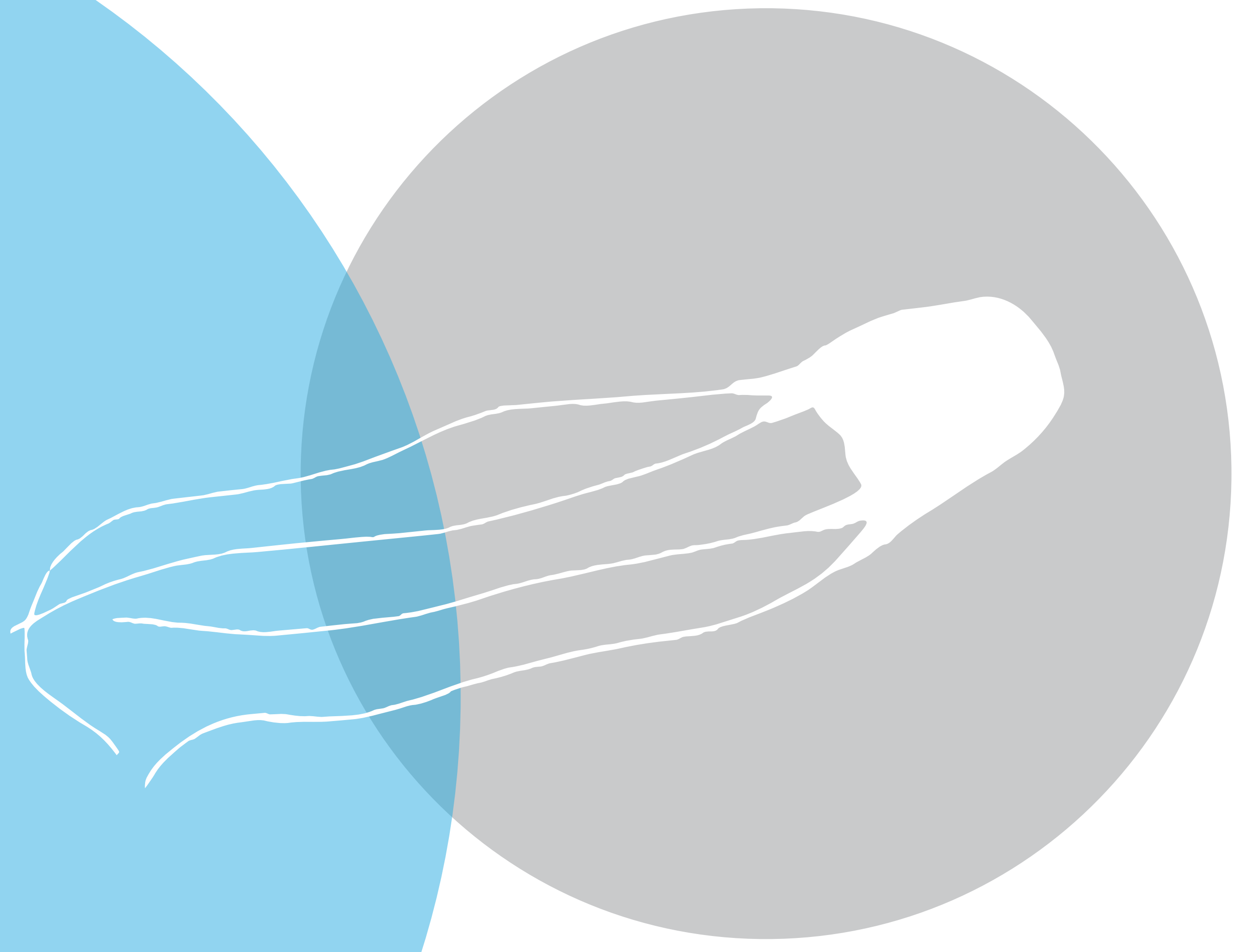
Biology, ecology and ecophysiology of the box jellyfish *Carybdea marsupialis* (Cnidaria: Cubozoa)



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Biology, ecology and ecophysiology of the box jellyfish *Carybdea marsupialis* (Cnidaria: Cubozoa)

Biologia, ecologia i ecofisiologia de la cubomedusa *Carybdea marsupialis* (Cnidaria: Cubozoa)

Melissa Judith Acevedo Dudley

Memòria presentada per optar al grau de Doctor per la Universitat Politècnica de Catalunya (UPC), Programa de Doctorat en Ciències del Mar (RD 99/2011).
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The design in the cover is a modification of an original drawing by Ernesto Azzurro.

"There is always an open book for all eyes: nature"

Jean Jacques Rousseau

"The growth of human populations is exerting an unbearable pressure on natural systems that, obviously, are on the edge of collapse […] the principles we invented to regulate our activities (economy, with its infinite growth) are in conflict with natural principles (ecology, with the finiteness of natural systems) […] Jellyfish are just a symptom of this situation, another warning that Nature is giving us!"

Ferdinando Boero (FAO Report 2013)

Thesis contents

Summary / Resumen

Over the last years, the sightings of the cubomedusa *Carybdea marsupialis* have increased in the Mediterranean Sea and this has been linked to an increase in its abundance. Consequently, this phD thesis addresses some questions regarding the possible causes and effects of this phenomenon. Firstly, the taxonomy and distribution of the species have been revised and updated. Moreover, laboratory experiments were conducted to study the development and ecophysiology of this animal. These results were complemented with field studies on the gut contents and trophic markers of *C. marsupialis*. Finally, the results of a four years monitoring in the coast of Denia (Spain), as well as the sightings of the species reported along the Mediterranean, provided solid evidence on the main factors affecting the distribution of *C. marsupialis*. Overall, the species seems to be favoured by high nutrient inputs from anthropogenic origin, and other human activities as coastal constructions.

Los avistamientos de la cubomedusa *Carybdea marsupialis* han aumentado en el Mar Mediterráneo en los últimos años, hecho que ha sido atribuido a un incremento en su abundancia. El objetivo de esta tesis doctoral es responder algunas preguntas relacionadas con las posibles causas y efectos de este fenómeno. En primer lugar, se han actualizado la taxonomía y la distribución de la especie. Además, se han llevado a cabo experimentos relacionados con su desarrollo y ecofisiología. Estos resultados se han complementado con estudios de sus contenidos estomacales y marcadores tróficos en el campo. Finalmente, un monitoreo durante cuatro años en la costa de Denia (España), junto con los avistamientos de esta cubomedusa en el Mediterráneo, han proporcionado evidencias sólidas acerca de los factores principales que afectan la distribución de *C. marsupialis*. En general, la especie parece verse favorecida por el aporte de nutrientes de origen antropogénico, y por otras actividades humanas como las construcciones costeras.

General introduction

Cubozoans, or box jellyfish, have received considerable attention from scientists and authorities from coastal areas because several species of this group represent a serious threat for human health. Numerous fatalities in tropical and subtropical coastal regions have been attributed to box jellyfish, also called cubomedusae or sea wasps (Bentlage et al. 2009; Keesing et al. 2016). The most venomous species are found in Australia, where they can cause important socio-economic losses (Bailey et al. 2004).

Box jellyfish have been considered inconspicuous animals in the Mediterranean. However, in July 2008 a cubomedusae population outbreak was detected in Denia (NW Mediterranean, Spain), reaching unusual very high densities in some turistic beaches (Bordehore et al. 2011). The region is a popular turistic area, specially during the summer months, and several beach users were affected due to the presence of these organisms in the coast. That year, the Red Cross emergency services recorded more than 3,330 jellyfish stings along 17 km of coastline (Bordehore et al. 2011). This event had a significant impact on the media, wich highlighted “the detection of a new exotic species in the Mediterranean” identified as the cubomedusae *Carybdea marsupialis* (Van den Berg 2010). The sting of this species has been described to be painful but non fatal to humans, producing dermatitis (Peca et al. 1997). However, a sting case that resulted in cutaneous and systemic manifestations have been recently reported (Bordehore et al. 2015a). This escenario triggered the adoption of a monitoring strategy in order to develop management recommendations (Bordehore et al. 2011). Consequently, the LIFE CUBOMED project (www.cubomed.eu), comprising this and other PhD thesis (Bordehore 2014; Canepa 2014), started in 2010 with that purpose. The first necessary step was the correct identification of the species and the determination of its natural range of distribution. A significant progress in the taxonomy of cubozoans has taken place in the recent years (Gershwin 2005, Bentlage et al. 2010), thus we started our investigations from that point in order to properly determine the origin of the cubozoan species present in the Mediterranean. Afterwards, we proceed with the compilation and clarification of some basic and lacking information about the biology and ecology of the species in order to elucidate the possible drivers of the blooming episodes.

Cubozoan life cycle

As other cnidarians, cubozoans are characterized by alternation of benthic polyp and free-swimming medusa generations. The investigations in this thesis are focused on the cubomedusa stage; polyps have been observed in the wild only once for a carybdeid species (Studebaker 1972). We unsuccessfully searched for the polyps of *C. marsupialis* in the sea during the samplings conducted in the framework of the LIFE CUBOMED project. Moreover, during the PhD I have carried out several trials to rear a culture of *C. marsupialis* polyps in the laboratory; I managed to obtain the planulae

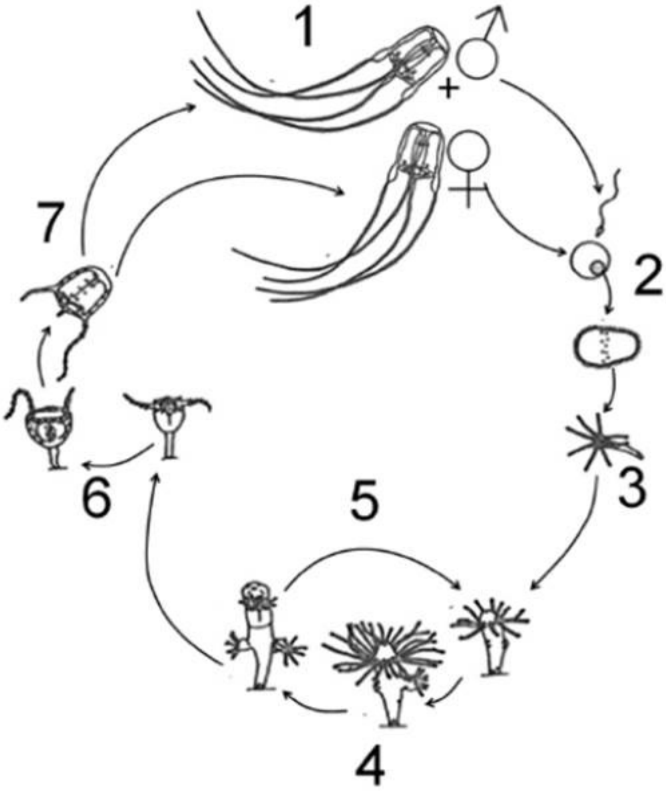

Fig. 1 Life cycle of *Carybdea*. (1) Male and female mating; (2) release of the fertilized egg into de water column; (3) settlement of planula larva on substrate after aprox. 2 days; (4) benthic polyp phase; (5) new polyps budding from existing polyps; (6) polyp metamorphosing into juvenile medusa; (7) release of juvenile cubomedusa. Drawing adapted from Studebaker (1972), University of Puerto Rico. Source: Bordehore et al. 2015b.

larvae and the primary creeping polyps, but unfortunately the polyps did not survive. Hence, the knowledge for this life stage is still limited.

Although the life cycle has not been completely clarified for *C. marsupialis* in the Mediterranean, similarities with another *Carybdea* sp. (= *C. xaymacana*, formerly considered as *C. marsupialis*, as demonstrated in Chapter 1 in this thesis) from Puerto Rico (Fig. 1) have been theorized (Studebaker 1972; Bordehore et al. 2015b).

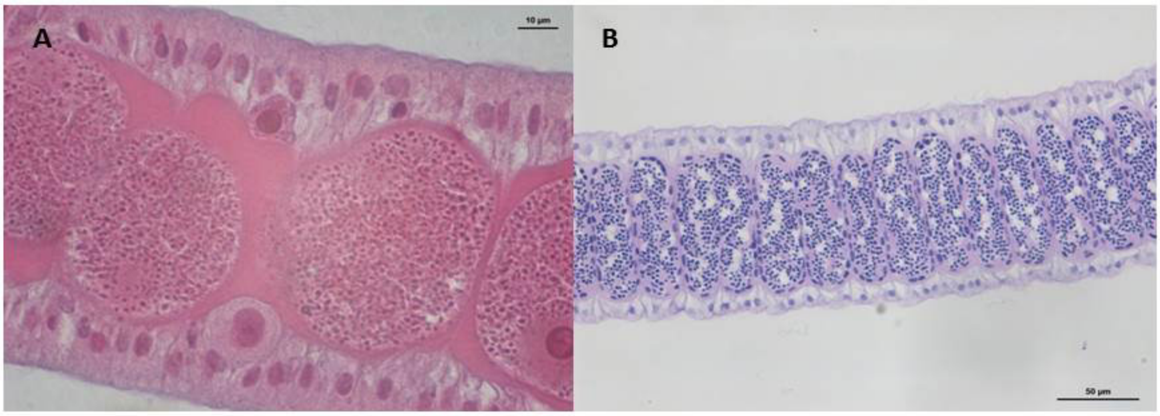

Fig. 2 Gonads of *Carybdea marsupialis*: (A) female gonads cross-section, scale bar = 10 µm; (B) male gonads cross-section, scale bar = 50 µm. Pictures: Jimena García (USP).

Cubomedusae are dioecius, the male and female gonads can be distinguished under a stereomicroscope; male gonads look smooth and finger-print like, while female gonads present a granulated surface (Fig. 2). The Caribbean *Carybdea* sp. is reported to be ovoviviparous: the spermatozoa are released into the water, enter the gastrovascular cavity of female via the manubrium, and fertilize the ova. Zygotes remain there until blastulae are released and develop into swimming planulae (Studebaker 1972; Arneson and Cutress 1976) (Fig. 1).

Once planulae larvae settle, it transforms into a crawling, two-tentacled primary polyp (Fig. 1). This creeping polyp will find a suitable substratum, and grow and develop into a multi-tentacled polyp capable to generate new polyps by asexual reproduction (Fig. 1). The mature polyps can also produce juvenile cubomedusae through the process of metamorphosis. Two types of metamorphosis exist in some carybdeid polyps: in ~45-50% of the cases the entire polyp transforms into the medusa (Fig. 1); whereas in the second type, the metamorphosing polyp leaves behind a regenerative remnant (Straehler-Pohl and Jarms 2005). This fact is unknown in the concrete case of the species studied here. Although similarities between *C. marsupialis* and the caribbean *Carybdea* sp. (= *C. xaymacana*) have been hypothesized, some particularities due to the seasonal variation caracterisc of temperate areas are expected.

Vision and behaviour

Cubozoans are unique jellyfish because of the possession of complex eyes. Their particular vision influence both their biology and ecology (Gershwin et al. 2013). Cubozoans have 24 eyes gruped in four rhopalium (Fig. 3). In each rhopalium they have two complex eyes with lens, retina and cornea. In addition, two pairs of simple ocelli are placed along the sides of the lensed eyes (Gershwin et al. 2013). Some eyes are orientated for looking up through the water surface, and others are orientated for looking downward at underwater structures and shadows (Gershwin et al. 2013). Garm et al. (2012) proposed that having different eye types for different visual tasks might require less neural processing than if all the information would pass through one eye. The rhopalia in Cubozoa coordinate the contractions of the umbrella and allow directional swimming in response to stimuli (Garm et al. 2007; Colin et al. 2013). Moreover, the statolith present inside the rhopalium, keeps the cubomedusa orientated vertically (Garm et al. 2012).

Cubozoans use their vision to navigate the environment and search for prey and mates (Gershwin et al. 2014). Experimental studies indicate that cubozoans are able to form images and react to shades, shapes and light colours. Therefore, they present complex behaviours such as hunting, evasion, navigation, courtship, and copulation (Garm et al. 2012; Colin et al. 2013). Coates (2003) provided a good literature review on the visual ecology and relevant functional morphology of cubozoans. Cubozoans are commonly found in nearshore habitats (e.g. sandy beaches, kelp forests, mangroves, and coral reefs), and they use their vision to navigate these complex habitats (Martin 2004). The movement of juvenile stages (especially the recent detached medusa) would be mainly influenced by the water currents. However, as these juveniles grow, they are able to overcome weak currents to select a suitable habitat or environment (Canepa 2014).

The positive phototropism of cubozoans has been reported several times. They are easily caught by light attraction (Larson 1976; Gershwin et al. 2013). This fact has strong implications for safe management (Gershwin 2005).

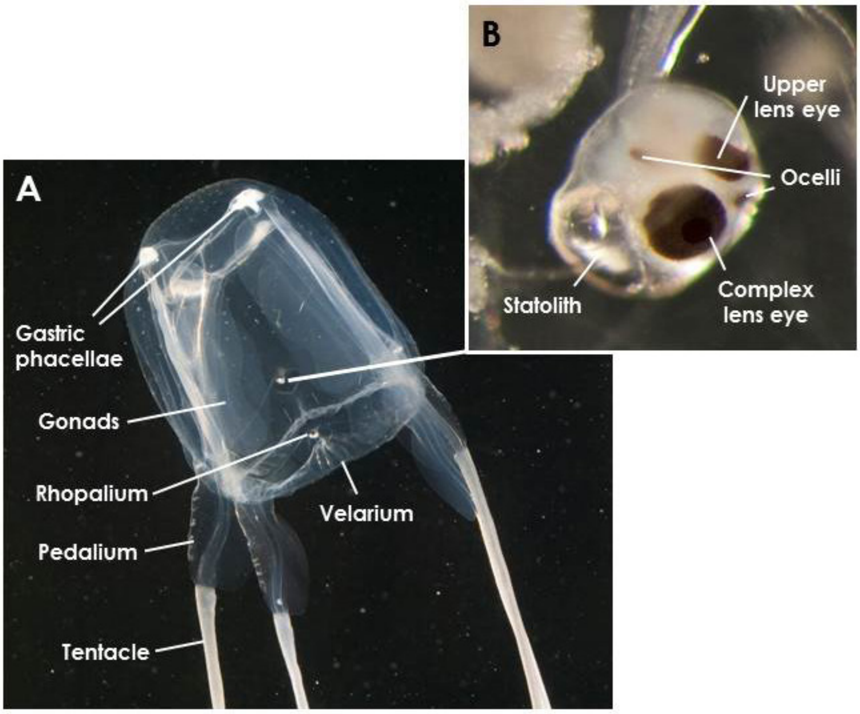

Fig. 3 *Carybdea marsupialis* morphology. (A) General aspect and morphology; (B) detail of the rhopalium structure. Pictures: Eduardo Obis.

Feeding ecology

These sensory capabilities of cubomedusa may enable them to orient and forage in specific habitats with high prey densities (Buskey 2003). In consequence, they increase their encounter rates with prey. This foraging strategy (i.e. cruising predators) requires a high level of swimming control and performance (Colin et al. 2013). Most cubozoan species show a foraging behaviour, where medusae swims with the tentacles extended, and once prey is entangled, the tentacles are introduced in the bell and the prey is grasped by the manubrium (Larson 1976; Hamner et al. 1995; Matsumoto 1995). Once the prey is within the bell cavity, a cessation of the cubomedusae swimming has been observed, and an extracellular digestion of the prey is produced by the gastric cirri (Larson 1976).

Different feeding and activity patterns have been reported in box jellyfish. On the one hand, some species (i.e. *C. xaymacana*, *C. rastonii*, *Copula sivickisi*) have been observed to rest close to the bottom during the day, but being more active in the water column during dusk and night (Larson 1976; Studebaker 1972; Matsumoto 1995; Martin 2004). In contrast, other carybdeid species, such as *Tripedalia cystophora*, presented the opposite pattern: active swimming near the surface during the day and near the bottom at night (Garm et al. 2012). The different behaviours observed seem to be an adaptation to the activity patterns of the available prey (i.e. copepods and other crustaceans) in their respective habitats (Garm et al. 2012).

Prey preferences of some cubozoan species have been studied by stomach content analysis. Conant (1897) made one of the first studies on the biology of cubomedusae, and he described *Carybdea* sp. (= *C. xaymacana*) to capture and swallow relatively large fish. Larson (1976) studied the feeding behaviour and functional morphology of cubomedusae, with particular focus also on *Carybdea* sp. from Puerto Rico (= *C. xaymacana*). He reported the diet of cubomedusae to consist mostly of crustaceans and fish, although polychaetes were seasonally important. Lai (2010) reported data on gut content analysis of *C. rastonii*, where crab zoea, shrimp larvae, copepods, arthropods and fish larvae were the most common prey. For some species, such as *Carukia barnesi* (Underwood and Seymour 2007), a general trend towards an increasing proportion of larval fish with increasing jellyfish body size has been observed. A similar ontogenetic shift in diet was noted for *Chironex fleckeri*, but not for the smaller *Chiropsella bronzie* (as *Chiropsalmus* sp.) (Carrette et al. 2002). Therefore, such a shift does not appear to be universal in the Cubozoa (Gershwin et al. 2013).

The trophic ecology of *C. marsupialis* has not been previously clarified, and the consequences of the possible interactions (predation, competition, etc.) between this species and other marine organisms are unknown.

Potential drivers of jellyfish blooms

Population explosions of gelatinous zooplankton are referred to as "blooms", and are considered as deviations from "normal" plankton dynamics (GFCM 2011). Although there is no robust evidence that supports a global rise in jellyfish blooms (Condon et al. 2013), the number of reports for certain jellyfish species has increased in many coastal ecosystems worldwide in the recent decades (Brotz and Pauly 2012; Gibbons and Richardson 2013). Concretely, proliferations of gelatinous zooplankton are a natural phenomenon in Mediterranean waters, but they seem to have become more frequent over the last years (CIESM 2008). This increase in the occurence of blooms at a local scale is seen as a potential indicator of a state shift in marine ecosystems (Graham, W.M., Kroutil 2001; Mills 2001; Purcell 2005; Purcell et al. 2007; Richardson et al. 2009), attributed not only to the natural conditions but also most probably to anthropogenic disturbance activities (Purcell et al. 2007; Dong et al. 2010),

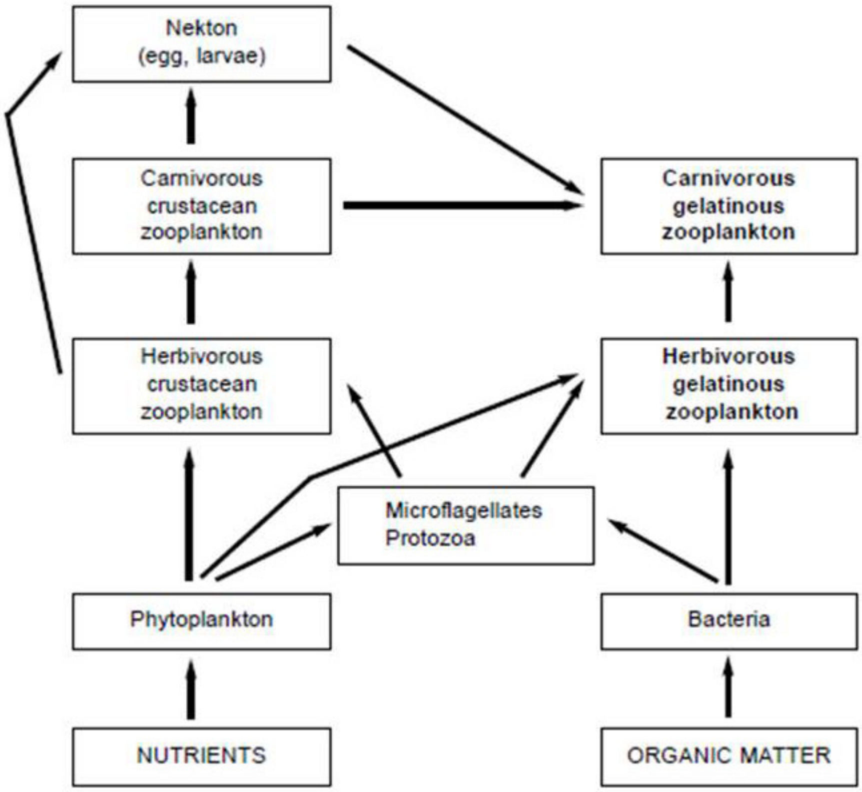

Fig. 4 Simplified food web forming the basis of the pelagic food chain. Gelatinous animals are indicated in bold. By feeding on different compartments of the pelagic food web and acting as "sinks", they are able to generate strong perturbations on the transfer of mater in the pelagic ecosystem. Source: CIESM 2001.

although more evidence is still needed. Multiple biotic and abiotic factors have been suggested as possible drivers of the apparent increase of jellyfish blooms: depletion of predators and competitors of jellyfish by overfishing, accidental translocations, eutrophication, human modification of the coast, and climate change (Mills 2001; Purcell et al. 2007; Duarte et al. 2012; Purcell 2012). Global warming has been considered as a factor increasing jellyfish populations because temperature could affect their distribution, growth and reproduction (Richardson et al. 2009). Moreover, causal relationship between variation in jellyfish population and some environmental factors have been stablished for some species: changes in nutrient dynamics; changes in waterfront constructions, as they provide increased attachement surface to the polyps; variation in other organisms which could be preys (e.g. copepods, cladocerans, ictioplankton…etc.) or competitors as zooplanktivorous fish; and changes in water tempeture, which regulate life cycles (Fujii et al. 2011).

Overall, human-induced impacts appear to be promoting jellyfish blooms to the detriment of other marine organisms. Human activities may be shifting the balance from highly evolved ecosystems dominated by fish (which control jellyfish through competition or predation), to the more ancient state dominated by jellyfish, reminiscent of the Palaeozoic (Richardson et al. 2009). These two pathways normally coexist in the food web (Fig. 4), but under certain conditions one can predominate over the other. Usually, the trophic path ending up with fish prevails and determines what we consider as a "normal" situation (Boero 2013). The other pathway, favouring herbivorous or carnivorous gelatinous zooplankton (Fig. 4), can go through episodic success (Boero 2013). Jellyfish may not only compete with fish for planktonic crustaceans, but also prey on fish larvae and eggs (Purcell and Arai 2001). Therefore, significant changes in the secondary production would cause a change in the relationship between jellyfish and zooplanktivorous fish production (Fig. 4). When abundant, medusae may deplete zooplankton stocks due to intense predation (Olesen et al. 1994). This may lead to secondary effects, which can greatly modify the biological structure of a system, hence some jellyfish species are considered keystone predators of the community (Olesen et al. 1994). The kind of interactions between Cubozoans and their potential competitors and prey are poorly known (Kingsford and Mooney 2014), and also in the particular case of *C. marsupialis* these have not been described yet.

More than 500 jellyfish species can be found in the Mediterranean Sea, both native and invasive, including 30 species of Ctenophores (Çinar et al. 2014), and also Cnidarians belonging to different classes: 457 species of Hydrozoans (Gravili et al. 2013, 2015), 20 species of Scyphozoans (Mariottini and Pane 2010), and only one Cubozoan species (Chapter 1 in this thesis). While several gelatinous organisms appear to be increasing in recent decades, these increases are not uniform throughout the basin (Brotz and Pauly 2012). Warmer temperatures due to climate change could benefit some species of jellyfish by increasing distribution, altered phenology, increased reproductive rates, and decreased mortality (Brotz and Pauly 2012). Other jellyfish populations may be influenced by coastal development; several species of jellyfish have been shown to benefit from eutrophication, and similar mechanisms may be acting in the Mediterranean Sea (Brotz and Pauly 2012). However, while the increased eutrophication of the Mediterranean Sea may have benefitted some jellyfish populations, the effects may be damaging others species. That means that different groups of jellyfish will respond differently to environmental and anthropogenic impacts (Purcell 1999).

Box jellyfish outbreaks

Quantitative data on the spatial and temporal distribution of Cubozoa is scarce, but seasonal variations in abundance are well known for some few species, as *Chironex fleckeri* from Northern Australia (Gordon and Seymour 2012) and *Alatina moseri* from Hawaii (Chiaverano et al. 2013). High concentrations of box jellyfish are relatively rare and blooms are typically found at small spatial scales

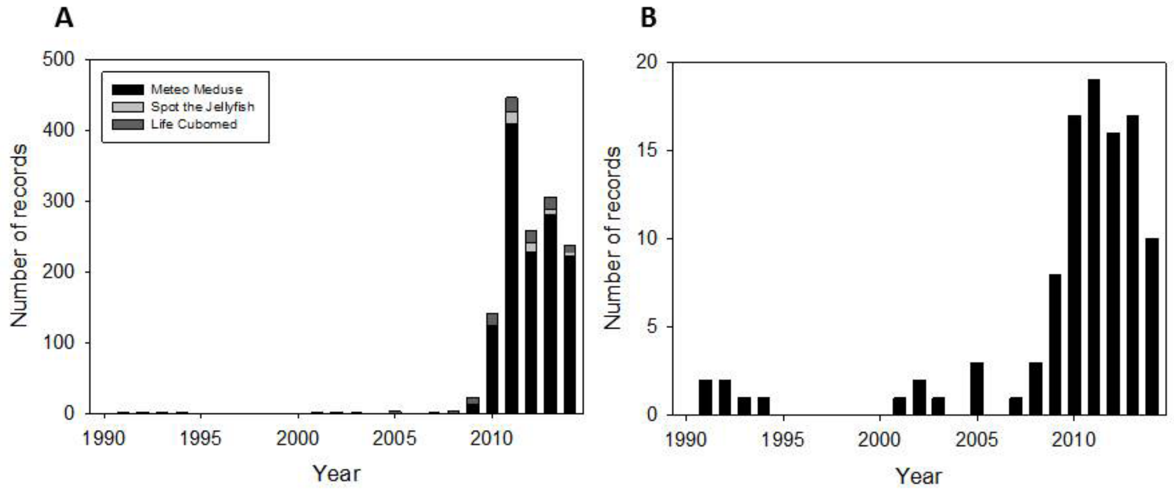

Fig. 5 Records of *Carybdea msrsupialis* along the Mediterranean Sea since 1990. (A) Compiled from different citizen science initiatives; (B) reports registered through LIFE CUBOMED project.

(Kingsford and Mooney 2014). Because of that, the effect of predation have been theorized to be intense but unimportant in affecting the population dynamics of prey (Kingsford and Mooney 2014).

Since 2008, the density of *C. marsupialis* has remained relatively high around coastal waters of Denia, with average density of approximately 5 to 10 individuals 100 m^{-3} (Bordehore et al. 2015b). High densities of *C. marsupialis* have been also reported from the Italian project Meteo Meduse (http://meteomeduse.focus.it), and from Malta as part of the Spot the Jellyfish initiative (www.ioikids.net/jellyfish). Moreover, two iniciatives have been developed along the Spanish coast: ENPI-Medjellyrisk project (www.jellyrisk.eu) and LIFE CUBOMED project (www.cubomed.eu). Thousands of records of the species along the Mediterranean coast have been compiled from these citizen science approaches which allowed a better evaluation of its distribution (this thesis). The results show an increase in the number of sightings of this conspicous species in the last years (Fig. 5). Particularly, *C. marsupialis* have been reported to be very abundant in correspondence with coastal defenses along the Adriatic Italian coast (Boero 2013). In addition, blooms of *C. marsupialis* have been also recently registered for the first time in Tunisia (Gueroun et al. 2015). However, it is not known if these recent increases in cubomedusae will be sustained in future, and which environmental factors are causing them.

This phD thesis adresses some questions regarding this sudden increase in the abundance of the cubomedusae *Carybdea marsupialis* and the possible causes and consequences of this phenomenon. Firstly, we pretend to clarify if this would be a case of an exotic species introduced in the Mediterranean or not. In addition, we wanted to elucidate the seasonality and development of *C. marsupialis* populations in the NW Mediterranean coasts, as well as to understand the trophic role of this cubozoan and the interactions with other marine organisms. The main question formulated is whether the overabundance of *C. marsupialis* would be a normal event or it could be indicating a shift in the ecosystem state. We hypothesized coastal eutrophication and other human impacts – as translocation, habitat modification and climate change – to be possible processes enhancing these episodes.

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Objectives and thesis outline

The main objective of the thesis has been to acquire new knowledge on the biology and ecology of the nowadays outbreaking, but scarcely known, Mediterranean box jellyfish: *Carybdea marsupialis*. The final aim is to support the management recommendations to be developed in the frame of LIFE CUBOMED project (LIFE08 NAT/ES/0064).

A general review on the current knowdledge about jellyfish blooms and cubozoans ecology is compiled in the **General Introduction**. We also introduced other key topics of the thesis, as box jellyfish life cycle, and their particular swimming and feeding behaviour.

In order to elucidate the possible causes and consequences of *C. marsupialis* overabundance in the Mediterranean, and determine whether it is a normal event or an environmental status indicator, the research has been organized in four chapters, corresponding to scientific publications (already published, submitted and in preparation for the submission).

In **Chapter 1**, “Revision of the genus *Carybdea* (Cnidaria: Cubozoa: Carybdeidae): the identity of its type species *Carybdea marsupialis*” (Article I, submitted to Zootaxa), we revised the identity of this cubozoan species, clarifying the real distribution of *C. marsupialis*, which has been often mistaken. We looked into literature, from ancient manuscripsts to lastest publications, and compared samples from different locations aroud the world, using both morphological and genetic tools. The main objective of this chapter was to determine the extension of the *C. marsupialis* metapopulation to be studied in the frame of this thesis, as well as to clarify the exotic or native status of the species in the Mediterranean sea.

Chapter 2, “Maintenance of *Carybdea marsupialis* (Cnidaria: Cubozoa) in the laboratory” (Article II; published in JEMBE), has been developed based on laboratory experiments and observations, and provides the groundwork to the subsequent chapters. In this chapter, we developed and tested two aquariums especially designed to culture specimens of *C. marsupialis* under controlled conditions. This gave us the opportunity to observe the development of the cubomedusae, as well as to quantify the feeding and growth rates of the species.

In the next section, **Chapter 3**, “Trophic ecology and potential predation impact of *Carybdea marsupialis* in the NW Mediterranean” (Article III; in prep.), we investigated the feeding habits of the species in the coast of Denia (W Mediterranean, Spain). By analysing the gastric contents, we determined the main preys of this box jellyfish, calculated prey selectivity indexs, and clarified the diurnal patterns in its feeding activity. We complemented this approach with molecular techniques (i.e. stable isotopes analysis) in order to obtain more information regarding the tropic ecology along its ontogeny. Finally, the potential predation impact of *C. marsupialis* over zooplankton community was estimated by using the digestion times obtained in laboratory experiments, as well as field data on cubomedusae and prey densities.

In the last chapter, **Chapter 4**, "Seasonality, interannual variation and distribution of *Carybdea marsupialis* (Cnidaria: Cubozoa) in the Mediterranean coast: influence of human impacts" (Article IV; in prep.), we studied the seasonal and interannual variability in cubomedusae abundance along the coast of Denia (W Mediterranean, Spain). Environmental and cubomedusae data were recorded during four years (i.e. from 2010 to 2013) on the basis of a monthly sampling, and we explored the correlation of *C. marsupialis* abundance with different environmental parameters monitored. Moreover, a Species Distribution Model (SDM) was developed using the sightings recorded in different citizen science databases from the Mediterranean region (ENPI-Medjellyrisk and LIFE CUBOMED projects). A forecasting for the probability of presence of this species along the Mediterranean under current conditions was obtained and inspected.

The main results extracted from the different chapters are related and argumented in the **General Discussion**. In this section we address the initial questions of the thesis, and discuss the role of *C. marsupialis* in Mediterranean coastal food webs, its invasiveness potential, and correlation with anthropogenic impacts on the sea shore. To conclude, we summarize and highlight the main **Conclusions** of the thesis.

CHAPTER 1

Revision of the genus *Carybdea* (Cnidaria: Cubozoa: Carybdeidae): the identity of its type species *Carybdea marsupialis*

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(Submitted to *Zootaxa*)

Abstract

While records of *Carybdea marsupialis* in the literature suggest a worldwide distribution of this species, the identification of some of these records has been questioned recently, as has the validity of some nominal *Carybdea* species. We inspected material of all known species of *Carybdea* from multiple locations (i.e. Spain, Algeria, Tunisia, Puerto Rico, California, Hawaii, Australia, South Africa, and Japan) using morphological and genetic tools to differentiate *Carybdea* species as well as understand their evolutionary relationships. We observed morphological differences between adult medusae of Mediterranean and Caribbean *C. marsupialis*; the most obvious differences were the structure of the phacellae, the structure of the pedalial canal knee bend, and the number and structure of the velarial canals. The characters of the adult Mediterranean specimens agree with the description provided by Claus (1878) for individuals of *C. marsupialis* from the Adriatic Sea (Italy); specimens from the Caribbean (Puerto Rico) agreed with the description of *C. xaymacana* by Conant (1897). Significant differences between both species were also observed in the newly detached medusa stage. Further, we found that a polyp culture of "*Carybdea marsupialis*" originated in Puerto Rico, is neither *Alatina alata* nor *C. marsupialis* but *C. xaymacana*. We also revised and described specimens from a population in California that had been referred to as *C. marsupialis* or *C. rastonii*, and determined that they probably represent a new species of *Carybdea*. Although *C. marsupialis* is considered the only species of Cubozoa to occur in the Mediterranean, specimens collected in Algeria and Tunisia demonstrate that species of Alatinidae may also be present in the Mediterranean. Our investigations indicate that *Carybdea* spp. are more restricted in their geographical distribution than has been recognized historically, and confirm that the 'old species' *Carybdea arborifera* Maas 1897 from Hawaii, *Carybdea branchi* Gershwin and Gibbons 2009 from South Africa, *Carybdea brevipedalia* Kishinouye 1891 from Japan, *Carybdea marsupialis* Linnaeus 1758 from the European Mediterranean Sea, *Carybdea rastonii* Haacke 1886 from South Australia, and *Carybdea xaymacana* Conant, 1897 from the Caribbean Sea are valid and not synonyms with each other. A taxonomic key to all currently valid species is provided, and a neotype for *C. marsupialis* is designated.

