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Variazioni temporali a lungo termine nella posizione trofica del Grande Squalo Bianco *Carcharodon carcharias* nella provincia costiera di KwaZulu-Natal, Sud Africa

Long-term temporal variations in the trophic position of Great White Shark *Carcharodon carcharias* along KwaZulu-Natal coast, South Africa

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Riassunto

Gli elasmobranchi sono pesci cartilaginei che occupano i livelli intermedi e superiori nelle reti trofiche marine. Tuttavia, le popolazioni sono minacciate da sovrasfruttamento delle risorse alieutiche globali, portando ad un depauperamento delle reti trofiche. Nonostante la loro rilevanza ecologica, le informazioni disponibili riguardanti l'ecologia trofica degli elasmobranchi rimangono ancora insufficienti. Una completa conoscenza delle abitudini alimentari e dell'uso dell'habitat è essenziale per lo sviluppo di piani di conservazione adeguati. Lo squalo bianco *Carcharodon carcharias* è classificata come "vulnerabile" all'interno della Lista Rossa dell'IUCN (International Union for the Conservation of nature) dal 2018. L'obiettivo di questo studio è stato quello di rilevare, tramite analisi isotopiche, i possibili cambiamenti avvenuti nelle abitudini alimentari e nella nicchia trofica della popolazione residente nelle acque oceaniche antistanti la regione di KwaZulu-Natal, Sud Africa. In questo lavoro di tesi sono state condotte delle analisi dei contenuti di isotopi stabili di C e N, con la conseguente determinazione dei valori di δ^{13}C e δ^{15}N. I valori di questi rapporti isotopici sono utilizzati per ottenere informazioni sulle principali prede assimilate negli ultimi mesi di vita dell'animale e sulla posizione trofica dell'animale stesso. In questo studio, le vertebre di individui di squalo bianco prelevate da animali spiaggiati lungo le coste di KwaZulu-Natal tra il 1987 e il 2014,

sono state divise in due gruppi temporali in base alla data di campionamento: G1 (organismi campionati tra il1987 e il 1996) e G2 (organismi campionati tra il 2005 e il 2014). La prima analisi condotta su queste vertebre è stata la stima dell'età attraverso un metodo di osservazione diretta delle bande di crescita. I risultati ottenuti sono stati successivamente comparati a quelli ottenuti da un metodo di calcolo inverso dell'età a partire dalle lunghezze alla forca dell'animale, presente per ogni singolo campione. Le analisi isotopiche sono state effettuate prelevando materiale dal birth mark (BM), che fornisce i potenziali valori isotopici delle madri, e dall'ultima banda di crescita (GB) che fornisce i valori isotopici dell'animale considerato.

La correlazione tra i valori di $\delta^{13}C$ e le lunghezze degli animali non è risultata essere significativa, dimostrando che non vi è stato un cambio nell'uso dell'habitat con l'aumentare della taglia. Tuttavia, correlazioni significative sono state registrate per i valori di $\delta^{15}N$, confermando l'esistenza di uno shift ontogenetico nella dieta degli animali. Sono state riscontrate differenze nei valori di $\delta^{13}C$ e $\delta^{15}N$ di BM e GB dei due gruppi temporali, ma nessuna di esse è risultata significativa. La modellizzazione delle nicchie trofiche condotta tramite il pacchetto SIBER del software R ha evidenziato cambiamenti nella posizione delle nicchie sia considerando il BM che considerando il GB in entrambi i gruppi temporali. Grazie agli output ottenuti è stato possibile evidenziare uno shift ontogenetico nella dieta degli

individui di taglia superiore a 250 cm appartenenti al gruppo G2 a sostegno della teoria secondo la quale un completo shift potrebbe avvenire intorno ai 300 cm, come dimostrato in pubblicazioni precedenti. Infine, le simulazioni condotte con il pacchetto simmr del software R utilizzando valori isotopici di prede ottenuti dalla letteratura, è stato dimostrato che le prede principali dello squalo bianco sono otarie, pesci pelagici e altri squali ma che il loro contributo proporzionale nella dieta dello squalo stesso sembra essere diminuito nei tempi più recenti a favore del contributo dato da prede come i clupeidi. Ciò potrebbe essere dovuto ad un effetto sinergico tra l'impatto della pesca, che ha impoverito la rete trofica diminuendo la disponibilità di prede e gli effetti dei cambiamenti climatici che hanno probabilmente spinto le otarie a spostarsi alla ricerca di habitat più adatti.

Abstract

Sharks are cartilaginous fish that occupy the intermediate and apex levels of the trophic web. Due to the overexploitation of the global fishery resources, and a decrease in prey availability together with an increase of bycatch, several shark populations are facing a demographic decline. Despite the essential ecological role of these animals, the available information regarding their trophic ecology is still not sufficient. A deeper knowledge about the feeding habits and their habitat use, together with their ecological role in marine ecosystems, are essential for the development of conservation strategies. In this study, the Great White Shark *Carcharodon carcharias*, listed as "vulnerable" by the IUCN (International Union for the Conservation of nature) Red List from 2018, was taken under consideration. In particular, this study focuses on the population that inhabits the oceanic waters along the KwaZulu-Natal coast, South Africa. here, we aim to investigate the possible changes occurred over time in the trophic niche of *C. carcharias*. This type of information can be obtained through the analysis of stable isotopes of C and N, resulting in the determination of values of $\delta^{13}C$ and $\delta^{15}N$ that are useful to obtain data about the main preys ingested in the last months before the death of the animal and about their trophic position. In the present study, several vertebrae samples from individuals stranded along the KwaZulu-Natal coast between the 1987 and 2014 were collected and divided

into two temporal groups depending on the year of capture: G1 (1987-1996) and G2 (2005-2014). Firstly, an age estimation using direct band count method on cross-sectioned vertebra, subsequently compared with a back-calculated method obtained from the fork length, here present for each individual. The SIA (Stable Isotope Analysis) was performed analysing material collected from the birth mark (BM) and the last growth band (GB) in order to obtain a comparison in between the potential isotopic signatures of the mothers and those of their own offspring. The correlation between the Total Length (TL) and the values of $\delta^{13}C$ showed no significant correlation, demonstrating that no change in the habitat use were recorded with the increasing growth. Nevertheless, a significant correlation was recorded for values of $\delta^{15}N$, showing the existence of an ontogenetic shift in the diet of the species. Several differences were observed in the values of $\delta^{13}C$ e $\delta^{15}N$ measured in BM and GB of the two temporal groups, but any of these resulted to be significant. The analysis of the trophic niches highlighted the presence of an ontogenetic shift in the diet of animals with a TL higher than > 250 cm in G2 confirming what is reported in previous published studies that observed a complete ontogenetic shift around 300 cm of TL. Finally, simulations conducted with the simmr package of the R software using isotopic values of prey obtained from the literature that the main prey of the white shark are sea lions, pelagic fish and other sharks but their proportional contributions in the diet seem to have decreased in more recent times in

favour of that of prey such as clupeids. This could be due to a synergistic effect between the impact of fishing, which has depleted the trophic web by decreasing the availability of prey, and the effects of climate change, which has probably driven sea lions to move in search of more suitable habitats.

1 Introduction

1.1 Elasmobranchs and their conservation

Elasmobranchii is one of the subclasses of cartilaginous fish that belong to the parvphylum Chondrichthyes, together with the subclass of Holocephali. The taxa include more than 1,107 species around the world between sharks, rays, skates and chimaeras; of those, 432 species are sharks (IUCN SSC-Shark Specialist Group). Elasmobranchs are widely distributed, with habitat ranging from high latitudes to tropical areas, and from coastal to deep waters (Camhi, 2008; Das et al., 2022). Several species engage seasonally migration from coastal waters to open ocean (Priede et al., 2006). The class is characterized by a high variability in size, feeding and reproductive strategies, spacing from species like *Rhincodon typus* (Smith, 1828), a large-body pelagic shark that can reach 21 m of total length - TL - (Stevens, 2007; Hsu et al., 2014), to deep-water species like *Etmopterus perryi* (Springer & Burgess, 1985) with ca. 20 cm of TL.

Based on their lifestyle, sharks can be categorized in bottom species, that live in strict association with the sea bottom, and pelagic species, highly mobile species, not strictly associated to the sea bottom (Camhi, 2008). Pelagic-shark species that spend their life, in whole or in part, in the ocean basin showing seasonal inshore migrations to feed and breed, are defined as

"oceanic" species (Compagno, 2008). Those species can be recognized as epipelagic, confined from the surface to about 200 m depth where sunlight irradiance allows photosynthesis; mesopelagic, below the epipelagic zone, from 200 to 100 m depth where light irradiance is lower; bathypelagic, inhabiting the dark zone below 1000 m depth; or semipelagic, including all the shark species migrate through oceanic waters but primarily concentrate close to the coast (Compagno, 2008).

Over the past century, the relentless growth of human population and the increasing demand of natural resources, led to the intensification of the industrial fishing activities and unintentional catch, usually referred to as bycatch (Lotze and Worm, 2009). The International Union for Conservation of Nature (IUCN) Red List reported that at least 17.4% of elasmobranchs are listed as near threatened or globally threatened, with risk of local extinction in many areas of the globe (**Fig 1.1**). Dulvy et al. (2021) used published Red List global-scale data from 2013 to 2021 assessments to provide an update status on chondrichthyans: results revealed that 32.6% are identified as threatened, with 15% listed as Vulnerable (VU), 10.1% Endangered (EN), and 7.5% Critically Endangered (CR).

Species Group	No. of Species Assessed under Red List™ Criteria	No. of Species Classified as Threatened*	Critically Endangered	Endangered	Vulnerable	Near Threatened	Least Concern	Data Deficient
RAYS	578	**120**	13	33	74	58	158	242
SHARKS	482	**79**	10	20	49	55	150	198
CHIMAERAS	47	**1**	0	0	1	2	25	19
Total Assessed	**1,107**	200	23	53	124	115	333	**459**

Fig 1.1 Threatened IUCN Red List Categories = Critically Endangered, Endangered & Vulnerable. IUCN Red List Assessment Results, 2019 (from iucnssg.org).

Between the 1950 and 2003, the number of shark and ray landings have increased by 227%, reaching a peak of over 900,000 tonnes/year in the 2013 (FAO 2013b). The following decline of 15% in 2011 was recognised as a result of a decrease in the fishing pressure rather than new protection measures (Davidson et al., 2015). The global increase of the shark fin market represents one of the major drivers of shark's population decline together with illegal, unreported and unregulated fishing (IUUF). The Food and Agriculture Organization (FAO) of the United Nation reported that 121,641 tonnes (USD 379.8 million) of chondrichthyan meat was imported only in 2011 (Dent and Clarke, 2005). A more detailed analysis of the trade flow through the Hong Kong fish market, proved that the annual trade could be between 26 to 73 million of sharks (median of 38 million of sharks), significantly higher than the data reported by FAO (Clarke et al., 2006)

1.2 Target species: **Carcharodon carcharias**

1.2.1 Distribution

The great white shark (hereafter, GWS), *Carcharodon carcharias* (Linnaeus, 1758), is a large cartilaginous fish that inhabit our oceans, including the Mediterranean Sea. Defined as "apex predators", its presence or absence leads to important modification on the food web structure and, in turn, of marine ecosystems (Estes et al., 1998¸ Ferretti et al., 2010).

Due to the increasing population declines, primarily attributed to target overfishing and bycatch (Baum 2003; Gubili et al. 2011; Chapple et al. 2011; Blower et al. 2012; Nasby-Lucas and Domeier 2012; Towner et al. 2013), GWS is globally listed as Vulnerable by the IUCN Red List (Fergusson et al. 2009). Classified inside the order of Lamnidae, its distribution covers mainly temperate and subtropical regions (**Fig.1.2**), with a preference for high temperatures, as showed from some records in the Coral Sea (Last and Stevens, 1994), Papua New Guinea (Burgess and Callahan, 1996), the central Pacific (Taylor, 1985; Compagno, 2001), northern Brazil (Gadig and Rosa, 1996), and the tropical southwest Indian Ocean (Cliff et al., 2000).



Fig.1.2 Great white shark *Carcharodon carcharias* (Linnaeus, 1758) distribution (www.GBIF.org).

Carcharodon carcharias juveniles occur primarily in coastal waters, including coastal bays and estuaries, while adults are more concentrated in offshore waters, probably in favour of increasing feeding opportunities (Bernvi, 2016). Otherwise, several movements occur between the coastal shoreline and the open waters to feed and reproduce. Juveniles are usually confined to the neritic zone (Dicken & Booth 2013) where warm waters support a better growth; some individuals engaged more movements to different neritic habitat, expanding their trophic niche from sub-tropical to temperate waters (Boustrary et al. 2002; Bonfil et al., 2005;). Also, adult females of GWS have been recorded in the neritic area during summer months in Western Cape, South Africa (Koch et al., 2013).

In recent years, several transoceanic migrations have been recorded between California to Hawaii (Boustany et al., 2002) and from South Africa to Western Australia (Bonfil et al., 2005). Transoceanic migrations were previously unknown or only hypothesised for males. Bonfil et al., (2005) have demonstrated the direct linkage between the South African and Australian white shark populations, confirming philopatry and demonstrating that also females are capable of long-distance movements, engaging transoceanic migrations to give birth. The assumption of large quantities of lipid rich preys' pre-migration (Del Reye et al., 2013), together with the endothermic capacity, are necessary to migrate and dive in deep and cooler waters with high availability of preys (Bernal et al. 2012, Bonfil et al. 2005, Boustany et al. 2002). A young of the year (YOY) can travel for 700 km (Weng et al., 2007) or even 1800 km (Bruce et al., 2019) away from their nursery area. In this case, insular marine areas are essential for GWS juveniles to gain experience in movements between inshore and offshore areas before starting their first long- distance migrations (Hoyos-Padilla et al., 2016).

Mature white sharks are able to dive into cold and hypoxic oceanic waters (Nasby-Lucas et al., 2009) below depth of 500 m and 1200 m (Bonfil et al., 2005; Weng et al., 2007; Francis et al., 2012) for few minutes, thanks to their physiological adaptation and thermoregulation. Many white sharks have been tracked to dive in water temperatures of 6.8°C in Australia, and 2.8°C

in New Zealand for 10-15 min/dive (Block et al., 2011; Bruce et al., 2012; Francis et al., 2012).

1.2.2 Morphology

The GWS is a cartilaginous fish and a ram-ventilating endothermic predator (Bonfil et al., 2005). *Carcharodon carcharias* is characterized by a spindle shaped body with coloration that changes from whitish to greyish with growth (Gauna and Sternes, 2024). Onate-González et al. (2016) have proposed four different size categories based on reproductive stage: young-of-the-year (YOY) ranging from 120 cm to 175 cm TL, juvenile ranging from 175 cm to 300 cm TL, sub-adult for all the individuals ranging from > 300 cm to maturity, and adult for mature sharks > 360 cm TL for males and > 480 cm TL for females.

White sharks show kinetic cranium characterised by a hyostylic jaw connected, and not fused, to the cranium, allowing a protrusion mechanism during predatory activity (Wilga, 2005). The upper and lower jaw are characterised by multiple rows of teeth made of a matrix of fluor-apatite ($Ca_5(PO_4)F$) with hydroxyapatite ($Ca_5(PO_4)OH$) and carbonate-apatite ($Ca_5(PO_4,CO_3,F)(OH,F)$) (Moller et al., 1975; Daclusi and Kerebel, 1980). Theet are continually replaced over the years, only the outer rows are functional, while the replacement teeth lie towards the rear (Vannemann et al., 2000).

Several fins can be recognised (**Fig.1.3**): the first large dorsal fin originate over the pectoral inner margins, while the second dorsal fin, smaller than the first one, is located above the peduncular area. Ventrally, two large pectoral fins can be easily recognised, followed by two pelvic fins, a small fin and a lunate shaped caudal fin. Fins' individual-morphology, and in particular the first dorsal fin, is largely used in photographic identification for studies on spatial distribution, habitat-use, and migration (Bonfil et al., 2005). Fins are constituted by cartilage and collagen fibres; these types of fibres have a slower growth compared to the skeletal muscle and the total length of the shark (Lingham-Soliar, 2005).

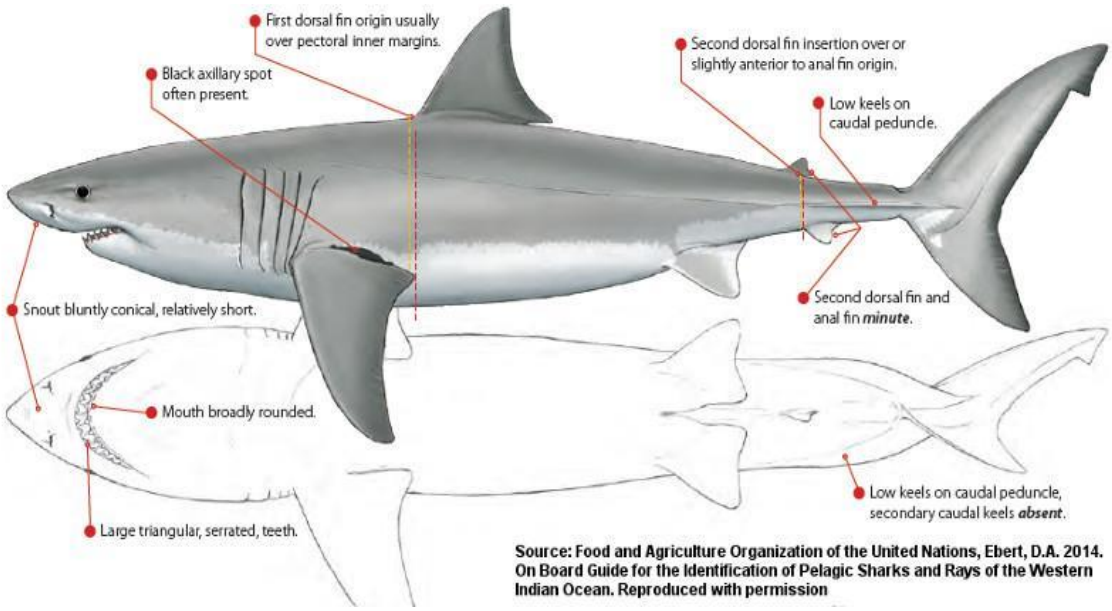


Fig.1.3 *Carcharodon carcharias* body morphology (FAO).

