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DIPARTIMENTO SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale Biologia marina

Uso delle risorse e dell'habitat da parte dei giovanili (Y-O-Y) dello squalo grigio (*Carcharinus plumbeus*) nel Mare Adriatico settentrionale

Resource and habitat use of the Y-O-Y sand bar shark (*Carcharinus plumbeus*) in the northern Adriatic Sea

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Summary

RIASSUNTO

Gli squali sono pesci cartilaginei che occupano i livelli intermedi ed apicali della rete trofica marina. In tutto il mondo vengono sovrasfruttati come oggetto di pesca commerciale e sportiva e sono una delle porzioni più consistenti del by-catch (catture accidentali), insieme a mammiferi marini e tartarughe. Nonostante la rilevanza ecologica degli squali, le informazioni riguardanti la loro ecologia trofica restano limitate. Comprendere meglio le loro abitudini alimentari e il loro ruolo negli ecosistemi marini è essenziale per sviluppare strategie di conservazione efficaci. A causa della pesca eccessiva e della perdita dell'habitat, la specie in esame in questo studio, *Carcharhinus plumbeus*, è stata classificata come specie "in pericolo" dalla IUCN (International Union for Conservation of Nature) a partire dal 2007. Questo tipo di informazioni possono essere ottenute tramite due metodi principali: l'analisi del contenuto stomacale (Stomach Content Analysis, SCA) e l'analisi degli isotopi stabili (Stable Isotope Analysis, SIA). L'analisi del contenuto stomacale fornisce informazioni dettagliate a breve termine, esaminando direttamente ciò che gli squali hanno mangiato di recente e identificando le prede consumate. Al contrario l'analisi degli isotopi stabili del C e del N e la determinazione dei valori di $\delta^{13}C$ e $\delta^{15}N$ permette di identificare le

prede maggiormente assimilate negli ultimi mesi di vita dell'animale, in quanto i loro valori riflettono le fonti alimentari e la posizione trofica e fornendo informazioni sulla dieta a lungo termine. Per il lavoro presentato in questa tesi, sono stati raccolti campioni di tessuto muscolare, pinne e vertebre di esemplari giovanili (da neonati ad un anno di vita) della specie *Carcharhinus plumbeus* catturati accidentalmente dai pescatori nella zona antistante Cervia, nel Nord Adriatico, durante la stagione estiva dal 2019 al 2021. Sui tessuti raccolti sono state svolte le analisi degli isotopi stabili di azoto e carbonio. I risultati ottenuti hanno permesso di ottenere informazioni sulla composizione isotopica di muscoli, pinne e vertebre dello squalo grigio, determinando la loro posizione nella rete trofica e avanzare ipotesi sull'area di provenienza delle madri. Inoltre, è stato possibile verificare i diversi tempi di turnover dei tessuti analizzati, che (come dimostrato da studi precedenti) sono più rapidi per muscoli e pinne, e più lenti per le vertebre. I valori di $\delta^{13}C$ e di $\delta^{15}N$ ottenuti suggeriscono che le madri degli esemplari giovanili di squalo grigio catturati nell'area di costa antistante a Cervia e Marina di Ravenna provengano da un'area geografica differente, possibilmente l'Adriatico centrale. Le analisi isotopiche hanno evidenziato un cambiamento significativo nella baseline della rete trofica per gli anni 2020 e 2021, probabilmente legata ad un maggior apporto di acque continentali del fiume Po nell'anno 2019.

Infine, i contenuti stomacali hanno confermato che la dieta di questi individui giovanili è poco variabile ed è costituita principalmente da piccoli pesci ossei principalmente bentonici e da cefalopodi.

ABSTRACT

Sharks are cartilaginous fishes that occupy the intermediate and apical levels of marine food webs. Worldwide, together with marine mammals and turtles, they are among the most common bycatch species and are overfished as targets in both commercial and recreational fisheries. Despite the ecological significance of sharks, information regarding their trophic ecology is still limited. Gaining a deeper comprehension of their feeding habits and role in marine ecosystems is essential to develop conservation strategies. Due to overfishing and habitat loss, the species under consideration in this study, *Carcharhinus plumbeus*, has been classified as "endangered" by the IUCN (International Union for Conservation of Nature) since 2007. Stable isotope analysis (SIA) and stomach content analysis (SCA) are the two main methods that provide information on a species trophic ecology. SCA analyzes what sharks have just eaten and identifies the prey consumed to offer precise short-term information. On the other hand, SIA allows to determine the assimilated food in the last months of the animal's life, as their values reflect food sources and trophic position and providing information on long-term diet. For this thesis, samples of muscle tissue, fins, and vertebrae were collected from juvenile (newborn to young-of-the-year) specimens of the species

Carcharhinus plumbeus accidentally caught by fishermen in the area in front of Cervia, North Adriatic Sea, during the summer season from 2019 to 2021. Nitrogen and carbon stable isotope analyses were carried out on the collected tissues, to determine their position and advance hypotheses on the area of origin of the mothers. Furthermore, it was possible to verify the different turnover times of the tissues analyzed, which (as shown in previous studies) are faster for muscles and fins, and slower for vertebrae. The $\delta^{13}C$ and $\delta^{15}N$ values obtained suggest that the mothers of juvenile sandbar shark specimens caught in the coastal area in front of Cervia and Marina di Ravenna come from a different geographic area, possibly the central Adriatic. Isotopic analyses showed a significant change in the food web baseline for the years 2020 and 2021, likely related to an increased input of continental waters from the Po River in the year 2019. Lastly, stomach contents confirmed that the diet of these juvenile individuals is little variable and consists mainly of small, mainly benthic bony fish and cephalopods.

Chapter 1. Introduction

1.1 Elasmobranchii

1.1.1 The class Elasmobranchii

The term Chondrichthyes (from Greek language, “χόνδρος” cartilage, and “ἰχθύς” fish) refers to fish belonging to the parvophylum of aquatic vertebrates of marine origin, whose main characteristic relies on their cartilaginous skeleton.

The Chondrichthyes are a monophyletic group comprising 1282 species distributed throughout the world's oceans; this group is divided into two classes: Holocephali (chimaeras; 56 species) and Elasmobranchii (sharks and batoids; 1226 species) *(Compagno et al., 2005*; *Nelson*, *2016*).

According to the FishBase website, the Elasmobranchii are more differentiated than the Holocephali: there are 12 orders and 54 families (Fig. 1.1), while Holocephali consist of 1 order and 3 families.

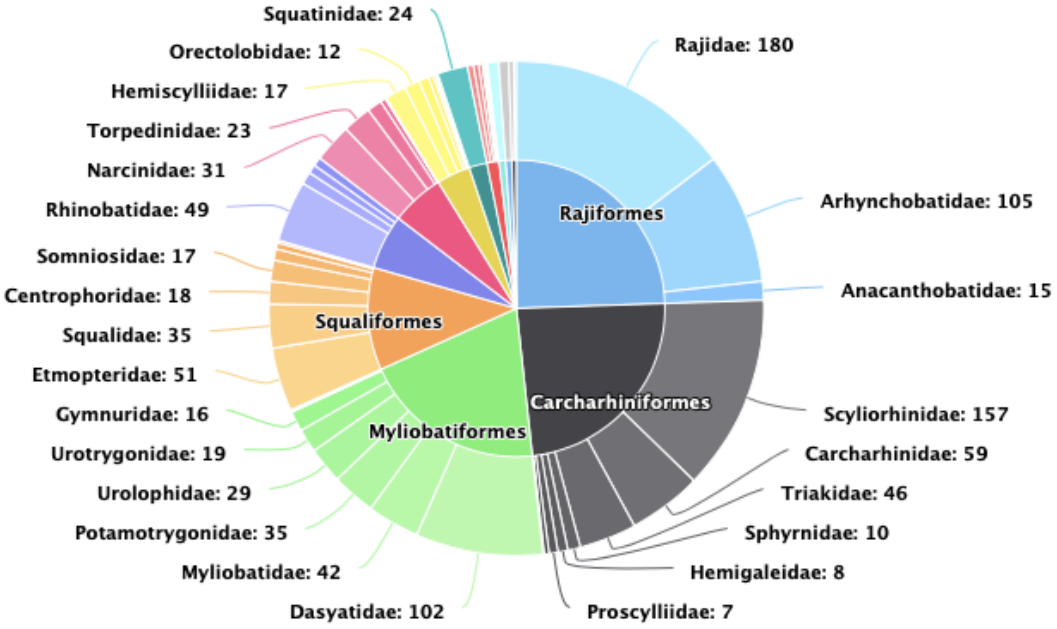


Fig 1.1 The class Elasmobranchii in Catalogue of Life Checklist, classified in the different orders and families, with number of known species (Catalogue of Life: *www.catalogueoflife.org*).

In the class of Holocephali, the palatal quadrate is completely fused to the neurocranium, and the non-replaceable teeth are fused into three pairs of mineralized tooth plates (*Maisey, 1986; Didier, 1995*). On the contrary, in the class Elasmobranchii, they have many rows of constantly growing teeth and an upper jaw that is not attached to the skull.

In this last class, the subclass Neoselachii can be divided into two infraclasses (*Ebert and Dando,2020; Worms: www.marinespecies.org*) (Fig. 1.2):

- Selachii: have an elongated cylindrical body (slightly indented), gill openings on the side of the body, pectoral fins clearly separated from the head and a developed tail, suitable for propulsion (these are good swimmers);
- Batoidea: are essentially sharks with flattened morphology back ventrally as an adaptation to benthic life. Their tails can be long or short (shear or threadlike), they have gills in the ventral portion of the body instead of an anal fin and massive, often disc-shaped pectoral fins.

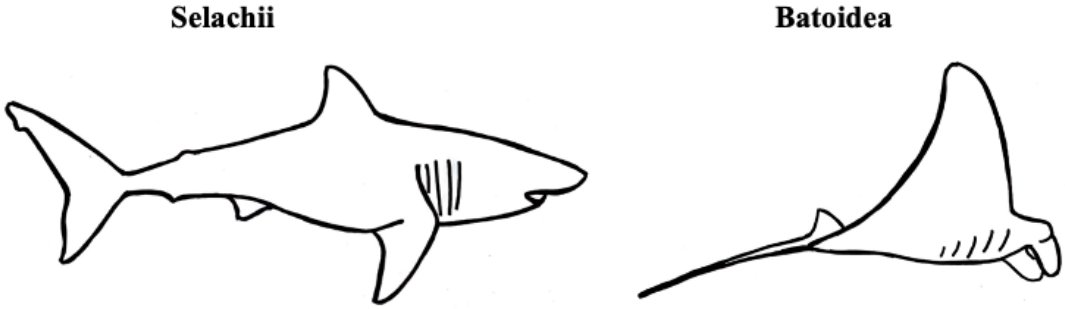


Fig 1.2 Simplified diagram of Selachii and Batoidea.

1.1.2 General anatomy of elasmobranch

MUSCOLOSKELETAL SYSTEM

The chondrichthyan skeleton is made up of lighter and more flexible material than bone, called cartilage.

Despite being made of a different substance than bone, this kind of skeleton is just as strong and resilient but lacks the latter's distinctive weight.

Elasmobranch skeletons are composed of different kinds of cartilage: it mostly consists of hyaline cartilage, but also of prismatic cartilage. Hyaline cartilage is naturally elastic and has a relatively simple structure made up of rounded or blunt cells, whereas prismatic cartilage is denser and has a more complex structure organized into tesserae.

Prismatic cartilage tiles resemble mosaic pieces and are covered by a perichondrium made of collagen and proteoglycans: proteoglycans could absorb water and produce swelling, whereas collagen serves as a strengthening factor. (*Dean and Summers, 2006; Klimley, 2013*).

The skeleton is divided into axial and appendicular (*Compagno, 1999; Klimley, 2013*). The axial skeleton is made up of the chondrocranium,

which supports the head and protects the brain and sensory organs, as well as the vertebral column, which supports the body, tail, and caudal fin. From front to back, chondrocranium may be distinguished into four sections: the ethmoidal region, the orbital region, the optic region, the occipital region; from this last section, the spinal column then articulates.

The vertebral column is made up of a succession of hourglass-shaped vertebrae (Fig. 1.3) that are made from areolar cartilage and extends from the chondrocranium's attachment point to the end of the upper lobe of the caudal fin.

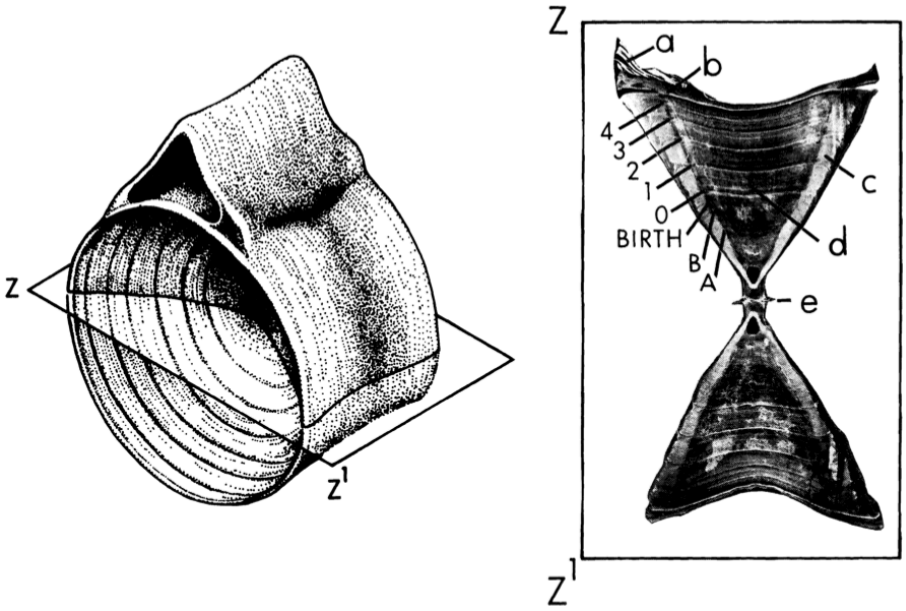


Fig. 1.3 Shape of a *Carcharhinus* sp. vertebra and longitudinal section.

A, B: prebirth mark; 0, 1, 2, 3, 4: year mark; a: membrane elastic external; b: margin; c: corpus calcareum; d: intermedialia; e: notochordal remnant; Z-Z': plane of section (*Casey et al., 1985*).

The tissue of the vertebrae consists of monucleated cells with plenty of cytoplasm (known as chondrocytes) and have their exteriors mineralized and calcified and are surrounded by perichondrium.

Vertebrae articulate whit each other, and each of them consist of a haemal arch, which is located in the lower zone of each vertebra and contains and protects the blood circulation, and the neural arch, which is located in the upper zone.

The central cartilaginous part is annually deposited in two concentric circles, one of which is more mineralized than the other (the deposition of which is species-specific) (*Compagno, 1999; Dean and Summers, 2006; Klimley, 2013*).

The general requirements for defining an annual growth mark are as follows: the mark must cross the whole intermedialia and visibly extend into the corpus calcareum. It must also occupy a separate narrow opaque zone relative to wider surrounding transparent zones in the intermedialia.

The first distinguishable mark that occurs out of focus and corresponds with a shift in the corpus calcarean's angle is referred to as the birth mark (*Casey et al, 1985*).

The efficiency of the elasmobranchs' movements is not only favored by their flexible but robust skeleton (as just seen), but also by their musculature, which is made up of tissues that alternately contract and relax.

Muscle tissue is made up of myomeres and myosepta: myomeres are the muscle segments made up of overlapping muscle fibers in a 'zigzag' pattern, which are located at the side of the animal's body and run along its entire length. The myosepta are directly responsible for the transfer of energy necessary for the lateral movement of the body, which are thin sheets of connective tissue attached to the derma that separate the myomeres. Since the propulsion of swimming is provided by the horizontal lateral movement of the tail, the transfer of energy is reciprocal: myomeres contract in a wave that travels down the body, while the myomeres on the opposite side of the body relax at the same time (*Liem and Summers, 1999; Klimley, 2013)*. In other words: the contraction of the myomeres on one side of the body pulls the myosepta on that side towards each other, causing a curvature, while the spinal column prevents the body from shortening. As opposed to if the muscle segments were oriented vertically, the overlapping myomeres' zigzag folding enables force to be produced over a longer length.