Keywords: box jellyfish, *Carybdea murrayana,* morphology, tree of life, identification, cryptic species, geographic distribution, development.

Introduction

Massive numbers of the box jellyfish *Carybdea marsupialis* were detected at some beaches of Denia (Alicante, Spain) in 2008 (Bordehore et al. 2011). This event had a significant impact on the media, with news incorrectly highlighting "the detection of a new exotic species in the Mediterranean" (Van den Berg 2010). After these events, we initiated a focused study of *C. marsupialis* to better understand its biology and ecology, and to identify the main factors that could stimulate the proliferation of this species.

The scientific literature suggest that *C. marsupialis* has a large geographic distribution, including the Mediterranean Sea (Linnaeus 1758; Péron and Lesueur 1810; Lamarck 1816; Claus 1878; Haeckel 1882; Ranson 1945), the East Atlantic Ocean along the West Coast of Africa (Mayer 1910; Kramp 1955a, b; Kramp 1961), the Gulf of Mexico (Loman 2004; Segura-Puertas et al. 2004), the Caribbean Sea (Studebaker 1972; Larson 1976), and the Pacific Ocean including the coast of California (Larson 1990; Larson and Arneson 1990; Sánchez-Rodríguez et al. 2006). In 2008, Kazmi and Sultana detected carybdeid specimens in Pakistan's Gwadar Bay (Arabian Sea), which they also identified as *C. marsupialis*. However, Gul et al. (2015) investigated the cnidarians found in coastal Pakistan and suggested that the Gwadar Bay carybdeid identification may not be accurate. Due to its vast distributional area, some authors (Fenner 1997; Gershwin 2005; Bentlage et al. 2010) pointed out that there might be more than one species united under the same species name. Most recent studies on *C. marsupialis* have focused on the cnidome and toxicity of this species, with special interest on human health effects (Rottini et al. 1995; Avian et al. 1997; Peca et al. 1997; Di Camillo et al. 2006), rather than dealing with its geographic distribution. The purpose of this paper is to clarify the identity of *C. marsupialis* and that of its close relatives.

Although the present records of *C. marsupialis* suggest a worldwide distribution, the taxonomic identity of several populations has been questioned (see Fenner 1997 for the Californian population; Gershwin 2005 observed Caribbean, Mediterranean, and Australian specimens; Bentlage et al. 2010 discussed the identity of several species of *Carybdea* based on molecular genetic data). Identification and differentiation of carybdeid species has suffered some confusion since the first descriptions of *C. marsupialis* by Plancus (1739) and Linnaeus (1758) from the Adriatic and Mediterranean Sea; *C. marsupialis* became the type species of the genus, and afterwards several publications classified carybdeid specimens from many different locations as "*Carybdea marsupialis*". However, the original manuscripts of Plancus (1739) and Linnaeus (1758) only contain brief descriptions of the species and lack specific details to distinguish this species from other *Carybdea* species.

Historically, some researchers such as Maas (1903, 1910), Bigelow (1909, 1938), Mayer (1910), and Kramp (1961) questioned the validity of the diverse number of carybdeid species and considered most of them as varieties of *Carybdea marsupialis* or *Carybdea rastonii*. Studebaker (1972) described the life cycle of cubozoan specimens from Puerto Rico, identifying them as *Carybdea marsupialis* using the arguments of Bigelow (1938). Since the publication of Cutress and Studebaker (1973) on the development and metamorphosis of this Caribbean species, research on polyps from that culture of Puerto Rico has contributed much to the current knowledge about carybdeid biology and ecology (Larson 1976; Stangl et al. 2002; Fisher and Hofmann 2004; Straehler-Pohl and Jarms 2005, 2011). But recently, Bentlage et al. (2010) found that the genetic sequence derived from a polyp culture identified as *Carybdea marsupialis* reared at the Zoological Institute of the University of Hamburg, fell within the family Alatinidae rather than the family Carybdeidae. This fact hinted to a possible confusion concerning the identity of this culture of polyps in Hamburg, originally obtained in Puerto Rico and afterwards used for life cycle experiments (e.g. Studebaker 1972, Cutress and Studebaker 1973, Stangl 1997, Stangl et al. 2002, Straehler-Pohl and Jarms 2005) as well as molecular studies

(Collins 2002; Collins et al. 2006). We agreed with Bentlage et al. (2010) that an "inclusion of *C. marsupialis* from close to its type locality in Italy in future phylogenetic studies should also help shed light on this issue". Moreover, Bentlage et al. (2010) showed that some *Carybdea populations* were a case of several species being united under the name.

A review of published studies revealed discrepancies in the morphology, anatomy, genetics, and biology among geographically distinct specimens identified as *C. marsupialis*. Misattribution and the presumption that a given species at a specific locality is *C. marsupialis* hinder resolving species-specific biology and ecology, including factors that influence presence, abundance, and blooming or spawning.

The objective of this study was to unravel the taxonomy and distribution of this genus using morphological and molecular genetic tools. The main focus was to identify the possible differences between *Carybdea* spp. from the Mediterranean, the Caribbean, the Pacific, and African coasts. Additionally, we developed an identification key for all currently valid species of *Carybdea*.

Materials and methods

Species list and cultivation

Comparative studies of specimens named *Carybdea marsupialis* from several different locations worldwide were conducted. As the documented distributional range of *C. marsupialis* seems to overlap with the one of *C. rastonii*, we incorporated the populations of this species that includes the recently resurrected species *C. arborifera* and *C. brevipedalia*, as well as the recently described species *C. branchi*. We inspected material of all known species of the genus *Carybdea* from multiple sampling locations (i.e. Spain, Algeria, Tunisia, Puerto Rico, California, Hawaii, Australia, South Africa, and Japan) and compared their morphology.

Moreover, when ethanol preserved tissue was available, molecular genetic studies were conducted in order to help differentiate species. By combining genetic analyses with morphological inspections of the medusa development of *Carybdea* from the Mediterranean and from the Caribbean sea we were able to unravel the identity of the controversial polyp culture from Puerto Rico [provided by Ronald Petie from University of Copenhagen, but originally sampled in La Parguera, Puerto Rico by Werner et al. (1971) and Cutress and Studebaker (1973), and transferred to University of Hamburg]. While most taxonomic observations and measurements were performed on preserved material, we examined all developmental stages of medusae of the Mediterranean specimens alive, as well as recently detached Puerto Rican cubomedusae. Specimens labelled as *C. marsupialis* (from Puerto Rico, Algeria and Tunisia) were requested as a loan from the National Museum of Natural History (Smithsonian Institution, Washington DC, USA): five (5) specimens from Puerto Rico (USNM 54457, 54458, 54461); One (1) specimen from Algeria (USNM 56659), and two (2) specimens from Tunisia (USNM 54378). These specimens were compared with individuals from the Mediterranean (Denia, Spain) in the Natural History Museum of Barcelona [Museu de Ciències Naturals de Barcelona; formerly Zoology Museum of Barcelona (MZB)], Spain (MZB 2015-1701; MZB 2015-1701, MZB 2015-4801, 4802, 4803, 4804, 4805, 4806). Additionally, we compared the anatomy, morphology, and genetics of other *Carybdea* specimens which have been deposited in the Natural History Museum of Barcelona: *Carybdea* sp. from California (MZB 2015-1702, provided by George I. Matsumoto); *C. arborifera* from Hawaii (MZB 2015-1703, provided by Angel Yanagihara); *C. branchi* from South Africa (MZB 2015-4807, provided by André C. Morandini and Sergio N. Stampar); *C. brevipedalia* from Japan (MZB 2015-1704, provided by Sho Toshino and Shin Kubota), and *C. rastonii* from southern Australia (provided by Jamie Seymour).

Medusa stages

Developmental stages of medusae were also studied. Specifically, different stages of *Carybdea marsupialis* from the Mediterranean population were collected by surface trawling in the summer (from June to September) of the years 2010 to 2013 at some beaches located in Denia (Spain). Three nets of different mesh size were used: 200 µm, 500 µm, and 4 mm (mouth area 0.13, 0.16, and 0.26 m^2 respectively). Most of the specimens were fixed in a mixture of 4% formalin and seawater while some medusae were maintained in a specially designed aquarium following the procedures described by Acevedo et al. (2013).

To obtain juvenile medusae of the Caribbean "*Carybdea marsupialis*" population for morphological comparison and genetic sequencing, polyps from Puerto Rico [provided by Ronald Petie from University of Copenhagen, but originally sampled in La Parguera, Puerto Rico by Werner et al. (1971) and Cutress and Studebaker (1973)] were maintained in the laboratory (Institut de Ciències del Mar – CSIC) in 250 mL glass bowls containing natural sea water as described by Jarms et al. (2002). The induction of metamorphosis was triggered by salinity reduction to 32 psu as described by Canepa et al. (2013). The newly detached medusae were maintained as described above for the Mediterranean Sea population. However, we were not able to grow newly detached medusae of the Puerto Rican population to a size bigger than 5 mm.

"Gonads" in Cubozoa: For this taxonomic group, the term "gonad" is used to refer to the areas where gametes are formed (Marques and Collins 2004; Bentlage et al. 2010; Morandini and Marques 2010, Straehler-Pohl et al. 2014).

Measurements

Measurements were taken with Medid callipers (1/20mm; ± 0.05 mm), and under a calibrated stereoscopic microscope (Leica S8APO) for the smaller life cycle stages (< 10 mm).

Standard measurements were used (Gershwin 2005, 2006; Straehler-Pohl 2014): bell height (BH) = measured from the apex of the bell to the velarial turnover; diagonal bell width (DBW) = distance between opposite pedalia at level of pedalia joining bell; interpedalial diameter (IPD) = distance between opposite pedalia (outer pedalial wing edges) at the level of the bell turnover; interrhopalial diameter (IRD) = distance between opposite sense niches; interrhopalial width (IRW) = distance between adjacent rhopalia; distance between opposite gastric cirrus (CW); we also measured pedalia width (PW) and length (PL).

Photographs were taken under the same conditions with digital cameras (Canon Powershot G12, Canon EOS 550D, or Olympus E3).

Molecular study and DNA analysis

DNA was extracted from polyps and single tentacles removed from the specimens using InstaGene (Bio-Rad) or DNAdvance (Agencourt®) kits following the manufacturer's protocol. Genes were amplified using PCR (see details in Stampar et al. 2014). PCR products were purified with the AMPure® kit (Agencourt®). The PCR primers CB1 (forward: TCGACTGTTTACCAAAAACATA) and CB2 (reverse: ACGGAATGAACTCAAATCATGTAAG) (Cunningham and Buss 1993) were used to amplify part of the mitochondrial 16S gene (expected fragment of 435 to 681 bp). Purified PCR products were made ready to sequencing using the BigDye® Terminator v3.1 kit (Applied Biosystems), with the same primers and temperature conditions of the PCR´s reactions. The sequencing procedure was carried out on an ABI PRISM®3100 genetic analyser (Hitachi).

Sequences were assembled and edited (removing ambiguous base calls and primer sequences) using Geneious™ 7.1 (Drummond et al. 2011). Multiple sequence alignments were inferred using MUSCLE with default parameters (Edgar 2004). New sequences were submitted to GenBank (Table 1). Uncorrected pairwise distances were calculated in MEGA6 (Tamura et al. 2013).

Maximum likelihood phylogenetics were inferred under the general time reversible model of sequence evolution with gamma rate heterogeneity (GTR+GAMMA) using RAxML (Stamatakis et al. 2008); confidence in the resulting phylogenetic tree topology was estimated using 500 non-parametric bootstrap replicates. Maximum parsimony analysis was conducted in Mega 6 (Tamura et al. 2013). In the case of maximum parsimony, trees were obtained by the Close-Neighbor-Interchange (CNI) algorithm under the Kimura two parameter (K2P) model (Nei and Kumar 2000) and node support estimated using 500 bootstrap replicates. Bayesian inferences were performed using MrBayes 3.2 (Ronquist and Huelsenbeck 2003) as implemented in Geneious™ 7.1 (Biomatters Limited, Auckland, New Zealand) under the GTR+GAMMA model (chain length = 1100000, subsampling frequency = 200, burn-in length = 100000 and random seed 27265).

Systematic account

Genus *Carybdea* Péron and Lesueur (1810)

Carybdea Péron and Lesueur, 1810: 332.
Charybdea L. Agassiz, 1846: 174 (synonyms, nomenclature)

Several authors between Milne-Edwards (1833) and Mayer (1910) used a variant spelling, *Charybdea*, which was later rejected. Since Kramp (1961) the usage of *Carybdea* was re-established and unified.

Diagnosis: Medusae of *Carybdea* Péron and Lesueur (1810), the only genus within Carybdeidae (Lesson 1843), can be differentiated from all other carybdeid families by their possession of a heart-shaped rhopaliar niche ostium with a single, upper covering scale and no lower scales (Gershwin 2005; Bentlage et al. 2010; Bentlage and Lewis 2012). To date there are 7 accepted species of *Carybdea*, with *Carybdea marsupialis* being the type species.

Type species: *Carybdea marsupialis* Linnaeus 1758, by subsequent description and designation by Claus (1878) and Haeckel (1880; 1882).

Remarks: Carybdeidae is a monogeneric family containing the genus *Carybdea*. Péron and Lesueur (1810) established the genus *Carybdea*, and Claus (1878) and Haeckel (1880; 1882) named *C. marsupialis* as the type species. *Carybdea* represents the oldest cubozoan genus, and numerous cubozoan species were assigned to this genus prior to recent taxonomic revisions (reviewed in Bentlage and Lewis 2012).

We inspected specimens of all known species of the genus *Carybdea* from different locations, with the main focus on *C. marsupialis* from the Mediterranean.

Recent classification of the seven carybdeid species considered for this study (following Mayer 1910; Kramp 1961; Mianzan and Cornelius 1999; Gershwin 2005; Calder 2009; Straehler-Pohl and Jarms 2011, World Register of Marine Species - WoRMS, present study):

Phylum Cnidaria Verrill, 1865
 Subphylum Medusozoa Petersen, 1979
  Class Cubozoa Werner, 1973
   Order Carybdeida Gegenbaur, 1857
    Family Carybdeidae Gegenbaur, 1857
     Genus *Carybdea* Péron & Lesueur, 1810
      Carybdea arborifera Maas, 1897
      Carybdea branchi Gershwin & Gibbons, 2009
      Carybdea brevipedalia Kishinouye, 1891
      Carybdea marsupialis (Linnaeus, 1758)
      Carybdea murrayana Haeckel, 1880 *
      Carybdea rastonii Haacke, 1886
      Carybdea xaymacana Conant, 1897

(*) Samples were not available for inspection. Therefore the taxonomic literature served as the basis for all comparisons.

Note that *Carybdea morandinii* was not considered for this revision of *Carybdea*, as it belongs to a different family as *Alatina morandinii* (Straehler-Pohl and Toshino 2015).

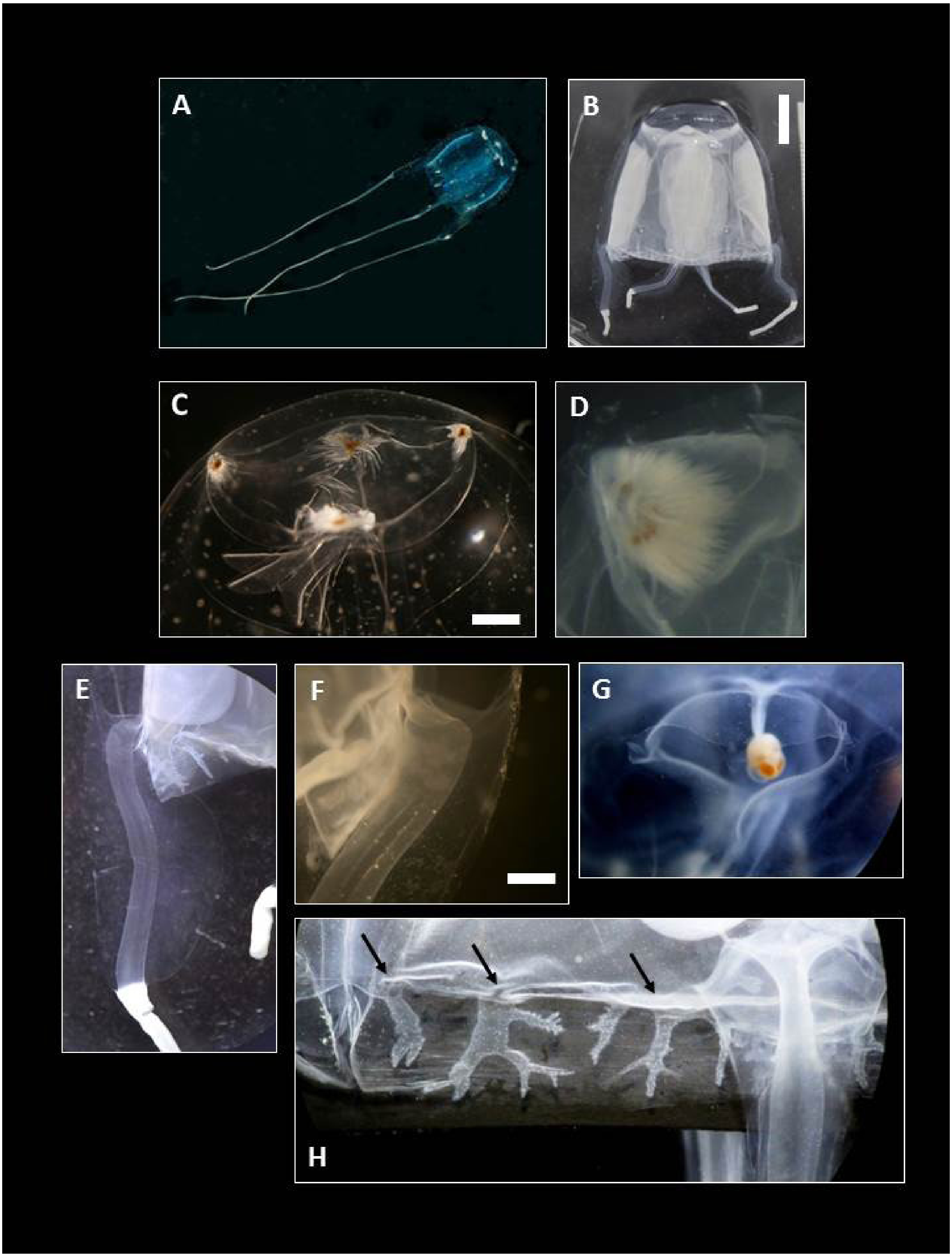

Fig. 1 General aspects of *C. marsupialis*; (A) Habitus alive (photographer: C. Casanellas) and (B) preserved (Neotype: MZB 2015-1701); (C) Aboral view of gastric cavity and (D) close up of eppaulette shape phacellae; (E) Pedalium and (F) pedalial knee bend; (G) Rhopalium; (H) Detail of the velarial canals (3 per quadrant; black arrows) (photographs of different *C. marsupialis* individuals C-H: E. Obis). Scale bar: 1 cm.

Carybdea marsupialis (Linnaeus, 1758)

Figs. 1A-H; 2A-F; 3A-D

Synonyms: While it is possible that we missed some mention of *Carybdea marsupialis* (Linnaeus 1758) in the literature, a comprehensive survey of the literature yielded the following selection of synonyms.

Urtica soluta marsupium referens: Plancus 1739: 41-42 (description in Latin), Fig. 5; (not valid as first species description under Art. 3 of the International Code of Zoological Nomenclature, which establishes that "No name or nomenclatural act published before 1 January 1758 enters zoological nomenclature, but information, such as descriptions or illustrations, published before that date may be used").

Medusa marsupialis: Linnaeus 1758: 660 (short note/description). Modeer 1791: 32.

Oceania marsupialis: Eschscholtz 1829: 101 (list of synonyms, description of species based on Plancus' drawing).

Marsupialis planci: Lesson 1843: 268 (description); Agassiz 1846: 224 (nomenclature); Agassiz 1862: 174 (synonyms, nomenclature).

Charybdea marsupialis: Milne-Edwards 1833: 248 (description), plates 11, 12; Gegenbaur 1857: 215-217 (description, discussion of structures); Agassiz 1862: 174 (nomenclature); Claus 1878: 6-56, plates 1-5 (throughout, anatomy and microanatomy of medusa, comparison with *Charybdea* (*Tamoya*) *haplonema* and *Tamoya* (*Chiropsalmus*) *quadrumana* (*quadrumanus*); Haeckel 1880: 442; Haeckel 1882: 92 (historical overview), 96-98 (comparison with *Carybdea murrayana*); Haacke 1886: 596, 598, 600, 605 (comparison with *Carybdea rastonii*); Mayer 1910: 507 (description), 508 (synopsis of the species of *Carybdea)*.

Carybdea marsupialis: Kramp 1961: 305 (description, list of synonyms); Di Camillo et al. 2006: 705-709 (cnidome); Daly et al. 2007: 151 (overview, first cubomedusa described by Linnaeus 1758); Brinkman 2008: 3 (tree of life), 166 (research overview).

Neotype (hereby designated)**:** Natural History Museum of Barcelona (Museu de Ciències Naturals de Barcelona): 1 adult female (MZB 2015-1701), preserved in 70% ethanol, collected by M. J. Acevedo, October 6th 2010, Denia, Spain. BH = 25.4 mm; DBW = 31.5 mm; IRW = 14.6 mm; GW = 16.8 mm; PL = 10.5 mm; PW = 4.7 mm. Bell cuboid, wider than high, thick mesoglea, few nematocyst warts; apex domed, with a constriction at level of gastric phacellae; phacellae epaulette shaped, single rooted; heart-shaped rhopalial niche ostium, one upper covering scale; velarial canals 3 per octant, canals flanking frenulum unforked, middle canals biforked, canals flanking pedalia multiple branched; tentacles four (4); pedalia knee bend rounded, no appendage, with irregular nematocyst bands on the outer keel; ripe female, gonads milky whitish.

The original manuscripts of Plancus (1739) and Linnaeus (1758) contain brief descriptions of *C. marsupialis*, and no details of any name-bearing type specimen. Moreover, we could not find any evidence to the existence of any type material in the scientific literature reviewed in this publication. In order to define this nominal species objectively, and following the definition and rules set forth under Article 75 of the International Code for Zoological Nomenclature (ICZN 1999), we suggest specimen MZB 2015-1701 from Denia (Spain, NW Mediterranean) deposited in the Natural History Museum of Barcelona (Museu de Ciències Naturals de Barcelona) to be designated as the neotype of *C. marsupialis*, since no holotype, lectotype, syntype, or prior neotype is believed to exist.

Other material examined:

Spain: Natural History Museum of Barcelona [Museu de Ciències Naturals de Barcelona; formerly Zoology Museum of Barcelona (MZB)]: Forty four (44) specimens from the same collection location as the neotype, separated into 6 different developmental stages, accession numbers MZB 2015-4801 (Stage A), MZB 2015-4802 (Stage B), MZB 2015-4803 (Stage C), MZB 2015-4804 (Stage D), MZB 2015-4805 (Stage E), MZB 2015-4806

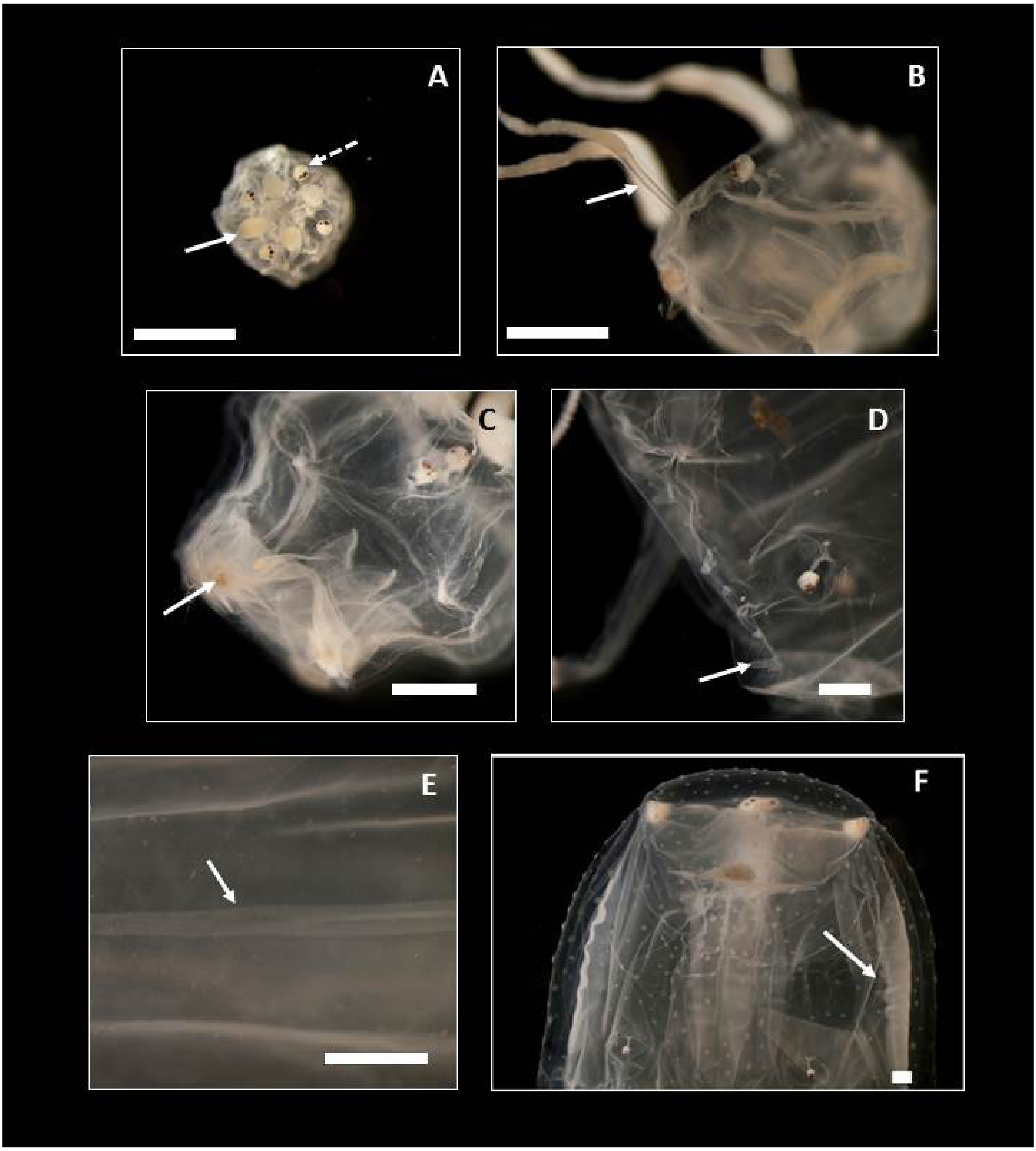

Fig. 2 Developmental stages *C. marsupialis* (preserved specimens); (A) Oral view, Stage A : short tentacle (solid arrow) and rhopalium (dashed arrow); (B) Stage B: starting the development of pedalia; (C) Stage C: Gastric phacellae completely developed; (D) Stage D: Start of development of velarial canals; (E) Gonadal tissue appearance, stage E; (F) Adult stage F with ripe male gonads. Photographer: E. Obis. Scale bar: 1 mm.

(Stage F), Denia (Spain), collected during August, September, October 2010 and June 2011.

The Netherlands: Naturalis Biodiversity Center [formerly Royal Museum of Natural History (Rijks-Museum)], Leiden: from the Mediterranean, without exact sampling location, 2 specimens (No. 1 and 2) no sampling date, (Stiasny 1919).

USA: Smithsonian Institution National Museum of Natural History (NMNH), Washington: from the Mediterranean, Naples Zoological Station (Italy), no exact sampling location, 1 specimen (USNM 19346), conserved in formalin, no sampling date; also from the Mediterranean, Pescara (Italy), 3 specimens

(USNM 1155726, 1155728, 1155729), preserved in ethanol, collected by Christina Di Camillo, no sampling date, identified by Bastian Bentlage in 2009.

Diagnosis: Gastric phacellae epaulette shaped, single rooted; Velarial canals 3 per octant; multiple branched; Pedalia knee bend rounded, no appendage. Apex thick, domed, with a constriction at the level of the gastric phacellae.

Description:

Adult medusa: (Figs. 1A-H, 3A-D): Bell sturdy, cuboid, slightly wider than high (BH: DBW ratio less than or equal to 1:1, Fig. 1A, B), interradial furrows shallow, highly transparent with few whitish nematocyst warts sparsely scattered on bell from apex (very small warts) to bell margin (big warts along interradial furrows), amount of warts varies between specimens (i.e. some individuals present few or almost lack warts, others have profuse warts); apex, thickened, domed, with slight horizontal constriction at level of gastric phacellae; bell height up to 40.5 mm high, bell width up to 40 mm (DBW).

Pedalium (Fig. 1E), simple, unbranched, flattened, scalpel-shaped, measures approx. 1/3 the bell height in length, situated in each interradial corner, with irregular white nematocyst bands on outer keel of pedalium, smaller warts scatter outer half of pedalium; in some mature medusae margin of inner keel of pedalium sometimes undulated. Pedalium carrying single tentacle, tentacles light brownish pink colour when contracted, extended resemble bead-chains with white nematocyst-battery “pearls” on pale pink tentacle “string”. Pedalial canal with rectangular knee without any hook or thorn appended to outer knee bend (Fig. 1F), slightly tapering at the upper end, straight (not bending) throughout the length of the pedalium but slightly curving towards the inner pedalial keel in the middle part, ellipsoid in diameter with sharp outer keels.

Rhopalium (Fig. 1G) located inside heart-shaped rhopalial niche ostium with triangular upper covering scale, without lower covering scale; some specimens present covering scale with nematocyst mammilation (1 or two warts), but not the neotype; approx. 1/5 of bell height up from bell margin; rhopalium with 6 eyes (2 median lens eyes+ 2 lateral slit eyes + 2 lateral pit eyes).

Velarium (Figs. 1H) with some small nematocyst warts, containing 3 velarial canals per octant (i.e. 6 v.c/quadrant), slim in width, very sharply pointed tips, deeply forked, slightly lobed with smooth margin, canals flanking frenulum are the simplest, mostly unforked, only few dents, middle canals, seldom more than 2 main branches, only single side branches, canals flanking pedalia bases, most complex with 3 to 4 main branches and several side branches.

Four-lobed, cruciform manubrium without nematocyst warts (≥1/4 BH in length), connected to a small, flat (lens-shaped) stomach; stomach communicates perradially with 4 gastric pockets leading into velarial canals. Gastric phacellae (Fig. 1C), 4, epaulette-shaped, mounted on four, conspicuously raised stomach corners; filaments brush-like, tightly bundled, originating from a single root, deeply branched at some distance from the root, with numerous short gastric filaments; phacellae brownish-orange in colour, also after preservation (Figs. 1C, D).

Gonads paired, narrow leaf-shaped, separated by perforated interradial septum, extending from stomach rim to pedalium, tapering at level of rhopalia and towards stomach rim; ripe gonads milky whitish.

Developmental stages: The growth of young medusae to adult has been studied. We classified the individuals of *C. marsupialis* captured in Denia (Spain) from June to October 2010 into 6 different developmental stages, using both size and different morphological characters as indicators of the development of the animals. The six stages were named from A to F (Figs. 2A-F). We monitored the development of small cubomedusae into the subsequent stages (Acevedo et al. 2013), so we confirmed they belong to the same species *C. marsupialis*.

Stage A (MZB 2015-4801, n=10): Although the metamorphosis of the polyp has not been observed and described yet, very small medusae (< 2mm DBW; supposed recently detached from the polyp) have been caught in the field (Denia, Spain). Initial stage A (Fig. 2A): whitish-colourless, tetraradial, spheroid to cuboid umbrella with large round warts irregularly dispersed over entire exumbrella; bell height up to 1.4 mm, bell width up to 2.2 mm. Tentacles, 4, without pedalia, resembling pearl-string with white, spherical nematocysts batteries. Velarial canals and rhopaliar niche ostia, not yet developed, rhopalium with 6 eyes (2 median complex lens eyes + 2 lateral slit eyes + 2 lateral pit eyes). No gastric filaments.

Stage B (MZB 2015-4802, n=12): Mean bell height (BH) 1.5 (± 1.0) mm, mean bell width (DBW) 2.1 (± 0.7) mm; although an overlap in size with stage A exists, the main difference is the appearance of gastric filaments, four, one in each stomach corner; velarial canals not yet developed; rhopalial niche with scale, pedalia, 4, begin to develop (Fig. 2B).

Stage C (MZB 2015-4803, n=8): Mean BH 2.1 (± 1.7) mm, mean DBW 3.1 (± 1.6) mm; gastric phacellae, completely developed (Fig. 2C); velarial canals not yet developed; pedalia, 4, still developing.

Stage D (MZB 2015-4804, n=8): Mean BH 4.3 (± 4.2) mm, mean DBW 6.4 (± 3.9) mm; velarial canals, begin to develop (Fig. 2D); pedalia development completed; gonads, appearance of central axis.

Stage E (MZB 2015-4805, n=2): Mean BH 15.8 (± 9.1) mm, mean DBW 19.1 (± 10.7) mm; velarial canals and pedalia completely developed; gonads, developing but not yet mature, distinction of sex not yet possible (Fig. 2E).

Stage F (MZB 2015-4806, n=4): Mean BH 23.7 (± 2.1) mm, mean DBW 28.8 (± 2.7) mm; Gonads, mature, sex distinction possible (males: finger-print appearance; females: oocytes) (Fig. 2F).

Remarks: The medusa stage can be found in coastal waters (~0.5-10 meters depth) along sandy beaches with a gentle slope where seagrass meadows (*Posidonia oceanica*) and green algae (*Caulerpa prolifera*) coexist on rocky and sandy bottoms in the Mediterranean Sea (Bordehore et al. 2011). It is also common to observe this species in canals or harbours. The medusae have been observed swimming near the surface both during day and night. However, they seem more active feeders during the night, preying on zooplankton and ichthyoplankton. They can be observed especially during the night, when attracted to a light source (Acevedo et al. 2013).

The sting of the medusae causes a severe pain, a burning sensation, erythematous-vesicular eruption, and local oedema (Peca et al. 1997). Bordehore et al. (2015) described the first published case of systemic effects after contact with this species.

When mature medusae of both sexes aggregate for reproduction around mid-October, spermatozoa are released into the water to fertilize the eggs inside the female medusa, as has been observed for other species of *Carybdea* (Studebaker 1972 for *C. xaymacana*; Matsumoto 1995 for *C. rastonii*). The animals are oviparous and the fertilized eggs are shed into the water (this study).

The development of the polyp stage is not completely known. Up to now it has been possible to observe the settlement of the planulae and their transformation into the primary polyp (with 2 or 3 tentacles), but after 2-3 months of maintenance they died before reaching adult polyp size (this study). Therefore, any asexual reproduction in this species remains unknown, but similarities with the development, budding, and metamorphosis described by Studebaker (1972), Stangl et al. (2002), Fisher and Hofmann (2004), and Straehler-Pohl and Jarms (2011) for *C. xaymacana* (= former *C. marsupialis* from Puerto Rico) are expected. The metamorphosis of polyps is hypothesized to start around the 1st – 2nd week of May in the Western Mediterranean, as tiny medusae (1-2 mm DBW) have been caught from around mid-May to July during the

Fig. 3 Characters for identification in *Carybdea* species (species in chronological order of appearance in the text): *C. marsupialis* (A-D): A: adult medusa (preserved), B: epaulette-shaped gastric phacellus, C: Pedalial canal with rounded knee bend without appendage, D: octant of velarium with 3 canal roots, note slender, very sharp tipped velarial canals; *C. xaymacana* (E-I): E: adult medusa (preserved), F: single rooted gastric phacellum, original drawing of Bigelow (1938), G: gastric phacellus, H: pedalial canal, note knee bend with appended peak (arrow), I: octant of velarium, note two broad, biforked, sharp-tipped velarial canals; *Carybdea* sp. from California (J-M): J: adult medusa (preserved), K: single stemmed, single rooted gastric phacellus, L: pedalial canal knee bend with appended thorn (arrow), M: octant of velarium, note two multiple branched, round tipped velarial canals. Scale bar: 1 cm.

monitoring of the species performed on the coast off Denia for 5 years (2010-2015). Adult medusae reproduce in late October to early November, and the last medusae of the season were collected in November.

