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Sexual Conflict Resolved by Invasion of a Novel Sex Determiner in Lake Malawi Cichlid Fishes

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Abstract

Sex determination mechanisms differ among animal species, but it is not clear how these differences evolve. New sex determiners may arise in response to sexual conflicts, which occur when traits benefit one sex but hinder the other. Here we identified the genetic basis for the orange-blotch (OB) color pattern, a trait under sexually antagonistic selection in the cichlid fish of Lake Malawi, East Africa. The OB phenotype is due to a *cis*-regulatory mutation in the *Pax7* gene. OB provides benefits of camouflage to females, but disrupts the species-specific male color patterns used for mate recognition. We suggest that the resulting sexual conflict at *OB* has been resolved by selection for a novel female sex determination locus, promoting its invasion into populations with an ancestral male sex determination system.

When an autosomal mutation modulates a trait to the benefit of one sex and the detriment of the other, the resulting sexually antagonistic selection creates a genetic conflict. This conflict can be resolved if expression of the phenotype is limited to the sex that gains a fitness benefit from the mutation. (1, 2). Sexually dimorphic pigmentation is a hallmark of the radiation of cichlid fishes in Lake Malawi, one of the three major East African Great Lakes (Malawi, Tanganyika, and Victoria). Competition for females has resulted in sexual selection for brightly colored, conspicuous males (Fig. 1B). In contrast, female coloration is generally drab and brown, making them inconspicuous to predators (3) (Fig. 1A).

An exception to uniformly brown pigmentation in female rock-dwelling cichlids in Lake Malawi is the orange-blotch (OB) phenotype (4). OB is found almost exclusively in females, and usually co-segregates with the more widespread brown barred (BB) female phenotype in local populations (3). The OB phenotype is characterized by dark melanophore blotches of variable number and size on a background of orange xanthophores (Fig. 1A, and fig. S1). The blotching provides an alternative form of crypsis by background-matching the mottled rock substrate that the cichlids inhabit (Fig. 1C; Movie S1). The variable high-contrast blotching may also provide camouflage by disruptive coloration, independent of background matching (5). OB males are rare and their characteristic nuptial colors are highly disrupted (Fig. 1B) (4). Given the strong reliance on specific male nuptial color cues for mate recognition and attraction (6), the disruption of pattern is predicted to lower the fitness of OB males. The OB phenotype has been observed in more than 20 species in four genera in Lake Malawi. Similar sex-associated blotched phenotypes are found in cichlids from Lake Victoria, but the cichlids of Lake Tanganyika appear to lack comparable phenotypes (7).

The *OB* locus was previously localized to a 5 cM region of Malawi cichlid linkage group five (LG5) (8). In the absence of a cichlid genome sequence, we took a comparative mapping approach to refine the map interval, using the *Tetraodon* genome as a guide (Fig. 2A, and fig. S2B). We identified additional markers in the region by aligning cichlid

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genome survey sequences to the *Tetraodon* genome (9, 10), and resequencing a panel of cichlid DNA to identify polymorphisms. Genotyping these new markers confirmed the broad-scale synteny between these species.

Pedigreed families from several species of the genera *Labeotropheus* and *Metriaclicma* were genotyped for newly identified microsatellite markers on LG5 (46 families, 678 individuals, 349 OB individuals; table S1). Breakpoint analysis localized *OB* to an interval of less than 1 cM, corresponding to a region from 3.9–4.0 Mb on *Tetraodon* chromosome 11 (Fig. 2A). We then used association mapping in natural populations to pinpoint the causative locus. Single nucleotide polymorphism (SNP) markers were developed spanning the region, including an intronic SNP marker for each annotated gene within the *OB* interval. We measured linkage disequilibrium (LD) between *OB* and SNPs within natural populations of *Labeotropheus*, *Metriaclicma*, and *Tropheops* from the northern and southern parts of Lake Malawi (Fig. 2B). Within each population, a peak of LD was found in a region overlapping the interval defined by breakpoint analysis (Fig. 2C–G). Given the shared pattern of association across study populations, we created a lake-wide mapping panel to increase the effective size of the mapping population. This lake-wide panel contains BB and OB individuals from 36 distinct populations of 12 species segregating OB (Table S2). Analysis of marker data across these populations increased resolution and statistical significance of LD beyond that available from any single population (Fig. 2H).

The maintenance of strong LD in the lake-wide mapping panel suggests the presence of a single, shared haplotype of SNP genotypes across the flock. Indeed, we identified a common haplotype signature found largely intact in OB individuals of all species, indicating a single origin of the causative *OB* mutation in Lake Malawi (Table 1). The *OB* haplotype block extends from *Akr7A2* to *Pax7* in most individuals, and based on comparative genomic information includes at least five genes (Fig. S2). Comparison to outgroup species suggests that BB chromosomes represent the ancestral haplotypes across this interval (Table S3). The single blotched cichlid examined from Lake Victoria also carries an ancestral haplotype, suggesting an independent evolutionary origin for the Victorian OB-like phenotype (Table 1, *Neochromis* ‘blotch’ morph). Our finding of a single origin of *OB* in Lake Malawi cichlids contrasts with a previous phylogenetic analysis that suggested multiple origins (11).

