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An identification key for Chondrichthyes egg cases of the Mediterranean and Black Sea

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Abstract
Chondrichthyan egg cases are important elements for species-specific identification and also provide a valuable aid in determining a species spatial distribution, as well as for defining spawning areas. Considering the absence of a general key for the identification of the egg cases of the Mediterranean Chondrichthyes, this work aims to fill this gap by presenting a species-specific key based on morphological features of the egg case. The key was developed primarily analysing fresh egg cases dissected from the oviduct, egg cases collected from the seabed or found dried lying on the seashore, after species confirmation by DNA analysis. Original data were integrated with information scrutinized from literature. In order to improve species identification, a protocol for the standardized acquisition of morpho-biometric and meristic features is also provided as a pre-requisite for the appropriate use of the identification key. The total width and length included the horns, when they are not broken, are the parameters that best explain the assignment of the egg case to a specific species.

Keywords: *Taxonomy, biometry, morphology, chondrichthyans*

Introduction

Cartilaginous fishes are characterized by a K-type reproductive strategy with slow population growth and low resilience to biomass reduction, due to natural causes or over exploitation (Ricklef 1979; Hoenig & Gruber 1990; Ramírez-Amaro et al. 2020). The reproductive modes of cartilaginous fishes are very complex and articulated in different biological phases such as birth, growth, maturation and reproduction, with two main strategies: oviparous with development of the embryo external to the mother's body and viviparous with development of the embryo internal to the mother's body (*sensu* Hamlett 2005).

Oviparity, allows the production of one or more egg cases. In fact, the females of some species are able to retain up to 10 capsules in the oviducts before being deposited (Hamlett 2005). The embryo develops inside the egg case, and after consuming all of the yolk, emerges from the capsule with the same body shape as an adult (Hamlett 2005).

Some sharks (e.g. Scyliorhinidae, Heterodontidae, Orectolobidae), and all skates and chimaeras, are

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oviparous, producing a relatively thick capsule that protects the developing embryo from external factors while the embryo develops over several months to years, depending on the species (Hamlett 2005; Stevenson et al. 2007; Ebert & Stehmann 2013).

In the Mediterranean, even if the presence of some species is doubtful, there are at least 22 oviparous chondrichthyans (Serena 2005; FAO 2018a, 2018b). These include: four sharks with two species each in the genera *Scyliorhinus* and *Galeus*; 16 skates species: three *Dipturus*, one *Rostroraja*, four *Leucoraja* and eight *Raja* genera; one belonging to the genus *Chimaera*, one to the genus *Hydrolagus*. A further egg case belonging to the resurrected species *Scyliorhinus duhamelii* (Garman 1913) (Soares & De Carvalho 2019) has not been considered, since the species must be definitively confirmed for the Mediterranean (Serena et al. 2020).

Production of egg cases occurs throughout the year, with peaks in certain seasons. In the Mediterranean these peaks mainly occur in spring-summer (Serena et al. 2010). The deposition of egg cases is carried out in specific areas (egg case nursery areas) (Hoff 2016), towards which the cartilaginous fish migrate for hatching and where the females tend to concentrate in large groups (Springer 1967; Serena 2011). Being that spawning is strictly connected to ecological conditions, females usually utilize the same places every year (Castro 1993; Hoff 2009).

The taxonomic use of egg cases has been well documented and many authors have described the morphology of the egg cases by providing useful working tools (e.g. Clark 1922, 1926; Springer 1939; Ishiyama 1958; Cox 1963; Hitz 1964; Templeman 1982; Koob & Summers 1996; Howard 2002, 2017; Iglesias et al. 2002; Ebert 2005; Ebert et al. 2006, 2008; Treloar et al. 2006; Ebert & Davis 2007; Stevenson et al. 2007; Mabragaña et al. 2009, 2011; Concha et al. 2012; Ishihara et al. 2012; Maia et al. 2015; Bor 2016; Gordon et al. 2016; Porcu et al. 2017).

Due to the important role of Chondrichthyes related to biodiversity, conservation, and fishery ecology, here we develop a dichotomous key for taxonomic identification of egg cases based on their morphological, biometric and meristic features. This key is based on personal experiences combined with other descriptive keys of chondrichthyan egg cases (Iglesias et al. 2002; Treloar et al. 2006; Ebert & Davis 2007; Stevenson et al. 2007; Mabragaña et al. 2009; Ishihara et al. 2012; Gordon et al. 2016).

Material and methods

Data sources

Data and material collected over 35 years of Italian and International trawl surveys (GRUND and MEDITS respectively) conducted with the aim of evaluating fishery resources in the Mediterranean along with information gathered from research programs such as “Oceanografia e fondi Marini”, have provided numerous egg cases belonging to various species of sharks, skates and chimaeras (Bertrand et al. 2000; C.N.R 1979; Relini et al. 2010; Serena 2014; Follesa et al. 2019; Spedicato et al. 2019). Since it was not possible to collect egg cases for all chondrichthyan species living in the Mediterranean area, data were integrated from images provided by colleagues from outside the Mediterranean Sea.