GWS show a typical locomotion defined as thunniform mode. Considered one of the most productive aquatic locomotion strategies, the movement is

produced by the lateral undulation of the posterior part of the body, while the anterior part is static, reducing friction through the water (Sfakiotakis et al. 1999). The thrust generated by a lunate shaped caudal fin allows to reach a speed of 35-42 km/h and predatory breaching over 2.4 m above the surface (Martin & Hammerschlag, 2012).

Five pairs of large gills are easily recognizable in this species; due to the absence of the branchial operculum, characteristic feature of teleost, shark's gills result more vulnerable to injuries. Gill arches support one or two gill filaments; these structures are designed as one-way water's flow while the blood flow in the opposite direction inside the filaments. This mechanism is defined as the counter-current system. A pair of spiracles, located between the eye and the gill slits, are responsible for waters' entrance and gill's ventilation.

GWS own a highly specialised multi- functional sensory system that allows them to detect and respond to different outputs of biotic and abiotic environmental stimuli.

The multi-functionality of the system permits the use of one or more senses when others are unusable, e.g. high turbidity in water does not allow a clear vision (Stein et al., 2005). The level of specialisation of each system depends on the species and its lifestyle. Mechanosensory, chemoreceptors and electroreceptors are the three most important systems used to detect preys, predators and water movements. The lateral line (**Fig. 1.4**) is a short-distance

mechanosensory structure constituted by groups of sensory hair cells called neuromasts (Maruska 2001). These types of cells can detect low frequency water movements, such as vibrations produced by other organisms, as well as direction and velocity of water currents (Boord & Campbell, 1977; Kalmijn 1989; Montgomery et al., 1995). Neuromasts are distributed all over the dorsoventral surface of the head, trunk and some groups around the spiracles, ventral to the mouth and anterior to the gills (Tester & Kendall, 1967; Peach & Marshall, 2009).

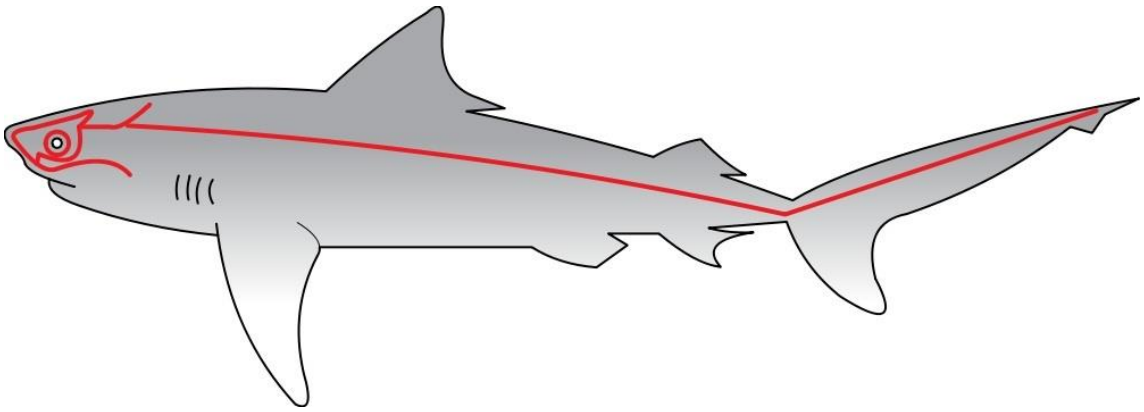


Fig. 1.4 General representation of the lateral line (in red) in *C. carcharias*.

Chemoreceptors are primarily associated with intraspecific interactions and communication, reproduction and food recognition (Harte & Collin, 2015). The Ampullae of Lorenzini are specialized electroreceptors able to detect weak electrical potentials produced by other organisms. They play an essential role during prey localization and capture, and to avoid predators. As extremely sensitive systems, Ampullae of Lorenzini cover an important

role in orientation and navigation; the information used are generated by differences in electric potential due to changes in water currents and their own body as they move through the Earth's magnetic field (Kalmijn 1978; Paulin 1995).

Another important characteristic of GWS is the endothermic capacity, defined as the ability to regulate body temperature independently from the environmental temperature. To permit migrations through different habitat temperatures, great white sharks developed several regional body specialisations to control and reduce conductive heat loss such as the closeness between vertebral column and red muscles (Carey et al., 1985), and the counter-current capillary network. Also known as *retia mirabilia*, this system extends between the red muscles and subcutaneous lateral vessel (Bernal et al., 2012) and is able to generate a temperature gradient that allow the circulation of cold oxygenated blood from the gills to the brain and eyes. The return of deoxygenated blood to the heart (Carey et al., 1971; Alexander 1998), increase the metabolic efficiency of red muscles of about 3-5 °C above the environmental water temperature (Carey et al. 1982; Tricas & McCosker 1984). Red muscles are vital for the sustainment during swimming and pre-adaptation to new habitats. Neonates have a poorly developed and homogenous red muscle distribution with a heat production doubled than in adults. Large skin surface area works as an insulator, but young present a skin layer that is not developed enough to avoid an elevated

heat loss; for this reason, neonates and juveniles are confined to warmer coastal zones (Bernvi, 2016).

1.2.3 Growth

The age determination of large and vulnerable species and the knowledge of demographic parameters as age-specific length, sex-maturity length and age at maturity, are fundamental for constructing appropriate conservation and management plans. In marine populations, growth patterns are usually influenced by environmental conditions; with the increasing worldwide loss of elasmobranch populations, the necessity of models to predict the effects of environmental changes is always greater. The determination of age and growth in marine organisms can be carried out in several ways. Between the most common techniques we can distinguish the analysis of calcified structures, such as otoliths or vertebrae, mark and recapture methods with the injection of oxytetracycline, and back calculated growth rates using known ages or length (Wintner & Cliff, 1997; Shrader et al. 2006). Unfortunately, many of these methods are not always conclusive due to the variability across species (Cailliet and Goldman 2004). Otoliths are the most used in this type of study: the calcium carbonate is incorporated and mineralized in the form of aragonite in daily and annual rings that can be counted to determine individual age. However, elasmobranchs lack these

hard parts; other calcified structures such as vertebrae have been used (Wintner & Cliff, 1997; Tanaka et al., 2011; Natanson & Skomal, 2015). Elasmobranchs vertebrae are biomineralized structures made of hydroxyapatite. The incremental growth occurs in annual deposition of concentric rings. The annual periodicity forms an alternated band pattern of high (opaque) and slow (translucent) rate of mineralization (**Fig.1.5**). A growth band is defined as one band pair constituted by one translucent and one opaque band,

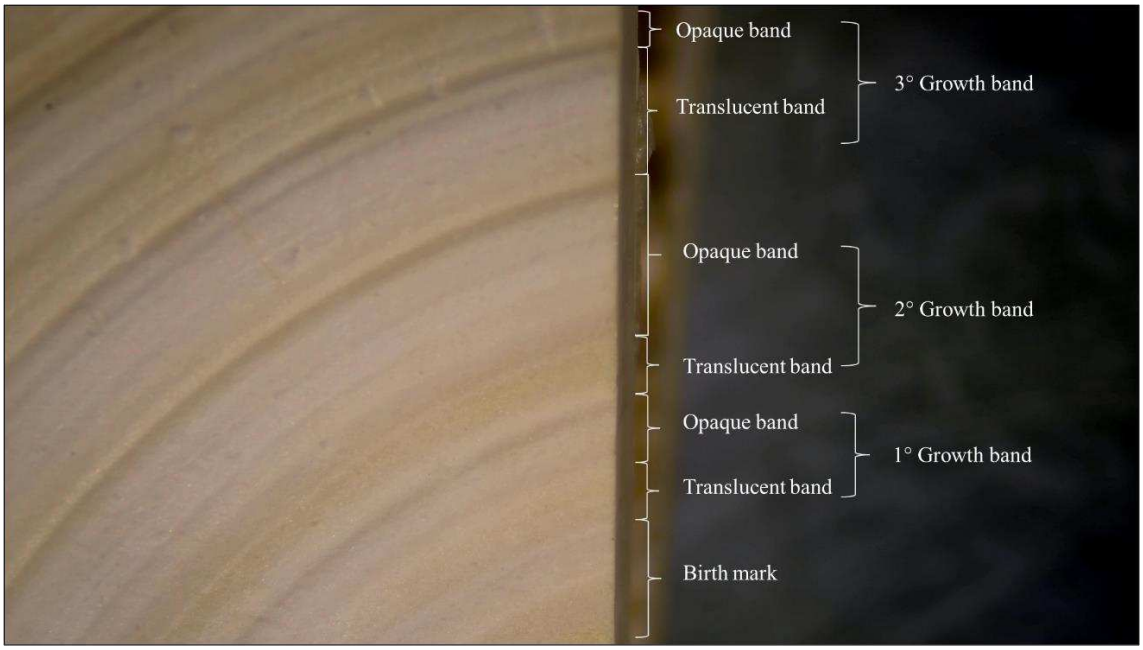


Fig. 1.5 Opaque and translucent bands of sample RB940.

respectively a period of slow growth, in winter, and a period of fast growth, during summer season. Fluctuation of environmental conditions, such as decrease in temperature or low food availability, could alter the band deposition, leading to the creation of false ring layers not fully completed.

The wrong count of these zones can lead to a wrong age estimation. Nowadays, there is not a specific procedure for the count of the vertebra growth bands but is usually represented as the mean of several reads.

1.2.4 Reproduction

GWS are gonochoric species characterised by a marked sexual dimorphism due to the presence of two claspers (sexual organs) in male individuals. The reproduction is internal, and the copula is violent: the male blocks the female clinging on and biting her, for this reason is common to observe mature females with several scars and bites all over the body. The copula ends when the males insert one of the claspers in the female's sewer. Aplacental viviparous species such as *C. carcharias*, are characterised by a slow growth and tardive sexual maturity. Size at maturity vary widely within the sexes: 3.5- 4.0 m TL for males, corresponding to 8-10 years circa, and 4.5- 5.0 m TL in females of about 12-15 years. Males reached sexual maturity earlier.
White sharks exhibit two reproductive strategies: lecithotrophy, where yolk is produced by the mother and sequestered in the yolk-sac for embryonic nutrition, and matrotrophy, with the production of yolk supplements such as uterine secretions (histotrophy) and eggs (oophagy), necessary to sustain embryonic development (Hamlett et al., 2005). Sato et al. (2016) provide clear evidence of uterine secretion of lipid-rich uterine fluid, often referred

as “uterine milk”, in *C. carcharias*. Lipid secretions decrease with the course of gestation as the uterine epithelium modified its structure and function changing the composition of uterine fluid from the early to the late gestation (Jensen et al., 2002). Like other lamnoids, white sharks perform the behaviour of oophagy; embryonic sharks consumed in uterus eggs storing the yolk in the stomach as an available source of nutrition (Uchida et al., 1996; Sato et al., 2016; Ebert et al., 2021). The consumption of nutritional (unfertilized) and not developed, in the early gestation period, is favoured by the presence of functional embryonic teeth (Gilmore, 1993) that are used for puncturing the capsule and feed directly in the maternal uterus (Gilmore, 1993; Francis and Stevens, 2000; Shimada, 2002).

Embryos development depends on at least three sources to reach the maximum size *in utero*: an initial phase of yolk-sac yolk, followed by lipid-rich uterine milk and a final phase of nutritional eggs.

The reproductive cycle is constituted by 18 months of gestation, followed by 18 months resting period. Parturition occurs between spring and summer, mostly in temperate coastal locations. Neonates body size widely differs from a range between 104 cm to 175 cm TL (Boldrocchi et al., 2017). Based on the abundance of the young of the year and the observation of pregnant females, several nursery areas are confirmed worldwide (**Fig. 1.6**): the coasts of Southern California, USA (Anderson et al., 2021; Gauna and Sternes, 2024), Sebastian Vizcaino Bay (Orñate-González et al. 2017) and Cedros

Island near Baja California, Mexico (Tamburin et al. 2020), Edremit Bay, Turkey (Kabasakal and Gedikoğlu 2008; Kabasakal, 2020), Corner Inlet and Port Stephens, Eastern Australia (Bruce et al., 2012; Spaet et al., 2020), Salisbury Island, Daw Island, and the Cocklebiddy region, the Great Australian Bight, South- West Australia (Anderson et al., 2021; O'Connel et al., 2023), the Sicilian Channel, Italy (Fergusson et al., 1996, 2002) and the Eastern coasts of South Africa (Cliff et al. 1989, 1996; Kock and Johnson 2006; Dicken and Booth 2013).

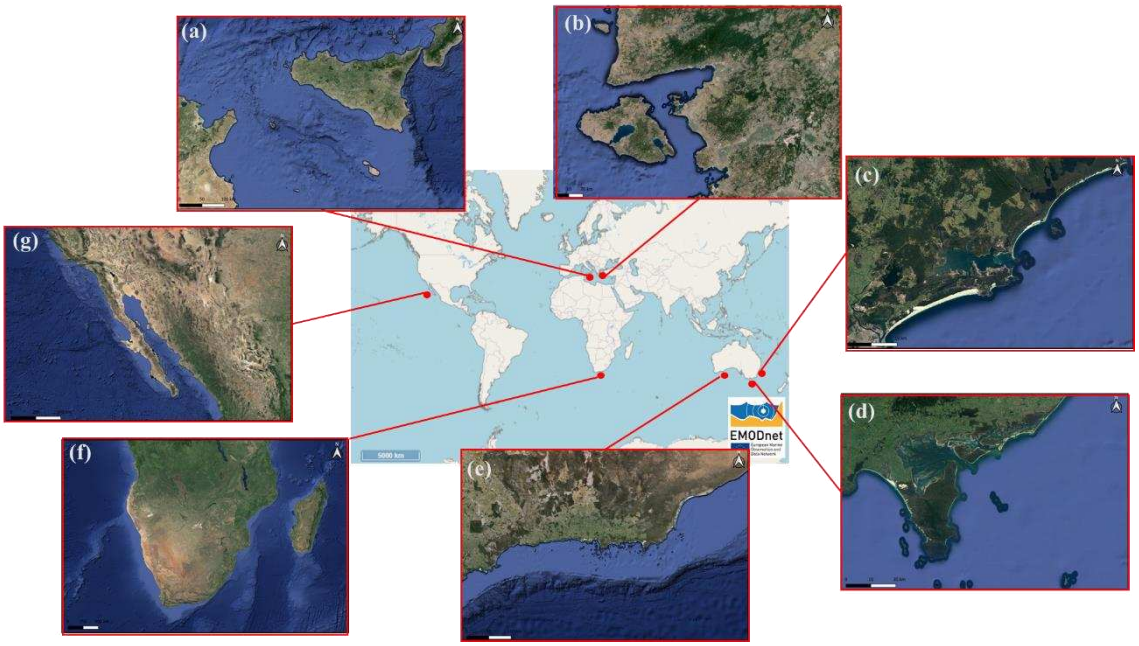


Fig. 1.6 Map of the *C. carcharias* nursery areas: (a) Sicilian Channel, Italy; (b) Edremit Bay, Turkey; (c) Port Stephens, Eastern Australia; (d) Corne Inlet, Eastern Australia; (e) Great Australia Bight, South-West Australia; (f) South Africa; (g) Sebastian Vizcaino Bay and Cedros Island, Mexico.

1.2.5 Feeding and trophic ecology

The constant increase of unsustainable industrial fishing activities, together with the lack of species - specific management plans and directives, have led to a drastic decline of several stock of marine predators (Myers and Worm, 2003, 2005; Hutchings and Baum, 2005). As apex predator, GWS cover the top of the marine food web (French t al., 2018), contributing to the balance of the marine ecosystem with a top-down control on lower levels. The depletion or decline of such predators result in different cascading effects along the trophic chain (Ferretti et al., 20110). The negative interactions resulted from the removal of predators lead to the increase of intermediate trophic level fish (Steneck, 2012), inducing what it is called the "mesopredators release" hypothesis (Ritchie and Johnson, 2009).

Great white shark exhibit size-based ontogenetic diet shift occurring within the first 5-6 years (Tricas and McCosker 1984; Klimley 1985; Estrada et al., 2006). As many large predatory sharks, GWS present a generalist feeding behaviour with different prey items (Hussey et al., 2012). Spacing within the functional prey categories and diversity, the feeding behaviour of GWS shows a high degree of plasticity, improving the ability of this species to face and respond to short and long-term shift in prey's distribution (Skomal et al., 2012). Different near-shore and off-shore predatory strategies are exhibit within the same population and among individuals (Kim et al., 2012). In

relation to the different availability of preferred prey, seasonal prey abundance, and energetic individual requirements, dietary specialisation (Estrada et al., 2006; Carlisle et al., 2012; Kim et al., 2012; Hamady et al., 2014; Christiansen et al., 2015), and sexual differences in movement patterns are observed (Pardini et al. 2001; Weng et al. 2007; Jorgensen et al. 2010; Bruce and Bradford 2012; Domeier and Nasby-Lucas 2012; Kock et al. 2013). Well documented records from pop-up archival satellite tags have recorded various foraging modes such as feeding in the surface mixed layer, on the seabed, and at different depths (Dewar et al., 2004; Weng et al., 2007; Nasby-Lucas, 2009). These behaviours are confirmed by the presence of several prey categories, from demersal to pelagic teleost, reported in the stomach content analysed by Tricas and McCosker (1984) and Klimley (1985). The shift to bigger prey is directly related to the increasing size of the individual, together with the source availability; Estrada et al. (2006) proposed the range size within the switch should occur, with a shift from yolk to fish in newborn between 91 cm – 153 cm, and to marine mammals in individual > 300 cm TL.

