Modular Inheritance of Color Pattern in Lake Malawi Cichlid Fishes

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Modular Inheritance of Color Pattern in Lake Malawi Cichlid Fishes

A Capstone Project Submitted in Partial Fulfillment of the Requirements of Distinction in Biology at Syracuse University

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Honors Capstone Project in Biology

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Abstract

This study attempts to elucidate the mechanisms that underlie the evolution of pigmentation patterns among Lake Malawi cichlids. For decades researchers have sought to provide a theoretical model of cichlid speciation based on the evolution of male nuptial patterns and female preferences, yet our understanding of how color patterns change is limited. Here, we performed exploratory statistical analyses on an F2 hybrid population to characterize the inheritance of color traits. Specifically, we sought to uncover patterns of modularity and levels of integration among color traits. Because cichlids are sexually dimorphic we did our analyses on males and females separately. We found that although males and females share a common pattern of modularity, the level of trait integration is stronger in females. We hypothesize that sex-specific levels of trait integration may be a means by which the cichlid system overcame sexual conflict associated with the need for males to be flashy and females to remain cryptic. As a result of this innovation, species-level diversity based on male nuptial coloration could explode to the levels we see today.
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1. Introduction

There is no better model to elucidate the factors involved in the evolution of vertebrate biodiversity than ray-finned fishes. No other vertebrate group is comparably old and species rich as the Actinopterygii (ray finned fish; Hurely et al., 2007). Much of this diversity belongs to the Teleost lineage, which is characterized by innovations in jaw and tail morphology (Long, 1996). The fundamental unit at which we measure biodiversity is the species and, even with roughly 27,000 species of Teleost, as many questions remain unanswered about the origins of fish diversity as there are species to study (Nelson, 2006). In the present period of rapid and uncertain global change, the drive to better understand where biodiversity comes from is stronger than ever.

The process by which a new species arises is speciation. Understanding the mechanisms of speciation allows us to better understand the origins of biodiversity. This study utilizes the most remarkable extant example of a vertebrate adaptive radiation—the cichlid fishes of the Lake Malawi and their extraordinary diversity in color patterns—to answer questions about how species evolve. In the following sections I will (1) define and discuss adaptive radiations, (2) introduce the study system, as well as the importance of color traits, and (3) discuss the concepts of integration and modularity as they apply to evolvability (i.e., the potential for populations to evolve) of the Lake Malawi cichlid species flock.
(i) **Adaptive radiations**

An ‘adaptive radiation’ describes a lineage that has diverged rapidly and into many species from a common ancestor. These lineages are sometimes referred to as ‘starbursts’ because species have evolved so rapidly that resolving their phylogenetic relationship is difficult (Fig. 1) (Schluter, 2000).

Adaptive radiations usually begin with an ecological opportunity (empty niche, for example) that presents itself to the founding population. Thus, according to the most generally accepted theories, in order to achieve a diversity of ecologically adaptive phenotypes, the driving force in the evolution of an explosively radiating clade must begin with divergent natural selection (Schulter, 2000). However, not all groups radiate to the same extent when presented with similar opportunities. This leads to the question of what intrinsic factors trigger the explosion of diversity associated with some clades but not others?

Notably, ecology is not the only force that drives adaptive radiations. “Opportunities” can also present themselves as shifts in behavior (e.g., mate choice, sensory bias, or cognitive ability; see Tebbich et al., 2010 for example). This study utilizes the observation that patterns of divergence among Lake Malawi cichlids are heavily influenced by sexual selection on male nuptial patterns. Specifically, by studying the potential for these color patterns to respond to selection (i.e., their evolvability), we aim to elucidate potential mechanisms for rapid species divergence. In the next section I more formally introduce the
system, and provide a discussion of the selective pressures that operate on pigmentation patterns in Malawi cichlids.

(ii) Lake Malawi Cichlids:

The Cichlidae family, thought to contain as many as 3000 species, is the most species rich vertebrate family on the planet (Kocher, 2004). The cichlids within the East African great lakes are the most successful extant vertebrate adaptive radiations, with the endemic flock of Lake Malawi cichlids, for example, representing as much as a third of the species diversity found in the family (Fig. 2) (Kornfield and Smith, 2000). Moreover, this level of species richness has evolved in a little as 1 million years (Kornfield and Smith, 2000). Many aspects of cichlid biodiversity have been explored in an attempt to uncover the mechanisms behind their explosive diversity, usually with a focus on “key innovations” (Albertson et al., 2003; Streelman et al., 2003; Carelton et al., 2005). This study aims to add to this body of literature by examining the radiation in the context of color pattern diversity.

Cichlid pigmentation

Pigmentation is often studied across taxa because it is a genetically and developmentally tractable trait with explicit biological importance. The male nuptial colors shown in Figure 3 represent examples of phenotypes that, like most pigmentation patterns, are relatively easy to quantify. Evolutionarily, pigmentation patterns can be influenced by any number of selective pressures. In
the Lake Malawi cichlid system, it is generally accepted that sexual selection plays a large role in the evolution of male nuptial colors (Kocher, 2004). The emergence of sexual selection as an influence on a flashy male ornament is dependent on females of the population being able to perceive the ornament, and the freedom of males from natural selection for crypsis (Protas and Patel, 2008). Several key ecological attributes of the Lake Malawi system support the model that flashy male pigmentation patterns have evolved under sexual selection.

