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First record of a male of *Kryptolebias hermaphroditus* Costa, 2011 (Cyprinodontiformes: Cynolebiidae)

Waldir M. Berbel-Filho[1,2], Helder M. V. Espírito-Santo[3] and Sergio M. Q. Lima[1]

During an ichthyological survey in September 2015 at the Ceará-Mirim River estuary, Rio Grande do Norte State, northeastern Brazil, we collected a male of *Kryptolebias hermaphroditus*, *a* cynolebiid species that had been previously described as containing exclusively self-fertilizing hermaphrodites. This is the first record of a male in this species, over 140 years after the discovery of the mangrove rivulid species from Brazil. Our discovery reinforces the need for more studies in *K. hermaphroditus*, as well as the potential of this species as a model for evolutionary studies due to its unique mating system.

Durante uma amostragem ictiológica em setembro de 2015 no estuário do rio Ceará-Mirim, Estado do Rio Grande do Norte, Nordeste do Brasil, nós coletamos um macho de *Kryptolebias hermaphroditus*, uma espécie de rivulídeo que foi descrita como contendo apenas hermafroditas auto-fertilizantes. Este é o primeiro registro de um macho dessa espécie, mais de 140 anos depois da descoberta das espécies de rivulídeos de manguezais do Brasil. Nossa descoberta reforça a necessidade de mais estudos em *K. hermaphroditus*, assim como, o potencial desta espécie como um modelo para estudos evolutivos devido ao seu sistema reprodutivo único.

Keywords: Hermaphroditism, Mangrove killifish, Mid-Northeastern Caatinga ecoregion, Mixed-mating system, Sexual dimorphism.

Introduction

Although the overall taxonomy of *Kryptolebias* Costa, 2004 is confusing (Costa, 2006), the *K. marmoratus* species group is well supported by both molecular (Tartarenkov *et al*., 2009) and morphological data (Costa *et al*., 2010). The species comprising this group, *K. marmoratus* (Poey, 1880), *K. ocellatus* (Hensel, 1868) and *K. hermaphroditus* Costa, 2011, inhabit estuarine areas associated with mangroves, ranging from southeastern United States of America to southern Brazil (Costa, 2011). Besides this unusual brackish habitat, these species are also unique among aplocheiloid killifishes due to the absence of females, which are replaced by hermaphrodites (Costa *et al*., 2010).

Indeed, self-fertilizing hermaphroditism is the dominant mode of reproduction in *Kryptolebias marmoratus* distributed from Florida to Venezuela (Tatarenkov *et al*., 2011) and *K. hermaphroditus* occurring in the Atlantic Forest mangroves of Brazil (Lira *et al*., 2015). This clade represents the only occurrence of self-fertilizing hermaphroditism among vertebrates (Tartarenkov *et al*., 2009; Costa *et al*., 2010). Because of its reproductive mixed-mated system, *K. marmoratus* has been a popular model species for embryological, physiological, behavioral and genetic studies (Avise & Tatarenkov, 2015). While males are rare in *K. marmoratus*, they are considered to be absent in *K. hermaphroditus* (Costa, 2011; Tatarenkov *et al*., 2011), despite this species having been known at least since 1868 (previously known as *K. ocellatus*) and numerous museum and unpreserved specimens have been examined (Costa, 2011). Due to the absence of males in fish collections, and following a re-examination of the holotype of *K. ocellatus*, the monomorphic hermaphrodite species from Brazil was described as *K. hermaphroditus* (Costa, 2011).

Hermaphrodites of both species (*K. marmoratus* and *K. hermaphroditus*) typically have a black ocellus on the caudal peduncle and a dark-grey body side (Soto & Noakes, 1994; Costa, 2011). In *K. marmoratus*, males exhibit a bright orange coloration on the body flank and fins, an absent or poorly-defined ocellus on the caudal peduncle, and black margins on the caudal fins (Davis *et al*., 1990; Soto & Noakes, 1994; Costa, 2011).

[1]Laboratório de Ictiologia Sistemática e Evolutiva, Departamento de Botânica e Zoologia, Universidade Federal do Rio Grande do Norte, Av. Senador Salgado Filho 3000, 56078-970 Natal, RN, Brazil. (SMQL) smaialima@gmail.com, (WMBF) waldirmbf@hotmail.com (corresponding author)
[2]Department of BioSciences, College of Science, Swansea University, SA2 8PP, Swansea, Wales, United Kingdom
[3]Post-Doctoral Program, Instituto Nacional de Pesquisas da Amazônia, Av. André Araújo, 2936, Petrópolis, 69067-375 Manaus, AM, Brazil. espiritosantohm@gmail.com

In a recent collection in the Ceará-Mirim River estuary, we found a *Kryptolebias hermaphroditus* individual with a male phenotype. Considering that this species has been described as being completely composed of hermaphrodites, the main objective of the present study is to record and describe the first occurrence of a male *K. hermaphroditus*.