Typical prey include pelagic and demersal finfish such as yellowfin tuna *Thunnus albacares*, mackerel *Scomber scombrus*, swordfish *Xiphias gladius* (Casey and Pratt, 1985; Sambilay 1990; Bruce, 1992; Fergusson 1996; De Maddalena & Zuffa 2009), other chondrichthyans like shortfin mako *Isurus*

oxyrinchus, common thresher *Alopias vulpinus* (Cortés, 1999) and the blue sharks *Prionace glauca* (Casey and Pratt, 1985; Cliff et al., 1989; Fergusson 1996), sea turtles (Long, 1996; Fergusson et al., 2000), pinnipeds such as the California sea lion *Zalophus californianus*, Cape fur seal *Arctocephalus pusillus* and grey seal *Halichoerus grypus* (Ainley et al., 1981, 1985; Le Boeuf et al., 1982; Tricas and McCosker, 1984; Bruce, 1992; Klimley et al., 1992; Long et al., 1996; Williams, 1999; Gallon et al., 2007; Costa et al., 2010), cetaceans like the bottlenose dolphin *Tursiops truncatus*, long-beaked common dolphin *Delphinus capensis* and Bryde's whale *Balenoptera edeni* (Arnold, 1972; Cliff et al., 1989; Long, 1991; Bruce, 1992; Fergusson, 1996; Rohr et al., 2002; Fallows et al., 2013) and some invertebrates such as squids and crustaceans (Klimley, 1985; Compagno, 2001). Together with the diet shift, also an ontogenetic change in teeth morphology occurs: individuals < 300 cm TL show sharply, narrow pointed teeth with coarse or no serration well adapted to feed on fish, and individuals > 300 cm show basally broader, serrated teeth well adapted to cut mammal skin (Estrada et al, 2006). The transition to high caloric and nutritious preys, supported by constant changes in teeth morphology, is necessary to sustain the daily energy requirement to support growth, reproduction and migrations, thus reflecting in an increase in the trophic level position (Del Raye et al., 2013). In addition, few *in situ* observations have recorded GWS feeding on whale carcasses (Curtis et al., 2006; Dicken, 2008). Scavenging is a typical foraging behaviour of white

sharks and become essential during migrations events. Even if can be challenging to reach marine mammal's carcasses, it was demonstrated a preference for this type of feeding behaviour, rather than predation. Considered as rich-lipid systems, it was estimated that 30 kg of whale blubber can sustain the elevated metabolic needs of a 4.5 m GWS for about 1.5 months (Carey et al., 1982; Bernvi, 2016). Fallows et al. (2013) recorded an initial feeding preference for the whale caudal peduncle and fluke, before moving along the rest of the body's carcass. The consumption of these type of high caloric nutrients can be considered as a large portion of adult white shark's diet, while small preys always persist.

1.3 Stable Isotope Analysis (SIA)

During the last century, stable isotope analysis (SIA) has become a powerful tool to enhance the knowledge of diet, trophic position and spatial- pattern distribution of marine species at community and, more importantly, at individual level. SIA assume that the analysis of carbon (C) and nitrogen (N), and their stable isotope ratios, respectively $\delta^{13}C$ ($^{13}C/^{12}C$) and $\delta^{15}N$ ($^{15}N/^{14}N$), can be transferred in a predictable manner as they reflect prey's isotopic values in their predator's tissues (Peterson & Fry, 1987; Fry, 2008). The $\delta^{13}C$ values are used to identify animal-use habitat and migration patterns as they determine the benthic or pelagic, marine or terrestrial origin

of the organic matter assimilated. The $\delta^{15}N$ values are representative of the diet and foraging ecology of an organism, providing its trophic position, and more in general, the food-web structure (Fry, 2008; Hussey et al. 2012). These values usually increase by 0-1‰ for $\delta^{13}C$ and 3-4‰ for $\delta^{15}N$ (Peterson & Fry, 1987; Hobson & Welch, 1992; Estrada et al., 2003; Fry, 2008; Hussey et al., 2012).

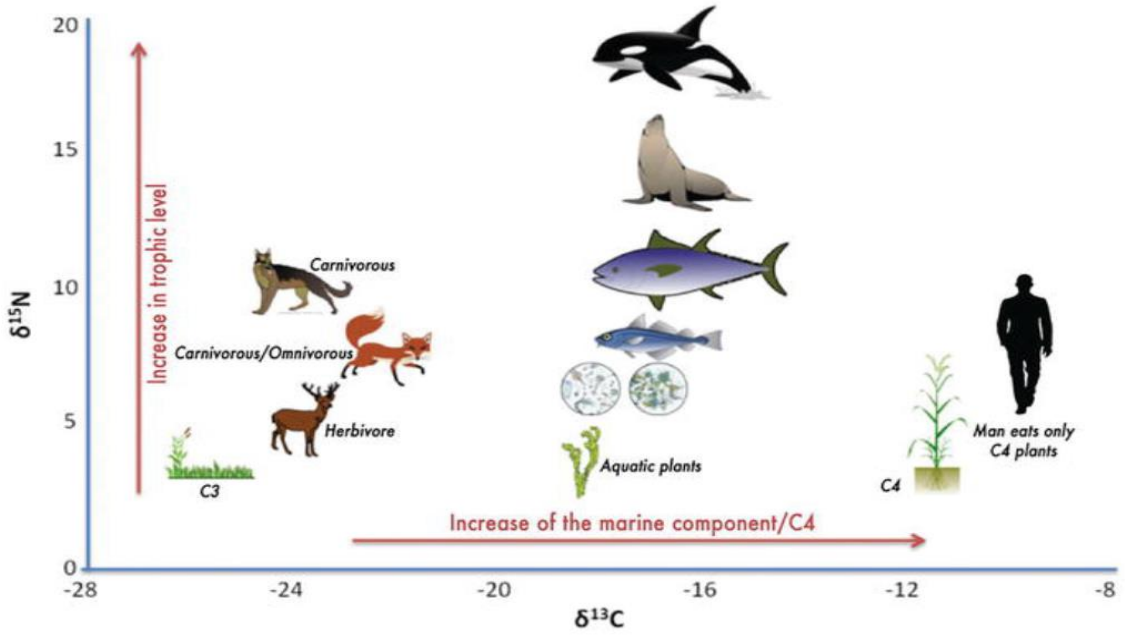


Fig. 1.7 Trophic chain's representation related to $\delta^{13}C$ and $\delta^{15}N$ variations in marine and terrestrial ecosystems.

For $\delta^{15}N$, increasing values are associated with the consumption of larger and high trophic level prey (Papastamatiou et al., 2010; Hussey et al., 2011), resulting in a shift in the isotopes values in consumers. Consequently, carnivores show a higher trophic position level than other organisms (**Fig.1.7**). In any case, every type of switch in diet or food preference, due to

seasonal migration of prey or competition caused by a niche overlap, will be detectable. For elasmobranchs soft tissues such as muscles, whole blood, blood cells and plasma from several individuals of different size, maturity, and sex are usually analyzed. These types of samples are obtained with minor invasive and non-lethal techniques as a biopsy punch, essential for the study of vulnerable and endangered species. Stable isotopes incorporation can be affected by a faster protein turnover in more metabolically active tissues like blood plasma and liver (MacAvoy et al., 2001 Carleton & Martínez del Rio, 2005; Bergamino et al., 2011). Other techniques include the analysis of stable isotope signals in vertebrae. These cartilaginous structures, mainly composed of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and collagen, exhibit a low turnover rate that allows to obtain more information on the variation of the trophic ecology of elasmobranchs during the entire duration of their life (Herzka & Holt, 2000). Differently from tissues, that provide short-term information relatable only to previous days/weeks before the catch, vertebrae analysis can provide an insight representation of feeding preferences over long time periods (Hussey et al., 2012). The slow accretion and deposition at discrete intervals of material along the vertebral centrum, allow to relate each deposition rings to a specific year (Campana et al., 2002; Estrada et al., 2006; Kerr et al., 2006). The SIA results will show different isotopic values based on prey preference and migrations to different foraging areas during a specific time's interval (Abrantes & Barnett, 2011; Dale et al., 2011; Hussey

et al., 2011a). Nevertheless, this type of sampling has several concerns (Hammerschlag & Sulikowski, 2011). In fact, it is important to consider that stable isotope values are in constant change as prey's values can vary temporally and spatially as the results of an increase in organic carbon and nutrient inputs within systems.

The stable isotopes values, resulted from the SIA, can be included inside Bayesian models such as SIMMR and SIBER. The mixing models produced, are actively used to analyse the temporal variability of the trophic niches within consumers and preys (Jackson et al., 2011).

2 Aim of the study

The objectives of this study are to investigate the trophic ecology and feeding preferences of the Great White Shark *Carcharodon carcharias* using Stable Isotopes Analysis on vertebrae. We focus primarily on:

- Age estimation of each individual, through the reading of bands in vertebrae.
- Differences in the trophic position of the species across a 35 years' period, by analysing the vertebrae of two different groups of individuals, the first one caught between 1987-1996 *vs*. the second one from 2005-2014, considering different TLs and sexes.
- Ontogenetic shift in the feeding ecology and possible relationship with TL and sex.
- Differences or similarities in the feeding ecology between each individual and its own mother, through the comparison of the signal of the birth mark *vs*. growth band.

3 Materials and methods

3.1 Study area

The Republic of South Africa is straddled between the Atlantic Ocean and the Indian Ocean, in the Southern Hemisphere. The encounter of two oceanic-water masses generates several climate events that can have negative or positive effects on ecosystems and species- patterns distribution. The coasts of South Africa are seasonally affected by two different water currents: during the austral summer (from December to February), the intensifying of the South easterly trade winds generate the Benguela Current, colder and rich in nutrients, that enters the bay of Cape West coast. In the austral winter (June to August), the Agulhas Current, warmer and poor in nutrients, flows offshore along the East Coast from North to South, pushed by the dominant Easterlies (**Fig. 3.1**).

The coast of South Africa offers multiple areas of upwelling (Lutjeharms 2006, Xavier et al. 2007), resulting in cold nutrient-rich water rising from the bottom to the ocean surface (Basterretxea and Aristegui, 2000). The increase in high primary productivity, growth, and biomass of several taxa (Menge, 2000) are the main drivers associated with the distribution of both predators and prey species (Field et al. 1998).

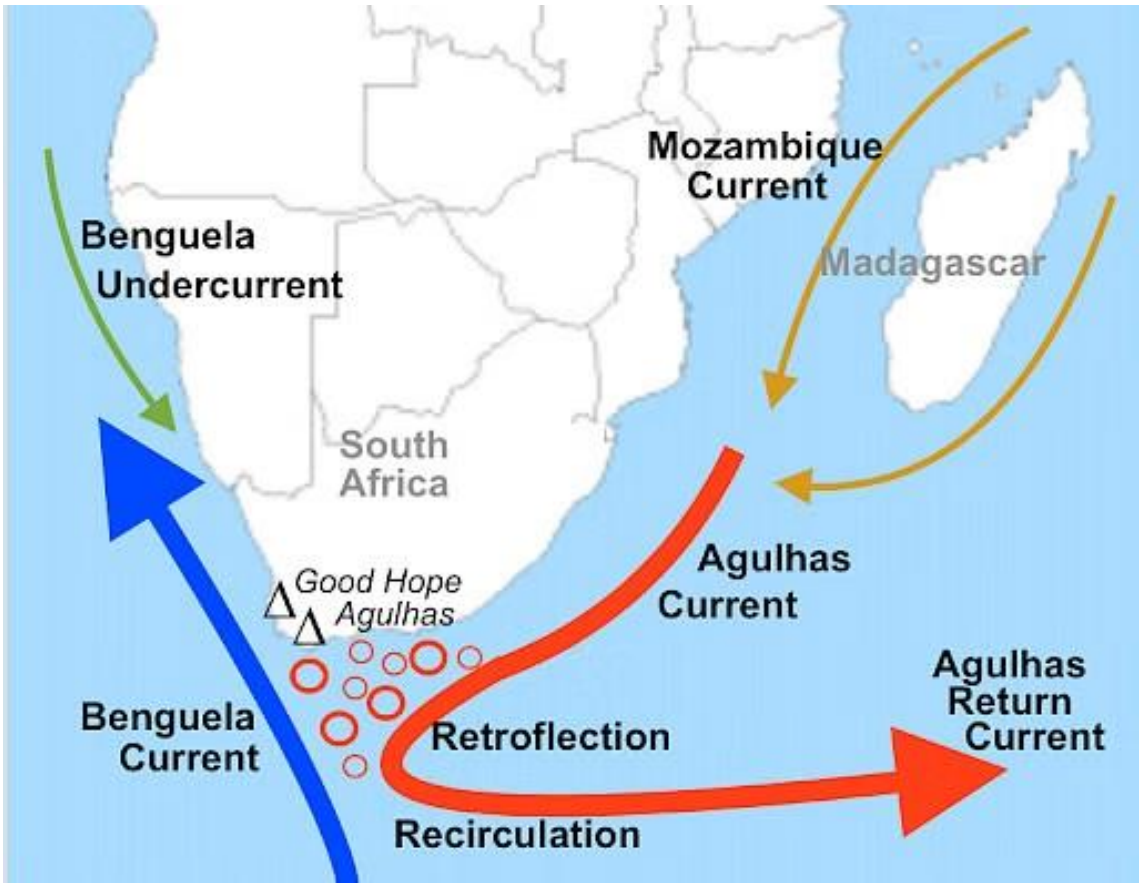


Fig. 3.1 South Africa's current: the Agulhas Current and the Benguela Current (from PREPP.in).

Bernvi (2016) showed that the 80% of the individuals caught from the shark control-program were juveniles or neonates, and only the 3% were adults. These results are linked to the warm areas of the South African Eastern Coast that show a higher abundance of juvenile white sharks, with over 96% being neonates or juveniles in Algoa Bay (Dicken et al. 2013; Dicken & Booth 2013). Sub-adults and adults are more abundant on the Southern Coast of the Western Cape, between Mossel Bay and False Bay, where nutrition rich upwelling areas and Cape fur seal colonies are present.

This study is focused on the KwaZulu - Natal province coasts, Western Indian Ocean (**Fig. 3.2**). South Africa has one of the most diverse shark faunas in the world: 15% of the world species, with 34 endemic species (27%) (Compagno, 2000). The elevated density of dangerous shark species in this area forced the government to introduce, in 1952, a shark-control program to reduce the incidence of shark attacks along Cape waters and KwaZulu-Natal coasts (Cliff et al., 1991). The bath-nets that were initially set on, generate an average catch of 1354 sharks per year, of which 39 were white sharks. All the individuals trapped, even if alive, were killed. In 1990, the Shark Research Centre of the South African Museum started a tagging program to fill the lack of information about habitat-use, behaviour, population structure and the status of the stock of sharks, including white sharks. In April 1991, the government granted full protection to *C. carcharias*, considering as illegal any type of activity that could result in their catch or kill (Compagno, 1991). In the 1999, the council of the United Nations Food and Agriculture Organization (FAO) promoted the International Plan of Action for Conservation and Management of sharks (IPOA-Sharks), suggesting that all the member parties should voluntarily develop a National Plan of Action for the Conservation and Management

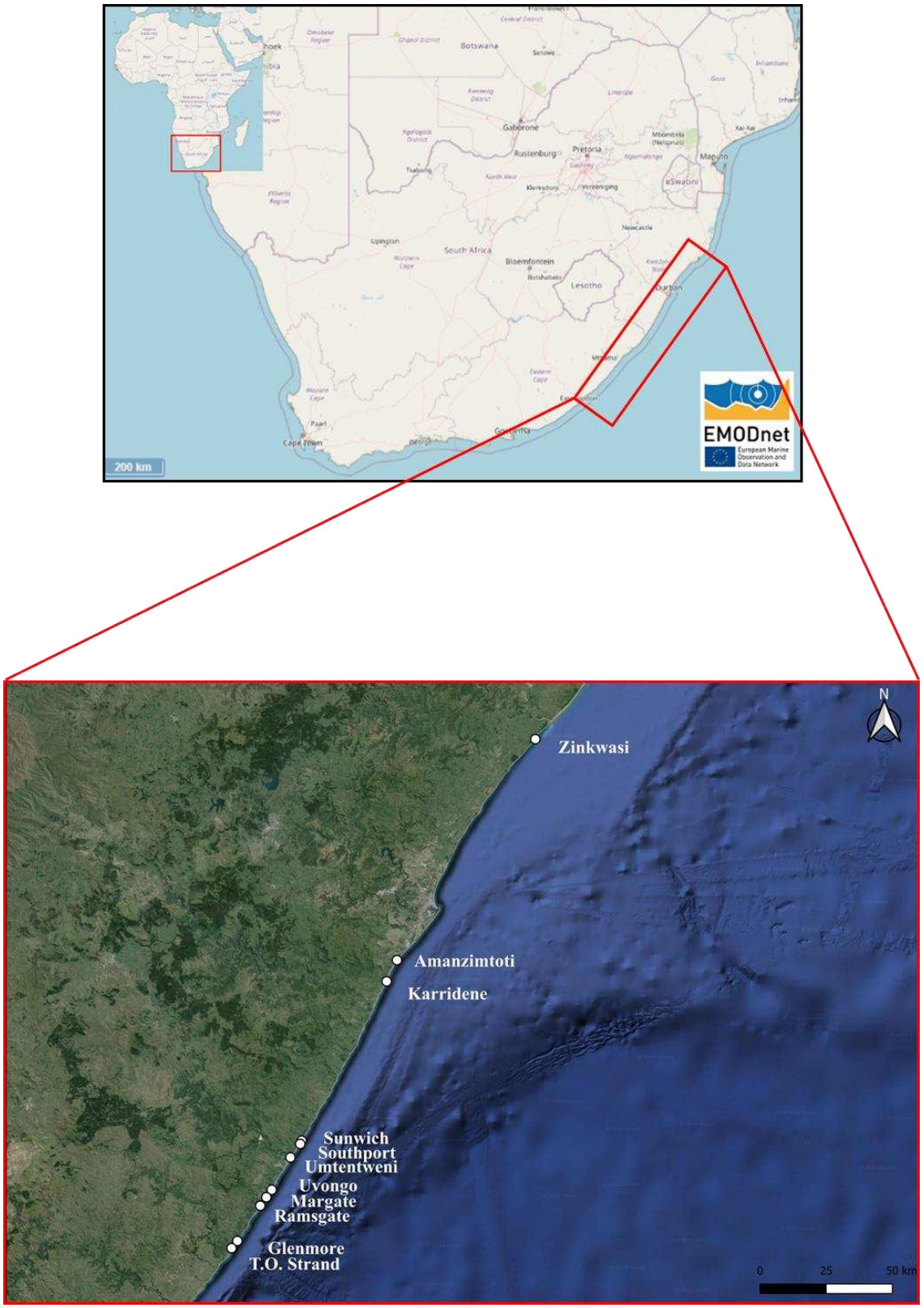


Fig. 3.2 KwaZulu Natal province, South Africa. In the red rectangular: the study area and some of the stranding sites.

of Sharks (NPOA-Sharks) based on the principles of the Code of Conduct for Responsible Fisheries. The South African NPOA-Sharks focus on the assessment of human-related threats on shark populations and their essential habitats, reducing by-catch and improving protection on vulnerable and threatened/endangered stocks. Between 1999 and 2004, the government decide to reduce the number of bath-nets administered by the KZNSB because of the high rate of by-catch of large sharks, such as the great white shark *C. carcharias*, tiger shark *Galeocerdo cuvier* (Peron & Lesueur, 1822), bull shark *Carcharinus leucas* (Muller & Henle, 1839) (Cliff et al. 1988, Cliff et al. 1996, Cliff & Dudley 2011; NPOA-Sharks, 2013). In addition, between 2005 and 2007, several experimental drumlines were introduced (NPOA-Sharks, 2013).