*Cichlid ecology, in brief*

Although birds of prey will actively feed on cichlid species from the near shore habitat, generally low predation has freed pigmentation patterns from natural selection for crypsis (Konings, 2007). Moreover, evolution in clear waters has encouraged the emergence of a female visual sensory bias based on pigmentation (Seehausen, 1997; Knight et al., 1998).

Figure 4 shows flashy male color patterns relative to uniform female coloration, it also demonstrates the degree of sexual dimorphism present in the lake. This disparity in color pattern between the sexes likely has a very rich evolutionary history. In the next section I discuss the role sexual selection, and permutations thereof, can play on the evolution of sex-specific pigmentation patterns and the effect this can have on the evolution of the entire lineage.
Sexual selection refers to pressures exerted on individuals as a result of the
pursuit of reproductive opportunities (Andersson, 1994). These pressures can be
intrasexual and intersexual. Intrasexual selection is a strong influence in systems
where males compete for access to females. The traditional notion that females
are choosy and males are promiscuous falls under the umbrella of intersexual
selection (Trivers, 1972). Across systems, the interactions between males and
females exist along a continuum from little discernable conflict to what
researchers have termed sexually antagonistic coevolution (Arnqvist and Rowe,
2005).

Sexually antagonistic coevolution describes situations in which the reproductive
strategies of one sex appear to adversely affect the fitness of the other sex, and
either sex evolves as the process of natural selection moves each toward their
respective optima (Chapman, 2006). For example, the evolution of a female
preference for a male ornament of a certain condition reduces the fitness of those
males who do not possess a suitable ornament. Conflict can arise if males evolve
a mechanism whereby they produce an ornament that is a dishonest signal of
fitness (Holland and Rice, 1998). Moreover, female fitness can be adversely
affected if the expression of a ‘flashy’ male ornament is not strictly sex-linked in
a system in which there is pressure for females to remain cryptic. In other words,
sexual antagonism results when males and females share a common genome, but
when a trait (e.g., color) is under different selective pressures in each sex.
The cichlids of Lake Malawi represent a system in which the flashy coloration of males may be in conflict with the need for females to remain cryptic (Fig. 4). Researchers have generally accepted an important role for coloration in terms of the production of species diversity in Lake Malawi (Dominey, 1985; Mckaye, 1991; Danley and Kocher, 2001). Large numbers of rapidly evolved species can arise in a system where female preferences and mate recognition changes rapidly, and male color patterns can respond very rapidly as well (Lande, 1981).

However, little is known about how “evolvable” these traits are (e.g., the freedom with which they can change in response to selection), and whether males and females possess different levels of evolvability.

We suggest that the explosion of many types of complex male color patterns, and the concomitant expression of cryptically color patterns in females, may have been facilitated by the ‘resolution’ of conflict between the expression of male and female color patterns. If male and female color patterns can change separately, the conflict between crypsis and flashiness can be reduced. The question becomes: How easy or difficult is it for color patterns to change in response to differential selection pressures? In other words, what is the evolvability of cichlid color patterns, and is it different between sexes?

Comment [RCA3]: Again, poor choice in females. Unless, of course, yellow is cryptic ;)
Integration and modularity describe the functional, developmental or genetic correlation between traits, or lack thereof, between groups of traits. Phenotypic traits that tend to vary together within individuals are integrated. Groups of traits that vary together, but show less correlation to other traits are referred to as modules (Fig. 5) (Klingenberg, 2008). Discussions of integration and modularity are often contextualized in terms of the ability of traits to respond to change.

Integration can affect the evolution of traits in two ways: (1) It can influence the time it takes change to manifest; and (2) It can affect the direction of change in phenotypic space. How traits evolve stronger or weaker correlations between other traits (integration/modularity) is not completely understood, and has been a topic of intense investigation for years (Cheverud, 1996; Murren et al., 2002; Badyaev and Foresman, 2004; Hallgrimsson et al., 2006).

Theoretically, separating traits into different modules allows variation to manifest to a greater degree than if traits were tightly correlated to other functions. This is because many, independent correlations among traits provide many independent axis of variation on which selection can act. In this way, modularity can be an efficient way to maintain variation in a population for a complex phenotype. Instead of requiring complexity to be built from scratch when change occurs in the environment (new mate preferences, for example), correlations among traits can lead to large phenotypic changes generated by relatively simple genetic changes. Indeed, this maintenance of variation itself could be a trait on which
selection acts, thus driving the evolution of integration and modularity (i.e., the evolution of evolvability) (Wagner and Altenberg, 1996; Kischner and Gerhary, 1998; Pigliucci, 2008)

Exploring integration and evolvability through a hypothetical example of dog and hyena limbs

As integration evolves, it can affect the speed and direction of evolutionary change. In a simple, theoretical example demonstrated in Figure 6, two traits in the domestic dog experience correlated changes. A linear regression can characterize a major axis of variation between two traits (here, between fore and hind limbs). If the direction of selection is parallel with this axis of variation, one might expect evolution will occur rapidly. Alternatively, if the axis of selection is perpendicular to this axis evolution is expected to occur slowly (if at all) because there is simply less variation to take advantage of. The ease with which populations of the domesticated dog can travel along this axis of variation is a likely reason why breeders have so rapidly developed breeds of dogs with limb lengths as disparate as the Pembroke Corgi and the Scottish Deerhound. The lack of variation present perpendicular to this axis could also theoretically explain why dog breeds with radically different fore- and hindlimb lengths do not exist. In other words, the limb lengths in dogs are tightly integrated. This integration facilitates rapid change, but it constrains the direction of that change through phenotypic space. Alternatively, if the variation in fore- and hindlimbs did not exhibit a major axis of variation, the apparent constraint seen in dogs might no
longer be as severe. That is, limb lengths could change across many dimensions of phenotypic space. Hyenas represent an example in which fore- and hindlimb lengths are likely broken into two modules, each with their own, relatively independent variation on which selection can act. Thus, we would predict that patterns of modularity in limb length would differ between the Canidae and Hyaenidae, with the former being more integrated and the later more modular.

