

## Effects of Vitamin C Deficiency on Body Shape and Skull Osteology in *Geophagus brasiliensis*: Implications for Interpretations of Morphological Plasticity

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**Morphological plasticity in fish and other vertebrates is usually attributed to the bone and muscle remodelling that results from exposure to different environmental regimes requiring different kinematic responses, such as feeding on different diets. I examined the extent of morphological plasticity that could be induced by feeding *Geophagus brasiliensis*, fish known to respond via plasticity to different foods, diets identical in consistency, but which differed in the amount of vitamin C they contained. Vitamin C is crucial to proper bone and connective tissue development, and there are numerous qualitative reports of deformation related to vitamin C deficiency. In this experiment, fish fed the vitamin C deficient diet differed in caudal peduncle measurements and had shorter snout and oral jaw measurements than fish fed the vitamin C sufficient diet. These results suggest that both nutritional differences between diets and mechanics of feeding on different diets in different ways can induce morphological plasticity.**

**T**HERE are a growing number of reports of phenotypic plasticity of jaw, skull, and body shape in vertebrates (Collins and Cheek, 1983; Witte, 1984; Wimberger, 1991). Those that involve trophic morphology usually infer that differences in the way that an organism captures or processes prey lead to the induced morphological differences. A heretofore unexplored alternative explanation for the morphological differences is that differences in nutrition between diets lead to morphological

differences. In this paper, I examine the effect of vitamin C deficiency on jaw, skull, and body shape in *Geophagus brasiliensis*, a species known to be morphologically plastic when fed different diets (Wimberger, 1991, 1992), to determine whether the morphological differences were due to feeding behavior or nutritional effects.

Differences in both body shape and jaw and skull morphology were previously induced in two species of the cichlid genus *Geophagus* by feeding juveniles either chironomid larvae or

brine shrimp nauplii (Wimberger, 1991, 1992). Feeding behavior of the fish fed the two diets was different (Wimberger, 1991). Fish fed brine shrimp had relatively longer and shallower heads and relatively shorter and shallower bodies than fish fed chironomid larvae. The differences induced by these two diets were similar to those one would expect from functional considerations. For instance, fish feeding on more evasive prey (like the brine shrimp in these experiments) tend to have more acutely angled snouts and more fusiform bodies. Fish needing to maneuver while feeding on benthic prey (like the chironomid larvae) generally have deeper, rounder bodies (Webb, 1984). Fish that exert greater biting force have been inferred to have blunter snouts, shorter dentigerous areas of their jaws, and deeper suborbital areas (Barel, 1983).

However, nutritional deficiencies, particularly of certain vitamins and minerals, can also affect morphology during development (Halver, 1984). For instance, ascorbic acid (vitamin C) deficiency has profound morphological consequences for fish and has been qualitatively documented in coho salmon, rainbow trout, channel catfish, yellowtail tuna, guppies, and carp (Halver et al., 1969; Sakaguchi et al., 1969; Halver, 1984). Vitamin C deficiency leads to scoliosis and lordosis of the spine (abnormal lateral or dorsiventral curvature), sometimes actually resulting in broken backs (Lim and Lovell, 1978); hyperplasia of collagen and cartilage; resorbed opercles; abnormal support cartilage in the gill, spine, and fins; hyperplasia of the jaw and snout; and internal hemorrhage (Halver, 1984). Vitamin A deficiency can lead to decreased growth, exophthalmia, and thickening or deformation of the gill arches and operculum (Halver, 1984). Mineral deficiencies can lead to deformation of skull bones which become shorter and rounder (Hepher, 1988). Both calcium and phosphorus deficiencies can lead to cranial deformities (Lall, 1984).

