OCURRENCE AND IDENTIFICATION OF ISTIOPHORIDAE LARVAE AND XIPHIIDAE EGGS OFF THE SOUTHEASTERN BRAZILIAN COAST

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ABSTRACT

This study had the aim to identify and relat the occurrence of billfish larvae and eggs in the Southeastern coast of Brazil. During the summer in two seasons (2012/2013 and 2013/2014) 74 surface trawls were done using an ichthyoplankton net off the coast of Vitória-ES, Rio de Janeiro-RJ and Ilhabela-SP and 391 fish larvae were collected. The identification of the billfish larvae was done by molecular biology using the DNA barcode (COI gene). During the 2012/2013 season, five sailfish and two white marlin larvae were identified and in the 2013/2014 season, two sailfishes, two white marlin larvae and two swordfish eggs were identified. The occurrence of billfish larvae shall be further studied, so that inferences about the area and period of spawning and development of early life stages of these fish can be made more accurately.

Key words: barcode; Istiophoridae; Xiphiidae; swordfish; marlin; sailfish, ichthyoplankton

OCORRÊNCIA E IDENTIFICAÇÃO DE LARVAS DE ISTIOPHORIDAE E OVOS DE XIPHIIDAE NA COSTA SUDESTE BRASILEIRA

RESUMO

O presente estudo teve como objetivo identificar e relatar a ocorrência de larvas e ovos de peixes-de-bico na costa sudeste do Brasil. Durante duas temporadas no verão (2012/2013 e 2013/2014) 74 arrastos de superfície foram feitos utilizando uma rede de ictioplâncton na costa de Vitória-ES, Rio de Janeiro-RJ e Ilhabela-SP, de modo que 391 larvas de peixes foram coletadas. A identificação das larvas de peixes-de-bico foi feita por biologia molecular utilizando o método do DNA barcode (gene COI). Durante a temporada de 2012/2013, foram identificados cinco larvas de agulhões-vela e duas de agulhão-branco. Na temporada 2013/2014, duas larvas de agulhões-branco e duas de agulhões-vela, além de dois ovos de espadarte foram identificados. A ocorrência de larvas de peixes-de-bico deve ser detalhadamente estudada, para que inferências sobre a área e período de desova e desenvolvimento das fases iniciais de vida destes peixes possam ser feitas com mais precisão.

Palavras-chave: barcode; Istiophoridae; Xiphiidae; espadarte; marlim; agulhão-vela, ictioplâncton.

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INTRODUCTION

The Istiophoridae species present in the Southwestern Atlantic are the blue marlin (*Makaira nigricans*, LACÉPÈDE, 1852), sailfish (*Istiophorus platypterus*, SHAW, 1792), white marlin (*Kajikia albida*, POEY, 1850), longbill spearfish (*Tetrapturus pfluegeri*, ROBINS and DE SYLVA, 1963) and roundscale spearfish (*Tetrapturus georgii*, LOWE, 1841); besides the Xiphiidae swordfish (*Xiphias gladius*, LINNEAEUS, 1752) acoording to AMORIM *et al.* (2011). These fish are captured by longlines on commercial fishing and by trolling on sports fishing, especially during the spawning period on the coast of Brazil, from November to March (ARFELLI and AMORIM, 1981; AMORIM and ARFELLI, 1984, 1987; ARFELLI *et al.*, 1986; AMORIM *et al.*, 2011). In the Atlantic, several authors observed the occurrence of females in the final stages of gonadal development as well as the presence of billfish larvae in different seasons (VOSS, 1953; UEYANAGI *et al.*, 1970; MATHER *et al.*, 1972; SHOMURA and WILLIAMS, 1975; ARFELLI and AMORIM, 1981; AMORIM and ARFELLI, 1984, 1987; ARFELLI *et al.*, 1986; POST *et al.*, 1997; LUTHY *et al.*, 2005; TIDWELL *et al.*, 2007; AMORIM *et al.*, 2011).

TIDWELL *et al.* (2007) state that information about the billfish first stages of development, nursery areas, larvae habitat preferences and eating habits is still scarce, especially for the Southern Atlantic. Studies on Istiophoridae and Xiphiidae larvae are most concentred in the Northern Atlantic, such as those by DE SYLVA (1963), JOLLEY (1977), BAGLIN (1979), HARVEY (1990), SOUZA *et al.* (1994), DE SYLVA and BREDER (1997) and RICHARDSON *et al.* (2009).