Reported distribution: Mediterranean Sea

Spain:

Denia: between Racons-Molinell River (38°53′09″N, 0°02′14″E) and 2 km south of Denia harbour (38°50′55″N, 0°02′14″E) (Bordehore et al. 2011).

Catalonia: canals of the harbour in Empuriabrava, Badalona, Sitges, l'Ampolla and Alfacs Bay (LIFE CUBOMED project database, www.cubomed.eu).

Southern coast of Spain: Valencia, Gandía, Oliva, Jávea, Sta. Pola, St. Pedro del Pinatar, Almería, Málaga and Cádiz (LIFE CUBOMED project database, www.cubomed.eu).

Italy:

Tuscany, Ligury (western Italian coast) (Bordehore et al. 2011);

Numana harbour (Riviera del Conero, Ancona, Adriatic Sea) (Di Camillo et al. 2006);

Fano (Boero and Minelli, 1986);

Gulf of Venice (Mizzan, 1993)

Gulf of Trieste in October 1998 (Bettoso, 2002)

Tunisia:

Hammamet beach (Gueroun et al. 2015)

Malta:

St. George's Bay (Birżebbuġa), Msida, Ta' Xbiex and other marinas and harbours (Pulis 2015).

Carybdea xaymacana Conant, 1897

Figs. 3E-I; 4B, D, F

Material examined: Five (5) specimens from Smithsonian Institution (USNM 54457, 54458, 54461); newly detached medusae and polyps from the cultures of R. Petie, member of the working group of Ass. Prof. A. Garm, University of Copenhagen, Denmark, originally sampled by B. Werner, C. E. Cutress, and J. P. Studebaker (1971) in Puerto Rico.

Diagnosis: Gastric phacellae epaulette shaped, single rooted; Velarial canals 2 per octant; biforked; Pedalia knee bend volcano-shaped to triangular.

Description:

Adult medusa: Bell (Fig. 3E), highly transparent, colourless, bell-shaped, scattered with very small colourless nematocyst warts; mammilation scatters bell from apex to bell margin (bigger nematocyst warts at interradial furrows, smaller nematocyst warts on bell sides): apex plane convex (Fig. 3E), slight horizontal constriction near the top present. Bell height up to 24 mm, bell width up to 27 mm.

Pedalium, simple, unbranched flattened, scalpel-shaped, measures approx. 1/3–1/2 bell height in length, situated in each interradial corner, with irregular, white nematocyst bands on outer keel of pedalia, very small warts scatter outer half of pedalia, carrying single, flesh coloured tentacles in the interradial corners of the bell rim, pedalial canal strongly depressed at base (diamond-shaped in diameter), going straight through pedalium, showing a volcano-shaped to triangular knee bend with small peak appended to outer knee bend (Fig. 3H).

Rhopalium located inside heart-shaped rhopalial niche ostium, with triangular covering scale ("angle" rounded), scale scattered with very small nematocysts, approx. 1/7 to 1/8 of bell height up from margin; rhopalium with 6 eyes (2 median lens eyes + 2 lateral slit eyes + 2 lateral pit eyes).

Velarium (Figs. 3I), with very small nematocyst warts, containing 2 velarial canals per octant, broad in width, forked at tips, sharp tips, seldom dendritic, at times with more than two branches; canals flanking frenulum simpler, sometimes unforked, canals flanking pedalia more complex with 2 to 3 main branches and sometimes with single side branches.

Four-lobed, cruciform manubrium without nematocyst warts, 1/3 - 1/2 bell height in length

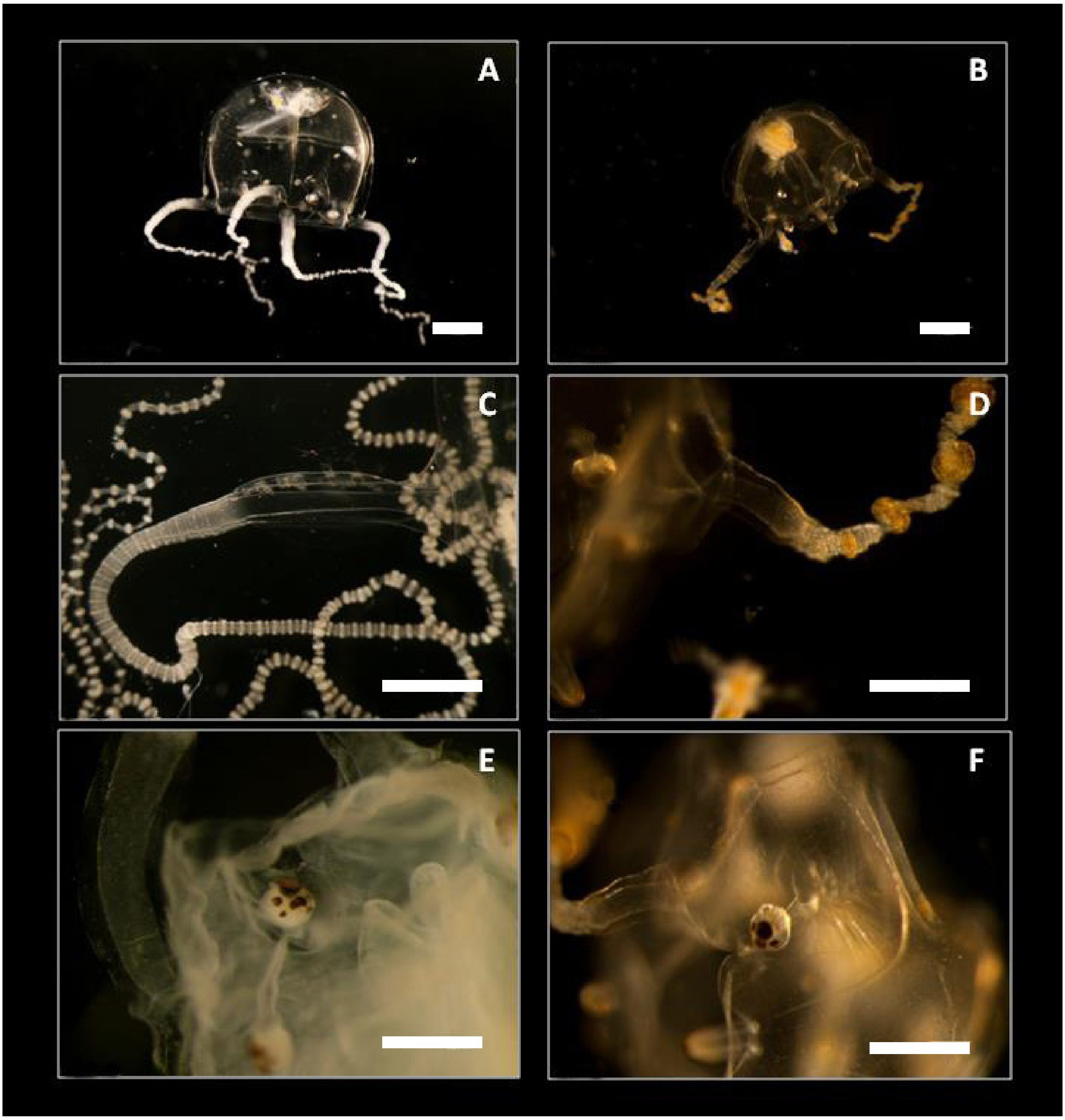

Fig. 4 Comparison between smaller stages of *C. marsupialis* (left column) and *C. xaymacana* (right column); (A) and (B) General view of the small cubomedusae, 4 tentacles in *C. marsupialis* and 2 tentacles in *C. xaymacana*; (C) and (D) Comparison of the tentacles; (E) and (F) Detail of the rhopaliar niche. Photographs taken to alive specimens (with exception of E): E. Obis. Scale bar: 1 mm.

and connected to flat and shallow stomach; stomach communicates perradially with 4 gastric pockets leading into velarial canals. Gastric phacellae (Fig. 3F), 4, epaulette-shaped, mounted on four stomach corners, consisting of 30-35 filaments per quadrant originating from a single root (Figs. 3F, G).

Gonads paired, narrow leaf-like, separated by unperforated interradial septum, extending from stomach rim to bell margin, tapering toward stomach rim and bell margin; differences in the gonads between both sexes can be observed under the microscope, as for other carybdeid species; ripe gonads milky whitish.

<u>Newly detached medusa and further development</u>: Bell (Fig. 4B), yellowish, tetraradial, slightly pyramidal shaped with large,

ovoid (0.14 mm x 0.06 mm) nematocyst warts, both sides of the interradial furrow; bell height up to 1.4 mm, bell width up to 1.2 mm.

Tentacles, 2 (primary) (Fig. 4B), located opposite without pedalia, resembling pearl-string with spherical, orange coloured, and lens-shaped, white nematocyst batteries (Fig. 4D); during further growth the medusa develops a second pair of opposite tentacles between primary tentacles.

Rhopalial niche ostia not yet developed, rhopalium with 6 eyes (Fig. 4F) (2 median lens eyes + 2 lateral slit eyes + 2 lateral pit eyes).

Gastric filaments, 4, one per quadrant.

Remarks: The species *C. xaymacana* Conant 1897 was first reclassified as *C. marsupialis* var. *xaymacana* by Mayer (1910) and then as *C. marsupialis xaymacana* by Bigelow (1938). Later, Kramp (1961) and Studebaker (1972) listed both *C. xaymacana* and *C. murrayana* as synonyms of *C. marsupialis*, but Gershwin (2005) hinted at the separation of these species.

Reported distribution: Caribbean Sea.

Carybdea sp. from Pacific Ocean (California)

Figs. 3J-M

Material examined: Five (5) different sized individuals from Santa Barbara (California) collected 20 meters west of Goleta Pier in 5 meters of water; collected by Shane Anderson, October 21, 1998; preserved in 5% formalin. Two (2) specimens deposited in the Natural History Museum of Barcelona (Museu de Ciències Naturals de Barcelona), Spain (MZB 2015-1702).

Diagnosis: Gastric phacellae epaulette shaped, single rooted, single stemmed; velarial canals 2 per octant; multiple branched; pedalial knee bend with thorn-like appendage.

Description:

Adult medusa: Bell (Fig. 3J) highly transparent, nearly cuboid, marked interradial furrows, bell densely scattered with white nematocysts of irregular shapes and sizes; bell height up to 20 mm, bell width up to 31.5 mm.

Pedalium, simple, unbranched flattened, scalpel-shaped, situated in each interradial corner measures approx. 2/3 bell height in length, outer wing, scattered with round to oval warts of different sizes, white nematocyst bands on outer keel; inner wing, without nematocyst warts; pedalium carrying one white to flesh coloured tentacle (preserved specimens). Pedalial canal, diameter diamond shaped with sharp keels, broad at base, flaring slightly from knee bend towards distal end, slightly tapering at distal end, tentacle insertion broader than distal end of canal; going straight to slightly curved through pedalium, showing a volcano-shaped to triangular knee bend with small, thorn-like appendage on outer knee bend (Fig. 3L).

Rhopaliar niche ostium heart shaped with triangular covering scale, small nematocysts on scales on rare occasions, approx. 1/5 of bell height up from margin; rhopalium with 6 eyes (2 median lens eyes+ 2 lateral slit eyes + 2 lateral pit eyes).

Velarium, in general free of nematocyst warts, only canal roots might show some scattered, small warts, containing 2 broad velarial canal roots per octant, giving rise to 2 to 3 branched velarial canals, canals slim in width, forked at tips, tips rounded, slightly dendritic or lobated, often with more than two side branches; canals flanking frenulum simpler, biforked to triforked with few, straight side branches; canals flanking pedalia more complex, root with 2 to 3 main branches with several side branches, resembling antlers (Fig. 3M).

Four-lobed, cruciform manubrium without nematocyst warts, 1/3 - 1/2 bell height in length and connected to flat and shallow stomach; stomach communicates perradially with 4 gastric pockets leading into velarial canals. 4 gastric phacellae (Fig. 3K), epaulette-shaped, mounted on four stomach corners, consisting of one circular root per quadrant that gives rise to one stem that splits into branches with several brush-like filaments.

Gonads paired, broad leaf-like to arrowhead-shaped, separated by unperforated interradial

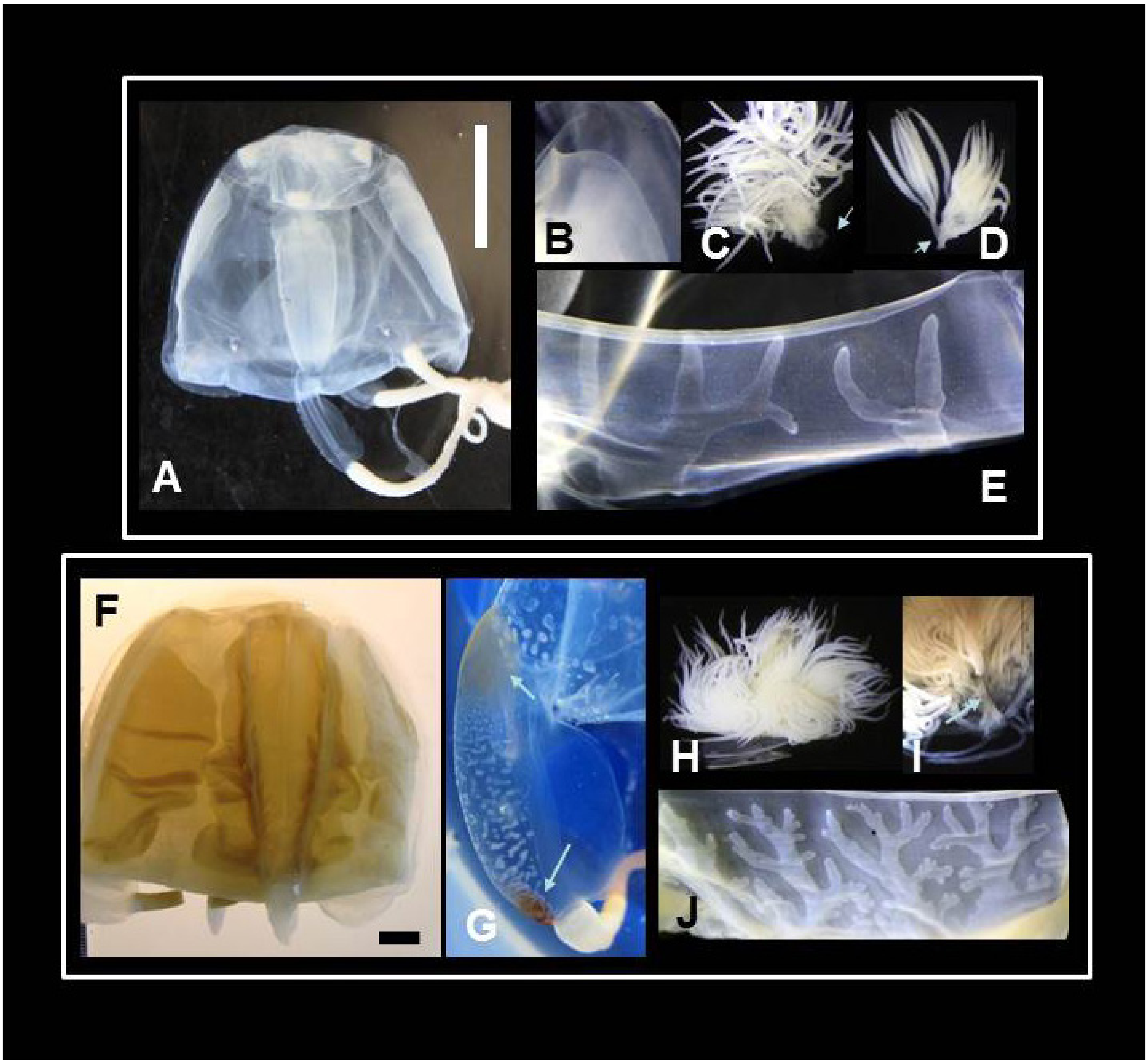

Fig. 5 Characters for identification in *Carybdea* species (species in chronological order of appearance in the text; preserved specimens): *C. arborifera* (A-E): A: adult medusa (preserved), B: rounded pedalial canal knee bend, C: Epaulette-shaped gastric phacellus, note multiple stems of gastric filaments (arrow), D: two short-stemmed (arrow), brush-shaped gastric filaments, E: octant of velarium with 2 velarial canal roots, note broad and quite simple branching structure of velarial canals; *C. branchi* (F-J): F: adult medusa (preserved), G: colourful pedalium of living specimen, H: Epaulette-shaped gastric phacellus, I: Close up of base of gastric phacellus, note single root as origin of gastric filaments (arrow), J: octant of velarium with 3 velarial canal roots, note complex, lobated branching structure of velarial canals. Scale bar: 1 cm.

septum, extending from stomach rim to bell margin, tapering towards stomach rim and bell margin, lateral margins overlap; sexes separate but unimorph; ripe gonads milky whitish.

Remarks: The specimens from California were first mentioned by Stiasny (1922) and identified as *Carybdea rastonii* citing Maas (1903, 1910), Bigelow (1909) and Mayer (1910) who had proclaimed *Carybdea arborifera* to be a synonym of *C. rastonii*. This identification was later adopted by Satterlie (1979) and Satterlie and Spencer (1979). In 1990, Larson and Arneson collected cubozoans off Scripps pier, La Jolla (California). Larson who had observed "*Carybdea marsupialis*" in Puerto Rico (Larson 1976) applied the name to reclassify the carybdeid species from California (Larson 1990). Larson and Arneson (1990) also examined *C. rastonii* specimens collected at the type locality of the species (i.e. Gulf of St. Vincent, Australia). In addition, they inspected carybdeid medusae of *Carybdea xaymacana* from the Bahamas, misidentified as "*C. marsupialis*". The authors concluded that the specimens collected in La Jolla fit more closely the diagnosis of *C. marsupialis* as described by Bigelow (1938) than the one of *C. rastonii* of Maas (1897). This diagnosis was adopted by Satterlie and Nolen (2001). Other authors did not agree with this reclassification, as the morphological characters did not completely fit (Fenner 1997, Gershwin 2005).

Reported distribution: Pacific Ocean (California).

Other *Carybdea* species

Carybdea arborifera Maas, 1897

Figs. 5A-E

Material examined: Ten (10) different sized specimens from Kewalo Basin (Hawaii), May 29, 2013, collected by Angel Yanagihara; preserved in 5% buffered formalin. Five (5) specimens deposited in the Natural History Museum of Barcelona (Museu de Ciències Naturals de Barcelona), Spain (MZB 2015-1703).

Diagnosis: Gastric phacellae epaulette shaped, single rooted, multiple stemmed; velarial canals 2 per octant; biforked to multiple branched; Pedalial knee bend rounded; no appendage.

Description:

Adult medusa: Bell (Fig. 5A), blunt pyramidal, highly transparent, mesoglea thin, regularly scattered with small, colourless, nematocyst warts, from apex to bell margin. Bell height, up to 30 mm, bell width, up to 33 mm.

Pedalium simple, unbranched flattened, scalpel-shaped, outer keel lined with rows of white nematocyst warts, carrying single, pale pink coloured homogeneous banded tentacle; pedalial canal diamond-shaped in diameter, rounded knee bend without appendage (Fig. 5B).

Rhopalium located inside heart-shaped rhopalial niche ostium; few, very small, round nematocyst warts on upper scale; rhopalium with 6 eyes (2 median lens eyes + 2 lateral slit eyes + 2 lateral pit eyes).

Velarium (Fig. 5E), free of nematocyst warts, containing 2 velarial canal roots per octant, velarial canals, broad, biforked to multiple branched, rounded tips; canals flanking frenulum, small, mostly bi-forked, canals flanking pedalia, larger, more complexly branched: bi-forked with additional bi-forked side branches.

Gastric phacellae (Fig. 5C), 4, epaulette-shaped, on four stomach corners, single rooted, gastric filaments (Fig. 5D), brush-shaped, multiple short stems attached to single root. In two specimens mysid shrimp and a polychaete worms were found in the stomach.

Gonads, leaf-shaped, extending from stomach to velarium.

Remarks: Maas (1903, 1910), Bigelow (1909), and Mayer (1910) considered *C. arborifera* to be a synonym of *C. rastonii*. However, the molecular genetic phylogeny of Bentlage et al. (2010) demonstrated that *C. arborifera* and *C. rastonii* are two different species.

Reported distribution: Pacific Ocean (Hawaii).

Carybdea branchi Gershwin & Gibbons, 2009

Figs. 5F-J

Material examined: Seven (7) living adult individuals collected by the authors for gross morphological examinations; five (5) preserved adult individuals for detailed morphological observations, Hout Bay (South Africa); collected by André C. Morandini & Sergio N. Stampar, May 05, 2013; preserved in 5% formalin. One (1) specimen deposited in the Natural History Museum of Barcelona (Museu de Ciències Naturals de Barcelona), Spain (MZB 2015-4807).

Diagnosis: Robust and well sculpted body; gastric phacellae epaulette shaped, single rooted, multiple stemmed; velarial canals 3 per octant; complexely branched; pedalia knee bend

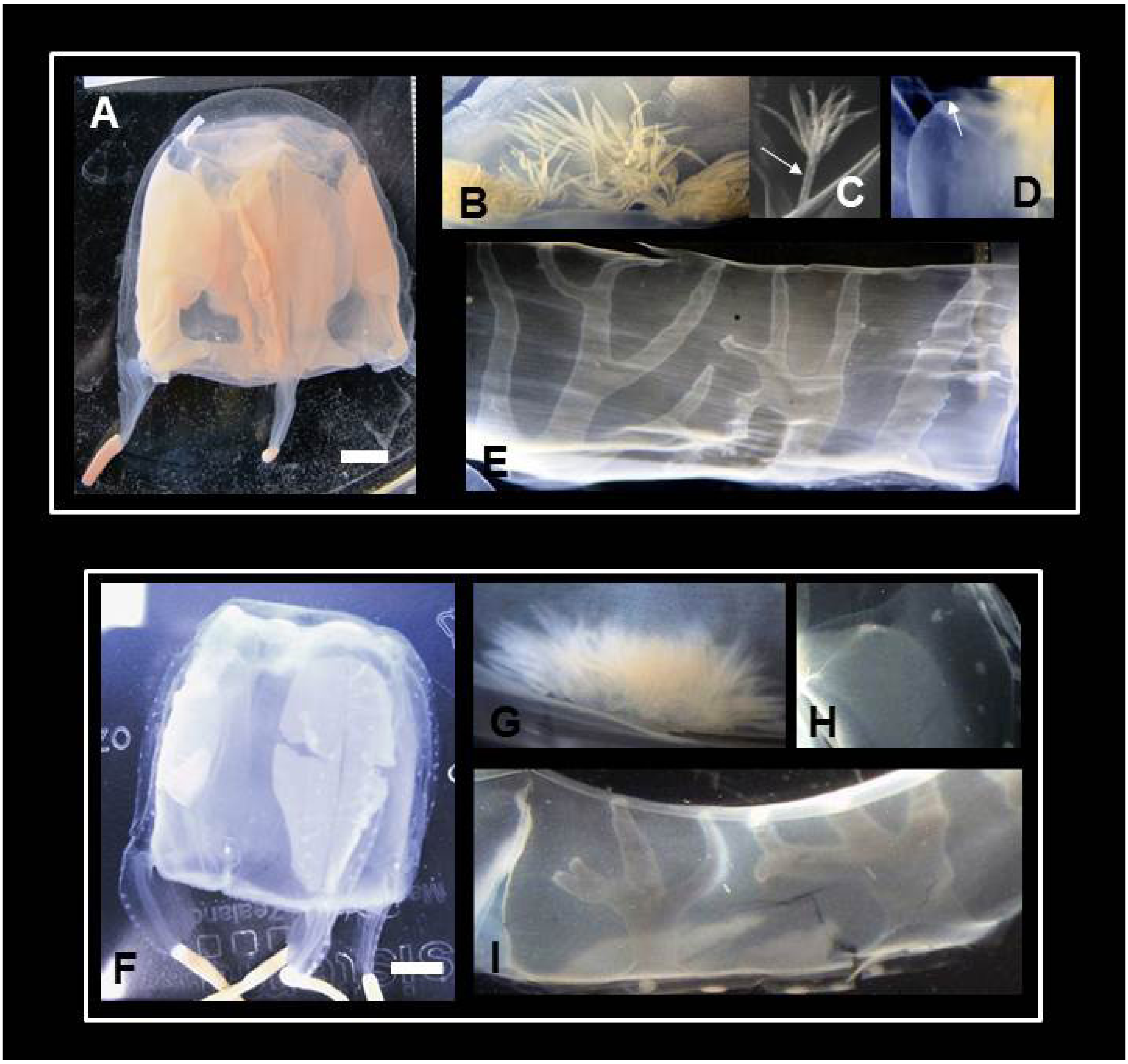

Fig. 6 Characters for identification in species of the family Carybdeidae (species in chronological order of appearance in the text; preserved specimens): *C. brevipedalia* (A-E): A: adult medusa (preserved), B: line-shaped gastric phacellus, C: gastric filament, note long stem (arrow), D: peaked (arrow) pedalial canal knee bend, E: octant of velarium with 3 canal roots; *C. rastonii* (F-I): F: adult medusa (preserved), G: line-shaped gastric phacellus, H: pedalial canal with rounded knee bend, I: octant of velarium, note two broad, blunt tipped velarial canals. Scale bar: 1 cm.

upwards turned volcano-shaped. The brownish pigmentation of the phacellae and pedalia is characteristic of this species.

Description:

Adult Medusa: Bell (Fig. 5F), highly transparent, gastric phacellae, yellowish to reddish-brown, pedalia light brown at outer wing base, reddish brown at distal end of outer wing – pigmentation fades very fast after preservation; cube-shaped, densely scattered with white nematocyst warts of different shapes and sizes; mammilation scatters bell from apex to bell margin (bigger nematocyst warts at interradial furrows and bell margin, smaller nematocyst warts on bell sides and apex); apex plane convex, thick mesoglea, no horizontal constriction near the top present. Bell height 50-70 mm high, bell width up to 65-90 mm (preserved specimens).

Pedalium (Fig. 5G) simple, unbranched, flattened, scalpel-shaped, brownish colour marks on base and distal end of outer wing, measures approx. 1/2 bell height in length, situated in each interradial corner, outer keel densely scattered with irregular, white nematocyst warts, carrying single, flesh coloured tentacle; pedalial canal stark depressed at base, square in diameter with lateral keels, going straight through pedalium, showing a volcano-shaped, upwards turned knee bend without appendage.

Rhopalium located inside heart-shaped rhopalial niche ostium. Orifice with triangular upper covering scale with pointed tip, two narrow, longish, lower scales, creating a compressed or narrow heart-shaped orifice (resembling almost Y-shaped); very small, round nematocyst warts scattered on scale; approx. 1/4 to 1/5 of bell height up from margin; rhopalium with 6 eyes (2 median lens eyes+ 2 lateral slit eyes + 2 lateral pit eyes).

Velarium (Fig. 5J), free of nematocyst warts, containing 3 velarial canal roots per octant, canals dendritic, some side branches tend to grow in centripetal direction (growth directed away from velarial margin, defined by Thiel 1970); canals flanking frenulum are very small, short, bush- or tree-like branched, canals originating from middle root and canals flanking pedalia are complexer with 2 to 3 dendritic, lobed main branches and several dendritic, lobed side branches.

Manubrium (1/2-2/3 bell height in length) with conspicuously long and broad mouth arms (2/3 manubrium length), tips rounded, without nematocyst warts, and connected to flat and shallow stomach; stomach communicates perradially with 4 gastric pockets leading into velarial canals. Gastric phacellae (Fig. 5H), 4, epaulette-shaped, mounted on four stomach corners, consisting of 15-20 brush-shaped filaments per quadrant, multiple stalked, stalks of filaments tightly aligned (Fig. 5I), originated from one root.

Gonads paired, broad leaf-like to ovoid shape, separated by unperforated interradial septum, extending from stomach rim to bell margin, tapering slightly towards stomach rim and bell margin; sexes separated but unimorph.

Remarks: Gershwin & Gibbons (2009) described *C. branchi* as a new species from South African waters. Previously, *C. branchi* had been reported in the literature as either *Carybdea alata* (*Alatina alata*) or *Tamoya haplonema*.

Reported distribution: South Africa (South Atlantic).

Carybdea brevipedalia Kishinouye, 1891

Figs. 6A-E

Material examined: Three (3) adult specimens, from Aburatsubo Bay, Kanagawa (Japan), collected by Sho Toshino, October 24, 2011; preserved in 5% formalin. Two (2) specimens deposited in the Natural History Museum of Barcelona (Museu de Ciències Naturals de Barcelona), Spain (MZB 2015-1704).

One (1) adult specimen, female, from Shirahama, Wakayama (Japan), collected by Shin Kubota, 1995.

Diagnosis: Gastric phacellae horizontal linear, gastric filaments multiple rooted, long stemmed; velarial canals 2 per octant; complexly branched; pedalial knee bend volcano-shaped, appended with a sharp peak.

Description:

Adult medusa: Bell (Fig. 6A), highly transparent, colourless, bell-shaped, regularly scattered with very small, colourless, equal-sized nematocyst warts, from apex to bell margin; apex convex, mesoglea thick, slight horizontal constriction near the top present. Bell height up to 35 mm high, bell width up to 33.5 mm (IPD).

Pedalium, simple, unbranched flattened, scalpel-shaped, approx. 1/3–1/2 bell height in length, situated in each interradial corner; outer wing scattered with very small, round warts, outer keel with irregularly shaped, white nematocyst bands; inner wing free of nematocyst warts, overhanging tentacle insertion; pedalium carrying single, white to flesh coloured tentacles in preserved specimens. Pedalial canal, diameter diamond shaped with sharp keels, flat and narrow at base, tapering below knee bend, flaring slightly towards mid-section, tapering towards distal end and flaring at tentacle insertion; going straight to slightly curved through pedalium, showing a slightly volcano-shaped to triangular knee bend with a sharp peak appended (Fig. 6D).

Rhopaliar niche ostium heart shaped, with triangular covering scale with distinct volcano-shaped tip; few small, round nematocyst warts on scale; approx. 1/6 to 1/7 of bell height up from margin; rhopalium with 6 eyes (2 median lens eyes+ 2 lateral slit eyes + 2 lateral pit eyes). .

Velarium (Fig. 6E), free of nematocyst warts, containing 2 velarial canal roots per octant, canals slim in width, seldom lobed, some side branches tend to grow in centripetal direction, rounded canal tips; canals roots flanking frenulum, giving rise to 2 main canals, 1st canal simple, slightly lobed, without additional side branches, 2nd main canal deeply forked into 2 branches with 0-2 additional side branches, canals roots flanking pedalia, giving rise to 1 main canal, deeply forked into 3 branches, with 0-2 additional side branches.

Very short, four-lobed, cruciform manubrium with sharply pointed mouth arm tips, without nematocyst warts, 1/5 bell height in length, connected to flat and shallow stomach; stomach communicates perradially with 4 gastric pockets leading into velarial canals.

Gastric phacellae (Fig. 6B), 4, horizontal linear, in four stomach corners, consisting of 10-15 single, long-stemmed, brush shaped filaments (Fig. 6C) per quadrant.

Gonads paired, arrowhead-shaped, (narrow at base, widening in the first third then tapering towards the upper end to a sharp peak) separated by unperforated interradial septum, extending from stomach rim to bell margin, tapering distinctly towards stomach rim and at rhopalial niche level, flaring towards marginal rim; sexes separated but unimorph; ripe gonads milky whitish to flesh coloured in preserved specimens.

Remarks: Bentlage et al. (2010), Bentlage & Lewis (2012), and Toshino et al. (2015) indicated that *C. brevipedalia* Kishinouye 1891 (formerly *C. rastonii*) should be listed as the only species of the genus *Carybdea* inhabiting Japanese waters.

Reported distribution: Japan.

Carybdea rastonii Haacke, 1886

Figs. 6F-I

Material examined: One (1) unregistered, preserved specimen, from Mirimbula (Victoria, Australia), collected by G. Hood, March 03, 2000; One (1) unregistered, preserved specimen, from Waterloo Bay (South Australia), collected by J. Seymour, February 1999.

Diagnosis: Gastric phacellae horizontal linear, gastric filaments with multiple roots, short stemmed; velarial canals 2 per octant, triforked; pedalial knee bend rounded, no appendage.

Description:

Adult medusa: Bell (Fig. 6F), highly transparent, colourless, slightly higher than wide, cuboid to almost cubical in shape, regularly scattered with colourless, nematocyst warts, from apex to bell margin, more dense on the bell sides than on the bell edges; nematocysts roundish to oval with different diameters, largest approx. 0.5 mm in diameter, velarium without nematocyst warts; apex plane to round convex, mesoglea thick, slight horizontal constriction near the top present. Bell height 30-35 mm high, bell width 20-30 mm (interrhopalial diameter).

Pedalium simple, unbranched flattened, scalpel-shaped, approx. 1/3–1/2 bell height in length, situated in each interradial corner; outer wing scattered with large, white to light brown nematocyst warts or bands of nematocysts (approx. 0.5 mm in length) covering the outer keel of the pedalia; inner wing free of nematocyst warts; pedalia carrying single, white to flesh coloured tentacles in preserved specimens, in living specimens pale pink to brownish when contracted, resembling bead-chain when relaxed with white nematocyst-battery rings on a pale pink tentacle “string”. Pedalial canal slightly tapering at upper end, rounded knee bend without any hook or thorn appended (Fig. 6H),

Specimen	Locality	Genbank accession	Citation
Carybdea arborifera **(SS216)**	Honolulu, Hawaii	KT288229	This study
Carybdea arborifera **(SS217)**	Honolulu, Hawaii	KT288230	This study
Carybdea arborifera **(SS218)**	Honolulu, Hawaii	KT288231	This study
Carybdea arborifera	Honolulu, Hawaii	GQ849096	Bentlage *et al.* 2010
Carybdea branchi **(SS195)**	South Africa	KT288232	This study
Carybdea branchi **(SS196)**	South Africa	KT288233	This study
Carybdea marsupialis **(CM20)**	Italy	KT288245	This study
Carybdea marsupialis **(CM21)**	Italy	KT288234	This study
Carybdea marsupialis **(CM02)**	St. Pola, Spain	KT288235	This study
Carybdea marsupialis **(CM06)**	St. Pola, Spain	KT288246	This study
Carybdea marsupialis **(CM08)**	St. Pola, Spain	KT288247	This study
Carybdea marsupialis **(CM19)**	St. Pola, Spain	KT288248	This study
Carybdea marsupialis **(CM09)**	Almadrava, Spain	KT288236	This study
Carybdea marsupialis **(CM12)**	Almadrava, Spain	KT288237	This study
Carybdea marsupialis **(CM13)**	Almadrava, Spain	KT288238	This study
Carybdea marsupialis **(CM23)**	Almadrava, Spain	KT288239	This study
Carybdea marsupialis **(CM25)**	Almadrava, Spain	KT288240	This study
Carybdea marsupialis **(CM10)**	Denia, Spain	KT288241	This study
Carybdea marsupialis **(CM14)**	Denia, Spain	KT288242	This study
Carybdea marsupialis **(CM15)**	Denia, Spain	KT288243	This study
Carybdea marsupialis **(CM22)**	Denia, Spain	KT288244	This study
Carybdea marsupialis **(CM07)**	Rasset, Spain	KT288249	This study
Carybdea marsupialis **(CM16)**	Rasset, Spain	KT288250	This study
Carybdea marsupialis **(CM17)**	Rasset, Spain	KT288251	This study
Carybdea marsupialis **(CM24)**	Rasset, Spain	KT288252	This study
Carybdea marsupialis **(SS183)**	L'ampolla, Spain	KT288253	This study
Carybdea xaymacana **(SS182)**	Puerto Rico	KT288254	This study
Carybdea brevipedalia **(SS213)**	Japan	KT288255	This study
Carybdea brevipedalia **(SS214)**	Japan	KT288256	This study
Carybdea **sp.**	Australia	GQ849115	Bentlage *et al.* 2010
Carybdea rastonii	Australia	GQ849116	Bentlage *et al.* 2010
Tamoya haplonema **(SS162)**	Ubatuba, Brazil	KT288257	This study

TABLE 1 Taxa included in this study with sampling area of the analyzed material and GENBANK accession.

going straight through the pedalium, diamond-shaped in cross-section with sharp outer keels at proximal end up to velarium level then turning circular in cross-section towards distal end.