The first indication that nutrition actually played a role in some of the shape differences between fish fed chironomid larvae and brine shrimp nauplii was that 5% of the *G. brasiliensis* raised on a diet of frozen chironomid larvae (Wimberger, 1991) exhibited lordosis, a symptom of vitamin C deficiency. Fish raised on chironomid larvae very rarely showed slight hemorrhaging at fin bases, also a sign of ascorbic acid deficiency. Nutritional analysis showed that there was no detectable ascorbic acid in frozen chironomid larvae. In contrast, though there are no published data for the vitamin C content of brine shrimp (*Artemia*) nauplii, larval fish raised on brine shrimp nauplii manifested no

symptoms of vitamin C deficiency (Leger et al., 1986). *Artemia* cysts contain a stable form of ascorbic acid which is rapidly mobilized into L-ascorbic acid (the active form) upon hatching (Leger et al., 1986). In this study, I attempted to determine the morphometric effect of vitamin C deficiency because previous studies of plasticity (Witte, 1984; Meyer, 1987; Wimberger, 1991) have not attempted to determine quantitatively the morphometric effects of any nutritional deficiency.

#### METHODS

To determine the relative importance of jaw function and nutrition in diet-induced morphological change, I constructed identical flake food diets except that one contained vitamin C in what should have been a sufficient amount (Halver, 1984; Hepher, 1988) and the other contained no vitamin C. If kinematic differences in food capture and processing are the only important variables in determining morphology, fish fed the two flake food diets should manifest no morphological differences. The morphological differences between the two treatments should be due exclusively to differences in vitamin C content of the diets. The flake food diet used was synthesized with the assistance of G. Ketola of the United States Fish and Wildlife Service Tunison Fish Nutrition Laboratory who provided a purified diet (Table 1). Because L-ascorbic acid oxidizes, I added 400 mg/kg of a stable form of ascorbic acid (L-ascorbyl-2-polyphosphate, or AsPP) in addition to 100 g/kg of normal L-ascorbic acid to the vitamin C supplemented diet (hereafter the diet to which vitamin C was added will be referred to as C+ and the diet to which no vitamin C was added will be referred to as C-). Other studies indicate that AsPP is an effective source of vitamin C for fish (Wilson et al., 1989). Data from other fish show that no deficiency syndromes appear with anywhere 50–150 mg/kg ascorbic acid in the diet (Halver, 1984; Hepher, 1988); thus the amounts used in this experiment should have been adequate. The slurries were drum dried in Cornell University's Animal Sciences Food Laboratory and then flaked. The food was stored in a -10 C freezer to reduce oxidation.

All fish used were from two successive broods from a single pair of *G. brasiliensis*. The young were taken from their parents when they were approximately 8–10 mm standard length. I put five to seven fish each in 19-liter compartments of 38- or 57-liter aquaria. Each aquarium was randomly allocated a vitamin C treatment. The fish were fed twice daily. I used 221 fish for the

TABLE 1. THE INGREDIENTS OF THE PURIFIED DIET USED TO CONSTRUCT THE FLAKE FOOD DIETS USED IN THE EXPERIMENT.

	% of diet
Fish Meal	53.8
Brewer's Dried Yeast	5.0
Soybean Flour	10.0
Blood Meal	10.0
Choline	0.4
Lecithin (liquid)	3.0
Pastry flour	10.0
Shrimp meal	2.0
Gelatin	3.0
Mineral Mix	0.16
Red Tobis Oil	2.0
Vitamin Mix	0.5
Vit A Palmitate	10,000 IU
Vit D3	4000 IU
Hetrazeen	10 mg/kg
DL-alpha-Tocopherol	780 IU
Thiamin HCl	40 mg/kg
Riboflavin	30 mg/kg
D-Ca-Pantothenate	150 mg/kg
Niacinamide	150 mg/kg
Pyridoxine HCl	20 mg/kg
D-biotin	3 mg/kg
Folic Acid (folacin)	15 mg/kg
Vit B12 in Mannitol	0.2 mg/kg
Ethoxquin (Santoquin)	150 mg/kg
Inositol, i, meso, myo	1000 mg/kg
Added to Vitamin C supplemented diet:	
L-ascorbyl-2-polyphosphatase	400 mg/kg
L-ascorbic acid	100 mg/kg

body shape analysis and 211 fish for the skull and jaw osteology analysis. Some young from the same broods were placed on brine shrimp nauplii diets to check for nondiet related morphological problems. These fish manifested no morphological abnormalities. I obtained developmental trajectories by harvesting fish on the nutrition treatments from 15–38 mm in length. These fish were euthanised in a dilute solution of tricaine methanesulfonate, fixed in 10% buffered formalin, and transferred to 55% ethyl alcohol.

