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Hematocrit and Hemoglobin Determination in the Mangrove Killifish, Kryptolebias marmoratus

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HEMATOCRIT AND HEMOGLOBIN DETERMINATION IN THE MANGROVE KILLIFISH, *KRYPTOLEBIAS MARMORATUS*

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ABSTRACT

Hematocrit, hemoglobin concentration, and mean cell hemoglobin concentration (MCHC) were determined in the mangrove killifish, *Kryptolebias marmoratus*, a tropical fish that can undergo emersion for >1 month when they leave the waters of mangrove forests and find refuge in moist, terrestrial habitats. Hematocrit was 27.67% ± 1.22 (mean ± s.e.m.), hemoglobin concentration was 7.41 ± 0.52 g/dL, and MCHC was 26.47 ± 0.93 g/dL. A linear relationship was present between hematocrit and hemoglobin in *K. marmoratus* (R^2 = 0.833; $P < 0.001$) . A significant correlation between collected blood volume and standard length was present ($P = 0.001$). Limitations of the assays used to measure hemoglobin and hematocrit in this small fish species are discussed. Hematocrit and hemoglobin concentrations are within ranges determined for other air-breathing fishes and show the feasibility of measuring hematological parameters in this small fish during emersion.

Keywords: Mangrove killifish, *Kryptolebias marmoratus*, hemoglobin, hematocrit, mean cell hemoglobin concentration, MCHC

INTRODUCTION

The mangrove killifish, *Kryptolebias marmoratus* (Order Cyprinodontiformes; Family Rivulidae), is a self-fertilizing hermaphroditic fish (1) found in the highly variable environment of waters surrounding mangrove forests from Florida to South America (2) where they face physiological stresses due to changes in water level, salinity, oxygen, and temperature that are typical of the mangrove ecosystem (3). In this environment *K. marmoratus* has evolved the adaptation of emersion, which enables this fish species to leave its aquatic environment and survive on land for periods >1 month (4) in response to increased levels of hydrogen sulfide, decreased water temperature, and aggressive encounters with other *K. marmoratus* (2, 4, 5). To prevent desiccation during emersion *K. marmoratus* must find refuge in moist habitats such as under detritus and inside decaying logs (2, 6) or they will die after a few hours of air exposure if in a dry habitat (7, Grove, personal observation).

Kryptolebias marmoratus has adapted to this amphibious life through physiological and anatomical modifications. *Kryptolebias marmoratus* re-

lies on volatilization of ammonia across their skin to excrete nitrogen when emersed (8, 9), and an epidermal capillary bed is present (10), which most likely aids in gas exchange in a terrestrial environment. Reversible changes in gill morphology occur during emersion that while most likely do not enable the gills to function as a gas exchange organ in air, may play another role such as helping to prevent desiccation or keeping the lamellae from fusing so that the gills will be functional when the fish returns to water (7). Finally, metabolism increases, as measured by CO_2 excretion, in individuals emersed in the laboratory, which may be an indication of the energetic cost required to complete these physiological and anatomical changes that occur as an individual transitions from breathing water to breathing air (7).

Our lab is interested in examining if the oxygen carrying capacity of blood changes during periods of emersion as the fish makes the necessary physiological adjustments to switch from an aquatic to a terrestrial environment. The majority of oxygen is carried bound to hemoglobin (Hb), an oxygen binding protein found in red blood cells of most vertebrates. The oxygen carrying capacity of blood can be altered by adjusting the intracellular environment of red blood cells to change hemoglobin's affinity for oxygen, by changing hematocrit (Hct) and hemoglobin concentration, or by changing isoform expression (11, 12, 13). As a preliminary step we adapted protocols to measure hematological properties of blood from *K. marmoratus* and report for the first time hematocrit and hemoglobin concentrations.

