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New record of *Hexanchus griseus* in the northwestern Mediterranean Sea with insights into its biology and feeding ecology

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Abstract

On 15 March 2021, a male *Hexanchus griseus* was incidentally captured by a commercial bottom trawler at 550-730 m depth in the Gulf of Valencia (Spain). The specimen measured 251.8 cm in total length, weighed 86.2 kg and its reproductive system was fully developed, characterizing it as the smallest mature male bluntnose sixgill shark recorded in the Mediterranean Sea. The specimen hosted the ectoparasitic copepod *Demoleus heptapus*. Remains of a *Stenella coeruleoalba* calf were identified in its stomach content by applying molecular methods. The feeding behaviour of *H. griseus* as either actively preying on live cetaceans or as scavenging on carcasses is reviewed and discussed to better understand the species' role in marine food webs.

Keywords: bluntnose sixgill shark; reproductive biology; predation; scavenging behaviour; stomach content; striped dolphin.

Introduction

The bluntnose sixgill shark, *Hexanchus griseus* (Bonnaterre, 1788) is a widely distributed deep-sea shark species that inhabits worldwide temperate, tropical, and boreal waters, ranging from near-surface depths to at least 2500 m deep (Ebert, 1994). It is classified as a species of Least Concern in the Mediterranean Sea (Soldo *et al.*, 2016), but Near Threatened at a global scale (Finucci *et al.*, 2020). In the northwestern Mediterranean Sea, it is captured with low frequency in longlines and bottom trawls (Guijarro *et al.*, 2012; Nuez *et al.*, 2021), with the catch occasionally being commercialised but not considered in fisheries statistics (Barría & Colmenero, 2019). The species spends most of the daytime in deeper areas and undertakes vertical migrations towards shallower areas during night-time, influenced by foraging opportunities (Andrews *et al.*, 2009; King & Surry, 2017).

The diet and feeding habits of *H. griseus* have been studied throughout its distribution range, including the Mediterranean Sea, using both stomach contents and stable isotopes. Such studies suggest that it is an apex

predator, which forages on diverse prey taxa across marine food webs, including teleosts, cephalopods, marine mammals, elasmobranchs, and crustaceans throughout its range (Ebert, 1994; Celona *et al.*, 2005; Barría *et al.*, 2015; Reum *et al.*, 2020). Furthermore, resource partitioning has been detected between juveniles, which mainly target invertebrates and small fishes, and adults, which mainly feed on larger fishes and marine mammals (Ebert, 1994; Barría *et al.*, 2015). *Hexanchus griseus* is considered an active epibenthic predator that also scavenges opportunistically (Ebert, 1994; Andrews *et al.*, 2009). However, its capacity to actively prey on marine mammals remains uncertain (Ebert, 1994; Heithaus, 2001).

Material and Methods

On 15 March 2021, a male *H. griseus* was incidentally captured in the Gulf of Valencia (northwestern Mediterranean Sea) off El Grao de Castellón, Spain (39.60°N, 0.42°E), by a commercial bottom trawler

operating at 550-730 m depth. The specimen was landed dead on board and kept for further scientific examination. Total weight was recorded, and 38 morphometric measurements were taken to the nearest millimetre following Compagno (1984). The prevalence and intensity of ectoparasites on different locations of the specimen's body were also recorded. The animal was dissected, and its reproductive status was determined following the criteria provided by the ICES Advisory Committee (2010) based on the macroscopic examination of the gonads as well as the clasper morphometric criteria provided by Ebert (2002a) and Capapé *et al.* (2004). Sperm was collected via abdominal massage and dissection of the seminal vesicle to determine sperm motility and viability following García-Salinas *et al.* (2021).

In order to determine diet, the stomach contents were examined; these were composed of the remains of a likely single specimen of the family Delphinidae, specifically the right flipper and part of the thoracic region. These remains were examined to determine their decomposition status and analysed for multiple scavenger marks. The species identification was accomplished using molecular tools to genotype both the mtDNA control region and a set of 22 microsatellite loci. DNA was extracted from two pieces of muscle collected from the same dolphin fragment, more specifically, from the right flipper, using a silica-based kit. Since samples were only taken from a single fragment, the possibility that all fragments belonged to the same individual could not be tested. The mtDNA D-Loop region was amplified and sequenced following the protocol described in Gray *et al.* (2018).