Integration and modularity in cichlids

Adaptive divergence as a result of natural selection on modular structures in the cichlid mandible has been implicated in the rapid ecological niche-filling seen in the African great lakes (Albertson et al., 2005). However, ecological divergence alone cannot account for the species richness found in Lake Malawi. Instead, we observe that diversity in pigmentation patterns may account for a significant portion of the population and species level diversity. This observation is illustrated in Figure 7, where closely related species differ strikingly in their color patterns. Even within species, color patterns can be very different.

Allender et al. (2003) suggested that male color patterns are composed of recurring traits, and that new patterns can be generated by small changes in those traits rather than building a new complex pigmentation pattern from scratch. These observations are consistent with the idea that pigmentation patterns in male cichlids may operate as modules. However, female pigmentation patterns are not changing with the same specificity in regard to individual traits. We suggest that
the levels or patterns of integration between males and females may differ, and it is the evolution of these differences that may have contributed to the ‘resolution’ of a sexual conflict between the pressure for males to be flashy and females to remain cryptic.

In the current project we used a F2 hybrid population to address whether there are discernable patterns of modularity in cichlid color traits, and whether the strength and/or pattern of integration differs between sexes. We demonstrate that variation in color traits is modular, and that patterns of modularity are largely the same between males and females. Alternatively, the level of integration between color traits was different between the sexes. This suggests that differential selective pressures are influencing the levels of integration but not necessarily the patterns of modularity. All in all, our data demonstrate differential evolvability between the sexes, and we suggest that this might account for at least part of the resolution to the sexual antagonism among Lake Malawi cichlids.

2. Materials and Methods

(i) Specimen and color data collection

Modularity and integration analyses were performed on an F2 population generated from a hybrid cross between *Labeotropheus fueliborni* (LF) and *Tropheops* ‘red cheek’ (Trc) (Fig. 8). Parentals of both species were wild caught from Lake Malawi Makenjila and Chismulu for LF and Trc, respectively (Fig. 2). Lab reared populations were collected and sacrificed in accordance with IOCCA
protocols. Euthanized fish were placed in an ice bath and photographed using a digital camera (Canon EOS). Fish were subsequently placed in (-)epinephrine solution and photographed again. The ice treatment insured maximum quantification of black color traits, whereas treatment of fish in (-)epinephrine solution, at a concentration of 1 mg/ml, allowed for maximum quantification of ‘red/yellow’ color traits. Melanocytes are constricted as a result, and the pigments beneath are more visible (Fig. 9). In total, 699 F₂ individuals were phenotyped.

(ii) Trait quantification

Photographs were taken with a piece of white plastic in the field to allow for correction of any color imbalance or discoloration caused by variation in lighting or water quality. RAW photographs were rotated, color-corrected, and converted into either JPEG or Photoshop formats in Adobe Photoshop CS3. All trait quantification was carried out in Photoshop as well. The cichlid color pattern was broken into 16 individual color traits, as demonstrated in Figure 10. ‘Black’ (BLK) and ‘red/yellow’ (RY) traits were measure for the Dorsal, Pelvic, and Anal fins. ‘Red/yellow’ was measured for nine regions on the flank (A-I). The number of Egg Spots on the anal fin was quantified. Standard length was measured in Photoshop as well. The majority of color traits were quantified as the percent of pigmented pixels across the region of interest. The total pixels in a fin were found via selection of the whole fin (for example) with the ‘lasso tool’. ‘Black’ and ‘red/yellow’ pigments were selected using a standard range of either RY or BLK colors. Two traits were not measured as percent: (1) The amount of ‘black’ on the
dorsal fin was found by ranking the amount of pigment present between each ray that runs in a distinctive bar along the fin (Fig. 10); and (2) Egg spots were counted as either ‘whole’ spots or ‘half’ spots.

(iii) Statistical analysis
The resultant dataset was size-corrected via least-squares regression on standard length, and all subsequent analyses were performed on residuals. Two complimentary statistical analyses were used to explore questions about the pattern and strength of modularity and integration. Hierarchical cluster analysis revealed the most likely pattern of modularity. Principal components analysis (PCA) corroborated these pattern, but was also used to provide insight into the strength of integration. All regressions and analyses were done using Systat 12.

Hierarchal cluster analysis
Using the residual dataset, hierarchal cluster analysis was performed using Ward linkage and Euclidean distance. The analysis was performed on the entire F2 population, and male and females separately.

