



Oceanographic variables and stomach content of stranded whale shark (*Rhincodon typus*) on the South Java Coast

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Abstract. Spatial oceanography parameters can be used as indicative variables on the presence of stranded whale shark *Rhincodon typus*. The current study was intended to provide scientific proof of the natural feed or diet of the whale shark. DNA barcoding was used to identify species of whale shark and their natural diet was determined from the stomach content. The value of SST at the site of stranded whale shark observation is 25.8°C. The sub-ST at various depths is: 25°C at 75 m, 24-25°C at 100 m and 23-25°C at 125 m. The chlorophyll-a content of 1.959 mg m⁻³ is assumed to be associated with small crustacean abundance. DNA analysis confirms the stranded whale shark is *R. typus*. This first attempt of DNA analysis of the natural diet from stomach content is dominated by Crustacean of the species *Solenocera crassicornis*. Minor natural diets are *Paguridae* and *Emerita emeritus*. In a sample of this megafauna, the study finds that the number of the guanine-cytosine (GC) genome pair of both *S. crassicornis* and *R. typus* is the same, namely 24 GC pairs. This finding is assumed to play an important role in the relationship of *R. typus* to its natural diet, being indicative for the genomic role in the adaptation to the sea water thermoregulation and diet variation during the oceanic migration.

Key Words: chlorophyll-a, sub-surface-temperature, whale shark, DNA barcoding.

Introduction. The whale shark (*Rhincodon typus*) is the largest endangered fish and a long-lived species from the ancient Elasmobranchii clade. In the study of Meekan et al (2020), the size of whale sharks can reach up to 14.5 m long. Whale sharks primarily inhabit the belt circles of the globe between 30 north and 35 south latitudes. They have a distinct distribution, covering the cosmopolitan tropics and the warm subtropical waters and are rarely seen in areas with SSTs below 21°C (Rowat & Brooks 2012; Sequeira et al 2012). The International Union for Conservation of Nature has classified them as endangered species. It is a solitary, slow-swimming species native to Thailand, Mahadeva, the Philippines, Taiwan, Indonesia, Christmas Island, India, Pakistan and Sri Lanka. In Indonesia, whale sharks are in the conserved status (Ministry of Marine and Fisheries 2013). First discovered in South Africa by Andrew Smith, the whale shark belongs to the Rhincodontidae family, Elasmobranchia group and Chondrichthyes class.

A phylogenetic analysis of clusters revealed that whale shark genomes would be aged roughly 333 million years (Weber et al 2020). In terms of feeding, whale sharks are classified as planktivorous and have a filtering organ on their pharyngeal arches, allowing them to consume small organisms (Motta et al 2010), such as zooplankton, small fishes and crab eggs. Previous studies investigated their feeding on plankton in the Mexican coastal waters of the Caribbean Sea (de la Parra Venegas et al 2011) and on zooplankton

during the mass spawning of corals in the Ningaloo Reef, Australia (Taylor 1996), and in Qatari waters of the Arabian Gulf (Robinson et al 2013). Whale sharks are known to display diel vertical movement behaviour, as evidenced by those found in the Indian Ocean, in the Seychelles (Rowat & Gore 2007) and especially during planktonic blooms found in Madagascar (Jonahson & Harding 2007). The distribution of chlorophyll-a is widely employed to estimate planktonic abundance in that area (Durán-Campos et al 2019). Sequeira et al (2012) stated that the migratory and distribution pattern of *R. typus* around the world remains to be an estimation, and they hypothesized that whale sharks inhabit zones with a limited temperature range. To this day, the efficiency of an indicative oceanographic variable for the event of stranded whale sharks' presence, which is an oceanic migratory species, remains unclear. There is very limited spatial data and knowledge on the oceanographic variables that contribute to stranded whale sharks' localization.