The *OB* haplotype is a genetic marker allowing us to make additional conclusions about the relationship between genotype and phenotype. Individuals in some populations are heavily pigmented with melanophore blotches, whereas individuals in other populations lack blotches almost entirely (the orange or “O” morph). Sex-linked white-blotch and white morphs (“WhB” and “Wh”), similar to OB and O morphs but lacking orange background coloration, also exist in the Malawi flock (Fig. S1). Individuals of these morphs also carry the *OB* haplotype, and can be definitively attributed to the *OB* locus (Table 1). Discrete morphs with different degrees of blotching sometimes segregate within a population, covering a similar phenotypic range as that found across species. OB individuals from the three study populations segregating multiple blotch phenotypes are all heterozygous for the same SNP haplotype block (Fig. 2C, 2D, and 2F), suggesting that differences between morphs are not due to gene dosage. Discrete morphs may result from allelic variants within the *OB* haplotype, or genetic modifiers of *OB* segregating in some populations.

The peak of LD in the lake-wide mapping panel corresponds to a non-coding SNP within a *Pax3/7* subfamily gene. These transcription factors play key roles in specifying pigment cell lineages from neural crest precursors (12, 13). Gene phylogeny and conserved synteny identify the OB-associated gene as cichlid *Pax7* (Fig. S2). OB individuals have significantly higher expression of *Pax7* in tailfin, regardless of the degree of melanophore blotching or relative numbers of other pigment cell populations (Fig. 3A). Allele-specific expression

analysis demonstrates that the *OB* allele of *Pax7* is specifically upregulated (Fig. 3B, C). Since no *OB*-associated differences were found in *Pax7* coding sequence from the species used for expression analysis, we suggest that *cis*-regulatory differences in *Pax7* account for the *OB* pigmentation phenotype (14).

In addition to overt pigmentation differences between BB and *OB* individuals, close examination reveals cell-level differences in tailfin melanophores. Melanophore number is reduced in three-month-old *M. 'kompakt'* *OB/+* individuals relative to BB broodmates (BB ($n = 7$), 414 ± 173 ; *OB/+* ($n = 10$), 118 ± 77 ; Welch's *t*-test: $p = 0.0032$). In contrast, contracted melanophore size is larger in *OB/+* individuals (BB ($n = 7$), 26.5 ± 8.6 pixels²; *OB/+* ($n = 10$), 50.6 ± 14.3 pixels²; *t*-test: $p = 0.0013$). The finding that upregulation of *Pax7* in cichlids is correlated with a fewer-but-larger melanophore phenotype is particularly interesting given that downregulation of *Pax3* in zebrafish produces a more-but-smaller melanophore phenotype (13). In both cichlids and zebrafish, higher *Pax3/7* dosage correlates with development of fewer, larger melanophores.

In our analysis of families and natural populations, we have been unable to genetically separate *OB* from the female sex determiner (*W*) known to be in the same region of LG5. Only one species in our laboratory stocks, *M. 'kompakt'*, occasionally produces *OB/+* males. However, these *OB* males inherit the maternal *OB* haplotype intact, and when bred, their *OB*-carrying chromosome determines female sex in offspring (Table S4) (14). We conclude that these *OB* males are genetically female at the *W* locus and sex-reversed by another mechanism. Mating of *M. 'kompakt'* *OB/+* males to *OB/+* females produces viable *OB/OB* homozygotes in expected Mendelian ratios (21 of 89 individuals from five broods; $\chi^2 = 1.36$, $p = 0.5067$). Thus *OB* homozygotes are fully viable, at least on some genetic backgrounds. However, we have not observed *OB* homozygous individuals in natural populations (0 of 199 wild *OB* individuals), which suggests that *OB* males have low mating success.

The maintenance of the *OB* haplotype over evolutionary timescales might be explained by selection for tight linkage between *OB* (*Pax7*) and the *W* sex determiner (15). Linkage and suppression of recombination between *OB* and *W* might be further increased by a *W*-specific inversion encompassing both genes, a rearrangement of the kind hypothesized to be an early stage of sex chromosome evolution (16). However, no obvious candidate gene for sex determination is within the interval (14), and we found no evidence of structural rearrangements.