Sequences were aligned with odontocete mitogenomes from GenBank and a Bayesian phylogenetic tree was reconstructed using Geneious R7 (www.geneious.com). A total of 22 microsatellite loci were amplified through two multiplex PCR reactions by using primers that amplify across cetacean species (Bourret *et al.*, 2008). Successful amplifications were genotyped using an ABI 3500 genetic analyser and allele sizes were determined in Geneious R7. A principal component analysis was carried out using GenAlEx 6.5 against closely related species as determined by the mtDNA phylogenetic tree. A more detailed version of the methods section is available in the supplementary material.

Following the molecular identification of the items found in the stomach content, we developed a length-length relationship (LLR) to estimate the total length (TL) of the ingested dolphin from the flipper length (FL) by using linear regression models (Steudel & Beattie, 1993). The regression was based on morphometric data from the "Valencian Marine Mammal Stranding Network" (VMMSN) database. The dolphin's age was estimated indirectly based on its TL following Calzada *et al.* (1997).

Results

The male specimen of *H. griseus* had a TL of 251.8 cm and weighed 86.2 kg (Fig. 1A). The morphometric measurements are presented in Table 1. The specimen was characterised as an actively spawning adult based on the macroscopic observation of rigid claspers (Fig. 1B),



Fig. 1: (A) Specimen of *Hexanchus griseus* examined; (B) detail of the claspers; (C) general view of the reproductive system with a black arrowhead marking the position of the sperm-filled seminal vesicles, and detail of the ductus deferens; (D) *Stenella coeruleoalba* remains found in the stomach including the side of the thoracic region and the right flipper.

Table 1. Morphometric measurements of the male specimen of *Hexanchus griseus* following Compagno (1984). Values are expressed in cm and as percentages of the total length (%TL).

Morphometric characteristics	cm	%TL
Total length (TL)	251.8	100
Fork length (FOR)	235.6	95.6
Pre-caudal length (PRC)	177.2	70.4
Head length (HDL)	40.1	15.9
Head height (HDH)	20.2	8.0
Pre-orbital length (POB)	10.4	4.1
Pre-narial length (PRN)	6.8	2.7
Pre-oral length (POR)	7.7	3.1
Pre-spiracular length (PSP)	26.9	10.7
Pre-branchial length (PGI)	33.8	13.4
Mouth length (MOL)	15.2	6.0
Mouth width (MOW)	28.5	11.3
Inter-gill length (ING)	10.1	4.0
Eye length (EYL)	4.9	1.9
Nostril width (NOW)	2.6	1.0
Internarial space (INW)	11.7	4.6
Interorbital space (INO)	20.2	8.0
Eye spiracle space (ESL)	12.9	5.1
Pre-first dorsal length (PD1)	134.6	53.5
First dorsal anterior margin (D1A)	19.1	7.6
First dorsal height (D1H)	12.0	4.8
First dorsal base (D1B)	18.6	7.3
Pre-pectoral length (PP1)	52.0	20.7
Pectoral anterior margin (P1A)	32.1	12.7
Pectoral height (P1H)	24.8	9.8
Pectoral base (P1B)	18.9	7.5
Pelvic anterior margin (P2A)	16.5	6.6
Pelvic posterior height (P2H)	17.2	6.8
Pelvic base (P2B)	20.8	8.3
Anal anterior margin (ANA)	11.7	4.6
Anal height (ANH)	11.6	4.6
Anal base (ANB)	12.5	5.0
Dorsal caudal margin (CDM)	73.2	29.1
Lower post-ventral caudal margin (CPV)	19.2	7.6
Caudal peduncle length (CPH)	10.9	4.3
Clasper inner length (CLI)	22.1	8.8
Clasper outer length (CLO)	12.5	5.0
Pelvic girdle to tip claspers (CL)	41.3	16.4

enlarged and swollen testes, highly convoluted paired *ductus deferens* (Fig. 1C), and seminal vesicles containing abundant sperm, which flowed with slight pressure on the abdominal area. Spermatozoa were mature and showed a motility value of 87.4% and a viability value of 79.8% (Fig. S1).

