Mediterranean Marine Science

Vol 24, No 3 (2023)

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doi: 10.12681/mms.31158

Short Communication

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
www.hcmr.gr
DOI: http://doi.org/10.12681/mms.31158

Pregnant female and near term embryos of the honeycomb stingray *Himantura uarnak* (Chondrichthyes – Dasyatidae) from Mersin Bay, northeastern Mediterranean

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Contributing Editor: Fabrizio SERENA

Received: 21 August 2022; Accepted: 17 July 2023; Published online: 14 September 2023

Abstract

The honeycomb stingray *Himantura uarnak* (Gmelin, 1789) is one of the non-indigenous species in habiting eastern Mediterranean Sea. A single pregnant female was accidentally captured in March 2022 in the commercial purse seiner fishing. The taxonomic identification of this species has been confirmed by using DNA barcoding. The present study provides the first account of a gravid female and near term embryos *H. uarnak*, captured off the coast of Mersin Bay, Türkiye. Data of near term embryos, suggests that parturition occurred in either end of March or early April.

Keywords: *Himantura uarnak*; Lessepsian Species; Mersin Bay; Mediterranean Sea; Reproduction.

Introduction

The honeycomb stingray *Himantura uarnak* (Gmelin, 1789) is a large benthopelagic batoid species inhabiting the coastal waters of Eastern Mediterranean Sea as well as from Red Sea, and tropical part of Indo-Pacific, south-eastern Africa and the continental shelf of northern Australia (Golani *et al*., 2006; Ali *et al*., 2010). This ray is actually one of two species belong to the genus *Himantura* inhabiting the eastern Mediterranean, and both of them similarly entered via Suez Canal into the Mediterranean Sea (Başusta *et al*., 1998). *Himantura uarnak* has been recorded along other Mediterranean coasts by Mouneimne (1977, in Lebanon), El Sayed (1994, in Egypt), Basusta *et al*., (1998, in Türkiye) and by Ali *et al*., (2010; 2013, in Syria). This species is listed as an endangered (EN) at the global Red Lists level by the IUCN (Sherman *et al*., 2021). Reproduction information of the honeycomb stingray is poorly known. Except for a few studies reporting its occurrence and some biometric data, very little is known about this species in the Mediterranean region. As such, the aim of this paper is to record the honeycomb stingray in Mersin Bay to elucidate important information on its reproductive biology.

Material and Methods

Fish sample was caught by purse seiner, at 16 m depth, off Mersin Deli Burun Coast of Türkiye (36.43035° N, 34.54001° E) (Fig. 1). The male and female embryos were preserved at the Museum of Fisheries Faculty, Firat University (FFM-FISH/2022-1; FFM-FISH/2022-2) (Fig. 2). Sex identification was determined by the presence/absence of a clasper. Total length (TL) was measured as a straight-line distance from the tip of the rostrum to the end of the tail, disc length (DL) as a straight line distance from tip of the rostrum to the end of the anal fin and disc width (DW) as a straight line distance between the tips of the widest portion of pectoral fins. Body weights of adult female and embryos (W) were recorded to the nearest gram in the laboratory.

DNA sampling

Samples taken from the muscle tissue of two of the fish whose morphologies were found to be the same were left in eppendorfs containing 70% ethyl alcohol and kept at -20°C (Sağlam *et al*., 2021).

Genomic DNA isolation

Tissues were crushed and washed at least 5 times with 1XPBS solution to completely remove alcohol and then Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) protocol was applied.

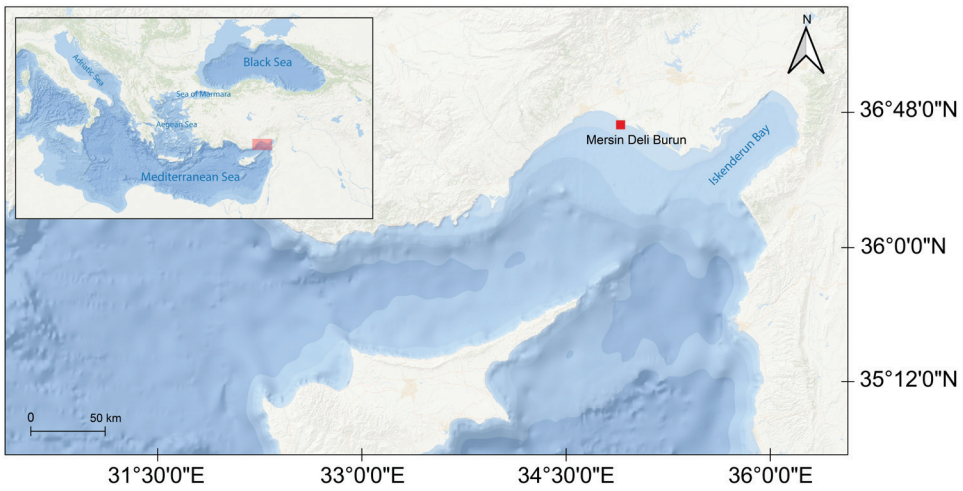

Fig. 1: *Himantura uarnak* fishing area, Deliburun, Mersin Bay, north-eastern Mediterranean.