Processing of the collected egg cases was carried out both on fresh (eggs extracted from female uteri) and on those beached or found on the sea bottom. For dry samples, it was needed a rehydration, which was achieved by immersing capsules in seawater for at least 30 minutes. Fresh or rehydrated capsules were stored in 70% alcohol. This allows species identification to be verified through genetic analysis of the egg cases or its contents afterwards. The mitochondrial Cytochrome Oxidase I (COI) gene, commonly used for DNA barcoding and widely applied in studies on Mediterranean elasmobranchs (Kousteni et al. 2015; Frodella et al. 2016; Cariani et al. 2017; Vella et al. 2017; Ferrari et al. 2018) also constitutes an efficient tool in the identification of their egg cases at species level (e.g. the case of *Raja asterias* [Catalano et al. 2021]), especially where the biological contents are better-preserved (Massi et al. 2018; Catalano 2020).

Morphology, biometry and meristics of the egg cases

In order to optimize the collection of information for each egg case, we utilized an updated scheme of morphological features of the egg cases for three chondrichthyan groups (sharks, skates and chimaeras). Using the features identified in the Figures 1–3, a detailed guide to facilitate biometric or meristic measurements is given in order to make any future statistical analysis more reliable. Moreover, the egg case surface can show a furry surface texture constituted by rows of low rounded spines (Hoff 2009; Concha et al. 2010; Gordon et al. 2016). Due to the complexity of the analysis of these features, especially for beached egg cases, we have not included this feature in the identification key proposed here.

Proposed identification key for the egg cases

In order to organize a useful identification key of the chondrichthyan egg cases, the detailed and

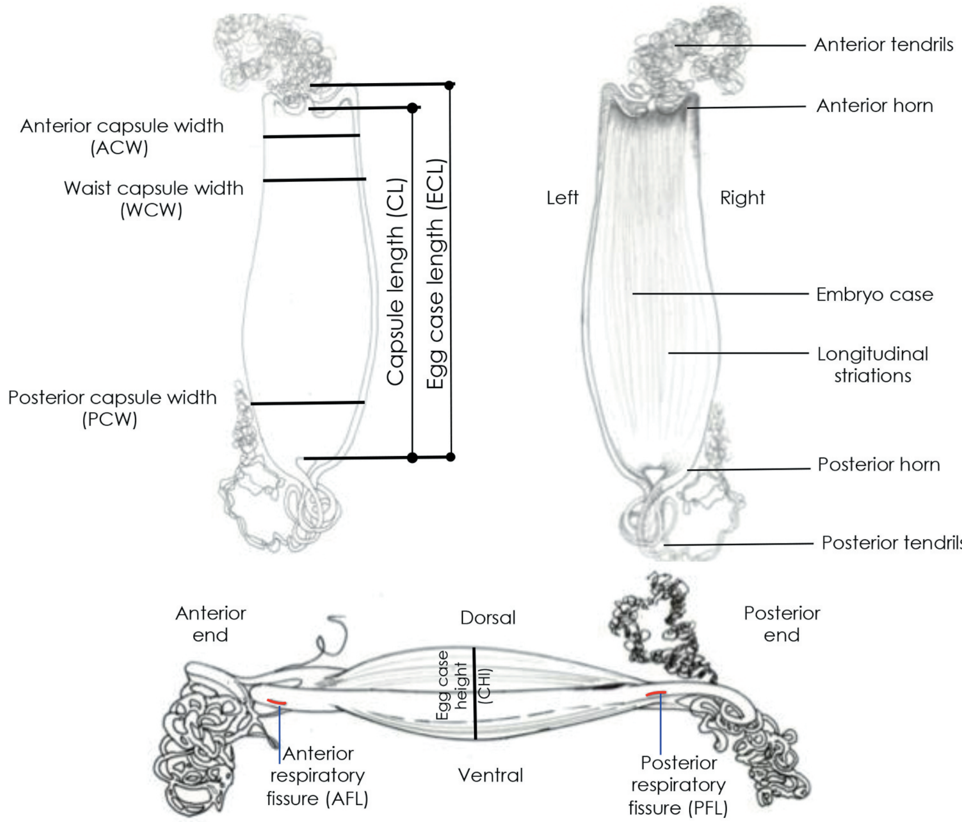

Figure 1. Morphology, terminology and biometry of the Mediterranean shark egg cases considered in the text.

diagnostic characters described below for the three groups, shown in Figures 1–3, are utilized. The measurements are expressed in millimetres (mm) and detected using calliper. Another important diagnostic feature might is also be the structure of the capsule surface, the texture that characterizes it, the presence of filaments on the keel and on the horns, etc.