A new approach uses both spatial SST and Sub-ST to identify the seawater temperature at the swimming layer and at the stranding site of the whale shark. Spatial chlorophyll-a data is assumed to be correlated to the planktonic abundance in the seawater column. Plankton is the main diet of the whale sharks. A spatial database had been developed earlier as a monitoring system for the migration track of cetaceans, as well as for mapping their mortality rate (Maro et al 2021a,2021b; Hartoko et al 2023). The current knowledge gap justifies a systematic approach to monitoring the giant whale shark's oceanic migration track. Thus, it is critical to employ large-scale oceanic spatial data, such as SST and mainly sub-ST satellite data, which is highly important in determining the temperature of the sub-surface seawater. The design and development of satellite data models in monitoring systems, the analysis methodologies, the optimization of monitoring networks and of data handling or quality and assurance procedures, all help to effectively minimize operational costs (Pringgenies et al 2021). The innovative aspect of this study is the use of DNA sequencing to identify and scientifically confirm the natural feed or diet of the whale shark, the mega fauna of the ocean.

Material and Method

Study area. The study area is the South Java in the Indian Ocean from 05°S,100°E–20°S,130°E (Figure 1). The stranded whale shark was discovered on September 19th 2020, at Congot Beach in Yogyakarta, South Java (marked with a red triangle), at the coordinates 7°54'04.1"S, 110°01'58.8"E (Figure 1).

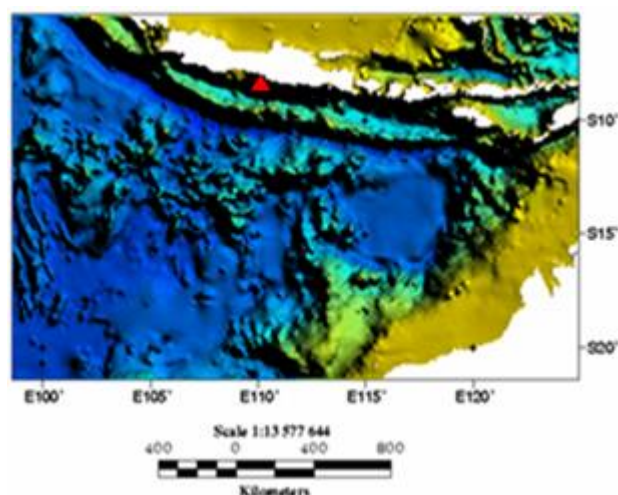


Figure 1. Area of study of the Indian Ocean and the coordinates of the stranded whale shark found on September 19th 2020 (marked with a red triangle).

Spatial data. The numerical ASCII data for SST and chlorophyll-a were obtained from <https://oceandata.sci.gsfc.nasa.gov/cgi/getfile/AQUA-MODIS>. The Sub-ST data were collected in September 2020 from the Copernicus Marine Environment Monitoring Service

(CMEMS) (<https://marine.copernicus.eu>) at depths of 75, 100 and 125 m. The SST, chlorophyll-a and Sub-ST measurements were taken using monthly data from September 2020, which coincided with the stranded whale shark observed on September 19th. The SST and chlorophyll-a used 16,062 numeric ASCII data coordinates. Three layers of depth, 75, 100 and 125 m were used for the sub-ST data, each using 37,000 coordinates. All three ASCII datasets were processed using the geostatistical Kriging method to generate the spatial data encompassing the area of study. The spatial layers were then superimposed on the Indian Ocean bathymetry dataset.

A stranded whale shark was found on Congot Beach in Yogyakarta, South Java. The soft stomach flesh and stomach content were sampled from the stranded whale shark over a 12-hour period. The sampling process was completed prior to the burial process in accordance with the national standard operating procedure in the instance of a stranded cetacean or whale shark on the beach. A kg of its stomach content was collected, which was then shimmered into 70% alcohol for morphological identification and frozen for species identification using the DNA barcoding method (Figures 2A, B, C and D).

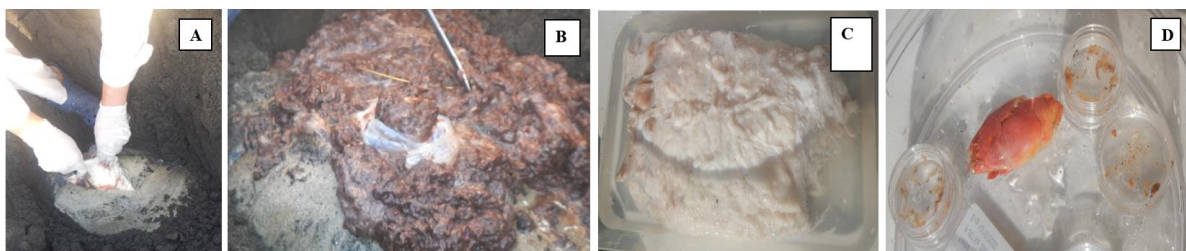


Figure 2. (A) Dissection of the whale shark stomach, (B) sample of the stomach content, (C) slice of stomach blubber, (D) samples of Crustaceans found in the stomach content.