Elasmobranch muscles are a combination up of two different types of muscular fibers: white muscle and red muscle.

The red muscle is characterized by the presence of numerous blood vessels, works by aerobic metabolism, has a plenty of mitochondria and high myoglobin concentrations. Red blood cells carry oxygen to the mitochondria, where (thanks to the Krebs cycle) ATP is produced, so the oxygen supply is slow but continuous, making red muscle functional for constant and prolonged movement.

The white muscle, on the other hand, acts to anaerobic metabolism, whereby the ATP required to function is obtained from the breakdown of glycogen by glycolysis: this leads to the production and accumulation of lactic acid (the terminal product of anaerobic metabolism). White muscle is therefore employed for sprints and quick motions and needs a lengthy recovery period.

Considering where these two types of muscle fibers are located, in relation to the vertebral column of the animal, the infraclass Selachii can be divided into Carangiform and Thunniform (Fig. 1.4; Fig.1.5). The former has a large mass of white muscle in their innermost portion, which is then covered by a smaller portion of red muscle. The latter, on the other hand, have a muscle structure fundamentally different from the former; as a

result, they can swim continuously for extended periods of time (*Shadwick, 2005; Klimley, 2013*).

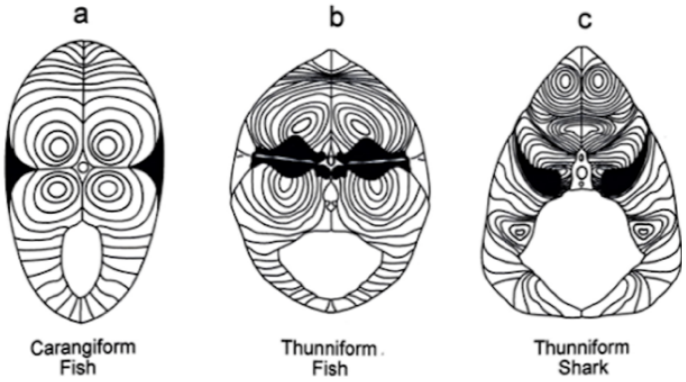


Fig. 1.4 Cross- section of a carangiform like (a), tuna-like (b) and thunniform shark-like (c), with an indication of the location of white (clear color) and red muscle (solid color) (Klimley, 2013).

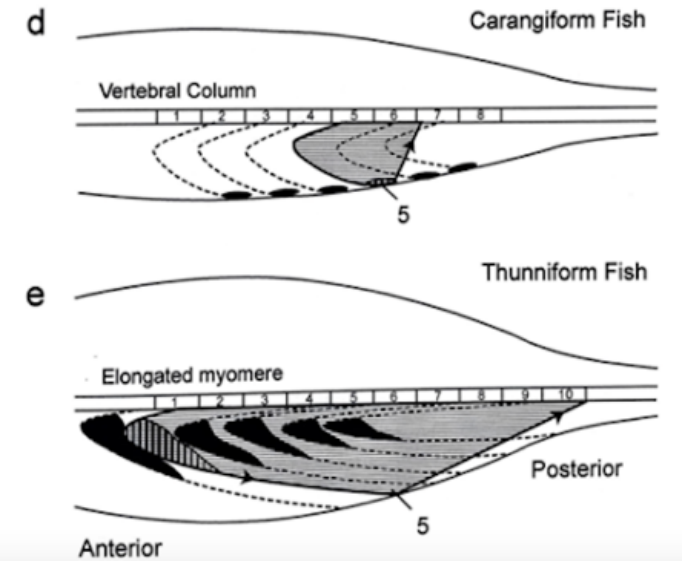


Fig. 1.5 Longitudinal section of the carangiform (d) and thunniform shark, showing the attachment of myomeres to the vertebral centra (*Klimley, 2013*).

Elasmobranchs, most notably sharks, are highly adapted to predation due to their morphological and physiological traits: each species has a distinctive set of teeth that can quickly alter.

Since they are susceptible to loss with each attack, the teeth develop in parallel rows like a conveyor belt. The mouth is ventrally located and has well-articulated, functional teeth; the jaw, since it has no connection to the skull, changes in size, shape, and direction depending on the kind of food (Fig. 1.6).

Fig. 1.6 Skull of an elasmobranch.

For finest biting pressure, the upper jaw muscles are very powerful; to maximize the penetration of the bite, the rotation of the teeth, and the removal of bigger portions of the prey, the jaw opens to several degrees (*Whitenack and Motta, 2010*).

INTEGUMENTARY SYSTEM

The skin is not part of the musculoskeletal system; however, it does contribute to the effectiveness of movement: when swimming, hydrostatic pressure supports skin, which functions like a tendon, transmitting the driving force throughout the body during muscle contraction (*Wainwright et al., 1978; Klimley 2013*). During acceleration, the energy stored elastically in the stretched skin on the convex side is released when the muscles on the concave side are contracted.

The skin is made by a thin layer of epidermis on the surface, followed by a thicker and more resistant layer of dermis. These layers together provide the animal with a sort of exoskeleton. Dermal denticles (Fig. 1.7), which resemble teeth more than fish scales, are small, flat, V-shaped scales that cover the surface of sharks.

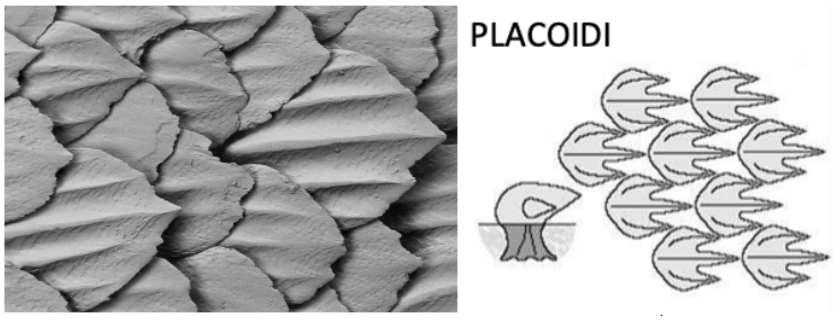


Fig. 1.7 Shark's dermal denticles (ocean.si.edu; dryads.units.it).

The shark can swim more quickly and silently thanks to these denticles' reduction of drag and turbulence.

The epidermis is also rich in mucipar cells, which are high producers of proteoglycans that minimize friction with water during movement.

SENSORY SYSTEM

Sharks have impressively diverse, highly specialized sensory systems: shark can recognize and react to a variety of both of biotic/abiotic stimuli in its immediate surroundings at various spatial scales thanks to each of its sensory pathways (Fig. 1.8):

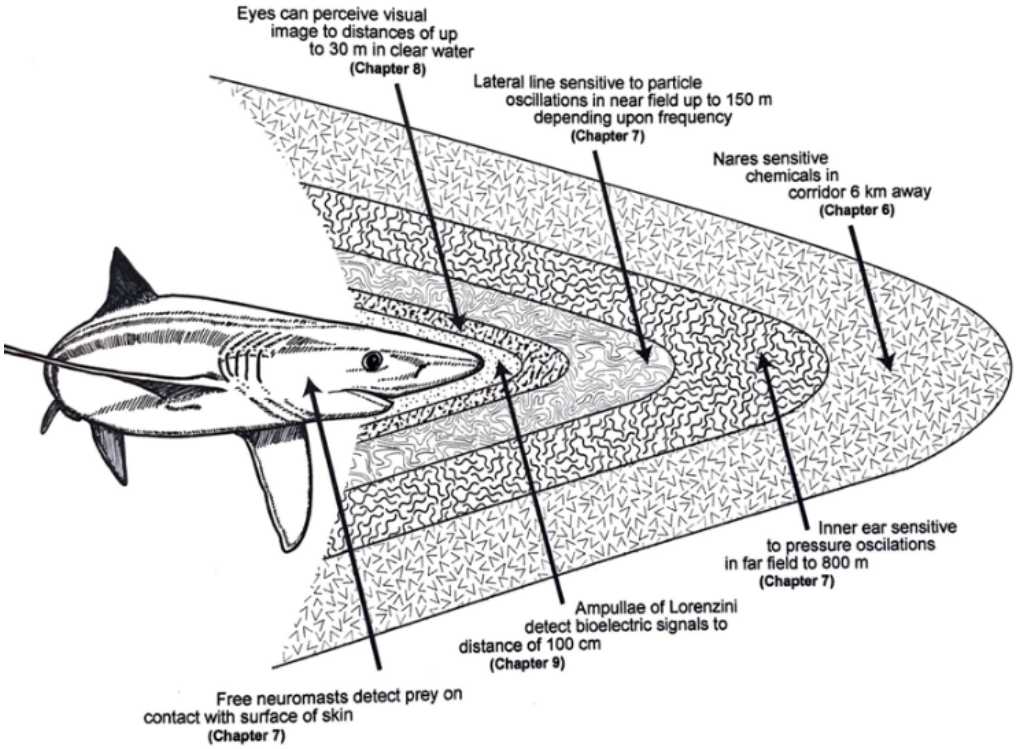


Fig. 1.8 Distance hierarchy of sensory stimuli that attract sharks to their prey/mate/predator (Klimley, 2013).

- Sense of smell: the nose detects compounds in a corridor six kilometers distant;

- Sense of hearing: the inner ear is sensitive to oscillations in a range within 800 m;
- Lateral line is sensitive to stimuli in a field up to 150 m;
- Sense of sight: In clear water, the eye can detect visual images up to 30 meters away;
- Ampullae of Lorenzini: can detect bioelectric signals up to 100 cm distance;
- Free neuromasts detect prey on contact whit skin.

The employment of many functionally separate sensory systems enhances the likelihood that a particular stimulus or item will be recognized and/or accurately identified as well as providing redundancy if one or more senses are rendered inoperative (*Stein et al., 2005; Klimley, 2013; Hart and Collins, 2015*).

1.1.3 Feeding and trophic ecology

Sharks are often classified as top-predators (i.e., secondary or tertiary consumers), although they can also occupy lower levels of the trophic web (and act as mesopredators): their place in the marine food web is affected by species, age, sex, and diet.

There are several feeding methods (Fig 1.9), including filter-feeding, which is used by basking (*Cethorinus maximus*) and whale (*Rhincodon typus*) sharks, suction crushing, which is used for example by carpet sharks (order Orectolobiformes), and efficient raptorial techniques, which are used by white (*Carcharodon carcharias*) and tiger (*Galeocerdo cuvier*) sharks (*Compagno, 1990; Compagno et al., 2005; Wilga et al., 2007; Ferretti et al., 2010*).

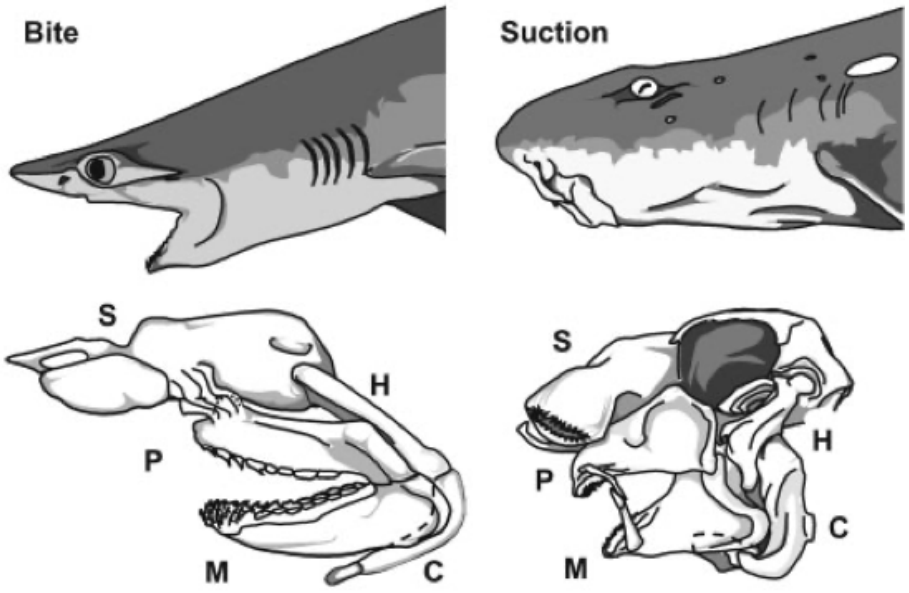


Fig. 1.9 Schematic representation of raptorial techniques (on the left) and suction crushing method (on the right) (Wilga et al., 2007).

Sharks are typically active predators, regardless of where they are located within the marine food web (either tertiary or quaternary consumers), who employ the same three-stage predation strategy of approaching, seizing, and handling prey (*Motta and Wilga, 2001; Klimley, 2013*).

According to these studies, sharks often eat opportunistically and asynchronously on the most abundant kind of prey, which is mostly other fish (*Motta and Wilga, 2001; Klimley, 2013*).

Understanding trophic interactions within marine ecosystems requires knowledge of the nutrition of sharks since their abundance can affect the community structure and prey habitat use. Elasmobranchs play a key role in maintaining the structure and functionality of food webs, making them essential components of the marine benthic environment (*Libralato et al., 2006; Baum and Worm, 2009; Barria et al., 2015*).

Due to their K-species traits, which include high longevity, slow development, low fecundity, and late attainment of sexual maturity, they are extremely vulnerable to ecological changes, overfishing, and habitat degradation (*Stevens et al., 2000; Myers and Worm, 2003; Ferretti et al., 2010; Dulvy at al., 2014; Barria et al., 2015; Tserpes et al., 2015*). Due to these features, elasmobranchs are regarded as reliable ecosystem health

indicators (*Steven et al., 2000; Baum and Worm, 2009; Barria et al., 2015*).

Numerous shark species that are at the top of the food chain, or nearby, serve as keystone predators, maintaining the equilibrium with other competitors and sustaining a high degree of ecological biodiversity (*Ritchie and Johson, 2009; Jachowski et al., 2020; Motivarash et al., 2020*). Many investigations have demonstrated how a substantial decrease in these species' biomass may outcome in an intricate series of "top-down cascade effects" (*Cortés, 1999; Stevens et al., 2000; Ferretti et al., 2010*).

The 'top-down' effect (Fig. 1.10) in a pelagic food web, can be summed up in the following manner: when apex predator populations, such those of sharks, decline, there is an increase of secondary predators such as foraging fish. This, in turn, increases the predation pressure on zooplankton, causing it to decline and primary producers to rise. This imbalance at the ecosystem level, over time, may result in the local or global extinction of some species (*Cury et al., 2003*).

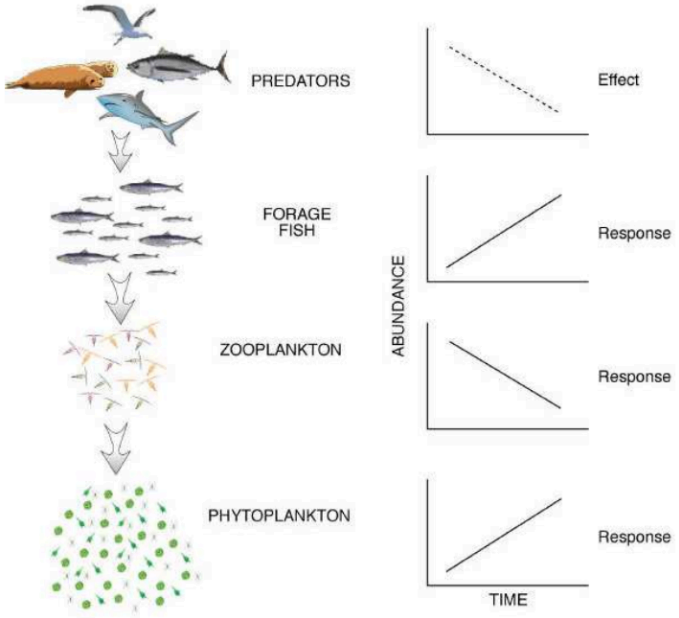


Fig. 1.10 Top-down control in a simplified food chain (*Cury et al., 2003*).