Sharks. It is possible to explain in detail the various components that describe the shape of the eggs belonging to the different species following the descriptions used by Ebert et al. (2006), Flammang et al. (2007) and Concha et al. (2010) (Figure 1). Therefore, the following measurements (in mm) of the egg cases were made: anterior border width (ABW), anterior capsule width (ACW), waist capsule width (WCW), posterior capsule width (PCW), posterior border width (PBW), anterior respiratory fissure length (AFL), egg case height (CHI), posterior respiratory fissure length (PFL), capsule length (CL) and egg case length (ECL).

Skates. It is possible to detail the morphological measurements of the egg case of skates by referring to Treloar et al. (2006), Ebert & Davis (2007) and Stevenson et al. (2007) (Figure 2), which suggest that some key measurements (mm) are to be taken, as used in this study: egg case length (ECL), anterior (proximal) horn length (AHL), posterior (distal) horn length (PHL), maximum egg case width (MAW), lateral keel width (LKW). In this study we suggest slightly different references: total length with horns (TL), making sure that the horns were not broken, capsule length without horns (CL), capsule width without keels (CW), superior field width (SFW) and inferior field width (IFW).

Chimaeras. Egg cases of Chimaeridae are not well documented, and the differences between capsules belonging to different species were not very evident. However, the number of respiratory pores on each lateral side of the egg case provides a good identification feature for this group (Figure 3).

Also, for chimaera egg cases, as well as for sharks and skates, we suggest recording the main measurements (mm): total length (TL), central spindle length (CSL), peduncle length (PL), filament length (FL), capsule height (CH), capsule width (CW).

The dichotomous key proposed here, mainly takes into account the morphological features of the egg

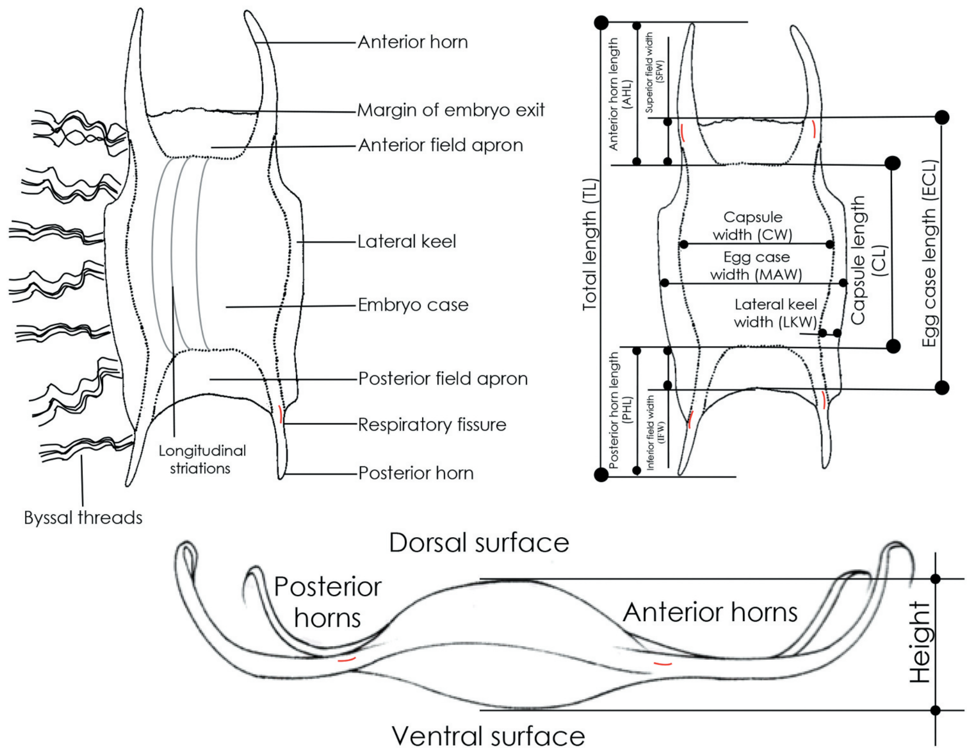

Figure 2. Morphology, terminology and biometry of the Mediterranean skate egg cases considered in the text (Partially from Stevenson et al. 2007).