DNA isolation and extraction. The sample code is AH-1. Before the isolation and extraction processes, a sample of soft tissue from the whale shark's abdomen was washed thoroughly using a low Tris-EDTA (LTE) buffer solution to remove the alcohol preservative used earlier. The sample was then extracted using a silica column of ZR Tissue and Insect DNA MiniPrep, Zymo Research, D6016. Gene partitioning was performed using mitochondria DNA (mtDNA). The PCR-Primer used is cytochrome oxidase-I (COI), to identify for PCR products with species barcoding of ~700 base-pair. Genomic DNA using ZR-Tissue and insect DNA-MiniprepKit Zymo Research namely D6015. The PCR amplification using MyTag HS-Red Mix of Bionline, namely BIO-25048, applied with bi-directional sequencing method.

PCR gene amplification. A segment of the target gene was amplified using a polymerase chain reaction unit. The primer used is a universal primer (FishF2-t1, FishR2-t1, VF2-t1 and FR1d-t1) (Ward et al 2005; Ivanova et al 2007) consisting of a mixed solution of 25–50 μ L MyTaq Red Mix Bionline volume, 0.5–5 μ L forward primer, 0.5–5 μ L reverse primer, 1–5 μ L DNA template and 25–50 μ L nuclease-free water. The PCR unit for amplification has a pre-denaturation condition temperature of 96°C maintained for 3 min. This technique uses agarose gel with 1.5% concentration diluted in a buffer Tris-Acetate-EDTA (TAE) with SYBR green as a colouring agent.

DNA fragments sequentiation and phylogenetic analysis. DNA analysis was performed using the software for Molecular Evolutionary Genetic Analysis (MEGA) for nucleotide alignment. The nucleotide alignment results were then compared to the GenBank National Centre for Biotechnology (NCBI) database using the Basic Local Alignment Search tool (BLAST). The construction for the phylogenetic analysis was accomplished using the MEGA-X software for the neighbour-joining tree evolution, based on a Kimura-2 parameter model with gamma distribution and with 1,000 bootstrap replications (Kimura 1980). The number of guanine-cytosine (GC) genome pairs in whale

sharks and the diet derived from the stomach content would be utilised to identify any potential epigenetic regulation (Weber et al 2020).

Results

Spatial oceanography. The examination of the monthly AquaMODIS satellite data in September 2020 revealed that the chlorophyll-a content on the position of stranded whale sharks ranges from 1.24 to 1.99 mg m^{-3} (Figure 3A). The spatial analysis using the AquaMODIS data in September 2020 found that the SST at the position of the stranded whale shark at Congot Beach, Yogyakarta, South Java, ranges from 25.86 to 26.36°C (Figure 3B).