Material and Methods

In September 2015, during a nocturnal exploratory fieldwork being conducted with the objective of designing an ecological study on *Kryptolebias hermaphroditus* at the Ceará-Mirim River estuary (05°40'25.9"S 35°14'14.5"W, see Lira *et al*., 2015 for a map and detailed description of the area), we observed a bright orange individual of *Kryptolebias* (Fig. 1). This specimen together with specimens exhibiting a more typical hermaphroditic appearance, were captured by hand and with the help of a hand sieve (50 x 60 cm, 1 mm mesh), under permit #30532-1/2011 issued by ICMBio/SISBIO. The fishes were euthanized using clove oil, fixed in formalin solution, transferred to an 70% alcohol solution and then deposited in the ichthyological collection of Universidade Federal do Rio Grande do Norte (UFRN 3774, 8 ex.). Some other individuals, as well as a small fin-clip (TIUFRN 3481; tissue collection of UFRN), were directly stored in 96% ethanol for molecular analysis.

Measurements and counts for the orange individual follow Costa (1995). *Kryptolebias* species identification was carried out using the key proposed by Costa (2009), and later confirmed using Costa (2011). To identify male traits, we used the information described for the closely related species *K. marmoratus* (Davis *et al*., 1990; Soto & Noakes, 1994; Costa, 2011).

Results

All specimens were identified as *Kryptolebias hermaphroditus* on the basis of having 1-4 teeth in the vomer and a color pattern with silvery or pale golden spots on the dorsal part of the flank. The male individual had bright orange coloration on the body flank and fins (mainly dorsal, anal and caudal), and black margins on the anal and caudal fins (Fig. 1), as in males of *K. marmoratus*.

Comparing male and hermaphroditic phenotypes of the captured specimens, both have a dark ocellus on the caudal peduncle; however, the ocellus is faint on the male specimen. The hermaphrodites consistently exhibit a brown or grey flank coloration, as well as hyaline unpaired fins with pale grey dots on the basal portion, whereas the male individual exhibits unpaired fins with orange coloration (Fig. 2).

Description. Morphometric data of male (30.1 mm SL) in Table 1. Urogenital papilla cylindrical in male, pocket-like shape in hermaphrodites (Fig. 3). Dorsal fin rounded. Anal fin sub-trapezoidal, distal margin slightly damaged; anal-fin rays 4 and 5 longer than other anal-fin rays. Caudal fin oval, deeper than long. Pectoral fin short and rounded. Pelvic fin elliptical. Pelvic-fin bases medially separated by short interspace. Dorsal-fin origin at vertical between 9th and 10th anal-fin rays. Dorsal-fin rays 8; anal-fin rays 11; caudal-fin rays 30; pectoral-fin rays 13; pelvic-fin rays 6.

Scales small, cycloid. Frontal squamation E-patterned. Longitudinal series of scales 47; transverse series of scales 13; scale rows around caudal peduncle 25. Contact organs absent.

Fig. 1. *Kryptolebias hermaphroditus*, male, UFRN 3774, 30.1 mm SL (three days after sampling); Ceará-Mirim River estuary, Extremoz, Rio Grande do Norte, Brazil.

Fig. 2. *Kryptolebias hermaphroditus*, male (below) 30.1 mm SL, hermaphrodite (above) 35.5 mm SL, UFRN 3774, Ceará-Mirim River estuary, Extremoz, Rio Grande do Norte, Brazil.

Table 1. Morphometric data of the *Kryptolebias hermaphroditus*, male (UFRN 3774).

Standard length (mm)	30.16
Percents of standard length	
Body depth	17.11
Caudal-peduncle depth	13.23
Predorsal length	73.18
Prepelvic length	62.10
Length of dorsal-fin base	12.33
Length of anal-fin base	15.78
Caudal-fin length	25.86
Pectoral-fin length	17.90
Pelvic-fin length	6.63
Head length	25.10
Percents of head length	
Head depth	59.31
Head width	71.07
Snout length	29.33
Lower-jaw length	23.51
Eye diameter	26.55