To measure differences in fish raised on the two diets, I first digitized body shape landmarks on all the fish. All digitizing was done using a Cohu 4810 Solid State Camera with a Nikkor 55 mm 1:2.8 macrolens. The video image was processed by a PC VisionPlus board. The data acquisition program was CODA (Haake, 1986). The points were then used to generate a truss (Bookstein et al., 1985), which gave fairly even

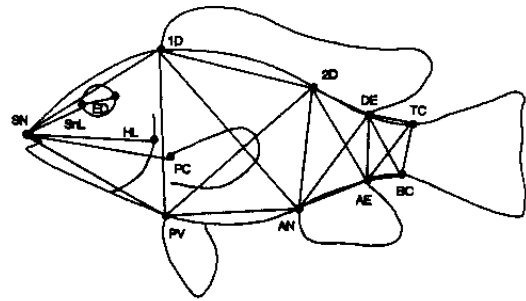


Fig. 1. The points that were digitized and the measurements taken on each fish.

two-dimensional coverage of both the length and depth dimensions along the whole body of the fish, as well as a few traditional measurements such as eye diameter and head length. Points difficult to see with the video camera were marked under a Wild M5A dissecting microscope with insect minuten pins. The digitized points were snout tip (SN), origin of the first dorsal fin (1D), origin of the second dorsal fin (2D), insertion of the dorsal fin (DE), top of the caudal peduncle (TC), the midpoint of the caudal peduncle (PC), bottom of the caudal peduncle (BC), insertion of the anal fin (AE), origin of the anal fin (AN), origin of the pelvic fin (PV), origin of the pectoral fin (PC), front of the eye, back of the eye, and back of the head as defined by the most caudal point of the operculum. The truss and the distances generated from these points are shown in Figure 1. Abbreviations used in the tables for the measures are combinations of the two-letter abbreviations for each point except for eye diameter (ED) and head length (HL).

The fish were then cleared and stained for cartilage and bone with alcian blue and alizarin red, respectively (Dunn, 1983; Taylor and van Dyke, 1985). I marked the following points (Fig. 2) of the skull and jaws on a piece of paper using a camera lucida on a Wild M5A dissecting microscope: (1) the top of the ascending process of the premaxilla, (2) the rostral tip of the premaxilla, (3) the most caudal point of the descending process of the premaxilla, (4) the rostrorodorsal point of the maxilla, (5) the posteroventral point of the maxilla, (6) the most rostrorodorsal point of the lacrimal where it abuts the orbit, (7) the most caudal point of the lacrimal where it abuts the orbit, (8) the quadrate-articular joint, (9) the dorsocaudal point of the coronoid process of the dentary, (10) the rostral tip of the dentary, (11) the posteroventral point of the angular, (12) the caudoventral point of the interoperculum, (13) the hyomandibula-

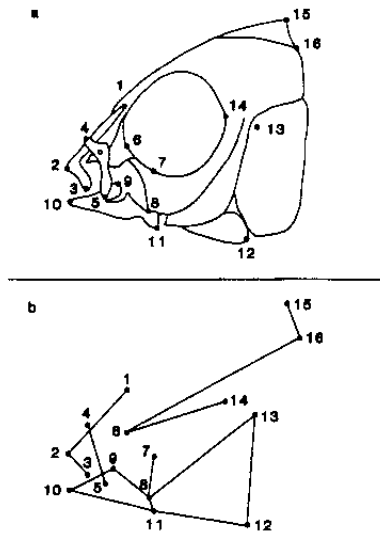


Fig. 2. (a) The skull of *Geophagus brasiliensis*. The indicated points were digitized. The numbers correspond to the descriptions in Methods. (b) The measurements taken on each skull.

operculum joint, (14) the back of the orbit directly opposite point 7, (15) the most dorsal point of the occipital crest, and (16) the ventral point of the occipital crest. The fish were not digitized directly because some of the points were easily visible only through the dissecting scope.