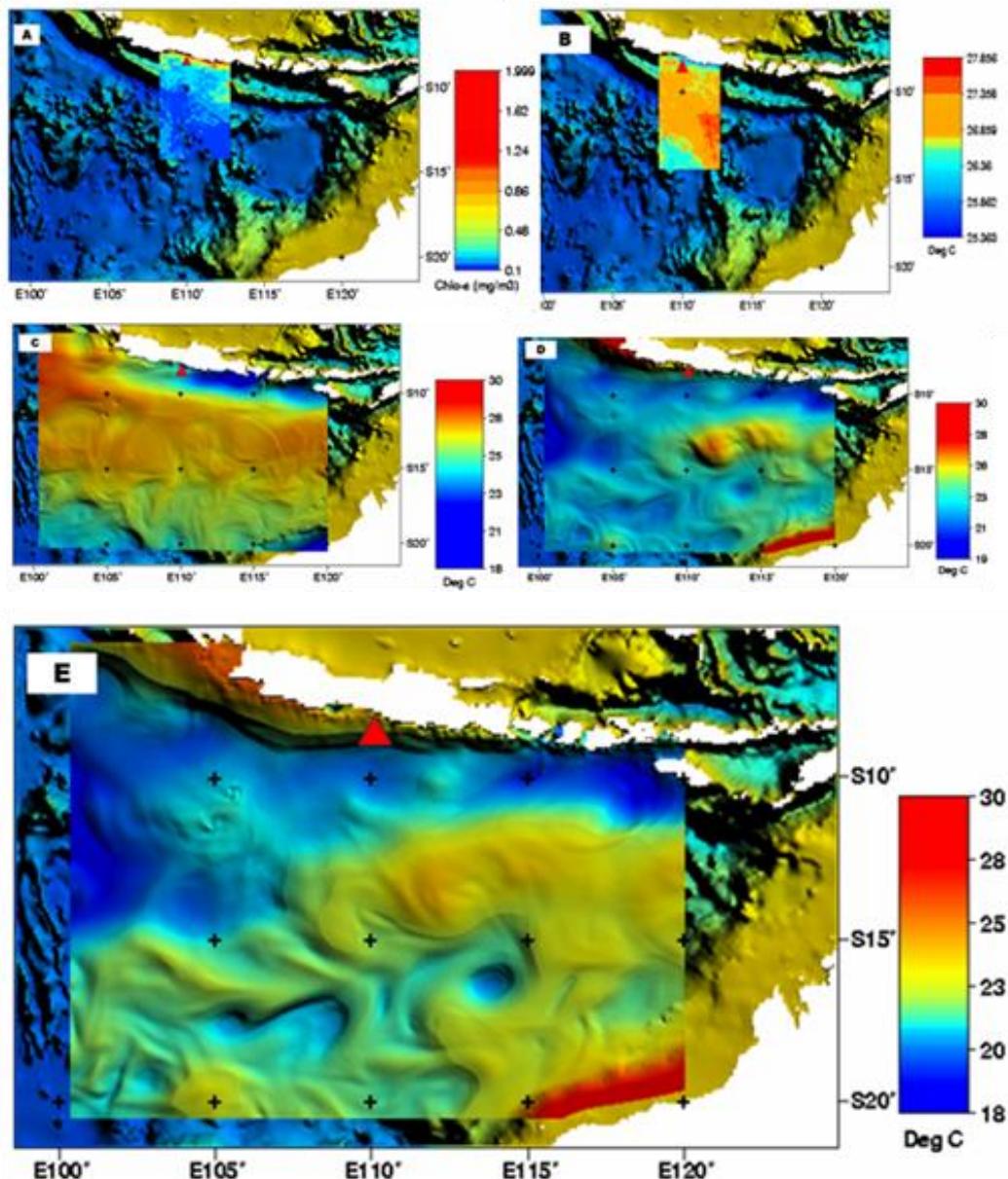


Figure 3. (A) Chlorophyll-a, (B) AquaMODIS SST, (C) Sub-ST at the depth of 75 m, (D) Sub-ST at the depth of 100 m, (E) Sub-ST at the depth of 125 m in the stranded whale shark found on September 19th, 2020.

The Sub-ST of the CMEMS data along the migration track of the stranded whale shark, during the east season of May–October 2020, is: 23–25°C at 75 m depth (Figure 3C), 23–26°C at 100 m depth (Figure 3D) and 23–25°C at 125 m depth (Figure 3E). This data

confirmed that the stranded whale shark is migrating, following the seawater column, seeking for seawater temperatures ranging from 23 to 25°C. The spatial data of SST and the three Sub-STs at depths of 75 m, 100 m and 125 m suggest the presence of the induced upwelling event (Figures 3C, D and E). The effects of an upwelling event include the nitrification of the upper seawater column, high chlorophyll-a content, primary productivity and planktonic abundance. Figures 3C, D and E clearly demonstrate the extent of the impact of the upwelling event that induced eddies.

Molecular biology. The result of the gel agarose electrophoresis visualization from the whale shark sample is presented in Figure 4, with a barcode COI base-pair (bp) of 700. The PCR sequence assembly is presented in Table 1.