The specimen hosted the ectoparasitic copepod *Demoleus heptapus* (Otto, 1821), which was attached to the outer skin of the gill slits (N = 13) and around the cloaca (N = 11). All parasites were females ranging between 1.22-1.31 cm (mean = 1.25 ± 0.03 cm) of total length.

The stomach contained freshly ingested remains of the right flipper and two parts of the thoracic region of a specimen of Delphinidae. Both soft and hard parts of the remains had a colouration pattern typical of the striped dolphin *Stenella coeruleoalba* (Meyen, 1833) (Fig. 1D). No other food remains were found in the stomach content. The results from the mtDNA analysis showed that the stomach content samples (Genbank accession numbers OP902517 and OP902518) grouped more closely with *S. coeruleoalba*, with a pairwise similarity percentage of 96.2 % (Fig. S2). Microsatellite PCA grouped the Delphinidae remains more closely to the samples from *S. coeruleoalba* (Fig. S3), confirming the identity of the remains. Although only one of the three parts of the remains was examined genetically, the size, colouration, decomposition status and anatomical concordance suggest that all three parts belonged to a single individual. However, this cannot be concluded with complete certainty.

The relationship between total TL and FL for *S. coeruleoalba* resulted in the following equation:

$$TL = 3.214 * FL^{1.225} \quad (N = 262, R^2 = 0.87) \\ \text{for sexes combined}$$

The ingested flipper measured 20.3 cm (Fig. 1D) and the specimen was calculated to reach 128.4 cm in TL according to the above LLR equation. Therefore, it was inferred as belonging to a dolphin with an estimated age of 1 year.

Discussion

This study presents basic morphometric characteristics of the smallest adult-active male *H. griseus* yet recorded in the Mediterranean Sea, according to the scientific literature (prior smallest individual of 270 cm TL; Vella & Vella, 2010). The presence of ectoparasites, particularly of *D. heptapus*, is also recorded for the first time in *H. griseus*. Although *D. heptapus* has been previously reported in the western-central Mediterranean (Chartosia *et al.*, 2018), no other published data is available on the prevalence of this parasite or other ectoparasite species on *H. griseus*. Furthermore, the feeding interaction between this shark species and dolphins is supported by novel analyses for species identification and determination of scavenging marks.

The feeding activity of *H. griseus* upon cetaceans has been reported on multiple occasions across its distribution

Table 2. Foraging interactions between *Hexanchus griseus* and cetaceans reported in scientific literature. Total number of specimens examined (N); range of total length (TL) of shark specimens (specified in brackets the TL range of specimens containing cetaceans remains in their stomachs); frequency of sharks with cetacean remains in their stomach contents (specified per size classes when stated); cetaceans species found in the diet, classified as: Unidentified cetacean (Uc), Unidentified Delphinidae (Ud), *Physeter macrocephalus* (Pm), *Globicephala melas* (Gm), *Stenella coeruleoalba* (Sc) and *Tursiops truncatus* (Tt); and study location.

Reference	N	TL (cm)	Frequency (%)	Cetaceans in diet	Location
Present study	1	251.8	-	Sc	NW Mediterranean
Mulas <i>et al.</i> (2021)	19	75.9–518.0	17.0	Uc	NW Mediterranean
Kabasakal (2006)	12	250-600 (360-523)	25.0	Ud	NE Mediterranean and Sea of Marmara
Kabasakal (2004)	9	66-550 (500)	11.1	Ud	Sea of Marmara
Henderson <i>et al.</i> (2003)	12	66-165 (165)	8.3	Uc	NW Atlantic
Morey <i>et al.</i> (2003)	26 [§]	-	15.4 [‡]	Pm; Gm; Sc; Ud	NW Mediterranean
Ebert (1994)	137	< 120 - > 200 (120 - >200)	0 (<120 cm) 6.3 (120-200 cm) 18.2 (>200 cm)	Ud; Tt	SW Atlantic and SW Indian
Clark & Kristof (1990)	7	220-358	14.3	Uc	NE Atlantic
Ebert (1986)	15	110-421	6.7	Uc	NE Pacific

