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The relationship between carotenoid type and skin color in the ornamental red zebra cichlid *Maylandia estherae*

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Abstract. This study examined the effects the dietary carotenoids astaxanthin, lutein, and Spirulina had in the skin color on red zebra cichlids (*Maylandia estherae* Konings 1995). We found that skin color was dependent on pigment type and concentration. Astaxanthin in the diet increased the red-orange color in the skin of the red zebra cichlid while Spirulina intake increased the orange and yellow tones. Lutein imparted a light-yellow tint. The results suggest that carotenoids may play an important role in animal color polymorphism and speciation, which may help explain different empirical and theoretical perspectives on color diversity in fish. Appropriate use of a wide variety of carotenoids in the diet can bring large economic benefits to farmers as fishes with an assortment of coloration are a priority for fish enthusiasts.
Key Words: *Pseudotropheus*, pigmentation, sexual dichromatism, speciation.

Introduction. The collection and keeping of ornamental fish is primarily for the home aquarium and backyard pond. Therefore, skin color is the most important criterion used for their selection, although body shapes, behavioral patterns, and other environmental adaptations are important as well (Chapman 2000). However, color perception is highly subjective and there is no preferred standard color. Of practical importance to the trade is that skin color in fishes originates and is obtained principally from colored chemicals or pigments in the food they eat. In other words, to achieve or enhance certain colors of the fish, specific pigments and amounts must be added to their diet. These pigments are principally carotenoids of which some 600 types have been described. When carotenoids bind to proteins or lipoproteins they also form complexes of carotenoproteins and carotenolipoproteins. Carotenoids and their complexes produce biological pigments that can be used to display the visible spectral colors from red and orange, to yellow, green, blue, and violet. Carotenoids are synthetized only by algae, plants, and some microbes but are accumulated and become available in the natural foods fish eat such as plankton, worms, and shellfish. Lutein, zeaxanthin, and astaxanthin are among the most potent of the carotenoids tested for coloring in fishes, which are also manufactured synthetically and available commercially (Torrissen et al 1989; Wallat et al 2005). Typically, lutein and zeaxanthin are yellow pigments while astaxanthin is considered a red pigment. Astaxanthin carotenoprotein complexes give off purple-blue and green colorations.

Fishes use color primarily for signaling during courtship, mating or other intrasexual communication, and for threatening displays, camouflage, and protection from predators by scaring them or warning that the animal or some part of it is poisonous (Theis et al 2012). Body color, together with the physics of light penetration, well-developed parental and territorial behavior patterns and remarkable feeding specializations have promoted sympatric speciation and adaptive radiation to proceed in just a few hundred thousand years in cichlid fish species in the great lakes of east Africa (Fryer & Iles 1972; Turner 1994; Barlow 2000; Kocher 2004; Seehausen & Schluter

2004; Terai et al 2006). Cichlids are among the most colorful of freshwater fishes. Particularly those of Lake Malawi in Africa where at least some 450 species have been described. The 'mbuna' or rock-dweller fish are a large group of very colorful cichlids that live among piles of rock along the lake shoreline. The red zebra cichlid (*Maylandia estherae* Konings 1995), is very popular among aquarium fish ethusiasts and common in pet stores. Despite being called red zebra cichlid, they are mostly orange in color. However, the species displays a wide range of color variation of yellows, oranges, peach, and reds; they can also have yellow spots on the end tips of the dorsal and anal fins. Typically the male is more vividly colored than the female and many individuals can be bright blue. In their natural habitat, this cichlid grows to about 9 cm in total length, although they have been known to reach 15 cm in captivity (Fryer & Iles 1972).

