; Ribbink *et al.*, 1983; van Oppen *et al.*, 1998; Konings, 2007; Roberts *et al.*, 2009; Svensson *et al.*, 2011) and established the chromosomal linkage of each to XY and/or ZW sex loci. Our results support the concept that cichlid chromosome 5 hosts a female heterogametic (ZW) locus coincident with QTL for (female-associated) OB patterning (Streelman *et al.*, 2003; Roberts *et al.*, 2009; Ser *et al.*, 2010), as well as male-associated blue color. We find strong evidence for additional sex-determining loci, including two male heterogametic (XY & X″Y″, where X″Y″ is linked to blue color) loci on chromosome 7, plus complimentary X′Y′ and Z′W′ loci on unlinked chromosomes (20 and 3, respectively). Taken together, these QTL account for over 50% of the variance in sex in this pedigree (LOD = 53.36, $P<10^{-10}$).

Our data are informative to consider in the context of a recent study by Ser *et al.* (2010). In that analysis, families of multiple rock-dwelling Malawi cichlids from the genus *Metriaclima* were screened for the effects of putative sex determiners on chromosomes 5 and 7 (Albertson, 2002; Streelman *et al.*, 2003). These sex systems were shown to be widespread among *Metriaclima* populations and to interact epistatically in some families. Importantly, these loci alone could not account for sex in all families, leading to the prediction that additional sex determiners segregate across the Malawi flock. Our analysis addresses that prediction and pinpoints three additional putative sex determiners in Malawi rock-dwelling cichlids. Our quantitative genetic approach similarly allows the partitioning of phenotypic variance to each locus and formally evaluates the epistatic relationships between the segregating sex systems. Notably, the power of our intercross design to accurately model phenotypic effects (including moderate-effect loci) and epistasis is the product of numerous fully informative SNP loci and nearly complete genotypic plus phenotypic data for ~350 F_2 (Broman and Sen, 2009).

A complex polygenic system for sex

We used the inferred mode of action, epistatic interactions and phenotypic variance explained by sex QTL to arrange these loci in a hierarchical scheme of sex determination (Figure 3). All WW (5@26.3) individuals were female (20.4% of individuals), while ZW and ZZ genotypes showed slightly skewed sex ratios (female:male 54:46%:43:57%, respectively). The expectation in a purely ZW system is that the W allele confers female sex, yet we do not observe this pattern; only homozygous W genotypes are unanimously female. This discrepancy highlights the importance of additional loci affecting sex and the genetic interactions contributing to sex determination in Malawi cichlids.

The sex of ZW individuals is strongly influenced by genotype at the distal chromosome 7 QTL (7@67.2), which we call the primary XY. ZWXX individuals are predominantly female (94%), ZWYY are mostly male (72%) and ZWXY individuals showed a roughly equal sex ratio (53:47% female to male). Notably, all but 3 of the 82 ZW males are XY or YY at a minimum of two of the three putative XY loci (XY, X′Y′, X″Y″), and over half are XY or YY at all three. Similarly, XY genotypes influence the sex of ZZ fishes; ZZYY individuals are all male, while ZZXX F_2 are 85% female. Additional QTL for sex have a refining role in sex determination and/or act to 'settle ties' between the primary ZW and XY loci. For instance, ZWYY individuals who inherit a Y′Y′ genotype from the putative X′Y′ locus (20@28.1) are 100% male. ZWYY individuals who inherit an X′Y′ from 20@28.1 plus a Z′W′ from the putative second ZW locus at 3@27 are predominantly male (90%); however, ZWYYX′X′—Z′W′ genotypes are 89% female. Likewise, the sex of ZZXY individuals is influenced by genotypes at the X″Y″ locus (7@44.2) such that ZZXY—Y″Y″—fishes are 100% male.

Because our F_2 population is finite in size, certain genotypic combinations did not appear frequently enough to quantitatively evaluate the effects and/or interactions among component loci. However, modeling sex determination in the context of QTL haplotypes (Figure 3) allows us to explain sex in ~82% of F_2 offspring. We cannot discount the effect of environmental influences on sex determination in fishes (Baroiller *et al.*, 2009), and we accept that these effects in cichlids in particular are not well described. The conditions in which we conducted our intercross experiment were well controlled in terms of light, temperature and other physical water parameters. However, social, dietary and yet unmeasured factors may still account for variance in sex, such as the small but significant effect of brood in our analysis (Table 1).