Principal components
PCA was performed on the entire F2 population residual dataset, and males and females separately. This study utilized a specialized interpretation of PCA in order to shed light on the strength of integration among individual color traits. We determined the level of integration among the traits in the F2 population, and
males and females separately, by looking at the amount of variation explained by
PC1, and the degree to which the amount of variation dropped off along
subsequent PCs. This can be visualized by plotting the component axes against
their eigenvalue. When traits used in a PCA are tightly correlated (i.e.,
integrated), they load heavily on PC1, and it is in these instances when PC1
accounts for a large percentage of the total variation (i.e., high eigenvalue). Thus,
the amount of variation associated with PC1 can be used as a proxy for the level
of integration (Peres-Neto and Magnan, 2004; Parsons and Robinson, 2006;
Pavličev et al., 2009).

3. Results

(i) Hierarchical cluster analysis
Hierarchical cluster analysis performed on the entire F$_2$ population showed our
individual color traits formed three clusters: (1) RY on the fins (anal, pelvic, and
dorsal); (2) Egg Spots and ‘black’ on the fins and; and (3) Regions A, B, C, D,
and G on the flank (Fig. 11, 1).

Males:
These general clusters show up when the analysis was performed on males only,
although some are slightly broken up (Fig. 11, 2). Cluster 1 is intact compared
with the total F$_2$ analysis. However, RY pelvic associates with these black traits.
Cluster 3 is composed of regions A-C and E, F, and I. Regions D and E group
together, independent of all other traits. Clusters of red traits are nearest to each
other, except in the case of red on the pelvic fin. Groups of colors generally represent the deepest divergences in the cluster hierarchy.

**Females:**

The cluster pattern is somewhat different in females (Fig. 11, 3) Cluster 1, however, is intact. Cluster 2 is composed of RY dorsal and pelvic. The flank traits fall out into two different clusters. Cluster 3a is made of regions A-C. Cluster 3b is made up of regions D-F and H and I. RY anal associates most closely with Cluster 3b, though it appears fairly independent. Traits are clustering according to color.

**(ii) Principal components**

*All the Fish:*

When performed for the entire $F_2$ population, the PCA extracted 5 PC axes that explained 75.51% of the variation in cichlid color traits ($PC_1=37.68$, $PC_2=14.50$, $PC_3=11.48$, $PC_4=6.21$, $PC_5=5.64$) (Fig. 12a). $PC_1$ loaded positively for RY traits on the flank and anal and dorsal fin, and negatively for BLK pelvic. $PC_2$ loaded positively for BLK fin traits, Egg Spots, RY pelvic, and regions A, D, and G. The second axis loaded negatively for RY anal fin. $PC_3$ loaded negatively for regions A-C, and positively for RY fin traits. $PC_4$ loaded Egg Spots and RY dorsal negatively, and BLK anal and dorsal positively. The fifth and final axis under consideration for this study loaded region H negatively, and RY pelvic loaded positively.
Males:

Our PCA of the male population included five PC axes that explained 68.31% of the variation (PC1=30.09, PC2=12.83, PC3=11.54, PC4=7.50, PC5=6.35) (Fig. 12b). The first axis loaded positively for RY anal and dorsal, and regions A-G and I. PC2 loaded positively for RY fin traits, black anal and dorsal, and regions E and I. PC2 loaded negatively for regions A-C. PC3 loaded positively for all BLK traits, RY pelvic, and regions A. The fourth axis loaded negatively for RY pelvic and dorsal, regions F and G, and region I. PC5 only loaded Egg Spots positively.

Females:

For the PCA run on the female population we considered five PC axes that explained 82.86% of the variation (PC1=48.59, PC2=12.94, PC3=10.53, PC4=6.20, PC5=4.90) (Fig. 12c). PC1 loaded RY anal and dorsal, and all flank regions, positively. PC2 loaded positively for RY fin traits and regions G and H, and loaded negatively for regions A-C. Only Egg Spots and the BLK fin traits loaded on PC3, both positively. PC4 loaded Egg Spots negatively, and RY anal fin and BLK dorsal positively. The final axis loaded RY pelvic and BLK anal fin positively and BLK pelvic negatively.

(iii) Integration comparisons

Females are more integrated than males. PC1 in the female-only dataset accounts for 48.59% of the variation while the first axis in males is responsible for 30.09%
of the variation. Females are also loading more traits onto PC1 (males=10; females=11), and the loadings for each trait were higher for females. A first PC axis which loads most of the traits, and accounts for the majority of the variation in the dataset, is the hallmark of an integrated set of traits (Fig. 12d-f). This occurs when the dataset is tightly correlated. We can conclude that since the female dataset allows for a higher eigenvalue on PC1 than the same analysis performed on males, female color traits are more correlated, and thus more integrated.

4. Discussion
Our results indicate that the striking sexual dimorphism in pigmentation patterns that characterizes Lake Malawi cichlids is influenced by differential levels of integration among the color traits. Females are more integrated with respect to coloration than males, but both sexes demonstrate generally similar patterns of modularity. These findings shed light on the way males and females have been able to overcome the sexual conflict with respect to color. We break this discussion into several parts including: (1) the factors that have possibly influenced the patterns of modularity we elucidated; (2) the distinct pressures on males to evolve greater modularity; (3) the evolution of strong integration in females; and (4) the impact differential levels of color trait integration between the sexes has on the resolution of sexual conflict in the Lake Malawi cichlid system.
(i) Patterns of modularity

Results of the hierarchal cluster analysis suggest that males and females share similar patterns of modular inheritance of color traits. The most consistent modules between the sexes include (1) ‘black’ on the fins (pelvic, anal, and dorsal) and egg spots; (2) ‘red/yellow’ on the fins; and (3) ‘red/yellow’ on the flank. According to the cluster analysis the black fin cluster is the most conserved module between males and females. The ‘red/yellow’ clusters are a bit more variable, drawing slightly different flank and fin traits into different groups in males and females. There are several potential explanations for the evolution of these particular patterns.