Since skin color in ornamental fishes can be enhanced by feeding them diets supplemented with pigments, the objective of this study was to measure the effectiveness of artificially supplementing pelleted fish feed with natural occuring carotenoid pigments to enhance and possibly change skin color in the red zebra cichlid. Although, there is a large body of scientific literature on the uses and relationship between diet pigmentation and muscle and skin coloring for trout and salmon, the information is scarce for ornamental fish, which target only a few of the most popular species like color carp and goldfish (Iwahashi & Wakui 1976; Lovell 1992; Duncan & Lovell 1993; Chapman 1997; Shahidi et al 1998; Paripatananont et al 1999; Gouveia et al 2003; Wallat et al 2005; Wang et al 2006; Harpaz & Padowicz 2007; Yasir & Qin 2010; Ho et al 2013a, b; Imués-Figueroa et al 2012). In contrast you will find an abundance of informative articles and tips in aquarium hobby magazines, written by experienced enthusiasts and biologists, on how to artificially enhance the color of ornamental fish through various means. Access to published scientific evidence on the uses of pigments to influence coloration in ornamental fish will enable farmers to enhance quality, add value consistently to their fish, and increase the probability of attracting a buyer. Given the importance of skin color and feeding strategies on cichlid speciation, studies on the interactions between skin color and the pigments they obtain from the food they eat, (which are involved in vision, behavioural ecology, physiology, immunology), may help to better interpret the empirical and theoretical basis of their evolutionary biology.

Material and Method

Fish and experimental conditions. The fish used in this experiment were juvenile red zebra cichlids *M. estherae*; hatched within the same week and approximately 3-months in age (May 2012). Fish were donated by an ornamental fish farm in Miami, FL and transported to the laboratory of Fisheries and Aquatic Sciences, University of Florida, Gainesville, USA. All the fish were prophylactically treated with a sodium chloride salt bath (1%) for several hours; acclimatized for three weeks until the experiments began, in two tanks (162 L, 90 x 40 x 45 cm) with 200 fish stocked per tank. After acclimation, 20 fish per tank were randomly distributed into 16 aquarium plastic tanks (31.5 L, 30 x 30 x 35 cm). Three sides of each tank were covered with a black plastic sheet to reduce outside disturbance that may have resulted in stress to the fish. Each aquarium contained an air-driven double sponge filter that provided constant aeration, and both mechanical and biological filtration. About one-third of the water was exchanged every week, and replaced with filtered freshwater from a well, kept in a reservoir tank at room temperature. Water quality conditions in each tank were monitored and maintained at 24-26°C, a dissolved oxygen level of 5-7 mg L^{-1}, pH of 7.0-7.1, and the fraction of un-ionized ammonia was never detected above 0.01 mg L^{-1}. The room where the experiment was conducted, was illuminated with a fluorescent light set to a 12 h light (1200 lux) and 12 h darkness cycle. The experiment had a duration of 90 days.

Experimental diets. Fish were divided into four experimental groups, with four replicates for each treatment. One group was offered the control diet that only contained basic feed ingredients without or little carotenoid pigments and expected to have little effect on coloration outcome. The other three diets were prepared using the same

ingredients as the control diet but with the addition of carotenoid pigments from different natural sources. One diet was supplemented with the red pigment astaxanthin extracted from algae (Cyanotech Corporation, Kailua-Kona, Hawaii). The yellow lutein pigment contained in corn protein concentrates (CPC, Cargill Corn Milling, Wayzata, Minnesota) was incorporated to another diet. Spirulina (Carbon Capture Corporation, La Jolla, California) was added to the fourth diet.

The red, orange, and yellow pigments were chosen because of their potency to impart skin color in other fishes and they correspond to the colors of the species in nature. These particular pigments also share biochemical similarities that may provide insight on the importance of metabolic processes in color development and implications on color dependence speciation theory.

The corn protein concentrates and Spirulina themselves are highly nutritious ingredients. Spirulina is a type of blue-green algae used as a feed supplement in many animal industries including aquaculture, and contains high amounts of the blue-phycocyanin pigment, and the orange beta-carotene from which zeaxanthin is formed. Lutein and zeaxanthin have identical chemical formulas (isomers), and astaxanthin biosynthesis can proceed from zeaxanthin. Ingredients containing the pigments lutein, and those from Spirulina were incorporated into the diets at 12%, as they are complete protein sources and contributed an important proportion to the diet. As a sole ingredient, almost purified astaxanthin was added to the diet at 0.3%, to produce an acceptable colored skin (Lovell 1992 and our laboratory experiences). The basal diet was formulated based on previous ornamental cichlid fish nutrition studies (Royes et al 2006), and investigations in our laboratory. The diets were formulated to be isocaloric and isonitrogenous using wheat flour (34.3%) and sardine meal (19.8%) as the major sources of protein and the extra energy coming from menhaden fish oil.