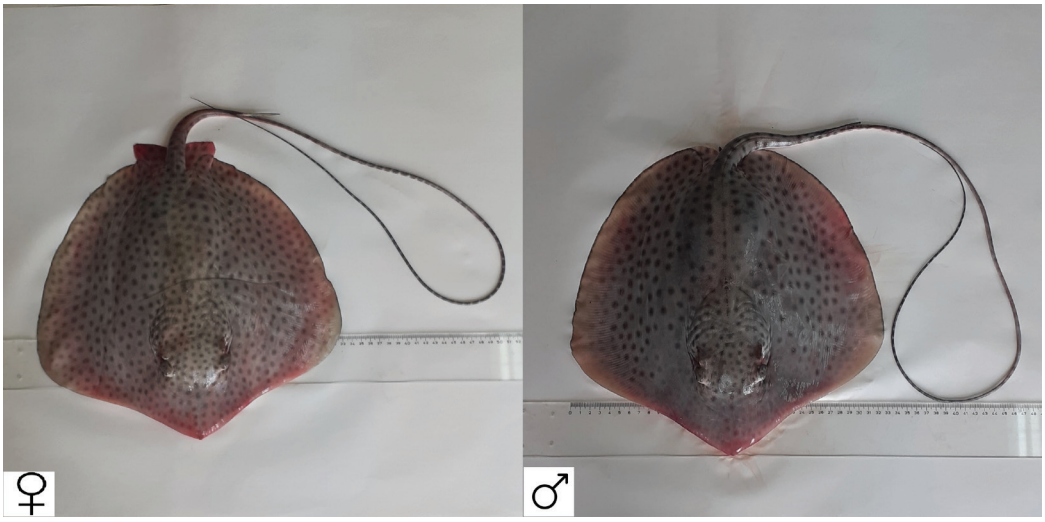

Fig. 2: Female and Male *Himantura uarnak* embryos.

PCR Amplification and Sequencing

Amplification process was performed using the 620bp region FishF1 and FishR2 primers (Ward *et al.,* 2005). The PCR reaction was carried out as a total volume of 25 µl. In this volume, 1X PCR buffer, 2.5 mM MgCl2, 1.25 pmol forward and 1.25 pmol reverse primer, 50 µM dNTPs, 0.6 U Taq polymerase, 50 ng purified DNA sample, and 15 µl ddH2O were used. The conditions used in the PCR reaction were: Predenaturation step at 95 ˚C for 5 min following the 35 cycles of (30 sec/94˚C, 30 sec/50˚C, 60 sec/72˚C) and a final extension step (10 min/72˚C). 2% stained ethidium bromide agarose gel was used in order to check the band quality in gel electrophoresis and electrophoretically separated PCR products were visualized under UV. The PCR bands with good results were then removed and were purified by using of QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Performing the sequence analysis of the samples was carried out by using forward primers used in the PCR process, through sanger service procurement (BM Laboratory Systems) (Doğan Barata *et al*., 2023).

Alignment and Phylogenetic Analysis

The chromatogram quality was checked with the use of FinchTV 1.4. (http://www.geospiza.com). The sequence ends were trimmed to a final length of 638 bp by comparing the published sequences using "BLAST" (http://www.ncbi.nlm.nih.gov/BLAST/) search. The alignment was applied using published reference sequences retrieved from NCBI Pubmed were used as outgroups. The sequences were then uploaded to MEGA X (Kumar *et al.,* 2018). ClustalW was used for sequence alignment. *Dasyatis marmorata* with GenBank number KF808194 was used as the outgroup.