In benthic systems, the removal of apical predators also has top-down effects. When reef sharks are removed from coral reefs, groupers and other secondary predators that feed on herbivorous fish—such as parrot fish (*Scaridae)*— seem to increase in number (*Desibiens et al., 2021*).

A decrease in this population consequently results in less grazing pressure, which raises the algal cover with detrimental effects for corals (*Desibiens et al., 2021*).

The widespread of mesopredators, known as mesopredator release hypothesis, could arise from the disappearance of apex-predator sharks (*Jachowski et al., 2020*).

The hypothesis states that an uncontrolled and unexpected increase in mesopredators would result from a decline in the biomass of "apex predators" (*Ritchie et al., 2009*).

This phenomenon may result in the extinction of prey based in areas where they are exposed to attack (*Polis and Holt, 1992*), in addition to decreasing the target species for fisheries (*Baum and Worm, 2009*). They are therefore referred to as "keystone species" due to their essential role.

Despite playing a significant ecological function, there are scarce information about the trophic ecology of some species. In fact, studying some elasmobranchs is challenging due to their inherent biological traits such as: inherently low population concentrations, typical elusive and highly mobile behavior, and long-distance migrations. For the management of populations and the effective conservation of sharks, an in-depth knowledge of their trophic ecology, food and foraging is considered essential (*Heupel and Simpfendofer,2010; Klimley, 2013*; *Maithc and Heithaus, 2014; Gračan et al., 2017*).

1.1.4 *Carcharhinus plumbeus*

BIOLOGICAL FEATURES

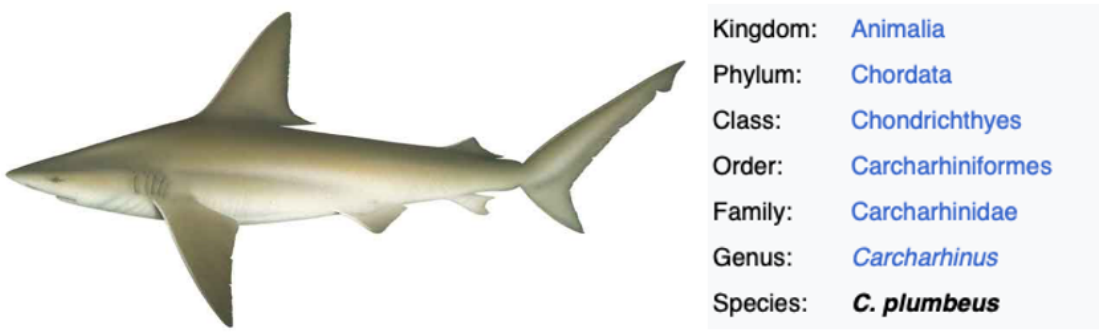


Fig. 1. 11 Portrayal and scientific classification of *Carcharhinus plumbeus (elifeproject.eu)*.

Also known as sandbar shark, *Carcharhinus plumbeus* (Fig. 1.11) can reach a maximum length of about 3 meters, although the average length is between 2 and 2.5 meters; females of *C. plumbeus* tend to be slightly larger than males (*specieaspim.it; Compagno, 1984; Ebert et al. 2013; Rigby et al., 2021*).

These sharks have a sleek and elongated body, typical of the family Carcharhinidae, and this body shape allows them to move swiftly trough the water.

As its name implies, the upper part of the body is grayish-slate or dark gray in color, the belly is usually off-white or yellowish white; on the flanks, the line of contact between the two shades crosses the head at eye level (slightly above or below) and runs shaded in the center of the flanks,

along the animal's caudal peduncle the line become shaper and more defined (*specieaspim.it; Compagno, 1984; Ebert et al. 2013*).

There are two dorsal fins: the anterior one is large and tall and has a concave posterior margin. It has a triangle-like shape with a rounded apex, a free flap at the base of the fin.

The second dorsal fin is considerably smaller than the former, and it is inserted on the back at the ventral insertion point of the anal fin. The dimensions of the second dorsal are in fact very similar to those of the anal fin.

The latter has a distinctive form and is little bigger than the second dorsal fin. Its posterior border is internally angled, creating a dimple in the middle and an apex that points posteriorly. The anal fin is placed near to the pelvic fins, which are tiny and have a trapezoidal form; they feature a free flap that is more or less evident. The caudal fin is asymmetrical: the top lobe is about three times as long as the lower lobe and has a subterminal incision. The lower lobe is long and inclined downward posteriorly. This type of shape helps the animal move quickly and agilely. The pectoral fins are relatively elongated, highly falcate, and wide. They enter the flanks just behind the fourth and fifth gill openings

(*specieaspim.it; Compagno, 1984; Last et al., 2010; Ebert et al. 2013; Rigby et al., 2021*) (Fig. 1.12).

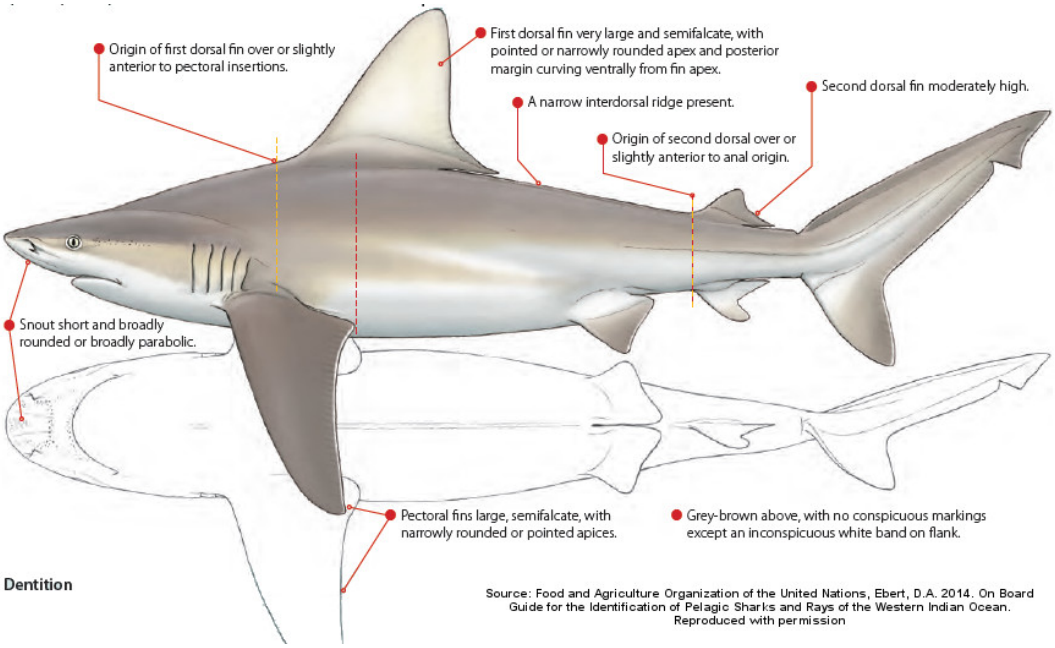


Fig. 1.12 *Carcharhinus plumbeus* morphology (FAO: *www.fao.org*)

This type of shark has a brief, rounded snout, large eyes with a contractile pupil and nictitating membrane; this species lacks the spiracle, and the five gill slits are straight (not curved) and perpendicular to the animal's longitudinal axis, at the base of the pectoral fin, the final one or two overlap.

The ventral portion of the head's outer margins is where the nostrils are situated. The nasal blades of this species are quite short and poorly formed.

Wide and forming a rounded arch, the mouth is slightly regular and has prominent labial grooves (*specieaspim.it; Compagno, 1984; Ebert et al. 2013; White et al., 2017*).

The oral cavity houses the upper and lower dental arch, both of which have approximately thirty teeth each; the upper teeth are generally triangular, laterally compressed and flattened, making them broader along one of the axes. They also have a cusp that is inclined on one side and a blade along the edges. The lower teeth are noticeably smaller than the upper teeth, although they are nonetheless flattened, have an extended cusp with a thin base, are pointed and symmetrical (not inclined), and have a finer blade than the upper teeth (Fig. 1.13) (*Bergam et al., 2017; Compagno, 1984; Compagno, 1999)*.

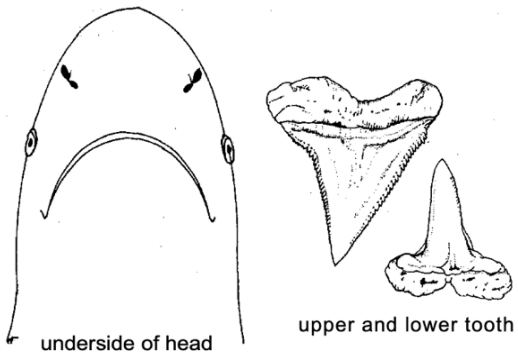


Fig. 1.13 Representation of the ventral portion of the head and the two different types of teeth of a *Carcharhinus plumbeus* (Compagno, 1984).

HABITAT AND GEOGRAPHICAL DISTRIBUTION

The sandbar shark, *Carcharhinus plumbeus*, is a species that can adapt to a variety of marine environments, and it is found all around the world (*Consoli et al., 2004; De Silva et al., 2006; White et al., 2006; Dragičević et al., 2010; Last et al., 2010; Sutaria et al., 2015; Rigby et al., 2021*).

The Sandbar Shark is a demersal and pelagic shark found in tropical and temperate seas on the continental shelf from near inshore to a depth of 280 meters.

The Atlantic, Indian, and Pacific oceans are all part of its wide geographic range (Fig. 1.14). It may be found all over the world, including Europe, South Africa, Australia, Mexico, and Brazil. The ideal temperature range for these sharks is typically 22 to 27° C (*Ebert et al., 2013; Sutaria et al., 2015; SEAFDEC, 2016; Weigmann, 2016; Hylton et al., 2017; White et al., 2017; Rigby et al., 2021*).

Fig. 1.14 Geographical distribution of *Carcharhinus plumbeus* (*Rigby et al., 2021*).

REPRODUCTIVE BIOLOGY

Carcharhinus plumbeus reaches sexual maturity relatively late, it takes between 10 and 12 years for males and 12 to 14 years for females to attain sexual maturity. Reproduction is viviparous, which implies that the

embryos grow inside the mother and give birth as fully developed pups (*Joung and Chen 1995; Baremore and Hale 2012; Rigby et al., 2021*).

Typically, a pregnancy lasts between ten and twelve months, furthermore, where the sharks are located and live affects how long the pregnancy lasts.

Giving birth often takes place between March and August in the boreal hemisphere and between (October) December and February in the southern hemisphere.

Calving usually takes place in shallow waters: female usually seek out for nurseries where to raise their young, where they are properly safe from attacks by sand shark predators. The number of puppies in a litter varies but is typically between 4 and 14; just hatched puppies range in size from 40 to 70 centimeters. The cubs have the ability to care for themselves and leave their mother after birth (*Cliff et al., 1988; Hazin et al., 2007; McAuley et al., 2007; Geraghty et al., 2016; Rigby et al., 2021*).

The age at first maturity for females ranges from 8 to 16 years, yet the oldest specimens documented were between 21 and 27 years (*Casey et al., 1985; Sminkey and Musick 1995; Joung et al., 2004; Romine et al., 2006; Hale and Baremore, 2013; Geraghty et al., 2016*).

From the time of birth until late fall, juvenile individuals have an aptitude to stick around in shallow waters. They gather after this second period and

swim out to deeper and warmer waters. Sharks return to shallower and colder waters, where they first appeared when the warm season begins. For around five years of their lives, juveniles make these migratory movements. These are short-lived movements that do not last as long as real migrations (*specieaspim.it; Nelson et al., 2016; Saïumldi et al., 2005*).

FEEDING ECOLOGY

Carcharhinus plumbeus mostly consumes a wide range of marine species: it mostly eats crustaceans, small fish, and squids, but the species was also observed to feed on little sharks. It could consume food at any time of day, although it seems to be most active at night (*McElroy et al, 2006; Ellis and Musick, 2007*).

Its diet changes based on the types of prey that are available in its environment. Grey sharks may adapt to various forms of feeding depending on the situation and are typically thought to be an opportunistic predator (*McElroy et al, 2006; Ellis and Musick, 2007; Nelson et al., 2016*).

Numerous benthic fish species, including mullets, groupers, croakers, moray eels, snakefish, fish of the genus *Trichiurus*, the tusk fish *Brosme brosme*, barracudas, sea breams, soles, plaices, toadfish of the family Batrachoididae, sea catfish of the family Ariidae, and porcupine fish, are

found in its diet (*McElroy et al, 2006; Ellis and Musick, 2007; Nelson et al., 2016*).

The species also consumes other cartilaginous fish such as small hammerhead sharks (*Sphyrna tiburo*), eagle rays (*Rhinoptera bonasus*), spiny dogfish (*Squalus acanthias*), skates, and rays of various species (*McElroy et al, 2006; Ellis and Musick, 2007; Nelson et al., 2016*).

IUCN CLASSIFICATION, THREATS AND CONSERVATION STRATEGIES

Due to excessive fishing practices and habitat loss, the conservation status of this species has been classified as Endangered by the International Union for Conservation of Nature (IUCN) since 2007, both in the global (*Rigby et al., 2021*) and Mediterranean (*Ferretti et al., 2016*) assessments, and its population has drastically decreased in several regions of the world (Fig. 1.15).

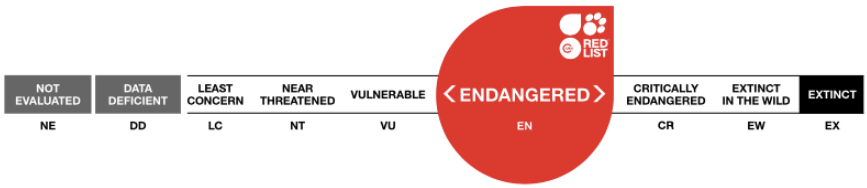


Fig. 1.15 IUCN classification of *Carcharhinus plumbeus* (IUCN).

The species is caught for its high-value flesh and fins in artisanal, commercial, and recreational fisheries. Worldwilde unless restrictions

forbid retention, the species is retained as target and bycatch. Population trend statistics specific to species indicate population declines of more than 50% and more than 80% in South Africa and the United States, respectively, during the last three generation lengths (60–78 years) (*Rigby et al., 2021*).

The species was quite abundant and widely distributed in the southern Mediterranean and the Levant Sea, and largely documented in the Sicilian fish markets in the 80s. However, the sandbar shark strongly decreased by 50–79% over the last three generation lengths (60–78 years) in the Arabian and Mediterranean Seas, and exploitation by fishery is the cause of these dramatic reductions (*Rigby et al., 2021*).

Plans for management and conservation have been created to save this species. In a few nations, there are management strategies specifically designed for *C. plumbeus*. For example, the Atlantic Fishery Management Plan for Consolidated Highly Migratory Species made the fishery of sandbar sharks illegal in US Atlantic seas in 2008, except for the research purpose. Prohibited species must be returned to the water as soon as possible, to reduce potential harm on them (*sedarweb.org; Rigby et al., 2021*).

The International Plan of Action for the Conservation and Management of Sharks (IPOA-Sharks), which is a component of the Code of Conduct for Responsible Fisheries, was developed by FAO in 1999. The IPOA-Sharks highlights that the harvesting of chondrichthyan fishes must be regulated to guarantee biodiversity conservation and the preservation of ecosystem structure and function. It must also be economically viable, ecologically sustainable, and utilize all the deceased sharks' body parts (*Bradai et al., 2012*). Signatory countries are required under this action plan to develop and implement a National Plan of Action for the conservation and management of sharks (*Bradai et al., 2012*).

The United Nations Environment Programme (UNEP) has developed an exclusive Plan of Action for the conservation of cartilaginous fish in the Mediterranean (*Bradai et al., 2012*).

The IUCN Centre for Mediterranean Cooperation, the Shark Specialist Group, and the contracting parties to the Barcelona Convention collaborated on the creation of this strategy, which was approved in November 2003. Since it is the first regional plan of action on sharks that UNEP has created, this is a highly important step.