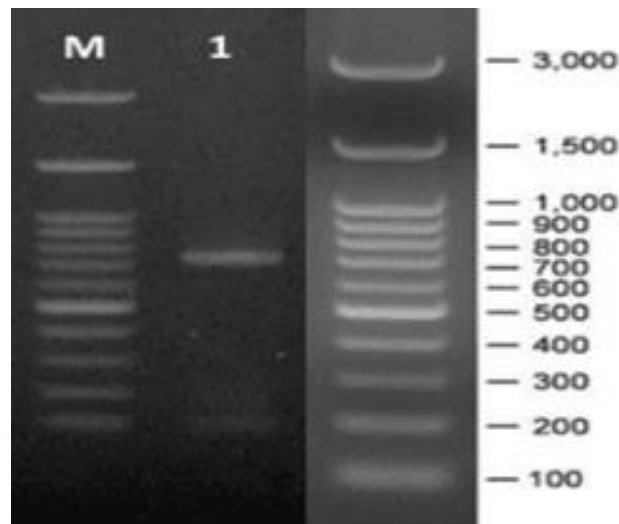


Figure 4. PCR result of whale shark tissue sample with 1.5% gel agarose; M = Marker, 1 = sample code AH-01.

Table 1
Percentage of similarity for the sample AH-01 of the whale shark

Sample code	Species	Query cover	Identical	Accession no
AH-01	<i>Rhincodon typus</i>	94%	96.66%	EU398993.1

The species of sample AH-01 was identified as *R. typus*, accession no. EU398993.1, with an identical or similarity percentage of 96.66% and a query cover of 94%. This was the result of the molecular gene identification, using COI into BLAST and NCBI databases, revealing the DNA nucleotide sequence according to the GenBank database (Table 1). Phylogenetic analysis in this specific case was based on the 699 bp sequence length of the gene COI. The outcome of the neighbour-joining method's phylogenetic analysis indicates a very noticeable distinction between the *Centrophorus atomarginatus*'s outgroup and the ingroup (Figure 5). The BLAST result, as shown in Table 2, confirmed that the sample code AH-A is *Solenocera crassicornis* with a query cover of 92%, accession No. KU324657.1. Using the small crustacean of *S. crassicornis* as the neighbour-joining tree in Figure 6, phylogenetic tree analysis of gene COI was conducted. The other five clades are as follows: clade-1 of *Scopelopheirus schellenbergi*, clade-2 of *Eurythenes gryllus* and *Neocervinia* sp., clade-3 of *E. gryllus* and *Desmosomatidae* sp., clade-4 of *Plesitacanta kannu* and clade-5 of *Lysmata seticaudata*.

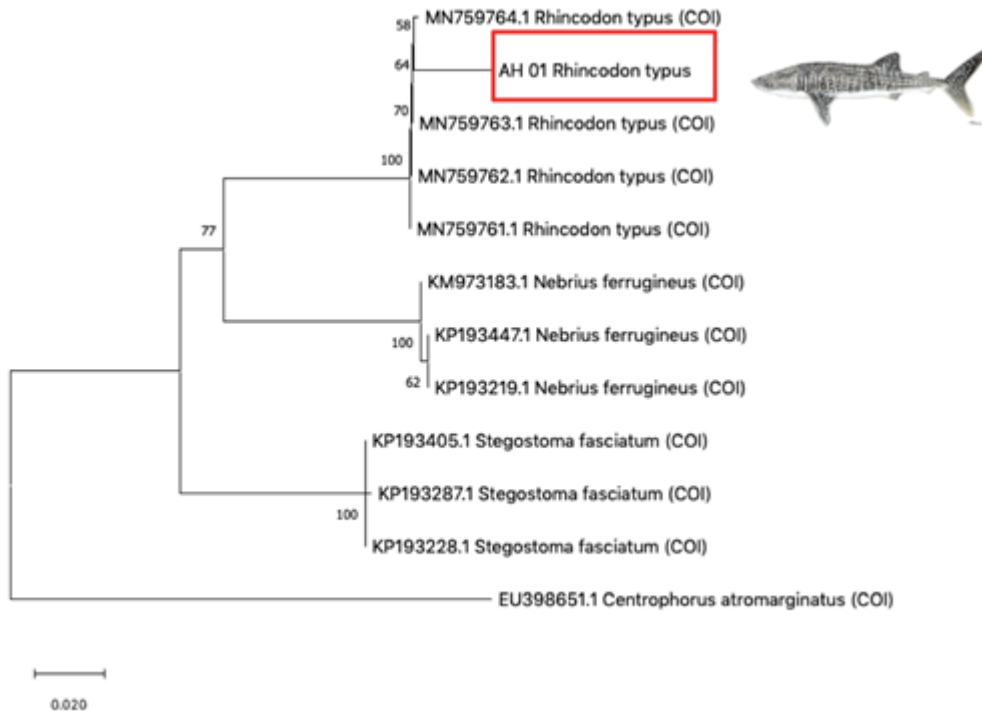


Figure 5. Phylogenetic tree of the gene COI (neighbour-joining tree) of the whale shark.