Data Analysis and Haplotype Network Analysis

A total of 76 sequences were analyzed using two sequences obtained in this study and 74 sequences of *H. uarnak* from GenBank to construct the haplotype network. DnaSP 6 was used to calculate population diversity indices (haplotype number (H), haplotype diversity (Hd), nucleotide diversity (π)), neutrality indices (Tajima's D and Fu's statistics), Fu and Li's D, and F tests (Rozas *et*

al., 2017). DnaSP 6 was also used to generate output formats, including the NEXUS file format. Networks were created using the TCS criteria method (Clement *et al*., 2000), which provides an agglomerative approach in which clusters are gradually joined by one or more connecting edges by means of PopART-1.7 software (http://popart.otago.ac.nz) (Leigh & Bryant, 2015).

Results and Discussion

On 16 March 2022, a gravid female specimen of *H. uarnak* (Fig. 3) was accidentally captured by a small-scale purse seiner, off Mersin Deli Burun coast of Türkiye.

Fig. 3: Pregnant female, *Himantura uarnak*, 132.7 cm Disc Width, (broken tail).

Morphometric measurements

All the diagnostic characteristics and colour pattern observed on the specimen agree with the descriptions of Golani *et al*. (2006). Some morphometric characters were measured and are presented in Table 1. The exact total length (TL) of the specimen could not be measured because the part of its tail with the thorn was cut by the fishers for security reasons. However, the measurement taken from the tip of the snout to the end of the remaining part of the tail was 143 cm. The other measurements made were 132.7 cm disc width (DW) and 121.6 disc length (DL). The weight of the fish was 65.45 kg (Table 1), and it was observed that the colours and patterns of the adult individuals differed from those of the embryos and juveniles.

Sequence analyses and phylogenetic tree

The species in the study was determined morphologically as *H. uarnak*. Sequence results obtained were found to be compatible with *H. uarnak* after blasting in NCBI. Species of the genus *Himantura* that we obtained at NCBI were sequenced by country and trimmed in their program to align. Sequences uploaded to Mega X program at 638 bp and by choosing the best model, a maximum likelihood phylogenetic tree was created. The species named H1 and H2 that taking from Iskenderun

Table 1. Morphometric measurements of pregnant female and embryos of the honeycomb stingray (*Himantura uarnak*) from the Mersin Bay (north-eastern Mediterranean).

Sex	Gravid Female	Embryo 1 (Female)	Embryo 2 (Male)
Measurements	cm	cm	cm
Total Length (Broken tail)	143	125.7	123.4
Disc length	121.6	33.7	32.0
Disc width	132.7	33.6	33.4
Tail	-	95.7	93.6
Thorn length on the tail	-	5.3	4.9
Diameter of eye ball	2.2	1.22	1.23
Cloaca Anterior to tail tip	-	97.1	93.5
Snout to anterior cloaca	-	28.4	27.4
Head length (ventral)	-	15.4	15.2
Preoral length	-	7.2	7.1
Rostrum to 1st gill openings	39.2	11.5	11.6
Rostrum to 2nd gill openings	42.7	12.5	12.7
Rostrum to 3rd gill openings	46.5	13.6	13.7
Rostrum to 4th gill openings	49.5	14.6	14.7
Rostrum to 5th gill openings	53.3	15.6	15.6
Clasper	-	-	3.7/08
weight (kg)	65,450	1,513	1,488