Carcharhinus plumbeus is one of the numerous protected shark species, whose exploitation is to be limited, that have been included to the list in

Annex III of the Barcelona Convention (approved in 2009) (*Bradai et al., 2012*).

The sandbar shark continues to contribute to an important percentage of elasmobranch bycatch, notably in the Adriatic, despite the previously mentioned programs (*Bradai et al., 2012*).

A better understanding of the species' diet would be required in addition to its preferred places to reproduce and its nursery areas, since this would enable the development of more effective protection measures to save this endangered species. This would make it possible to protect foraging sites or even limit/prohibit fishing on this species.

1.2 Stomach content and stable isotope analysis

The stomach content analysis (SCA) has historically been employed for investigating the trophic ecology of marine organisms (*Hyslop, 1980; Cortés, 1999; Barria et al., 2015*); such a type of analysis has drawbacks, including the possibility that the results could be altered by the high frequency of empty stomachs and the fact that many items found in stomachs are among the hardest to digest, and thus they are overestimated.

Furthermore, a significant number of stomachs must be analyzed to gather accurate information on eating patterns: obtaining a significant quantity of samples for uncommon, threatened, and protected species as some elasmobranchs would be challenging and negatively affect the species.

In addition to the investigation of stomach contents, the analysis of the stable isotopes of nitrogen and carbon has been employed, in the last forty years, to explore the trophic ecology of marine organisms (*Estrada et al., 2006; Hussey et al., 2010; Shiffman et al., 2012; Fanelli et al., 2023 and references cited therein*).

For evaluating nutritional and trophic connections in marine environments, stable isotopes analysis (SIA), more commonly of nitrogen and carbon, can address multiple drawbacks of SCA: as SIA reflects assimilate food, fewer samples than for SCA are needed. Moreover, for SIA small amounts of tissue are necessary, and thus they can be taken through biopsies (by using punch) and thus avoiding sacrificing the animal. However, this technique cannot be used to identify individual prey. Furthermore, as the isotopic values of predator reflect those of assimilated nutrients from consumed prey integrated over extended time periods, this approach allows to gain information on long-term nutrition (*Domi et al., 2005; MacNeil et al., 2006; Shiffman et al., 2012*). SIA

therefore contributes to a deeper comprehension of a species' function in the ecosystem, which could end up in management and conservation interventions (*Hussey et al., 2012*).

The biochemical principle at the base of the use of SIA in dietary studies is that the heavier, rarer isotope is retained throughout ordinary metabolic processes while the lighter, more prevalent isotope is eliminated. Specific heavy-to-light isotope ratios that allow one to deduce the food and/or habitat a species rely on, are indicative of resource utilization (*Fry, 2006; De Necker, 2017*).

Carbon and nitrogen are the two stable isotopes that are most-commonly used, respectively $\delta^{13}C$ ($^{13}C/^{12}C$) e $\delta^{15}N$ ($^{15}N/^{14}N$) (*France 1995; Vander Zanden et al., 1999*). The origin of the digested organic matter, whether pelagic or benthic, marine or terrestrial, is identified using the $\delta^{13}C$. At each trophic level, the $\delta^{13}C$ increases by 0 to 1‰. In general, more positive $\delta^{13}C$ values are indicative of benthic food sources, while more negative ones point out to pelagic sources (*Wada et al., 1991*).

The trophic level of a species is assessed by the $\delta^{15}N$, which displays an enrichment of ca. 3–4 ‰ at each level. Herbivores display lower $\delta^{15}N$ values than carnivorous predators, and thus in general, the lowest the value

of a species the closest it is to the basal source (*DeNiro and Epstein, 1978; Fry and Sherr, 1984; Wada et al., 1991; McCutchan et al., 2003)* (Fig. 1.16).

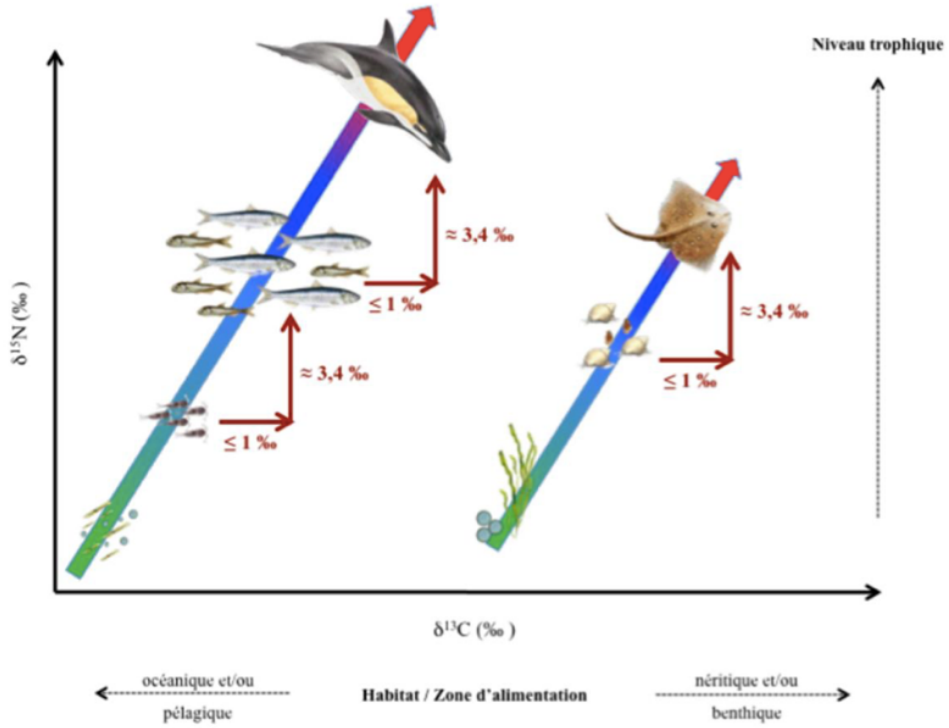


Fig. 1.16 A schematic diagram of the use of $\delta^{13}C$ and $\delta^{15}N$ for the reconstruction of marine food webs (pelagic on the left and benthic on the right). The red arrows represent the trophic enrichment factors for $\delta^{15}N$ (vertical arrows) and $\delta^{13}C$ (horizontal). The benthic food web represented by the ray as apical predator is characterized by enriched $\delta^{13}C$ values, while the pelagic one, with the dolphin as apical predator presents more depleted $\delta^{13}C$ values. Primary producers (both benthic and pelagic) show low $\delta^{15}N$ values while predators (dolphin and ray) high $\delta^{15}N$ values.

The analysis of the stable isotope contents in inert tissues like vertebrae appears to be useful in revealing dietary changes and/or habitat use as a

result of ontogenetic and/or sexual changes in the animal, making it possible the determination of the trophic level of each specimen and serving as chemical tracers for migration over large distances or between different environments, such as estuaries *vs*. open sea (*Fisk et al., 2002; Estrada et al., 2003; Domi et al., 2005; Estrada et al., 2006; Kerr et al., 2006; Wolf et al.,2009; Kim et al., 2012*).

For elasmobranchs, the soft tissues used for SIA include muscle, whole blood, red blood cells, plasma, and liver (*Hussey et al., 2012*). Given that most elasmobranchs grow more slowly than teleosts do, muscle tissue is one of the most frequently used tissues because it represents an integrated long-term measure of the feeding habits and habitat use. Additionally, muscle tissue from several individuals of various sizes, sexes, or maturity stages can be sampled to offer a comprehensive understanding of the trophic ecology of a species throughout its lifespan (*Papastamatiou et al., 2010; Abrantes and Barnett, 2010; Borrell et al., 2011; Hussey et al., 2010 and 2012*).

Chapter 2. Aim of the study

The aim of the present study is to define the diet of *Carcharhinus plumbeus* through the analysis of young-of-the-year specimens that have been collected as bycatch in the northern Adriatic Sea, through the integration of SCA and SIA.

Moreover, through the analysis of the vertebral center, which include the birth mark and thus represent the signature of the mother, we also try to identify the area where the mothers of the specimens analyzed come from and thus the habitat use of adult sandbar shark in the northern Adriatic sea. This last objective will be also achieved through the comparison of the isotopic signals of muscle and vertebrae's core.

Chapter 3. Materials and Methods

3.1 Study area

3.1.1 Adriatic Sea

The Adriatic Sea is a semi-enclosed basin between the Italian and Balkan peninsulas. Six countries face the Adriatic Sea: Italy, Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, and Albania, to the southeast, the Adriatic Sea is connected to the Ionian Sea through the Otranto channel.

The Adriatic Sea spans an area of roughly 135,000 km^2, orienting from southeast to northwest over a length of about 800 km and a width of around 150 km (*Orlic et al., 1992; Russo and Antegnani, 1996*).

Due to its hydrographic features, the basin may be split into three sub-basins: Northern, Central, and Southern Adriatic.

The maximum depth is 1233 m in the southern part, while the average depth is 252.5 m: 73% of the basin is shallower than 200 meters. The northern Adriatic has an average depth of 35 m (Fig. 3.1).

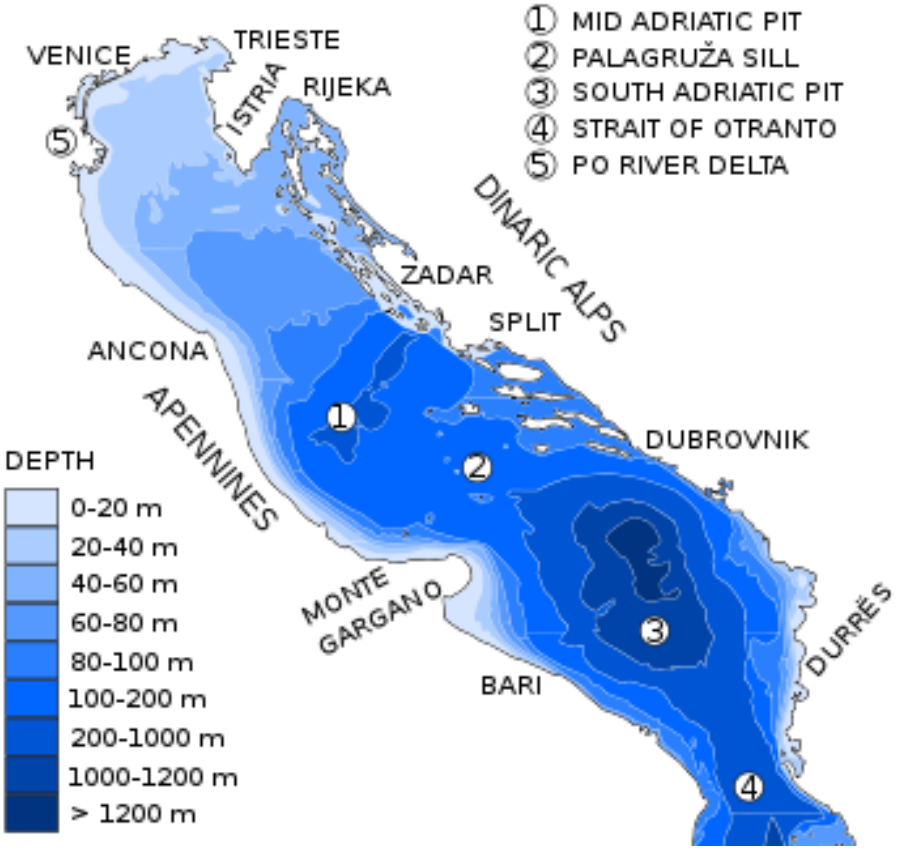


Fig. 3.1 Bathymetry of the Adriatic Sea and indications of major depressions indicated by (1) and (3) (Wikipedia).

The circulation of the entire Adriatic basin is cyclonic, with counterclockwise movement: the water current runs northward along the eastern shore of the Adriatic basin before traveling southward along the Italian side.

Sea surface temperatures of the Adriatic Sea fluctuate significantly from season to season and rises in the surface waters during the summer, ranging from 22°/25°C in summer to 7°/14°C in winter (*Kružić et al., 2012*).

The salinity is between 38 and 39 psu (practical scale unit): due to the freshwater intake from the Po and Adige rivers, it decreases in the Northern Adriatic and increases with depth.

The northern and central Adriatic Sea is characterized by a wide continental shelf and sandy-muddy bottoms in its western side. River inputs, mainly from the Po River, and water circulation control the sedimentology of the western basin, while rocky shores are dominant in the eastern one.

The FAO Area 37 for the Mediterranean Sea includes the Western (37.1), Central (37.2), and Eastern (37.3) Mediterranean subareas. The Adriatic Sea is divided into two GSAs (geographic subareas identified by the GFCM): the GSA17 indicate the North and Central Adriatic and the GSA 18 includes the South Adriatic, both correspond to the FAO sub-Division 37.2.1 (Fig. 3.2).

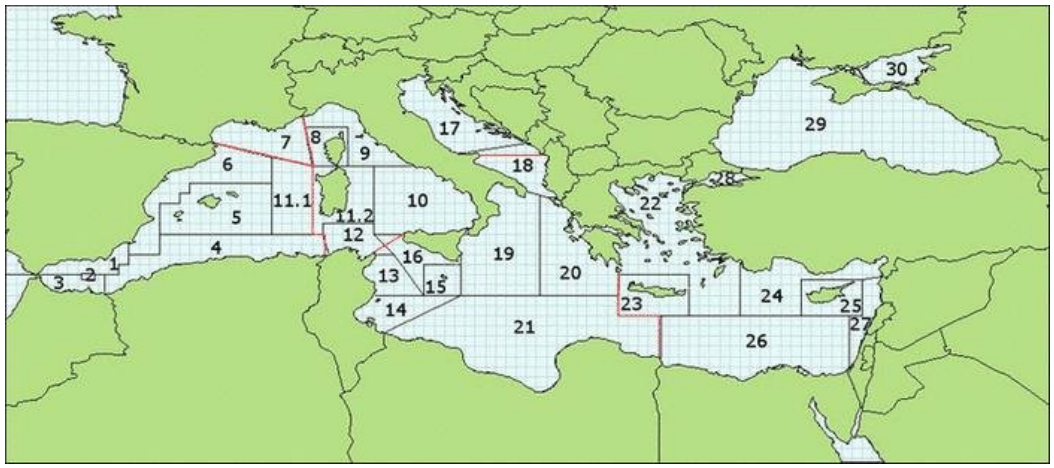


Fig. 3.2 Subdivision of the Mediterranean into GSAs according to the GFCM (*FAO*: *www.fao.org*).

3.1.2 Cervia – Marina di Ravenna ISRA (Important Shark and Ray Area)

Specimens used in this study were collected in the Northern Adriatic Sea, more precisely, the region of interest (Fig. 3.3) stretches 6 nautical miles from the coast, between the cities of Cervia and Marina di Ravenna (Emilia-Romagna, Italy). This area hosts an important parturition area for a threatened shark species, the sandbar shark *Carcharhinus plumbeus*, and due to this in 2023 it was declared an Important Shark and Ray Area - ISRA (*IUCN SSC Shark Specialist Group. 2023. Cervia-Marina di Ravenna ISRA Factsheet. Dubai: IUCN SSC Shark Specialist Group*).

Fig. 3.3 Map of the study area.

This region, which lies on the continental shelf of the northwestern Adriatic Sea, is characterized by shallow (from 0 up to 25 m depth) and productive waters (because of the presence of the Po River delta plume), and muddy-sandy bottoms.

The area is characterized by strong seasonal changes, which affects the migration patterns, distribution, and abundance of many marine species. Low salinity, low water temperature, and high productivity are all effects of the Po River plume (*Fonda Umani, 1996; Marini et al., 2008; Lipizer et al., 2014*) which occurs mostly in late autumn and winter.

Moreover, the study area is part of the Northern Adriatic Ecologically and Biologically Significant Marine Area (EBSA) established by the Convention of Biological Diversity (CBD 2023) and is close to a biological protection zone ("Zona di Tutela Biologica" ZTB) formed by a series of gas platforms and partially buried pipelines, where fishing activities are forbidden.