Table 2

BLAST results based on the NCBI database on the sample

Code	Species	Query cover (%)	Identification (%)	Accession no
AH_A	<i>Solanocera crassicornis</i>	92	81.30	KU324657.1
	<i>Pleistacha kannu</i>	99	76.69	MH425628.1
	<i>Solanocera crassicornis</i>	61	81.90	KP136603.1
	<i>Lysmata seticaudata</i>	87	73.81	KF006318.1
	<i>Eurythenes gryllus</i>	99	71.69	AY830436.1
	<i>Scopelocheirus schellenbergi</i>	99	71.37	AY830439.1
	<i>Desmosomatidae</i> sp.	89	72.81	KJ736166.1
	<i>Neocervinia</i> sp.	79	71.58	MF077931.1
AH_C	<i>Emerita emeritus</i>	96	96.20	KR047035.1
	<i>Daldorfia horrida</i>	99	81.90	NC049029.1
	<i>Rimicaris chacei</i>	99	81.90	MT270758.1
AH_L	<i>Paguridae</i> sp.	92	78.39	MH3399319.1
	<i>Decapoda</i> sp.	92	78.08	MH338371.1
	<i>Paralomis africana</i>	99	76.83	HM020907.1
	<i>Orconectes carolinensis</i>	98	76.94	KU168750.1
	<i>Nihonotrypaea harmandi</i>	99	76.58	LC221567.1
	<i>Cambarellus patzcuarensis</i>	99	76.78	JX127945.1
	<i>Leptestheria cortieri</i>	94	77.23	MN553636.1
<i>Procambarus tolteca</i>	99	76.37	JX127966.1	

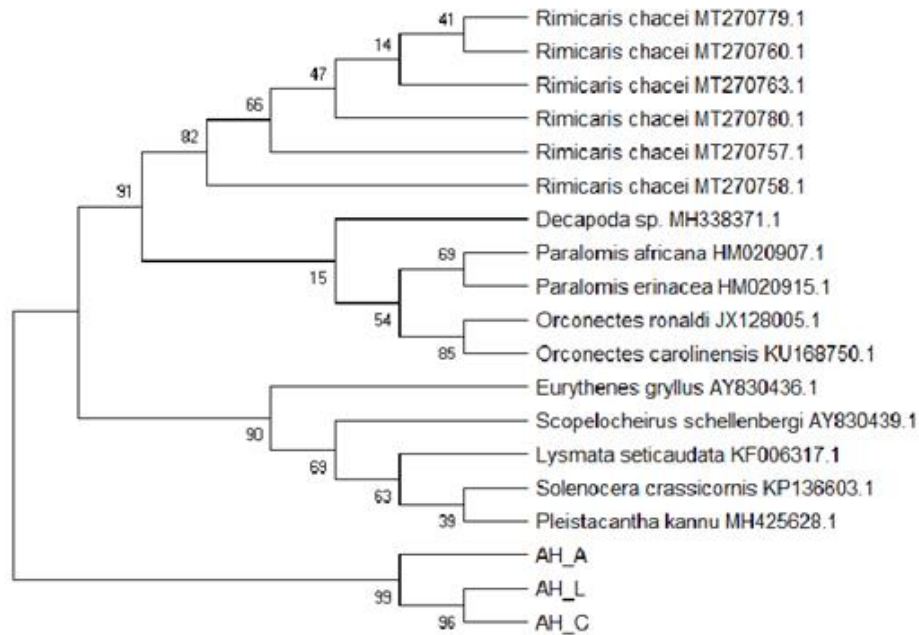


Figure 6. Phylogenetic tree of the three small crustaceans in the group of Super Class of Crustaceans, which were confirmed as the natural diet of the whale shark, as found in its stomach content.