Developmental effects on color pattern modularity

The development of pigmentation patterns in fishes is distinct from the process in mammals, though they share some genetic mechanisms. Whereas in mammals colors are deposited in the integument by one cell line which synthesizes many different types of pigments, fish color patterns are the results of many different types of pigment cells (chromatophores) derived from neural crest cells. The arrangement of melanophores (Mel) produces black patterns, while red/yellow patterns are most likely due to xanthophores (Xan). An additional cell line, the irridephores, contributes to the typical blue or silver iridescence characteristic of fish, but this type of pigmentation was not considered in this study (Kelsh, 2004).
The development of pigmentation patterns in teleosts is best understood in zebrafish. In this system, after an initial population of Mel and Xan differentiates from a common pigmentation stem cell precursor they migrate ventrally through the skin into a larval pattern. During metamorphosis, those pigment cells already in the skin are necessary for recruiting more pigment cells (both differentiated and precursor cells) into the proper patterns, and maintaining pigment cell identity (Parichy and Turner, 2003).

Since black and red/yellow colors are produced by distinct lineages of cells, the origin of black versus red/yellow modules could be the result of the developmental pathways that produce different colors in fish. We would therefore hypothesize that individual color traits derived from the same population of cells would be more similar than color traits of a different cell lineage. We suggest the developmental relationship between the cell lines that produce black and red/yellow coloration plays a significant role in the modularity we observe in the cluster analysis and PCA.

Ecological pressures
The evolutionary origin of the decoupling of color traits may include natural pressures. We believe this is particularly true for the flank region. Natural selection may have the most influence over the color traits composing the flank because it represents the greatest area if pigmentation on the cichlid body, and is continuously visible to potential predators from the side and from above.
Alternatively, fin traits, such as the dorsal fin, can be collapsed against the body when they are not being used for display purposes. Through the evolution of a separate module for the flank, natural selection is able to act on those traits independently from traits, which might play a more explicit role in mate choice and recognition. With independently evolving color modules, changes on the flank can occur as a result of a varying environment without adversely effecting traits involved in mate recognition.

**Sexual selection**

Considerable work has been devoted to the role of nuptial patterns in female mate-recognition and mate-choice, and theories have been proposed suggesting that rapid evolution of both female choice and male color can explain the level of species diversity found in Lake Malawi (Seehausen and van Alphen, 1998; Danley and Kocher, 2001; Couldridge and Alexander, 2002; Haesler and Seehausen, 2005; Kidd et al., 2006; Pauers et al., 2010). Here, we find that males exhibit a more modular color pallet while females are more integrated, which begs the obvious question of whether sexual selection can act at the level of modularity (Badyaev, 2004). While anecdotal, the observation that black fin traits form a module with red/yellow egg spots (traits with presumably disparate developmental origins) suggests that sexual selection may underlie this pattern of modularity. Specifically, the function of egg spots is believed to be to insure paternity; they are used to lure females who are picking up recently deposited eggs in their mouth toward a male’s vent for fertilization (Hert, 1989).
Moreover, there has been considerable work in cichlids and other organisms that has demonstrated that levels of melanism (e.g., black pigment) play a critical role in male courtship displays (Jawor and Breitwisch, 2003; Roulin, 2004; Maflil et al., 2011). This suggests that egg spots and black fin pigmentation operate together because they are used within a very specific timeframe and during a very specific behavior that is influenced by sexual selection. Some distantly related cichlids from Lake Tanganyika have long, bright structures on the ends of their pelvic fins (i.e., the fin with which egg spot number consistently clusters with) that might serve a similar purpose as egg spots, so one could speculate there are phylogenetic reasons for the consistent pelvic fin association, which was unexpected considering egg spots are expressed on the anal fin (Salzburger et al., 2007).

In theory, the degree to which male modules manifest themselves in females should present a problem. It is generally expected that females who care for young will demonstrate some degree of cryptic coloration (Protas and Patel, 2008). As mouthbrooders, Lake Malawi cichlid females are under natural selection to remain cryptic, and any significant persistence of flashy male modules could represent a threat to their fitness. Conversely, expression of dull female patterns could adversely affect a male’s chance of acquiring matings. In the next section we discuss how differential levels of integration found between sexes could resolve this conflict.
(ii) Levels of integration

Our results indicate that females are much more integrated than males. PC1 in the female dataset accounts for 54% of the total variation while the same axis in males is only responsible for 30% of the total variation. Even though the sexes share a similar pattern of modularity (i.e., similar traits load on the same axes), the PCA suggests that there is a tighter association between traits in females compared to males. This means that changes in the pigmentation phenotype for females will have a more global effect across the body. Such uniform changes could help keep females cryptic in a changing environment.

During the evolution of the cichlid flock, Lake Malawi has undergone a series of depth changes, some of which have occurred relatively rapidly. Changes in depth on the order of 500 m represent radical shifts in the habitat into which females must blend (Cohen et al., 2007). Tight integration among color traits provides a firm axis of variation along which populations of females can move quickly. In addition, a high degree of modularity in males could facilitate similarly rapid changes in color patterns to optimize conspicuousness in a dynamic environment.