3.2 Samples collection

For sandbar sharks, the Cervia-Marina di Ravenna area is a key reproductive site. During the reproductive season, which runs in summer, from the (July to September, several newborns are recorded in this area.

Neonatal sandbar sharks and less Young-of-the-Year (YOY) sandbar sharks were recorded as bycatch of gillnet fisheries during investigations of small-scale fishery vessels operating in this area from 2019 to 2021 (Fig. 3.4). Many specimens (i.e., newborns) had an umbilical scar that was either freshly open or partially open.

Every year in July and August, data were gathered daily. Each year, the daily landings from a certain number of fishermen were examined. Overall, 20 specimens (19 newborns and 1 YOY) were collected in the region in 2019, 14 (13 neonates and 1 YOY) in 2020, and 17 in 2021 (Fig. 3.4).

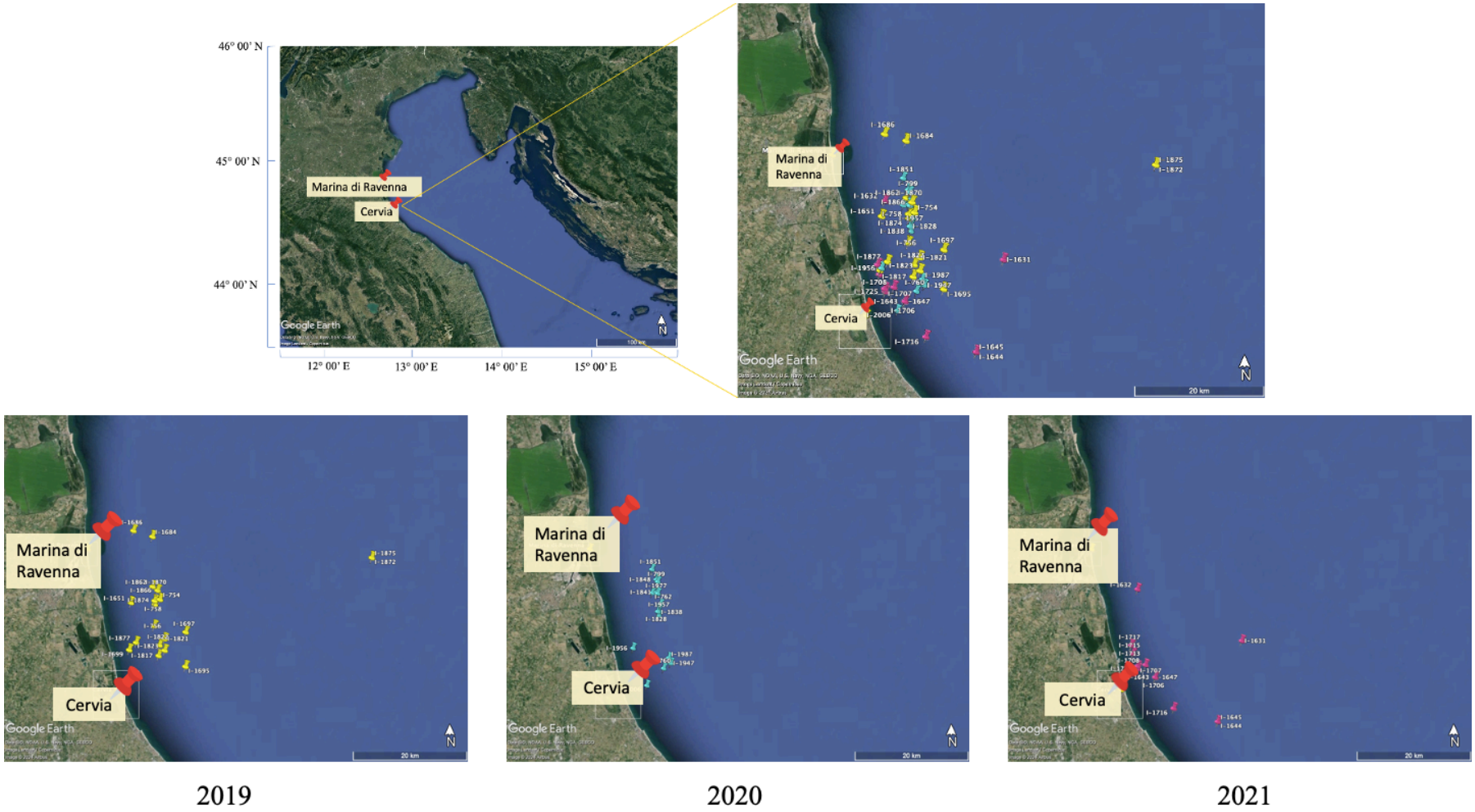


Fig. 3.4 Map of the capture area: pins represent all the individuals who had been captured between 2019 and 2021(Google Earth).

The collected specimens were all weighed and measured, more specifically the following information were recorded: total length (TL, cm), body weight and stomach weight.

For conducting stomach content analysis (SCA), each stomach was extracted from the body and immediately frozen till following laboratory analyses.

After collecting tissue samples from fins, muscles, and vertebrae from each specimen, these were prepared for subsequent stable isotope analysis.

3.2.1 SCA (stomach content analysis)

After the collection, the whole stomach of each specimen was weighed (stomach weight, or SW, in grams); next, the stomach' contents were removed, opened in a Petri dish) and the stomach's wall was weighed, to obtain, by subtraction the wet weight of the stomach contents (*Fanelli and Cartes, 2010*). Then, stomach contents were analyzed and identified to the lowest taxonomic level, as possible.

A visual estimation of the percentage of replenishment and the stage of prey digestion (on a scale 4-degrees scale from 1- poorly digested prey, to 4 - prey almost totally digested), have been assessed for each open stomach.

3.2.2 SIA (stable isotopes analysis)

Stable isotope analysis is believed to be an excellent approach for assessing diet as, despite not being able to directly identify prey, it provides information on long-term nutrition and a deeper knowledge of a species' role within the ecosystem *(Domi et al., 2005; MacNeil et al., 2006; Hussey et al., 2012; Shiffman et al., 2012)*.

Analysis of the isotopic composition of multiple tissues that have different turn-over time frames is typically related to one another, as the present study depicts *(Caut et al., 2013)*.

The tissue samples were originally divided into three pieces to provide a total of three replicates for the isotopic analysis of the muscles and fins.

The first replicate (R1) was selected for the procedure to be used for the subsequent investigation, while the other two replicates (R2 and R3) were stored for potential future examination.

The harvested muscle was then dried for a period of 24 hours at 60 °C (*Fanelli et al.,2011*); after drying, it was ground into a fine powder using a mortar and pestle (Fig. 3.5).

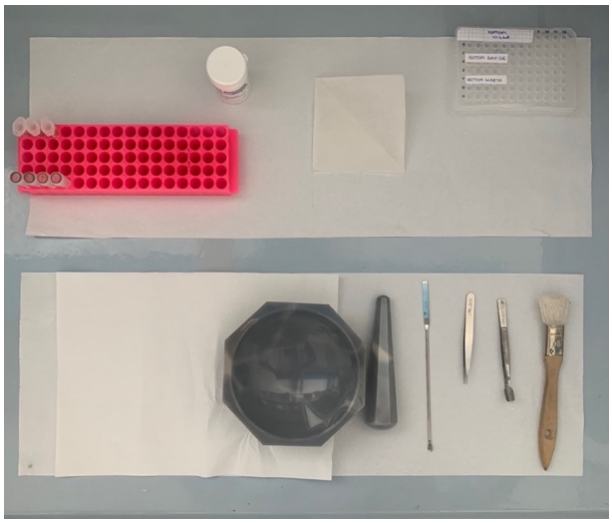


Fig. 3.5 Workstation with numbered racks and tools.

Before each usage and in between samples, deionized water was used to clean the different equipment. Using a precise balance (10^{-5} g), approximately one microgram (0.7 micrograms at the lowest and 1.2 micrograms at the most) of powdered tissue

was measured and put into tin capsules (Fig. 3.6, 3.7 and 3.8).

Fig. 3.6, 3.7 and 3.8 Stage of filling the tin capsule and weighing in the scale to five decimal places.

After being filled, the capsules were properly sealed and stored in a numbered rack at -20°C. Then, capsules were automatically loaded into an Elemental Analyzer (Thermo Flash EA 1112) coupled, through a continuous-flow, with an isotope ratio-mass spectrometer (IRMS, Thermo Delta Plus XP) at the University of Palermo's lab of Stable Isotope Ecology, for the determination of the %TN and %TOC (through the Elemental Analyzer) and of $\delta^{13}C$ and $\delta^{15}N$.

The ratio of stable isotopes has been expressed, in relation to international reference standards (atmospheric N_2 for $\delta^{15}N$ and PeeDee Belemnite for $\delta^{13}C$), as $\delta^{13}C$ or $\delta^{15}N$: $[(R_{campion}/R_{standard})] *10^3$

where R = $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

3.2.3 Vertebrae analysis

One of the main activities carried out in this thesis was also the setting up of a protocol for sample processing using a Micromill (Fig. 3.9).

Fig 3.9 Micromill MM2 device

Since vertebrae reflect the unique characteristics of organisms throughout their lifespan, by analyzing them we can identify ontogenetic variations in the diet; they also possess a high concentration of organic compounds and are metabolically inactive (*Calliet et al., 1986; Campana et al., 2002*).

Thanks to the concentric banded growth, it is possible to identify the "core" of the vertebra, which is indicated by the "birth mark," a little groove or protrusion; when analyzed, the birth mark yields information about the mother's isotopic fingerprint (*Carlisle et al., 2015*; Fig. 3.10 and 3.11).

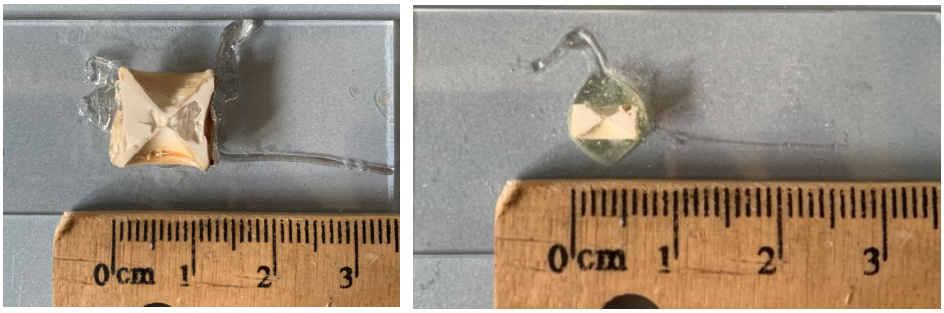
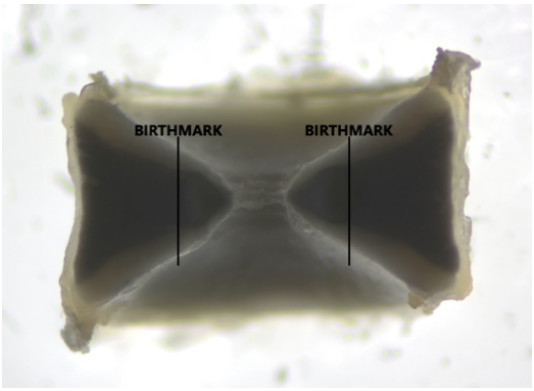


Fig. 3.10 Image of the vertebra of a specimen of *Mustelus mustelus* compared to the vertebra of *C. plumbeus* from the samples examined, along with a measurement of comparison.



3.11 Vertebra of *C. plumbeus* with birthmark identification.

As they directly reflect the isotopic composition of prey, each of the different development bands are the consequence of the deposition of cartilaginous material by exogenously acquired nutrients. They indeed represent a direct reflection of the prey's isotopic structure (*De Niro and Epstein, 1978 and 1981; Schoeninger and De Niro, 1984; Fry, 1988*).

However, as elasmobranchs possess a cartilaginous endoskeleton partially calcified (hydroxyapatite), this might affect the isotopic ratio (*Porter et al., 2006*). Consequently, hydroxyapatite must be removed from the sample to examine the inorganic fraction of carbon and nitrogen since it has a distinct isotopic signature.

To protect the integrity of collagen, EDTA (ethylenediaminetetraacetic acid) is often employed for this purpose (*Tuross et al, 1988; Koch, 2007*) instead of the more classic HCl that is used to remove inorganic carbon from muscle samples A (*Tuross et al, 1988; Kim and Koch, 2012*).

Thus, For SIA on vertebrae, first each vertebra was dissected along its longitudinal axis, then vertebral samples were processed as follows:

- Each sample (after being previously welded onto a slide with bicomponent glue) was examined under a microscope to determine the birthmark.
- After that, ca, 3 mg of material was collected from the vertebral core with a bistoury to recover.
- The obtained sample was gathered on the slide using a piece of weighing paper, put inside an Eppendorf, previously calibrated, and weighed to ensure that ca.3 mg was collected.

- 1.5ml of 0.25 M EDTA was added to the sample. The sample was left at room temperature from five to seven days (depending on the size of the sample), to demineralize it.

To remove inorganic carbon from vertebrae, EDTA treatment was modified based on the requirements of this investigation. Initially, 0.75 ml of the 0.50M EDTA solution were used alongside with 0.75 ml of deionized water according to the procedure.

Given the young age of the individuals analyzed and, consequently, the lower degree of calcification of the vertebrae, it was decided to decrease the molarity of the EDTA solution to 0.25M to perform a gentler decalcification based on the amount of material remaining at the end of the treatment week as by the original protocol of Kim and Koch (2012).

The removal of EDTA after the incubation time followed these steps:

- The sample was centrifuged for one minute at 8000–12000 rpm (rounds per minute), depending on the sample size. The necessary rpm increases with decreasing size.
- A glass Pasteur was used to remove the EDTA from the Eppendorf, leaving the sample at the bottom of the tube. Then1.5 ml of deionized water was added, vortexed first and repeated centrifuging while

retaining the prior settings. This procedure was continued to rinse with deionized water five to ten times (*Galindo-Rosado et al., 2023*).

- After that, the last rinse was accomplished using a glass Pasteur, and then all the water was removed.
- The sample was removed from the Eppendorf and put into a tin capsule that had been previously weighed.
- Finally, the opened capsule was placed on the isotope rack, and baked for about an hour.

3.3 Data analysis

3.3.1 Statistical analysis

To test whether the isotopic signals were different among the various tissues considered, a univariate PERMANOVA analysis *(Anderson et al., 2008*) was done with the factor "tissue" as fixed with three levels (corresponding to muscle, fin and vertebrae).

To test for differences among years and between sexes, a two-way crossed design with "year" (fixed with three levels, corresponding to 2019, 2020 and 2021) and "sex" (fixed, with two levels) was used. Then univariate PERMANOVA tests were performed on the Euclidean distance matrices of untransformed values of TL, δ^{15}N and δ^{13}C of fins and vertebrae.

Since muscle tissues were only sampled in 2019, a one-way design, considering only sex was used in this case.

After that, to test for differences on the diet of small sandbar sharks among years, a one-way PERMANOVA test was run on the "Modified Gower" resemblance matrix of presence/absence prey data, with "year" as fixed factor.

Significance was set at $p=0.05$ and p-values were obtained using 9999 permutations of residuals under "unrestricted permutation of raw data", in

case of the one-way design and "permutation of residuals under a reduced model", in case of the two-way design (*Anderson*, *2001*).

If some of the factors resulted significant, pairwise comparisons were carried out.

The software PRIMER6 & PERMANOVA+ was used to perform both univariate and multivariate statistical analyses (*Clarke and Gorley, 2006*).

3.3.2 Mixing models

Isotopic niche widths between and within communities have been assessed using the SIBER package (Stable Isotope Bayesian Ellipses in R 3.5.3) (*Jackson et al., 2011*) in R Studio (R Development Core Team 2021), and to determine the SEA (Standard Ellipse Area, p interval = 0.40 to include the 40% of our data), TA (Total Convex Hull Area), and SEA_C (SEA adjusted for limited sample size) (*Layman et al., 2007*) for each of the communities, i.e. the different groups of specimens analyzed (per years).