Rhopalium located inside heart-shaped rhopalial niche ostium, with triangular covering scale; few small, round nematocyst warts on scale; approx. 1/6 to 1/5 of bell height up from margin; rhopalium with 6 eyes (2 median lens eyes + 2 lateral slit eyes + 2 lateral pit eyes).

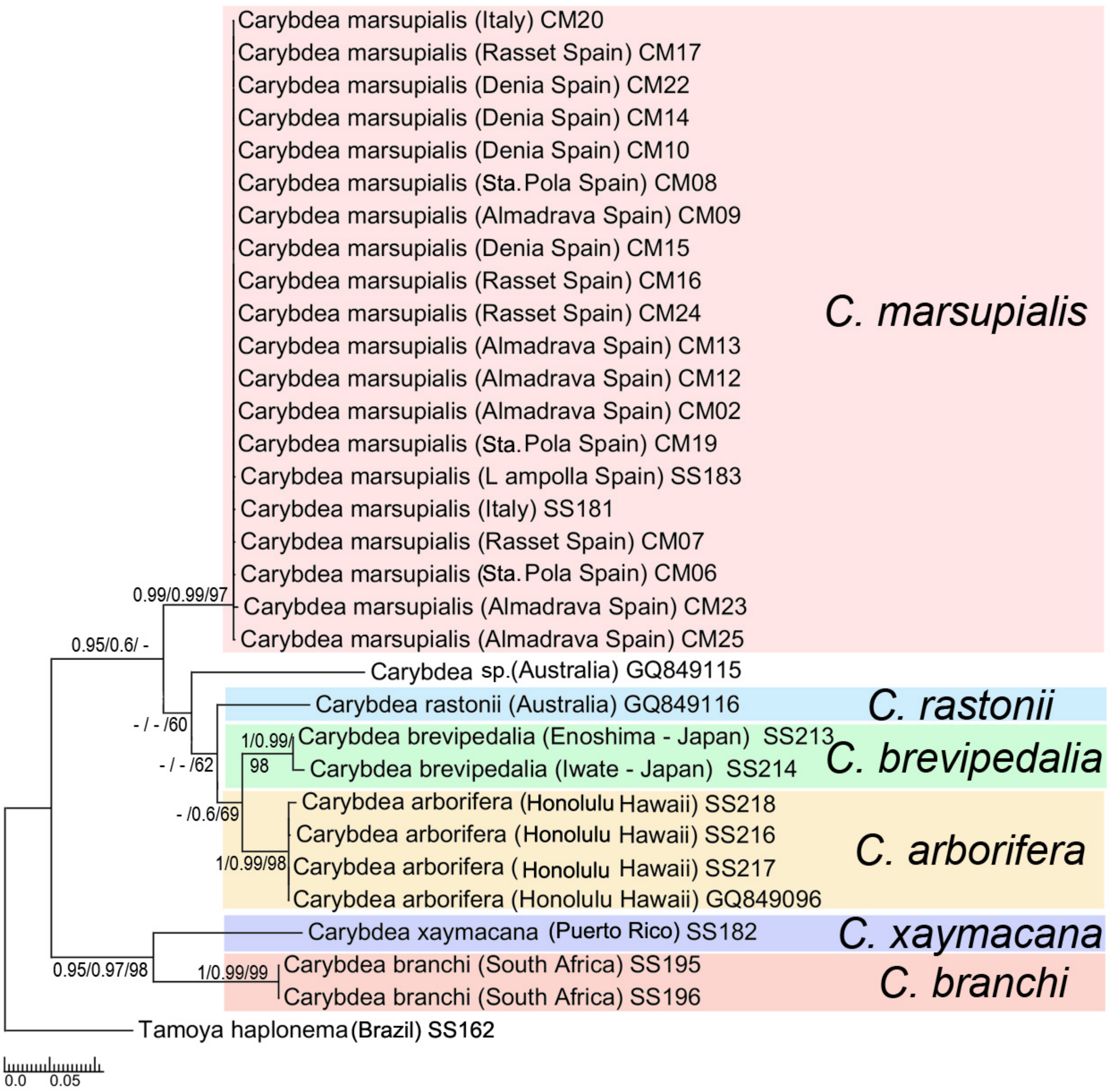

Fig. 7 Phylogenetic reconstructions (Maximum likelihood) of the analyzed specimens of *Carybdea* using the mitochondrial marker 16S. Numbers on the branches represent the estimated values of Bayesian inference (BI), maximum parsimony (MP) and maximum likelihood (ML) respectively. Note: The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-1776.8808) is shown. Branch with less than 50% support in annoted with (-).

Groups						
C. branchi		0.022	0.022	0.024	0.024	0.024
C. rastonii	0.339		0.023	0.017	0.016	0.016
C. xaymacana	0.233	0.344		0.025	0.025	0.025
C. marsupialis	0.269	0.215	0.312		0.020	0.019
C. arborifera	0.297	0.196	0.320	0.188		0.016
C. brevipedalia	0.304	0.200	0.323	0.178	0.108	

TABLE 2 Estimates of Evolutionary Divergence (p-distance - gray) over Sequence Pairs between Morphology Groups. Note: The number of base differences per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 347 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

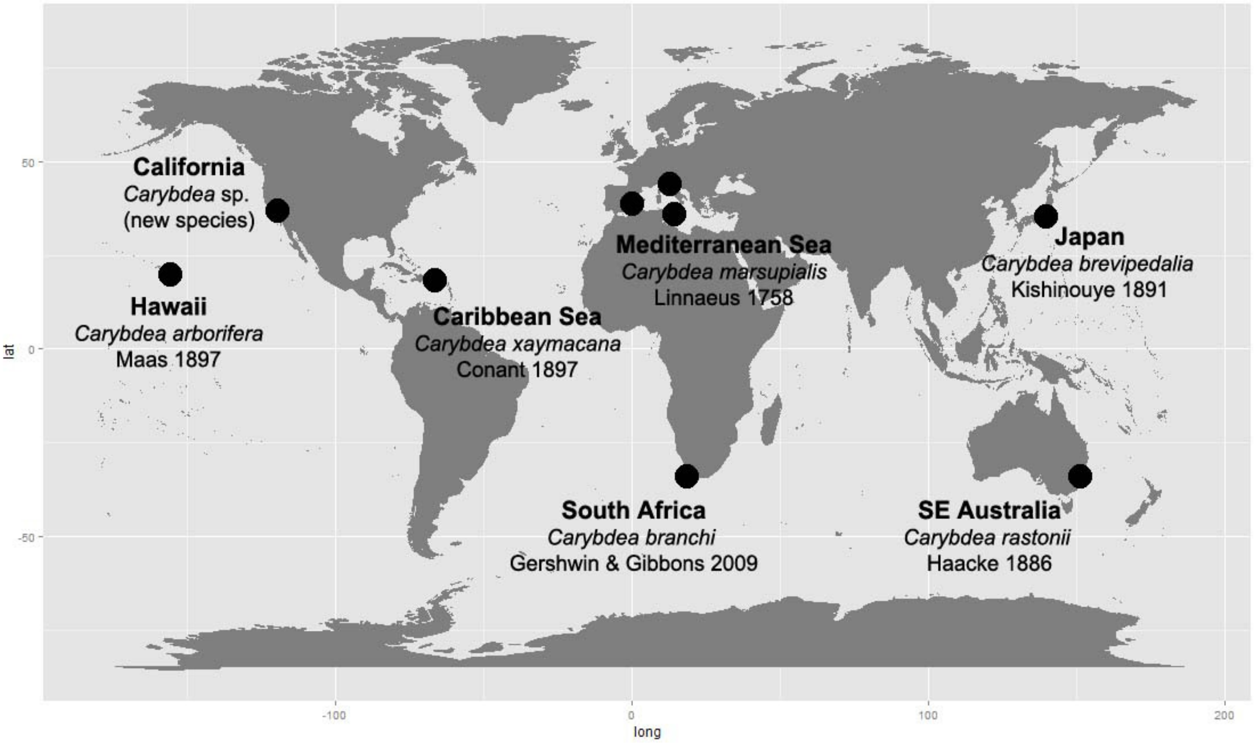

Fig. 8 Revised identification of *Carybdea* species. The results indicate that several species within Carybdea which were considered invalid or synonyms are indeed valid and recognizable species with distinct and restricted geographic distributions.

Velarium (Fig. 6I), free of nematocyst warts, containing 2 velarial canal roots per octant, canals broad in width, main canal tri-forked, square tipped, side branches sometimes bi-forked; canals flanking frenulum slightly smaller than canals flanking pedalia, all canals equally complex.

Four-lobed, cruciform manubrium with long, straight and blunt mouth arms, 1/2 to 3/4 of bell height in length, without nematocyst warts, connected to large stomach; stomach communicates perradially with 4 gastric pockets leading into velarial canals.

Gastric phacellae (Fig. 6G), pale pink to brownish coloured, 4, horizontal rows, in four stomach corners, consisting of ca. 12-15 single, short-stemmed, brush shaped filaments per quadrant.

Gonads paired, narrow leaf-like to blunt spear-head-shaped, separated by interradial septum, extending from stomach rim to bell margin, tapering towards stomach rim, tapering at rhopalia level, and broadening again towards bell margin; sexes separated but unimorph; ripe gonads yellowish to flesh coloured in preserved specimens.

Remarks: Haacke (1886) gave a detailed description of a species that he had sampled in St. Vincents Gulf in Southern Australia and which he named *C. rastonii*. Later, the species *C. arborifera* Maas 1897 and *C. brevipedalia* Kishinouye 1891 were reclassified as "geographic races" (Bigelow 1909) or local varieties ("lokale Varietäten") (Maas 1897, 1910) of the same species and synonyms of *C. rastonii* (Maas 1903, 1910; Mayer 1906, 1910; Bigelow 1938; Kramp 1961). This gave *C. rastonii* a wide distribution throughout the Pacific Ocean on both the eastern and western margins [Japan, Philippines, Taiwan, Guam (USA), California (USA), Hawaii (USA)] (e.g. Yatsu 1917, Matsumoto 1995, Kingsford and Mooney 2014). But Gershwin (2006) demonstrated that the Japanese population has a distinctive cnidome compared to *C. rastonii* from Australia. Moreover, Bentlage et al. (2010) showed that the populations from Hawaii and Japan were a case of several species being united under the name *C. rastonii* and resurrected the species *C. arborifera* (Hawaii) and *C. brevipedalia* (Japan).

Reported distribution: Australia.

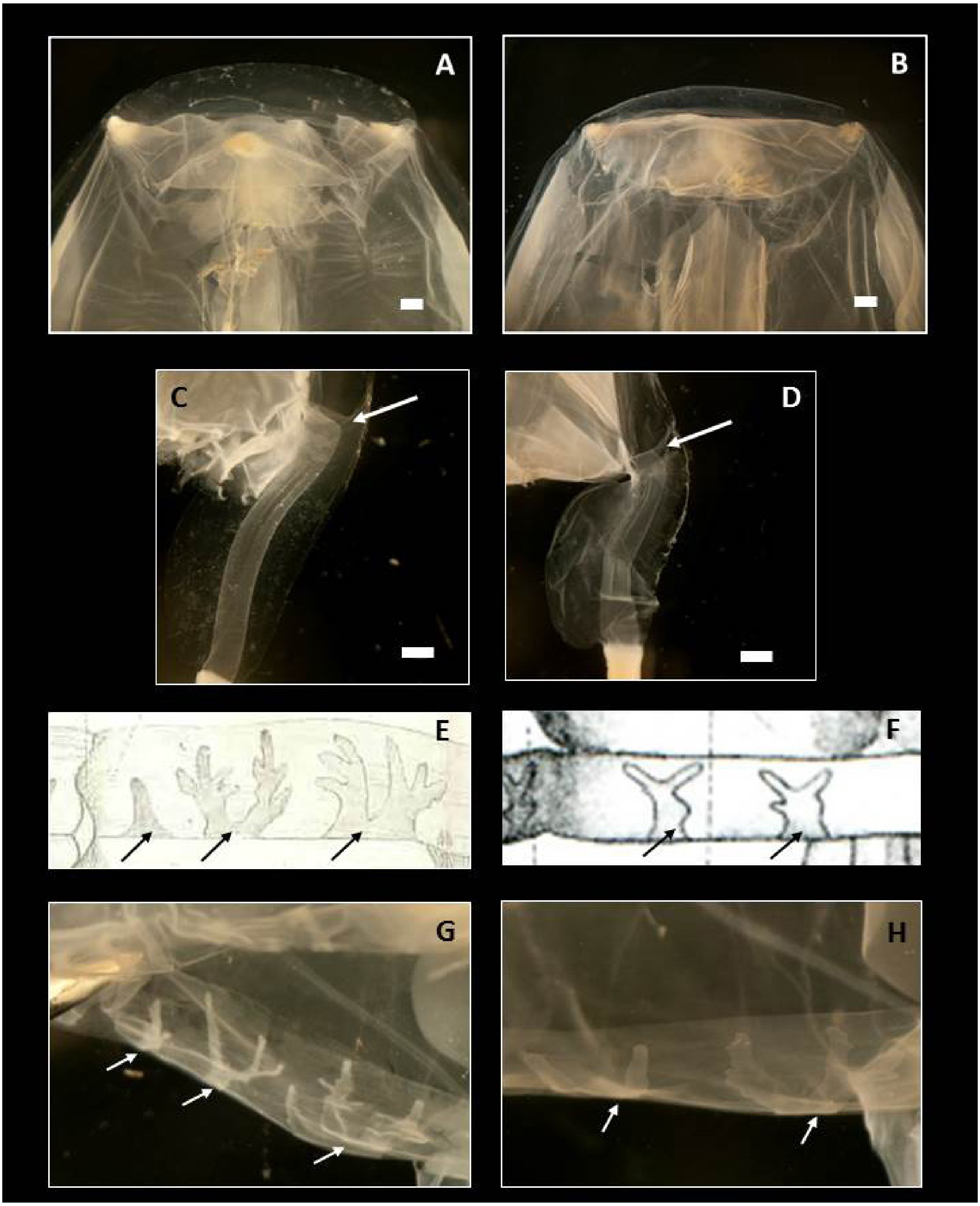

Fig. 9 Comparison between adult *C. marsupialis* and *C. xaymacana* (preserved specimens); (A) Thick apex and gastric phacellae of *C. marsupialis*; (B) Upper exumbrella and phacellae of *C. xaymacana*; (C) Pedalial canal with rounded knee bend (arrow) in *C. marsupialis* and (D) Volcano-shaped to triangular knee bend (arrow) in *C. xaymacana*; (E) Original line drawing of *C. marsupialis* velarial canals (arrows) by Claus (1878); (F) Original line drawing of *C. xaymacana* velarial canals by Conant (1898); Velarial canals on the specimens observed in this publication: (G) *C. marsupialis*, 3 velarial canals (arrows) per octant (= 6 per quadrant) with different complexity; (H) *C. xaymacana*, 2 biforked velarial canals (arrows) per octant (= 4 per quadrant). Photographs: E. Obis. Scale bar = 1 mm.

Author	Collection site	Named species	Actual species
Mayer 1910	Hawaii	*C. rastonii*	*C. arborifera* Maas 1897
Stiasny 1922	East Pacific	*C. rastonii*	"Californian *Carybdea*" (new species)
Studebaker 1972	Puerto Rico	*C. marsupialis*	*C. xaymacana* Conant 1897
Larson 1976	California	*C. marsupialis*	*C. xaymacana* Conant 1897
Larson & Arneson 1990	California	*C. marsupialis*	"Californian *Carybdea*" (new species)
Stangl *et al* 2002	Puerto Rico	*C. marsupialis*	*C. xaymacana* Conant 1897
Fisher & Hofmann 2004	Puerto Rico	*C. marsupialis*	*C. xaymacana* Conant 1897
Martin 2004	California	*C. marsupialis*	"Californian *Carybdea*" (new species)
Straehler-Pohl & Jarms 2005	Puerto Rico	*C. marsupialis*	*C. xaymacana* Conant 1897
Kazmi & Sultana 2008	Arabian Sea	*C. marsupialis*	*Alatina cf. grandis* Agassiz & Mayer 1902
Gray *et al.* 2009	California	*C. marsupialis*	"Californian *Carybdea*" (new species)
Straehler-Pohl & Jarms 2011	Puerto Rico	*C. marsupialis*	*C. xaymacana* Conant 1897

TABLE 3 References and corrected identifications.

Molecular genetic results

The molecular approach conducted in this study intended to complement the morphological analysis. Our data from the mitochondrial 16S rRNA gene supports the species delimitations based on morphological differences between the species of *Carybdea*. In Figure 7 a phylogenetic reconstruction (Maximum Likelihood) is presented with the terminals sampled for this study (Table 1). Six clades are consistent with our interpretation of the morphological data. Of particular interest is that *C. xaymacana* from the Caribbean Sea (historically referred to as *C. marsupialis*) is only distantly related to the material from the Mediterranean Sea identified as *C. marsupialis*. Moreover, the molecular results show the specimens from Italy and Spain to belong to the same species (i.e. *C. marsupialis*). Further, there is one sequence corresponding to a *Carybdea* species from Australian waters that differs from *C. rastonii* in South Australia for which identity and distribution have not been clarified yet (Gershwin 2005; Bentlage et al. 2010).

Molecular divergence for the same mitochondrial marker was estimated via p-distance. The distance between the groups defined by morphology was evident and in this case, the *C. rastonii* group showed considerable consistency (Table 2). The p-distance values were between 0.339 and 0.108 among morphological groups (Table 2). The standard deviation also remained consistent for all comparisons always between 0.016 and 0.025.

Discussion

We have observed differences (both morphological and genetic) between *Carybdea* specimens from different regions. Our results confirmed the conclusions drawn from the molecular phylogenetic analysis of Bentlage et al. (2010) that several species names within *Carybdea* that were considered invalid or synonyms of other species (i.e. *Carybdea arborifera* from Hawaii, *Carybdea brevipedalia* from Japan, *Carybdea marsupialis* from the Mediterranean Sea, *Carybdea rastonii* from South Australia, and *Carybdea* *xaymacana* from the Caribbean Sea) are indeed valid and recognizable species with distinct geographic distributions (Fig. 8).

Differentiation of Mediterranean and Caribbean *Carybdea*

Our observations on the characters of the Mediterranean specimens coincide in every aspect with the detailed description of Claus (1878) of individuals of *C. marsupialis* from the Adriatic. On the other hand, adult specimens from the Caribbean (Puerto Rico) agreed with the original description of *C. xaymacana* by Conant (1897). The most obvious differences between Mediterranean and Caribbean adult specimens are the structure of the phacellae, the structure of the pedalial canal knee bend and the number and structure of the velarial canals (Fig. 9).

The phacellae of the Mediterranean *C. marsupialis* are more robust and profused than those in *C. xaymacana*, having several trunks arising from the main root (cauliflower shape) which is connected to the floor of the stomach (Figs. 3E, G). By contrast, the phacellae in the medusae of the Caribbean *C. xaymacana* have only a single stalk each and fewer gastric filaments per phacellum (Figs. 3F, H).

The pedalial canal knee bend in the Mediterranean *C. marsupialis* is rounded, showing no appendage (Figs. 3C, 9C) contrary to the volcano to triangular knee bend of the Caribbean *C. xaymacana* which shows an appended sharp peak (Figs. 3H, 9D).

The Mediterranean *C. marsupialis* typically possesses 3 velarial canal roots per octant (Figs. 3D, 9E, G) while the Caribbean *C. xaymacana* has only 2 velarial canal roots per octant (Figs. 3I, 9F, H), all giving rise to one branching canal per root. Bigelow (1938) interpreted the number of velarial canals as representing an ontogenetic series, but as shown for *C. brevipedalia* (former *C. rastonii* from Japan) the ontogenic development of the velarial canals begins with a fixed number of canal roots from which the main velarial canals arise and only the profusion and complexity of the growing canals differ ontogenetically but not the number of the roots. Also Gershwin (2005) stated that the number of canals is constant throughout development in these two species. In addition, the structure of the velarial canals of both populations is different: the Mediterranean *C. marsupialis* shows canals with very slim, delicate, dendritic canals with short, slim lobations, while the Caribbean *C. xaymacana* shows broad and less complexly branched canals.

Species identification within the genus *Carybdea* is based mainly on the different shapes of gastric phacellae (and structures of their filaments) and by the number and shape of the velarial canals (Gershwin 2005; Gershwin and Gibbons 2009; Bentlage and Lewis 2012) of sexually mature medusae. Data on the different developmental stages are scarce, but important in order to clearly establish species boundaries. Differences between both Mediterranean and Caribbean specimens were observed in the young medusae (Fig. 4) of the earliest, newly detached stage, such as umbrella colour [colourless in Mediterranean specimens (Fig. 4A); brown in Caribbean specimens (Fig. 4B)]; number of gastric filaments (1 per quadrant in Caribbean, none in Mediterranean); number, colour, and structure of tentacles [4, white, homogeneous banded in Mediterranean (Fig. 4C); 2, opposed-located, white-orange coloured, heterogeneous banded in Caribbean (Fig. 4D)]. The rhopalia niche (Fig. 4E, F) is not very useful for interspecific comparisons between small cubomedusae since the shape of this structure is still developing.

In addition, there are differences between the species from both localities in the number of bioactive proteins isolated from *C. marsupialis* from the Mediterranean (Rottini et al. 1995) and from *C. xaymacana* species from the Caribbean (Sanchez-Rodriguez et al. 2006).

All these results demonstrate that the two “populations” of *C. marsupialis* of the Mediterranean Sea and of the Caribbean represent in fact two distinct species. The species from the Mediterranean Sea is the originally described *C. marsupialis*, and the one from the Caribbean, including the polyp population from Puerto Rico described here, belongs to *C. xaymacana* described by Conant in 1897. Therefore, all publications on Caribbean specimens may contain *C. xaymacana* and not *C. marsupialis* (see Table 3). Also Loman (2004) and Segura-Puertas et al. (2009) reported *C. marsupialis* from the Gulf of Mexico. However, a more detailed description (especially on the number and shape of the velarial canals) would be necessary to ensure the identity of those specimens.

Other cubozoans from the Mediterranean and Arabian Sea

It seems that the Mediterranean coasts and harbours host more than one cubozoan species, and more than one family could be found, at least temporarily. Specimens from North Africa labelled as "*Carybdea marsupialis*" (from the Smithsonian Institution collection) do not fit with the description of this species. The specimen from Algeria (USNM 56659) differed from *C. marsupialis* in the number and shape of the velarial canals, which were noted to be several parallel branches, very similar to the canal pattern of "*Charybdea alata*" Stiasny 1939. This specimen does not fit the characters of *C. marsupialis* or any carybdeid species known from African coasts (e.g. *C. branchi*), but rather appeared to be a member of the Alatinidae family considering its velarial canals.

Although the 2 specimens from Tunisia (USNM 54378) were in bad condition and some structures were destroyed due to preservation and previous dissections, some characters could be examined that separate them from *C. marsupialis*. Specimen 1 had a larger size (DBW = 52.4 mm) than *C. marsupialis*, and both differed in the shape of their rhopaliar niche ostia, which was not heart-shaped in the Tunisian specimen. But the most conspicuous features are the combination of the number and shape of the velarial canals and the gastric phacellae that are more crescentic than epaulette-shaped. These characters hint to an *Alatina* species and needs further investigation to clarify its identification. Specimen 2 also had a larger bell (DBW = 51.3 mm) and velarial canals that were similar in number (3 per octant) to the ones of *C. marsupialis* but different in their structure. Next to that, a thorn-like appendage noted on the pedalial canal bend and the additional spikes along the sharp outer keel of the pedalial canal of the Tunisian specimen differed from the smooth pedalial canals with rounded knee bend of *C. marsupialis*.

Both Tunisian specimens are also unlikely to be members of the same species as all above-mentioned characters differed between them. The first one seems to belong to Alatinidae and the second could belong to Carybdeidae but its characters do not fit well with *C. marsupialis*. Nonetheless, *C. marsupialis* has been recorded recently from the eastern Tunisian coast, accompanied by a detailed description that leaves little doubt as to its identity (Gueroun et al. 2015).

Few references exist for the carybdeids in African waters. During the Challenger Expedition, Haeckel (1882) recognized significant differences between an Atlantic Ocean carybdeid specimen sampled on the Western Coast of Africa and *Carybdea marsupialis* from the Mediterranean Sea. Therefore, he considered the West African form a separate species and named it *Carybdea murrayana*. Ranson (1945) reported *Carybdea marsupialis* (Linnaeus, 1758) off Algiers, North Africa, but it is not clear if this report really refers to *C. marsupialis* – a population exists in Tunisia (Gueroun et al. 2015) – or if it might be assigned to *C. murrayana*, or even to *C. branchi*. The range limits of these two species are not currently understood (Gershwin and Gibbons 2009).

Furthermore, in 2008, several cubozoan specimens were collected in Gwadar (Pakistan, Arabian Sea) and were also identified as "*Carybdea marsupialis*" (Kazmi and Sultana 2008). However, the huge bell size of up to 190 mm in length and up to 120 mm in width, as well as the pictured velarial canal patterns are completely unusual for *C. marsupialis*. Stiasny (1939) found in the collection of Dr. J. H. Ziesel an alatiniid specimen from Kamaran (southern end of the Red Sea) which he identified as "*Charybdea alata*" due to its T-shaped rhopaliar niche ostia, its crescentic phacellae, and its velarial canal pattern. This canal pattern (see Fig. 10) is identical to that shown in Kazmi and

Sultana (2008) and we conclude that the Gwadar specimens are more likely to be a species of the Alatinidae family. Based on its size and the shape of the pedalia (Bentlage 2010) it might be *Alatina grandis* Agassiz and Mayer 1902, which was also sighted several times in the Northern Arabian Sea at the coast of Pakistan (Shahnawaz Gul, personal communication in 2014). Recently, Gul et al. (2015) list the cnidarians from Pakistan and put doubt on the record of *C. marsupialis* in these waters.

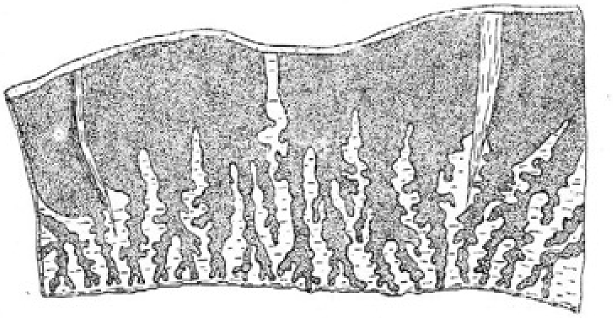

Fig.10 Velarial canal pattern of "*Charybdea alata*" Stiasny (1939) from Kamaran (Red Sea). This canal pattern is identical to that shown in the supposed "*Carybdea marsupialis*" described by Kazmi and Sultana (2008) in Pakistan waters (more probably an alatinid species).

Identity of *Carybdea* from California, USA

Stiasny (1922) found similarities between Californian specimens and *Carybdea arborifera* from Hawaii which is the closest location where carybdeid species have been found, but he identified the Californian cubozoans as *Carybdea rastonii*. This identification was later adopted by other authors (Satterlie 1979, Satterlie and Spencer 1979), but, as mentioned before, the molecular phylogeny of Bentlage et al. (2010) demonstrated that *Carybdea arborifera* and *Carybdea rastonii* are two different species and gave us reason to doubt the identification of the Californian population as *Carybdea rastonii*. Our results show that the Californian population is distinct from *C. rastonii* from Australia due to the shape of the gastric phacellae which are one-rooted and epaulette-shaped in the Californian specimens (Fig. 3K) and not horizontal linear as in *C. rastonii* (Fig. 6G) or *C. brevipedalia* (Fig. 6B). *C. arborifera* is distinguishable from the Californian population by its gastric phacellae which are similarly epaulette shaped but consist of a cluster of long-stemmed, brush-shaped filaments with multiple stems that are attached to one root in *C. arborifera* (Figs. 5C, D) while the filaments in Californian specimens are shorter and single stemmed (Fig. 3K), even though they also originate from a single root.

On the other hand, other authors identified the specimens from California as *C. marsupialis*. Larson (1976) applied the name "*Carybdea marsupialis*" from Puerto Rico to California to reclassify the carybdeid species there (Larson 1990). Later, other authors did not agree with this reclassification because the morphological characters did not completely fit (Fenner 1997; Gershwin 2005). In order to clarify this issue, we compared medusae of *Carybdea marsupialis* from the Mediterranean and *Carybdea xaymacana* from the Caribbean with the medusae from the Californian coast (Fig. 3). The specimens from California differed distinctly from both *C. marsupialis* and *C. xaymacana*, especially in the shape and number of velarial canals − *C. marsupialis* has 3 velarial canal roots per octant (Figs. 1H, 3D, 9E, G) while the specimens from California show only 2 canal roots per octant (Fig. 3M), equal to the number of canal roots of *C. xaymacana* (Fig. 3I, 9F, H). The velarial canals themselves are very broad, quite simple, biforked with sharp tips in *C. xaymacana*. By contrast, the velarial canals from the Californian specimens are branched more profusely and rounded at their tips. Lastly, *Carybdea branchi* possesses 3 velarial canal roots per octant (Fig. 5J) according to our investigations [contrary to the 2 velarial canal root per octant described by Gershwin & Gibbons (2009)]. The Californian specimens are unlikely to be *Carybdea branchi* because of their smaller size, their lack of colour, and the possession of 2 velarial canals per octant instead of 3. *Carybdea murrayana* Haeckel 1880, another carybdeid species from West African coasts, also possesses more than 2 velarial canal roots.

Species	Revised Distribution	Gastric phacellae	Velarial canals	Pedalial knee bend
C. rastonii	Australia	Horizontal linear; gastric filaments multiple rooted, short stemmed	2 v.c./octant; triforked	Rounded, no appendage
C. brevipedalia	Japan	Horizontal linear; gastric filaments multiple rooted, long stemmed	2 v.c./octant; complexely branched	Slightly volcano-shaped, appended sharp peak
Californian *Carybdea* sp.	California	Epaulette shaped; single rooted, single stemmed	2 v.c./octant; multiple branched	Thorn-like appendage
C. xaymacana	Caribbean Sea	Epaulette shaped; single rooted, single stemmed	2 v.c./octant; biforked	Volcano-shaped to triangular
C. marsupialis	Mediterranean Sea	Epaulette shaped; single rooted, multiple stemmed	3 v.c./octant; multiple branched	Rounded; no appendage
C. branchi	South Africa	Epaulette shaped; single rooted, multiple stemmed	3 v.c./octant ; complexely branched	Volcano-shaped, upwards turned
C. arborifera	Hawaii	Epaulette shaped; single rooted, multiple stemmed	2 v.c./octant; biforked to multiple branched	Rounded; no appendage

TABLE 4 Comparison of characters between *Carybdea* species; v.c. = velar canals.

In summary, we conclude that the Californian carybdeid does not belong to any of the species of *Carybdea* but represents a new species that will be described separately.

Comparison of *Carybdea* specimens from different populations worldwide

We confirmed the validity of the seven species of the genus *Carybdea* and developed an identification key to facilitate future species identification. Differences in the structure and number of velarial canals, the structure of the pedalial canal knee bends and the shape and composition of the gastric phacellae were observed and form the basis of the identification key presented here (Table 4). The presence or absence of velarial warts could be an artefact due to preservation, so we do not consider this as a genuine character of the species.

Identification key for species of the genus *Carybdea*

1 Gastric phacellae, horizontal linear (multiple rooted) 2
- Gastric phacellae, epaulette shaped (single rooted) 3

2 Gastric filaments, short-stemmed, multiple rooted (Fig. 6G); pedalial canal, knee bend rounded, no appendage (Fig. 6H); 2 velarial canal roots/octant (Fig. 6I): canals, triforked, scarcely branched, square tipped .... *Carybdea rastonii*

- Gastric filaments, long-stemmed, multiple rooted (Figs. 6B, C); pedalial canal, knee bend volcano-shaped to triangular, with appended sharp peak (Fig. 6D); 2 velarial canal roots/octant: canals, complexly branched, sharp tips (Fig. 6E) .... *Carybdea brevipedalia*

3 Gastric phacellae, single rooted, single stemmed 4
- Gastric phacellae, single rooted, multiple stemmed 5

4 Gastric phacellae, single rooted, single stemmed (Fig. 3K); 2 velarial canal roots/octant: canals, multiple-branched, rounded tips (Fig. 3M); pedalial canal, knee bend with thorn-like appendage (Fig. 3L) .... Californian *Carybdea* sp.
- Gastric phacellae, single rooted, single stemmed (Figs. 3F, G); 2 velarial canal roots/octant: canals broad, biforked, simple to scarcely additionally branched, sharp tips (Fig. 3I); pedalial canal, volcano-shaped to triangular knee bend, with small peak-like appendage (Fig. 3H) .... *Carybdea xaymacana*

5 3 velarial canals per octant 6
- 2 velarial canals per octant: broad, biforked to multiple branched, rounded tips (Fig. 5E) gastric phacellae, single rooted, multiple stemmed (Fig. 5C), filaments, short stemmed (Fig. 5D), unconstrained aligned, brush-shaped, originating from one root; pedalial canal, rounded knee bend, no appendage (Fig. 5B) .... *Carybdea arborifera*

6 Gastric phacellae, single rooted, multiple stemmed (Figs. 3B), filaments, short stemmed, brush shaped, brown coloured at “brush base”; pedalial canal knee, rounded, no appendage (Fig. 3C); 3 velarial canal roots/octant: canals, slender, multiple branched, denticulated, very sharp tips (Fig. 3D) .... *Carybdea marsupialis*
- Gastric phacellae, single rooted, multiple stemmed (Figs. 5H, I), filaments, long stemmed, complexly branched, tightly aligned, originating from one root (Fig. 5I); 3 velarial canal roots/octant: canals, complexly branched, lobated, rounded tips (Fig. 5J); pedalia canal, volcano shaped, upturned knee bend with no appendage; brownish coloured spots located over gastric phacellae, pedalia bases and tentacle insertion (Fig. 5G) .... *Carybdea branchi*

Phylogeny/Biogeography

Our molecular phylogeny indicates the genus organization into two major clades. The first clade unites the two lineages (species) of the Atlantic and Caribbean Sea, *C. branchi* and *C. xaymacana*. Correspondence and similarities between the South East Atlantic fauna and the Caribbean Sea fauna is known for some species (e.g. Stampar et al. 2012; Stampar and Morandini 2014). This pattern has been linked to the South Atlantic currents (e.g., the South West African Benguela Current) uniting with the South Equatorial Current that reaches the South East American coast in Brazil (Berger and Wefer 1996; Berger et al. 1999). The second clade includes species that occur in the Mediterranean Sea and the Indo-Pacific, *C. marsupialis*, *C. rastonii*, *C. brevipedalia*, and *C. arborifera*. This connection seems surprising considering the isolation of the Mediterranean Sea in relation to the Indo-Pacific. However, historically there had been a connection between these two areas as the Mediterranean Sea is considered a relict of the Tethys Sea, which connected the early Atlantic and Pacific Oceans before the Miocene; some species have been hypothesized to have survived the Messinian crisis [e.g. the sea-grass *Posidonia*, as suggested by the fact that representatives of this genus are restricted to the Mediterranean and Southern Australia (Boero and Bouillon, 1993)]. In this way, the fauna of the Mediterranean Sea has much similarity with parts of the Indo-Pacific fauna (see more in Coll et al. 2010). In addition, we should take in consideration other anthropogenic factors such as ballast waters or increase of substrate availability for the polyps. These could influence the distribution and connection of species that are reported to occur near harbours and marinas such as box jellyfish.

We suggest that the observed present-day distribution of the species of *Carybdea* (Fig. 8) is the result of the dispersal of their common ancestors and subsequent speciation. The separation of the *Carybdea* species into two major clades (Fig. 7), one grouping the Atlantic species (*C. branchi* and *C. xaymacana*) separately from the remainder of the species, is congruent with that obtained by Bentlage et al. (2010).

Identity of the culture of polyps from Puerto Rico

Bentlage et al. (2010) found a sequence data from "*C. marsupialis*" polyps, from a culture maintained in the lab of Bernd Schierwater at the University of Hannover (Germany) (the culture was originally obtained from Gerhard Jarms at the University of Hamburg), to fall within Alatinidae rather than Carybdeidae. As *Alatina alata* [formerly described as *Carybdea alata* (Bigelow 1938)] can be found in Puerto Rico, Bentlage et al. (2010) suggested the possibility that the culture in Hamburg actually contained the polyp stage of a species of *Alatina* rather than *Carybdea*. However, when comparing the early life histories of the well-studied *Carybdea* cultures from Puerto Rico (Studebaker 1972; Werner et al. 1976; Stangl 1997; Stangl et al. 2002; Straehler-Pohl 2001, 2009; Straehler-Pohl and Jarms 2005, 2011) with *Alatina* cf. *moseri* from North Queensland/Hawaii (Carrette et al. 2014) or with *Carybdea alata* from Puerto Rico (Arneson and Cutress 1976), we observed these polyps to be different from those of the genus *Alatina*. Moreover, we extracted DNA from the culture of polyps in Copenhagen (derived from the culture in Hamburg) and generated new sequence data from these extracts. Our results, both morphological and molecular, indicate that the Puerto Rican polyps in this culture are neither *A. alata* nor *C. marsupialis* (which exists only in the Mediterranean Sea) but *C. xaymacana* originally described by Conant (1897). Up to now, *C. xaymacana* is the only species of the genus *Carybdea* recognized from the Caribbean Sea. We suggest that multiple polyp cultures exist in labs around the world that are labelled with the same species name (i.e. *C. marsupialis*), but in fact contain different species of Alatinidae and *Carybdea*. This seems a plausible explanation of the different sequences obtained from this and previous studies. Therefore, we highlight the importance to provide morphological descriptions together with the molecular sequences to help clarify the identity of these polyp cultures.