The proximate compositons of the diets are given in Table 1 and were determined using standard methods of food analysis (AOAC 1990). The carotenoids in the feed were extracted based on Torrissen & Naevdal (1984) methods, and total concentrations determined spectrophotometrically in chloroform using extinction coefficients ($E^{1\%}$, 1 cm) of 1692.2 at 485 nm for astaxanthin, 2540 at 487 nm for zeaxanthin, 2369 at 454 nm for lutein, and 2330 at 462 nm for β-carotene (Neal & Joseph 1992).

Table 1

Proximate composition of the feed pellets offered to the experimental fish. Diet-1, control with no carotenoid pigments added; Diet-2, containing astaxanthin; Diet-3, with lutein; Diet-4, supplemented with Spirulina

Diet	*Calories*	*Protein (%)*	*Fat (%)*	*Carbohydrate (%)*	*Ash (%)*	*Moisture (%)*
1. Control	391	41.4	9.6	35	7.1	7.3
2. Astaxanthin	390	41.6	9.3	35	7.3	7.1
3. Lutein	389	41.4	9.6	34	7.0	7.1
4. Spirulina	394	41.5	10.1	34	6.6	7.6

The diets were manufactured at the U.S. Fish & Wildlife Service Bozeman Fish Technology Center, Bozeman, MT, USA. Raw ingredients were weighed, reduced in size with a grinder, blended, extruded into pellet form, and dried; the size of the final pellets was 0.8 mm. There were apparent color differences between the diets (Figure 1). Fish were hand-fed to apparent satiation twice a day (10 AM and 4 PM), except on Sundays. Feces and uneaten feed were removed from each tank daily.

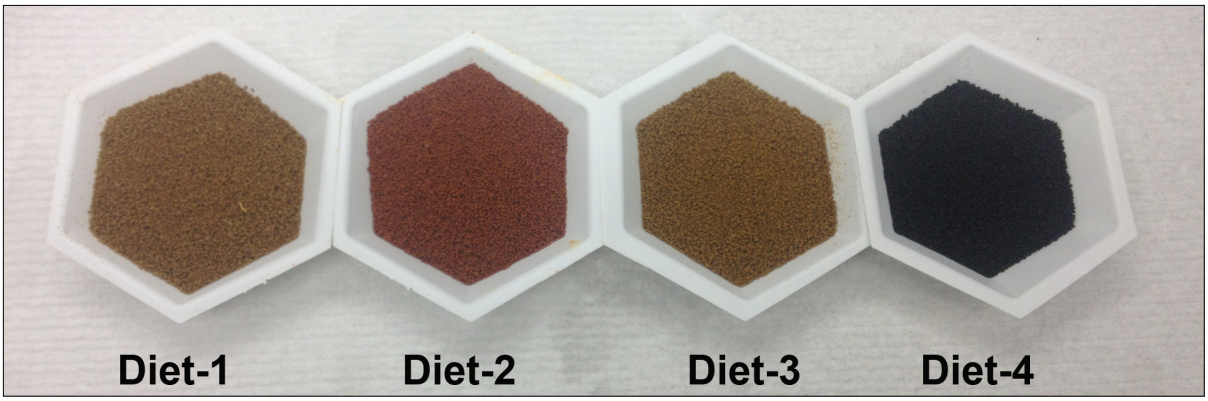

Figure 1. The color of experimental Diet-1, control with no carotenoid pigments added; Diet-2, containing astaxanthin; Diet-3, with lutein; Diet-4, supplemented with Spirulina.

Skin color analysis. We modified a color machine vision sytem routinely utilized in our laboratory to objectively measure skin color changes in live ornamental fish (Wallat et al 2002). The major components included a water-filled glass tank to place the fish in. The tank was placed inside a portable light box for product photagraphy (Bestlight Studio), illuminated from above with a circular fluorescent white light tube (6500K) covered with a sheet of polarizing filter (Rosco). Photographs were taken with a Sony MVC-CD500 digital camera equipped with a 6x zoom glass lens, circular polarizing filter, and mounted on a mini tripod. The camera was on manual exposure setting with the lens aperture at f/5, a focal length of 13.9 mm, and exposure time of 1/15 s. Each fish was removed briefly from its aquarium and placed inside the photography tank where it was photographed. At least three pictures were taken from the right and left side of each fish.