The morphological identification of the billfish larvae is troublesome because lack of distinctive characteristics between the species. However, DNA based techniques have assisted larvae identification accurately (MCDOWELL and GRAVES, 2002; LUTHY *et al.* 2005, HYDE *et al.*, 2005). According to HERBERT *et al.* (2003) the sequence of a single gene (mitochondrial COI) could be used to identify and distinguish species. The present study confirms the occurrence of billfish larvae and eggs in the superficial water mass off the Brazilian coast, Southwestern Atlantic, using barcode DNA. This finding has important implications for the conservation of billfish species by determining breeding grounds in Southwestern Atlantic.

METHODS

Study area and Larvae Collection

The area of study is located in the southeastern coast of Brazil, encompassing the continental shelf, from 40 to 200 m depth off the coast of Vitoria (20º19'S 40º20'W), Rio de Janeiro (22º54'S 43º12'W) and Ilhabela (23º46'S 45º21'W) (Figure 1). The area is a traditional oceanic area for billfish sport fishing. With the support of the sport fishing anglers (SCHMIDT *et al.*, 2015) from the Espirito Santo Iate Clube (ICES), the Rio de Janeiro Iate Clube (ICRJ) and the Ilhabela Yacht Club (YCI), it was possible to embark during the oceanic fishing seasons from 2012/2013 and 2013/2014 to collect the biological material. The collections were made from October/2012 to January/2013 in the first season and from November/2013 to February/2014 in the second season. The trips for collection were made on fishing tournament or training days. The collection stations were limited by the area in which the fishing vessels worked.

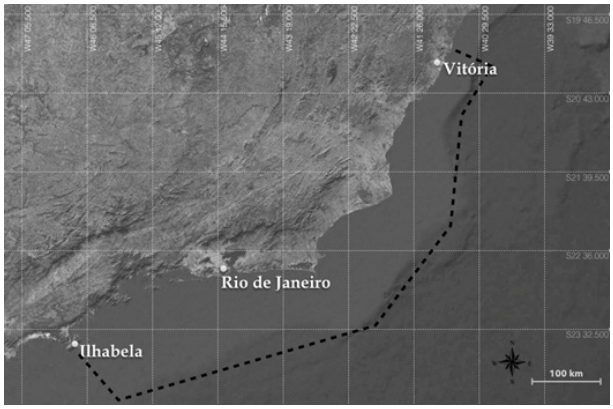

Figure 1. Representation of the study area. The area inside the segmented black line is a traditional oceanic area for billfish sport fishing.

The larvae were collected with surface trawls (about one meter deep) using a 1.0 m diameter and 2.90 m long conical ichthyoplankton net made of 500 µm mesh in the body and 600 µm in the cup. The average speed of the trawls was 2 knots. There were made a total of 74 surface trawls, 14 during the 2012/2013 season and 50 during the 2013/2014 season. Each sampled station was numbered and the following data was taken: geographic position, date and time, air temperature, weather conditions,

sea surface temperature – SST and depth. When possible, the wind speed was also checked using an anemometer. Figure 2 shows the geographic distribution of the collection stations in the area of the study.

The samples with remaining sea water were first preserved in a solution with 95% alcohol for material conservation (LUTHY *et al.*, 2005) and all the fish larvae were separated from the accompanying planktonic organisms. The probable Istiophoridae larvae were identified based on the four backwards-facing spines with a pronounced snouth (FAHAY, 2007). Then those larvae were photographed measured (total length) and separated for later DNA extraction and specific identification. Eggs morphologically similar to those described by HYDE *et al.* (2005) as possible billfish were separated from the samples for testing.

Electronic microscopic scan was performed using Istiophoridae larvae. Some precautions were necessary for the electronic microscopic scan: setting the larvae with 3% Glutaraldehyde and 1% Osmium tetroxide; dehydration was done in 50% to 100% alcohol in each weighing; the sample was glued with carbon glue (so there is interaction with the electron) and inserted into a device with gold, palladium or silver.