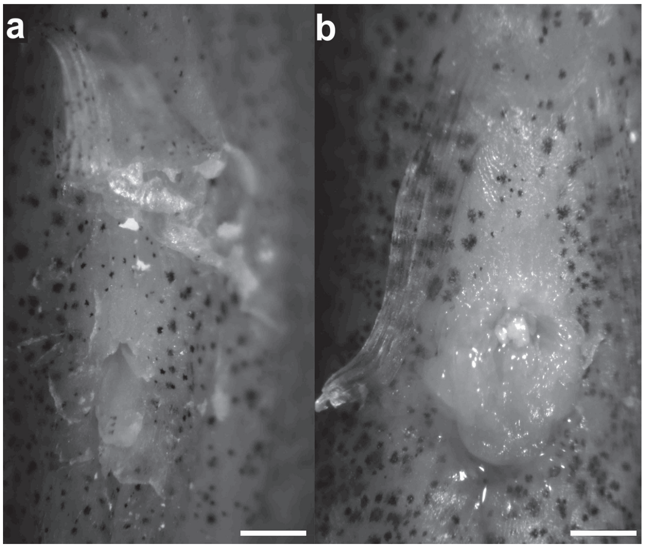

Fig. 3. Urogenital papillae of *Kryptolebias hermaphroditus* in ventral view; UFRN 3374: a, cylindrical shape in male, 30.1 mm SL; b, pocket shape in hermaphrodite, 35.5 mm SL. Scale bars = 1 mm.

Coloration. Flank dark grey on anterior and dorsal portion of head, from greyish to orange on posterior and ventral portions. Side of the body intensely pigmented with faint dark grey stripe between postorbital region and caudal-fin base, and small irregular somewhat zigzag pattern composed of small orange spots more concentrated on anterior half of body side. Inconspicuous dark grey humeral blotch; rounded dark spot with faint yellowish grey margin on dorsal end of caudal peduncle. Side of head light brown on dorsal portion, pale golden with dark grey dots ventrally. Jaws grey. Iris brown, with narrow pale yellow margin around pupil. Dorsal, anal and caudal fins hyaline with dark orangish-brown on basal and medial portions of fins; black stripe on distal anterior margin of anal and lower portion of caudal fin. Pectoral and pelvic fins hyaline. Bright orange coloration faded a little after sampling, probably due to stress and non-natural conditions (Figs. 1-2).

Ecological notes. The sampling locality had clear water and the fishes could be observed using flashlights. The *Kryptolebias hermaphroditus* male was captured in an ephemeral pool at higher elevations among the estuary's mangroves. This pool had at least four fiddler crabs (*Uca maracoani* Latreille, 1802) burrow openings and, prior to our approach, the male fish was resting on the water's surface near one of these burrows. During our sampling attempts, the fish repeatedly hid and re-appeared among the crab burrows, appearing periodically in different openings until it was caught. Time between reappearances was about one minute.

Large (23.2-35.5 mm SL) hermaphrodites were also collected in the same area as the male, in shallow pools formed in the edge of the mangroves; in the inner and deeper pools, *Poecilia vivipara* Bloch & Schneider, 1801 was the dominant species. *Guavina guavina* (Valenciennes, 1837), potential predator of *Kryptolebias hermaphroditus*, were also active during the night and observed entering the same large crab burrows. Salinity in these pools, previously recorded in January 2015 at the same site, was 34-36 ppm during the low tide.

Discussion

Over the 140 years, since the description of *Kryptolebias ocellatus* (as was long called hermaphroditic species who was later described as *K. hermaphroditus*) from a mangrove in Rio the Janeiro, no males of this hermaphroditic species from the Brazilian coast have ever been found (Costa, 2011). However, potential miswritten on previous literature (Costa, 2006; Lira *et al*., 2015) needs to be clarified for a better understanding of the issue. Both studies used the subjective term "rare" while discussing the existence of males in *K. hermaphroditus.* Costa (2006) had already mentioned two reports of potential *K. hermaphroditus* males based on pictures and brief descriptions of live specimens with light grey and dark orange spots on the flanks, grey humeral and caudal peduncle spots and dark grey to black distal zones of unpaired fins. However, this author recognized that this material was not preserved for study in any scientific collection and needed posterior confirmation. Posteriorly, Costa (2011) described *K. hermaphroditus* as only composed by hermaphrodites, discarding the relevance of the two previous unconfirmed reports. In both cases male phenotypes were reported for Rio de Janeiro, where *K. ocellatus* (males and hermaphrodites, previously known as K. caudomarginatus Seegers) occurs in syntopy with *K. hermaphroditus* making these records more doubtful. The potential records described above, the presence of males on populations of *K. marmoratus* (Avise & Tatarenkov, 2015), as well as the cryptic and complex mangrove microhabitats inhabited by *K. hermaphroditus*, may have led Costa (2006) and Lira *et al*. (2015) to not fully discard the presence of males of *K. hermaphroditus*, despite extensive sampling and no reliable scientific evidence of males have been made available up to that moment.