MATERIALS AND METHODS

Specimens

All animal care and use protocols were approved by Valdosta State University's Institutional Animal Use and Care Committee. *Kryptolebias marmoratus* were maintained in brackish water (17‰ artificial seawater, Instant Ocean) at 28°C under a constant photoperiod 14 hr:10 hr (light: dark) in Valdosta State University's Aquatic Facility where they were fed newly hatched brine shrimp daily. Individuals ranged in size from 2.1-3.8 cm with a mass of 0.17-0.78 g.

Hematocrit and Hemoglobin Determination

Individuals were anesthetized in 17‰ artificial seawater with 125 mg/L MS-222 neutralized with sodium bicarbonate for 3-13 minutes; the time to anesthetization increased as size of fish increased. After anesthetization the caudal fin was severed posterior to the anal fin, and blood was collected from the caudal vein using a heparinized capillary tube (length: 75 mm, inner diameter: 0.5-0.6 mm). Collected blood volume ranged from 1.3 – 7.8 µl, and the entire blood volume was used in subsequent Hct and Hb determinations. Because of the small blood volume, replicates from a single individual were not possible. For Hct determination, capillary tubes were centrifuged for five minutes in a microhematocrit centrifuge (Damon/IEC), and Hct was determined using a microcapillary tube reader (Damon/IEC). As a second method

of determination, capillary tubes were scanned, image size was increased to 400%, and Hct was measured from these images using a Vernier caliper.

Hemoglobin content was measured based on the cyanmethemoglobin method (14, 15). Briefly, blood was extracted from capillary tubes immediately after Hct determination. Human hemoglobin (Pointe Scientific) was used to create a standard curve. Drabkin's reagent (Fisher Scientific) was added to standards and blood at ratio of 1:250 (sample:Drabkin's reagent) and incubated at room temperature for 30 minutes. Absorbance was measured at 540nm, and total hemoglobin concentration in blood samples was determined from the standard curve. Mean cell hemoglobin concentration (MCHC) was calculated using the following equation:

$$\left(\frac{[Hb]\,g/dL}{Hct\%}\right) * 100 = MCHC\,g/dL$$

Statistical Analyses

Data are presented as means ± s.e.m. Regression analysis was performed to determine if a linear relationship was present between variables.

RESULTS

Preliminary results showed that determination of Hb from blood samples used immediately after collection was not different from blood samples used first to measure Hct (data not shown); thus Hb and Hct were determined from one blood sample taken from each individual. A linear relationship was present between Hct and Hb ($R^2 = 0.557$; Figure 1). Two data points from individuals were determined to be outliers, and the strength of the linear relationship increased when removed from analysis ($R^2 = 0.833$; Figure 1). Hct and Hb values of these outliers were removed from further statistical analysis. Hct of *K. marmoratus* (n = 13) was 27.67% ± 1.22, hemoglobin concentration was 7.41 ± 0.52 g/dL, and mean cell hemoglobin concentration (MCHC) was 26.47 ± 0.93 g/dL. These values were within the ranges determined for blood Hb concentration and Hct of other air-breathing fishes (Table I).