[§] Total number of stranded cetaceans with bite marks of *H. griseus*

[‡] Percentage of the studied stranded cetaceans with bite marks of *H. griseus*

range (Table 2), however, genetic tools have never been used before to identify the preyed species. Therefore, data resolution for species-specific interactions is low, coming exclusively from beached cetaceans that have marks of predation by *H. griseus* (Morey *et al.*, 2003; Ebert, 1994). Our results using molecular tools confirm the findings of previous studies showing that *H. griseus* feeds on *S. coeruleoalba* (Morey *et al.*, 2003) – in this case, on a calf. This dolphin species feeds mostly on the continental slope of the Gulf of Valencia and exhibits occasional incursions into continental shelf areas, overlapping with the geographical and bathymetric ranges of *H. griseus* (Cañadas *et al.*, 2002; Gómez de Segura *et al.*, 2006; Aznar *et al.*, 2017).

Foraging interactions between *H. griseus* and cetaceans have been determined as scavenging events using underwater cameras and analyses of carcasses (e.g., Morey *et al.*, 2003; Aguzzi *et al.*, 2018). Therefore, it is largely assumed that scavenging is the only cause of such interactions. *Hexanchus griseus* exhibits slow and sluggish movements most of the time (McNeil *et al.*, 2016) and has strong dognathic heterodonty dentition associated with generalist feeding and scavenging behaviour (McNeal *et al.*, 2016). In the present study, the *H. griseus* specimen was captured during a historical peak in the number of stranded specimens of *S. coeruleoalba*, with 13 specimens reported from February 15 to March 15, 2021 (VMMSN, unpublished data). Considering that most dolphin carcasses never reach the shore (Moore *et al.*, 2020), the increased mortality may reflect a higher abundance of *S. coeruleoalba* carcasses in deep waters, making them more available for *H. griseus* to feed on. Nevertheless, previous studies have suggested that the frequency of cetacean occurrence in the *H. griseus* diet appears too high to result solely from scavenging, indicating that this species could be involved in stealthy hunting (Ebert, 1994; Heithaus, 2001). Indeed, *H. griseus* exhibits vertical migratory patterns, shifting towards more productive shallower areas during the night (Andrews *et al.*, 2009; King & Surry, 2017; Coffey *et al.*, 2020). During this period, it exhibits increased activity and vertical dynamics, indicating that at night it preys on mobile prey dispersed in the water column (Comfort & Weng, 2015; Coffey *et al.*, 2020). The species also displays positive buoyancy, which may be adaptive in approaching prey undetected through near-motionless upward gliding (Nakamura *et al.*, 2015). Despite its general slow swimming behaviour, *H. griseus* can also make striking movements and rapid bites (McNeil *et al.*, 2016). Indeed, its large body mass provides sufficient thermal inertia to slow heat loss, enabling occasional high activity levels (i.e., bursts). Such bursts could be used in ambushing strategies and may provide *H. griseus* with a significant thermal advantage over prey during feeding events (Coffey *et al.*, 2020). Additionally, it has been demonstrated that *H. griseus* uses biting to trap living prey, aided by suction forces created by the upper jaw protrusion, followed by sequential processing bites using the saw-like teeth from the lower jaw to ease ingestion (McNeil *et al.*, 2016). This set of adaptations recently

described and grouped here indicate that *H. griseus* may be not only a scavenger but also a capable active predator.