Discussion. A 12 m whale shark washed up on Congot Beach, Yogyakarta, South Java. In the seawater surrounding the stranded whale shark, the chlorophyll-a content analysis from the AquaMODIS data was found to be 1.959 mg m^{-3} (Figure 3A). On September 19, 2020, the SST found at the position of a stranded whale shark at Congot Beach, Yogyakarta, South Java, was 25°C (Figure 3A). Based on the CMEMS data analysis, it appears that the whale shark was following the SST pattern at the depths of 125 m and 100 m, then vertically migrating at the depth of 75 m and ultimately at the sea surface, with seawater temperatures ranging between $23\text{--}25^\circ\text{C}$, until the point where it got stranded (Figures 3B and C). This phenomenon is also supported by the study of Wirasatriya et al (2020). Whale sharks have the appearance of a large fish, and they benefit from their body temperature stability, allowing them to move in an environment with wide temperature variations (Nakamura et al 2020). Furthermore, the distribution of whale sharks was also determined to be in cosmopolitan tropical or warm subtropical zones and rarely in areas with SST less than 21°C (Weber et al 2020). A study on SST using SeaWiFS and NOAA-AVHRR data also reported that whale shark habitat in the northeastern Arabian Sea was found to be within the $23\text{--}26^\circ\text{C}$ range, in February and March 1998–2000 (Kumari & Raman 2010). The Indian Ocean provides a crucial habitat for whale sharks, with numerous large and well-documented aggregations (Norman et al 2016). The spatial data of SST and the three Sub-STs at depths of 75, 100 and 125 m suggested induced upwelling events (Figures 3C, D, E). This is also corroborated by the earlier study of Pranowo et al (2005). According to Utamy et al (2015), the upwelling event has the following impact: nitrification of the upper seawater column, high chlorophyll-a content, primary production and planktonic abundance. Another impact of the upwelling event is also the formation of oceanic eddies, as clearly exhibited in Figures 3D and E, with enhanced Eddy development from August and peak in October as also previously reported by Zu et al (2022).

Natural diet and stomach content of the whale shark. The result of the first DNA analysis of the stomach content of the stranded whale shark *R. typus* on the tropical Congot Beach in Yogyakarta, South Java, is presented in Table 1. The phylogenetic tree shown in Figure 6 confirmed that the whale shark's natural diet consists of three small crustaceans, with *S. crasicornis*, as the most dominant species, and the minor species comprised *Emerita emeritus* and the hermit crab Paguridae. The three species in the diet

were in a group along with other eight clades of small crustaceans, namely, (1) *Rimicaris chacei*, (2) Decapoda sp., (3) *Paralomis africana* and *P. erinacea*, (4) *Orconectes ronaldi* and *O. carolinensis*, (5) *Eurythenes gryllus*, (6) *Scopelocheirus schellenbergi*, (7) *Lysmata seticaudata*, (8) *Solenocera crassicornis* and *Pleistacantha kannu*. According to the study of Dineshbabu & Manissery (2009), the *Solenocera choprai* itself mostly feeds on zooplanktonic small crustaceans, decapod crustaceans, polychaetes and foraminifer shells. In addition, Meekan et al (2009), using DNA, confirmed that *R. typus* had been feeding on *Gecarcoidea natalis* larvae. The whale sharks spend most of their vertical habitat as the key control factor of diel vertical migration (DVM) for zooplankton feeding activity (Sims et al 2005). The proposed theory suggests that the DVM behaviour observed in the planktivorous megamouth shark, *Megachasma pelagios*, is likely driven by their search for a constant level of ambient light (Nelson et al 1997). The basking shark, *Cetorhinus maximus*, demonstrated the influence of zooplankton distribution, density and characteristics on foraging behaviour (Sims & Quayle 1998). Tarling et al (2002) found an abundance of *Calanus finmarchius* in the sub-temperate seawater of the English Channel at depths ranging from 50 to 80 m, as well as Euphausiids, *Meganyctiphanes norvegica* and *Thysanoessa raschii*, at depths ranging from 50 to 100 m. Sampaio et al (2018) revealed that in Brazilian seawater, whale sharks feed on Geryonidae crab larvae and ingested plastic debris. Young whale sharks of 4m length, from the Cenderawasih Bay National Park population, which is considered a vital habitat, feed on small fish near fisherman lift nets, in the sea surface layer (Himawan et al 2015). Montero-Quintana et al (2021) observed whale sharks feeding on schools of small fish, specifically anchovies, in four locations: Bahia de Los Angeles, Baja, California and Mexico. In comparison to other large oceanic migratory groups, the carnivorous whale, *Physeter catodon*, in the Savu Sea East Nusa Tenggara, Indonesia, was revealed to feed on the small squid *Sepioteuthis lessoniana*, as determined from the stomach content of the whales in this region, via DNA analysis, to be the natural diet (Pringgenies et al 2021). As confirmed by Motta et al (2010), whale sharks feed on nektonic plankton such as squids, krill, micro-algae and small fish.