The TA gives an indication of the variety of food items on which the species can feed while SEA_C contains approximately 40% of the data within a set of bivariate data and thus represents the core area for a population or community (*Layman et al., 2007; Jackson et al., 2011*).

Layman metrics, which provide quantitative measures of the trophic structure of a community, were also calculated. Specifically, $\delta^{13}C$ range (CR), $\delta^{15}N$ range (NR), mean distance to centroid (CD) were obtained running SIBER (*Layman et al., 2007*), using R version 4.0.5 (*R Core Team, 2021*). CR provides information on the diversity of the resources at the base of the trophic web with higher values that indicate multiple basal carbon sources; NR gives information on the trophic length of the community and CD estimates trophic diversity within a food web and is a function of the degree of species spacing lower numbers indicate that distinct taxa are exhibiting similar ecological functions (*Jackson et al., 2011*).

Chapter 4. Results

4.1 Biological features of *Carcharhinus plumbeus* specimens

For the present study, 51 specimens of *C. plumbeus* of TL ranging from 42 cm to 90 cm were analyzed.

In 2019 and 2020, the percentage of captured males and females was similar, whereas in 2021, there is an increase in the percentage of captured males (Fig 4.1).

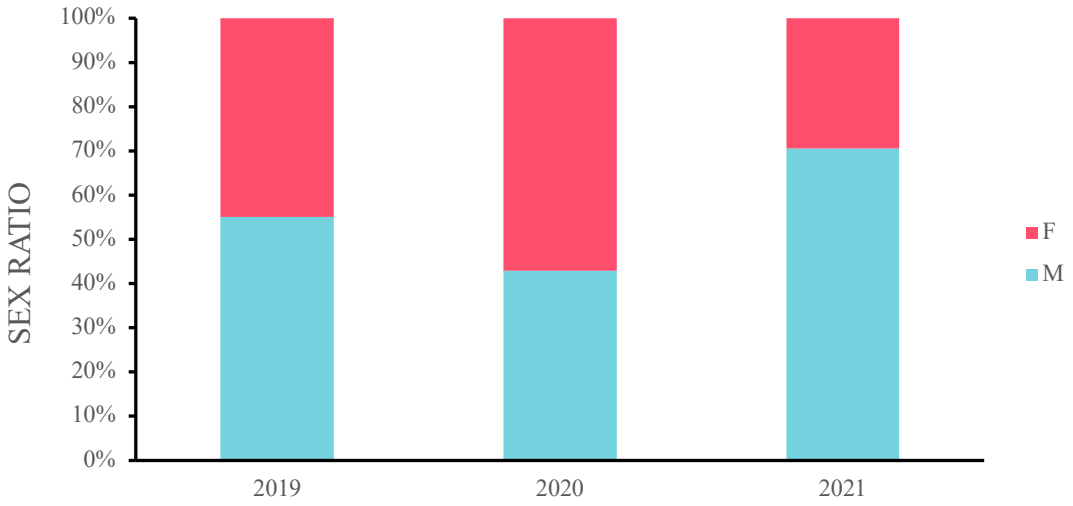


Fig. 4.1 Sex-ratio of *C. plumbeus* specimens collected in 2019-2021.

More precisely, of the 20 specimens taken in 2019, 11 were males and 9 were females, in 2020 6 males and 8 females were caught and, in 2021, 12 males and 5 females.

The total length (TL) distribution was similar between males and females and across years (Fig 4.2).

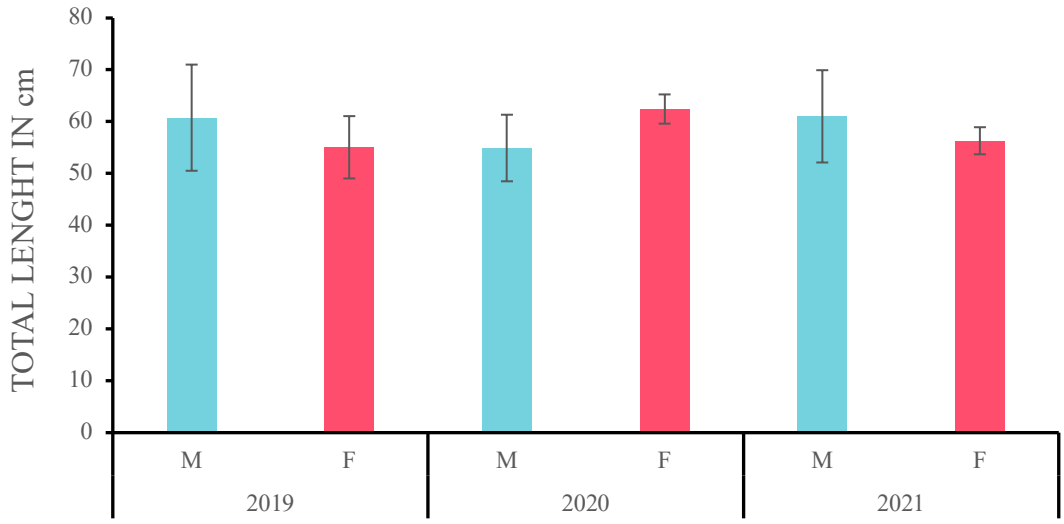


Fig. 4.2 Average length of male and female divided per year (pink columns represent female and the blue ones represents the male).

The univariate PERMANOVA carried out on TL showed significant differences only for the interaction term "year x sex" (Table 1a).

The pairwise comparison carried out on this factor showed that the TL of females varied significantly between "2019 *vs* 2020" and "2020 *vs* 2021" (Table 1b).

Table 1. Outcomes of the PERMANOVA main test (a) and pairwise comparisons (b) for TL. df = degrees of freedom; MS = mean square; Pseudo-F = statistic F; t = statistic t for pairwise comparisons; P(MC) = probability level with Monte Carlo test.

a) Source	**df**	**MS**	**Pseudo-F**	**P(MC)**
Year	2	22.96	0.63	0.53
Sex	1	0.84	0.02	0.88
Year × Sex	2	128.28	3.54	**0.04**
Residuals	43	36.20		
Total	48			

b)	**Within level 'M' of factor 'Sex'**	
Groups	t	P(MC)
2019 vs 2020	1.16	0.27
2019 vs 2021	1.03	0.32
2020 vs 2021	1.49	0.16
	Within level 'F' of factor 'Sex'	
Groups	t	P(MC)
2019 vs 2020	2.78	**0.02**
2019 vs 2021	0.44	0.67
2020 vs 2021	4.17	**0.002**

Once examining the individual weight data, the same general pattern as the TL data is evident, indicating that there is little variation in body weight between the sexes (Fig. 4.3). In 2019 and 2021, males are larger than females, while in 2020 females are larger than males**.**

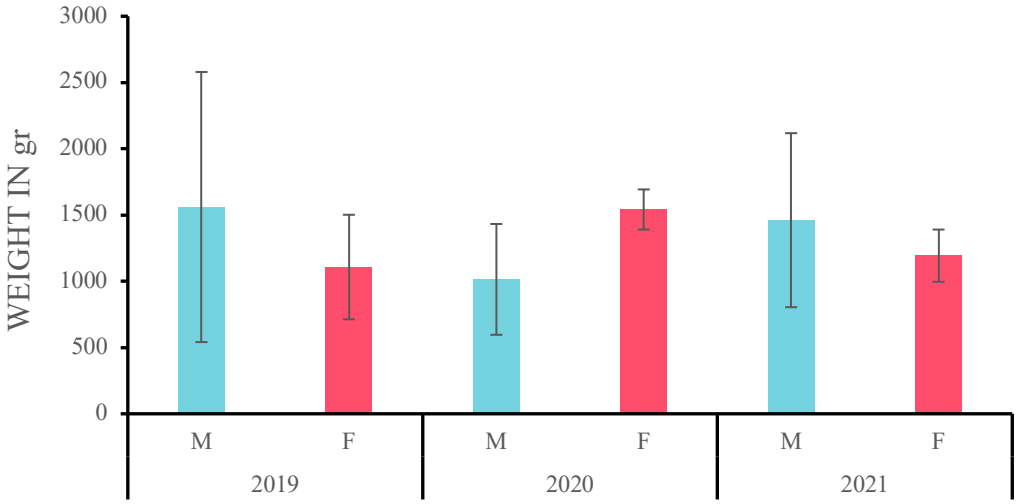


Fig. 4.3 Average weight of male and female divided per year (pink columns represent female and the blue ones represents the male).

Similarly to TL, also Weight significantly differed for the interaction term (Table 2a) and pairwise comparison carried out on this factor showed that the weight of females varied significantly between "2019 *vs* 2020" and "2020 *vs* 2021" (Table 2b).

Table 2. Outcomes of the PERMANOVA main test (a) and pairwise comparisons (b) for WEIGHT. df = degrees of freedom; MS = mean square; Pseudo-F = statistic F; t = statistic t for pairwise comparisons; P(MC) = probability level with Monte Carlo test.

a) Source	**df**	**MS**	**Pseudo-F**	**P(MC)**
Year	2	82566	0.44585	0.6
Sex	1	9196	4.97E-02	0.8
Year × Sex	2	6.07E+05	3.2804	**0.04**
Residuals	43	1.85E+05		
Total	48			

b)	**Within level 'M' of factor 'Sex'**	
Groups	t	P(MC)
2019 vs 2020	1.40	0.18
2019 vs 2021	0.87	0.39
2020 vs 2021	1.51	0.15
	Within level 'F' of factor 'Sex'	
Groups	t	P(MC)
2019 vs 2020	2.63	**0.02**
2019 vs 2021	0.45	0.66
2020 vs 2021	3.27	**0.01**

The length frequency distribution was unimodal with a peak around 60 cm for both sexes, except for few males larger than 70 cm (Fig. 4.4).

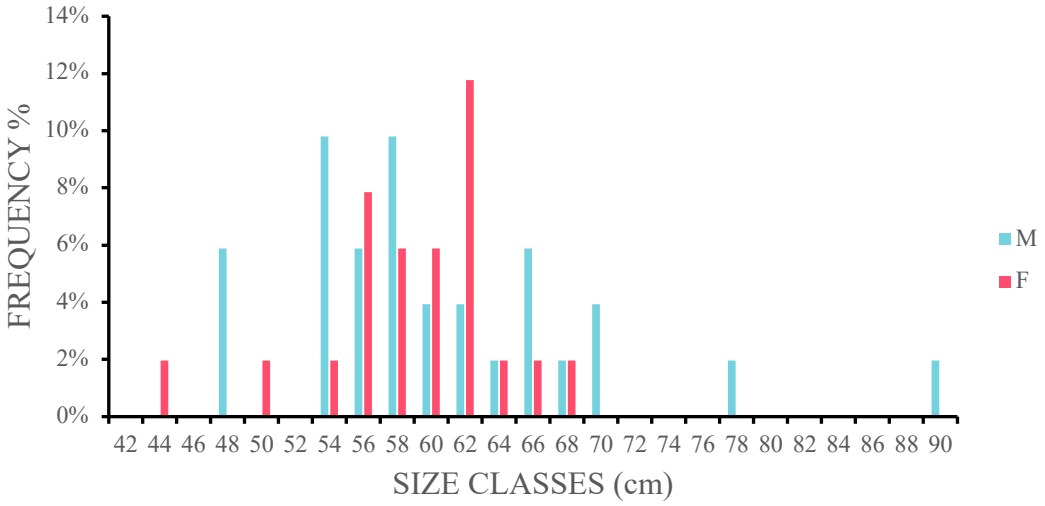


Fig. 4.4 Length frequency histograms (LFDs) for all the C. *plumbeus* organisms that were sampled (N = 51) (pink columns represent female and the blue ones represents male).

4.3 Differences in isotopic signature of the tissue analyzed

The δ^{15}N values of fin and muscle were higher than those of vertebrae, while the δ^{13}C values of vertebrae were generally more positive that those of fins and muscle (Fig. 4.5).

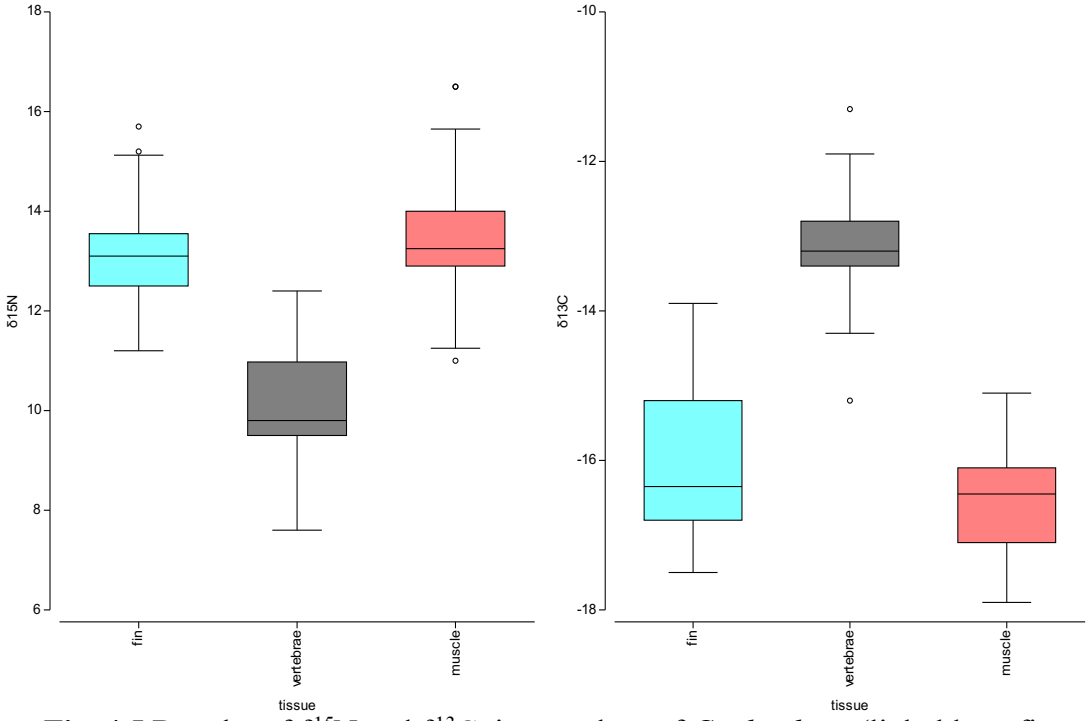


Fig. 4.5 Boxplot of δ^{15}N and δ^{13}C tissue values of *C. plumbeus* (light blue= fin, grey=vertebrae, red= muscle)

Univariate PERMANOVA analysis run on δ^{15}N and δ^{13}C values, separately, for factor “tissue" showed significant differences for both (Table 3a).

The pairwise comparison relative to both isotopic signals showed a highly significant difference between the signal relative to "vertebrae *vs*. fins" and "vertebrae *vs*. muscle" (Table 3b, Fig. 4.5).

Table 3. Outcomes of the PERMANOVA main test (a) and pairwise comparisons (b) for $\delta^{15}N$ and $\delta^{13}C$ of tissue values. df = degrees of freedom; MS = mean square; Pseudo-F = statistic F; t = statistic t for pairwise comparisons; P(MC) = probability level with Monte Carlo test.

a)

	$\delta^{15}N$			
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**
tissue	2	132.12	121.5	**0.0001**
Residuals	110	1.0874		
Total	112			

	$\delta^{13}C$			
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**
tissue	2	130.59	209.09	**0.0001**
Residuals	110	0.62456		
Total	112			

b)

	$\delta^{15}N$		
Groups	**t**	**P(perm)**	**P(MC)**
fin *vs* vertebrae	14.742	0.0001	**0.0001**
fin *vs* muscle	2.0252	0.0489	0.0473
vertebrae *vs* muscle	11.045	0.0001	**0.0001**

	$\delta^{13}C$		
Groups	**t**	**P(perm)**	**P(MC)**
fin *vs* vertebrae	17.695	0.0001	**0.0001**
fin *vs* muscle	2.0587	0.0455	0.0428
vertebrae *vs* muscle	20.542	0.0001	**0.0001**

4.4 Changes in stable isotope composition in *Carcharhinus plumbeus*

Regarding muscle tissues, samples were available only for 2019. For this year the mean values of $\delta^{15}N$ and $\delta^{13}C$ were 13.6 ± 1.4 ‰ and -16.6 ± 0.7 ‰, respectively.