The distances generated from the digitized points (Fig. 2) were length of the ascending process of the premaxilla (Asc Proc, 1–2), length of the descending process of the premaxilla (Desc Proc, 2–3), snout length (Snout, 2–6), maxilla length (Maxilla, 4–5), suborbital depth, the distance from the caudoventral point of the lacrima to the quadrate-articular joint (Suborb D, 7–8), the distance from the quadrate-articular joint to the coronoid process (Quad-Cor, 8–9), the distance from the quadrate-articular joint to the caudoventral point of the angular (Quad-RA, 8–11), the distance from the coronoid process to the dentary tip (Cor-LJTip, 9–10), lower jaw length, the distance from the rostral tip of the dentary to the caudoventral point of the angular (LJ, 10–11), eye diameter (Eye Diam, 6–14), the distance from the front of the eye to the bottom of the occipital crest (Head L, 7–16), the distance from the quadrate-articular joint to the hyomandibula-operculum joint (Quad-Hm, 8–13), the distance from the caudoventral tip of the angular to the caudoventral point of the interoperculum (RA-IO, 11–12), the distance from the caudoventral corner of the interoperculum to the hyomandibula-oper-

culum joint (IO-Hm, 12–13), occipital crest height (Occ Cr, 15–16).

These measurements were originally chosen (Wimberger, 1991, 1992) because they were shown to vary in response to different diets in previous studies (Witte, 1984; Meyer, 1987) or have been shown to be functionally important for some aspect of feeding (Anker, 1974; Westneat, 1990). The primary objective of this study was to compare the differences induced by vitamin C deficiency to differences induced in previous studies on *G. brasiliensis* (Wimberger, 1991, 1992). Thus, I used most of the same measures in this study as in the previous studies. The reasons for choosing the measurements are given in more detail in Wimberger (1991, 1992).

All measurements were natural-log transformed so that variances were independent of means. A total of 221 *G. brasiliensis* were used in the following analysis. Fish in both treatments spanned the whole range of lengths considered. The data were analyzed using analysis of covariance (ANCOVA, Sokal and Rohlf, 1981), with the log of standard length as the covariate, looking for a diet effect (difference in intercepts between diets) or a size-by-diet interaction (a difference in slope between diets). I centered standard length (the size covariate) at 25 mm standard length, the size at which I centered previous analyses (Wimberger, 1991, 1992). Thus, a significant diet effect indicates a significant difference in intercept at 25 mm. I used the natural log of standard length as the covariate, rather than Principal Component 1 or other derived size variable, in order to incorporate my sampling scheme into the statistical analysis (I sampled fish by standard length and not by Principal Component 1). It is easier to understand the meaning of relative size increase with a discrete variable, such as standard length, as opposed to a composite variable, such as Principal Component 1.

All analyses were done using SYSTAT (Wilkinson, 1987) or Statistical Analysis System (SAS Institute, 1985). Partial sums of squares (Type III in SAS GLM) were used to calculate F-statistics and significance levels. I adjusted the alpha-level accepted as denoting significance for each measurement with Bonferroni's Multiple Comparison Adjustment (Neter et al., 1985) for all analyses because 21 body shape measurements and 17 skull and jaw measurements were taken on the same fish and, thus, were not independent. However, I treated each set of measurements separately to make them comparable to my previous studies. The adjusted alpha-level used to indicate significant differences was  $P < 0.003$ . The Bonferroni adjustment decreases the

TABLE 2. THE SLOPES AND INTERCEPTS OF FISH FED VITAMIN C SUPPLEMENTED (C+) AND DEFICIENT (C-) FLAKE FOOD DIETS AND THEIR STANDARD ERRORS (SE). The significance values (*P*) are for differences between treatments. The Bonferroni adjusted alpha level denoting significance is 0.003. See Methods for abbreviations of the measurements. Asterisks denote significant treatment difference.