Figure 2. Geographic distribution of the collection stations. Each point represents a trawl, collected off the coast of Ilhabela and Rio de Janeiro Cities (Map A) and on the coast of Vitoria City (Map B).

Molecular Identification

DNA extraction and amplification of the gene COI by PCR.

The total DNA extraction of the billfish larvae and eggs tested was carried out using a DNeasy Blood & Tissue – Qiagen kit. Larvae smaller than 4.0 mm were completely macerated for DNA extraction, just as for the eggs. Larvae larger than 4.0 mm were partially used. In general, the tail was cut and then fragmented, avoiding the intestinal region, which is a possible area for contamination.

A fragment of approximately 650 pairs of bases from the mitochondrial gene COI (cytochrome c oxidase subunit I) was amplified using a pair of primers developed by WARD *et al.* (2005) FishF2 (5'TCGACTAATCATAAAGATATCGGCAC3') and FishF2 (5'ACTTCAGGGTGACCGAAGAATCAGAA3'), both universal for fish. The primers FishF1 (5'TCAACCAACCACAAAGACATTGGCAC3') and Fish R1 (5'TAGACTTCTGGGTGGCCAAAGAATCA3') were also tested, but they amplified a smaller number of samples.

The Polymerase Chain Reaction - PCR to amplify the fragment of gene COI was done from a mix containing 18.7 µl of ultra pure water; 1.5 µl of $MgCl_2$ (50 mM); 1.0 µl of KCl (50 mM); 1.0 µl of dNTP mix

(2 mM); 0.5 µl of each primer (10 µM); 0.3 µl of Taq DNA Polymerase (Fermentas Life Sciences) and 1.5 µl of the DNA template. The PCR was done in a Peltier Thermal Cicler PTC 200 (MJ Research). A negative control was used without the DNA template to indicate the absence of contamination. The program used was: 94ºC for 2 min; 35 cycles of 94ºC – 30 s, 58ºC – 1 min (annealing), 72ºC – 1 min and 72ºC – 10 min; finalizing with cooling at 4ºC.

The result of the amplification was observed in 1.5% agarose gel that was colored with GelRed and viewed under UV light in the photodocumenter ImageQuant 300 (GE Healthcare Life Sciences). The purification was done using ExoSap (ExoSap-it, GE Healthcare) in a mix containing 10 µl of the product of the PCR and 4 µl of ExoSap according to the program of 60 min at 37ºC and 15 min at 80ºC and the samples were prepared for sequencing.

Sequencing and data analysis

Purified PCR products were subjected to sequencing reaction with BigDye Terminator v.3.1 @ Cycle Sequencing kit (GE Healthcare Life Sciences) according to the protocols provided by the manufacturer and sequenced bidirectionally in a sequencer ABI PRISM 3730 DNA Analyzer (Applied Biosystems). Sequences were analised using the software BioEdit Sequence Alignment Editor (HALL, 1999) and CodonCode Aligner (CodonCode Corp., Dedham, MA, USA) to obtain a consensus sequences. The consensus sequences of each specimen was compared with the barcode sequences that already existed in the data banks of the Barcode of Life Data Systems – BOLD; Fish Barcode of Life (FISH-BOL) and with the Nucleotidae BLAST (GenBank - NCBI) tool for the analysis of similarity and identification of the species.

RESULTS

A total of 391 fish larvae were captured at 38 stations and from these only seven (1.8%) were morphologically identified as probable billfish. Five Istiophoridae larvae captured during the January/2013 off Vitoria were identified as sailfish (*Istiophorus platypterus*) by the PCR multiplex. Two additional Istiophoridae larvae were collected off Rio de Janeiro and were previously analyzed by multiplex PCR, identified as white marlin (*Kajikia albida*) according SCHMIDT *et al.* (2015), as shown in Table 1.

The other Istiophoridae larvae were stored for identification by mitochondrial gene COI barcode fragment sequencing. After sequencing, three more *I. platypterus* larvae were identified (3.3 mm, 4.1 mm and 3.2 mm) coming from Vitória (January 2013) and one *Kajikia albida* (3.1 mm) collected in November 2013 in Vitória. Three larvae were collected in Ilhabela: one *K. albida* (6.7 mm) and one *I. platypterus* (6.8 mm) collected in December 2013 and one *I. platypterus* (4.4 mm) collected in January 2014 at the shallowest station. A greater specific abundance was observed in January 2013 off the coast of Vitória where eight specimens of sailfish were collected at three collection points on the same sampling day. Of the 12 Istiophoridae larvae identified, only three presented a total length greater than 5.0 mm. The average temperature of the points where the larvae were found was 25.7 ºC (Table 1).