Sex, color and their co-evolution

Our analysis provides evidence for sex linkage of colors and patterns expected to favor male (blue color) versus female (OB pattern) fitness. The coincidence of QTL for OB color with the female-dominant ZW has been suggested by previous work (Streelman *et al.*, 2003; Roberts *et al.*, 2009; Ser *et al.*, 2010). Here, the W allele confers this color phenotype to all individuals possessing it. Of 246 OB (and O) individuals, 164 were female (66.7%) and all (100%) were genetically ZW (or WW), while the 82 OB males were exclusively ZW. OB has been suggested to function in disruptive crypsis, thereby conferring an advantage against predation (Holzberg, 1978; Konings, 2007; Roberts *et al.*, 2009). In nature, this could asymmetrically favor females, as males with this phenotype are almost never present (Streelman *et al.*, 2003; Roberts *et al.*, 2009). The rarity of OB males can be explained by reduced reproductive fitness, as OB presumably disrupts recognized male nuptial coloration (Holzberg, 1978; Seehausen *et al.*, 1999b; Kocher, 2004). Tight linkage between OB and ZW could have evolved in response to sexual conflict and might indicate the invasion of this sex system into an ancestral XY (Kocher, 2004; van Doorn and Kirkpatrick, 2007; Roberts *et al.*, 2009; Ser *et al.*, 2010). Intriguingly, because W shows higher penetrance for color pattern (OB) than for sex (that is, ZW individuals are always OB but can be male when they inherit Y alleles from other loci (above)), male OB offspring will continue to be produced in *mbuna* populations segregating multiple sex systems.

Blue color is emergent in these F_2; it is absent in the parental individuals but is present in males of *P. elongatus* (the dam's species) and many other LM cichlid species. The blue-barred phenotype is an important male nuptial color conferring mating success (van Oppen *et al.*, 1998; Svensson *et al.*, 2011). We report that blue color is found exclusively in male F_2, of both the BB and OB type (Figure 3). Blue OB may not be well recognized by females in the wild as the blotches disrupt the recognized male pattern (that is, blue-barred; Holzberg, 1978; Seehausen *et al.*, 1999b; Roberts *et al.*, 2009). After accounting for the effect of sex, we identify QTL for blue color on multiple chromosomes, including chromosome 5 (@27.9) near the ZW locus, and chromosome 7 (@44.2) at the putative X″Y″. Male coloration and other display traits are known to be in strong linkage with sex chromosomes in several other fish species (Lindholm and Breden, 2002; Basolo, 2006). Sexually antagonistic fitness drives such linkages because trait expression in males is beneficial for reproduction but detrimental to females due to increased predation and/or intraspecific aggression (Bull, 1983; Rice, 1987). Our data thus identify and bolster existing data supporting sex chromosome linkage for colors and patterns with presumed female versus male reproductive function. As the OB phenotype is putatively beneficial to females, and thus in tight linkage with the ZW locus, it is possible that linkage between sex loci and the blue phenotype is also a result of sexually antagonistic selection.

We interpret these findings in the context of Malawi cichlid evolution, within the confines of one important caveat. Because we map loci contributing to sex and color in an intergeneric cross, the loci and interactions we identify could be the result of this particular hybrid genetic background (Presgraves, 2007) and might not reflect the genetic basis of these phenotypes within natural populations. Generally, the consistency of our data with previous experiments between and within other Malawi rock-dwelling species (Albertson, 2002; Streelman *et al.*, 2003; Roberts *et al.*, 2009; Ser *et al.*, 2010) and

the peculiar genomic biology of Malawi groups (Loh et al., 2008; Stelkens et al., 2010) tends to argue against this interpretation. Furthermore, because the intercross we studied is made between species that likely exchange genes in the wild (as many rock-dwellers do), our findings are relevant to cichlid evolutionary dynamics.

Multifactorial sex systems are predicted to be unstable and thus transient (Bull and Charnov, 1977; Karlin and Lessard, 1986). Based on the diversity and lability of sex systems in fish lineages and the rapidity and extent of the LM cichlid radiation, the presence of such a complex assortment of sex determiners is perhaps not unexpected (Volff and Schartl, 2001; Nanda et al., 2003; Kondo et al., 2004; Otake et al., 2006; Cnaani et al., 2008; Ross et al., 2009; Ser et al., 2010). We note that Cnaani et al, (2008) have reported a ZW locus for sex on chromosome 3 in the ancestral tilapia (10–20 million years divergent from LM species flock). This ZW may correspond with the Z′W′ described here (3@27.0), suggesting that at least some of these sex-determining loci are evolutionarily conserved. Theory suggests that certain conditions may favor equilibrium and maintenance of both XY and WZ systems in the same genome (van Doorn and Kirkpatrick, 2010) Additional study is needed in *mbuna* cichlids, as well as from other evolutionary lineages in Malawi (and elsewhere in East Africa), to determine whether the sex systems identified here (and/or others) segregate widely across this species flock.

DATA ARCHIVING
SNP marker sequences are available in GenBank on the dbSNP website (accession numbers 51975064–519751979).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)