Nowadays, beach protective gillnets set from the KwaZulu-Natal Shark Board extend from Richards Bay to Port Edward. Nets are positioned 300-500 m from the shore, in waters between 10-14 m depth, and parallel to the coast. Beach nets consist of 214-304 m long and 6.3-7.3 m deep yellow polyethylene braided gill nets with a stretched mesh size of 51 cm. Nets are inspected every morning; trapped sharks found alive are tagged, when possible, and released. Experimental drumlines consist of a baited hook linked to a chain and connected to a floating device anchored adjacent to a beach protective net (Cliff et al. 1988, Cliff & Dudley 2011).

3.2 Vertebrae analysis

3.2.1 *Samples' collection*

The vertebrae samples were obtained from stranded white sharks along the KwaZulu-Natal coast, South Africa Eastern Cape, between the 1987 and 2014; no individual was sacrificed for this study. Vertebral centra were collected from the researchers of the KwaZulu-Natal Shark Board (KZNSB)-Marine Centre of Excellence, with the aim of future monitoring investigations and scientific research. Few data were collected *in situ* from the researchers: capture date (DD/MM/YY), stranding zone, fork length (FL) and sex. The fork length was measured as a strict line from the top of the snout to the bifurcation of the caudal fin. Every individual was tagged with a numerical code and the tag of the beach where it was stranded. For each individual, at least 3 vertebrae were collected from the dorsal-anterior region of the first dorsal fin. The samples were stored in a refrigerator at a temperature of -4°C. Authorization for using the vertebra for scientific purposes was granted by the KwaZulu-Natal Shark Board and South-African authorities, and the samples were regularly exported from South Africa and imported in Italy in strict accordance with the CITES requirements. The laboratory of marine ecology at Polytechnic University of Marche (UNIVPM), Ancona, required a specific CITES licence to import and work

with these samples. Samples were preserved inside a refrigerator box through all the shipping, in order to maintain them frozen.



Fig. 3.3 General morphology of a single vertebra of *Carcharodon carcharias*, from the specimen listed as SP14004, a female with a FL of 2.15 m stranded in September 2014.

In the laboratory, every vertebra was checked and divided per year of capture. The total of vertebrae received was 104; of these, only 36 (**Fig. 3.3**) were chosen for our analysis. Samples were divided in two blocks: the first block, mainly constituted by individuals collected between 1987 and 1996 (hereafter referred to as Group 1 or G1), and a second block represented by individuals collected between 2005 and 2014 (G2). To maintain the maximum intraspecific variability, each sample was chosen based on its FL size (in cm) and sex. Samples were stored frozen until the cleaning. Lots of

connective tissue was still attached to the cartilaginous bones. Using a scalpel and forceps, each vertebra was hand-cleaned under a biological laminar hood and dried in a stove at a temperature of 60°C for 72 hours to eliminate any residual tissue. Each vertebra was photographed and measured twice; firstly, when frozen and secondly, when dried.



Fig. 3.4 Frozen vertebra with evident small septa and one big septum.

We measured the diameter of every single vertebra and the number, diameter and the height for both the smaller and bigger septa (**Fig 3.4**). No substantial differences were recorded between frozen and dried samples.

3.2.2 *Cross sectioning*

Once dried, the vertebrae were cross-sectioned at the SIMAU department (Science and Engineer of Matter, Environment and Urbanistic) of UNIVPM. The cross-sectioning was necessary to carry on the analysis. Three new aluminium supports (**Fig. 3.5**) were built due to the different diameters of the cartilaginous vertebra: support 1 was 4 cm x 2.2 cm + 4 cm x 0.9 cm, support 2 was 5 cm x 3 cm + 5 cm x 1.2 cm, support 3 was 9.4 cm x 4.1 + 9.4 cm x 0.6 cm. The supports were made of two rectangular blocks of steel with two screws that allowed the regulation of the width, both on the vertical and horizontal axes. On the bottom of the support 3, a thin layer of wood was added to avoid a possible break of the vertebra due to the strength of the grip. Vertebrae were cross sectioned along the central longitudinal plan to obtain a thick section. Based on the diameter of the vertebrae, two different low-speed diamond saws were used: 1- Micracut151 (Metkon) saw with speed blade of 260 RPM, 2- Isomete (Buehler) saw with speed blade of 200 RPM (**Fig. 3.6**).



Fig. 3.5 The three new supports built for vertebrae of different diameters.

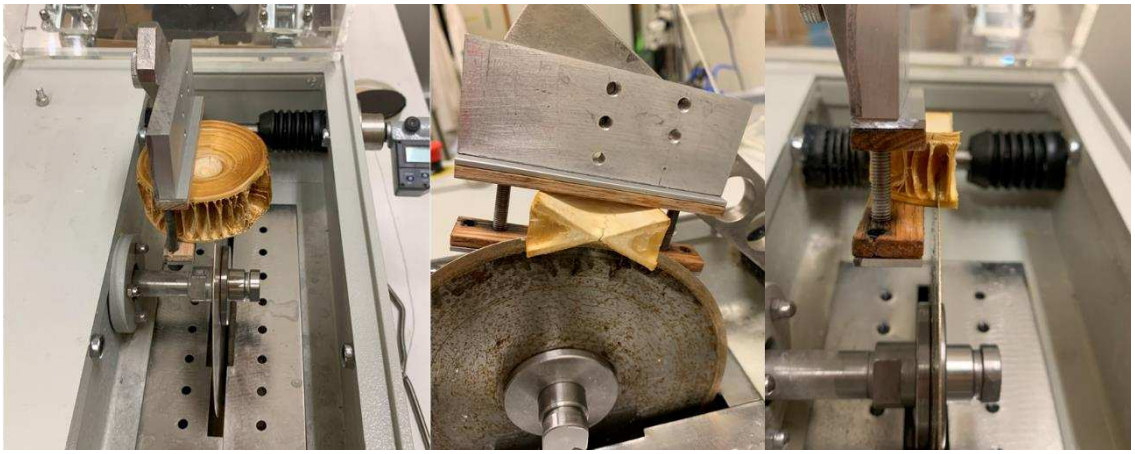


Fig. 3.6 Cross-sectioning with low- speed diamond saw.

3.2.3 *Total length back-calculation*

The total length (TL) values were obtained firstly from a back-calculation using data from the Natal Shark Board in Cliff et al. (1989), as follows:

TL = (FL +6.8357)/0.9517 (Casey and Pratt 1985)

Fork length (FL) is also defined as the 92% of the TL body (Kohler et al., 1996). PCL was back calculated from Cliff et al. (1989):

TL = (1.173 PCL – 4.9533)/0.9517 (Casey & Pratt, 1985)

Once obtained the back-calculated total lengths, the Length Frequency Distribution (LFD) was calculated with the software Microsoft Excel. The individuals were categorized in 5 length classes between 150 cm and 450 cm, based on the total lengths obtained. Each length class has a width of 50 cm.

3.2.4 *Age determination with growth-band counts and back calculation*

To assess age estimation for each sample considered in this study, it was necessary to identify the birth band, or birth mark (hereafter BM). This consists in the first opaque band observed after the focus and representing the first deposition about or soon after birth (Finucci & Maolagáin, 2021). This point is considered as the year zero. From this point, we start the reading of growth band (GB) along the vertebra's corpus calcareum. Each growth band is considered one year. Each vertebra was read by 3 readers using a Zeiss Microscope and the age was estimated as the mean of the three reads.

Three types of readings were made: 1- band count on a cross-section (**Fig. 3.7**), 2- count of circular band on the half-sectioned vertebra, 3- direct count on band pair without the microscope to test if there could be some differences in the readings linked to a wrong reflection of the artificial light on translucent bands. We noticed that microscope's artificial light can alter the colour, producing a light band coloration that are more difficult to recognize. Every cross-sectioned and half-sectioned vertebra was photographed for future readings.



Fig. 3.7 General view of a half cross-sectioned vertebra (on top) and the corresponding BM and GB.

An age estimation was obtained with the Von Bertalanffy Growth Function (VBGF) using the parameters from Wintner and Cliff (1999):

$$L_t = L_\infty [1-e^{-K(t-t0)}]$$

with

$$t = -1/K*ln(1-L_t/L_\infty) + t_0$$

where L_t = length at time t

L_∞ = maximum theoretical length

k = the rate at which L_∞ is reached

t_o = the theoretical number of growth rings at length zero

The function "cor.test" was run in the software R studio to perform a correlation test between the back-calculated TL and the two ages assessed with the two different determination methods (i.e. back-calculated years with VBGF *vs* TL and age determination with growth band counts *vs* TL). This was done considering both the overall data and the data divided by year of sampling (G1 and G2).

A t-student test was carried out on between the age values obtained by the two different age determination methods. The test was performed in order to verify if the visual counts on the vertebra's annuli made from the three readers in the laboratory were comparable or not with the VBGF back-calculated age.

3.2.5 *Stable Isotopes Analysis*

Stable isotopes analysis (SIA) was conducted to determine C and N isotopic ratios to make inferences on the ontogenetic shift in the diets of the

considered specimens, and to determine trophic habits of their mothers. First, it was necessary to attach the vertebrae on a hard surface. Each cross-section was mounted on a glass slide using thermoplastic adhesive (Crystalbond) at the IRBIM-CNR (Institute for Biological Research and Marine Biotechnologies- National Council of Research) of Ancona. After age determination, each vertebra was drilled in at least 2 different points using the MicroMill2: the birthmark (BM), to obtain information on the mother's diet and migration patterns, and the last band growth (GB), to study the individual's diet and assess the trophic position. Following Kim & Koch (2012), at least 3 mg of powder was drilled from each point and stored in a plastic Eppendorf. SIA requires that all the inorganic compounds must be eliminated before analysing the samples, thus a demineralization treatment is necessary. This was made with EDTA (0.5 M, pH=8), an organic chelating agent that binds to Ca^{2+} ions, and yielded more collagen than the common treatment with HCl (Tuross et al., 1998).

In each plastic vial 3 mg of powdered vertebra was weighed, and 1.5 ml EDTA (0.5M, pH=8) were added: the final solution was vortexed for 1 minute. After 1 week at room temperature, samples were centrifuged for 15 min, and EDTA was replaced, vortexed for 1 minute and left to react for another week. Once the samples were de-mineralized, we centrifuged them for 15 minutes, EDTA was removed with a pipet, and samples were rinsed

10 times with deionized water (DIW). A centrifugation of 1 minute was added between each rinse. The 1st, 5th, and 10th rinses received 10 min of sonication, and the 5th and 10th rinses soaked overnight. Centrifuge speed parameters were 8000 – 12000 rpm.

Once dried, every sample was newly weighted to determine the weight loss due to the EDTA treatment. For SIA, between 0.7 mg and 1.1 mg of powdered vertebra was weighted and collected into a tin capsule; in some cases, a lower quantity was used because of an excessive powder loss due to the EDTA treatment. The minimum relievable weight by the instrument used for the stable isotope determination is 0.4 mg. In parallel, for each treated sample, 1 mg of a non-treated sample was stored and conserved. All the weighted samples for SIA were collected in a rack and stored at -20 °C. Then, samples were analyzed through an elemental analyser (Thermo Flash EA 1112) for the determination of total carbon and nitrogen, and then analyzed for $\delta^{13}C$ and $\delta^{15}N$ in a continuous-flow isotope-ratio mass spectrometer (Thermo Delta Plus XP) at the Laboratory of Stable Isotopes Ecology of the University of Palermo (Italy). Stable isotope ratio was expressed, in relation to international standards (atmospheric N_2 and PeeDee Belemnite for $\delta^{15}N$ and $\delta^{13}C$, respectively), as:

$\delta^{13}C$ or $\delta^{15}N$: $R_{sample}/R_{standard} - 1 \times 10^3$

where R = $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Analytical precision based on standard deviations of internal standards (International Atomic Energy Agency IAEA-CH-6; IAEA-NO-3; IAEA-N-2) ranged from 0.10 to 0.19‰ for $\delta^{13}C$ and 0.02 to 0.08‰ for $\delta^{15}N$.

A t-student test was carried out on the $\delta^{13}C$ values obtained in the samples demineralized with EDTA and in untreated samples, to verify the efficacy of the applied demineralization's protocol in our specific case.

A correlation test was performed in the software R studio using the function "core.test" to investigate the correlation between the TL and the values of $\delta^{15}N$ and $\delta^{13}C$. The test considers both the overall data and G1 and G2 as separate temporal group.

3.3 Statistical analysis

The software PRIMER7 & PERMANOVA+ was used to perform univariate and multivariate statistical analyses on isotopic signals.

A two-way crossed design with two fixed factors was used to investigate differences among variables. The first factor was the "time group", presenting two levels (G1 and G2), and the "sex" with two levels (M and F). Furthermore, a two-way crossed design with the factors "year" and "size" presenting two levels (TL< 250 cm as small individuals and TL> 250 cm as medium individuals) was used to identify possible existing ontogenetic differences in the isotopic contents of the samples obtained by drilling the external part of each vertebra.

In all PERMANOVA main tests, significance was set at $p< 0.05$. Due to the lower number of samples, 9999 permutations of residuals under reduced model and the Monte-Carlo asymptotic distribution were used to obtain the p-values.

Univariate PERMANOVA (Anderson et al., 2008) main tests were performed on the Euclidean distance matrices of untransformed values of $\delta^{15}N$ and $\delta^{13}C$ of each vertebra, separately, and multivariate PERMANOVA main test were conducted on the Euclidean distance matrices of untransformed values of $\delta^{15}N$ and $\delta^{13}C$ taken together; significant results were tested with pairwise comparisons.

3.3.1 Isotopic niches

The SIBER package (Stable Isotopes Bayesian Ellipses in R) (Jackson et al., 2011) was run in the software 4.0.5 (R Development Core Team 2009). The model was carried out several times, considering different communities and groups within these communities (**Tab. 3.1**), in order to compare isotopic niches width.

	Temporal group GB	Sex-Temporal group BM	Sex-Temporal group GB	TL-Temporal group GB
Group	GB	G1 and G2	G1 and G2	G1 and G2
Community	G1 and G2	BM	GB	GBS and GBM

Tab. 3.1 Groups and communities used to model the trophic niches. BM= birth mark values; GB= growth band values; G1 and G2= temporal group; GBS= growth band values of small individuals; GBM= growth band values of medium individuals.

Subsequently, the model was used to calculate the Layman metrics for each of the community considered. The total area of the convex hull (TA), the corrected Standard Ellipse Areas (SEA_C), the mean distance to centroid (CD), and the mean nearest-neighbour distance (MNND). The TA gives indications on food sources variety on which the species can feed on, while SEA_C (which contains 40% of the data within a set of bivariate data) represents the core area for a population or community; CD is considered as a function of the degree of group spacing, and estimates the trophic diversity

within a food web; the MNND value provides a measure of density and clustering of species within the community.

Hussey et al. (2010) studies on δ^{15}N-δ^{13}C diet-discrimination factors on large sharks' soft tissues reported a difference of about -3‰ between values of δ^{13}C of vertebral cartilage and of muscle tissue of the same specimens. Considering this assumption, we corrected our vertebral cartilage values of δ^{13}C to estimate muscle tissue values. These values were considered for running mixing models. The Bayesian model SIMMR (Stable Isotope Mixing Models in R) (Parnell, 2021) was run in R software version 4.0.5 (R Development Core Team 2009), using the empirically derived trophic enrichment factors (TEF) reported for large sharks in de Necker (2017) ($\Delta\delta^{13}$C = - 0.9± 0.33 ‰; $\Delta\delta^{15}$N = - 2.9 ± 0.22 ‰) and muscle prey's δ^{15}N and δ^{13}C values obtained from literature (**Tab. 3.2**), in order to estimate the contribution of each trophic source to the diet of *C. carcharias.* As an upgrade to the SIAR (Stable Isotope Analysis in R) package, SIMMR allows the analysis of the high variability of stable isotopes for different prey species through complex mixing models using Markov-chain Monte Carlo modelling. These models collect data on consumers values and fit a Bayesian model of diet preference on a Gaussian function with a Dirichlet prior mixture distribution for the mean. According to Fanelli et al. (2015), each targeted value derived from a Gaussian distribution with an unknown mean

and standard deviation. Means' values are represented by a combination of the different isotopic values of preferred preys.

Before running SIMMR, the isotopic values of the potential food sources (**Tab. 3.2**) and the estimated muscles' isotopic contents of considered white sharks were plotted together, applying the correct TEFs to determine the best mixing polygon (Smith et al., 2013; Phillips et al., 2014).