In summary, given that the polyp development and metamorphosis are unknown, the life cycle of the original *C. marsupialis* is not completely resolved yet. However, similarities with the life cycle of *C. xaymacana* from Puerto Rico described by Cutress and Studebaker (1973) have been hypothesized (Canepa et al. 2013). Obtaining a culture of polyps from the Mediterranean *C. marsupialis* and studying their life cycle would be necessary to understand the entire biology and ecology of this species.

Conclusions

First records of *C. marsupialis* were from the Adriatic and Mediterranean region, and taking into account the later confusion regarding the identities of *Carybdea* species around the world, we consider specimens from the Mediterranean Sea (Denia, Spain) studied in this work to belong to the original *C. marsupialis*. We provided detailed descriptions and images to facilitate identification at different life stages.

Our taxonomic investigations, and those of Bentlage et al. (2010), indicate that *Carybdea* spp. are more restricted in their geographical distributions than has historically been recognized. We think that life cycle observations are a very valuable tool for taxonomy as they might help to clarify the identity of some cubozoan species.

The correct identification of specimens should be the basis for the development of correct ecological hypotheses and proper assessment of box jellyfish blooms. *C. marsupialis* in the

Mediterranean should be considered as a native species, a fact that should be taken into account when control measures or protocols against population outbreaks are developed and applied in that region.

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CHAPTER 2

Maintenance, feeding and growth of *Carybdea marsupialis* (Cnidaria: Cubozoa) in the laboratory

Acevedo MJ, Fuentes VL, Olariaga A, Canepa A, Bosch-Belmar M, Bordehore C, Calbet A (2013) *Journal of Experimental Marine Biology and Ecology* 439:84-91

Abstract

The box jellyfish *Carybdea marsupialis* has proliferated in some areas of the north-western Mediterranean Sea since July 2008. As for many species, controlled experimentation in the laboratory is needed to improve our knowledge about *C. marsupialis*, with the ultimate goal of extrapolating the knowledge gained to the marine environment. The aims of this study were to identify the optimal conditions (i.e. aquarium design, environmental parameters and prey type) for the growth and maintenance of this cubomedusa in the laboratory and, additionally, to quantify the feeding rates of the juveniles of this species. We were able to maintain healthy medusae for 140 days. During this time they reached the subadult condition (fact corroborated by observing the gonadal tissue), growing from 2 to 15 mm in diagonal bell width from June to November 2010, respectively. We observed a progressive shift in their preferred dietary composition as the individuals grew. The medusae fed on *Artemia salina* nauplii along the entire development. Other, larger, prey types (e.g. *Mysis* sp., *Acartia grani* copepods and adult *Artemia salina*) were progressively ingested at the same time as they increased their umbrella size. We also describe the clearance rates, ingestion rates, prey selectivity and digestion times of juvenile *C. marsupialis* on natural zooplankton and on the copepod *Acartia grani*. Growth and mortality rates were also calculated.

Keywords: cubozoa, feeding rates, jellyfish, prey, survival.

Introduction

Harmful jellyfish blooms are amongst themost conspicuous events in the oceans worldwide (Mills, 2001). Even though there is a very recent publication questioning the rise of gelatinous zooplankton in the world's oceans and bringing in the question of the media-driven public perception of such increase (Condon et al., 2012), other studies have demonstrated the existence of a significant growth in jellyfish abundance in coastal systems worldwide using analytic methods designed tominimize the effect of the bias reporting (Brotz et al., 2012). The periodicity of occurrence of some jellyfish species (both native and nonindigenous) has shortened in recent decades and the recurrence of blooms has increased in a local or regional scale, probably due to food web modifications and climate change (Daly Yahia et al., 2010; Kogovšek et al., 2010; Licandro et al., 2010; Mills, 2001; Purcell et al., 2007). Human activities are thought to be contributing to increasing jellyfish abundances in coastal waters worldwide, which are in turn affecting swimmers, fisheries, aquaculture and other coastal industries (Purcell et al., 2007). Reviews have proliferated recently speculating that jellies have benefited from human-caused changes, including climate change, eutrophication, overfishing, coastal construction, and species introductions (Purcell, 2012).

The species *Carybdea marsupialis* is the only cubozoan known to inhabit the Mediterranean Sea (Linnaei, 1758); it was recorded for the first time at the Adriatic Sea in 1878 by Claus (Di Camillo et al., 2006). Since then, *C. marsupialis* has been recorded in high densities several times in the Adriatic region (Avian et al., 1997; Boero and Minelli, 1986; Corbelli et al., 2003; Di Camillo et al., 2006). It was never considered that *C. marsupialis* could form blooms in the western Mediterranean; however, since July 2008, populations in at least two localities (Denia and Santa Pola beaches; east coast of Spain) along the NWMediterranean coast (~120 km apart) have increased reaching unusual very high densities in some beaches (Bordehore et al., 2011 for Denia; C. Bordehore unpublished data for Santa Pola).

It is particularly important to determine whether high densities of *C. marsupialis* in the NW Mediterranean will alter ecosystem function and/or biodiversity. Moreover, some details of the lifecycle of this species are unknown for the Mediterranean Sea. This cubozoan species has a metagenetic life cycle; we know that the cubomedusa stage is present from May to November in the NW Mediterranean region, but we still lack information on the cubopolyp stage, which is still to be found in the field or obtained in vitro. Considering the importance of benthic stages for jellyfish outbreak formations due to their asexual reproduction and resting capacity (Boero et al., 2008), it is vital to acquire data both on the polyp and the medusa stage of this species. The ability to maintain local *C. marsupialis* in aquaria will allow the development of in vitro experiments and assays, facilitating the comprehension of the role of *C. marsupialis* in coastal ecosystems and the consequences of future bloom events.

In our case, we successfully monitored the development and growth of the cubomedusa stage of *Carybdea marsupialis*; moreover, visually mediated behaviour and feeding behaviour could be studied in the laboratory in an appropriately designed aquarium. To date, there are no published evidences of cultivation of carybdeid medusa to maturity in captivity, except for *Tripedalia cystophora* (Straehler-Pohl and Jarms, 2011). The complete life cycles of different carybdeid species in La Parguera (Puerto Rico) have been described; however, no laboratory-raised *Carybdea* sp. medusa lived beyond 15 days (4mm in umbrella height (UH)) (Studebaker, 1972; Werner, 1971, 1983). Pioneering studies have also been conducted with other cubozoan species, especially in Australia (Hamner et al., 1995), where the most venomous species are present. Yamaguchi and Hartwick (1980) described the early life history of the sea wasp *Chironex fleckeri*. The largest medusae observed were 7 weeks old and exceeded 10 mm in UH, but they did not show any signs of branching in their pedalia (Yamaguchi and Hartwick, 1980). Hamner et al. (Hamner et al., 1995) were also able to maintain one individual of *Chironex fleckeri* over a 9-month period to sub-adult condition, describing in detail the swimming, feeding, circulation and vision of this Australian cubomedusa. Some adult individuals of different carybdeid species, such as *Carybdea sivikisi* (Hartwick, 1991; Lewis and Long, 2005) and Tripedalia cystophora (Buskey, 2003), have been kept in aquaria for a limited time with the aim of studying their behaviour and life cycles. Nevertheless, there is still very limited data on the early life stages of carybdeids. The present study represents the first long-term maintenance and growth of a carybdeid species from recent detached cubomedusa to subadult condition in the laboratory. We focus on the diet as one of the most important factors involved in survival of juvenile cubomedusae and we describe the feeding rates of *C. marsupialis* and dietary preferences of the species. These experiments will improve our knowledge of the trophic requirements of cubomedusae and they will identify areas for further research on the predatory impact of this species.

The aims of the present study were (1) to determine the optimum conditions for the maintenance and growth *C. marsupialis* in the laboratory, and (2) to assess the clearance, ingestion and digestion rates of juveniles of *C. marsupialis* by incubation experiments. In order to achieve these aims, medusae were collected from the field and an experimental culture of *C. marsupialis* was established.

Materials and methods

Medusae collection

We collected juveniles of *Carybdea marsupialis* from Rasset Beach (Alicante, Spain) on 16th June 2010. The medusae were caught very close to the beach (~2 m depth) by superficial trawling with a 200-μm plankton net (40 cm diameter) for 5 min at a speed of ~2 knots. The medusae collected were immediately transported in 25 L plastic containers filled with ambient seawater and without air spaces (to avoid damage) to the Institut de Ciències del Mar – Consejo Superior de Investigaciones Científicas (ICM-CSIC) in Barcelona, where they were kept in an especially designed aquarium.

Aquarium design and maintenance

The recent detached medusae were placed in a 140 L, black sided, aquarium especially designed with low, horizontal flow in a continuous flow-through seawater system. This type of aquaria prevents the attachment of the tentacles to the glass, which could cause death by starvation (Straehler-Pohl and Jarms, 2011). We generated a vertical (i.e. parallel to the lateral walls of the aquarium), laminar flow by coupling a vertical pipe with holes in the aquaria to the input stream and four buffer plates across at each corner of themain chamber of the aquarium. This mechanism prevented the medusae for getting stuck to the corners. The input stream was adjusted to achieve adequate water renewal and velocity so that the cubomedusae were kept in the water column away from the walls of the aquarium, and also allowing them to swim freely against the current. The output of the overflow water was in a chamber behind a 300-μm mesh screen, which allowed the passage of food debris but not medusae (Fig. 1A). Cubomedusae use to swim near to the bottom, especially during the ingestion of food. Therefore, it was necessary to clean the bottom of the aquarium every day in order to avoid the accumulation of leftover food. During summer 2012 we put on trial a new version of the aquarium (Fig. 1B) specially designed for the adult stages, with some changes and adjustments, which allowed us to maintain adult cubomedusae for more than 50 days. This 180 L aquarium has two separate circulation systems. The circular and laminar flow of the water in the main chamber, where the animals are kept, is also generated by coupling a vertical pipe with holes in the aquaria to the input stream and four buffer plates across at each corner of the main chamber of the aquarium. The secondary circulation system is located in the lower part of the aquarium, within a space separated from the main chamber using an acrylic plate of 5 mm thickness, with perforations of 50 mm which were covered with 500 μm mesh. This constant flow generates homogenization of the water, which prevents anoxia.

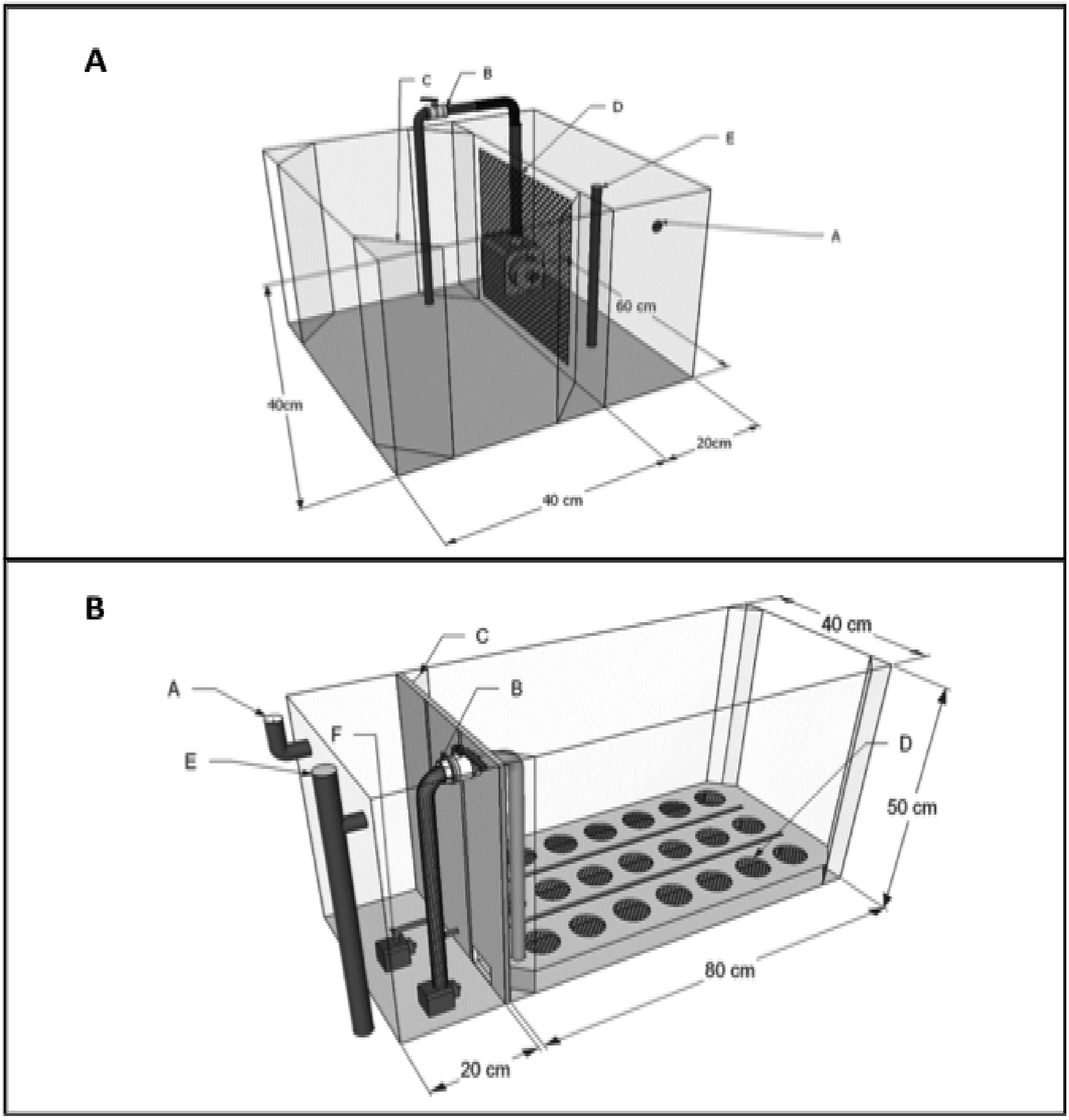


Fig. 1 Especially designed aquariums used to keep *Carybdea marsupialis* under controlled conditions. (A) Aquarium for the early stages: A = water inflow; B = overflow outlet water; C = buffer plate; D = vertical pipe with holes that creates horizontal laminar flow; E = 300-μm mesh; (B) Aquarium for the adult stages (> 1.5 cm diagonal bell width (DBW): A = water inflow; B = main circulation system composed by a pump coupled to a vertical pipe with holes that creates horizontal laminar flow; C = buffer plate; D = acrylic plate with perforations covered with 500-μm mesh; E = overflow outlet water; F = secondary circulation system.

Lighting is also crucial for the maintenance of cubomedusae because they have a well-developed sense of vision with 24 eyes, including simple and complex eyes that allow them to distinguish colours, form images (Coates, 2003), and even avoid objects (Hamner et al., 1995). For the smallest aquarium (Fig. 1A) we used three-beam central lightning (white light), controlled with a photoperiod of 12:12 h light:dark. The central vertical light shaft and the black sides and bottom of the aquarium kept cubomedusae away from any surfaces that could damage them. For the maintenance of the adult stages in the larger aquarium (Fig. 1B) we used also central lightning but with a blue coloured filter.

Both aquaria were kept with running seawater at the natural temperature and salinity of the NW Mediterranean Sea during throughout the period of the study. Consequently, we were able to monitor the growth and development of the cubomedusae under simulated natural conditions. Temperatures ranged from amaximumof 25.1 °C during summer, to a minimum of 17.2 °C in November when the cubomedusae died.

The number of live individuals and measurements of the diagonal bell width (DBW) and UH of the medusae were recorded every week. We decided to rely on these sorts of non-destructive biomass estimates to obtain growth rates, instead of weight, to maximize the final number of individuals. All measurements were conducted under a calibrated stereoscopic microscope until the juveniles reached 10 mm wide, when they could be easily manipulated and measured using callipers (Medid, 1/20 mm; ±0.05 mm).

Prey preference and feeding experiments

We maintained cultures of different prey to meet the feeding requirements of *C. marsupialis*. *Artemia salina* nauplii (~400 μm) were provided *ad libitum* every day as the main diet item to maintain the medusae. During the early days of maintenance we also tested to feed the recent detached medusae with cultured rotifers. The diet was supplemented every 2 days, either with natural plankton when available, or with adult copepods (the calanoid *Acartia grani*). Copepods were maintained in a 20 L transparent, acrylic cylinder at 20 °C in 5-μm filtered seawater and fed ad libitum with *Rhodomonas salina*. As the juvenile cubomedusae increased in DBW we added to the diet live *Mysis* sp. and adults of *Artemia salina*. *Mysis* sp. were kept in 5 L transparent beakers and fed with rotifers and pellet food. We also feed *C. marsupialis* with frozen *Mysis* sp. and *Artemia salina*.

Prey consumption by jellyfish has been estimated for several species using different approaches: (1) clearance rate experiments (incubations); (2) gut content analysis of field-collected specimens to measure digestion times; and (3) respiration rate experiments (Purcell et al., 2010). Here, three types of incubation experiments were conducted with juveniles of *C. marsupialis* to assess prey consumption: (1) incubations with different concentrations of natural zooplankton to investigate prey selectivity and to calculate clearance and ingestion rates; (2) incubations with different concentrations of *Acartia grani* to calculate clearance and ingestion rates; and (3) individual observations of gut contents over time to calculate digestion time. In addition,we present information on prey selectivity from visual gut content analysis of field-caught medusae.

For the natural plankton incubations, zooplankton were collected close to the coast of Barcelona (Spain), by vertical trawls, using a 300-μm Nansen net with a plastic bag as cod-end to avoid damage to the plankton. In the laboratory, different concentrations of the natural plankton were prepared by diluting the sample with 5-μm filtered seawater. Thirteen glass Nalgene bottles (1200 mL) were initially filled with 5-μm filtered seawater: two replicates for each control and three replicates for experimental treatment. In order to achieve the final concentration desired at each treatment, we calculated the volume of the corresponding plankton dilution necessary to add into the experimental bottles (i.e. 8 mL). The three concentrations used were 27, 58 or 90 organisms bottle^{-1} (22.5, 48.33 and 74.58 organisms L$^{-1,}$ respectively). For the control bottles, only 8 mL of the corresponding plankton suspension was added. An 8 mL aliquot from the initial sample was fixed with 4% formalin in seawater at time 0 h (t_0) to determine the start prey concentrations. At time 0 one medusa was placed in each of the nine experimental bottles. The bottles were incubated for 24 h at 24.5 ± 0.5 °C on a plankton wheel (0.2 rpm) to keep both medusae and prey suspended. At the beginning and at the end of the experiment, we measured the DBW of the cubomedusae, and filtered and preserved the zooplankton of all bottles for later quantification and identification. The zooplankton components analysed to determine prey selectivity were cladocerans, copepods and icthyoplankton (fish eggs and larvae). The final prey concentrations from each experimental treatment were compared to the final prey concentrations of the control treatments. If there was a significant difference (t-test $p < 0.05$) in the prey concentrations between the experimental and the control treatments then the individual clearance and ingestion rates were calculated. The clearance rate (CR: the volume of water filtered in L medusa^{-1} day^{-1}) for each incubation was calculated as:

$$CR = ([V/(n \times t)] \times \ln(C_0/C_t)) \times 24$$

where *CR* = clearance rate; *V* = volume of the container (L); *n* = number of cubomedusae; *t* = incubation time (hours); C_0 and *C* = initial and final number of prey (Purcell and Arai, 2001). The individual ingestion rates (IR) were then calculated as:

$$IR = CR \times C_m$$

where *CR* = clearance rate calculated; C_m = mean prey concentration calculated as:

$$C_m = \exp[\ \ln(C_0 \times C_t)/2]$$

A similar experimental design to that described above was used with the cultured copepod *Acartia grani* as the only available prey. This species of copepod was selected due to the fact that calanoid copepods were the principal prey item detected (23.52%) in the gut contents of 102 *C. marsupialis* captured during previous work (M. J. Acevedo, unpublished data). The experimental design was equal to that previously described; however, the three different prey concentrations prepared were 10, 20 or 40 copepods bottle−1 (8.3, 16.6 and 33.3 copepods L^{-1}, respectively). Similarly, at the beginning and at the end of the experiment, the DBW of the cubomedusae from the experimental bottles was measured. The copepods were filtered and fixed from all bottles for later quantification and the CR and IR for each treatment was calculated.

Because *C. marsupialis* are often observed in the field with empty gastrovascular cavities (M. J. Acevedo, unpublished data), we prepared an experiment with the aim of calculating the digestion time of this cubomedusan species. Three juvenile medusae (~10 mm in DBW) were chosen and placed in individual aquaria. When their gastrovascular cavities were empty, we gave one *Mysis* sp. shrimp to each of them. Then, we recorded the state of the prey item every 15 min until complete digestion was achieved. Digestion

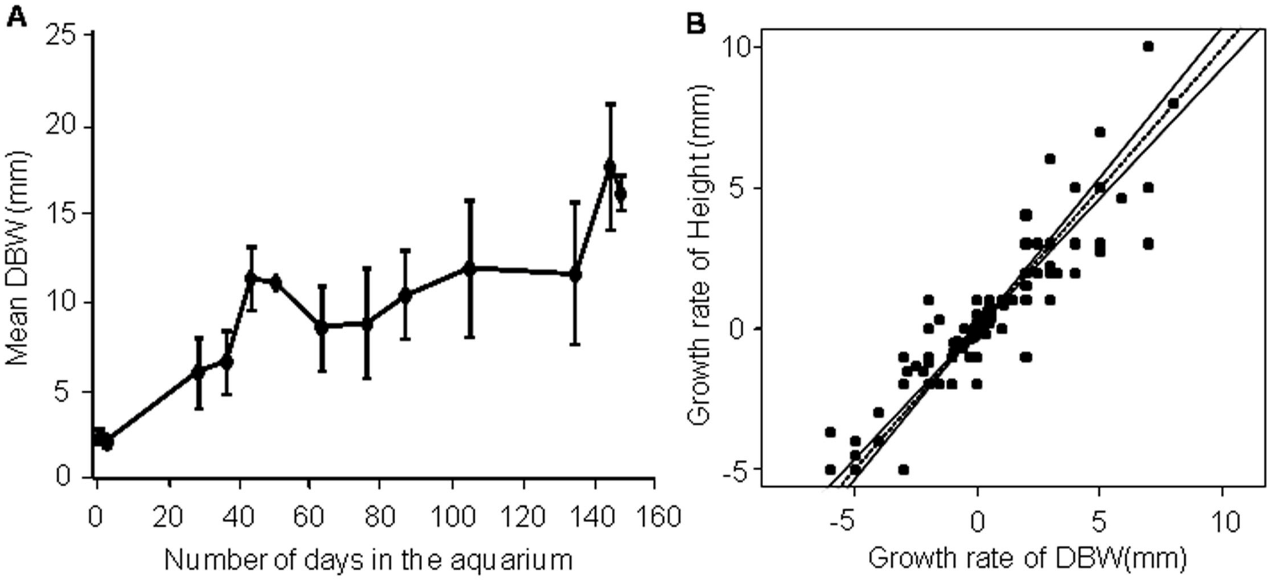


Fig. 2 Evolution of cultured *Carybdea marsupialis*. (A) Size evolution of *C. marsupialis* medusae DBW over the course of the study; (B) Linear regression of the diagonal bell width (DBW) and umbrella height (UH)-based growth rates ($R^2 = 0.87$; $p = 0.01$; slope = 0.99).

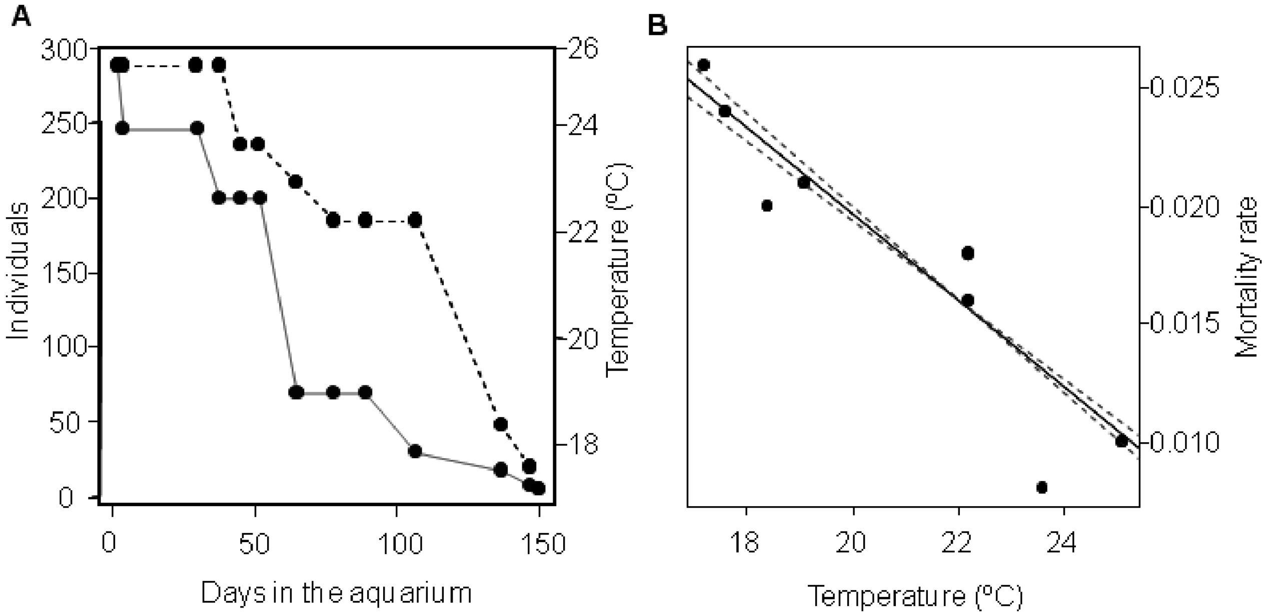


Fig. 3 Number of live *Carybdea marsupialis* medusae in relation to temperature over the course of the study. (A) Number of medusae and temperature variation over the study. Solid line: number of individuals, dashed line: temperature (°C); (B) Linear regression of mortality rates vs. temperature ($R^2 = 0.91$; $p = 0.01$; slope = - 0.0018).

time was the mean time taken to reach complete digestion for the three medusae. The end point for digestion was easy to determine due to the transparency of the bell of *C. marsupialis*.

Statistical analyses

In order to test the correlation between DBW and UH-based growth rates and between temperature and mortality rate, we used model II regression analysis, as the independent and dependent variables were estimated with comparable error (Laws and Archie, 1981). Model II regression analysis was performed using the Major Axis (MA) method, and confidence intervals were obtained with a total of 99 permutations. The analysis was conducted using the R package "lmodel2" (Legendre, 2011).

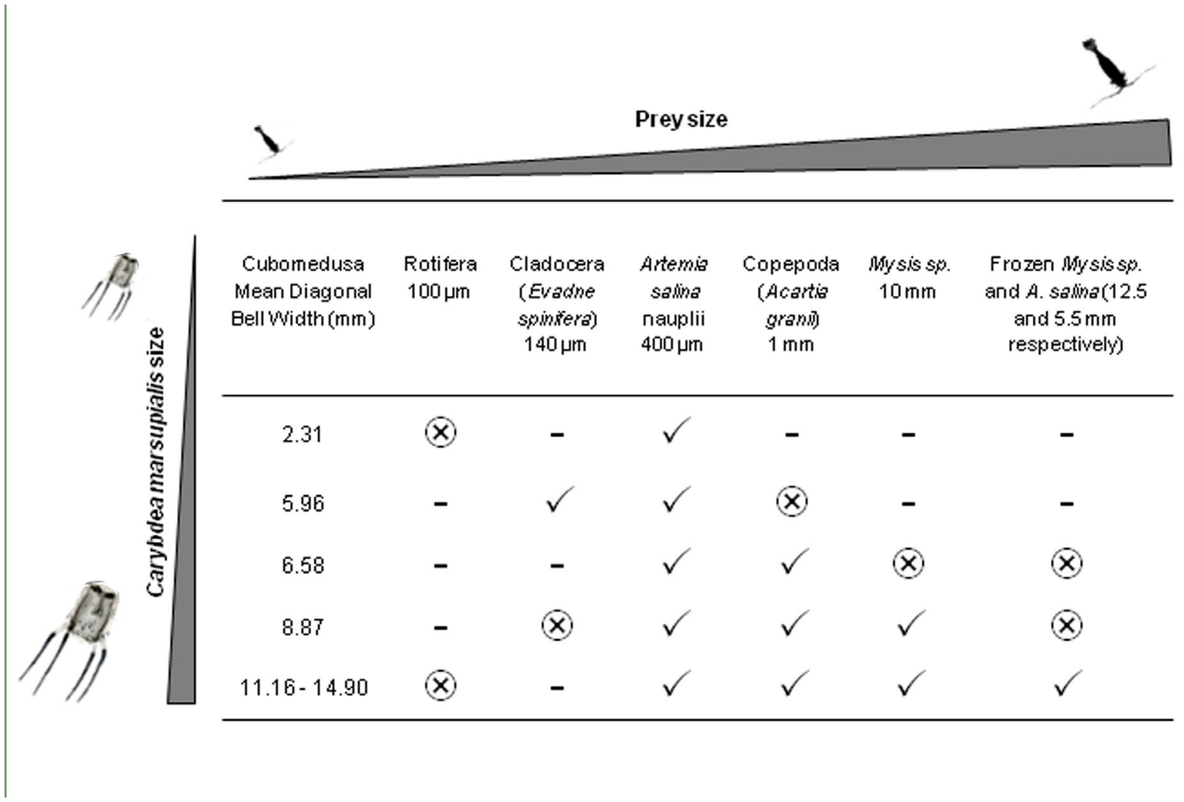
Multiple ANOVA analyses were conducted in order to test differences among ingestion rates on different prey type (i.e. copepods, cladocerans and icthyoplankton). The differences between controls and treatment bottles were proved using t-test in both feeding experiments (i.e., incubations with natural mesozooplankton and *Acartia granii* copepods).

Results

Sampling and medusae maintenance

During the sampling to collect *Carybdea marsupialis*, a total of 38.76 m^3 of water were filtered as calculated by the flowmeter readings. A total of 288 cubomedusae were collected (size: 2–3 mm in DBW), giving a density of 7.45 medusa m^{-3}. When collected, the cubomedusae had a mean DBW of 2 mm (n = 288). A total of 30 individuals reached a mean DBW of 15 mm (Fig. 2A), ~60 % of the maximum natural size observed in the sea; the mean DBW of the wild adults captured for gastric content analysis (n = 102) in 2009 was 25 mm. There was a significant linear relationship ($R^2 = 0.87$; p = 0.01; slope = 0.99) between DBW and UH growth ratios (Fig. 2B). Therefore, all further comparisons presented are based on DBW.

We were able to maintain 200 healthy medusae in the aquarium for ~60 days (Fig. 3A). We observed the higher mortality rates (i.e. 4% day^{-1}) during the first week of maintenance and acclimatization to the aquarium. After this period, the mortality rate decreased to 0.52 % until day 65th



Cubomedusa Mean Diagonal Bell Width (mm)	Rotifera 100 µm	Cladocera (*Evadne spinifera*) 140 µm	*Artemia salina* nauplii 400 µm	Copepoda (*Acartia grani*) 1 mm	*Mysis sp.* 10 mm	Frozen *Mysis sp.* and *A. salina* (12.5 and 5.5 mm respectively)
2.31	⊗	-	✓	-	-	-
5.96	-	✓	✓	⊗	-	-
6.58	-	-	✓	✓	⊗	⊗
8.87	-	⊗	✓	✓	✓	⊗
11.16 - 14.90	⊗	-	✓	✓	✓	✓

TABLE 1 Progressive shift in prey size and dietary composition of *Carybdea marsupialis.* Symbols: cross = non ingested; tick = ingested; dash = non-tested at this size.

of the culture (21th of August 2010) when gradually increased from 0.95 to 2.1 % day^{-1}, and survival percentage was 24 % of the initial population. Then, during September we observed a month of stability either in the number of cubomedusae (n = 70), temperature (~22 °C) and mortality rate (1.87 ± 0.25 % day^{-1}). Later, from the 107th day of the culture, temperature decreased to 19.1 °C, and at that moment only 10.5 % of the initial population remained alive. After 137 days (4.5 months) 18 healthy medusae remained; at this point they had reached the pre-adult stage growing from 2 to 15 mm in DBW. In November 2010 the temperature of the seawater in the flow-through system decreased to 17.8 °C, and after this decrease in water temperature, only two cubomedusae survived. We maintained them in another tank at 21 °C, where they were able to live for 3 more weeks. We renewed the water in this tank every 2 days. We could detect an association between survival rate and water temperature, where the decreasing trend in the number of live medusae were linearly related to the decrease in temperature (Fig. 3B; $R^2 = 0.91$; $p = 0.01$; slope = −0.0018).

Food supply

Different prey items were tested as a potential food source during the growth and maintenance of the medusae. We observed a progressive shift in dietary composition as individuals increased in DBW. *Artemia salina* nauplii were eaten by juvenile medusae of all sizes and progressively complemented with other prey types. After a few attempts with a new prey type, they fed the new items provided to them, and finally they ingested frozen food. Rotifers were not ingested by medusae of any stage (Table 1). When we fed individuals with prey items of similar size than the cubomedusae (i.e. *Mysis* sp.), at first they did not ingest them; however, after a few days the cubomedusae were capable to catch and immobilise these prey with their tentacles and introduce them into their gastrovascular cavity by flexing their tentacles towards manubrium. In addition, fish larvae (*Dicentrarchus labrax*) were offered several times to the adult cubomedusae kept in aquaria. The preys were cached and killed, but the jellyfish later refused to ingest either alive or dead fish.

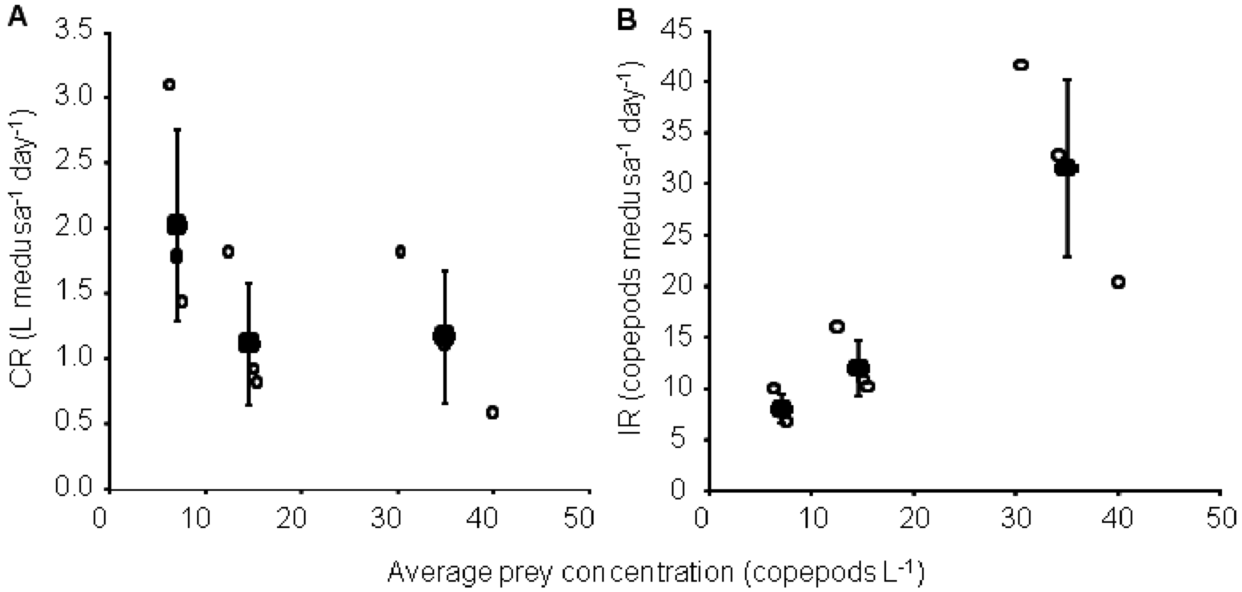


Fig. 4 Clearance (CR) and ingestion rates (IR) of *Carybdea marsupialis* feeding on natural mesozooplankton at different prey concentrations: (A) Clearance rate with natural prey (calanoid copepods); (B) Ingestion rate with natural prey (calanoid copepods). Grey-filled circles are individual values; black-filled squares are mean values ± SD for each prey concentration.