We suggest that *OB* originated once as a novel allele upregulating *Pax7* expression, and was incorporated into multiple species across four genera of the Malawi cichlid flock by sorting of an ancestral polymorphism and/or hybridization events after the radiation of species. The *OB* phenotype positively affects the fitness of females by providing an alternative form of crypsis against the mottled background of their habitat. Conversely, *OB* reduces male fitness by disrupting their nuptial pigmentation patterns. The genetic conflict created by sexually antagonistic selection on the *OB* phenotype may be resolved by sexually dimorphic expression of the trait. In the case of the *OB* locus, the sexual conflict has been resolved by tight genetic linkage with a dominant female sex determiner, rather than by evolution of modifier genes to sex-limit expression of the phenotype during development. Thus, the *OB-W* haplotype has invaded populations in a manner consistent with a model of sex chromosome evolution by sexual conflict (17).

The *OB*-associated ZW sex determining system is epistatically dominant to a putatively ancestral XY locus on LG7, responsible for sex determination in several other Malawi cichlids (Tables S5 and S6) (14). We propose that sexually antagonistic selection at *OB*

(*Pax7*) provided conditions facilitating the invasion of a tightly linked female sex determiner on LG5 to resolve the genetic conflict. Alternatively, the *OB* polymorphism may have arisen near a nascent female sex determiner, providing conditions for the *OB-W* allele to rise in frequency and persist in multiple lineages along with the ancestral XY sex determination system. In either case, the evolutionary success of the allele is the result of the interaction of the pigmentation and sex determination traits.

The *OB-W* locus is a nascent sex chromosome, and demonstrates the interactive evolution of sexual dimorphism and sex determination resulting from resolution of sexual conflicts. Sexually antagonistic selection is usually thought to arise from processes of sexual selection (18). Our results show that a novel trait increasing fitness under natural selection can readily create strong sexual conflicts, especially if the trait negatively impacts sexually selected characters in the opposite sex. Phenotypes rarely fall into a simple dichotomy of sexually and naturally selected traits, nor do they impact overall male and female fitness equally. Thus, multiple aspects of a phenotype can trigger sexual conflicts, and ultimately the evolution of new sex determiners. We suspect that similar genetic conflicts are important in the evolution of many other traits, and have likely contributed to the extraordinary rates of diversification and speciation in African cichlid fishes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

OB pigmentation affects different aspects of fitness in females and males. **(A)** Typical female BB (top) and OB (bottom) morphs, represented by *Tropheops* 'orange chest'. **(B)** Common BB male nuptial coloration (top) and rare OB male (bottom) of *Labeotropheus fuelleborni*. **(C)** OB (white arrow) and BB (black arrow) *L. fuelleborni* females are differently cryptic over the boulder reef habitat at Nankoma Island (13°53'30"S, 34°36'43"E; frame from Movie S1).

Figure 2. Genetic mapping in families and natural populations. (A) Individual recombinant chromosomes (rows) at the *OB* locus from pedigreed families, with “OB” and “BB” indicating genotypes of inherited maternal alleles, and empty blocks indicating non-informative markers. Marker names and Mb position on *Tetraodon* Chr.11 are indicated at top of marker columns. Breakpoint analysis across multiple recombinants defines an *OB* interval between markers *AKR7A2* and *SKI*; one recombinant refines the interval to *AKR7A2* and *MET4802*. Italics indicate gene-associated markers. (B) Collecting sites in Lake Malawi. The populations analyzed in panels C-G are marked with orange stars (c-g). Localities combined for the lake-wide mapping panel (H) are marked with red dots. (C-G)

Association of markers with OB and OB-like phenotypes in populations, graphed as LOD of association versus position on *Tetraodon* Chr.11. (C) *M. zebra* Makonde (24 BB, 24 WhB & Wh); (D) *M. zebra* 'blaze' Manda (24 BB, 16 OB & O); (E) *M. xanstomachus* Nankoma (26 BB, 22 OB); (F) *L. trewavasae* Thumbi West (24 BB, 24 OB & O); (G) *Tropheops* 'orange chest' Thumbi West (32 BB, 18 OB). (H) Multi-species lake-wide mapping panel (34 BB, 44 OB class; Table S2).

Figure 3.

Pax7 expression is increased in OB individuals independent of species and morph. **(A)** *Pax7* expression relative to BB expression level within each species, measured by quantitative real-time PCR of juvenile tailfin RNA and normalized to β -actin expression. OB genotypes and expressed phenotypes are indicated along the x-axis. **(B, C)** Allele-specific expression using RT-PCR of a size polymorphism in the 5'UTR of *Pax7*. **(B)** Representative size separation traces of products from +/+ and OB/+ individuals, with allele type and size indicated above peaks. **(C)** The ratio of OB- to non-OB-sized allele expression. All *p* values from pairwise *t*-tests.

Table 1

The most prevalent SNP genotypes at the *OB* locus in the four genera segregating OB and in outgroup genera. The most frequent OB and BB haplotypes are indicated separately at the bottom. SNP genotypes indicating the OB-specific haplotype signature are labeled in orange.

* Details of the species and localities sampled for this table are found in Tables S2 and S3.

[†] Intronic SNP markers labeled by gene are a subset of those used in LD analysis (Fig. 2, C-H).