A certified diffuse color standard (LabSphere, Spectralon) was also placed in the tank together with the fish to precisely describe, match or compare the colors in the skin, in the photographs. The entire procedure lasted approximately five minutes and did not require the fish to be anaesthetized. The LensEye Color Expert software (Version 10.1.7) was used to analyse and quantify color of the fish images; color classification using the CIELAB Color model.

Sampling design and statistical analysis. During the 21-d acclimation period, fish were fed the basal, control diet with no added carotenoid pigments. At the end of this period, ten fish from each of the two acclimation tanks were randomly sampled, weighed, measured, and photographed to establish a baseline for comparison. Fish were then divided into their respective experimental groups, and then offered the carotenoid-rich feed for 35-d at which time they were sampled again. After another 35-d, nine bright-colored and five light-colored fish were selected from each tank and analyzed for color differences and determining their sex. Gonads were dissected for viewing and routine histology (tissues fixed in 10% formalin, paraffin-embedded, and stained with H & E). The nutritional adequacy of the diet was assessed by comparative evaluation in measures of survival and growth performance in terms of body weight gain, and percentage body weight increase per day (SGR).

The data were analyzed using the program SPSS for Windows (Version 16). A one-way ANOVA and Tukey's HSD tests were used to determine if there were significant differences between the diets on survival and growth, and in skin color in fish being fed carotenoids of different types and concentrations. A chi-squared test was used to detect differences in sex ratio and fish having distinguishable color differences of darker and lighter shades. An alpha level of 0.05 was used and considered differences to be significant if $p < 0.05$.

Results. All fish readily accepted and consumed the food pellet that was offered to them, regardless of the pigment type incorporated into it. With the exceptation of two individuals that died during the first week, all fish survived and remained healthy from the beginning to the end of the trial. Fish in all treatment groups, gained significant

length and weight during the experimental period of 35 days. Body length and weight gains, specific growth rate, and survival were not significantly different among fish fed different diets (Table 2). Average body length and weight gains alone were greater than one standard deviation from the initial mean values 32 ± 3 mm TL and 0.61 ± 0.2 g. At the end of five weeks, fish averaged 43.2 ± 4 mm TL and 1.38 ± 0.5 g.

Table 2

The average body length (BLG) and weight gain per fish (BWG), specific growth rate (SGR), and survival of red zebra cichlid fed carotenoid-rich diets. No significant differences were observed between groups ($p > 0.05$)

Diet formulation	*BLG, mm*	*BWG, g*	*SGR, % day^{-1}*	*Survival, %*
Basal diet	9 ± 3	0.76 ± 0.4	2.3 ± 1.2	100
w/ Astaxanthin	11 ± 4	0.75 ± 0.3	2.3 ± 1.2	98.7
w/ Lutein	11 ± 3	0.75 ± 0.3	2.3 ± 1.3	100
w/ Spirulina	11 ± 4	0.81 ± 0.4	2.5 ± 1.3	98.7

Diets differed in carotenoid concentrations, with the most amount of pigments 348.7 mg kg^{-1} and 409.5-448.5 mg kg^{-1} respectively, extracted from the formulations that contained astaxanthin and Spirulina. Only moderate total carotenoid concentrations (42.2 mg kg^{-1}) were obtained when corn protein concentrates were added to the diet. No carotenoid pigments were detected in the basal, control diet. Although the diets varied considerably in the amount of pigments they contained, the type of pigment in the diet significantly affected skin color in the red zebra cichlid. At the initiation of the trial the fish were primarily light yellowish-brown in color. About two weeks thereafter, in fish fed the carotenoid-rich diets, clearly distinguishable differences in skin color were observed that covered at least 6% of their body surface; after five weeks, these fish had a distinct skin coloration (Table 3 and Figure 2).

Table 3

Predominant skin color in red zebra cichlid and averages of total carotenoid concentrations in the diet

Pigment source	*Initial color*	*Final color*	*Pigment amount*
Control	Light yellowish brown	Light yellowish brown	None detected
Astaxanthin	Light yellowish brown	Moderate orange	348.7 mg kg^{-1}
Lutein	Light yellowish brown	Dark yellow	42.2 mg kg^{-1}
Spirulina	Light yellowish brown	Dark orange-yellow	409.5-448.5 mg kg^{-1}

The red zebra cichlids fed the diet containing the carotenoid astaxanthin developed the most orange-red coloration. While those fed the diet containing corn protein concentrates (a lutein source) became light-yellow. Fish fed the Spirulina (a rich source of beta-carotene and zeaxanthin) diet became dark yellow-orange in color, had the most vivid colors, and most visible 'egg spots' on the anal fin (Figure 2). The cichlids fed the basal, control diet (without carotenoid pigments) had no significant changes in their skin coloration and lacked vivid colors.