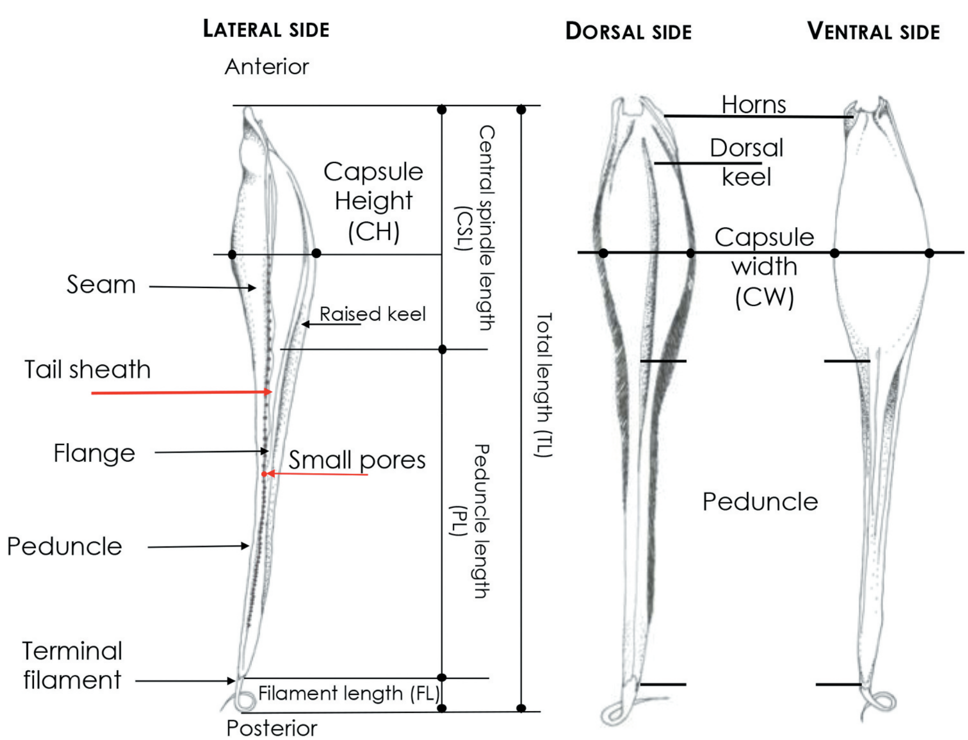

Figure 3. Morphology, terminology and biometry of the Mediterranean chimaera egg cases considered in the text.

cases. In this sense, a protocol for the standardized acquisition of morpho-biometric and meristic features is provided as a pre-requisite for effective use of the identification key (Figures 1–3). This protocol, with the inclusion of additional details, is provided according to Mancusi and Serena (2017) who described the egg case morphology of the Mediterranean species and the analysis procedures used in the laboratory. In particular, with regard to the egg case of the new chimaera for the Mediterranean *Hydrolagus mirabilis* (Collett, 1904) (Hassan 2013; Farrag 2016), it seems to be extremely difficult to find it in the wild. Therefore, in order to provide a good dichotomous key, the features of the genus *Hydrolagus* have been utilized, while for the detailed description of morphological features the egg case of *Chimaera monstrosa* Linnaeus, 1758 was used.

The use of taxonomy keys for determining the identification of egg cases to a particular species has been well documented and developed by Ishiyama (1958), Hubbs & Ishiyama (1968), Ishihara & Ishiyama (1985), Ebert (2005), Ebert & Davis (2007) and Stevenson et al. (2007). In this work, we try to adopt the same method to create a dichotomous key for identifying the egg cases of the species of sharks, skates and chimaeras that live in the Mediterranean basin. Furthermore, a simplified key for the correct identification at the genus level was also presented (Figure 4).

Results

We were unable to collect the egg cases of all the Mediterranean species. Nevertheless, we were able to fill the gaps with assistance from colleagues who provided photographs of missing images from Atlantic Ocean specimens. In particular, these colleagues provided us with photographs of *Leucoraja naevus* (Müller & Henle, 1841), *Leucoraja fullonica* (Linnaeus, 1758) and *Leucoraja circularis* (Couch, 1838), the latter from the Clark (1926) catalogue. The only egg case we were unable to obtain a sample from *H. mirabilis*, which was therefore left out of the key. Indeed, the research program allowed us to collect many egg cases of different chondrichthyans, taken directly from the female just before deposition. A total of 461 egg cases were collected and analyzed both from a morphological and statistical perspective (Mancusi & Serena 2017). In Annex I, for each individual species, the number of egg cases analysed are reported.

A preliminary multivariate analysis of biometrics of some chondrichthyan egg cases have suggested that their total width and length, in terms of absolute sizes, are the measures with the highest power for species identification. These two variables alone allow for a 96% separation of the correlation function of the species under study, obviously the horns must be unbroken (Mancusi & Serena 2017). Following the average value of the egg case width of some most representative species is shown (Table I).