(iii) Resolving sexual conflict via trait integration

Different levels of integration among the sexes represent an efficient way for variation to exist differentially between males and females. When this occurs different selective pressures can act on one sex without deleteriously effecting the other sex. The efficiency of trait integration becomes especially advantageous
when considered in a system characterized by diverse and complex sex-determination mechanisms (Ser et al., 2010). Sex determination across teleosts, including Lake Malawi cichlids, represents an area of active research because it is surrounded by uncertainty. What studies have shown for cichlids in particular is that sex-determination is the result of many interacting loci (Ser et al., 2010). Likewise, it is not unreasonable to expect that the structure of cichlid pigmentation patterns are also controlled by many loci (Barson et al., 2007). In a sex-determination system which is itself under active change, the recruitment of every male-color allele into sex linkage appears a prohibitive constraint on the path to resolve sexual conflict.

So far, the OB locus has been the only cichlid sex-linked color trait uncovered. In the WZ system, the OB locus is linked to the W loci on linkage group 5 and is responsible for producing the ‘orange blotch’ pattern in females (Roberts et al., 2009). This pigmentation pattern represents a complete breakdown in the modularity of pigmentation patterns, and is thought to persist in females because it confers a high degree of crypsis. The large effect of a single allele on such a complex phenotype hints at the possibility that loci which control the integration and modularity of traits can be few in number, but broadcast huge effects.

Recent discoveries made concerning the genetic architecture of sex-determination have led researchers to posit that the cichlid genome may be structured after Rice’s (1987) theory concerning the evolution of sex chromosomes in response to
intralocus sexual conflict (Roberts et al., 2009). Ser et al. (2009) specifically suggest a model of cichlid speciation preceded by the resolution of sexual conflict associated with sexual color dimorphism via the recruitment of color alleles into sex-linkage. They admit their hypothesis requires more information regarding the degree to which females express male color traits, and thus the present state of sex-linkage for color traits in the lake.

Our study addresses their uncertainty about the nature of how sexually dimorphic color traits have evolved in cichlids. We show that females and males express the same basic trait configuration, but that the level of correlation among color complexes is very different. With a sex-linked set of color integration alleles, females can rapidly produce broad changes in pigmentation across the body while male patterns remain unchanged. Males, on the other hand, can quickly produce novel patterns through specific changes to modules without building new nuptial patterns from scratch. These quick changes can free males from competition for finite resources (Pauers et al., 2008), and take advantage of females’ theoretically plastic mate choice. Populations of males who attract a novel female preference because of rapid adjustments in color modules could be the first step in generating a new sympatric, reproductively isolated species.

Therefore, we would amend Ser and colleagues’ model of rapid, sex-linked color pattern evolution by adding that loci controlling the degree of color trait integration should also be considered with respect to sex-linkage. Whether
differences in integration are due to the action of discrete sex-linked “integration loci”, or are rather due to pleiotropy between sex-determination and color trait integration remains to be seen. Regardless of the specific molecular mechanism(s), we propose that a key innovation underlying the evolutionary success of Lake Malawi cichlids involves the resolution of sexual conflict via sex-specific trait integration.

5. Conclusion

Our study set out to answer two fundamental questions about the way color patterns are inherited in Lake Malawi cichlids: (1) Are cichlids color traits modular; and (2) Do sex-specific differences exist in the levels of trait integration? Through our exploratory analysis we uncovered that complex pigmentation phenotypes demonstrated a modular form of inheritance. Because these fish are sexually dimorphic we analyzed males and females separately and found the modules were generally the same between sexes, but their levels of integration were very different. Ecological aspects of the system and factors concerning mating behavior suggest females and males are under very different selection pressure for their color patterns. The apparently fixed, widespread sexual dimorphism throughout the lake suggests any sexual conflict caused by these differential pressures has been largely resolved. Based on a model for cichlid species evolution posed by Ser et al. (2010), we suggest that the key to overcoming the constraint of sexual conflict was the evolution of sex-specific (and potentially sex-linked) levels of trait integration.
Further study should be aimed at understanding the mechanisms (genetic and developmental) through which modules can be broken down or rearranged between sexes and species. In addition, research will need to demonstrate that male modularity and female integration is a consistent pattern across Lake Malawi cichlids species. Finally, future work should focus on the eventual elucidation of the genetic basis for color trait integration in cichlids.
6. References


7. Figures

Figure 1

Hypothetical, unresolved phylogeny for an adaptive radiation (A) Well supported, hypothetical phylogeny (B). Over less time, the lineage in A evolves more diverse and disparate ecological resource utilization strategies than lineage B. In comparison, A represents an adaptive radiation.
Figure 2

Map illustrating the Rift Valley of Africa (inset). The wild-caught parentals used in this study were acquired from the locations specified on the illustration of Lake Malawi. Maps are not to scale, and for reference purposes only.
Figure 2

Nuptial colors of male Lake Malawi cichlids. (A) *Labidochromis caeruleus*. (B) *Cyanotilapia mbamba*. (C) *Pseudotropheus acei*. (D) *Maylandia zebra* (Zimbabwe Island). (E) *Melanochromis cyaneorhabdos*. (F) *Tropheops chinyamwezi*. 
Figure 4

Sexual dimorphism in Lake Malawi cichlids. (A) *Lodotropheus stuartgranti*. (B) *Maylandia lombardoi*. (C) *Melanochromis joanjohnsonae*.