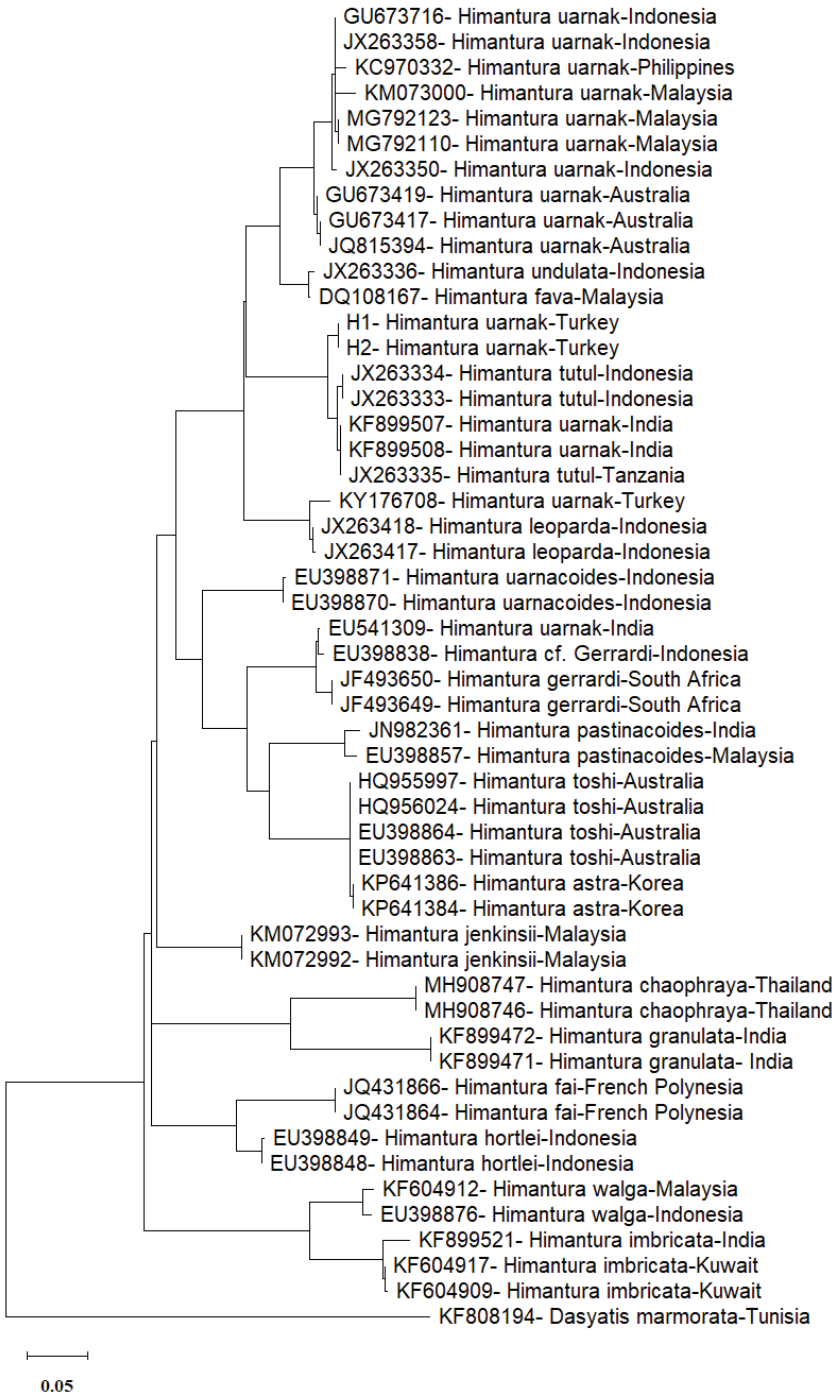

Fig. 4: Evolutionary phylogenetic tree of *Himantura uarnak* by Maximum likelihood method.

were 100% similar, and their similarities with the other species we discussed and on the basis of countries are shown in Figure 4.

Haplotype Network

All sequences of the Cytochrome c oxidase (COI) gene regions of *H. uarnak* species were obtained in NCBI. A total of 76 sequences were obtained and flush cut (398 bp). The 76 sequences obtained were aligned with the Mega X program. Then, DNAsp6 and PopArt 1.7 programs were used and 19 haplotypes (Number of haplotypes, h: 19; Haplotype diversity, Hd: 0.9242) were determined. In addition, Diversity was determined in neutrality indices and some molecular statistics (Table 2-3). The haplotype in Network tcs format was created in PopArt 1.7 program and shown in Figure 5.

Reproduction

The honeycomb stingray observed was a pregnant female carrying two developed embryos, one female and one male (Fig. 3), the former of which measured 125.7 cm in TL and 1,512.9 g in weight and the latter 123.4 cm in TL and 1,487.2 g in weight. These measurement values were higher than those reported for Leopard whiprays

Table 2. Diversity and neutrality indices obtained by using nucleotide data of *Himantura uarnak* COI gene.

n	H	hd ± SD	πd ± SD	Tajima's D	p value	Fu's Fs	FLD	p value	FLF	p value
76	19	0,924 ± 0,010	0,005772 ± 0,00597	-0,68912	P > 0.10	10,515	-2,90595	P < 0.05	-2,40005	P < 0.05

n: Number of isolates, *h*n: number of haplotypes; *h*d: haplotype diversity; πd: nucleotide diversity; SD: standard deviation; FLD: Fu and Li's D* test statistic; FLF: Fu and Li's F* test statistic.