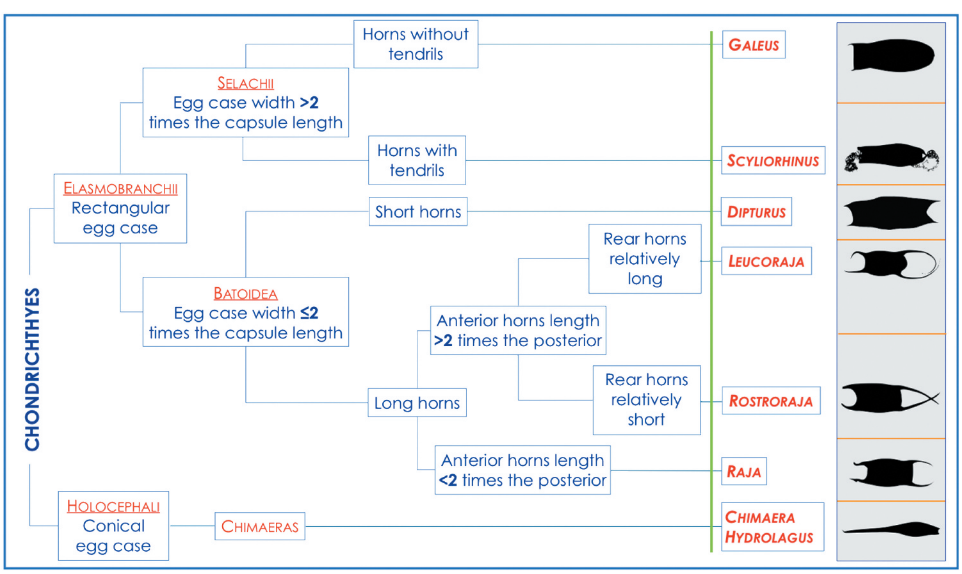

Figure 4. Simplified key for the correct identification at the genus level. The picture it is not a cladogram.

Table I. Main statistic of the egg-case width of nine skates: n = number of available specimens, avg = average width, sd = standard deviation, se = standard error, cl- and cl+ = 95% confidence limits, min and max = minimum and maximum observed values.

	Dipturus nidarosiensis	Dipturus oxyrinchus	Raja miraletus	Raja asterias	Raja polystigma	Raja undulata	Raja clavata	Raja brachyura	Rostroraja alba
n	1	15	9	7	2	8	20	2	6
min	7.5	4	2.15	2.7	3.4	4.2	3.9	6.9	8.1
max	7.5	6.2	2.6	3.1	3.6	4.6	5.6	6.9	11.9
avg	**7.5**	**5.5**	**2.4**	**2.9**	**3.5**	**4.4**	**4.6**	**6.9**	**10.5**
sd	0	0.6	0.1	0.2	0.1	0.2	0.3	0	1.4
se	0	0.17	0.05	0.06	0.05	0.05	0.08	0	0.57
cl-	7.5	4.1	2.1	2.5	3.2	4	3.8	6.9	7.1
cl+	7.5	6.8	2.7	3.3	3.8	4.7	5.3	6.9	13.8

Morphology of the egg case

The egg case structure is typically oblong with a dorsal and a ventral side, the first usually convex correlated to the inner surface of the oviduct where it remains for a certain period.

Overall, egg case surface structure varies among species and is useful for identification at species level especially in the case of the skates. In fact, the egg case surface can show rows of low rounded spines creating a corduroy texture or rows of long multifid spines creating a furry surface texture (Hoff 2009; Concha et al. 2010; Gordon et al. 2016). These features, however, were not considered in the definition of the identification key proposed in this work.

Sharks. The egg cases, jar-shaped, are light yellow or dark brown when they are fresh and can be covered with 12–15 or more longitudinal striations that are partly evident and reinforce the structure. The egg case may be of differential thickness and can be relatively transparent, making the embryo visible with its yolk sac. The anterior edge of the egg case is straight, while the posterior edge is semi-circular with the horns tending to unite. The anterior and posterior ends of the egg case continue with small extensions called horns which in some species are considerably reduced or very long, with filaments curling up into tendrils, useful for anchoring the egg cases to a suitable substrate. At the ends of the egg cases, before the beginning of the horns, there are small openings on the dorsal and ventral side, respectively, useful for oxygenation (Ishiyama & Ishihara 1977; Flammang et al. 2007) (Figure 5).

Skates. The egg cases are usually rectangular shaped with a horny process at each corner, an anterior margin, through which the embryo, in most cases, comes out and a more or less flattened posterior margin. The dorsal and ventral surfaces are relatively convex. The longitudinal sides of the capsule are generally equipped with keels or fringed. The lateral keels in some species can extend anteriorly and posteriorly to the horns. The size of the horns varies from shortest, measuring just some millimetres, to very long, measuring several times the length of the capsule, in many cases with tapered horns that become filamentous, thread-like or flatten out towards the tips. In some species the egg cases may have, over their surfaces, fibrous bisso-like laminae, which allow them to adhere to the seabed. In any case, this does not preclude that deep currents can move them even if for short distances. The lateral margins of some capsules, depending on the species, may have fixing fibres. The oxygenation is aided by the fissures present at the ends, before the beginning of the horns, both on the