Concerning muscle tissues and sex, the average values of $\delta^{15}N$ were 13.8 ± 1.3 ‰ and 13.4 ± 1.5 ‰ in males and females, respectively. The average values of $\delta^{13}C$ were -16.5 ± 0.8 ‰ and -16.7 ± 0.6 ‰ in males and females, respectively (Fig. 4.6).

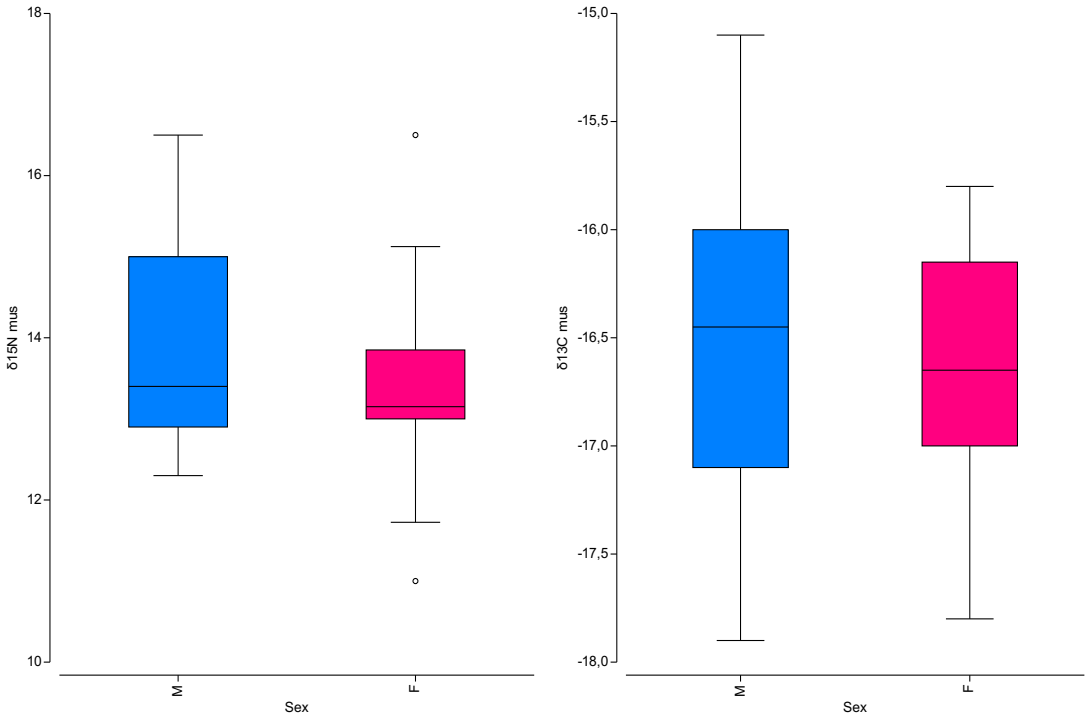


Fig. 4.6 $\delta^{15}N$ and $\delta^{13}C$ muscle values displayed in a box plot.

Univariate analyses were conducted on the $\delta^{15}N$ and $\delta^{13}C$ values of the different tissues, according to the one-way design described above for muscle and two-way for fins and vertebrae.

For muscle tissue, any significant difference was found for both $\delta^{15}N$ and $\delta^{13}C$ between sexes ($p>0.05$).

Regarding fins, the average values of $\delta^{15}N$ and $\delta^{13}C$ during the three years were 13.1 ± 0.9 ‰ and -16.0 ±1.0 ‰, respectively (Table 4, Fig. 4.7).

Table 4. mean ± standard deviation of $\delta^{15}N$ and $\delta^{13}C$ values of fins of *C. plumbeus* collected in 2019-2021 ± standard deviation.

		$\delta^{15}N$		**$\delta^{13}C$**	
YEAR	**SEX**	**MEAN**	**SD**	**MEAN**	**SD**
2019	M	12.9	0.4	-15.0	0.4
	F	12.6	0.5	-15.0	0.5
	ALL	12.8	0.5	-15.0	0.4
2020	M	13.4	1.1	-16.7	0.5
	F	13.1	1.1	-16.8	0.3
	ALL	13.2	1.11	-16.8	0.4
2021	M	13.0	1.2	-16.7	0.7
	F	13.3	0.5	-16.6	0.4
	ALL	13.2	1.0	-16.6	0.7

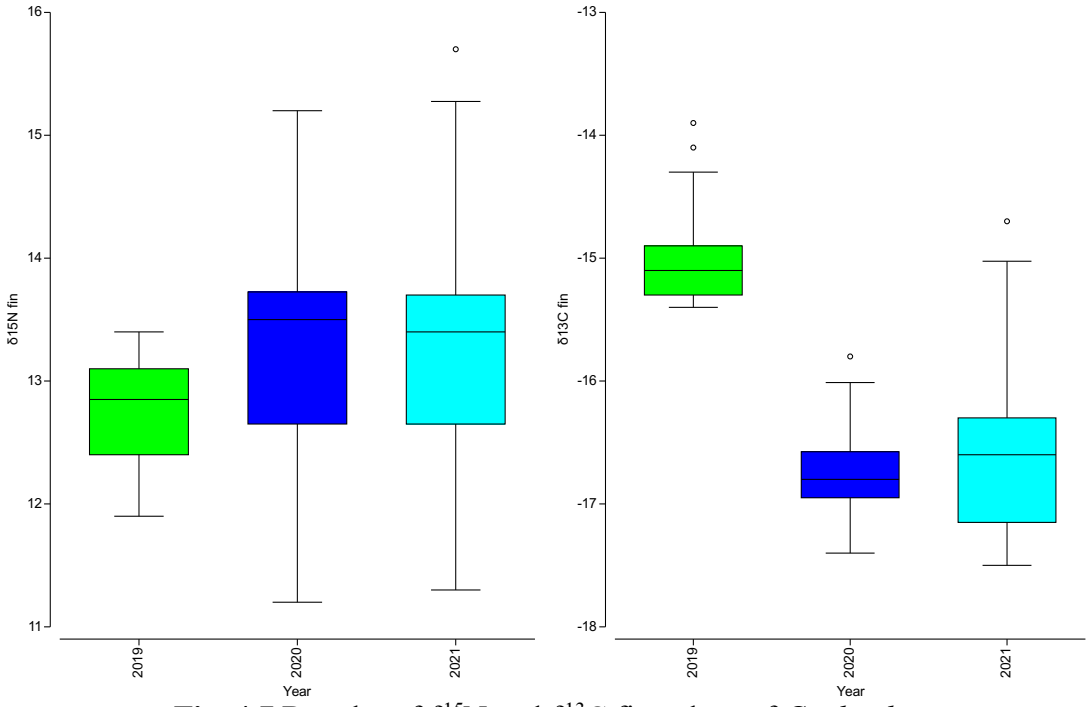


Fig. 4.7 Boxplot of δ^{15}N and δ^{13}C fin values of *C. plumbeus*

The univariate test on δ^{15}N values did not provide significant results for any of the factors tested. Conversely, the interaction term "year x sex" for the univariate PERMANOVA carried out on δ^{13}C values showed significant differences (Table 5a).

The pairwise comparison carried out on the “year” factor, showed significant values for the year 2019 (Table 5b).

Table 5. Outcomes of the PERMANOVA main test (a) and pairwise comparisons (b) for $\delta^{13}C$ of fin values. df = degrees of freedom; MS = mean square; Pseudo-F = statistic F; t = statistic t for pairwise comparisons; P(MC) = probability level with Monte Carlo test.

a)

Source	df	MS	Pseudo-F	P(perm)
Year	2	15.804	53.583	**0.0001**
Sex	1	0.01087	0.036855	0.8534
Year x Sex	2	0.053607	0.18176	0.8478
Residuals	42	0.29494		
Total	47			

b)

Groups	t	P(perm)
2019 vs 2020	10.877	**0.0001**
2019 vs 2021	7.9795	**0.0001**
2020 vs 2021	0.63001	0.5462

Concerning the isotopic composition of vertebrae, the mean $\delta^{15}N$ and $\delta^{13}C$ values were 10.1 ± 1.0 ‰ and -13.1 ± 0.6 ‰, respectively (Table 6, Fig. 4.8).

Table 6. mean ± standard deviation of δ^{15}N and δ^{13}C values of vertebrae of *C. plumbeus* collected in 2019-2021 ± standard deviation.

		δ^{15}N		δ^{13}C	
YEAR	**SEX**	**MEAN**	**SD**	**MEAN**	**SD**
2019	M	10.4	0.9	-12.9	0.4
	F	10.6	1.0	-13.2	0.2
	ALL	10.5	1.0	-13.0	0.4
2020	M	10.7	1.2	-13.4	0.4
	F	9.7	0.9	-13.2	0.4
	ALL	10.2	1.1	-13.3	0.4
2021	M	9.6	1.0	-13.1	0.9
	F	10.0	0.9	-13.2	0.6
	ALL	9.7	1.0	-13.1	0.8

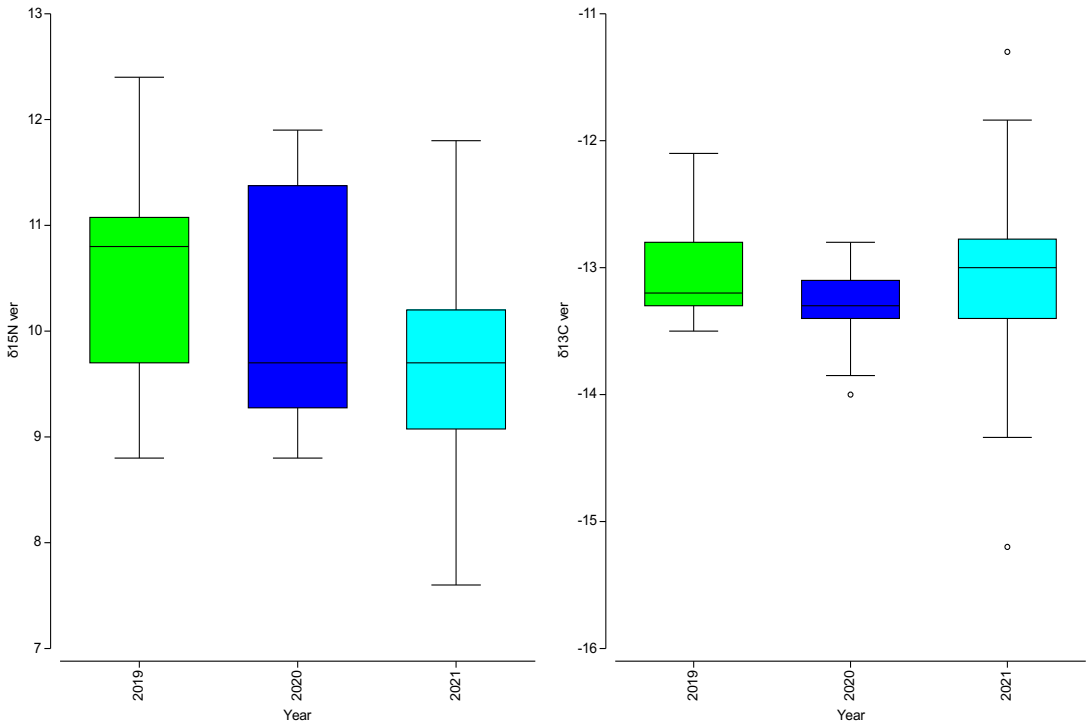


Fig. 4.8 Boxplot of δ^{15}N and δ^{13}C vertebrae values of *C. plumbeus*

The univariate analysis performed on the δ^{15}N and δ^{13}C values of vertebrae did not provide any significant results ($p>0.05$).

Then multivariate analyses were performed on the bivariate matrix of $\delta^{15}N$ and $\delta^{13}C$ values of fins and vertebrae, on the two-way design described above.

Concerning fins, a significant difference occurred in $\delta^{15}N$ and $\delta^{13}C$ values by year (Table 7a).

$\delta^{15}N$ and $\delta^{13}C$ values significantly differed for the interaction term and pairwise comparison carried out on this factor showed that the $\delta^{15}N$ and $\delta^{13}C$ values varied significantly between "2019 *vs* 2020" and "2019 *vs* 2021" (Table 7b).

Table 7. Outcomes of the PERMANOVA main test (a) and pairwise comparisons (b) for $\delta^{15}N$ and $\delta^{13}C$ of fin values. df = degrees of freedom; MS = mean square; Pseudo-F = statistic F; t = statistic t for pairwise comparisons; P(MC) = probability level with Monte Carlo test.

a) **Source**	**df**	**MS**	**Pseudo-F**	**P(perm)**
Year	2	16.773	15.239	**0.0001**
Sex	1	1.0511	0.95494	0.3579
Year x Sex	2	0.061722	0.056076	0.9924
Residuals	42	1.1007		
Total	47			

b) Groups	t	P(perm)	perms	P(MC)
2019 *vs* 2020	5.466	0.0001	9942	**0.0001**
2019 *vs* 2021	4.8236	0.0001	9942	**0.0001**
2020 *vs* 2021	0.35182	0.861	9945	0.8509

Layman metrics measured on fin isotopic values showed that specimens collected in the different years showed different values of the all metrics (Table 8). NR increased from 2019 to 2021, while CR values were more variable. TA and SEAc values also increased from 2019 to 2021, as well as CD (Table 8, Fig. 4.9). Additionally, while the 2019 SEAc is stretched along the x-axis, those of 2020-21 are stretched along the y-axis.

Table 8. Total Convex Hull Area (TA) and Standard Ellipse Areas corrected for small sample size (SEAc) values of *C. plumbeus*

	2019	**2020**	**2021**
TA	2.04	3.67	6.27
SEA	0.69	1.29	1.88
SEA_C	0.73	1.40	2.01
NR	1.50	4.00	3.90
CR	2.20	1.60	2.80
CD	0.58	0.91	0.88

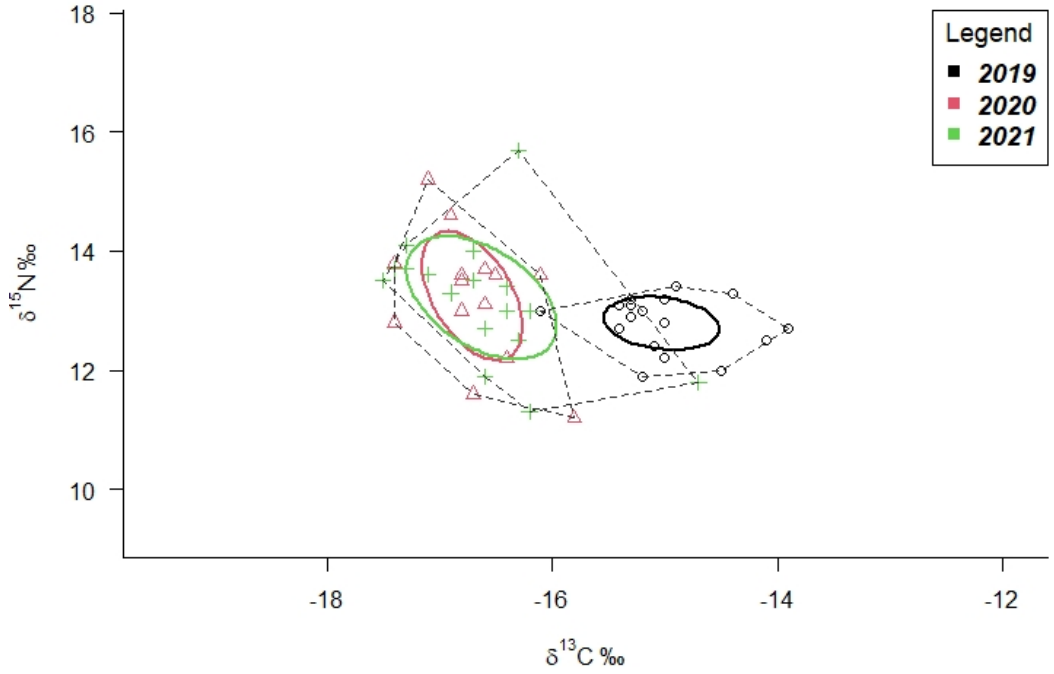


Fig. 4.9 δ^{15}N - δ^{13}C scatterplot with standard ellipses corrected for small sample size (p interval =40%) calculated on the contents

4.4 Analysis of diet composition based on SCA

Based on the stomach content analysis, the diet of *C. plumbeus* juveniles mainly rely on three phyla: Chordata, Mollusca, and Arthropoda.