Table 3. Diversity and some molecular statistics of *Himantura uarnak.*

Diversity Indices	Results of Word
Number of sequences	76
Selected region	1-398
Number of variable sites (S)	114
Total number of mutations, Eta	141
Number of Haplotypes (h):	19
Haplotype (gene) diversity (Hd)	0.924
Variance of Haplotype diversity	0.00011
Standard Deviation of Haplotype diversity	0.010
Nucleotide diversity (per site), Pi	0.05772
Sampling variance of Pi	0.0000356
Standard deviation of Pi	0.00597
Average number of nucleotide differences (k)	22.97088
Theta (per sequence) from Eta:	28.76755
Theta (per site) from Eta	0.07228

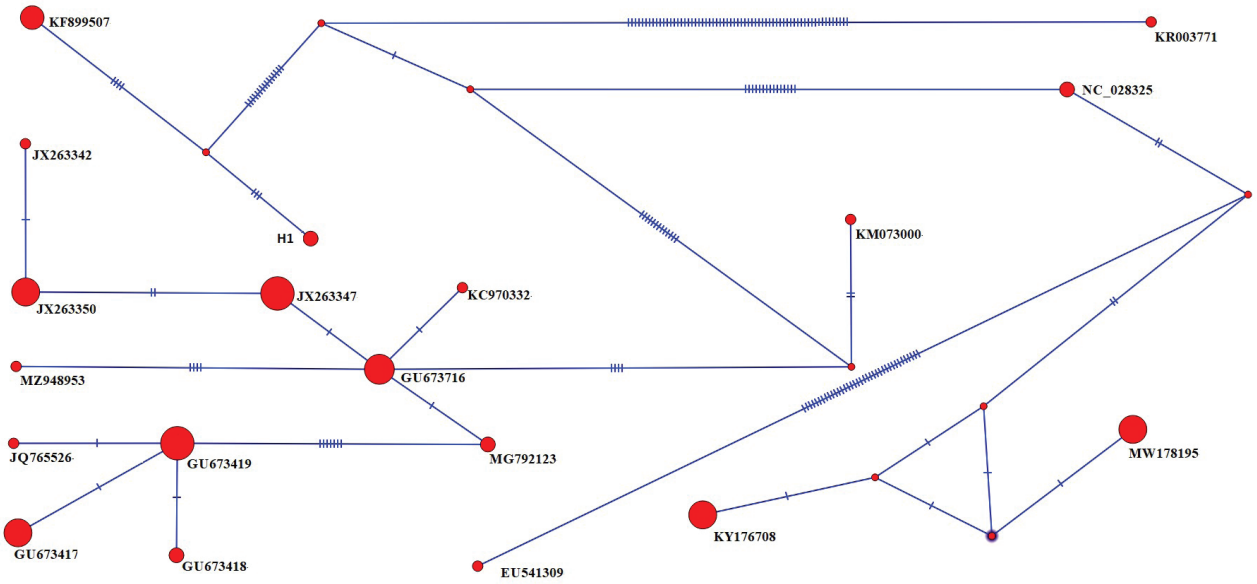

Fig. 5: Haplotype network of *Himantura uarnak* isolated according to the COI mtDNA gene region.

(*Himantura leoparda*) by Yucel *et al.* (2017) and Saad *et al.* (2021).

The honeycomb stingray specimen examined in the present work provides valuable data regarding the reproduction of this species in Mersin Bay. Capture of a pregnant female honeycomb stingray in Deliburun (Mersin Bay), may be a clear indication that the species has a breeding and nursery area in the vicinity. Recently, Tiraşin & Basusta (2018) studied a total of 129 (89 female and 40 male) incidentally caught Lusitanian cownose rays (*Rhinoptera marginata*) very close to the present study area. They noted that the dissections of the rays revealed that 36 female fish were gravid, each bearing one near-term embryo. After discussing their findings with other relevant studies, Tiraşin & Basusta (2018) concluded that the incidental catch of such a large number of fish, including many gravid specimens with near-term embryos and mature males together in one single haul, suggested that the rays were in a schooling formation when they were captured. The males might have been following the fe-

males so that they could maximise their chances of mating with them soon after parturition. Tiraşin & Başusta (2018) also argued that the location of the haul in Mersin Bay — a marine area in the vicinity of the estuaries of large rivers, the Tarsus and Seyhan prompts a hypothesis that these fish may be using this region as a reproduction and nursery area in the north-eastern Mediterranean. The previous observations of neonates, small juveniles and gravid females of Lusitanian cownose ray made in this region ((N. Başusta, pers. obs., 2021) provide additional support for this proposition. Similarly, honeycomb stingray may also be using the same region as a reproduction and nursery area. However, more additional studies and findings are needed to accurately describe this species potential reproduction and nursery area. According to the interviews with the fishermen, there is a small population of the honeycomb stingray in this region.

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