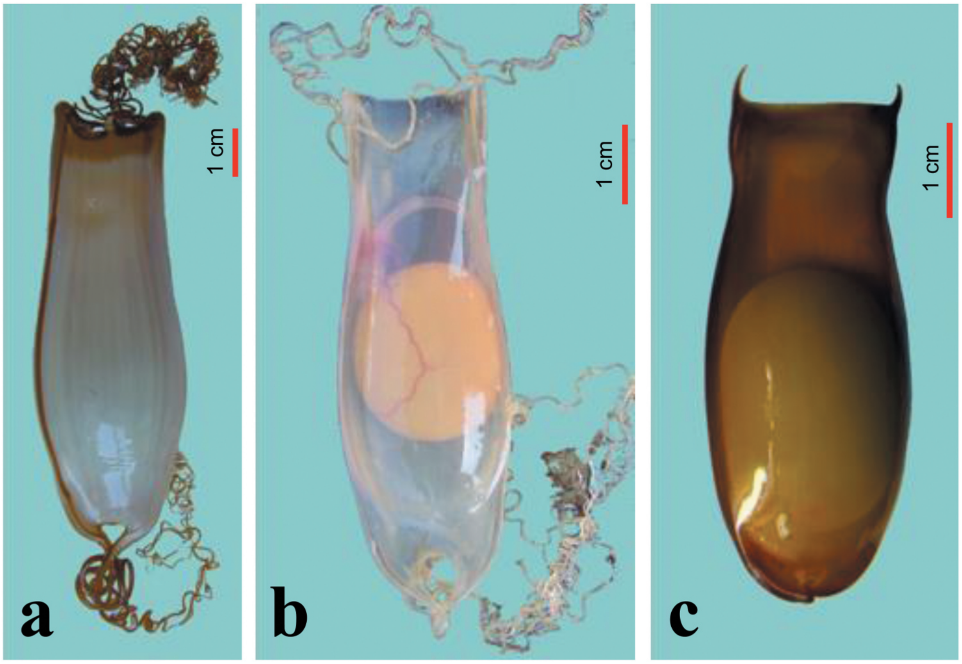

Figure 5. Three of the four egg cases from species of the two genera of Mediterranean sharks: a) *Scyliorhinus stellaris*; b) *Scyliorhinus canicula*; c) *Galeus melastomus* (by F. Serena).

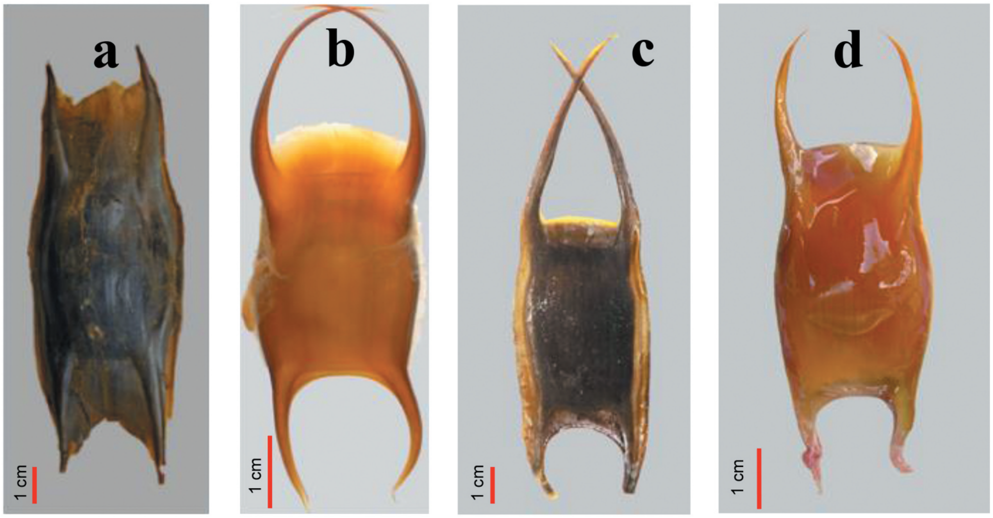

Figure 6. Egg cases of the four genera of Mediterranean skates: a) *Dipturus*; b) *Leucoraja*; c) *Rostroraja*; d) *Raja*. Photos by D. Massi (a); S. Iglesias (b); G. Morey (c); F. Serena (d).

dorsal and ventral sides (Ishiyama & Ishihara 1977; Flammang et al. 2007) (Figure 6).

Chimaeras. The egg cases are small, tapered and with little fringe. They have a tear drop-shape that thins in the back and becomes an elongated peduncle whose terminal filament often is inserted into the substrate. The dorsal surface has, in the middle part, a longitudinal keel. Along the edges of the central spindle and of the sheath or tail, there is a series of small pores which, after the deposition of the capsule, open to allow the oxygenation of the embryo (Dean 1906; Didier 1995) (Figure 7). The dorsal side has an evident keel starts from the end of the peduncle to just before the opening placed in the proximal side of the egg case. The ventral side is devoid of keel and