However, two distinct groups of fishes having tones of lighter and darker shades were visible in the fish fed the astaxanthin and spirulina rich diets, especially after an additional 35 d period of feeding the diets (Figure 3). The number of fish within a treatment group having light and dark skin coloration did not differ significantly from a 50:50 ratio. Macroscopic examination and histology of the gonads revealed light color fish were females and those with the vivid colors were males. Interestingly enough the color shade differences were not apparent, neither in the control group, nor in the lutein experimental group.

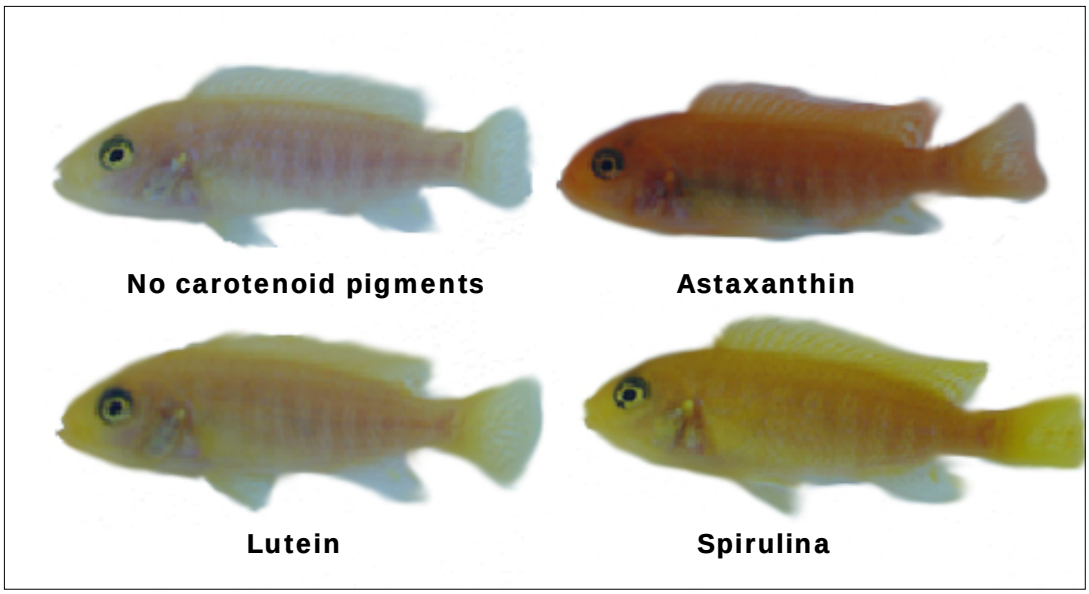

Figure 2. Skin coloration in red zebra cichlid, five weeks after being fed diets containing astaxanthin, lutein, and Spirulina. The basal, control diet without carotenoid pigments.

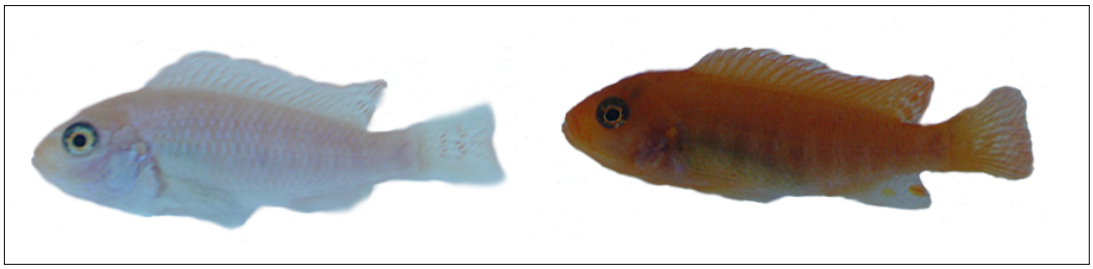

Figure 3. Two significantly distinct color tones were apparent in the fish fed the astaxanthin and spirulina rich diets.