Carcharhinus plumbeus mainly ate benthic fish, such as mullets and gobies, along with a few crustaceans, such as *Penaeus kerathurus*, and, to a slightly lesser extent, pelagic fish like *Sardina plichardus* (Fig. 4.10).

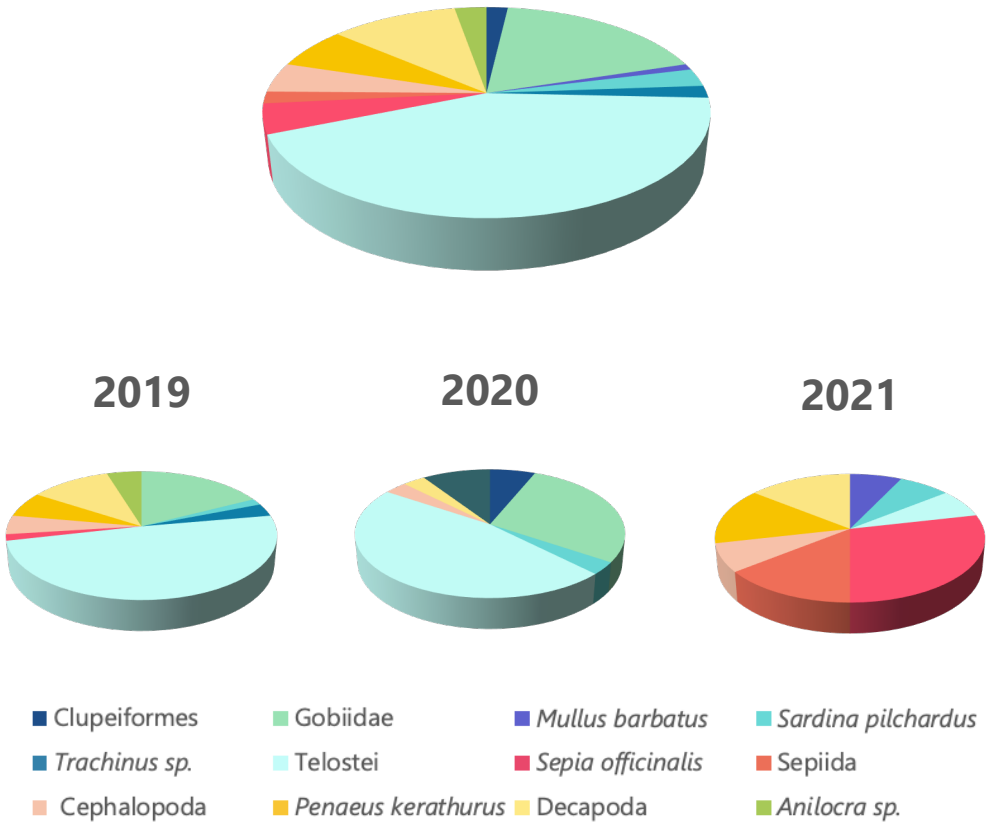


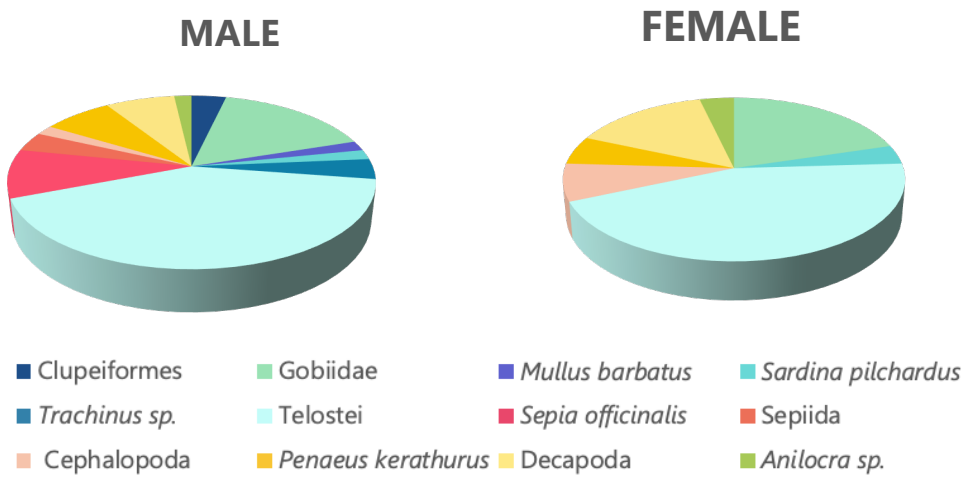
Fig. 4.10 (top) An overview of the prey identified in the stomach contents examined during the three-year study; (bottom) Percentage contribution of the different organisms that compose *C. plumbeus*'s diet in the three years.

Some changes occurred in the diet of the species during the three years.

Small bony fish accounted for most of the diet in 2019 and 2020, whereas the diet was more varied in 2021.

In 2021 sandbar sharks mostly consumed Mollusca species, with lesser amounts of organisms from the phylum Chordata and Arthropoda (which were not often included in the diet over the earlier two years).

The SCA conducted on males and females, separately indicated that small teleosts and gobies were ingested by both sexes, with a higher percentage of cephalopods (primarily *Sepia officinalis*) consumed by males, while decapods were mostly fed by females (Fig. 4.11).



4.11 Percentage contribution of different prey groups in the diet of males and females of *C. plumbeus*.

Chapter. 5 Discussion

5.1 Methodological approach

In this study, we needed to modify the protocol commonly used for the analysis of SIA in elasmobranchs vertebrae, proposed by Kim and Koch (2012), who suggested to use an EDTA solution at 0.5 M. Here, the protocol underwent modifications due to the lower degree of calcification of the vertebrae as a function of the young age of the captured individuals.

Because of this, we used an EDTA concentration of 0.25 M, which was proved to be more effective for treating such low-calcified tissue than the standard one (0.5 M).

Moreover, as documented in previous studies (*Hussey et al., 2012*), the isotopic signals of muscles and fins often have faster turnover rates than those of vertebrae, which are a more inert tissue. The results of the present study supported these findings; in fact, the analyses revealed that the values for the vertebrae differed more than those for the other two tissues.

5.2 Biological features of *Carcharhinus plumbeus*

This study investigated 51 specimens (newborns or Y-O-Y) of *Carcharhinus plumbeus* collected as by-catch by recreational fishers in the northern Adriatic Sea.

In this study, the TL range of the specimens collected ranged from 42 to 90 cm, and this confirms they were all newborns or Y-O-Y specimens, according to published studies (*Compagno, 1984; Cliff et al., 1988; Hazin et al., 2007; Geraghty et al., 2016; Rigby et al., 2021)*, stating that at hatching, pups can measure between 40 and 70 centimeters.

The average number of puppies produced by mothers is typically 4-14, with a sex ratio of 1:1 (*Joung, & Chen, 1995*), as confirmed by our findings with a sex ratio close to 1 during the whole sampling period.

5.3 The use of multiple tissues to highlight the trophic ecology of *Carcharinus plumbeus*

According to previous studies (*Kim et al., 2012; Di Lorenzo et al., 2020)*, the larger the size of a species the greater is its δ^{15}N value and thus its trophic level.

However, for different shark species, the relationship between δ^{15}N and TL is not always evident (*Kim et al, 2012*).

In this study any significant changes of δ^{15}N with size was observed, likely due to the small size of specimens analyzed, all belonging to newborns or Y-O-Y and thus possibly without any changes in the diet and habitat. Similarly, in other species of the genus *Carcharinus* (i.e. *C. longimanus*) a clear shift toward greater δ^{15}N values were observed later during the lifespan of the animal at ca. 7 years of age (*Shen et al., 2021*).

Similarly, δ^{13}C values did not change with TL, as the juveniles likely remain in the same, highly productive, area during their early life stages, while moving offshore later. Indeed, a study conducted on the co-generic species *Carcharhinus leucas*, although it can reach bigger sizes, reported a shift from mostly freshwater/estuarine diets to more marine diets with increasing length, with a loss of maternal signal in δ^{13}C values occurring at a length of about 100 cm (*Belicka et al., 2012*).

While any significant differences were observed regarding $\delta^{15}N$ values of *C. plumbeus* by sex and years for any of the tested tissues, $\delta^{13}C$ values were found to vary across years in fins but not in vertebrae.

The stable carbon isotope value provides information on the marine or continental nature of the carbon source, as well as whether it is more planktonic or more benthic (*Jennings et al., 1997; Rabehagasoa et al., 2012*). A more positive $\delta^{13}C$ (-13 to -17‰) reflects a species' reliance on the benthic food chain, while lower (more negative) $\delta^{13}C$ values, suggest a stronger dependence on a pelagic food web (*Jennings et al.,1997*).

Here we found significant difference between the $\delta^{13}C$ values of muscle and fin with those of vertebrae and the lack of difference in the $\delta^{13}C$ values of vertebrae across years. The first result suggested that while puppies and Y-O-Y inhabit and forage in the area where they have been captured (based on the $\delta^{13}C$ values of fin and muscle), their mothers (having analyzed the birth mark of the vertebrae, and thus the signatures of the mother), come from an area different from the parturition site (Cervia-Ravenna). The second result suggested that the mothers of the three generations' puppies (from 2019-2021) always come from the same area. The parturition site indeed is close to the Po River mouth, an area characterized by lower $\delta^{13}C$ values because of the strong continental input

(*Fanelli et al., 2022*) and the $\delta^{13}C$ values found in muscle and fins (fast-tun over) are compatible with the baseline of the northern Adriatic (*Fanelli et al., 2022*). On the other hand, the more enriched $\delta^{13}C$ values observed in the puppies' vertebrae were more aligned with the baseline signature observed in the Central Adriatic Sea (*Fanelli et al., 2022*).

Concerning the significant differences found in the $\delta^{13}C$ values of fins in 2019 vs. 2020-21, this can be explained by a change in the baseline from 2019 onward. Indeed, based on the average flow rates of the Po River (Fig. 5.1) over the three sampling years, an increase in river flows can be observed in 2019.

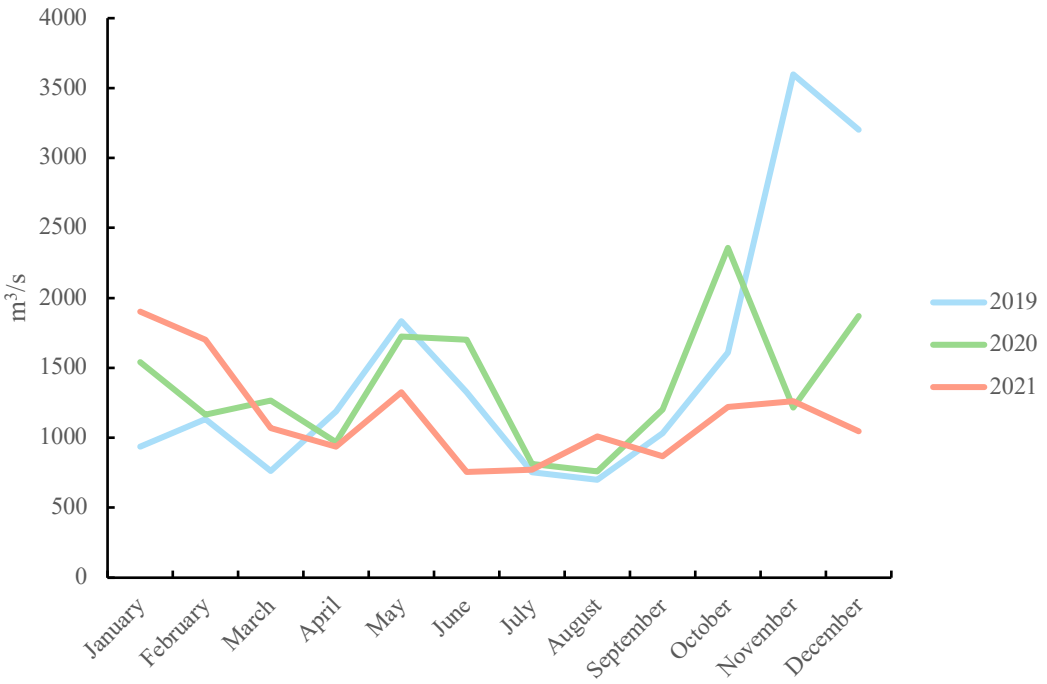


Fig. 5.1 Time trend of monthly average flow rates (cubic meters/second) of the Po River

Greater continental water input in 2019 likely affected the baseline, by depleting it, of the food web the 2020 and 2021 specimens rely on.

This is also supported by the Layman metrics, which showed greater TA and SEAc in specimens from 2020-21 compared to those of 2019.

This suggests that while specimens in 2019 probably rely on less variable food sources, those from 2020-21 depend on a wide array of basal sources characterized by a mix of terrigenous and marine inputs. Also, the CD values, as a proxy of trophic diversity, were greater in 2020-21 vs. 2019, confirming this high availability of prey, likely enhanced by greater nutrient inputs through the greater river flow recorded in 2019.

5.4 Diet composition based on stomach content analysis

Sandbar shark mostly consumes a wide range of marine species: fish (both Chondrichthyes and Osteichthyes) represent the primary food source, while crustaceans and cephalopods are secondary and sporadic prey.

Being an opportunistic species, the diet of *C. plumbeus* is dependent on prey that are easily available in its natural habitat. Dietary changes were noted and linked to specimen size (*McElroy et al, 2006; Ellis and Musick, 2007; Saidi et al., 2007; Nelson et al., 2016*).

In line with previous research (*Saidi et al., 2007; Nelson et al., 2016*), the diet of the specimens analyzed in this study was mostly composed of tiny bony fish, primarily benthic species, and cephalopods.

In this case study, significant variations in the diet had not been expected given the individuals' young age and geographic spread, nor for the year factor since food is probably always easily accessible being close to the Po river delta, an area characterized by high production level and nursery for several benthic fishes (*Bongiorni et al., 2018*).

Chapter 6. Conclusions

When this study began, in 2022, the area where the specimens of *C. plumbeus* here analyzed were collected, was not recognized as an essential fish habitat for the early life stage of the species, i.e. parturition and nursery. In 2023, the area of Cervia-Marina di Ravenna was declared as an Important Shark and Ray Area – ISRA, by IUCN.

However, the provenience of the mothers that arrived here, each year, to give birth is still unknown. Here, thanks to the use of multiple tissues for SIA, we may formulate some hypotheses that need further investigations to be confirmed:

1. The newborns and Y-O-Y live around the area of parturition and rely on similar food sources, belonging to the same trophic level, as ascertained by the lack of differences in the $\delta^{15}N$ values across years;
2. The mothers of these specimens do not live in this same area, as resulted by the more enriched $\delta^{13}C$ values of the vertebrae with respect to those of the other two tissues used, but probably inhabit more offshore bottoms in the Central Adriatic. The lack of differences in $\delta^{13}C$ values of vertebrae across years suggested that they all come from the same area;

3. There was a change in the baseline from 2019 to 2020-21 likely due to the greater river flow recorded in 2019 which affect the whole food web, as shown by the different $\delta^{13}C$ values of fins.

Further investigations, by tracking sandbar shark specimens and monitor their movements, together with eDNA analysis from opportunistic surveys such as the MEDITS, which cover the whole GSA17, are necessary to confirm the hypothesis on the provenance of adult females.

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