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Figure 5

Schematic of trait correlations found within and between two modules. Trait integration exists when variation between two traits is correlated. When groups of traits are more strongly correlated with each other than other traits, those groups form modules (gray dashed line). Adapted from Klingenberg, 2008.
Figure 6

The effect of trait integration and patterns of modularity on the evolution of complex characters. The dashed green boxes surrounding the limbs of the domestic dog and hyena represent hypothetical modules. The lengths of the fore and hind limbs in the dog are tightly correlated. Evolution occurs rapidly when selective pressure act in the direction of the major axis of variation. However, the evolution may occur in a restricted area of phenotypic space. Trait integration in the limbs of dogs can explain the rapid immergence of phenotypically disparate breeds of dogs such as the Pembroke Welsh Corgi and the Scottish Deerhound, while also suggesting why breeds with disparate limb lengths within the individual are rare. This pattern of integration may be broken in the distantly related Hyaenidae.
Figure 7

Illustration of partial Lake Malawi cichlid phylogeny following Allender et al., 2003. Branch tips represent populations while black boxes encompass individual species. Closely related populations have very different color patterns. Patterns are also divergent at finer scales than the species, demonstrated by the diverse patterns found in populations of the single species *Labeotropheus fuelleborni*. 
Figure 8

Figure 9

Male (left column) and female (right column) examples of ice bath (top row) and (-) epinephrine (bottom row) treatments in the F$_2$ population. Arrows indicate areas of significant change in the degree of ‘black’ due to the constriction of melanocytes.
Figure 10

Individual color traits comprising the complex cichlid color pattern, according to this study. (1) Male from the F$_2$ population. (2) Fin color traits: (a) pelvic fin, percent “black” and “red/yellow” were measured (b, c); (d) anal fin, percent “black” and “red/yellow” measured (e, f), and number of egg spots (star); (g) dorsal fin, percent red measured (h). (3) Flank (a) color traits, (b) illustrates the nine regions in which percent “red/yellow” was measured. (4) Quantifying “black” on the dorsal fin (a); (b) demonstrates the specific band of “black” of interest; (c) how the space between each ray was scored.
Output of hierarchal cluster analysis reveals similar patterns of modularity between males and females. (1) Analysis performed on entire F₂ population. (2) Analysis including only males. (3) Analysis including only females. (4) Putative modules according to cluster analysis: (a) “black” fin traits and egg spots; (b) “red/yellow” fin traits (c) flank traits A-C, E, F, I.
Figure 12

Output of Principal Components Analysis (PCA). (A) Analysis performed on entire F2 population. (B) Analysis performed on F2 males only. (C) Analysis performed on F2 females only. (D-F) Plots of eigenvalue on principal component axes. Charts correspond with PCA loadings above it.
Figure 13

Modularity and integration between males and females in the F$_2$ population. Males (top row) demonstrating the independent variation between fin traits and the flank. (a) and (b) have similar amounts of black and red/yellow on their fins, but the extent of red/yellow on the flank is very different. Females (bottom row) showing whole-body color trait integration. (c) is very red across all the entire individual, while (d) has little red in any of the regions measured. Both c and d both have similar amounts of black, however, suggesting certain independence (and thus modularity) is present in females as well.
8. Project Summary

In the present period of rapid and uncertain global change, the drive to better understand where biodiversity comes from is stronger than ever. There is no better model to elucidate the factors involved in the evolution of vertebrate biodiversity than ray-finned fishes. No other vertebrate group is comparably old and species rich. Much of this diversity belongs to a special sub-group of the ray-finned fishes called the teleosts. When most people think of their favorite fish, like tuna or bass, they are thinking about a teleost. This study utilizes a fascinating family of teleosts, the Cichlidae, to answer questions about how species evolve.

The process by which a species evolves is speciation. Understanding the mechanisms of speciation allows us to better understand the origins of biodiversity. The particular members of the family Cichlidae we are interested in are the cichlid fishes of Lake Malawi, one of the great rift lakes in east Africa. Lake Malawi cichlids represent the most successful living group of vertebrates. In 1-2 million years, the Lake Malawi cichlid flock has diverged into as many as 1000 species, representing a third of the species diversity in the Cichlidae family. Many aspects of biodiversity have been explored in an attempt to uncover the mechanisms behind their explosive diversity—including, but not limited to, jaw structure, tooth shape, and eye physiology. This study adds to the many perspectives by examining the radiation in the context of color pattern diversity.

Male cichlid fishes are exceptionally flashy. Females, however, are very dull because they care for young by mouthbrooding. They must remain camouflaged so as to avoid predation of themselves and their young. We refer to
these flashy pigmentation patterns as nuptial colors because they are very important throughout mating behaviors. In particular, reproductive isolation in east African cichlids is based on the visual cues provided by the male nuptial patterns. Thus, particularly in the Lake Malawi cichlid system, it is generally accepted that sexual selection plays a large role in the evolution of male nuptial colors. Sexual selection refers to pressures exerted on individuals as a result of the pursuit of reproductive opportunities, and they are often considered separately from the forces of natural selection which result from a varying environment. Sometimes, the interactions between males and females are relatively harmless. Other times, the interests of either sex seem to be in conflict. This type of sexual selection is often called sexual conflict, and a common metaphor for it is an “evolutionary arms race”.