	SLOPE (SE)		<i>P</i>	Intercept (SE)		<i>P</i>
	C+	C-		C+	C-	
<b>Perimeter measures</b>						
SN-1D	0.94 (.008)	0.93 (.009)	0.598	2.36 (.002)	2.36 (.002)	0.707
1D-2D	1.01 (.013)	1.04 (.013)	0.169	2.22 (.004)	2.22 (.004)	0.370
2D-DE	1.16 (.021)	1.16 (.022)	0.784	1.40 (.006)	1.37 (.006)	0.003*
DE-TC	1.14 (.011)	1.15 (.025)	0.821	1.21 (.007)	1.28 (.007)	0.0001*
TC-BC	1.14 (.011)	1.13 (.011)	0.327	1.34 (.003)	1.33 (.003)	0.212
BC-AE	1.03 (.022)	1.08 (.023)	0.103	1.27 (.006)	1.30 (.007)	0.0001*
AN-AO	1.14 (.020)	1.18 (.020)	0.196	1.51 (.005)	1.49 (.006)	0.144
AO-PV	0.98 (.008)	1.02 (.007)	0.0005*	2.38 (.002)	2.39 (.002)	0.001*
PV-SN	1.01 (.010)	1.03 (.010)	0.228	2.30 (.003)	2.31 (.003)	0.011
<b>Depth and diagonals</b>						
1D-AN	1.06 (.009)	1.07 (.009)	0.382	2.53 (.002)	2.52 (.003)	0.634
2D-PV	0.96 (.010)	0.94 (.011)	0.273	2.47 (.003)	2.47 (.003)	0.828
2D-AN	1.13 (.009)	1.16 (.009)	0.013	2.09 (.002)	2.09 (.003)	0.424
2D-AE	1.16 (.010)	1.17 (.011)	0.646	1.97 (.003)	1.95 (.003)	0.0001*
DE-AN	1.11 (.009)	1.15 (.009)	0.008	2.03 (.002)	2.02 (.003)	0.006
DE-AE	1.11 (.011)	1.13 (.011)	0.226	1.49 (.003)	1.47 (.003)	0.0001*
DE-BC	1.10 (.011)	1.12 (.011)	0.145	1.65 (.003)	1.67 (.003)	0.0001*
TC-AE	1.12 (.012)	1.13 (.012)	0.862	1.71 (.003)	1.72 (.003)	0.157
<b>Other head measures</b>						
SN	1.30 (.026)	1.27 (.027)	0.480	0.87 (.007)	0.86 (.008)	0.570
ED	0.84 (.015)	0.88 (.016)	0.065	1.24 (.004)	1.22 (.005)	0.020
HL	0.88 (.010)	0.91 (.010)	0.031	2.15 (.003)	2.14 (.003)	0.010
SN-PC	0.94 (.009)	0.97 (.009)	0.011	2.24 (.003)	2.24 (.003)	0.296

likelihood of finding a significant difference when there is none (Neter et al., 1985) and is thus conservative.

#### RESULTS

**Body shape.**—All but one of the measures that differed significantly between the two vitamin C treatments were caudal to the origin of the second dorsal fin (Table 2, Fig. 3). The measures from the second dorsal fin to the end of the dorsal fin and to the end of the anal fin, and the measure from the end of the dorsal fin to the end of the anal fin, were longer in C+ fish. The measures from the end of the dorsal fin to the top of the caudal peduncle and to the bottom of the caudal peduncle, and from the end of the anal fin to the bottom of the caudal peduncle, were shorter in C+ fish. Cranial to these measures, the distance from the snout to the pelvic girdle was longer in fish fed the C- diet.

**Jaw and skull morphology.**—There was a trend

toward the shortening of all skull and jaw measurements in C- fish (Table 3, Fig. 4). The following measures were significantly longer in fish fed the C+ diet: ascending process of the premaxilla, the maxilla, lower jaw length, eye

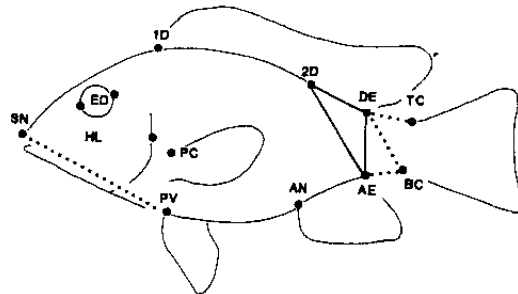


Fig. 3. Significant differences in body shape between C+ and C- fish. Solid lines indicate that the measurement was significantly longer in fish fed the C+ diet. Starred lines indicate the measurement was significantly longer in fish fed the C- diet.