Discussion. In this study, red zebra cichlids were fed carotenoid-supplemented diets, which induced skin color changes. Differences in skin color of the fish were analyzed using a modified color machine vision sytem that matched colors to the standardized L*, a*, and b* color space values. Using this method, multiple colors on individual or multiple fish can be objectively determined. Because this method is consistent color thorough, rapid, and non-lethal, we recommend it for live fish skin color analysis.

Many reports have demonstrated that skin color change over time depended on the level of carotenoid in the diet and differed among species (e.g., Duncan & Lovell 1993; Storebakken et al 1987; Chatzifotis et al 2005; Dharmaraj & Dhevendaran 2011; Ho et al 2014). The diets used in this study contained astaxanthin, lutein, and Spirulina as colorant pigments. After 14 days of feeding the diets, the change in skin color of the red zebra cichlids could be easily distinguished with the naked eye and the color started to fully develop from about day 35. In other studies the skin color on ornamental goldfish fed with an astaxanthin-supplemented diet started to differ from fish that were fed a control diet after 7 d (Paripatananont et al 1999). It took about 28 d for the orange-red skin color on oranda goldfish to develop when fed commercial diets that contained astaxanthin, lutein, or zeaxanthin (Wallat et al 2005). More astaxanthin was deposited in the skin of *Salmo salar* after 21 days of feeding a diet supplemented with astaxanthin compared to fish on a control diet (Storebakken et al 1987). Similarly, after a 21-day period of feeding on an astaxanthin-rich diet, high levels of astaxanthin (40 mg kg^{-1}) were also found in the skin of the gilthead sea bream *Sparus auratus* (Gomes et al 2002).

However, carotenoid supplementation had no positive or negative effect on their growth, survival or apparent health. Similar findings were also reported in other fishes and penaed shrimp supplemented with astaxanthin, β-carotene, or lutein (Bell et al

2000; Amar et al 2001; Boonyaratpalin et al 2001; Ramamoorthy et al 2010). Although astaxanthin did not appear to affect weight gain in ornamental goldfish, it lead to a significantly better survival (Paripatananont et al 1999). Given the abundant recent literature on the possible protective effect of dietary carotenoid intake, including antioxidant, anti-inflammatory, and immunological benefits for humans and terrestrial laboratory animals, further studies are needed as well in aquaculture. Carotenoids may also have an important role in speciation. The explosive radiation of cichlid species, especially in lake Malawi has been tightly linked to optimal foraging theory and sexual selection (Turner 1994; Barlow 2000). Since skin color is dependent on pigments found in the natural food the fish eat, the skin of individuals of the same species may acquire different hues and eventually differentiate into distinct populations.

This study examined the effects the dietary carotenoids astaxanthin, lutein, and Spirulina had in the skin color of red zebra cichlids. Astaxanthin in the diet increased the red-orange color in the skin of the red zebra cichlid while Spirulina intake increased the orange and yellow tones. Lutein imparted a light-yellow tint. However, an extensive review of the literature shows the main carotenoids fed in aquaculture are astaxanthin and canthaxantin, both from natural and synthetic sources. Although the greatest amount is incorporated into the feed for fish for human consumption, the same carotenoids are often added to the feed for ornamental fish regardless of what their natural color is, and the acquired skin color or tone is primarily red-orange (Imués-Figueroa et al 2012; Seyedi et al 2013). If used judiciously in aquaculture, the use of different carotenoids in the diet can bring large economic benefits to farmers who raise fish with different coloration. Color is a very important criterion for the fish consumer (Dharmaraj & Dhevendaran 2011). Supplementing the diet of farm fish with carotenoids can enhance skin color and increase their market value.

Conclusions. The results of this study not only stress that skin color in the red zebra cichlid is depedentent on pigment type and concentration, but suggest the important role carotenoids may have in animal color polymorphism, and speciation. In other words, carotenoids may help to integrate the empirical and theoretical perspectives underlying the bewildering diversity of colorful fishes, especially cichlids. The wide array of cichlid species has been tightly linked to optimal foraging theory and sexual selection. Therefore, there is a possibility that not only foraging differences but dietary carotenoid content of their diets be a driving force to the adaptive radiation of cichlid species in nature. However, since we also observed that male red zebra cichlids became more colorful as they aged, possibly as they reach sexual maturity, these results suggests that diet alone does not regulate speciation nor sexual dichromatism.

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