Sources		$\delta^{13}C$ ‰	SD	$\delta^{15}N$ ‰	SD	Mean $\delta^{13}C$ ‰	SD	Mean $\delta^{15}N$ ‰	SD	Authors
Beloniformes	*Hyporhampus capensis*	-22.32	0.31	14.1	0.28					Bergamino et al., 2014
Whales	*Eubalena australis*	-20.8	1.4	8.4	2.1	-23.2	3.39	7.7	0.98	Valenzuela et al., 2018
	Megaptera novaengliae	-25.6	0.1	7	0.1					Montanari et al., 2019
Clupeiformes	*Engraulis encrasicolus*	-17.41	0.45	10.83	0.43	-17.56	0.21	10.57	0.36	Horton and van der Lingen, 2019
	Stolephorus holodon	-17.72	0.21	10.32	0.23					
Seal+Teleosts+Sharks	*Poroderma africanum*	-14.9	0.33	15.5	0.52	-15.2	0.48	15.07	1.24	Caracausi et al., 2024
	Seal	-14.95	0.33	16.93	0.65					de Necker, 2017
	Demersal chondrichthyans	-14.69	0.25	14.64	0.48					
	Inshore sharks	-14.85	0.38	15.69	0.41					
	Inshore teleosts	-14.96	0.37	14.9	0.32					
	Offshore sharks	-15.82	0.01	15.65	0.15					
	Large offshore teleosts	-15.98	0.21	14.73	0.39					
	Small offshore teleosts	-15.5	0.26	12.58	0.44					

Tab. 3.2 $\delta^{13}C$-$\delta^{15}N$ values of the potential preys of *C. carcharias* obtained from the literature.

4. Results

In the present study, 36 individuals of *C. carcharias* were considered, respectively 20 females and 16 males. Specimens were divided in two temporal group: G1 (those sampled between 1987 and 1994) comprise 19 individuals, 11 females and 8 males, G2 (those sampled between 2005 and 2014) comprise 17 individuals, 9 females and 8 males (**Fig. 4.1**).

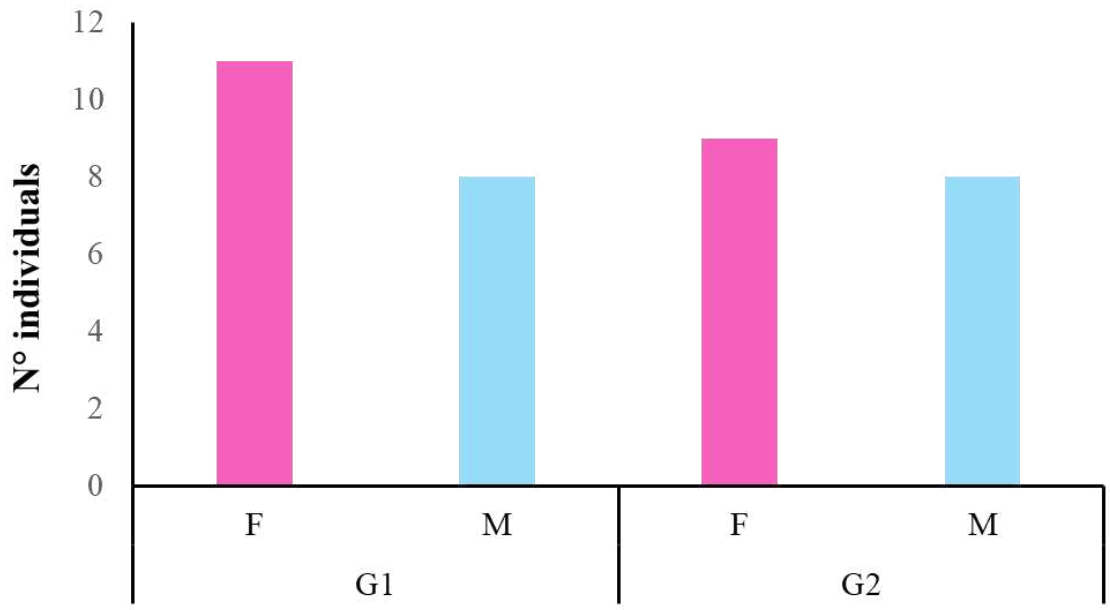


Fig. 4.1 Number of individuals of *C. carcharias* for both sex in G1 and G2. Number of individuals= 36. F=females; M= males.

4.1 Back calculated lengths and age validation

In **Tab. 4.1** the back-calculated lengths for each individual are reported. The table shows also the corresponding sex of each individual.

ID SAMPLE	FL (cm)	PCL (cm)	TL (cm)	SEX
AMA14001	217	195.05	235.20	F
BRI14012	219	196.75	237.30	M
BRI95013	175	159.24	191.06	M
GLN05009	136	125.99	150.08	M
KAR94022	205	184.82	222.59	F
M.G14001	180	163.50	196.32	M
M.G14016	167	152.42	182.66	M
M.G14018	189	171.18	205.77	F
M.G96041	137	126.84	151.14	M
MAR94002	146	134.52	160.59	F
MBG94023	235	210.39	254.11	M
P.E14010	272	241.93	292.99	M
R.B09035	200	180.55	217.33	M
R.B13039	240	214.65	259.36	F
R.B14054	175	159.24	191.06	F
R.B88008	214	192.49	232.04	F
R.B94018	280	248.75	301.39	F
R.B96080	173	157.54	188.96	F
RAM94010	137	126.84	151.14	F
S.P14004	215	193.34	233.09	F
SAN07005	358	315.25	383.35	M
SOB95012	196	177.14	213.13	M
ST.94011	270	240.23	290.89	M
SUN14004	195	176.29	212.08	F
T.O87013	215	193.34	233.09	M
T.O94010	224	201.01	242.55	F
TRA94012	229	205.28	247.80	M
UMT06006	189	171.18	205.77	M
UMT89019	197	178.00	214.18	F
UMT94009	260	231.70	280.38	M
UMT94011	160	146.45	175.30	F
UMT94015	229	205.28	247.80	F
UVO09004	142	131.11	156.39	F
ZIN09016	370	325.48	395.96	F
ZIN14026	270	240.23	290.89	F
ZIN94010	265	235.97	285.63	F

Tab. 4.1 Back-calculated lengths for each individual. Number of individuals =36. FL= Fork Length; PCL= Pre-Caudal Length; TL= Total Length.

4.2 Length Frequency Distribution (LFD)

Considering all the specimens, the Length-Frequency Distribution (LFD), shows a non-homogeneous trend along the 5 different length classes between 150 cm and 400 cm.

The LFD for the two temporal group G1 and G2 shows a similar frequency distribution (**Fig. 4.2**); the 41.67% of the total individuals collected are comprised within the length class 200-249 cm. There are no individuals present in the length class 300-349 cm for G2: same result for the length class 350-399 cm for G1.

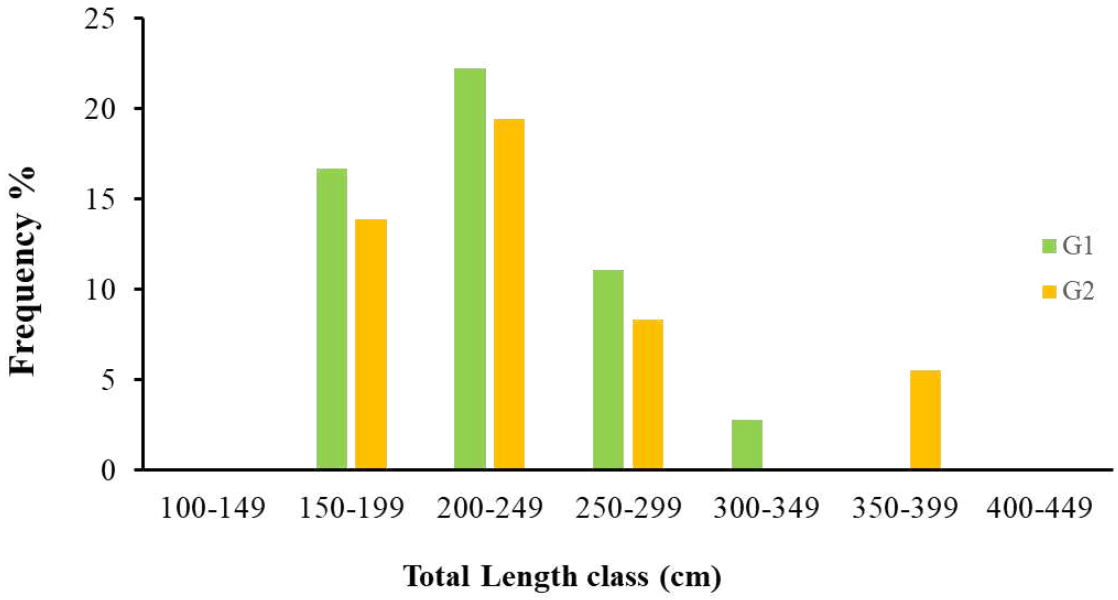


Fig. 4.2 Length Frequency Distribution (LFD) of *C. carcharias* for G1 and G2, Number of individuals = 36.

There are no individuals under the 150 cm TL, neither over 400 cm TL. The LFD by sex shows a strong predominance of females (55.55 %) in all the 5 length classes, with a peak within the length class 200-249 cm (**Fig. 4.3**). There are no males present in the length class 300-349 cm.

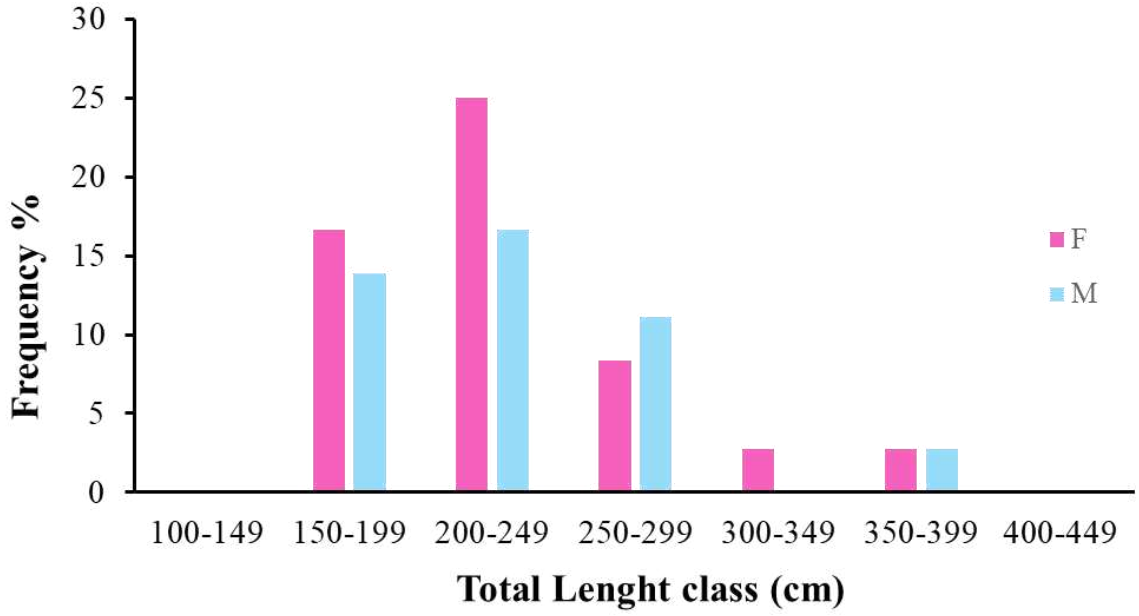


Fig. 4.3 Length Frequency Distribution (LFD) of *C. carcharias* for both sex, Number of individuals = 36.

The peak of frequency, highlighted in the length class 200-249 cm, was considered as the threshold to categorize the specimens in individuals with a TL < 250 cm and a TL> 250 cm TL, respectively indicated as Small and Medium animals.

4.3 Age determination

In **Tab. 4.2** the back-calculated years with the VBGF and the vertebra annuli's counts are reported.

ID SAMPLE	AGE (years)	AGE (vertebra's annuli)	SEX
AMA14001	4	4	F
BRI14012	4	4	M
BRI95013	2	3	M
GLN05009	1	1	M
KAR94022	4	2	F
M.G14001	2	3	M
M.G14016	2	1	M
M.G14018	3	2	F
M.G96041	1	2	M
MAR94002	1	1	F
MBG94023	5	3	M
P.E14010	7	4	M
R.B09035	3	3	M
R.B13039	6	3	F
R.B14054	2	2	F
R.B88008	4	3	F
R.B94018	8	5	F
R.B96080	2	2	F
RAM94010	1	1	F
SAN07005	14	7	M
SOB95012	3	3	M
SP14004	4	5	F
ST.94011	7	5	M
SUN14004	3	2	F
T.O87013	4	2	M
T.O94010	5	3	F
TRA94012	5	3	M
UMT06006	3	3	M
UMT89019	3	2	F
UMT94009	7	3	M
UMT94011	2	1	F
UMT94015	5	4	F
UVO09004	1	1	F
ZIN09016	16	9	F
ZIN14026	7	5	F
ZIN94010	7	4	F

Tab. 4.2 Age estimation from back-calculated VBGF and vertebra annuli's count.

The correlation between the means of the vertebra's annuli reads and the TL resulted to be significant both considering the overall data and considering G1 and G2 separately ($p < 0.001$) (**Fig. 4.4**).

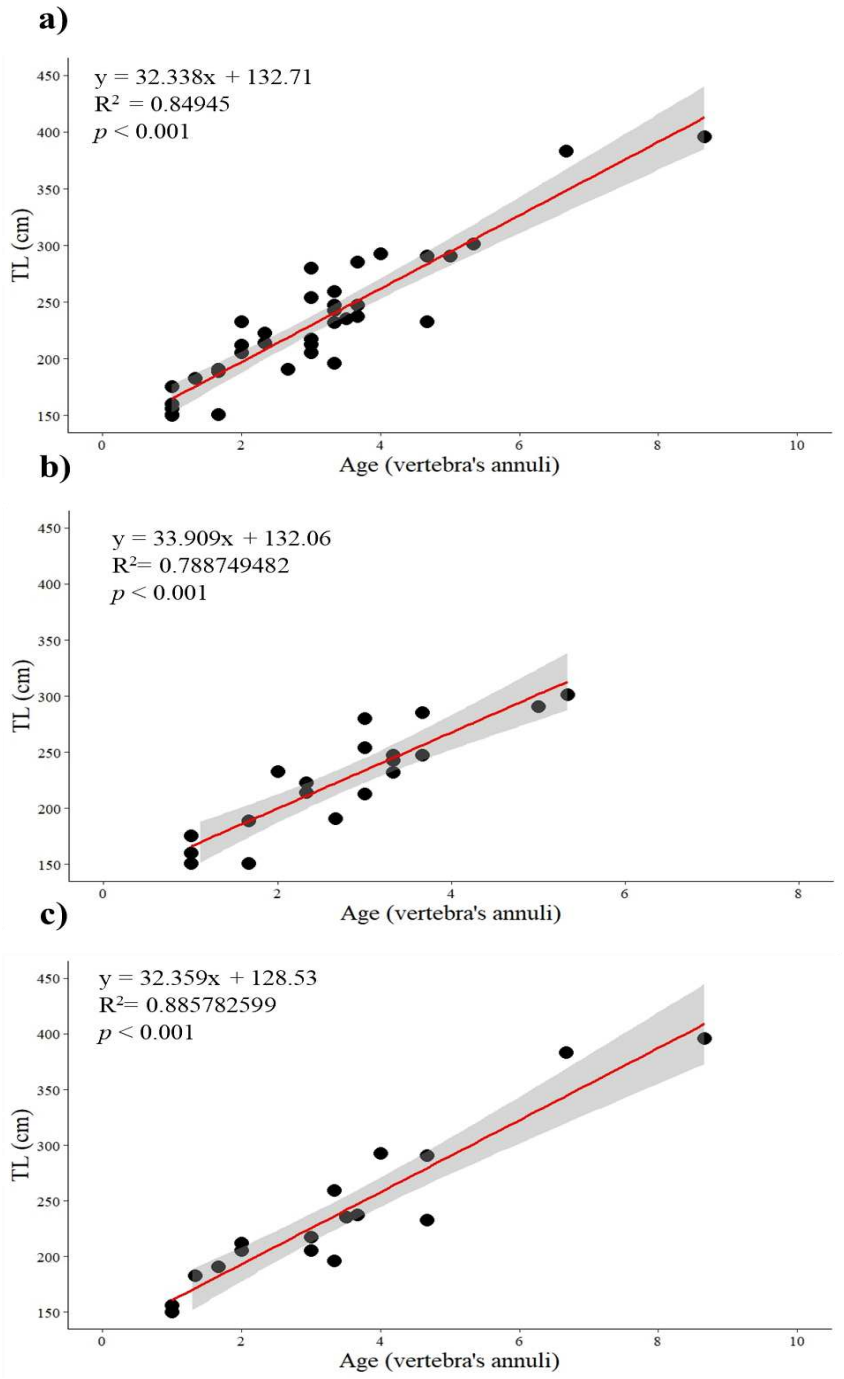


Fig. 4.4 Correlation of TL and the means of vertebra's annuli reads in a) the overall specimens, b) specimens belonging to G1 and c) specimens belonging to G2.