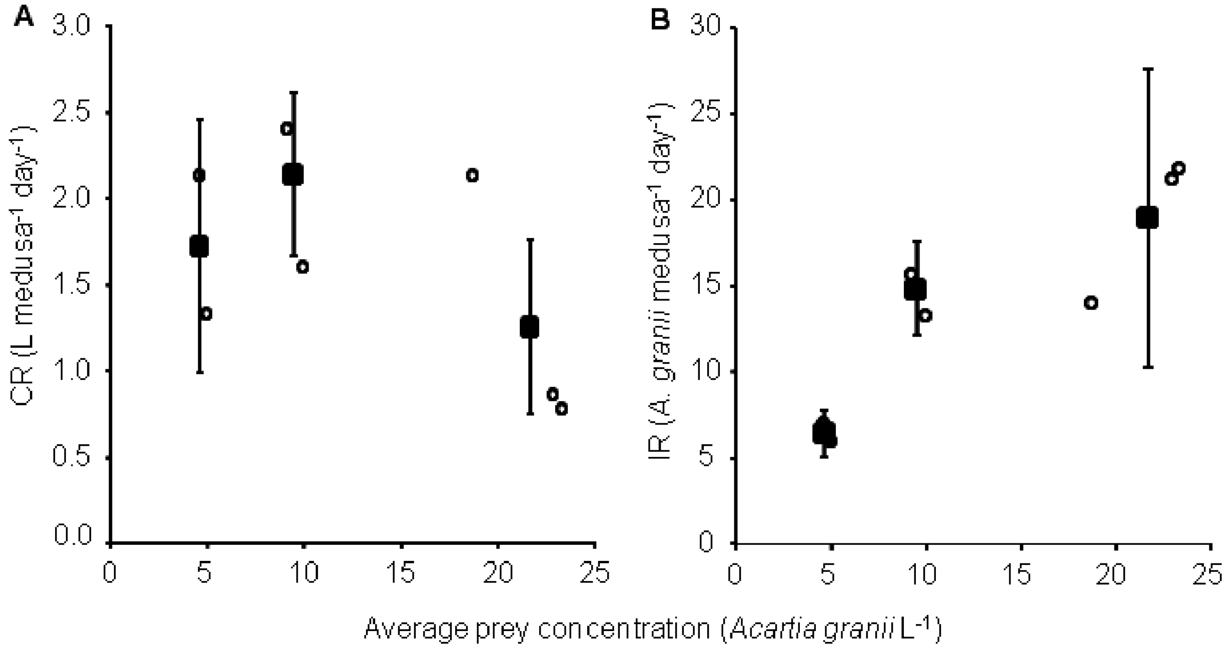


Fig. 5 Clearance (CR) and ingestion rates (IR) of *Carybdea marsupialis* feeding on *Acartia grani* at different prey concentrations: (A) Clearance rate with *A. grani*; (B) Ingestion rate with *A. grani*. Grey-filled circles are individual values; black-filled squares are mean values ± SD for each prey concentration.

Feeding experiments

When the medusae were offered natural assemblages of zooplankton they only significantly ingested copepods (MANOVA; $p < 0.01$) (i.e. no significant ingestion of cladocerans and icthyoplankton). Therefore, the actual prey concentrations for the three experimental treatments with natural zooplankton were 23, 53 and 88 copepods L^{-1}. The clearance rate of *C. marsupialis* feeding on naturally occurring species of copepods ranged from 0.89 to 2.1 L $medusa^{-1}$ day^{-1} (Fig. 4A). The ingestion rate on natural zooplankton increased with increasing prey (copepod) concentration, ranging from 8 to 31.6 copepods $medusa^{-1}$ day^{-1} (Fig. 4B).

The clearance rate of *C. marsupialis* feeding on *Acartia grani* averaged 1.7 L $medusa^{-1}$ day^{-1} (from 0.78 to 2.4 L $medusa^{-1}$ day^{-1}) at prey concentrations ranging from 8.33 to 33.3 ind L^{-1} (Fig. 5A). The ingestion rates increased with prey concentration from 6.5 to 19.0 copepods $medusa^{-1}$ day^{-1} (Fig. 5B). There was no evidence of feeding saturation at the prey concentrations tested. Given prey concentration decreased considerably along the incubations (from 40 to 100 % in 1 case; mean decrease 73.67 ± 15.94 %), we present the CR and IR based on average prey concentrations. Because final prey concentration decreased more than 50 %, and that fact may alter both the CR and IR (Båmstedt et al., 2000), we have to consider that the CR and IR we obtained from our incubations could be underestimated.

The digestion time of *C. marsupialis* feeding on *Mysis* sp. at 23 °C was 2.24 ± 0.63 h (mean±SD) for complete digestion to be achieved. During that time some indigestible wastes (i.e. exoskeleton) were expelled. The head and the eyes of the preys were the last part to be digested.

Discussion

Cultivation of *Carybdea marsupialis*

Previous attempts to cultivate carybdeids did not succeed in obtaining individuals larger than 5–6 mm in DBW (Straehler-Pohl and Jarms, 2011). Aquarium design, water quality, diet and feeding regimen are key factors for a successful maintenance of jellyfish species, and also box jellies. As it can be inferred from our experiments, horizontal flow of the water, central lightning and black sides are necessary in order to prevent the attachment of the tentacles to the aquarium, which could cause death by starvation. The size and the stage must be considered when feeding the cubomedusae in order to provide them the right range of prey size. It is also very important to feed box jellies with different types of prey, since feeding only on *Artemia salina* seems not to be nutritionally enough for supporting the development. We previously observed high mortalities of adult medusae caught from field that were fed with *Mysis* sp. (either alive or frozen) and juvenile fish (M. J. Acevedo, personal observation). This suggests that copepods could be a necessary component on the diet of large and small medusae. Moreover, a percentage of 23.5% of adult cubomedusae (~25 mm in DBW) caught from the field on previous occasions had copepods in their gastric cavities (M. J. Acevedo, unpublished data). Also Larson (1976) described the diet of cubomedusae consisting mostly of crustaceans and fish; crustaceans (mostly *Acartia* sp.) were eaten by all sizes of *Carybdea* sp.; however, small medusae seem to be more dependent on them than larger ones because their difficulty of capturing fish (Larson, 1976). Other carybdeid species, such as *Tripedalia cystophora*, has been registered to prey on dense swarms of the copepod *Dioithona oculata* in the mangrove prop-root habitat of Puerto Rico (Buskey, 2003). By completing the diet with natural mesozooplankton, or cultured copepods, the cubomedusae in our experiments were able to progress in their development. We also observed that an inadequate food ration resulted in bell deformation, but when feeding was increased the bell shape returned to normal. Feeding the adult cubomedusae was more difficult than the recent detached medusae, since they have a more complex feeding behaviour. Although adult individuals of this species are known to occasionally ingest fish in the field (Larson, 1976), cultured specimens refused the fish larvae (*Dicentrarchus labrax*) provided, either alive or dead. It was probably not the adequate fish species to feed this type of jellyfish and most of the adults died after 10 days due to starvation. During summer 2012, we maintained adult cubomedusae for more than 50 days feeding them with a culture of *Mysis* sp. which was kept in the same aquaria were the box jellies were maintained. A detailed analysis of the gastric contents in wild specimens will determine the key prey species for this medusa.

Despite the difficulties in maintaining juvenile cubomedusae in aquaria, we were able to sustain 18 individuals of *C. marsupialis* over a 4-month period (137 days), although only at ~60 % of the mean maximum natural size observed in the sea (M. J. Acevedo, unpublished data). This percentage of reduction in the cultivated adult size is similar to the ones obtained by Hamner et al. (1995) for *Chironex fleckeri*. Their *C. fleckeri* achieved 75% of their natural size, growing from 40 up to 120 mm in DBW, in comparison to the 160 mm in DBW attained in the sea. In our study, although the cubomedusae grew, they did not reach full sexual maturity; however, gonads started to develop as a thin line of gonadal tissue on either side of the septa. These individuals lived the same period of time than the assumed life span of *C. marsupialis* medusae (i.e. from late May to early November), but their development was much slower than the expected at sea. We suggest that captivity affected their growth and correct development. Among many possible causes, we think that they probably needed a higher energy intake (food) to reach full gonadal maturity.

The temperature affected the survival ofour cubomedusae during the period of maintenance in the aquarium, but individuals from the sea developed mature gonads during August at similar temperature. The slower growth rate of the cultured cubomedusae compared with wild individuals, in addition to the decrease in temperature under ~18 °C in November could be the reason why they did not reach sexual maturity in the aquaria. Field observations have shown a similar trend in decreasing densities of *C. marsupialis* with decreasing seawater temperatures (Bordehore et al., 2011). Sexton et al. (2010) have also indicated that low temperatures cause *Chrysaora quinquecirrha* to sink to the bottom and decreased their pulsation rate until they reach the limit of their temperature tolerance, at which point they would die. We observed a similar behaviour in individuals of *C. marsupialis* maintained in the aquaria, when the temperature of the seawater flow-through in the aquarium decreased they sank to the bottom and died.

Feeding behaviour

The present study has also improved our knowledge of the trophic requirements of cubomedusae. Given the few data available, it is not possible to determine the type of functional response of the IR; however, we decided it was relevant to the study because it provides an approximated vision on the amount of food these organisms require for growth.

It is difficult to compare clearance rates from the present work with other similar studies, since feeding experiments with cubomedusae are scarce. Because of this, we compare our results with the feeding rates of other gelatinous organisms, but must be taken into consideration that experimental conditions and swimming behaviour of the different medusae are not identical, and this may be an integrated part of the hunting technique which could produce differences between the CR of the species. As stated before, given the severe prey reduction during some incubations, it may well be our maximum rates are underestimated. Individuals of *C. marsupialis* of the size class we used (i.e., mean DBW of 10.5 mm; 0.76 g of WW) showed a mean CR of 1.57 L medusa^{-1} day^{-1} in our experiments of feeding on natural zooplankton and cultured *Acartia grani*. The estimated rates in this study are similar to the ones obtained for *Mnemiopsis leidyi* in the same WW range (i.e., individuals of 0.93 g of WW) had CR about 1.94 L medusa^{-1} day^{-1} (Acuña et al., 2011), although they are one order of magnitude lower than the ones obtained for other scyphomedusae species, such as *Pelagia noctiluca*, also with the same WW (i.e., individuals of 0.66 g of WW had CR of 1.94 L medusa^{-1} day^{-1}) (Acuña et al., 2011; Hansson et al., 2005; Madsen and Riisgård, 2010; Purcell, 1985).

Large-sized gelatinous organisms are a problematic group in incubation experiments, particularly those whose hunting mode is 'cruising entangling', as *C. marsupialis.* Experiments performed on such organisms without prior knowledge of their feeding behaviour are unreliable (Båmstedt et al., 2000). *Carybdea marsupialis* even if moderated in size requires high incubation volumes because has tentacles at least eight times longer than its diameter. This should be considered when trying to incubate the species.

Advantages and disadvantages of being transparent

Cubomedusae are transparent; therefore, holding any opaque object in their gastric cavities for long periods of time could make them more visible and vulnerable to their natural predators (e.g. turtles). Thus, the rapid digestion of prey items might favour them; indeed, cubozoans have very active digestive extracellular proteases that are produced by cirri and stomach wall (Larson, 1976) which enable them to digest prey quickly. There are other factors influencing digestion time, such as prey type and size, number of prey in the gut content, temperature, and species of predator (Ishii and Tanaka, 2001; Martinussen and Båmstedt, 2001).

In the present study, *C. marsupialis* were able to fully digest one *Mysis* sp. in 2.24 h at 23 °C. Puerto Rican *Carybdea* sp. digested one 20 mm fish in 3–4 h, continuously expelling wastes of prey during digestion (Larson, 1976). The Australian cubomedusa *C. fleckeri* has a digestion time ranging from 3.5 to 4.5 h at 30 °C (Hamner et al., 1995). Other gelatinous organisms, about the same size of the cubomedusae we used for incubation experiments, showed a wide range of DT from 2.16 h in the case of *Mnemiopsis leidyi* (feeding on copepods at 21 °C), 4.62 h for *Aurelia aurita* (feeding on *Calanus* sp. at 20 °C), to 6.55 h for *Cyanea* sp. (feeding also on *Calanus* sp. at 20 °C) (Martinussen and Båmstedt, 2001). The shortest DTs correspond to the most transparent species: *M. leidyi* and *C. marsupialis* (2.16 and 2.24 h respectively).

Their aforementioned transparent bell may not only aid predator avoidance, but may also benefit them as predators, combined with a developed sense of vision and very long extensile tentacles. Cubomedusae differ from scyphomedusae and hydromedusae, in that their rhopalia (sensory structures) contain simple eyes and complex eyes (which have an apparent cornea, a spherical lens and retinal cells connected to nerve fibres) (Yamasu and Yoshida, 1976). Several investigators have speculated that complex eyes of cubozoan medusae and large numbers of simple pigment-cup ocelli should make these organisms capable of detecting changes in the distribution of light and perhaps even a crude form of vision (Pearse and Pearse, 1978). The sophisticated photosensitive behaviour of these predatory medusae may assist them in locating and remaining in copepods swarms where their prey is plentiful. We observed a pronounced positive phototropism of the medusae that were kept in the aquarium, as it is stated for other cubozoan species; we selected blue light for maintaining the cubomedusae since this colour is supposed to produce changes in the behaviour of some boxjelly species that were interpreted as feeding behaviour (Gershwin and Dawes, 2008). *C. marsupialis* are ambush predators, relying on the prey to swim into contact with their nematocyst-laden tentacles for prey capture. However, it may be more of a challenge for ambush predators to locate prey patches (Buskey, 2003). Other gelatinous zooplankton predators that are known to exhibit behavioural responses to the presence of prey are the scyphomedusa *Aurelia aurita*, which directs their movements according to odours that are associated with prey (Arai, 1997). In the present study, juvenile *C. marsupialis* were able to capture *Artemia salina* and copepods by extending their long tentacles; however, larger prey, such as *Mysis* sp., had to be placed manually on themanubrium. Also some form of active selection for copepods is indicated when feeding on natural zooplankton, but more detailed experiments have to be conducted to prove that.

As it can be inferred from our results and observations, horizontal flow of the water, central lightning and black sides are necessary in order to prevent the attachment of the tentacles to the aquarium, which could cause death by starvation. The size and the stage of the cubomedusae must be considered in order to feed them with the right range of prey size. Moreover it is also very important to provide the box jellies different types of prey (i.e. *Artemia*, copepods and *Mysis*), since feeding only on *Artemia salina* seems not to be nutritionally enough for supporting the development.

Future studies are needed to determine the energetic requirements of the different stages of *C. marsupialis* in detail. Such studies could provide more information about trophic connections between *C. marsupialis* and other zooplankton, and therefore the potential predatory impact of *C. marsupialis* in the NW Mediterranean and other areas. Moreover, due to the importance of the polyp stage for jellyfish outbreak formations, it is vital to solve some key questions regarding to the polyp biology, ecology and location. This information will be vital if the ecological role of *C. marsupialis* and environmental issues arising from more frequent blooms of this species are to be understood.

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CHAPTER 3

Trophic ecology and potential predation impact of *Carybdea marsupialis* (Cnidaria: Cubozoa) in the NW Mediterranean

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Abstract

Gut contents and stable isotope analysis were used to study the trophic ecology and potential predation impact of a population of *Carybdea marsupialis* Linnaeus sampled in shallow coastal waters of Denia (38°51'55.60"N / 0° 0'41.40"E), Spain, from 2010 to 2015. The abundance of this box jellyfish was significantly greater during the night, although both diurnal and nocturnal captures revealed a similar proportion of about 50 % empty stomachs. Calanoid copepods were the most numerous prey in the stomachs (14 %), followed by gammarids (11.1 %), mysids (5.7 %) and isopods (3.4 %). Polychaetes and fish larvae were present in lower frequencies in the stomachs, but they contributed with higher proportion to the total carbon ingested. Prey selectivity indices showed a preference for nocturnal, epibenthic zooplankton, highlighting the species contribution to the bentho-pelagic coupling. Trophic-level (TL) estimates – based on δ^{15}N values – varied significantly along the progression of developmental stages. A mixing model indicated that epibenthic zooplankton contributed on average ~40-50 % to the diet of juveniles and adults, and fish larvae ~14 % and 20 %, respectively. The gut content/digestion time method showed that the species predation impact was higher during the night. Potential predation of this species has been observed to be intense but not important enough to regulate the populations of their prey. Furthermore, potential competition between *C. marsupialis* and juvenile fish may exist, as their diets strongly overlap (Percentage of Similarity Index = 85.4 %).

Keywords: cubozoans, *Carybdea marsupialis*, feeding behaviour, stable isotope analysis, SIAR, Isotopic Niche Breath, ontogenetic shift.

Introduction

The box jellyfish *Carybdea marsupialis* Linnaeus (1758) seems to have increased in abundance in both the Adriatic and the Mediterranean seas from the mid-1980s (CIESM 2008). Since 2008, blooms of this species have been detected and studied along the Spanish coast (LIFE CUBOMED project, www.cubomed.eu), as well as along other Mediterranean coasts, reaching unusually high densities at some beaches (Bordehore et al. 2011; De Donno et al. 2014; Gueroun et al. 2015). Considering the increasing presence, it has become important to determine whether high densities of *C. marsupialis* would alter ecosystem function and/or biodiversity. In this sense, questions regarding the biology and feeding ecology of this organism need to be answered to elucidate what is enhancing stocks of this native species to outbreak levels. The life cycle of *C. marsupialis* has not been completely described in the Mediterranean, but similarities with other *Carybdea* species have been hypothesized (Bordehore et al. 2015). *Carybdea* medusae are oviparous and dioecious, releasing fertilized eggs into the water column (Studebaker 1972). Settlement of the planula larvae on substrate takes place a few days after ova fertilization, and then the development of the benthic polyp phase initiates (Studebaker 1972). Later, polyps can reproduce asexually by budding (Stangl et al. 2002; Straehler-Pohl and Jams 2005). Mature polyps metamorphose and detach the cubomedusae, which grow and develop until the gonads mature, and finally reproduce completing the life cycle (Bordehore et al. 2015).

Diet studies can provide important information on the biology of a species and enable the construction of trophic models (Nogueira and Haddad 2008; Navarro et al. 2014). Although the knowledge of box jellyfish feeding habits is scarce compared to that for scyphozoans, there are some studies based on the analysis of stomach contents (see Kingsford and Mooney 2014 for a review). In general terms, the size of the prey a medusa captures depends on its own size. Small cubomedusae primarily prey on planktonic crustaceans, while larger individuals are able to prey on fish (Kingsford and Mooney 2014). An ontogenetic shift in preferred prey has been documented from gut content analyses of some species: the carybdeids *Carukia barnesi* (Underwood and Seymour 2007) and *Carybdea rastonii* (Lai 2010), and the chirodropids *Chironex fleckeri* (Carrette et al. 2002) and *Chiropsalmus quadrumanus* (Nogueira and Haddad 2008). Nogueira and Haddad (2008) observed an ontogenetic shift in *C. quadrumanus* diet, with smaller cubomedusae feeding on a wider variety of prey. Other evidence of developmental changes in the diet comes from age-specific toxins and injection mechanisms, which may enable predation on specific prey (Carrette et al. 2002, Underwood and Seymour 2007). Moreover, it has been suggested that cubomedusae have developed activity patterns that match the movement patterns of their prey (Matsumoto 1995; Gordon and Seymour 2009; Garm et al. 2012). Some cubozoans, like *Carybdea rastonii* (Matsumoto 1995) and *Copula sivickisi* (Garm et al. 2012), have been observed resting on the bottom during the day and actively swimming at night. Other species, including *Chironex fleckeri* (Seymour et al. 2004) and *Tripedalia cystophora* (Garm et al. 2012), have been reported to feed during daytime and to be less active on the bottom at night. Diel variation of behaviour has not been clearly described for *C. marsupialis*.

Some jellyfish species may compete effectively for planktonic prey and compromise the survival of other organisms such as fish larvae (Eiane et al. 1997; Purcell and Arai 2001; Lynam et al. 2006). Indeed, jellyfish can prevail under certain extreme conditions, like high water turbidity (Eiane et al. 1997). While most fish depend on visual sensing of their prey, jellyfish can also rely on tactile contact with prey. Most cubozoan medusae show a foraging behaviour, swimming with the tentacles extended to entangle their prey (Larson 1976; Hamner et al. 1995; Matsumoto 1995). In addition, the swimming behaviour of box jellyfish is characterized by more variable orientation and better maneuverability than exhibited by scyphozoans. These behaviours are directed by their complex lensed eyes, and may favour effective foraging by cubomedusae in complex habitats (Colin et al. 2013), which in contrast may offer high prey abundance (e.g. mangroves and littoral zone). Certainly, the structural complexity of the environments inhabited by cubozoans has a strong influence on their behaviour (Kingsford and Mooney 2014). For instance, the coastal area of Denia, where *C. marsupialis* can be found, is mainly composed of shallow sandy beaches with patches of *Posidonia oceanica* meadows (Bordehore et al. 2011; Bordehore et al. 2015). In the seagrass meadows, the main trophic fluxes are from primary producers (i.e. plant detritus, microphyta and macrophyta), via secondary producers (i.e. copepods, ostracods and gammarid amphipods) and decapod crustaceans, to fish, considered the highest-level consumers (Zupo and Stübing 2010). Such a rich environment might provide enough food supply to cubomedusae growth. However, basic information on the feeding biology of *C. marsupialis* and its trophic interactions is unavailable, preventing better understanding of its apparent increases and the trophic consequences of its proliferation.

In order to shed light on this issue, the main objectives of this study were: i) to reveal the feeding habits of *C. marsupialis* and possible daily cycles in its feeding activity; ii) to determine the diets of its developmental stages from recently detached to fully developed adults; iii) to investigate its trophic role in the *Posidonia oceanica* meadows, with special attention to the potential competition with fish larvae and juveniles; and iv) to estimate the predation impact of this organism on the pelagic communities of Denia. We used different approaches, including gut-content and stable-isotope analyses of medusae collected from the field, as well as laboratory experiments.

Materials and methods

Sampling

The abundance of *Carybdea marsupialis* Linnaeus medusae was measured in two different beaches (i.e. Raset 38°50’51.25”N/ 0° 6’36.81”E and Almadrava 38°51’55.60”N/ 0° 0’41.40”E) located at Denia, Spain (NW Mediterranean). This coastal area is mainly composed of shallow sandy beaches with patches of *Posidonia oceanica* meadows and the seaweed *Caulerpa prolifera*. Four samplings were conducted between the 25th of August and 13th of October 2011 at each beach. For each sampling date, collections were done in two moments: during the day (from 0800 to 1300 hr) and during the night (from 2100 to 0100 hr). Given the low number of specimens captured during the day, specimens collected in 2009 also in diurnal samplings were added to the gut contents analysis (n = 102). Before combining the data, we tested for differences in diet composition between individuals captured in 2009 and those of 2011 using multivariate permutational ANOVA. We did not detect statistically significant differences (pseudo-F = 0.177, p-value = 0.95).

We sampled along three coastal transects at each point, using nets pulled manually through shallow waters (between 0.5-1.5 m depth, 1 to ~15 m distant from the shoreline). Each tow was pulled by one person walking at a speed of ~0.3 m s^{-1}, along a distance of about 20 to 40 m parallel to the coastline. Tows were done simultaneously with three types of nets varying in mesh size, mouth size and length: 200-µm, 50-cm diameter (~3 m^3 filtered per transect), 160 cm length; 400-µm, 50-cm diameter (~5 m^3 filtered per transect), 155 cm length; 4-mm 50x50-cm square net (~12 m^3 filtered per transect), 65 cm length. Three different nets allowed inclusion of the whole size range of the species, as well as the co-occurring potential preys in the mesozooplankton. The net mouth was completely submerged in the water during the tow, and the distance to the sea bottom was maintained between 10 and 30 cm. A flow-meter (General Oceanics, 2030R standard flow-meter) was placed in the mouth of each net to calculate the volume of water filtered, and finally estimate cubomedusae and prey densities. Some cubomedusae were anaesthetized in seawater with menthol crystals and then rapidly preserved in seawater-formaldehyde solution (4 %) for later dissection and gut content analysis under a stereomicroscope. Other cubomedusae were transported to the laboratory for feeding experiments. In addition, samples for stable isotope analysis were collected (with the same procedure as described above) from 2010 to 2015: cubomedusae of different developmental stages, samples of their potential prey and particulate organic matter (POM; particles ranging from 0.7 to 200 µm retained in GF/F filters). These were immediately frozen at -20 °C prior to the analysis.

Stomach content analysis

Diagonal Bell Width (DBW, the distance between two opposite pedalia) and Bell Height (BH, from the top of the exumbrella to the margin, excluding the velarium) were measured, and the developmental stage was determined (i.e., recently detached DBW ≤ 2 mm; small 2 mm < DBW ≤ 5 mm; juveniles 5 mm < DBW ≤ 15 mm; and adults DBW > 15 mm). The specimens were dissected under the stereomicroscope through an incision in the constriction of the upper exumbrella, and the gastrovascular cavity and phacellae were exposed. The content was extracted and the prey were identified and measured.

Three parameters were calculated for the different prey observed in the stomachs (according to Espinoza and Wehrtmann 2008): the percentage of occurrence of a prey item among all prey registered (% O); the number organisms with that prey item in the stomach (N); the frequency of occurrence of the item in the total number of stomachs analysed (F_o). The total biomass contributed by each prey over all medusae captured was calculated as carbon biomass, using the carbon content of individual prey items obtained from the isotopic analysis.

Statistical analyses were performed using R (R Core Team, 2015). Generalized linear models (GLM) (with a poisson error distribution and a log-link function) were applied for testing the differences in the cubomedusa abundance between day and night. GLM using a binomial error distribution were applied to compare the proportions of empty stomachs from diurnal and nocturnal samples. In addition, the PRIMER & PERMANOVA 6 statistical package (Clarke and Gorley 2006) was used to analyse the dietary composition from all specimens captured during day and night samplings (i.e. all samples from 2009 and 2011 gut contents pooled), by applying three different tests: non-metric MDS was conducted to explore and visualize the data; multivariate permutational ANOVA to test the differences between the two groups; and SIMPER (Clarke 1993; Clarke and Warwick 2001) to identify the prey items that contributed to the similarity of the gut contents at a specific sampling moment (i.e. day and night). The routines were conducted using a Bray-Curtis similarity index matrix based on fourth-root transformed data.

Prey selectivity was estimated by the α index (Chesson 1978, Confer and Moore 1987):

$$\alpha = \frac{\left(\frac{r_i}{p_i}\right)}{\sum_{i=1}^{n}\left(\frac{r_i}{p_i}\right)}$$

where r_i and p_i are the proportions of prey *i* in the gut contents and in the field, respectively, and *n* is the number of prey taxa. Neutral or random selection (i.e. when the forage ratio for a prey type equals the mean forage ratio for all other types) would result in a constant $a_{neutral} = 1/n$. This index is density independent, and determines whether prey items were ingested in higher or lower proportion relative to what would be expected due to their abundance in the field (Pearre 1982). When the number of prey types is constant, α may be the most meaningful indicator of prey type preference; α values are sensitive to changes in the number of prey types, but not to changes in prey proportion (Confer and Moore 1987). Chesson's α index was first calculated for each tow, and then averaged by prey type and by sampling moment (i.e. day and night).

Stable isotope analysis

We determined the C and N stable isotope compositions of *C. marsupialis* of different sizes as well as those of their potential prey. Depending on the organism size, samples were treated individually or pooled to achieve the sufficient organic matter (i.e. N > 10 µg and C > 20 µg) to conduct the analysis (see Table 3 for further details of the different sources). In the case of cubomedusae smaller than 1.5 mm DBW, the pooling of several individuals was necessary (10 cubomedusae per sample, 5 replicates) (Table 3). After storage (at -20 °C), samples were oven dried (72 hours at 60 °C) and ground to a fine powder. Samples were weighed in tin cups, except for crustacean C samples, that were acidified (0.12 M HCl) in silver cups to remove carbonate minerals from their exoskeletons. Because most of the tissues analysed had low-lipid content, lipids were not extracted from the samples, but a mathematical correction was applied to δ^{13}C when the carbon-to-nitrogen (C: N) ratio was > 3.5 (Post et al. 2007). All of the samples were combusted at 1000 °C for analysis in a FlashEA1112 (ThermoFinnigan) elemental analyser (precision ± 0.2 ‰ for both δ15N and δ^{13}C results). The isotopic compositions were expressed in delta notation (δ ‰). Delta is a comparison of the ratio of the two dominant isotopes (^{13}C: ^{12}C and ^{15}N: ^{14}N) in the sample relative to international isotopic standards:

$$\delta X = \left[\left(\frac{R_{sample}}{R_{reference}}\right) - 1\right] \times 10^3$$

where X is either ^{13}C or ^{15}N, and R is the corresponding ^{13}C/^{12}C and ^{15}N/^{14}N ratio (Peterson and Fry 1987).

We tested possible effects of size and developmental stage of *C. marsupialis* specimens on their isotopic ratios. We applied Generalized Additive Models (GAM), due to the highly non-linear relationship observed between the variables (see Fig. 5). We also included other variables (i.e. month, year and beach) in order to ensure the reliability of combining results from different samplings.

Furthermore, we applied the SIAR Bayesian isotopic mixing model (Stable Isotope Analysis package for R, SIAR 4.2; Parnell and Jackson 2013) in order to estimate the contribution of different sources to the diet composition of *C. marsupialis*. One of the main issues in the application of mixing models is to choose the most appropriate $\Delta\delta^{15}$N and $\Delta\delta^{13}$C (trophic enrichment factors, TEF) for the organisms being studied (Phillips and Koch 2002). The only TEF estimates experimentally calculated for jellyfish were reported by D'Ambra et al. (2014) for *Aurelia* spp. (i.e. $\Delta\delta^{13}$C = +4 ‰ and $\Delta\delta^{15}$N = +0.1 ‰). However, the use of this $\Delta\delta^{15}$N resulted in unfeasibly high trophic positions for field collected jellyfish (Fleming et al. 2015). Moreover, we could not apply the $\Delta\delta^{15}$N = +0.1 ‰ because this value is lower than the precision limit of our analyses (i.e. ± 0.2 ‰). Overall, the values published by D'Ambra et al. (2014) contrast with the average TEFs of other organisms (Minagawa and Wada 1984; Hobson and Welch 1992; Vanderklift and Ponsard 2003; McCutchan et al. 2003), and those applied in some other studies on jellyfish (Frost et al. 2012; Nagata et al. 2015). Therefore, based upon a literature review, and in the absence of published TEF values for *C. marsupialis*, we estimated trophic enrichments as the difference between *C. marsupialis* isotope values (30% of the data were used), and those of the main prey items observed in their stomachs (i.e. copepods and amphipods). The calculated TEFs were $\Delta\delta^{13}$C = +1.4 ± 0 .9 ‰ and $\Delta\delta^{15}$N = +2.5 ± 0.8 ‰. We also tested a range of TEFs frequently applied in jellyfish studies (i.e. Post et al. 2002; McCutchan et al. 2003; Javidpour et al. 2016) to test the sensitivity of the mixing model to the variation in TEF value.

Regarding the sources to be included in the mixing model, these were determined based on the items observed in the gut contents. From these, the items better delimiting the mixing polygon (Phillips and Gregg 2003) were chosen. Finally, we grouped preys with similar isotopic signatures in order to avoid overabundance of sources (e.g. for 2 isotopes a maximum of 3-4 sources would be adequate) (Phillips and Gregg 2003; Phillips et al. 2005; Caut et al. 2008). Therefore, the mixing model included three sources: copepods, epibenthic zooplankton (i.e. mysis and amphipods together) and fish larvae (Sparidae, Mugilidae and Clupeidae). We also estimated the isotopic niche area of the different developmental stages of *C. marsupialis* as the Standard Ellipse Area of the SIAR package (Jackson et al. 2011), which is a

	Copepods		Mysis		Amphipods		Fish larvae	
Collection time	Day	Night	Day	Night	Day	Night	Day	Night
Total # preys in guts	153	25	10	12	4	65	2	0
Mean prey density in field (prey m^{-3})	8.6 ± 6.3	5.9 ± 12.2	5.6 ± 6.4	26.8 ± 27.8	0.2 ± 0.2	1.5 ± 1.3	0	0.04 ± 0.1
Digestion time (h)	1.3	1.3	1.3	1.3	1.3	1.3	3.7	3.7
Feeding rate (prey cubomedusa^{-1} h^{-1})	0.9	0.08	0.06	0.04	0.02	0.22	0.004	0
% Prey stock consumed h^{-1}	**1.9**	**2.6**	**0.2**	**0.3**	**2.3**	**25.7**	**0**	**0**
Potential daily predation (8 h day: 8 h night)	**15.1**	**20.7**	**1.5**	**2.2**	**18.5**	**206.1**	**0**	**0**

TABLE 1 Predation impact estimates. Number of guts analysed, n = 123 from diurnal samplings and n = 227 from nocturnal samplings. Average predator density: 0.17 ± 0.37 cubomedusae m^{-3} during day and 1.83 ± 3.28 cubomedusae m^{-3} during night.

representation of the species trophic-niche. This analysis has been previously applied for other gelatinous organisms (Nagata et al. 2015).

In addition, we estimated the trophic level (TL) of the different organisms (Hobson and Welch 1992; Nagata et al. 2015) relative to calanoid copepod values, which were assumed as the TL 2:

$$TL = \lambda + (\delta^{15}N_{consumer} - \delta^{15}N_{copepods})/\Delta\,\delta^{15}\mathrm{N}$$

where λ corresponds to the $\mathrm{TL}_{\mathrm{copepods}} = 2$, and $\Delta\delta^{15}$N the estimated increase in δ^{15}N per trophic level of 2.5 ± 0.8 ‰.

Competition and predation impact estimations

Two different aspects of *C. marsupialis* trophic ecology have been estimated. On one hand, dietary overlap and potential competition between *C. marsupialis* gut contents and the diet of post-larval juvenile fishes inhabiting *Posidonia* meadows (mainly Labridae, Pomacentridae, Scorpaenidae and Serranidae; data for fish published by Zupo and Stübing 2010) was calculated using the percent similarity index (PSI):

$$PSI = \left(1 - 0.5\sum_{i=1}^{n} |p_{ik} - p_{jk}| \times 100\right)$$

:

	n_p (%O)		N (Fo)		Total biomass (mg C)	
Prey category	**J**	**A**	**J**	**A**	**J**	**A**
Crustacea						
Copepods	7 (21.2)	168 (55.8)	7 (9.3)	41 (15.1)	0.01	0.3
Gammarids	13 (39.4)	56 (18.6)	11 (14.67)	26 (9.59)	0.6	2.8
Mysids	2 (6.1)	20 (6.6)	2 (2.67)	18 (6.64)	0.6	5.6
Isopods	3 (9.09)	9 (3)	3 (4)	9 (3.3)	0.8	2.5
Decapod larvae	2 (6.1)	3 (1)	2 (2.67)	3 (1.1)	2.8	4.2
Cumaceans	0 (0)	3 (1)	0 (0)	3 (1.1)	0	0.06
Exoskeletons	5 (15.15)	34 (11.3)	5 (6.7)	28 (10.3)	NA	NA
Polychaete	1 (3.0)	6 (2)	1 (1.3)	6 (2.2)	0.6	3.5
Fish larvae	0 (0)	2 (0.7)	0 (0)	2 (0.74)	0	5.9
TOTAL	**33 (100)**	**301 (100)**	**31 (41.3)**	**136 (50.18)**	**5.43**	**25.1**

TABLE 2 Prey items observed in *Carybdea marsupialis* stomachs collected in October 2009 (n = 102) and between August and October 2001 (n = 248); data differentiated for juveniles (J) and adults (A); n_p: number of prey; *% O*: percentage of occurrence; *N*: number of cubomedusae with the prey item in the stomach; F_o: percentage of stomachs with a given prey; Total biomass: as mg of carbon.