Active predatory behaviours upon cetaceans have also been observed for other Hexanchiform species. For example, the sevengill shark, *Notorynchus cepedianus* (Péron, 1807) exhibits sluggish and sinuous movements, using ambushing strategies either at night or in poorly lit areas with bursts of speed to capture large and elusive preys (Ebert 1991, 2002b). *Somniosus microcephalus* (Bloch & Schneider, 1801), *S. pacificus* Bigelow & Schroeder, 1944, and *S. antarticus* Whitley, 1939, other slow-swimming deep-water sharks, have also been suggested as active predators of marine mammals using speed bursts (Heithaus, 2001; Van Den Hoff & Morrice, 2008; Nielsen *et al.*, 2014). Thus, predation based on stealthy behaviours upon elusive prey may be a widespread strategy. However, such interactions are thought to depend on relative predator/prey sizes, with only larger sharks feeding on cetaceans, predominantly calves (Ebert, 1994; Heithaus, 2001) or potentially disoriented or sick individuals.

Neither scavenging fauna (e.g., lysianassid amphipods or brittle stars) were found in the stomach, nor other clear predatory marks on the dolphin remains were observed, which would have been expected in a carcass lying on the seabed (Bozzano & Sardà, 2002). Nonetheless, absence of such evidence would not necessarily indicate that the dolphin was captured alive, as *H. griseus* is believed to scavenge on carcasses at early stages of decomposition (Aguzzi *et al.*, 2018). Additionally, the remains were fresh, retaining the characteristic colouration of the skin, smoothness and consistency of the tissue, suggesting that the dolphin was consumed either alive or shortly after its death.

Although our data is not sufficient to determine whether the *H. griseus* dolphin consumption was the consequence of scavenging or active predation, further research on *H. griseus* feeding behaviour could provide more information about the nature of these interactions. Future research on the predatory behaviour of *H. griseus* on cetaceans is suggested to include the analysis of biting patterns, as active predation of pelagic sharks upon cetaceans is thought to be initiated near the genital slit and the caudal region (Walker, 2001). Parasitological studies could also shed light into the frequency of these interactions. The merocercoid stage of the cestodes *Clistobothrium delphini* and *Clistobothrium grimaldii* exhibit a high prevalence in *S. coeruleoalba* (Agusti *et al.*, 2005; Aznar *et al.*, 2007). It is known that the definitive hosts for *Clistobothrium* species are sharks, and indeed these parasites tend to accumulate in the blubber around the genital slit, the caudal peduncle and the abdominal cavity of the dolphins (Walker, 2001; Aznar *et al.*, 2007; Caira *et al.*, 2020). However, the definitive host species for these parasites remain unknown, and their presence in *H. griseus* would indicate a regular feeding interaction due to scavenging or predation.

Direct observations of *H. griseus* actively capturing live prey are difficult to obtain. Nevertheless, future research involving tracking, accelerometry and

underwater monitoring could provide further insight. This information combined with a further understanding of the species reproductive biology and parasitic fauna, would be essential to better understand the role of this apex predator in marine food webs, evaluate the magnitude of potential impacts with the ecosystem and inform management plans.

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Supplementary Data

The following supplementary information is available online for the article:

Supplementary Methods

Table S1: GenBank accession numbers and original references, for all reference mtDNA sequences used in the phylogenetic analysis showed in Figure S2.

Fig. S1: *Hexanchus griseus* spermatozoa stained to assess their plasma membrane integrity. SYBR-14 stains intact cells in green, and propidium iodide (PI) stains damaged cells in red. Stained samples were observed under a fluorescence microscope (Nikon Eclipse 80i) at 10x magnification.

Fig. S2: Bayesian phylogenetic tree based on cetacean mtDNA D-Loop sequences extracted from mitogenome datasets (see Moura *et al.*, 2013 for details). Posterior probabilities are presented next to the nodes. Only one of the mtDNA D-Loop sequences obtained from the suspected dolphin muscle found in the shark's stomach content was used for the reconstruction of this tree, and is shown in red.

Fig. S3: PCA based on 21 microsatellite loci genotyped from the dolphin muscle found inside the shark stomach, compared to the phylogenetically close species *Stenella coeruleoalba*, *Delphinus delphis* and *Tursiops truncatus*. Both shark stomach content extracts had identical genotypes, and grouped more closely to striped dolphin (*Stenella coeruleoalba*) than the other reference species.