In addiction to the described larvae, four fertilized eggs that presented the following morphological characteristics were tested: around 1.5 mm diameter, opaque white coloring in 95% ethanol with melanophores (pigmentation) uniformly distributed over the entire egg and one pigmented embryo in development circling the sphere, without oil globules. Of the four eggs tested, two did not amplify even in tests with other primers (FishF1 and FishR1) and variations in the PCR program, while the other two amplified and were identified after sequencing as *Xiphias gladius*. Both were collected in Vitória, the first in November and the second in December of 2013.

Figure 3 illustrates the morphology of two *I. platypterus* larvae in different stages of development collected in this study, along with one *K. albida* larvae and two eggs identified as *X. gladius*.

Figure 4 shows the details of the head of *K. albida* caught by SCHMIDT *et al*. (2015), while Figure 5 shows the photographs from the electronic microscopic scan of *I. platypterus* larvae, where it is possible to see the four bony spines in the head, two superior and two inferior, both turned backwards and the teeth all over the mouth.

In January 2013 trawls at Vitoria a great abundance of a peculiar larva was observed, which we initially thought morphologically very similar to the Istiophoridae, having spines on the head

facing back and around the same size, but without a pronounced snouth. Later DNA sequencing revealed that they were not Istiophoridae but Dactylopteridae of the species *Dactylopterus volitans*. There were 82 individuals captured in only five trawls and despite the greatest effort the following season, they were not captured again. Figure 6 shows the photographs from the electronic microscopic scan of these larvae, where it is possible to see the four bony spines in the head, two superior and two inferior, both turned backwards. The adition of these larvae showed us even more how difficult is to identify corectely Istiophoridae and Xiphiidae based on morphology only.

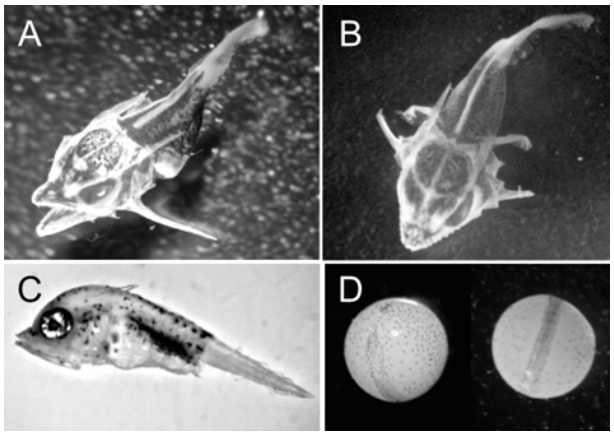

Figure 3. Sailfish larvae (A: 6.8 mm and C: 3.2 mm), white marlin larvae (B: 6.7 mm) and swordfish eggs (D: 1.5 mm).

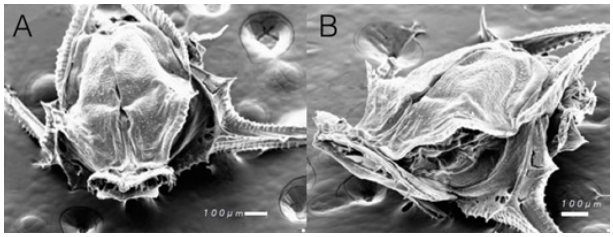

Figure 4. Electronic Microscopic Scan of *Kajikia albida* (11.6 mm). A: Front view; B: Lateral-front view of the hea*d.*