Genetic/DNA species barcoding and whale shark genome. All PCR sequence assembly results and genome tables were presented, which can be used for other further comparative studies. The guanine-cytosine (GC) genome pair of *S. crassicornis* and *R. typus* has the same amount of 24 GC pairs, and the diet of *S. crassicornis* is thought to have an influence on the epigenetic regulation of *R. typus*. Large-scale comparative genomic analysis has revealed valuable insights into the whale sharks' genome architecture and guanine-cytosine (GC) content. One notable finding is that the whale shark's metabolic rate in whale sharks is directly dependent on temperature. Reduced temperatures have been linked to lower basal metabolic rates and growth rates, and increased body sizes in whale sharks (Weber et al 2020). Understanding the correlations between distinct genomic traits is crucial in unravelling the complex interaction between genetic factors and phenotypic outcomes. Numerous studies have found that several major genomic traits show significant correlations with body size, temperature and lifespan. The genetic diversity study of a total of 70 individuals identified 51 single site polymorphisms and 8 regions containing indels, yielding 44 unique haplotypes. The study found high levels of haplotype and nucleotide diversity, indicating a complex genetic structure within the population. Furthermore, phylogenetic analyses revealed no distinct geographical clustering of lineages, implying a lack of population differentiation based on geographic location. The most prevalent haplotype was found to be distributed globally, indicating potential gene flow and genetic exchange across different populations (Castro et al 2007). According to Weber et al (2020), whale shark genetic evidence reveals the presence of at least two distinct populations that rarely intermix. A recent study that employed additional microsatellite loci to pinpoint distinct genetic variations between Atlantic and Indo-Pacific whale sharks supports these findings. Vignaud et al (2014) revealed that the genetic evidence of *R. typus* whale sharks are composed of at least two populations that rarely mix; they are the first to document a population expansion. Lou et al (2022) obtained 14,930 full-length transcripts of the *R. typus* utilizing the Oxford

Nanopore (ONT) platform to conduct a comprehensive analysis. By comparing the identified 714 novel transcripts and the FL-transcripts of *R. typus*, it is possible to predict the distribution patterns of alternative splicing analysis (ASS), which refers to mRNA precursors with multiple splicing, long non-coding RNA (LncRNA) prediction, polyA length analysis (polyAS), potential coding sequences and *R. typus* FL transcriptome methylation sites. A total of 31,021 coding sequences (97.86%) have obtained annotation data. The paper claims to have provided the first FL-transcriptome, which completes the annotated *R. typus* reference genome material. By comparing *R. typus* to large marine mammals like the bowhead whale, *Balaena mysticetus*, the longest-living mammal, the study of Keane et al (2015) found the adaptive genes in association with the DNA repair, cell-cycle regulation, immune resistance, thermoregulation and dietary adaptations.

Conclusion. The SST at the studied *R. typus* specimen's coordinates is 25°C, while the Sub-ST at the depths of 75, 100 and 125 m is in the 23-25°C range, exhibiting an upwelling event in the South Java Indian Ocean. The upwelling event induced nitrification, primary productivity and a chlorophyll-a content of 1.959 mg m⁻³ in the upper seawater column as well as the formation of seawater eddies. The main finding from the DNA analysis revealed that the dominant diet, based on the stomach content, is *S. crassicornis*, with Paguridae species and *E. emeritus* serving as minor diets. The GC genome pairs in *S. crassicornis* and *R. typus* is found to have the same number, 24 GC pairs, and the diet of *S. crassicornis* having a moderate effect on the epigenetic regulation of *R. typus*. This finding is assumed to be indicative of the *R. typus*'s genome relationship to its natural diet's genome adaptation to the seawater thermoregulation, due to the fluctuation of the seawater temperature and diet during the long-distance oceanic migration.

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Conflict of interest. The authors declare no conflict of interest.

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