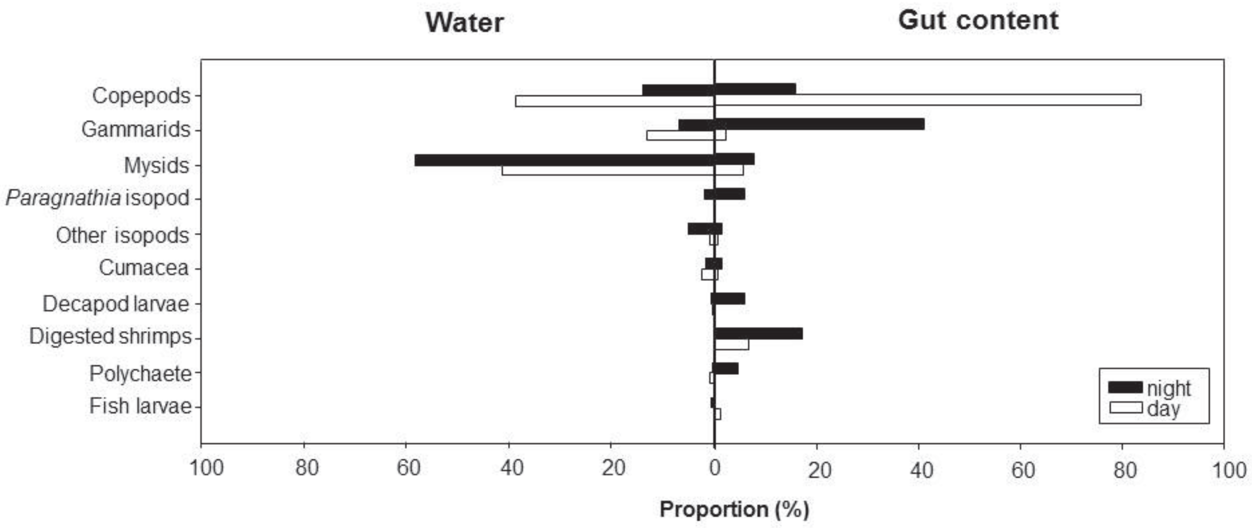

Fig. 1 Comparison of Percent of abundance in the water of the different prey items, and Percent of Occurrence (*% O*) in the gut contents of *C. marsupialis* captured during day (123 individuals) and night (227 individuals).

where p_i and p_j are the numerical proportions of the k^{th} prey species consumed by predator species *i* and *j*. Values over 60 % indicate high overlap (Wallace and Ramsey 1983; Brodeur et al. 2008).

On the other hand, predation impacts exerted by cubomedusae on different prey types (i.e. copepods, mysids, amphipods and fish larvae) were calculated with the following equation (Purcell 1992):

$$P = \frac{F \times M}{C_t} \times 100$$

where *P* = percentage of prey-item standing stock consumed d-1; *F* = individual feeding rate (number of prey items ingested cubomedusa-1 d-1); *M* = mean density of cubomedusae m-3; and *Ct* = mean density of prey m-3. Individual feeding rate was calculated from *F* = *G* /*Dt*, where *G* is the gut content (Total no. prey in guts / no. guts analysed) and *Dt* the digestion time (h). *F* was then multiplied by the number of hours a day cubomedusae spent for feeding. We conducted laboratory incubations with different prey items (i.e. copepods, mysids, amphipods and fish larvae) to estimate *C. marsupialis* digestion times. Cubomedusae were fed *ad libitum* during 20 min and afterwards transferred separately to different containers filled with 5 µm filtered sea water. The volume of the containers ranged from 250 ml to 5 L depending on the cubomedusa's size. After that, observations of gut content were registered every 10-15 minutes. The incubation finished when the prey was totally digested or the wastes (i.e. the exoskeleton in the case of crustaceans) were egested.

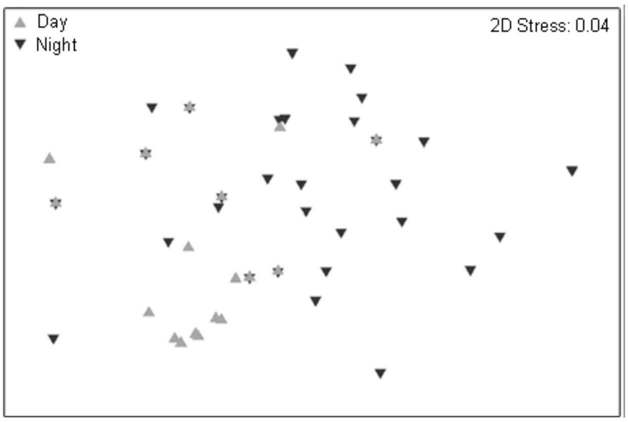

Fig. 2 MDS representation showing differentiation between diurnal and nocturnal stomach contents.

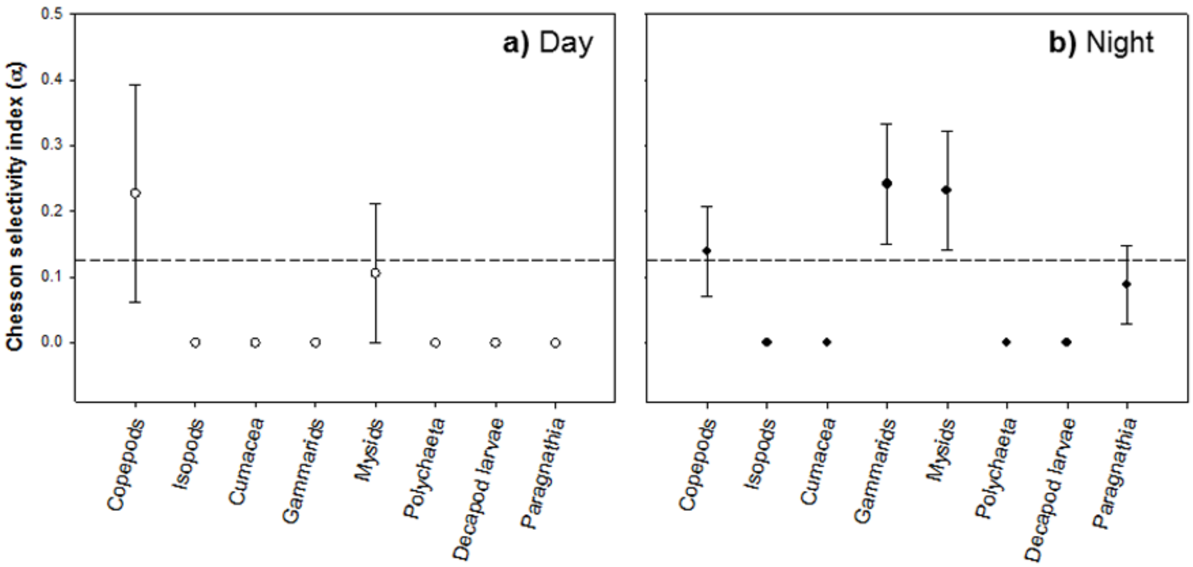

Fig. 3 Chesson's α prey selectivity index: Day (a) and night (b); mean values and SE bars. The dashed line corresponds to the a constant value (α neutral = 1/n; n is the number of prey taxa) which indicate neutral or random selection. Values above this threshold indicate positive selection of corresponding prey type.

Results

Abundance and gut contents

The abundance of medusae was significantly higher (Z = 8.93; N = 48; $P < 2.0e^{-16}$) during the night (1.83 ± 3.28 cubomedusae m^{-3}, n = 24) than during the day (0.17 ± 0.37 cubomedusae m^{-3}, n = 24) (Table 1). Considering only the nocturnal samplings, the highest density of 15.8 cubomedusae m^{-3} was recorded on 20th August 2011. The mean temperature and salinity registered in the sampling period were 27.3 ± 1.9 °C (n = 8) and 37.8 ± 0.2 (n = 8), respectively.

A total of 350 stomachs of *Carybdea marsupialis* collected in 2009 (n = 102) and 2011 (n = 248) were analysed (123 individuals captured during day and 227 during night). The DBW of the specimens varied between 6.1 and 41 mm, corresponding to juvenile and adult stages. Approximately half of the specimens (51.1 %) had empty stomachs, with no significant difference in the proportions of empty stomachs between day and night.

Crustaceans were the most abundant item in the guts of *C. marsupialis* (Table 2). Prey groups with substantial frequency of occurrence were calanoid copepods (14 %), gammarids (11.1 %), mysids (5.7 %) and isopods (3.4 %). Polychaetes and fish larvae were present at lower frequencies. Unidentifiable digested exoskeletons of crustaceans were registered in 9.7 % of the stomachs. The prey contributing the most to the total ingested biomass were decapod larvae, mysids, polychaetes, gammarids and fish larvae (Table 2). Copepods were found in 14 % of guts, but their contribution was low compared to other prey (e.g. mysids) because of their smaller size and carbon content.

The gut content analysis revealed differences in diet composition of the species and prey availability between day and night (Fig. 1). The MDS representation (Fig. 2) showed the distinction between gut contents of day versus night collections. This difference was significant based on the Permanova analysis (Pseudo-F = 9.19; N = 134; P = 0.0001). The SIMPER routine indicated copepods as the main daytime prey (average contribution of 76.4 % to the similarity in the diurnal group), and gammarids as the main prey during night time (50.7 % to the similarity). This difference was also reflected in

Taxonomic group/Species	Collection date	Replicates	δ^{13}C	δ^{15}N	TL	%C	%N	C:N ratio
POM	19/06/2015	Filters (2)	-22.0 ± 0.0	4.8 ± 0.1	NA	NA	NA	NA
Primary producers								
P. oceanica	05/08/2014	Pooled (5)	-12.8 ± 0.1	6.4 ± 0.2	1[a]	33.1 ± 1.6	1.7 ± 0.1	19.3 ± 0.3
C. prolifera	05/08/2014	Pooled (5)	-12.7 ± 0.4	5.3 ± 0.1	1[a]	38.7 ± 2.0	2.1 ± 0.0	19.1 ± 1.1
Crustacea								
Copepoda	19/06/2015	Pooled (5)	-19.8 ± 0.3	7.4 ± 0.8	2	1.3 ± 0.6	1.2 ± 1.2	4.8 ± 0.4
Mysidacea	05/08/2014	Individuals (9)	-19.0 ± 0.5	7.8 ± 0.5	2.2 ± 0.1	14.8 ± 5.6	10.4 ± 0.2	4.5 ± 0.4
Amphipoda	05/08/2014	Individuals (8)	-18.5 ± 1.3	7.2 ± 0.5	1.9 ± 0.1	2.9 ± 1.0	4.7 ± 0.8	5.0 ± 0.6
Isopoda	05/08/2014	Individuals (4)	-19.2 ± 0.2	9.2 ± 0.5	2.8 ± 0.3	20.2	5.6 ± 0.4	3.59
Polychaeta	05/08/2014	Individuals (2)	-17.3 ± 0.6	9.5 ± 0.3	2.9 ± 0.4	45.3 ± 0.7	10.6 ± 0.1	4.3 ± 0.1
Fish larvae								
Mugilidae	23/08/2013	Individuals (5)	-20.0 ± 0.4	8.4 ± 0.5	2.4 ± 0.2	40.9 ± 2.6	9.3 ± 0.5	4.4 ± 0.3
Clupeidae	18/06/2015	Individuals (2)	-19.1 ± 0.1	8.7 ± 0.1	2.6 ± 0.2	49.0 ± 0.5	13.2 ± 0.1	3.7 ± 0.0
Other fish larvae	05/08/2014	Individuals (2)	-18.5 ± 0.4	10.1 ± 0.5	3.0 ± 0.8	25.5 ± 0.9	6.3 ±0.5	4.0 ± 0.2
C. marsupialis								
Detached ($\leq$ 2 mm)	18/06/2010 + 19/06/2015	Ind (11) + Pooled (5)	-19.0 ± 0.4	10.0 ± 1.0	3.1 ± 0.5	9.3 ± 6.4	2.3 ± 1.6	4.0 ± 0.1
Small (2-5mm)	14/06/2011	Individuals (27)	-15.8 ± 0.4	5.9 ± 1.1	1.3 ± 0.3	6.0 ± 3.1	1.5 ± 0.7	3.9 ± 0.3
Juveniles (5-15mm)	08/08/2012	Individuals (14)	-16.7 ± 0.9	9.5 ± 1.1	2.9 ± 0.4	8.0 ± 3.4	2.2 ± 1.0	3.7 ± 0.3
Adults (> 15mm)	08/08/2012	Individuals (15)	-16.7 ± 0.5	10.1 ± 0.6	3.2 ± 0.5	10.6 ± 2.9	2.9 ± 0.8	3.6 ± 0.1

TABLE 3 Values (means ± SDs) of δ^{13}C, δ^{15}N, estimated trophic level (TL), %C, %N and C: N of Particulate Organic Mater (POM) for the main primary producers, the cubomedusa *Carybdea marsupialis* along with its potential competitors (fish larvae) and prey items (invertebrates and fish larvae). δ^{13}C values shown in this table are not corrected for lipid content. Replicates: indivuals or pooled; number of samples in parenthesis. [a] *P. oceanica* and *C. prolifera* are primary producers of the benthic trophic pathway.

Chesson's α prey selectivity index (Fig. 3), since copepods were the only prey positively selected during the day, while gammarids and mysids were preferred during the night.

Stable isotopes

No significant differences between years were observed for the cubomedusae isotopic values of either C ($F = 0.53$; $N = 72$; $P = 0.47$) or N ($F = 1.35$; $N = 72$; $P = 0.25$), which allowed for compiling the results from different sampling periods. GAM analysis indicated a significant effect of DBW on δ^{15}N (R-squared = 0.69; $N = 72$; $P = <2e^{-16}$) and also on δ^{13}C (R-squared = 0.73; $N = 72$; $P = <2e^{-16}$). Nitrogen isotope composition was different among cubomedusa stages: higher for recently detached individuals (mean δ^{15}N ± SD = 10.0 ± 1.0 ‰) and adults (10.1 ± 0.6 ‰), than for small (5.9 ± 1.1 ‰) and juvenile (9.5 ± 1.1 ‰) individuals (Table 3, Fig. 5). Isotopic values of different prey items and sources are also presented in Table 3. The majority of the prey items analysed (i.e. crustaceans, polychaetes, fish larvae) were placed between TL 2 and 3. Trophic-level estimates for *C. marsupialis* varied among the developmental stages: ranging from TL 3.1 ± 0.5 in recently detached specimens; decreasing to TL 1.3 ± 0.3 in small individuals; and increasing again to TL 2.9 ± 0.4 in juveniles and

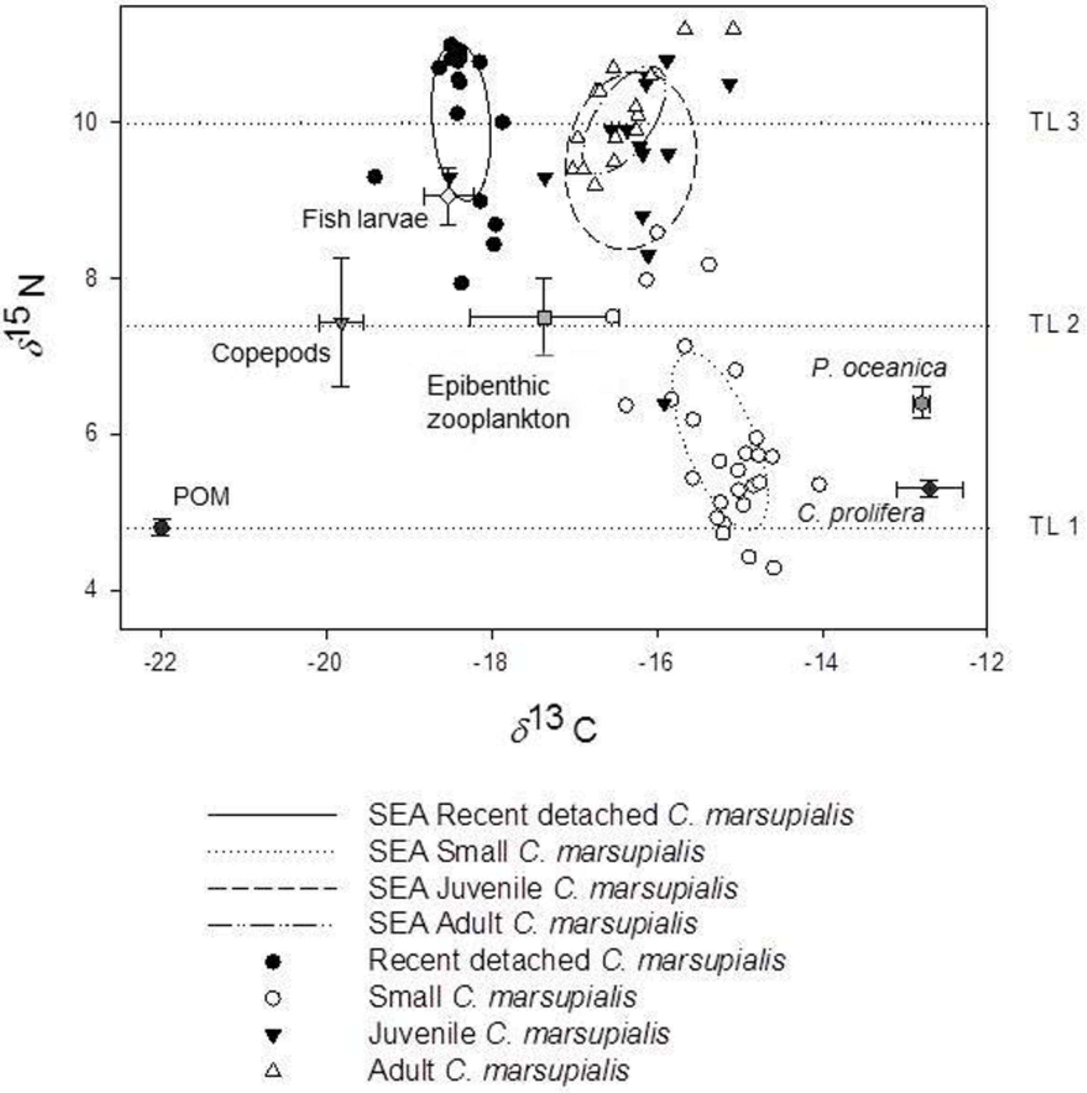

Fig. 4 Biplot and Isotopic Niche Breath (SEA: Standard Ellipse Area) of *C. marsupialis* stages. Horizontal dotted lines correspond to Trophic Levels (TL).

TL 3.2 ± 0.5 in adult individuals. Juvenile and adult cubomedusae had higher trophic position than co-occurring larval and juvenile fish. Moreover, the representation of the Standard Ellipse Area (SEA) (Fig. 4) showed different isotopic niche occupancy patterns of the developmental stages of *C. marsupialis*. Juvenile and adult individuals occupied the upper part of the plot and had a high degree of SEA overlap (36.2 %). Recently detached individuals presented a SEA located near the adults, whereas small individuals were placed in the lower part of the biplot.

The ontogenetic shift of isotopic ratios was also observed in the contributions of potential food sources to the species diet according to the SIAR analyses (Fig. 6 and supplement table SM 2). The mixing model (including copepods, epibenthic zooplankton and fish larvae) found feasible results for juveniles and adults, but not for recent detached and small specimens. The best adjustment was obtained when using the estimated TEF. Using TEFs extracted from literature (Post 2002; McCutchan et al. 2003; Javidpour et al. 2016) cubomedusae were placed out of the mixing polygon bounded by all sources, resulting in more diffuse solutions (SM 1 and supplement table SM 2). The major contribution to the assimilated diet of juveniles and adults was from epibenthic zooplankton (~50 and 42 % respectively), followed by copepods (~40 %), and fish larvae (~14 % for juveniles, and ~20 % in the case of adults).

Competition and predation impact

The PSI calculations suggest 85.4 % dietary overlap between *C. marsupialis* and juvenile fish diets. Both groups show a preference for epibenthic fauna (i.e. copepods, gammarids, isopods, mysidaceans, polychaetes and cumaceans) (Supplement table SM 3).

Further, the gut content/digestion time method was used to estimate cubomedusae predation impact. Digestion times varied between a minimum of 1.3 h for copepods and 3.75 h for fish larvae. Once a cubomedusa entangled a large prey that filled the stomach (e.g. a juvenile fish), it could not ingest another prey until the digestion of the first was completed (~3-4 h). Because of that, calculations of potential predation impact were based on the assumption that the individuals spent 8 h for feeding during day and 8 h during night (instead of 12:12). The predation impact estimated for copepods, mysids, amphipods and fish larvae differed for the diurnal and nocturnal samplings (Table 1). In general, impacts were higher at night because of the higher cubomedusa densities. In the case of copepods and mysids, the percentage of prey standing stock consumed per hour were of the same order of magnitude in the two periods (i.e. copepods: day 1.9 % h^{-1}, night 2.6 % h^{-1}; mysids: day 0.19 % h^{-1}, night 0.27 %). In contrast, nocturnal impact calculated for amphipods (25.8 % h^{-1}) was 1 order of magnitude higher than the diurnal one (2.3 % h^{-1}). The impacts on the larval fish stock were estimated to be null both during the day and during the night.

Discussion

Dietary composition from gut contents analysis

We described the first information on the feeding behaviour and potential trophic impact of the Mediterranean cubozoan *Carybdea marsupialis*, a species increasingly reported in this sea during the last decade (Bordehore et al. 2011; Gueroun et al. 2015). Crustaceans, such as copepods, gammarids, mysids and isopods were the main prey; polychaetes and fish larvae were less frequently observed in the guts. Similar results have been published for other carybdeid species (Larson 1976; Lai 2010). Box jellyfish produce strong digestive enzymes that digest most prey within a few hours (Larson 1976). Therefore, the time of collection influences the gut contents observed, especially considering that digestion times differ among prey types. Our data reveal a similar proportion of empty stomachs in diurnal and nocturnal captures. We discount the possibility of gut evacuation during sampling, because we towed hand nets at low velocity in order to avoid gut content loss inherent with collections made by more aggressive samplings (e.g. Barz and Hirche 2005). The higher densities of adult *C. marsupialis* observed at night may indicate concentration in shallow waters related with the nocturnal feeding, as has been reported for other cubomedusae (Arneson and Cutress 1976; Larson 1976). Underestimation of *C. marsupialis* density due to net avoidance during diurnal samplings may be unfeasible, since the swimming velocity of cubomedusae was observed to be insufficient to escape from the sampling nets.

Abundance and composition of prey in the guts and in the water column differed between day and night. Both the diet and the mesozooplankton available were more diverse at night, probably because some animals were emerging from the seabed. In the study area, this vertical displacement occurs over seagrass (*P. oceanica*) meadows and also unvegetated soft bottoms (Sánchez-Jerez et al. 1999; Fernandez-Gonzalez et al. 2014). In ecosystems where prey availability changes over the diel cycle, surveys of feeding should include night-time sampling. Sullivan (2014) found that night-emergent gammaridean amphipods contribute a substantial proportion of the daily carbon ration of the ctenophore *Mnemiopsis leidyi*. Pitt et al. (2008) provided evidence that *Catostylus mosaicus* derives most of its nutrition from night-emergent zooplankton, including *Lucifer* sp. and mysids. Furthermore, our results from stable isotope analysis demonstrated that epibenthic zooplankton were an important food source for juvenile and adult cubomedusae. While cubomedusae fed during both day and night, they derived more carbon at night because the emergent epibenthic zooplankton species were larger. Even though fish were not numerically abundant in the diet, they are probably important due to their high biomass, as Larson (1976) observed for *Carybdea* sp. from Puerto Rico. He also pointed out that polychaetes are probably a seasonal food, especially when they swarm during their reproductive period. We infrequently observed some large nereid polychaetes in gut contents. Seasonal changes in polychaete abundance have been reported in *C. prolifera* mats when compared to the seasonality observed in *P. oceanica* meadows (Box 2008). Maximum abundances of polychaetes in nearby habitats dominated by *C. prolifera* (Balearic Islands, W Mediterranean) were recorded in October (Box 2008), which is the reproductive season of cubomedusae in Denia (Bordehore et al. 2015). Consequently, the progressive decline of *P. oceanica* and its replacement by *C. prolifera*, which also produces changes in the crustacean and polychaete communities (Box 2008), might favour *C. marsupialis* proliferation. This substitution has also been observed in other areas in the NW Mediterranean basin, resulting from various

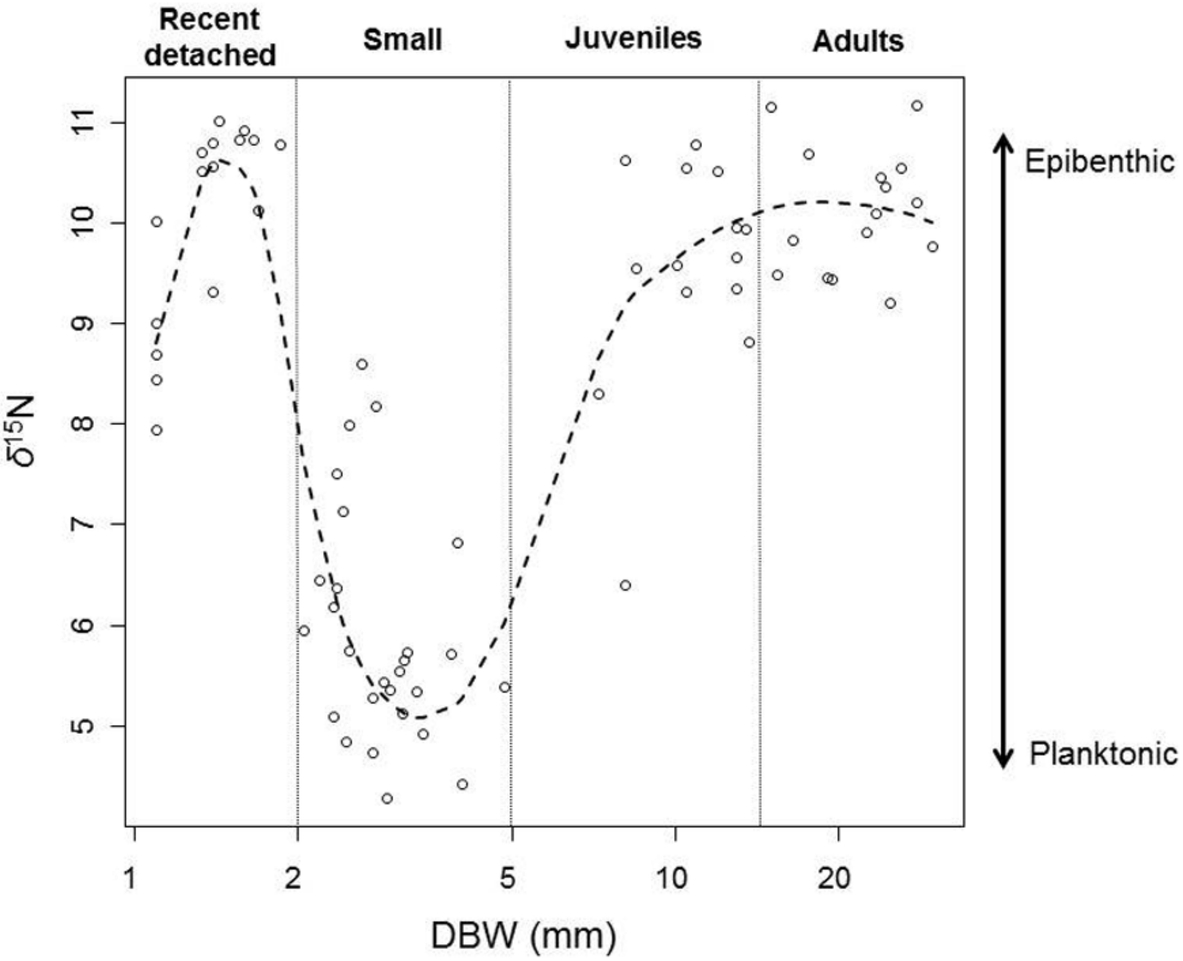

Fig. 5 Ontogenetic variation of $\delta^{15}N$. The dashed line corresponds to the smoothline (span = 0.5) adjusted to the scatterplot of the data (using the statistical platform R).

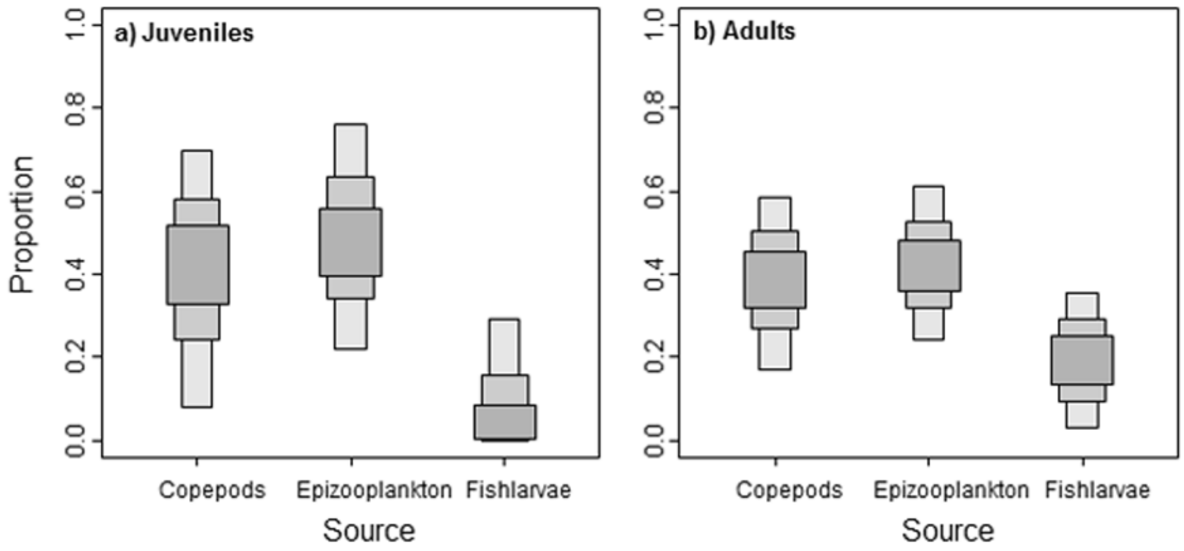

Fig. 6 Contribution of different sources to the diets of *C. marsupialis* at different developmental stages: Juveniles (a); Adults (b). A mathematical correction was applied before the development of the mixing model when the carbon-to-nitrogen (C: N) ratio was > 3.5, as established by Post et al. (2007).

environmental stresses including coastal eutrophication (Montefalcone et al. 2010).

Ontogenetic shift in diet and trophic role

Because we sampled in late summer-early autumn, the *C. marsupialis* collected for gut content analysis in 2009 and 2011 belonged to juvenile and adult stages. However, thousands of recently detached and small cubomedusae were collected by the overall LIFE CUBOMED project. Very few records of small medusae containing copepods, cladocerans or appendicularians have been compiled through 5 years of that sampling (i.e. 2010-2015) (Acevedo et al. unpubl data). A factor affecting the frequency of empty stomachs in the smaller specimens (< 5mm DBW), could be intraspecific competition for food because of their high abundance compared to larger ones. We suppose that cod-end feeding (e.g. Larson 1987) would be null or minimal because the samples were immediately preserved after the short tows. Moreover, microscopic food items (e.g. microzooplankton and POM) may have passed our stereomicroscope examination unnoticed, giving the impression of empty stomachs. For instance, other gelatinous organisms are nourished by microzooplankton (Omori et al. 1995; Malej et al. 2007) and POM (Båmstedt et al. 2001), especially during their initial developmental stages (Sullivan 2007 for *Mnemiopsis*).

We observed a progressive, ontogenetic shift in the diet, similar to previous laboratory observations (Acevedo et al. 2013). Crustaceans are eaten by all sizes of *C. marsupialis*, but younger stages could be more dependent on them. Indeed, *C. marsupialis* between 2 and 15 mm DBW fed on cladocerans and copepods in the laboratory (Acevedo et al. 2013). Ontogenetic feeding shifts have also been documented for the carybdeids *Carukia barnesi* (Underwood and Seymour 2007) and *Carybdea rastonii* (Lai 2010), showing that young cubomedusae initially prey on planktonic crustaceans, whereas larger and more mature individuals prey on fish. These changes in prey selectivity and diet composition could be linked to size related changes in bell shape, swimming and turning kinematics (Colin et al. 2013), and increasing ability to capture more motile prey, including age-specific changes in the cnidomes of some cubomedusae (Carrette et al. 2002). For example, adult *C. barnesi* extend and “twitch” the tentacles frequently to target larval fish (Courtney et al. 2015). The phototrophic behaviour of *C. marsupialis* as observed in other box jellyfish species (Buskey 2003), together with the swimming orientation and speeds of adult individuals, might enable them to prey on faster and larger prey. Ontogenetic variations in diet composition and prey selectivity have direct implications for growth rate and reproductive output in medusae (Graham and Kroutil 2001).

Using stable isotope analysis, we obtained a highly significant influence of cubomedusae size in the isotopic signal (δ^{15}N). The SEA showed differences in the trophic niche breath among the developmental stages. Although the SEAs of juveniles and adults overlap in some degree, the smaller area of adult individuals may reflect a more specialized feeding behaviour. In the case of the high isotopic values of recently detached *C. marsupialis*, these could be explained by the influence of polyp feeding behaviour; they have been hypothesized to be carnivorous suspension feeders, as other cubopolyps (Canepa et al. 2013; Toshino et al. 2014). It takes time to stabilize an individual's isotopic ratio and reflect the values of the new diet acquired from the metamorphosis. Recently, D'Ambra et al (2014) determined a period of 18–20 days for adult *Aurelia* spp. to reach an isotopic steady state on a laboratory diet. Turnover rates specifically for *C. marsupialis* are unknown. But, some delay is expected between dietary change and adjustment of isotopic values, which might be shorter in smaller individuals with faster growth rates (Fleming et al. 2015).

Scope and limitations of isotope mixing models

The ontogenetic shift was also observed in the contributions of potential sources to the species diet, which was dependent on the life stage. But, outcomes of isotopic mixing models should be interpreted with caution due to the potential sources of variation in discrimination factors and other limitations (Caut et al. 2008; Layman et al. 2012). Preservation and processing might modify the stable isotope composition of the samples. Fleming et al. (2011) concluded that freezing enriched δ^{15}N values in *A. aurita* by 2 ‰. Furthermore, oven-drying the samples at 60 °C may also enrich the δ^{15}N isotopic value compared to freeze-dried samples, which might lead to an incorrect estimation of the trophic position (Kogovsek et al. 2014). In the present study the preservation was unavoidable due to need of transportation of the samples from the sampling point to the laboratory. Despite we did not evaluate the possible effects of the processing methods, we used the same procedure for all the samples. Therefore, the possible change in δ^{15}N may be uniform across all samples. This may enable the relative comparison, whereas it would not be possible to combine and compare them with samples processed with different protocols. Uniform protocols would be needed for that (Fleming et al. 2011; Kogovsek et al. 2014).

Cubomedusae isotopic values did not differ among different years. Therefore, the differences observed among cubomedusae from different stages might reflect changes in the diet rather than shifts in the baseline. Vander Zanden and Rasmunssen (2001) suggested that using primary consumers (i.e. $\lambda = 2$) like copepods as a baseline might reduce the error in the estimation of TL. Realistic estimates of TL require accurate TEFs, and these remain problematic in the case of jellyfish (Fleming et al. 2015). Also, the isotope mixing models are highly sensitive to the discrimination factors used in model construction (Bond and Diamond 2011). Species-specific discrimination factors are not available, and the use of TEFs published for other species may not be adequate, because these vary among species, among tissues, and also among diets (Martínez del Río et al. 2009; McCutchan et al. 2003). It would be desirable to perform experiments for estimation of discrimination factors, but it is often unfeasible, and published values are frequently applied. In this case, it is wise to rely on a range of TEF values for related species fed on similar diets and measured on the same tissues, and conduct a sensitivity analysis to examine the effect of variation in TEF (Martínez del Río et al.

2009). From the published literature, we selected three different discrimination factors (and their standard deviation as a measure of uncertainty), and we also estimated a TEF from part of our data. Depending on the TEF used, our estimates of the diet composition were significantly different. We found that the best fit was obtained from the discrimination factor estimated as the difference in delta values between a food resource and a consumer.

Overall, mixing models are based on restrictive assumptions and provide estimates rather than exact values of contributions to consumer diet (Phillips and Gregg 2003; Caut et al. 2008; Martínez del Río et al. 2009). These provide information on assimilated foods (not just ingested foods), as well as time-integrated information, complementary to conventional methods (Caut et al. 2008). Concretely, the mixing model enabled to distinguish the contribution of the sources for juvenile and adult cubomedusae. However, this was not the case for the recent detached and small stages, probably because important food resources were missing for these stages (e.g. cladocerans or appendicularians as observed in laboratory experiments, unpubl data). As *C. marsupialis* continued its development, we observed a better adjustment of the model, which emphasized the ontogenetic shift in the diet observed from the gut content studies and feeding experiments.

In summary, understanding and estimating a consumer diet with stable isotopes is still complex, but combining these with other methodologies promotes better understanding of the feeding ecology of organisms (Shiffman et al. 2012). For instance, the results obtained from isotopes consistently agree and complemented the observations registered from gut contents analysis.

Resource competition with juvenile fish and potential predation impact

Percent similarity indices show high overlap between dietary compositions of adult cubomedusae and juvenile fish inhabiting seagrass meadows (i.e. Labridae, Pomacentridae, Scorpaenidae and Serranidae, according to Zupo and Stübing 2010). Both groups prefer epibenthic zooplankton and feed on crustaceans. However, to demonstrate competition we would require evidence of co-occurrence and resource limitation.