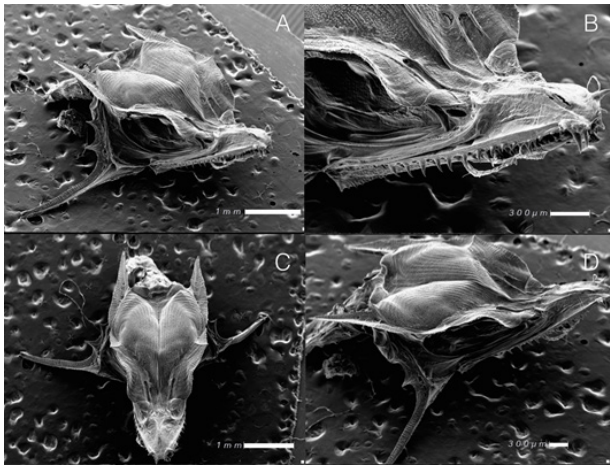

Figure 5. Electronic Microscopic Scan of two *Istiophorus platypterus* (A-B: 5.6 mm; C-D: 6.8 mm). A: Lateral-front view; B: Lateral of the mouth; C: Front view; D: Lateral view of the head.

Table 1. Description of the collection stations with the presence of Istiophoridae larvae (including those from SCHMIDT *et al.*, 2015 (*) and Xiphiidae (**) eggs. SST – sea surface temperature.

Date	Time	Latitude (S)	Longitude (W)	Location	Species (Number)	SST °C	Local depth m	Wind m s^{-1}	Length mm
11/18/11*	10:44am	23°48′630″	42°48′610″	Rio de Janeiro	*Kajikia albida (1)*	23.07	196	/	11.6
11/16/12*	2:30pm	23°41′020″	42°46′870″	Rio de Janeiro	*Kajikia albida (1)*	23.60	129	/	3.8
01/25/13*	08:40am	20°30′470″	39°53′323″	Vitória	*Istiophorus platypterus (5)*	25.4	100	4.0	4.5;4.9;5.7;5.9;8.1
01/25/13	01:55pm	20°22′173″	39°53′092″	Vitória	*Istiophorus platypterus (2)*	25.5	53	10.0	3.3;4.1
01/25/13	02:20pm	20°22′121″	39°53′356″	Vitória	*Istiophorus platypterus (1)*	25.5	53	6.0	3.2
11/20/13	11:30am	20°44′059″	39°56′453″	Vitória	*Kajikia albida (1)*	25.1	165	2.1	3.1
11/20/13	03:26pm	20°38′659″	39°55′743″	Vitória	*Xiphias gladius (1)***	24.9	67	1.5	1.5**
12/07/13	04:00pm	20°24′069″	39°52′793″	Vitória	*Xiphias gladius (1)***	26.2	62	1.9	1.5**
12/16/13	09:00am	24°15′000″	44°26′000″	Ilhabela	*Kajikia albida (1)*	24.6	141	/	6.7
					Istiophorus platypterus (1)				6.8
01/07/14	07:20am	23°52′000″	45°05′000″	Ilhabela	*Istiophorus platypterus (1)*	25.9	38.5	/	4.4

DISCUSSION

UEYANAGI *et al*. (1970) reported the presence of Istiophoridae mature gonads and larvae in oceanic areas of the South Midle Atlantic. However, there were no additional studies detailing these occurrences of larvae on the Brazilian coast. According to ARFELLI and AMORIM (1981), females sailfish were observed with mature gonads in southern Brazil from November to February. ARFELLI *et al.* (1986) also found mature gonads of white marlin females in the southern Brazil, from November to March. According to AMORIM *et al.* (2011) different youth sizes of sailfish found in southern Brazil.

The larvae collected (except one, 11.6 mm *K. albida*) were smaller then 10 mm (total length-TL) and according to LUTHY *et al.* (2005) this size range can only be identified by molecular analysis. Only post-larvae, larger then 10.0 mm (TL) could be identified based on morphology (GEHRINGER, 1956; DE SYLVA, 1963; FAHAY, 2007).

The identification by gene COI barcode fragment was considered satisfactory in this study since the sequenced fragments distinguished up to the taxonomic level of species of Istiophoridae and Xiphiidae in the data banks. Identification by COI can be highly precise when the most appropriate database for reference is used (HERBERT *et al*., 2003; DAWNAY *et al*., 2007).