The values of back-calculated years were significantly correlated with TL, both considering the overall data and G1 and G2 separately ($p < 0.001$) (**Fig. 4.5**)

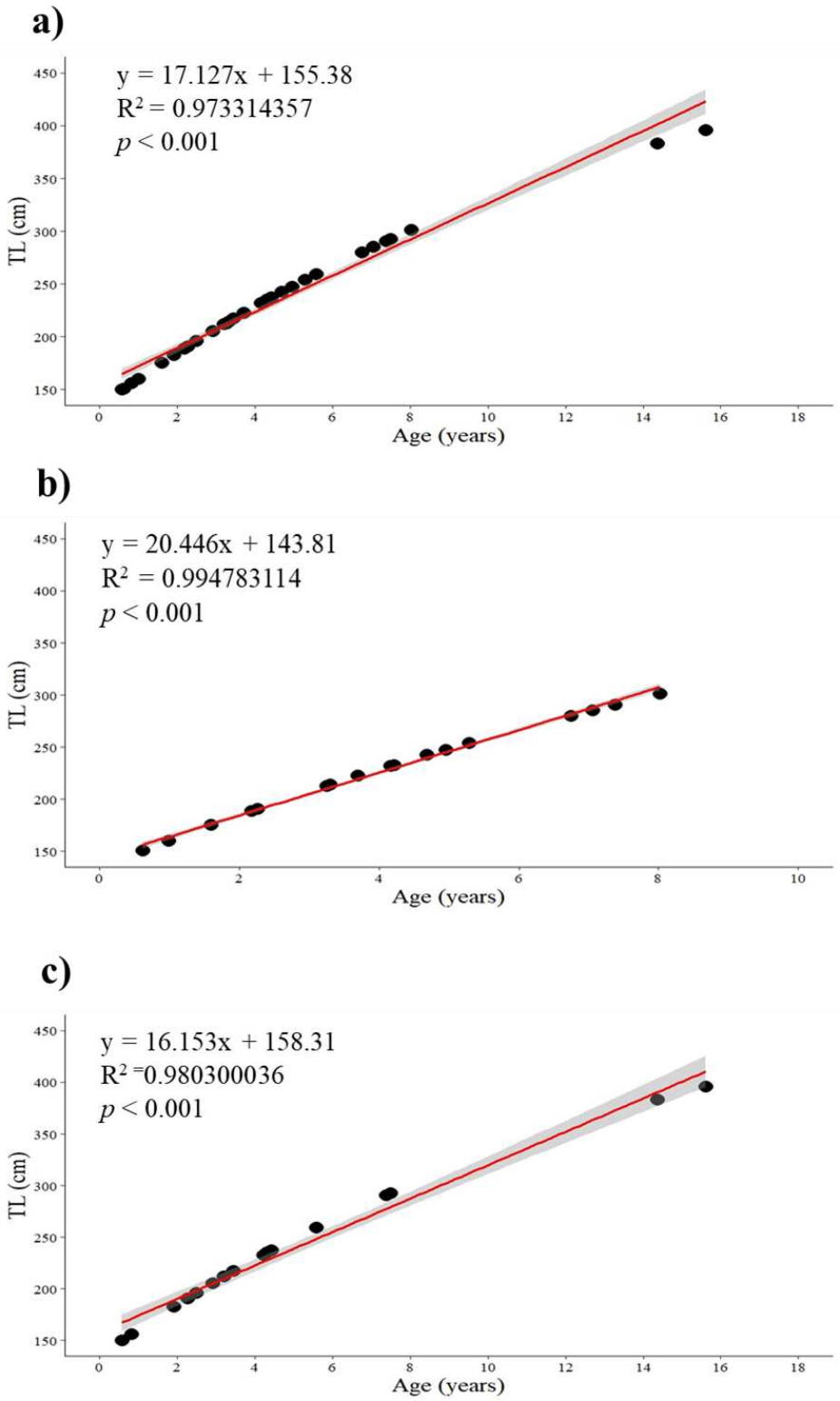


Fig. 4.5 Correlation of TL and back-calculated years with the VBGF on a) the overall specimens, b) specimens belonging to G1 and c) specimens belonging to G2.

The Student t-test carried out to compare the age values estimated with the two age estimation methods provided significant differences ($p<0.001$).

4.4 Stable isotopes analysis

4.4.1 $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures

The signatures obtained at the two drill positions BM and GB were also analysed within the two temporal group (G1 and G2).

Considering G1, the $\delta^{15}N$ mean ± standard deviation (SD) values were 14.4 ± 1.3 ‰ and 15.2 ± 1.2 ‰ for the BM and GB respectively, while $\delta^{13}C$ average values were -14.2 ± 1.4 ‰ and -13.5 ± 1.8, respectively BM and GB (**Fig. 4.6.; Tab. 4.3**).

Both $\delta^{15}N$ and $\delta^{13}C$ mean values for G2 were lower than those for G1, being $\delta^{15}N$ mean values 13.8 ± 1.4 ‰ and 14.9 ± 1.5 ‰ for BM and GB, respectively and $\delta^{13}C$ average values -15.2 ± 2.0 ‰ and -14.3 ± 1.7 ‰ for BM and GB, respectively (**Fig. 4.6.; Tab. 4.3**.).

A same trend was observed when considering the sex. The mean values of $\delta^{15}N$ and $\delta^{13}C$ recorded for G1 were 15.3 ± 1.5 ‰ and -14.0 ± 1.9 ‰ for females, while 15.1 ± 0.8 ‰ and -12.9 ± 1.6 ‰ for males (**Fig. 4.7; Tab. 4.3**). $\delta^{15}N$ and $\delta^{13}C$ mean values reported for G2 were generally lower, being

14.9 ± 1.3 ‰ and – 14.7 ± 1.2 ‰ for females, and 14.9± 1.9 ‰ and -13.7 ± 2.0 ‰ for males (**Fig. 4.7; Tab. 4.3**).

Tab. 4.3 Mean ± standard deviation of δ^{15}N and δ^{13}C of *C. carcharias.*

		δ^{15}N		δ^{13}C		δ^{15}N		δ^{13}C	
		BM		BM		GB		GB	
		MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
G1	F	14.3	1.4	-14.4	1.5	15.3	1.5	-14.0	1.9
	M	14.6	1.1	-13.9	1.3	15.1	0.8	-12.9	1.6
	< 250 cm	14.3	1.2	-14.2	1.6	15.1	1.0	-13.3	2.0
	> 250 cm	14.8	1.5	-14.5	1.1	15.4	1.7	-13.8	1.3
	ALL	14.4	1.3	-14.2	1.4	15.2	1.2	-13.5	1.8
G2	F	13.7	1.6	-15.8	1.8	14.9	1.3	-14.7	1.2
	M	13.9	1.4	-14.7	2.2	14.9	1.9	-13.7	2.0
	< 250 cm	13.5	1.4	-15.7	1.9	14.4	1.6	-14.5	1.6
	> 250 cm	14.6	1.2	-14.2	2.1	16.0	0.4	-13.7	1.8
	ALL	13.8	1.4	-15.2	2.0	14.9	1.5	-14.3	1.7

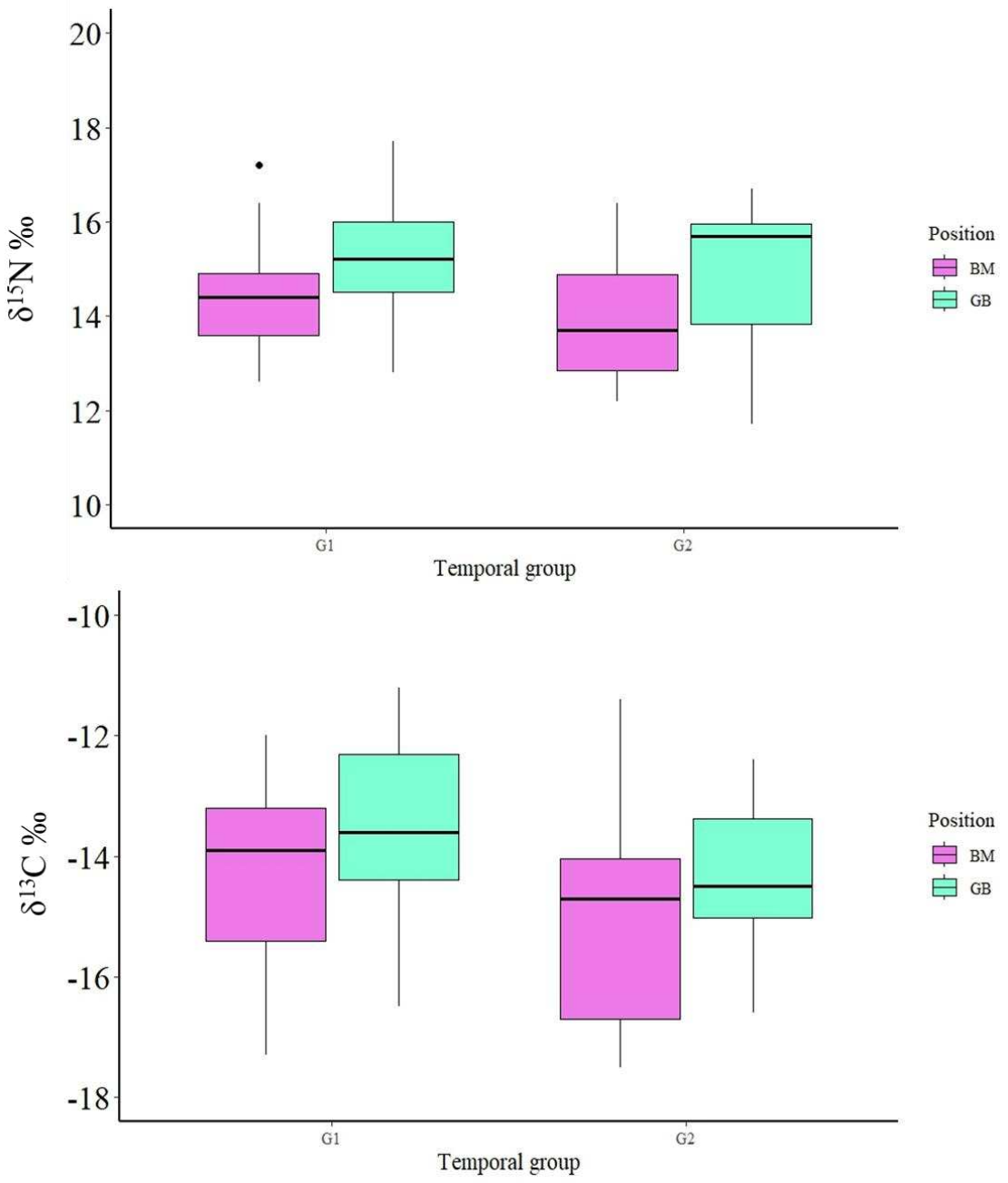


Fig. 4.6 Boxplot of values of $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) in the BM and GB of G1 and G2

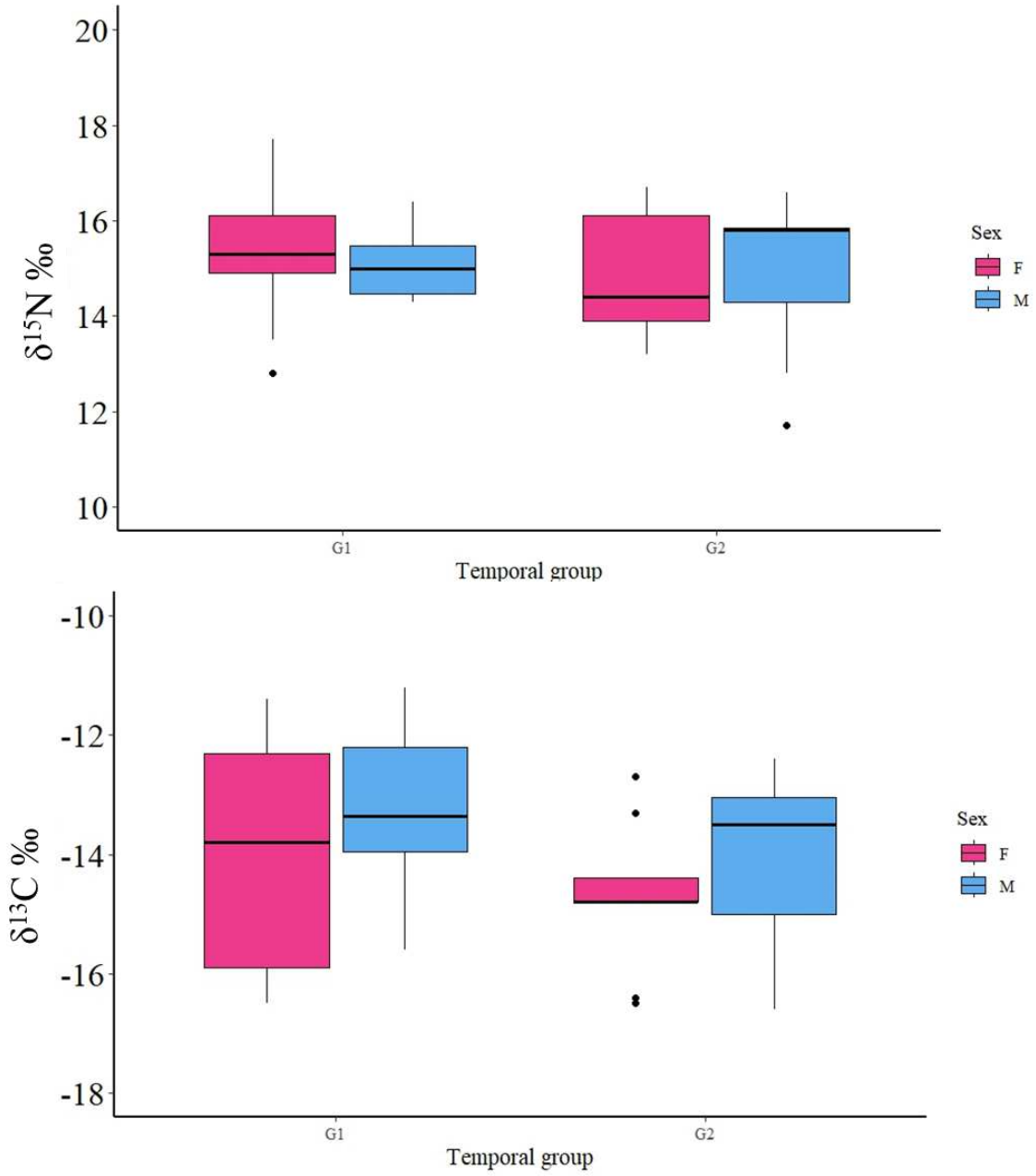


Fig. 4.7 Boxplot of values of $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) in the female F and male M of G1 and G2.

Considering the values of $\delta^{15}N$ and $\delta^{13}C$ in BM taken together, and $\delta^{15}N$ and $\delta^{13}C$ separately, there were no significant differences for the factors temporal group and sex, and for their interaction ($p > 0.05$) (**Tab. 4.4**).

		$\delta^{13}C$-$\delta^{15}N$		$\delta^{15}N$		$\delta^{13}C$	
	df	**MS**	**Pseudo-F**	**MS**	**Pseudo-F**	**MS**	**Pseudo-F**
Temporal group	1	7.82	$1.79^{N.S}$	2.46	$1.35^{N.S}$	5.36	$2.11^{N.S}$
Sex	1	3.05	$0.70^{N.S}$	0.11	$0.06^{N.S}$	2.94	$1.16^{N.S}$
Temporal group^{x}Sex	1	0.28	$0.06^{N.S}$	0.23	$0.13^{N.S}$	0.05	$0.02^{N.S}$
Residuals	31	4.37		1.82		2.54	
Total	34						

Tab. 4.4 Outcomes of the PERMANOVA main test for $\delta^{15}N$ and $\delta^{13}C$ taken together, and $\delta^{15}N$ and $\delta^{13}C$ separately, of BM. df = degrees of freedom; MS = mean square; Pseudo-F = statistic F; N.S = not significant.

Considering the values of $\delta^{15}N$ and $\delta^{13}C$ in GB taken together, and $\delta^{15}N$ and $\delta^{13}C$ separately, there were no significant differences for the factors temporal group and sex, and for their interaction ($p > 0.05$) (**Tab. 4.5**).

		$\delta^{13}C$-$\delta^{15}N$		$\delta^{15}N$		$\delta^{13}C$	
	df	**MS**	**Pseudo-F**	**MS**	**Pseudo-F**	**MS**	**Pseudo-F**
Temporal group	1	5.74	$1.32^{N.S}$	0.70	$0.37^{N.S}$	5.03	$2.08^{N.S}$
Sex	1	3.80	$0.87^{N.S}$	0.05	$0.03^{N.S}$	3.74	$1.55^{N.S}$
Temporal group^{x}Sex	1	0.11	$0.02^{N.S}$	0.07	$0.04^{N.S}$	0.03	$0.01^{N.S}$
Residuals	29	4.34		1.93		2.41	
Total	32						

Tab. 4.5 Outcomes of the PERMANOVA main test for $\delta^{15}N$ and $\delta^{13}C$, and $\delta^{15}N$ and $\delta^{13}C$ separately, of GB. df = degrees of freedom; MS = mean square; Pseudo- F = statistic F; N.S = not significant.

Considering the values of $\delta^{15}N$ and $\delta^{13}C$ in GB taken together, and $\delta^{15}N$ and $\delta^{13}C$ separately, there were no significant differences for the factors temporal group and size, and for their interaction ($p > 0.05$) (**Tab. 4.6**).

	$\delta^{13}C$-$\delta^{15}N$			$\delta^{15}N$		$\delta^{13}C$	
	df	**MS**	**Pseudo-F**	**MS**	**Pseudo-F**	**MS**	**Pseudo-F**
Temporal group	1	3.27	$0.79^{N.S}$	0.02	$0.01^{N.S}$	3.25	$1.29^{N.S}$
Size	1	6.44	$1.56^{N.S}$	6.44	$4.00^{N.S}$	0.00	$5.5E\text{-}05^{N.S}$
Temporal group^{x}Size	1	3.66	$0.89^{N.S}$	3.02	$1.88^{N.S}$	0.64	$2.5E\text{-}01^{N.S}$
Residuals	29	4.13		1.61		2.52	
Total	32						

Tab. 4.6 Outcomes of the PERMANOVA main test for $\delta^{15}N$ and $\delta^{13}C$, and $\delta^{15}N$ and $\delta^{13}C$ separately, of GB. df = degrees of freedom; MS = mean square; Pseudo- F = statistic F; N.S = not significant.