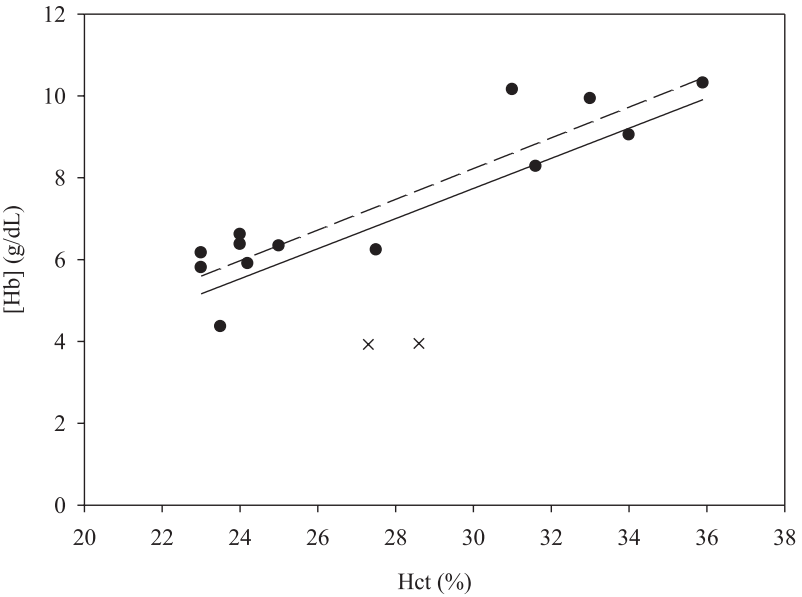

Figure 1: Relationship between Hct and Hb in *Kryptolebias marmoratus*. The regression for all data points (solid line) is [Hb] = 0.3679 Hct – 3.2997 (R^2 = 0.557; P = 0.001). When outliers (×) are removed the regression (dashed line) is [Hb] = 0.3754 Hct – 3.0368 (R^2 = 0.833; P < 0.0001).

Table I. Comparative Values of Hematocrit (%) and Hemoglobin (g/dL) for Air-Breathing Fishes

Species	Common Name	Hct (%)	[Hb] (g/dL)	MCHC (g/dL)	Ref.
K. marmoratus	Mangrove killifish	27.67	7.41	26.47	This study
Megalops cyprinoides	Indo-Pacific tarpon	37.6	12.06	31.70	16
Trichogaster trichopterus	Blue gourami	27.30	10.42	38.17	17
Clarias gariepinus	African catfish	41.39	13.50	30.27	18
Helcogramma medium	Twister	11.55	3.20	27.71	19
Dormitator latifrons	Pacific fat sleeper	15.5	39.1	25.23	20
Protopterus aethiopicus	African lungfish	27.4	7.4	27.01	21

No linear relationship was present between Hct and standard length (P = 0.32) or mass (P = 0.54). No linear relationship was present between Hb concentration and standard length (P = 0.11) or mass (P = 0.25). A significant

relationship between collected blood volume and standard length was present (P = 0.002, Figure 2).

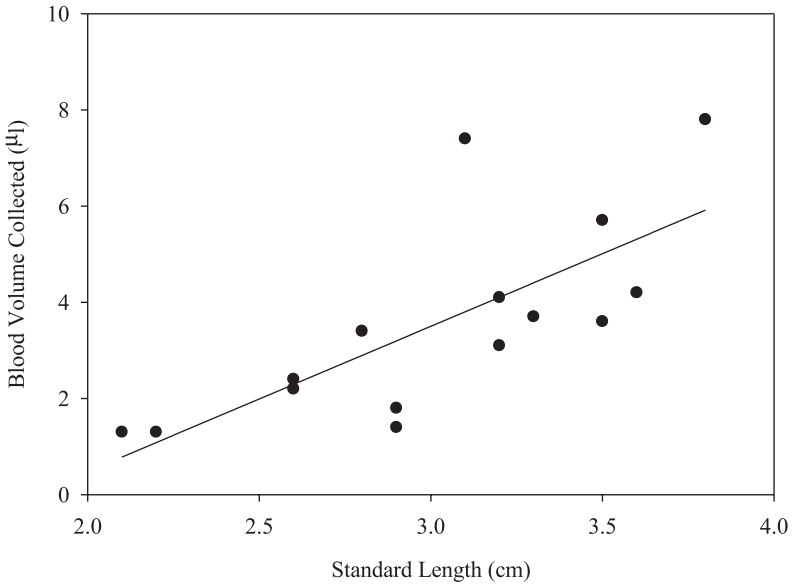

Figure 2: Relationship between standard length and blood volume collected from 15 individuals (R^2 = 0.54; P = 0.002).