Despite the sampling effort, the occurrence of billfish larvae was rare in other ichthyoplankton studies on the coast of Brazil. MAFALDA JR. *et al.* (2004) collected ichthyoplankton along the coast of Bahia in December 1993 and March 1994 and they captured 826 larvae identified in 33 families, none of which were Istiophoridae or Xiphiidae. A study of ichthyoplankton diversity in the Arvoredo Marine Reserve (SC) was carried out by RUTKOWSKI *et al.* (2011) with collections in the winter and summer of the years 1997/1998, 2007/2008 and 2008/2009, having collected 467 larvae identified in 19 families and again, there were no examples of Istiophoridae or Xiphiidae. KATSURAGAWA *et al.* (2011) made trawls in the Baía de Todos os Santos and Camamu (Northeastern Brazil) during the winter and summer of 2003, winter 2004 and summer 2005, identifying 11 families and once again, no billfish were found. Finally, BONECKER *et al.* (2012) collected ichthyoplankton on the coast of Espirito Santo and north of Rio de Janeiro between February and April and between August and September 2009 and identified billfish. In this study, the samples were collected at five depths: surface, 250m, 800m, 1200m and 2300m and a total of 10,978 fish larvae were collected. Seventy five families and 169 taxa were identified, and even so, only two specimen of Istiophoridae were collected, both in the superficial water mass, which could not be identified to a smaller taxonomic level (BONECKER *et al*., 2012). The present study, even with limited sampling effort, collected 12 billfish larvae and two eggs, more than any other studies from Brazilian coast previously described. It is important to notice that the identification of Istiophoridae larvae usually needs DNA analysis and maybe because of that the previous studies mentioned did not report those species.

The nursery area of the billfish larvae in the Gulf of Mexico is found to be widely studied and the points of high density of these fish were already covered in other studies. TIDWELL *et al.* (2007) did a sampling with 287 collection stations from May to September 2005 and 2006 and captured 2,587 billfish. The total length of the larvae varied from 2.2 to 31.0mm and the average temperature where the larvae were found by TIDWELL et al. (2007) was 27.5ºC; 1.8ºC higher than the average temperature of the present study.

During the months of June and July from 2006 to 2008 ROOKER *et al.* (2012) also found high densities in the Gulf of Mexico with ichthyoplankton trawls and captured 3,152 billfish larvae, 264 of which were swordfish (*Xiphias gladius*), identified by multiplex PCR. The authors observed that the sailfish larvae presented a very wide horizontal distribution, different from that of the white marlin, blue marlin and swordfish, which seem to be less tolerant of environmental variations. According to ROOKER *et al.* (2012), areas with a mixture of continental water masses, presenting low salinity and higher temperatures, characterize the environments with a rare presence of billfish larvae.

From July to November 2003, SERAFY *et al.* (2008) captured 19 live Istiophoridae larvae on the coast of Miami, Florida (US). The temperature varied from 28.8 to 30.4ºC and the total length was from 3.3 to 17.7 mm. In August 2005, SERAFY *et al.* (2006) tested the use of the Continuous Access Neuston Observation Net – CANON, a special kind of trawl net designed for capturing live billfish larvae, collecting a total of 104 larvae that were 3.5 to 12mm long, also on the

coast of Miami.

Istiophoridae and Xiphiidae larvae and eggs were also collected by HYDE *et al*. (2005) on the coast of Kona, Hawaii (US) on a scientific cruiser in May 2003 and another in July 2004, identified by multiplex PCR on board. The larvae captured by HYDE *et al*. (2005) were 57 swordfish and 8 blue marlins and there were 54 swordfish and 2 blue marlin eggs identified. The morphological description of the swordfish eggs by HYDE *et al*. (2005) was similar to the characteristics observed in the eggs collected in this study, which were then submitted to sequencing to prove the hypothesis of being from the *X. gladius* species.

Interestingly, none of the previously cited studies identified Dactylopteridae larvae as was done in the present study. *Dactylopterus volitans* was abundant in only one day of sampling but is another example of a species that lacks larval phase studies on the Brazilian coast.

Despite the low sample size, the present study confirmed through molecular identification for the first time in the Brazilian coast the occurrence of larvae of sailfish (*I. platypterus*), white marlin (*K. albida*) and swordfish (*X. gladius*) eggs. Further studies should be done in the area, amplifying the sampling months in order to capture larvae in more advanced stages of development of blue marlin (*M. nigricans*), longbill spearfish (*T. pfluegeri*), roundscale spearfish (*T. georgii*) and swordfish (*X. gladius*).

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