However, since the p-value for the factor 'size' was close to the significancy, a pairwise test for the factors temporal group, for pairs of levels of factor size was conducted. In this case, the pairwise test conducted for the term 'Temporal group×Size', for pairs of levels of factor Size (**Tab. 4.7**) showed no significant differences in the values of $\delta^{15}N$ of small and medium

specimens of G1 ($p > 0.05$), while significant differences in the values of $\delta^{15}N$ of small and medium specimens of G2 ($p < 0.05$) were recorded.

Term 'yexSI' for pairs of levels of factor '**SIZE**'

Within level 'G1' of factor 'Temporal group'

Groups	t	P(MC)
S, M	0.47	$0.64^{N.S}$

ithin level 'G2' of factor 'Temporal group'

Groups	t	P(MC)
S, M	2.25	0.04*

Tab. 4.7 Pair-wise test on the term 'Temporal group^{x}Size' for pairs of levels of factors 'Size' and 'Temporal group' within level G1 and G2. P(MC) = probability level with Monte Carlo test; N.S = not significant.

A significant and positive correlation was recorded between $\delta^{15}N$ values and TL ($p < 0.05$), while no significant correlation was found between $\delta^{13}C$ values and TL ($p > 0.05$) (**Fig. 4.8**). Analysing correlations between isotopic values of G1 and G2 taken separately and TL, a significant correlation was found only between $\delta^{15}N$ values and TL in G2 ($p < 0.05$).

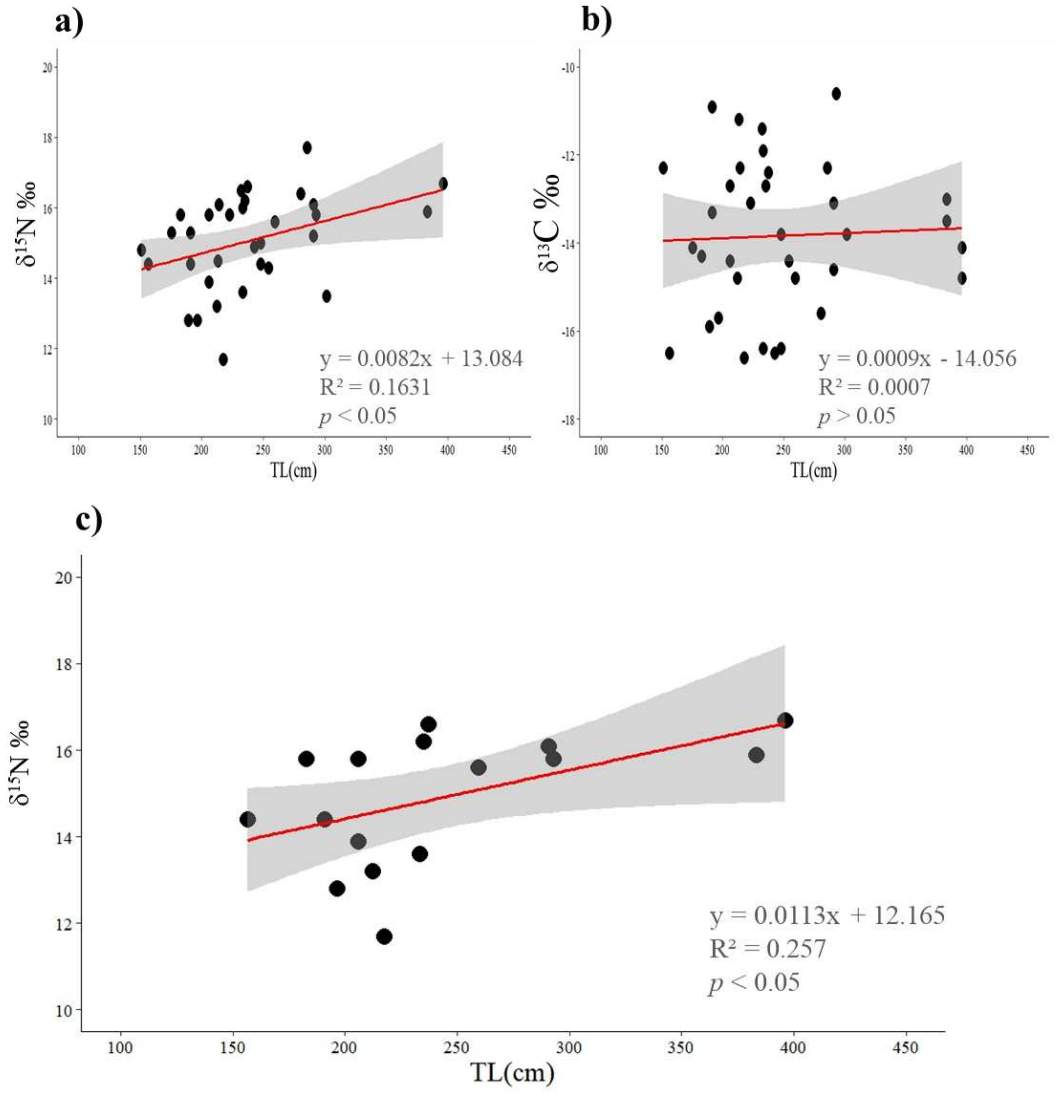


Fig. 4.8 Correlation of δ^{15}N (‰) and TL on a) the values of δ^{15}N (‰) and b) δ^{13}C (‰) in the overall specimens and c) values of δ^{15}N (‰) in the specimens belonging to G2.

4.4.2 SIBER outputs

SIBER was performed for the comparison of the trophic niches among several factors' combination, such as temporal group, TL and sex. Layman metrics were reported for each trophic niche.

Considering the isotopic values of BM, the two niches (G1 and G2) (**Fig. 4.9**) showed a more compressed shape for G1. The G2 trophic niche encompassed lower d15N values and was more stretched along the y-axis (wider spectrum of δ^{13}C values).

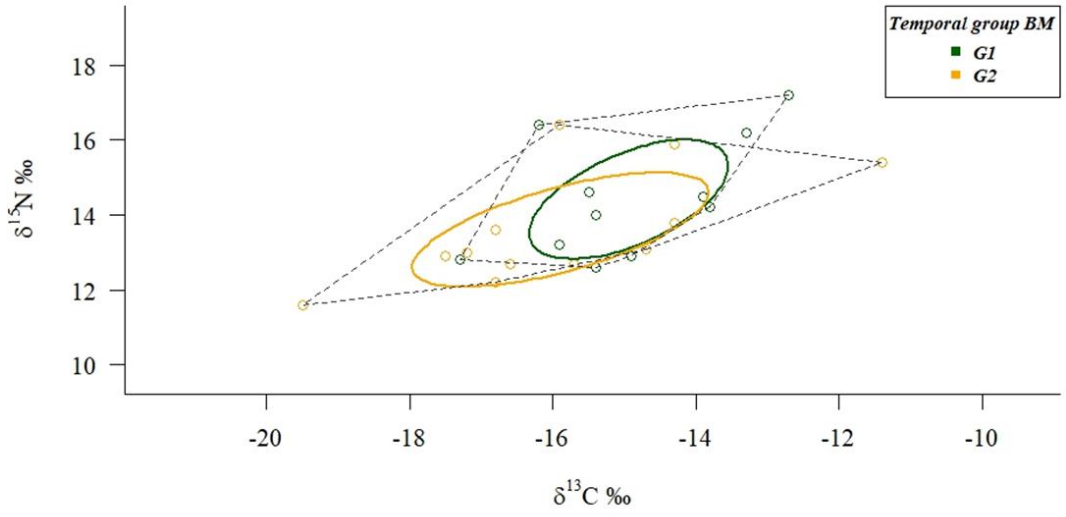


Fig. 4.9 δ^{15}N-δ^{13}C (‰) scatterplot with standard ellipse (p interval= 40 %) calculated for the BM of G1 and G2.

The SEA_C of BM G2 resulted to be wider than that of BM G1(**Tab.4.8**). CD and MNND values were higher in BM G2.

	BM G1	BM G2
TA (‰^{2})	12.2	16.2
SEAc (‰^{2})	6.2	8.1
dNrange	0.8	0.7
dCrange	0.3	2.8
CD	0.4	1.4
MNND	0.8	2.9

Tab. 4.8 Values of Total Convex Hull Area (TA), Standard Ellipse Areas Corrected for small sample size (SEA_C) and the Layman metrics calculated for the BM of G1 and G2.

Comparing the trophic niches obtained with GB isotopic values of specimens belonging to G1 and G2, the SEA_C resulted to be similar for both the position in the isospace and their width (**Fig. 4.10**).

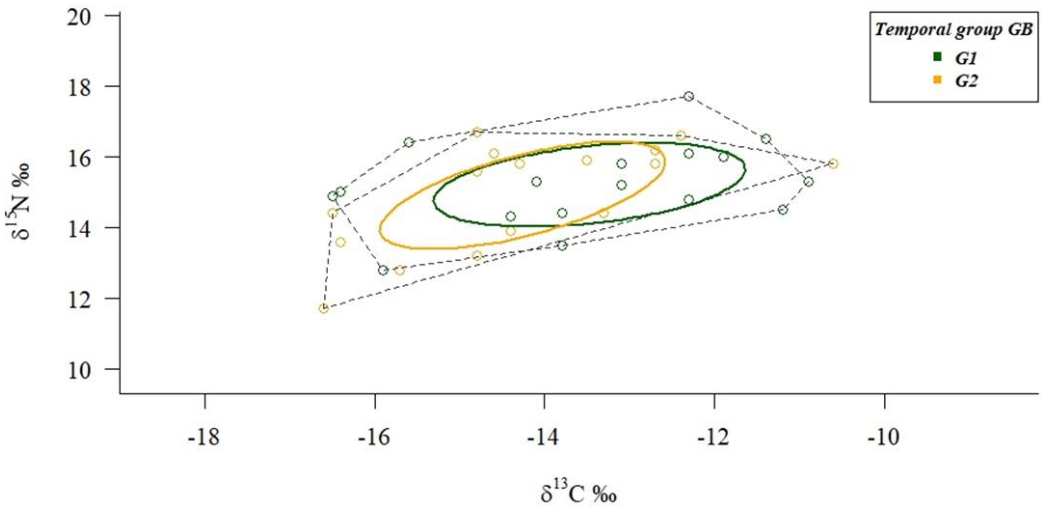


Fig. 4.10 δ^{15}N-δ^{13}C (‰) scatterplot with standard ellipse (p interval= 40 %) calculated for the GB of G1 and G2.

The width of the two SEA_C, showed in **Tab. 4.9**, was similar. The CD and MNND values were similar.

	GB G1	GB G2
TA (‰^{2})	16.9	14.4
SEAc (‰^{2})	6.6	6.5
dNrange	0.2	0.0
dCrange	1.1	1.0
CD	0.5	0.5
MNND	1.1	1.0

Tab. 4.9 Values Total Convex Hull Area (TA), Standard Ellipse Areas Corrected for small sample size (SEA_C) and the Layman metrics calculated for the GB of G1 and G2.

The trophic niche calculated for the two sexes showed a wider SEAc for females of GB G1, more stretched along both δ^{15}N and δ^{13}C values, while SEAc for females of GB G2 was narrower and shifted towards more negative values of δ^{13}C. In males, the trophic niches showed a different orientation

with G1 more stretched along $\delta^{13}C$ -axis, and G2 more stretched along both the axes (**Fig. 4.11**).

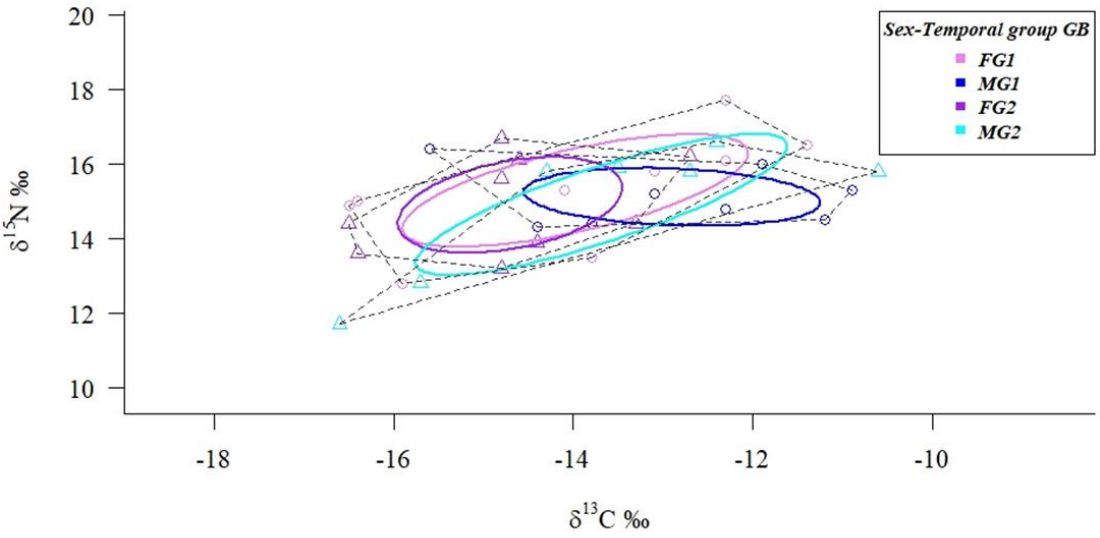


Fig. 4.11 $\delta^{15}N$-$\delta^{13}C$ scatterplot (‰) with standard ellipse (p interval= 40 %) calculated between G1 and G2 for both sexes (F and M).

The width of the four SEA_C showed different values (**Tab. 5.10**). The CD and MNND values were similar, except for GB G2 M that showed lower values than G1 M.

	GB FG1	GB MG1	GB FG2	GB MG2
TA (‰^{2})	12.0	6.6	8.3	9.1
SEAc (‰^{2})	7.6	4.5	5.2	8.0
dNrange	1.2	0.8	0.2	0.3
dCrange	0.8	1.4	1.6	0.8
CD	0.7	0.8	0.8	0.4
MNND	1.4	1.6	1.6	0.8

Tab. 4.10 Values of Total Convex Hull Area (TA), Standard Ellipse Areas Corrected for small sample size (SEA_C) and the Layman metrics calculated for the GB of females F and the males M of G1 and G2.

The trophic niches calculated considering the two TL ranges of specimens of G1 and G2 showed a partial overlap of trophic niches of small and medium specimens of G1. Contrarily, the two trophic niches of G2 specimens only partially overlap (**Fig. 5.12**).

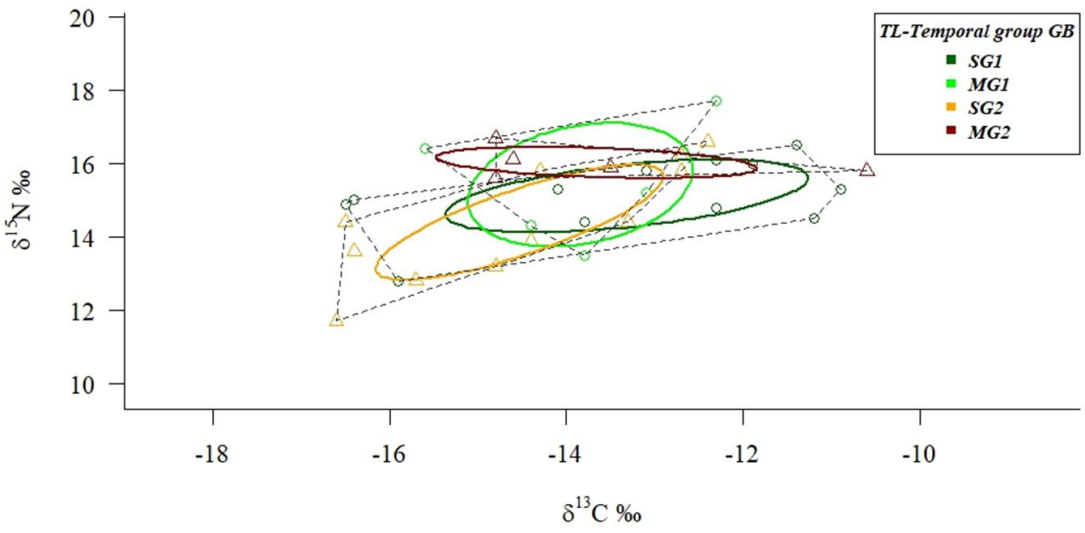


Fig. 4.12 δ^{15}N-δ^{13}C (‰) scatterplot with standard ellipse (p interval= 40 %) calculated between TL and temporal group.

Considering the different TL and the temporal group, the extension of the four SEA_C was similar, except for GB G2 M that showed a narrower niche (**Tab. 4.11**). The CD and MNND showed similar values, except GB G2 M that reported lower values than the other niches.

	GB SG1	GB MG1	GB SG2	GB MG2
TA (‰^{2})	11.3	10.3	6.6	2.3
SEAc (‰^{2})	5.9	6.1	5.1	3.0
dNrange	0.6	0.4	0.2	0.3
dCrange	1.1	0.9	0.5	1.7
CD	0.6	0.5	0.3	0.9
MNND	1.2	1.0	0.6	1.8

Tab. 4.11 Values of Total Convex Hull Area (TA), Standard Ellipse Areas Corrected for small sample size (SEA_C) and the Layman metrics calculated for the GB of small S and the medium M of G1 and G2.

4.5 Mixing model

Among all the food sources tested with the construction of the mixing polygon, four sources were identified as the best items to be used in mixing models (**Fig. 4.13**). Due to their very similar position in the biplot, isotopic contents of seals, sharks and teleosts (**Tab. 3.2**) were merged together to form a single vertex.