The recently collected specimen of *Kryptolebias hermaphroditus* with male appearance, described herein, matches the description of the putative *K. hermaphroditus* males mentioned by Costa (2006) and *K. marmoratus* males (Davis *et al*., 1990; Soto & Noakes, 1994; Costa, 2011). Although gonadal histological evidence was not surveyed in order to maintain the unique specimen integrity, its morphological characters support this first documentation of a male *K. hermaphroditus*. This evidence includes orange body and fins, faded ocellus, black margins of anal and caudal fins and cylindrical urogenital papillae (Costa, 2011). Once the color pattern of males is the most conspicuous character for differentiating cynolebiid species (Costa, 2003), the discovery of a *K. hermaphroditus* male may reveal important morphological differences between this species and *K. marmoratus*.

Regardless the presence of an ocellus on the caudal peduncle, it appeared faded in comparison to that of hermaphrodite individuals. According to Soto & Noakes (1994), the most distinctive characters attributed to *Kryptolebias marmoratus* males are: a bright orange overall coloration and the absence of a distinct caudal ocellus. However, they also considered that individuals with intermediate phenotypes (with orange color and a faded ocellus) could also be males. Davis *et al.* (1990) had previously mentioned that it is not possible yet to distinguish primary and secondary males, the latter of which result from sex change of functional hermaphrodites into males due to environmental conditions (Harrington, 1971). Huber (1992) cited the presence of ocellus in secondary males of *K. ocellatus*, which agrees with our specimen description. However, as discussed above, this report was not afterwards confirmed.

Primary males of *Kryptolebias marmoratus* can be readily produced in laboratories by inducing eggs to temperatures lower than those usually found in mangrove areas (<20°C), while secondary males can be induced by maintaining individuals at higher temperatures (>30°C), which are similar to those found on populations with higher rate of males (Turner *et al*., 2006; Earley *et al*., 2012). Their induction in the lab at naturally occurring temperatures suggests that, in the wild, secondary males may be the most ecologically relevant type of male (Turner *et al.,* 2006). This may also be the case for *K. hermaphroditus* in northeastern Brazil, where higher temperatures are predominant due to the Equator's line proximity.

Davis *et al.* (1990) demonstrated distinct male phenotypes within *Kryptolebias marmoratus*. One, from Twin Cays, Belize, exhibited a dark orange color pattern, an inconspicuous caudal ocellus, and caudal black margins along the dorsal, anal and caudal fins. The other one, from Florida, USA, exhibited a bright orange coloration almost devoid of dark chromatophores, except the head. Ellison *et al.* (2012) also found a male of *K. marmoratus* with orange coloration, faded ocellus and black borders on the anal and caudal fins in Belize; a pattern similar to that of the *K. hermaphroditus* male herein described.

Between 2011 and 2015, during extensive surveys at the Ceará-Mirim River estuary (Lira *et al*., 2015), we collected 107 hermaphrodites before finding this single male. This apparent rarity of males aligns with the extremely low degree of heterozygosis reported for populations of *Kryptolebias hermaphroditus* from southeastern Brazil (Tartarenkov *et al*., 2011). Together with absence of male specimens in all known populations (Costa, 2011; Lira *et al*., 2015), these findings suggest that self-fertilizing hermaphroditism is the major mode of reproduction in *K. hermaphroditus*. However, the persistent occurrence of male individuals in *K. marmoratus*, but at low densities (varying from 1% in Florida to 10-25% in Belize, Davis *et al*., 1990), suggests that sexual outcrossing serves an adaptive role within natural populations, possibly contributing to genetic diversity that helps the species to deal with new environmental pressures, such as parasites loads (Ellison *et al*., 2011, 2013).

The occurrence of self-fertilization in *Kryptolebias marmoratus* has made that species a popular model for population genetics, development, evolutionary biology, behavior, among others (for reviews see Earley *et al*., 2012; Taylor, 2012; Avise & Tatarenkov, 2015; Turko & Wright, 2015). Nonetheless, there have been relatively few studies on the South American lineage of *K. hermaphroditus*, possibly due to its misidentification as *K. marmoratus* (Seegers, 1984). Additionally, the difficulties of working in mangrove environments, which represent a complex, multi-dimensional, and cryptic habitat with variable environmental and physical features could also contributed to the scarcity of studies on the South American lineage (Taylor, 2012).

The discovery of a male specimen encourages more studies on *Kryptolebias hermaphroditus*. While researchers have addressed some aspects of systematics (Costa, 2006, 2011), evolutionary history (Costa *et al*., 2010; Tatarenkov *et al*., 2009, 2011), and geographic distribution (Sarmento-Soares *et al*., 2014; Lira *et al*., 2015), there is little available information regarding natural history, ecology and population dynamics. Despite the large gap in our knowledge of the biology of *K. hermaphroditus*, the recent studies above mentioned, including the present one, suggest some interesting perspectives for studies on evolution, morphology, behavior and physiology across the species' wide geographic distribution in the mangroves of the Brazilian coast.

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