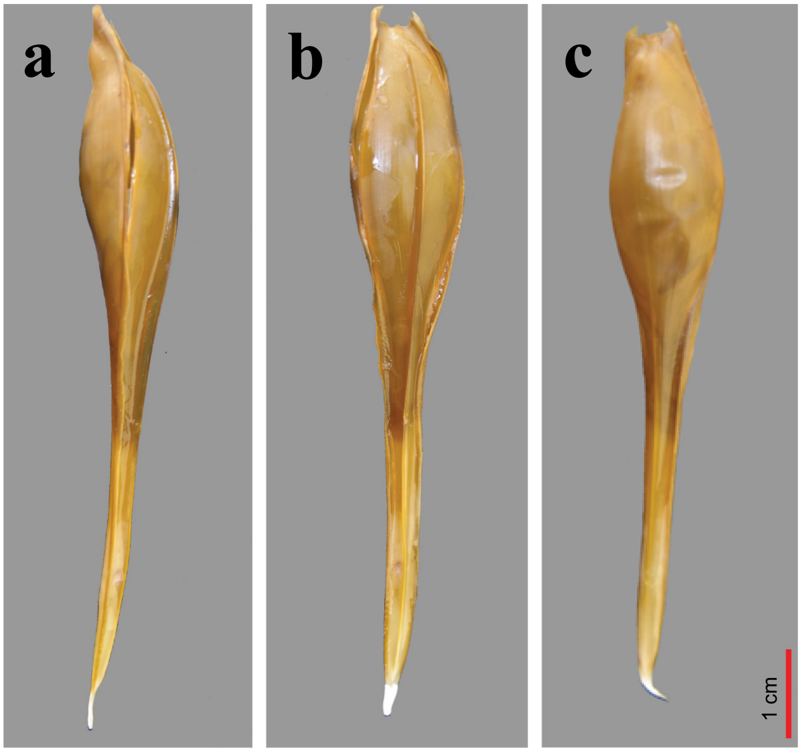

Figure 7. Egg case of *Chimaera monstrosa*: a) Lateral side; b) Dorsal side; c) Ventral side. The chimaera egg case comes out of the genital opening with the dorsal side facing downwards. Then, the ventral side faces upwards in direct contact with the mother's belly (by F. Serena).

highlights the presence of thin rugae flowing towards the distal end of the peduncle. At the thinner end of the egg case, there is a short filament that usually gets fixed in the mud or on some substrate (Bini 1967; Armstrong 1996; Hamlett 2005).

Identification key for egg cases

Eight genera of Chondrichthyes occur in our study area divided into three distinct groups: sharks, skates and chimaeras. The main morphological features that allowed their distinction are summarized below: in sharks, there are egg cases completely devoid of tendrils (*Galeus*) unlike *Scyliorhinus* which instead has long ones on both ends of the egg case. The egg case of *Dipturus* has very short horns on both ends, unlike *Leucoraja* whose horns are always very long both at the anterior and posterior of the egg case. *Rostroraja* instead shows an intermediate situation with very long anterior horns and relatively short posterior ones. *Raja* is instead characterized by a regular, almost squared capsule whose anterior and posterior horn are almost equivalent but never very long. Finally, *Chimaera* egg case is a sort of cone with the upper part swollen and the lower part tapering (Figure 4).

The identification key includes all of the known valid shark, skate and chimaera species in our study area (Table II, Annex I). Only in two cases do we have non-specific egg cases regarding the species to which they belong, they are *L. circularis* and *H. mirabilis*. For the former, we have inserted in the key an egg case described by Clark (1926) since it shows an undamaged egg case with complete horns. In the case of *H. mirabilis*, its egg cases were not available at all.

Discussion and conclusions

In order to better manage the chondrichthyan fish stocks, as well as to improve our knowledge of the conservation status of stocks and biodiversity, the recognition of certain zones as deposition, hatching and nursery areas is a priority. Being able to identify such nursery areas is one of the main requirements for the definition of sound actions required to conserve and enhance these Essential Fish Habitats (EFH) of certain species, intended as waters necessary for spawning, breeding, feeding or growth to maturity. The protection of such areas could be an excellent management measure in order to allow the renewal of elasmobranch populations (McMillan &

Table II. Identification keys to the chondrichthyans egg cases of the Mediterranean and Black Sea.