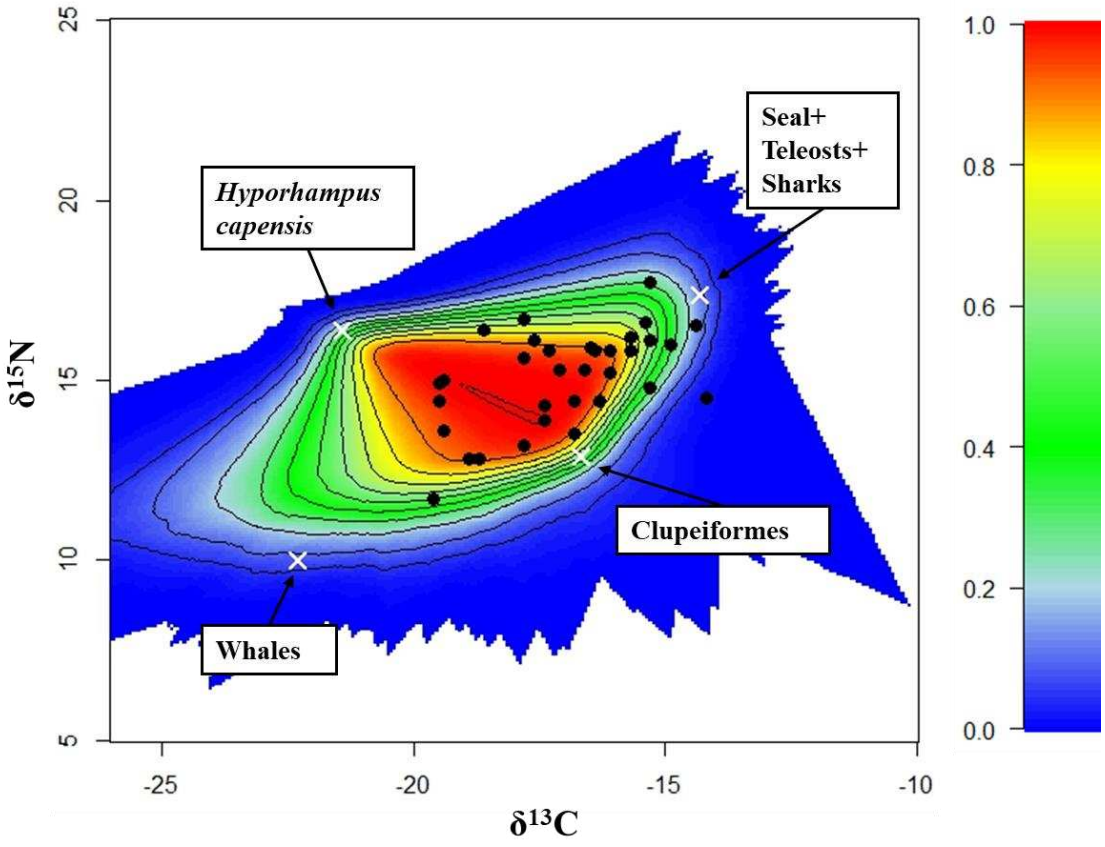


Fig. 4.13 Mixing polygon of the four potential food sources of *C. carcharias*.

Simmr model run to determine the diet of small individuals (< 250 cm) of G1, highlighted two principal souces: the group composed by seals, teleosts and sharks (56%) and Clupeiformes (18%) (**Fig. 4.14a**). A similar result was shown for G2, with seals, teleosts and sharks contributing 33% and Clupeiformes with 30% (**Fig. 4.14b**). The medium size individuals (< 250 cm) showed the same diet preference, as the small individuals, for G1 and G2, with showed a predominance of seals, teleosts and sharks, 41% and 44% respectively, followed by clupeiformes, 26% and 20% respectively (**Fig. 4.14c; Fig. 4.14d**).

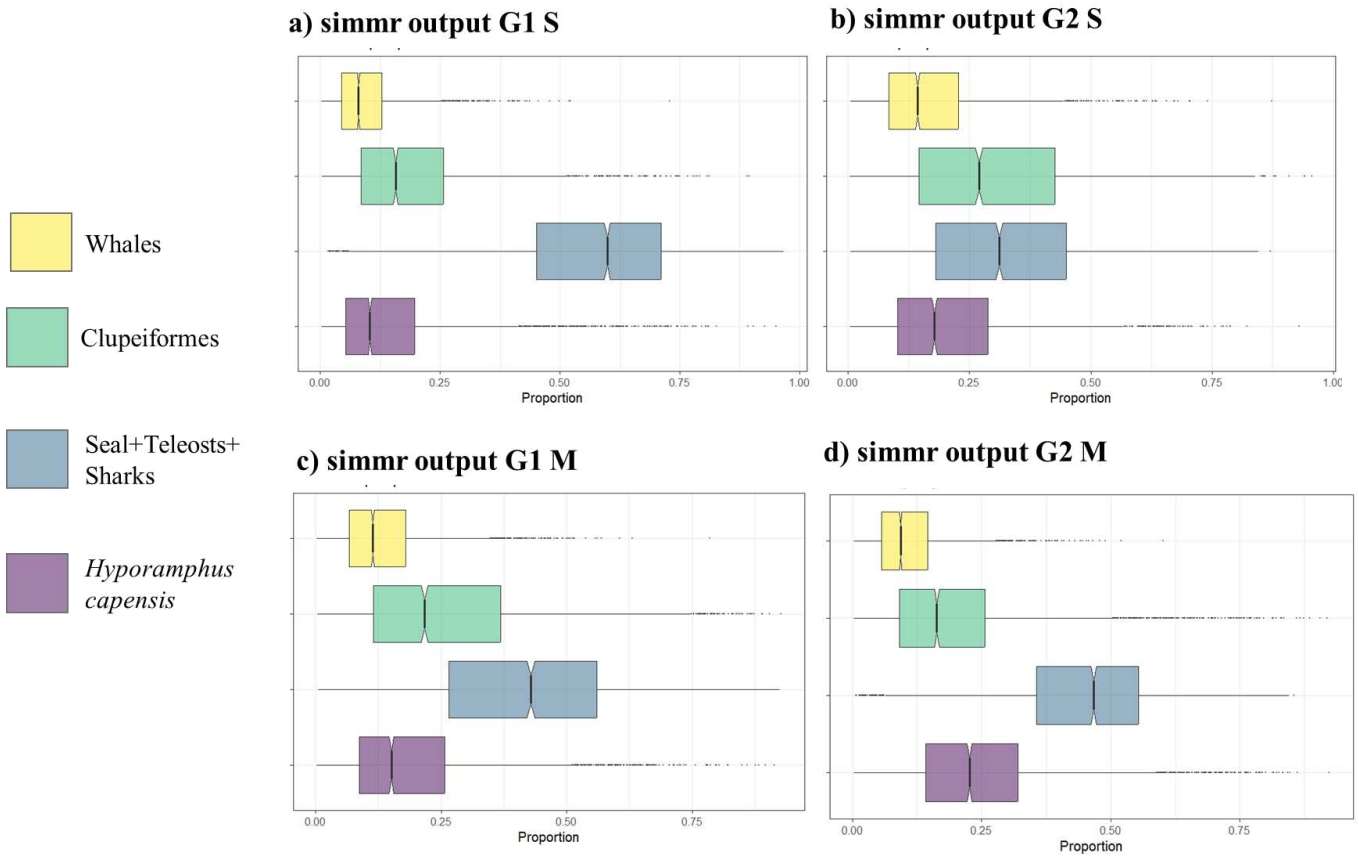


Fig.4.14 Posterior probabilities of the proportional contribute of each food source in the diet of *C.carcharias* in small (S) individuals in a) G1 and b) G2, and for medium (M) individuals in c) G1 and b) G2. The horizontal line within the boxes represents the median and the 95% credibility interval, boxplots represent 25th%, 50th% and 75th% percentile, while whisker lengths represent 1.5*interquartile range.

5. Discussion

5.1 Age estimation of Carcharodon carcharias

This study was conducted on 36 individuals of *Carcharodon carcharias* stranded along the South Africa Eastern coast. The TL of the collected individuals ranged from 150 cm to 395 cm. According to Onate-González et al. (2016), among the specimens we analysed, we can distinguish Y-O-Y (Young of the Year) (ranging from 120 cm to 175 cm TL), juveniles (175 cm to 300 cm), subadult (from 300 cm to maturity) and one male adult (> 360 cm TL); no mature females were found. Size at maturity can vary widely within the sexes, as published studies agreed: maturity in males occurs around 350-400 cm TL (Pratt, 1996), while most females mature around 450-500 cm TL (Francis, 1996).

The LFD showed a peak of frequency in the length class comprised between 200 and 249 cm. Considering that our specimens were collected as dead animals near the beaches or stranded on beaches themselves, this result confirms that warm coastal waters host mainly juveniles of *C. carcharias* that concentrate here due to the increasing forage opportunity, as reported in previous studies (Dicken & Booth 2013; Bernvi, 2016).

The growth of vertebral cartilage in elasmobranchs occurs as annual depositions of concentric rings, alternating between period of slow and fast growth. In this study, two methods for the age estimation were proposed.

Utilizing two different approaches for the age estimation, we were able to detect significant correlations between the vertebra annuli's count and the back-calculated years in correlation with the TL: in particular, we are able to assess that the TL positively increase with the age for G1 and G2 with both methods.

Significant differences were recorded between the age estimated with the two age determination methods in each considered specimen. In fact, after the 5th years of age, the band's marginal limits observed in each vertebra become less clear, and the formation of "false deposition rings" increases together with the probability of a wrong count, resulting in an underestimation of the age by the band count method. Thus, the two age estimation methods do not match. Within the first 5 years, the back-calculated years resulted as 1-2 years higher than the vertebra annuli's count. Moreover, together with the increasing growth, and the consequently band deposition, the overestimation of age become always greater: from the 5th year, the difference between the two methods increases from 3 to 7 years, with an overestimation due to the back-calculated method. To overcome these errors, staining techniques, implying the use of chemicals to enhance growth band visibility, such as Sefranin O, Alcian Blue, Crystal Violet and Silver Nitrate (Baususta et al., 2017), X-radiography (Ferreira and Vooren, 1991; Officer et al., 1996) and imaged with micro-computed tomography

(micro-CT) (Geraghty et al., 2012) were successfully used in the age determination in several elasmobranch species. Nevertheless, the success of each technique is often species specific, and slight modifications in technique may enhance or not the results (Goldman, 2005).

5.2 Considerations on the methodological approach

In the present study, for the preparation of samples for SIA, the protocol proposed by Kim and Koch (2012) was applied. This commonly used protocol consists in a one-week treatment in EDTA solution at 0.5M and pH=8. After the first analyses, very depleted signals of ^{13}C were found, together with very high values of C/N. As stated in the previously cited paper, high values of C/N in vertebra correspond to the still high presence of mineralized material. Therefore, as the protocol's guideline suggests, the EDTA treatment was extended to two weeks of duration. Nevertheless, any significant difference was recorded between $\delta^{13}C$ values determined in not treated and treated (2 weeks – treatment with EDTA) samples; these results highlight a high degree of mineralization in the cartilaginous structures of *C. carcharias* vertebra analysed in the present study. Because of the lack of significant differences, values of $\delta^{13}C$ obtained from non-treated samples were used for the subsequent statistical analysis and models.

5.3 Stable isotopes as a tool for trophic ecology assessment

Values of stable isotope signatures provide several information about the marine ecosystems, describing continental or marine origin of the food sources and their more pelagic or benthic reliance (Fry, 2008; Hussey et al. 2012), and providing information useful to identify the position of a species within a food web (Post et al., 2002).

In this study, the BM isotopic values were considered as the potential signals of the mothers (Christiansen et al., 2015). The values δ^{15}N found in vertebral BM are higher than those found in the GB, confirming that mature females occupy a higher trophic level compared to their own offsprings. In *C. carcharias*, female specimens reach the reproductive maturity at around 400 cm of TL (Onate-Gonzales et al., 2017). Mature females are known to move offshore. However, higher δ^{13}C values can be observed in waters with high phytoplankton productivity or warmer water closer to the equator (Graham et al., 2010). Major marine and marginal marine habitat types had distinct δ^{13}C values and could provide different basic carbon sources. Such variation can be transferred along the food chain and reflected in the tissues of top predators (Hobson et al., 1994). Here females probably migrate offshore but to colder waters and this can explain the ^{13}C depletion observed in the analysis (Shen et al., 2021). Consistently, in the BM of both G1 and G2 we found depleted values of δ^{13}C.

In the present study, no significant differences were reported in $\delta^{15}N$ and $\delta^{13}C$ values (both in BM and GB) for the tested factors, neither if taken together or separately.

Differently, considering temporal group and size, a slight significant difference was highlighted in the values of $\delta^{15}N$ of GB between small and medium individuals of the G2 group. A significant correlation was reported between the values of $\delta^{15}N$ and TL within G2. These assumptions taken together, highlight clear evidence of an ontogenetic shift in the diet at 250 cm TL, the threshold choose to distinguish between small and medium individual. This evidence denotes an individual's preference for higher trophic level preys with increasing length as previously demonstrated in literature (Tricas and McCosker 1984; Estrada et al., 2006).

The absence of a significant correlation between values of $\delta^{13}C$ and TL within the two temporal groups, indicates that the individuals between 150 cm TL and 300 cm TL likely remain in the same high productive area with a high prey availability of over the long period analysed.

5.4 Trophic niche estimates

SIBER outputs were used to investigate the differences or similarities between the trophic niches of G1 and G2, concerning also the possible variations in time related to sex and TL.

In the present study, considering the signals of the BM of the analysed specimens, it is notable that the trophic niche defined by the signals of BM of animals captured before 2000s occupied a higher trophic level and the mothers had a more selective diet. On the contrary, more recent mothers seems to have a wider and more generalist diet.

Conversely, concerning the signals of the growth band, the trophic niches of G1 and G2 were similar, with slightly depleted values of $\delta^{13}C$ in more recent specimens (G2), highlighting a shift of their diet versus preys with pelagic habits.

Concerning the sex, the models of trophic niches described an evident difference between males and females' niches within each time period. While in G1 males showed a niche stretched along the $\delta^{13}C$- axis but a constant trophic level, in G2 males' trophic niche has an opposite orientation, completely overlapping with the female's trophic niche of G1. Even if it is considered a generalist species, a dietary specialisation occurs in *C. carcharias* when the prey availability decrease (Christiansen et al., 2015).

Before 2000s, our results suggest the occurrence of an intraspecific partitioning of food sources by sex, with males that fed on higher trophic levels. On the contrary, in more recent years the diet of males is more similar to that of females, probably due to a lower availability of preys which needs further studies to be explained. Moreover, the actual niche of males is

completely overlapped with that of females of 2000s. This demonstrates a clear shift in the trophic ecology of the species in the area of KwaZulu-Natal. Moreover, the trophic niches of small and medium animals were clearly different in position and extension especially in more recent animals, confirming an ontogenetic shift around the 250 cm TL (Estrada et al, 2006).

Published researchs demonstrated the existance of an ontogenetic shift in the diet of the great white sharks. In fact, specimens with a total length less than 300 cm feed maily on teleosts and elasmobranchs, while in the diet of bigger animals, marine mammals, such as seals, give the highest contribution to the diet (Cliff et al., 1989; Weng et al., 2007; Hussey et al., 2012).

In the present study, simmr and SIBER outputs support the same conclusions about the diets of the analysed specimens. The preferred prey, in fact, remain the same for the two temporal groups, but they give a different contribution to the diet of white sharks, probably reflecting a different prey abundance due to drivers such as anthropogenic pressure and climate change. The synergistic effects of increasing demersal trawling pressure and increasing sea surface temperatures (SST) (Currie et al., 2019) along the South Africa's East Coast can lead to a decline in prey populations. When preferred preys abundances decrease, predators shift to less nutritious preys (Adame et al., 2020). Great white sharks are most likely associated with colonies of Cape fur seal (*Arctocephalus pusillus pusillus*) that populate

the Southern and Western Cape, thus they frequently move along the inshore regions of False Bay, Mossel Bay and KwaZulu-Natal (Johnson et al., 2009; Koch et al., 2012). As reported in Adame et al. (2020), pinnipeds' demographic decline in Californian coasts can be caused by a multi-decadal sea surface warming, resulting in a change in the diet of sharks from high to low quality preys. Similarly, along the KwaZulu-Natal coast, the general reduction of the abundance of several demersal species and the westward shifts of many stocks, together with a generally decreasing spatial extent of these community, was attributed to climate (environmental) driver rather than an anthropogenic (fishery) one (Currie et al., 2019). Such changes have affected in turn all the elements of the food web, up to seals and sharks. Considering that the preferred group of preys consists in seals, teleosts and sharks, the conclusions of this study can support the hypothesis that white sharks limit their movements around the Southern Cape, where a higher prey availability can sustain their food demand. In the meanwhile, Cape fur seals are forced to move more frequently towards the northern part of the coast, where this species finds a more suitable habitat, avoiding demographic decline. Nevertheless, any conclusion can be done considering only prey's values from literature, and further studies are needed to deepen the knowledge on th the trophic ecology of great white sharks that live along South Africa's coasts.

6. Conclusion

This study represents the first of its kind providing a new and deepen knowledge about the trophic ecology of the Great White Shark *Carcharodon carcharias* population that lives along the KwaZulu-Natal coast. The SIA on vertebrae, represented a powerful tool for these type of investigation.

- The band count method can be used as method for a prelimiary age estimation, but it can not be used as a real and effective method of age validation. It is important to take into account that this method does not provide the real age of an individual, but an estimation that can be affected by human errors during the band count. Over the 5^{th} year of age, it increases the probability of counting false rings . The back-calculated method from the total length can provide a more accurate age estimation.
- The SIA demonstrated substantial differences in the trophic position of individuals within the temporal groups G1 and G2, considering both TL and sex as discrimination factors.
- The ontogenetic shift was highlighted in the relationship between the temporal group G2 and the TL. The shift was evident at 250 cm TL andconfirming a possible complete diet shift at > 300 cm TL, as reported many published reasearchs. In South Africa population of *C. carcharias*, the sexual dimorphism in the diet is not evident.

- The comparison between the isotopic signal of BM that allowed to make inferences on the feeding ecology of the mothers that of GB that was used to investigate the feeding ecology of their offsprings, highlighted higher differences in the trophic position rather than in the food web baseline.

Further investigations are necessary and should be carried out considering a wider number of specimens, stable isotopes signatures of preys found in the stomach contents of stranded animals together with more detailed knowledge on white sharks movements between inshore and offshore ecosystems, through acoustic tagging, and how this is reflected in the variations of isotopic signals.

7. Bibliography

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