DISCUSSION

When emersed *K. marmoratus* individuals increase their reliance on skin over gills as the major respiratory structure, and individuals show increases in metabolism after two days of emersion (7), but it is not known if oxygen carrying capacity in this amphibious fish changes. Setting the stage for measuring Hct and Hb content in *K. marmoratus* during emersion we adapted techniques to measure these hematological parameters in *K. marmoratus* and report for the first time values for both Hb and Hb content in *K. marmoratus* living in an aquatic environment.

The main difficulty of examining blood parameters of *K. marmoratus* is the small blood volume that can be obtained from these small individuals. The blood samples collected during this study (1.3-7.4 µl) did not enable replication of Hb and Hct determinations from each individual. Values of Hb and Hct obtained from blood samples (1.3µl) taken from the smallest individuals (2.1 and 2.2 cm) were determined to be outliers in the regression analysis of the relationship between Hb and Hct. The MCHC was determined to be 14.38 and 13.81 g/dL for these two samples, which is lower than other calculated values for *K. marmoratus* individuals and lower than MCHC from other air-breathing fishes (Table I). We therefore concluded that there is a minimal

volume of blood that can be used in the assays described here to accurately measure Hb and Hct. To remove any error that is inherent in working with such a small volume of blood collected, future studies should utilize individuals > 3 cm to enable larger blood volumes (>2.5 µl) to be collected and used for subsequent analysis.

While our Hb and Hct values are similar to other air-breathing fishes (Table I), caution must be used when drawing any conclusions from comparisons between different studies and across species. Different blood sampling techniques result in different experimental errors. Acute sampling, which is the method employed in this study, does not allow the researcher to take repeated blood samples from the same individual, and it tends to over-estimate Hct due to red blood swelling that occurs during stress associated with capture, handling, and exposure to air. While chronic sampling, which requires the implantation of a cannula into a blood vessel, enables the researcher to take multiple blood samples and reduces the amount of stress in the fish, it tends to underestimate Hct due to anemia that is associated with both repetitive sampling and blood loss that can occur during surgery when the cannula is implanted or after surgery if blood leaks from the cannulation site (22). Acute sampling is the method of choice when working with small fish because of the difficulty of cannulation, and despite the drawbacks associated with the acute sampling method, careful implementation of the technique can still result in reliable data that can be used to examine physiological changes that occur in response to environmental changes.

Red blood cell swelling in fish can also occur due to the use of MS-222 that is a common anesthetic used on fish (23, 24). Preliminary experiments showed no discernable difference in Hct measured from blood samples from anesthetized individuals using 100 mg/L and 125 mg/L MS-222 (data not shown); 125mg/L was chosen because it shortened the time required to fully anesthetize individuals. Mean cell hemoglobin concentration (MCHC) was used to evaluate the degree of red blood cell swelling; a lower value of MCHC indicates swelling of red blood cells. Our calculated values of MCHC are similar to values in the literature reported for other air-breathing fishes (Table I) and help validate our methods for determining Hct and Hb in *K. marmoratus*.

Trends in hematocrit can be identified when fish species are grouped together in similar environmental categories and broad evolutionary groups and are examined on a gross level (22). Teleosts typically have higher hematocrit than elasmobranchs; active teleosts (*i.e.* scombrids) have higher hematocrit values than more sluggish, benthic fish. Warm-adapted fishes generally have higher hematocrit than temperate and cold-adapted fishes. Many of the extant air-breathing fishes live in tropical regions where water levels change significantly during the dry season resulting in hypoxic, warm pools. These fishes generally have higher hematocrit levels than temperate and cold adapted fishes (12, 22). The values of Hct and Hb reported here for *K. marmoratus* are consistent with those of other air-breathing fishes.

While it is not known at this point whether the relatively high hematocrit and hemoglobin concentrations are due to being adapted to warm environmental temperatures or being adapted to an amphibious life, it does set the stage to test the hypothesis that oxygen carrying capacity of the blood changes in this fish during periods of emersion.

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