The cichlids of Lake Malawi represent a system in which the flashy coloration of males may be in conflict with the need for females to remain cryptic. We are not the first set of researchers to consider the role color has played in cichlid evolution. In fact, a generally accepted model exists for rapid cichlid species evolution in which female mate preferences change rapidly, and male color patterns can respond very rapidly. However, little is known about how easy it is for color traits to change, and whether or not male and female traits can change separately. The hypothesis we explore in this study is that the explosion of many different kinds of flashy male nuptial patterns, and the ability of females to remain cryptic, may have been facilitated by the ‘resolution’ of conflict between the expression of male and female color patterns. If male and female
color patterns can change separately, the conflict between crypsis and flashiness
can be reduced. The question becomes: How easy or difficult is it for color
patterns to change in response to differential selection pressures?

To answer this question we draw upon the vast theoretical framework of
trait integration and modularity. These concepts are related, and surprisingly
straightforward. Traits that tend to vary together within individuals are
integrated. Groups of traits that vary together, but show less correlation to other
traits are referred to as modules. Integration can affect the evolution of traits in
two ways: 1.) It can influence the time it takes change to manifest; and 2.)
Integration can affect the direction of change in phenotypic space, which simply
means the way the shape of trait, such as a leg, is changing. Modularity allows
for these changes over time and space to occur relatively independently across an
animal. So, length of a human leg and arm can evolve differently, unlike in dogs
where all their limbs are the same length.

Some researchers have suggested that male cichlid color patterns are
composed of recurring traits, and that new patterns can be generated by small
changes in those traits rather than building a new complex phenotype from
scratch. These observations are consistent with the idea that nuptial patterns in
male cichlids may operate as modules. However, female colors do not change
from species to species with the same specificity in regard to individual traits. In
this study, we propose that either the levels or patterns of integration between
males and females may differ, and it is the evolution of these differences that may
have contributed to the ‘resolution’ of a sexual conflict between the pressure for males to be flashy and females to remain cryptic.

In order to elucidate patterns and levels of trait integration we quantified 16 individual color traits that were either black or red in a $F_2$ hybrid population. This population was generated by a cross between wild-caught Lake Malawi species *Labeotropheus fueliborni* and *Tropheops* ‘red cheek’.

Trait quantification involved finding the percent of target pixels in the region of interest, a fin for example, in Adobe Photoshop. Black and Red were measured for three fins: pelvic, anal, and dorsal. Red was also measured for nine regions on the flank, or the side of the fish, labeled A-I. We also counted the number of Egg Spots on the anal fin.

Two complimentary, exploratory multivariate statistical analyses were performed on our size-corrected dataset. Hierarchal cluster analysis was used to determine the most likely pattern of modularity. Principal components analysis (PCA) was used to corroborate this pattern, and to get an idea of the strength of integration in the dataset.

Using these powerful tools we found that males and females shared a pattern of modularity that follows: 1) Black fin traits and egg spots; 2) red fin traits; and 3) flank traits. Males and females did differ with regard to the level of integration the expressed. Females were much more integrated than males.

In terms of the pattern of modularity we observed, there are several possible explanations for their evolutionary origin, though this is certainly not exhaustive. Natural selection could have favored the evolution of a separate flank
module initially because it is the largest area which is continuously visible on the cichlid body. Modularity in this respect could allow for changes to occur in response to a varying environment without affecting any possible mating-display functions on the fins. Similarly, the formation of modules along color lines hints at the possibility that the developmental pathways which produce those colors could play a role in their evolution. Red pigment cells and black pigment cells represent distinct cell lineages. We would therefore hypothesize that individual color traits derived from the same population of cells would be more similar than color traits of a different cell lineage. Factors such as these predispose cichlids, in terms of male nuptial patterns, toward modularity. Sexual selection could reinforce these modules. If female preferences change rapidly but only slightly, populations of males with modules that can move through phenotypic space quickly, but specifically, are selected for.

The evolution of flashy male modules presents a problem, however. As mouthbrooders, Lake Malawi cichlid females are under natural selection to remain cryptic, and any significant persistance of flashy male modules represents a threat to their fitness. Conversely, expression of dull female patterns could adversely affect a male’s chance of acquiring matings. When we consider levels of integration, however, the path towards resolution of this conflict becomes clearer.

There are more associations between modules in females than in males. This means that changes in the color pattern for females have a more global effect across the body. Such uniform changes could keep females cryptic in a changing
And, the environment in which cichlids were evolving was changing a great deal. Lake Malawi has undergone a series of depth changes, some of which have occurred relatively rapidly. Tight integration among color traits means females can blend into new environment quickly.

Different levels of integration in the sexes represent an efficient way for variation to exist differentially between males and females. When variation exists in different ways between the sexes different selective pressures can act on males without deleteriously affecting females, for example.

With a sex-linked set of color integration alleles, females can rapidly produce broad changes in pigmentation across the body while male patterns remain unchanged. Males, on the other hand, can quickly produce novel patterns through specific changes to modules without building new nuptial patterns from scratch. These quick changes can free males from competition for finite resources and take advantage of females’ theoretically rapidly changing mate choice. Populations of males who attract a novel female preference because of rapid adjustments in color modules could be the first step in generating a new sympatric, reproductively isolated species. Thus, color trait integration could serve as a ‘key innovation’ in the generation of variation necessary to produce the wealth of biodiversity in the east African lakes we observe today.

In general, exploring the notion of biodiversity as a process which produces variation, and not simply as the number of species you can count, represents the direction we much head in our consideration of the topic if we truly want to preserve it indefinitely.