1a	Rectangular egg case	ELASMOBRANCHII			2
1b	Conical egg case	HOLOCEPHALI			21
2a	Egg case length more than 2 times the capsule width	SELACHII			3
2b	Egg case width less than or equal 2 times the capsule length	BATOIDEA			6
3a	Horns with filiform tendrils		*Scyliorhinus*		4
3b	Horns without filiform tendrils		*Galeus*		5
4a	Capsule length ≤ 75 mm			*S. canicula*	
4b	Capsule length ≥ 80 mm			*S. stellaris*	
5a	Total egg case length not exceeding 35 mm			*G. atlanticus*	
5b	Total length of the egg case greater than 40 mm			*G. melastomus*	
6a	Short horns or reduced to a simple shape. Capsule length ≥100 mm		*Dipturus*		7
6b	Long horns even more than the length of the capsule. Capsule length <100 mm				9
7a	Surface of the egg case not covered with fibers			*D. nidarosiensis*	
7b	Surface of the egg case densely covered with fibers				8
8a	Maximum width of the egg case less than 2 times the egg case length			*D.* cf *batis*	
8b	Maximum width of the egg case about equal 2 times the egg case length			*D. oxyrinchus*	
9a	Length of the anterior horns more than twice that of the rear				10
9b	Length of the anterior horns less than twice that of the rear		*Raja*		14
10a	Capsule length ≧ 40 mm. Anterior horns almost right shape		*Rostroraja*	*R. alba*	
10b	Capsule length < 40 mm. Anterior horns subcircular shape		*Leucoraja*		11
11a	Length of the anterior horns about 3 times that of the rear horns			*L. naevus*	
11b	Length of the anterior horns less than 3 times that of the rear horns				12
12a	Egg case length ≧ 30 mm				13
12b	Egg case length < 30 mm			*L. melitensis*	
13a	Egg case length ≧ 30 mm; < 50 mm. Anterior horn length greater than the egg case length			*L. circularis*	
13b	Egg case length ≧ 30 mm; < 50 mm. Anterior horn length shorter than the egg case length			*L. fullonica*	
14a	Lateral keel present				15
14b	Lateral keel absent				18
15a	Capsule width not equal to length. Non-square shape				16
15b	Capsule width almost equal to the length. Square shape			*R. clavata*	
16a	Egg case length ≧ 40 mm			*R. brachyura*	
16b	Egg case length < 40 mm				17
17a	Well-developed anterior apron. Not evident lateral keel			*R. miraletus*	
17b	Short anterior apron. Evident lateral keel			*R. asterias*	
18a	Capsule width ≧ 45 mm			*R. undulata*	
18b	Capsule width < 45 mm				19
19a	Egg case length up to 75 mm. Egg case width up to 40 mm			*R. montagui*	
19b	Egg case length not as above				20
20a	Egg case length < 50 mm			*R. polystigma*	
20b	Egg case length < 70 m; > 50 mm			*R. radula*	
21a	50 small respiratory pores along each side of opercular valve:		*Chimaera*	*C. monstrosa*	
21b	More than 80 respiratory pores along each side of opercular valve		*Hydrolagus*	*H. mirabilis**	

*egg cases not available.

Morse 1999; Serena et al. 2006). The observed presence of a large quantity of egg cases in a particular area, if repeated over time, is a way to determine whether an area is a spawning area that likely will subsequently concentrate there the newborn juveniles (Heupel et al. 2007; Hoff 2016; Rooper et al. 2019). Moreover, many skate species exhibit site fidelity, returning to the same nursery ground annually as reported for some species of *Bathyraja* spp. in the eastern Bering Sea (Hoff 2010) or for *Raja asterias* in the north Tyrrhenian Sea (Serena & Relini 2006). Therefore, the study of egg cases allows us not only to resolve taxonomic problems, but also to study the spatial distribution of habitat where the critical phases of species life cycle occur.

Thanks to their species-specific morphology, egg cases can be used to identify the species to which they belong. However, it is always difficult for the observer to attribute the parent species to the egg case, since little information on their morphology is often available, especially for some Mediterranean species. In these circumstances, we were forced to use egg cases of the same species collected in the Atlantic area (e.g. *L. fullonica*). In one case (e.g. *L. circularis*) even the original egg case of the individual studied is missing.

Indeed, regarding this last species very few images of fresh egg cases are available. However, some valid drawings or old images are usable from literature (Clark 1926; Mnasri et al. 2009). Despite this, we found it useful to produce a first dichotomous key for identifying the egg cases of the Mediterranean Chondrichthyes. A key that surely can be improved if we can collect and analyse more egg cases for each species, than those currently available.

Moreover, we are aware of the need to collect species-specific missing egg cases directly from the relevant pregnant females. Indeed, only in this way could we be completely sure of the real belonging of the egg cases to a certain species. In any case, genetic analysis provides useful help by directing choices in the best way. Distinguishing and assigning an egg case to a specific species represents a hard challenge. Nevertheless, considering two different methods simultaneously, such as genetic and morphologic approaches, this will help to achieve the goal of a better identification of the species to which the egg case belongs (Massi et al. 2018). For a genetic identification the mitochondrial COI gene can be used, while the main morphological features can be taken into account to compare the egg cases each other (Catalano 2020). In fact, the preliminary multivariate analysis of biometrics of the chondrichthyan egg cases suggested that the two main variables for species identification are total width and length. These variables even explain the 96% and certainly the width is the most reliable feature (Table II) (Mancusi & Serena 2017).

Finally, we must remember that the average values of the measurements mainly depend on the number of samples collected and analyzed, and/or the dimensions of the female. Indeed, the relationship between the total length of females and the total length of the egg cases has been demonstrated (Oddone et al. 2006).

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Supplementary material

Supplemental data for this article can be accessed here.

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Annex I.

List of the egg cases of each single species as identified in the dichotomous key (Table II). In brackets, the number of egg cases analyzed in the period considered. Regarding *Leucoraja circularis*, it was preferred to use the old image of Clark (1926) since it has shown unbroken horns. We were unable to find an original image of *Hydrolagus mirabilis* egg case. It seems that the egg capsule of this species has not yet been described.