We used the gut content/digestion time method to estimate the predator impact of *C. marsupialis* on different prey. The field-based gut content method is supposed to minimize artefacts that are difficult to avoid in laboratory experiments (Purcell 1997). Generally, digestion times range from 2 to 4 h for a variety of pelagic cnidarian species feeding on small crustaceans at various temperatures (Purcell 2009). We recorded digestion times of ~1.3 h when *C. marsupialis* were fed with copepods, mysis and amphipods at 23-24 ºC. The digestion of a 20 mm fish larva took 3-4 hours, as Larson (1976) reported for carybdeid medusae from Puerto Rico. Because of this, the potential predation impact of *C. marsupialis* varied depending on the type of prey considered, as well as the time of the day. According to Purcell (1997), predation impacts of < 10 % of prey standing stock consumed d^{-1} should cause insignificant prey population declines; however, predation removing > 20 % d^{-1} might cause reduction of zooplankton standing stocks. Potential predation by *C. marsupialis* was > 20 % d^{-1} over copepods and amphipods, which are also the main prey of many adult and larval fish. The evidence that these prey populations are significantly impacted by predation suggests competition between cubomedusae and postlarval-juvenile fish in the studied area. However, the density of amphipods may be underestimated due to the sampling method, since the density of these organisms may be greater on the leaf substratum, and lower in the water column (Sánched-Jerez et al. 1999). This would attenuate the effect of predation, resulting less intense than estimated. Furthermore, our results did not reveal predation impact on fish. But this seems to be a consequence of the sampling methodology, as fish larvae would escape from sampling nets during day. Overall, the influence of box jellyfish on the food web may be relevant in environments where they appear frequently and in great densities, as can occur in semi-enclosed coastal waters. According to our data and all the collected records along the Spanish Mediterranean coast, aggregations of *C. marsupialis* are usually found along shallow waters at small (i.e. few km) spatial scales (unpubl data). Therefore, the predation impact of this species might be patchy and intense, and remains unclear if this would be important enough to regulate plankton populations over larger spatial scales. Our calculations were based on mean density values. But, the estimated impact would be much higher if maximum cubomedusae densities recorded by Canepa (2014) at the coast of Denia (i.e., 6.6 juvenile individuals m^{-3} and 5.2 adult individuals m^{-3}) are considered.

Alterations in coastal pelagic food webs derived from nutrient enrichment might also influence trophic and competitive relationships, and thus the structure of marine food chains. Freshwater runoff could increase light attenuation directly (i.e. increase in dissolved and particulate organic matter) and by enhancing phytoplankton production due to N, P and Si discharge, as seen in the study area by Alventosa (2015). Coastal waters with high light attenuation may provide a regime favouring tactile planktivores (Eiane et al. 1997). Therefore, jellyfish as *C. marsupialis* may be favoured in this highly impacted ecosystem with low visibility. Furthermore, an increase in some excavator species inhabiting the sea grass *P. oceanica* and sea weed *C. prolifera* meadows due to nutrient enrichment and eutrophication (Box 2008) might also favour jellyfish more than fish. Since the feeding activity of fish is predominantly during daytime (Sánchez-Jerez et al. 1999), the nocturnally emergent species (e.g. decapods, amphipods, mysids, isopods and polychaetes) could be a valuable prey source for cubomedusae but scarcely exploited by fish larvae and juveniles.

Summary

This research contributes to the understanding of the trophic role of *C. marsupialis* from both bottom-up and top-down points of view. From a bottom-up perspective, we have determined the main prey of this box jellyfish, which may nourish and drive increases in the survival and reproduction of the cubomedusa population. From a top-down perspective, we demonstrated the potential competitive impacts of this cubomedusan, which occupies a similar trophic level as fish in *P. oceanica* meadows. Regarding the potential predation impact of the species, this can be intense, particularly during its patchy population outbreaks, but it remains unclear whether this would be important enough to regulate prey stocks over large spatial scales.

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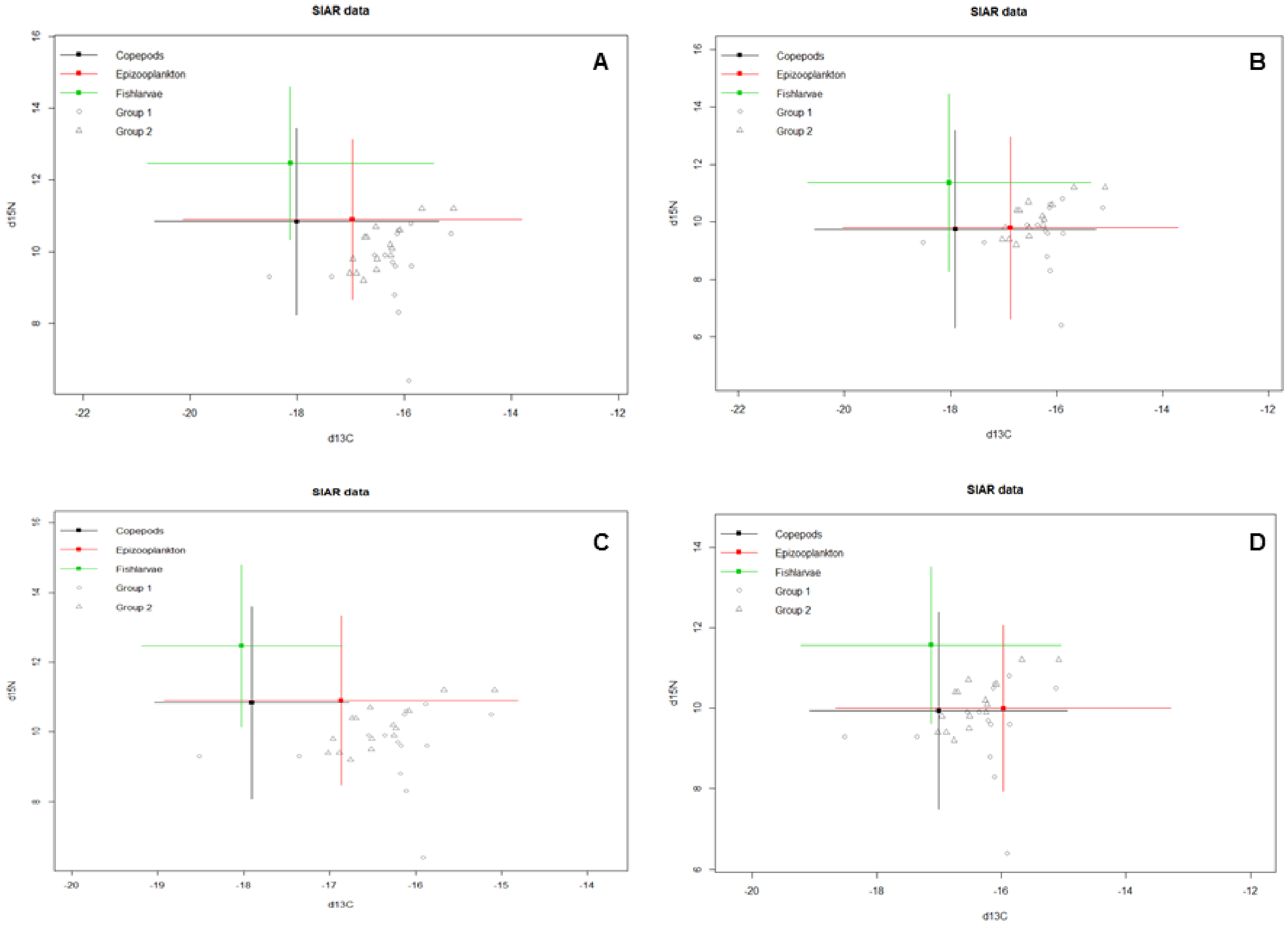
Supplementary material



SM 1 Graphical outputs of SIAR Mixing models using different TEFs: Post et al. 2002 (A); McCutchan et al. 2003 (B); Javidpour et al. 2016 (C) and the TEF estimated in this study (D).

TEF	**Contribution of prey sources (%)**					
	Copepods		**Epibenthic zooplankton**		**Fish larvae**	
	J	**A**	**J**	**A**	**J**	**A**
Post (2002) $\Delta\delta^{13}C = 0.4 \pm 1.3$ ‰ $\Delta\delta^{15}N = 3.4 \pm 1.0$ ‰	0 – 48 %	0.9 – 50 %	44 – 97 %	45 – 94 %	0 – 20 %	0 – 13 %
McCutchan et al. (2003) $\Delta\delta^{13}C = 0.5 \pm 1.3$ ‰ $\Delta\delta^{15}N = 2.3 \pm 1.5$ ‰	0 – 48 %	1.4 – 45 %	40 – 92 %	32 – 76 %	0 – 24 %	1.5 – 29 %
Javidpour et al. (2016) $\Delta\delta^{13}C = 0.5 \pm 0.5$ ‰ $\Delta\delta^{15}N = 3.4 \pm 1.1$ ‰	0 – 40 %	0 – 32 %	52 – 100 %	64 – 99 %	0 – 16 %	0 – 12 %
This study $\Delta\delta^{13}C = 1.4 \pm 0.9$ ‰ $\Delta\delta^{15}N = 2.5 \pm 0.8$ ‰	8.2 – 70 %	17 – 59 %	22 – 76 %	24 – 61 %	0 – 29 %	3 – 35 %

SM 2 Summary Output of the Mixing Model. Contribution (%) of different sources to the diet of *C. marsupialis* at different developmental stages: Juveniles 5< DBW ≤ 15 mm (J) and Adults > 15 mm DBW (A). (95 % adjustment).

Prey item	***C. marsupialis*** **% Abundance**	**Fish population % Abundance**[a]
Copepods	15.0	12.9
Gammarid amphipods	12.9	10.1
Isopods	2.9	4.3
Mysidaceans	22.1	2.3
Polychaetes	2.4	1.5
Cumaceans	2.5	0.3

SM 3 Comparison of *C. marsupialis* and postlarval-juvenile fish diets. [a] % of gut contents (Zupo and Stübing 2010).

CHAPTER 4

Influence of human impacts on and forecasting of the occurrence of the box jellyfish *Carybdea marsupialis* in the Mediterranean coasts

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Abstract

We investigated the possible causes producing the overabundance of the box jellyfish *Carybdea marsupialis* in the NW Mediterranean, with the aim of forecasting the probability of its occurrence along the basin. We based our study on monthly-recorded environmental data and cubomedusae density along the coast of Denia (Spain) from March 2010 to December 2013. Environmental variables included water temperature, salinity, inorganic nutrient concentrations (i.e. nitrate, nitrite, ammonium, phosphate and silicon), chlorophyll a concentration and zooplankton abundance and composition. In addition, the Land Use Simplified Index (LUSI) has been calculated and used as a descriptive variable of human influence; this index reflects the impact of nutrient inputs along the coast. Generalized Linear Models and Generalized Additive Models have been computed to understand the relationships between aforementioned environmental variables and *C. marsupialis* abundance. LUSI, nitrate, phosphate, chlorophyll a, copepod and cladoceran concentration showed a positive relation with the presence of this box jellyfish. Moreover, an ensemble platform in the form of a species distribution model (Biomod2 package for R software) has been fitted using the sightings of the species recorded through different citizen science databases from the Mediterranean region (ENPI-Medjellyrisk and LIFE+ Cubomed projects). A forecasting facility for the probability of occurrence of this species under prevailing environmental conditions was developed, and the results indicated that *C. marsupialis* inhabits coastal areas characterized by a high degree of human disturbance. This and other previous studies relating box jellyfish blooms with the intensity of human coastal activities need to be considered during the development of cubomedusae population management protocols.

Keywords: cubozoa, zooplankton, seasonality, population structure, cohort analysis, species distribution model, environmental status, citizen science.

Introduction

The abundance of the cubozoan *Carybdea marsupialis* has increased in some locations along Mediterranean coastlines in the last decade (Di Camillo et al. 2006; Bordehore et al. 2011; Boero 2013; Gueroun et al. 2015). One of the problems associated with these episodes is that this stinging species may represent a threat for human health (Peca et al. 1997; Bordehore et al. 2011; Bordehore et al. 2015b). This box jellyfish also may interact with other zooplanktivorous organisms, such as fish larvae, through competition and direct predation (Acevedo et al. submitted). Consequently, the factors influencing the abundance and distribution of this box jellyfish have been recently investigated (Canepa 2014; Bordehore et al. 2015a). Major conclusions of such studies pointed towards sea surface temperature, salinity, and phytoplankton abundance (measured as Chl-a concentration) as the main environmental drivers of *C. marsupialis* microscale distribution and seasonality (Canepa 2014). Certainly, the life cycle of Cubozoans seems to be in general controlled by the interaction of diverse factors, such as temperature, salinity, photoperiod, and abundance of prey (Gordon and Seymour 2012; Canepa et al. 2013; Kingsford and Mooney 2014). Cubozoan development, as in other cnidarians, alternates between an asexually reproducing polyp phase and a sexually reproducing medusa phase. Experimentation in the laboratory has shown that some physical factors (i.e. temperature, salinity and food supply) can trigger the metamorphosis of the polyps and, therefore, influence cubomedusae abundance and proliferation events (Straehler-Pohl and Jarms 2005; Gordon and Seymour 2012; Kingsford et al. 2012; Canepa et al. 2013). It has also been hypothesized that blooms of *C. marsupialis* in the Mediterranean might also be linked with the proliferation of artificial constructions and breakwaters (Bordehore et al. 2011; Boero 2013). The addition of hard substrates in coastal zones may be a key driver of jellyfish blooms, as they may provide a suitable habitat for the benthic polyp stage (Boero et al. 2008; Duarte et al. 2012). However, association of *C. marsupialis* polyps with these artificial structures has never been observed and corroborated.

Other factors, less addressed, could also be related to the outbreaks of *C. marsupialis* in the Mediterranean. For instance, the flow of nutrients into coastal waters from run-off might influence the abundance and distribution of box jellyfish as well (Canepa 2014; Bordehore et al. 2015a). Hence, anthropogenic nutrient inflow can lead to undesirable effects associated with eutrophication, as alterations in the food webs (Sebastiá and Rodilla 2013). This increase in nutrients generally leads to a greater biomass in all trophic levels, leading to extra food supply to polyps and to jellyfish, and thus to an augmented growth rate (Ishii and Bamstedt 1998; Chiaverano et al. 2013), as well as modified sexual (Lucas 1996; Lucas and Lawes 1998) and asexual reproduction rates (Straehler-Pohl and Jarms 2005; Han and Uye 2010; Purcell et al. 2012). Moreover, eutrophication is considered to be one important factor contributing to increased jellyfish proliferation in some areas (Mills 2001; Purcell et al. 2007; Richardson et al. 2009; Dong et al. 2010). Changes in the marine nutrient concentration as a direct consenquence of anthropogenic activities are currently taking place in the Mediterranean (Gadea 2016). Therefore, we hypothesized that nutrient loads could be also a key factor shaping the distribution of *C. marsupialis* in the Mediterranean, in combination with other environmental variables.

In this study, we progress from the microscale distribution level of the species towards a macroscale forecasting level. We firstly investigated the factors responsible for the proliferation of the cubomedusa *C. marsupialis* in a particular location of the NW Mediterranean coast (Denia, Spain). We used environmental data for temperature, salinity, nutrients concentration and chlorophyll concentration. Since the causative connection between chlorophyll a (Chl-a) concentration and cubomedusae abundance has not been previously clarified, we also included data on zooplankton abundance and composition. All these variables were used to explain the presence, seasonal and interannual variations in *C. marsupialis* numbers over a period of four years (2010-2013), which is the longest data set for this species recorded to date. Once the most important factors operating at the microscale were determined, we built a Species Distribution model (SDM) for the probability of its occurrence along the Mediterranean coast. For that purpose, we combined together sightings of *C. marsupialis* recorded in different databases, and those were associated with the corresponding environmental variables. The impact of nutrient inputs as a result of anthropogenic influence was considered as one of the most important drivers of the increased presence of box jellyfish in the area. Finally, we make some coastal management recommendations with the aim of reducing the abundance of this box jellyfish in Mediterranean coastal waters.

Materials and methods

Study area

Denia is located in the southern area of the Gulf of Valencia (0º 2' 47.7'' E; 38º 50' 37.0'' N, Western Mediterranean, Spain) (Fig. 1). The area has a Mediterranean climate, with a dry period in summer (June-August) and two rainy seasons, spring (March-May) and autumn (September-November) [16]. The study area is located in coastal waters in close proximity to the aquifer of Gandia-Denia. The hydrology of this coastal area has been modified for agriculture purposes (Gadea 2016). The addition of artificial fertilisers to crops (mainly citrus fruits and vegetables)

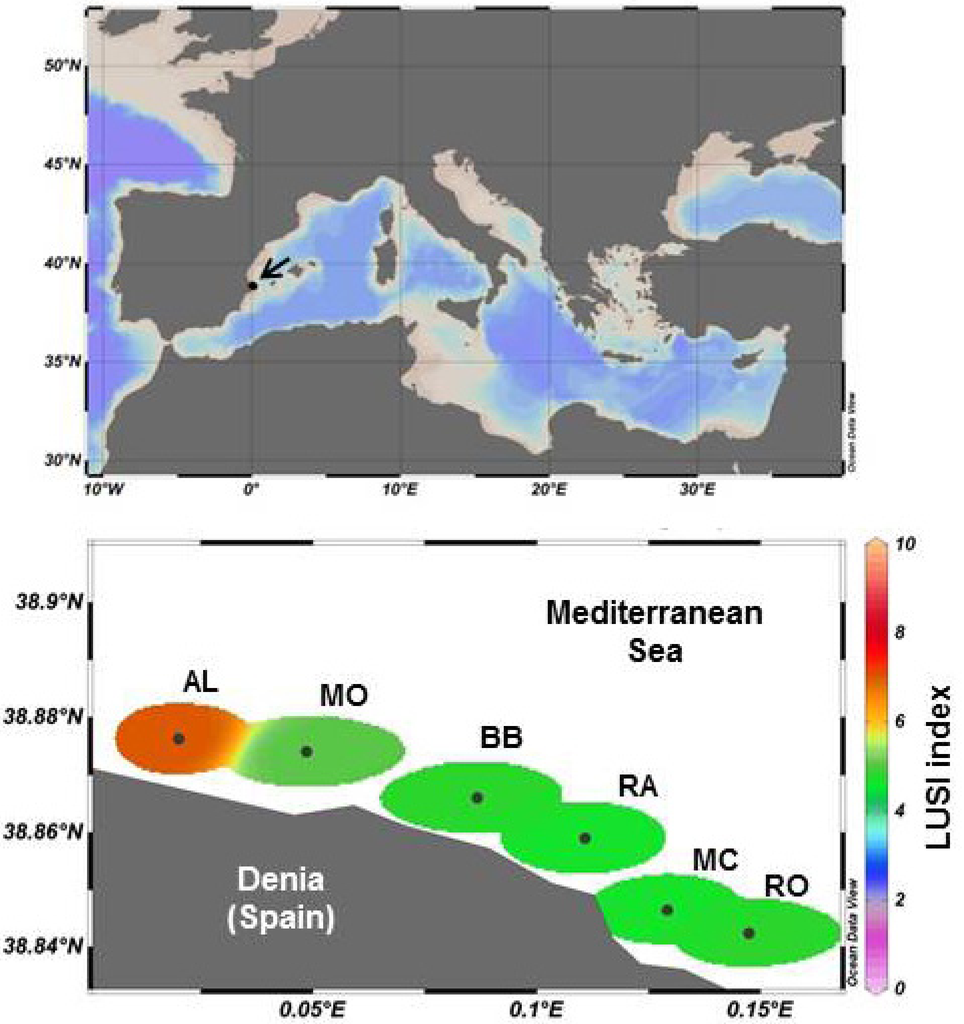

Fig. 1 Study area and Land Use Simplified Index (LUSI) along the coast of Denia. Stations: Almadrava (AL), Molins (MO), Blay Beach (BB), Raset (RA), Marineta Casiana (MC), Rotes (RO). Low LUSI value indicate the absence or only the slight influence of continental pressures on a water body; a high LUSI value indicate a strong influence of continental pressures.

is mainly conducted in late winter and early spring (Gadea 2016). Three rivers discharge in the coastal waters off Denia: the Racons River, Almadrava River and Alberca River. Racons River receives the water discharged from the wastewater treatment plant (WWTP) servicing Pego and El Verger Norte (Bordehore 2014). The Almadrava River receives the residual waters from WWTP El Verger-Els Poblets (Ballesteros et al. 2009). There is also another discharge point, from the WWTP Denia-Ondara-Pedreguer, via a marine outfall located 1300 meters off the Raset beach (Bordehore 2014). In addition, several breakwaters can be found along the coastline, and the harbour of the city has been extended on many occasions. Concretely, three stone breakwaters were constructed at l'Almadrava beach in 2005 (Bordehore et al. 2011). Moreover, tourism is one of the main economic activities in Denia. In summary, the Gulf of Valencia has suffered a progressive increase in the eutrophication pressure due to the increasing agricultural and demographic development of the area (Gadea 2016).

Sample collection

Environmental variables and zooplankton abundance were monitored in the coastal area of Denia from March 2010 to December 2013. Permits and approvals to conduct the field studies were obtained from the Spanish Ministry of Economy and Competitiveness and from the Generalitat Valenciana. Samplings were conducted with a monthly frequency from 6 stations along ~12 km of coast, from North to South: Almadrava, Molins, Blay Beach, Raset, Marineta Casiana and Rotes (Fig. 1). At each station, physical factors (temperature and salinity) were measured with a CTD (COMPACT CTD-Lite de JFE Advantech). Five litres of superficial seawater were collected for *ex situ* analysis of nutrients, particulate suspended material (PSM) and total Chl-a concentration. Zooplankton samples were collected through superficial horizontal net tows (250 µm mesh pore size) conducted for 10 minutes at ~1.5 knots. A flowmeter (KC Digital Flow Meter) was placed at the mouth of the net to quantify the seawater volume filtered. Cubomedusae were initially sampled every two to three weeks all year round from June 2010 to July 2011, and once the duration of the medusa season was determined, the sampling was only conducted from May to November in 2012 and 2013. Individuals were sampled using hand nets (200 µm, 500 µm and 4 mm mesh size), and the volume filtered was estimated by using flowmeters (General Oceanics, 2030R standard flow-meter). The sampled *C. marsupialis* individuals were preserved in 4 % formalin. In the laboratory, the Diagonal Bell Width (DBW, the distance between opposite pedalia) of each specimen was measured using callipers (Vernier, precision ± 0.05 mm). The sex of adult individuals was determined when mature gonads were present.

Sample processing

Immediately after the sampling, two 10-mL samples of seawater were frozen at -20 °C for nutrient analysis. This was later performed by the Nutrient Analysis Service at the Marine Science Institute (ICM-CSIC) with an AA3 (Bran + Luebbe) system (formerly known as Technicon). In the case of the PSM analysis, surface seawater was filtered through a pre-muffled and pre-weighed GF/F Whatman glass fibre filter (25 mm diameter), and afterwards preserved at -20 °C. At a later stage, PSM filters were dried at 60 °C for 24 hours and were finally weighed using a micro-balance (Mettler 143 Toledo MX5, precision 1 µg). The total PSM (mg L^{-1}) content (including both the organic and inorganic fraction) was calculated. Samples for Chl-a determination were filtered through a GF/F Whatman glass fibre filter (47 mm diameter) and preserved at -20 °C. The filters were analysed fluorometrically after 24 h acetone (90 %) extraction in darkness and in cold (4 °C) conditions. The reading was measured using a TURNER fluorometer at 670 nm. Chl-a measurements were always taken against a blank. Total Chl-a (µg L^{-1}) was calculated following Wasmund et al. (2006).

Zooplankton samples collected in 2010 and 2011 were analysed using digital image processing (Zooscan, http://www.zooscan.com) following the protocol described by Grosjean et al. (2004). In the case of the samples collected in 2012 and 2013, zooplankton organisms were identified under a stereomicroscope; subsamples were taken until at least 1000 zooplankton organisms per sample were counted and identified. Five groups were considered in the analysis: cladocerans, copepods, ichthyoplankton (i.e. fish eggs and larvae), mysids and decapod larvae. These were chosen due to their relevant contribution to the zooplankton community composition, and also because they have been demonstrated to be the main prey of *C. marsupialis* (Chapter 3, this thesis).

Population structure of *C. marsupialis*

Different *C. marsupialis* size classes were defined every mm, with class 1 containing individuals 0–1 mm Diagonal Bell Width (DBW). We differentiated between three additional developmental stages: small, with specimens having < 5 mm DBW; juveniles, having DBW values ranging between 5 and 15mm; and adults, characterised by > 15 mm DBW values. Size class histograms were constructed from monthly box jellyfish collections to determine if *C. marsupialis* has one or several recruitment episodes during the season. Potential cohorts were identified from the histograms applying the modal progression routine of FiSAT II (Gayanilo et al. 2005). This software decompose size-frequency distributions into several normal curves representing each cohort (Bhattacharya's method) and then applies an iterative process of the maximum likelihood concept (NORMSEP) to obtain parameter estimates, with distinct cohorts being distinguished when the separation index estimated for the modes was > 2 (Gayanilo et al. 2005; Milisenda et al. 2016).

Data analysis

Significant differences between annual environmental parameters values were determined using the non parametric Kruskal-Wallis test, and post-hoc pairwise multiple comparisions (Nemenyi test; library "stats" in R) were conducted to identify the year or years responsible for the significant differences. A set of Generalized Linear Models (GLM) and Generalized Additive Models (GAM) were applied to evaluate potential associations among variables, with cubomedusae density being applied as the response variable. GAM is a non-linear regression procedure that uses non-parametric smoothing functions to model the relationship among response and explicative variables (Zuur et al. 2009; Canepa 2014). The approach followed to select the optimal model was a forward selection. Among the fitted models, the one with the lowest Akaine Information Criterion (AIC) value was selected as the best model. The AIC value is a measure of goodness of the fit and complexity of the model (Zuur et al. 2009). Finally, model validation was conducted by the analysis of the residuals.

In the case of temperature and salinity, the values recorded at a water column depth ranging between 50 and 100 cm were used for the analysis. Indeed, the values between 50-60 cm depth represented the best adjustement for the Sea Surface Temperature (SST_11 Daytime) registered by MODIS satellite (Durà et al. 2014). Three sources of nitrogen were measured (NH_4^+, NO_3^-, NO_2^-; in µmol L^{-1}). These were afterwards also integrated in a single value Dissolved Inorganic Nitrogen (DIN). The nutrient ratios DIN:P, Si:P and Si:DIN were calculated to define the prevailing trophic conditions (Karydis 2009). In order to detect potential nutrient control, nutrient ratios involving DIN, P and Si concentrations were compared with Redfield ratios (DSi:DIN:DIP = 16:16:1). In additon, the average PSM content (µg L^{-1}), total Chl-a concentration (µg L^{-1}), and concentration (ind m^{-3}) of the different zooplankton groups, quantified at each sampling station and month, were added to the GLM and GAM analysis.

Moreover, we applied the coastal pressure index Land Use Simplified Index (LUSI) in order to evaluate the anthropogenic influence in the different stations (Flo et al. 2011; Camp et al. 2015), with the relevant index formula being the following:

LUSI = (score urban + score agricultural + score industrial + score typology) * correction number

LUSI index integrates the continental pressures that affect a water body, including information about urban, agricultural, industrial, and river influences. On the basis of these characteristics, a score is assigned to each pressure, and then all scores are summed up. A correction is applied to take into account the morphology of the coast that could accentuate or reduce the effects of the same pressures (Flo et al. 2011). A low LUSI value indicates the absence or only the slight influence of continental pressures on a water body; in contrast, a high LUSI value indicates a strong influence of continental pressures (Flo et al. 2011; Camp et al. 2015). LUSI values were calculated for the different beaches in the study area using the 2006 CORINE land cover map. An area demarcated by the limits of each beach and extending up to 1.5 km inland has been considered when assigning the category of each pressure to each water body (Flo et al. 2011). This index value was also added as an explicative variable to the GLM.

Species distribution model

We developed a species distribution model (SDM) based on the information obtained from the GLMs and GAMs models constructed for the coast of Denia. SDM models predicts potential distribution of a species along an area using both occurrence data and environmental information. We compiled *C. marsupialis* occurrence data from sightings recorded within different jellyfish sighting networks from the Mediterranean: the presence of jellyfish is being monitored along the Italian coast since 2008 through the initiative “Occhio alla medusa” (Spot the jellyfish; http://meteomeduse.focus.it) coordinated by the University of Salento and CNR-ISMAR; the Spot the Jellyfish citizen science campaign (www.ioikids.net/jellyfish) registers the presence of jellyfish in Maltese waters since 2010; also the LIFE CUBOMED (www.cubomed.eu) and ENPI-Medjellyrisk (www.jellyrisk.eu) projects have been recording sightings of *C. marsupialis* and other gelatinous organisms along the Mediterranean since 2010 and 2013 respectively. These platforms are populated by citizen observations, but a scientific validation process was conducted prior to acceding the same reports to the final list of sightings. The environmental data included in the model are raster files summarizing yearly averages of SST, salinity, Chl-a, nitrate and phosphate concentrations. These input parameters are available for download in the Bio-Oracle (Ocean rasters for Analysis of Climate and Environment) online database. Spatial resolution of griddle fields are 5 arcmin (9.2 km). We built a projection using different approaches: Generalized Linear Models (GLM), Generalized Additive Models (GAM), Multi Adaptative Regression Splines (MARS) and Random Forest (RF), after which we constructed an ensembled forecast (final predictive map) from the best fitted models (Georges and Thuiller 2013; Thuiller et al. 2014). Both presence and absence data are necessary to calibrate these models (Georges and Thuiller 2013; Thuiller et al. 2014); therefore it was necessary to add pseudo-absences to our data set (based on sightings, that are presence-only data). In order to extract a range of pseudo-absences, we applied a “random” strategy when formating the data (algorithm included in R package Biomod2). The TTS and ROC criteria were used for model evaluation. A similar approach has been used to study the distribution of other gelatinous species spreading within the Mediterranean, such as the ctenophore *Mnemipsis leidyi* (CIESM 2014).

Results

Environmental parameters

Water temperature values presented a seasonal pattern, ranging from 11.7 °C (winter) to 29.7 °C (summer) (Table 1 and Fig. 2). Significant differences in the annual mean temperature were

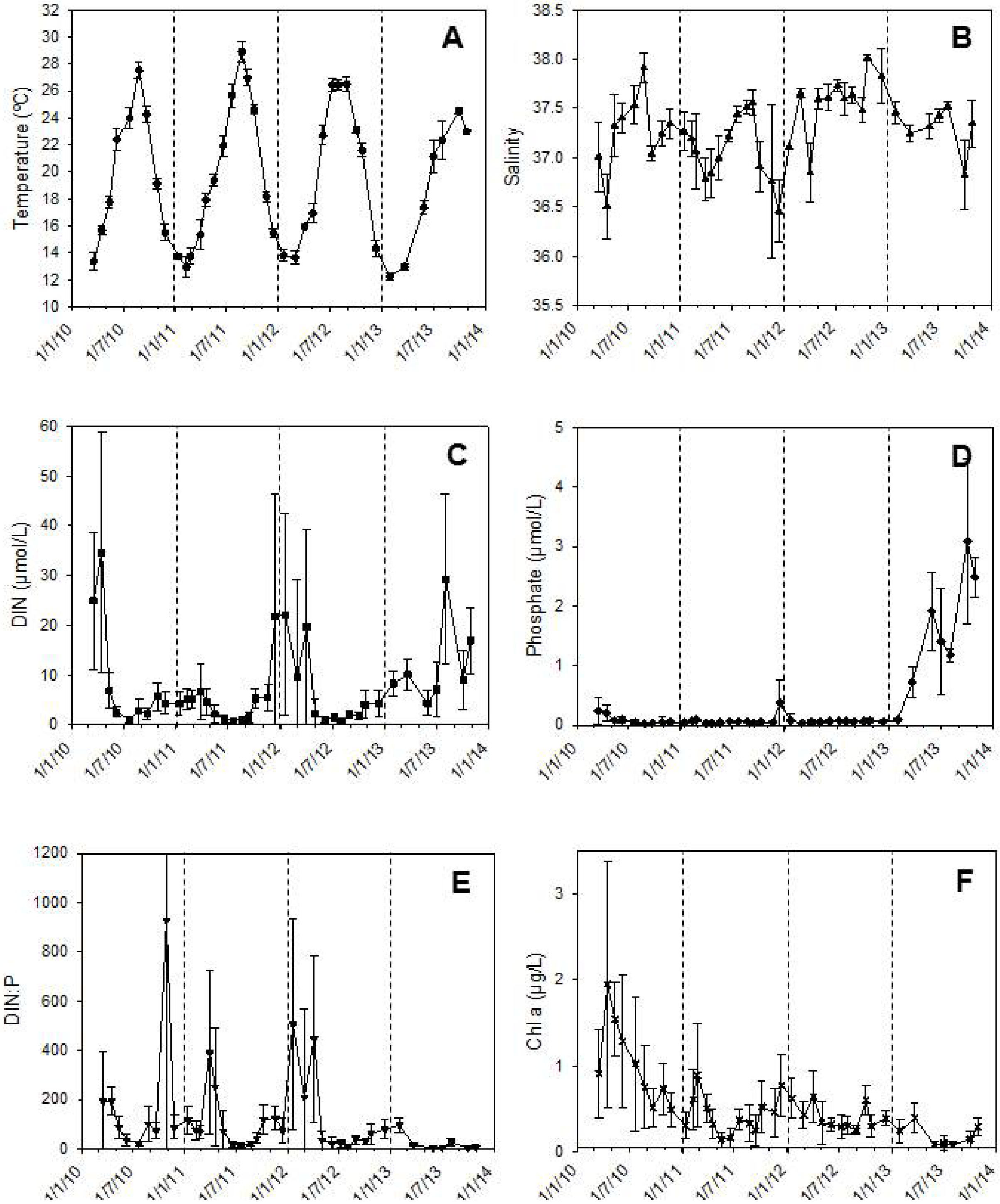

Fig. 2 Interannual variability of different environmental parameters registered in the coast of Denia. (A) Temperature (ºC); (B) Salinity; (C) Dissolved Inorganic Nitrogen concentration (µmol/L); (D) Phosphate concentration (µmol/L); (E) DIN:P proportion.; (F) Total Chlorophyll concentration (µg/L). Doted lines indicate separation between different years; x-axis, date indicated in D/M/Y.

Variables	Min.	Max.	Mean	Interannual variability	Chi-squared	p-value
Temperature (ºC)	11.70	29.69	19.74	Lower in 2013	2.63	< 0.001
Salinity	35.26	38.16	37.29	Higher in 2012	58.84	< 0.001
PSM (mg L^{-1})	2.86	38.35	18.87	Higher in 12/13	106.6	< 0.001
Ammonium (µmol L^{-1})	0.02	50.44	1.65	Higher in 2013	43.97	< 0.001
Nitrate (µmol L^{-1})	0.07	78.53	5.81	Lower in 2012	14.46	< 0.001
Nitrite (µmol L^{-1})	0.01	1.02	0.13	Lower in 2012	23.78	< 0.001
DIN (µmol L^{-1})	0.24	79.68	7.59	Higher in 2013	39.08	< 0.001
Phosphate (µmol L^{-1})	0.002	5.21	0.33	Higher in 2013	91.629	< 0.001
Silicon (µmol L^{-1})	0.01	38.11	1.80	Lower in 2013	71.02	< 0.001
DIN:P	0.72	3562.20	117.99	Lower in 2013	57.39	< 0.001
Si:DIN	0.002	4.05	0.58	Lower in 2013	90.33	< 0.001
Si:P	0.01	1456.20	42.61	Lower in 2013	78.11	< 0.001
Chla (µg m^{-3})	0.002	4.44	0.52	Decreasing trend	72.86	< 0.001
LUSI	3.75	7.5	5.21	Lower in 2012	61.35	< 0.001
Cladocerans (ind m^{-3})	0.00	2517.73	106.34	Lower 11/13	58.28	< 0.001
Copepods (ind m^{-3})	0.00	4954.08	323.82	Higher in 2012	28.26	< 0.001
Ictioplankton (ind m^{-3})	0.00	175.77	5.47	Lower in 2011	26.78	< 0.001
Mysids (ind m^{-3})	0.00	14.35	0.37	Higher in 2010	43.82	< 0.001
Decapod larvae (ind m^{-3})	0.00	245.23	4.05	Higher in 12/13	28.25	< 0.001
C. marsupialis (ind m^{-3})	0.00	6.44	0.17	Not significant	6.28	> 0.05

TABLE 1 Summary of environmetal variables. Min., max. and mean values, as well as the results from interannual comparison of the means (Kruskal-Wallis).

observed during the study, with lowest values recorded in 2013. A decrease (almost -2 ºC in the case of Marineta Casiana beach) in the summer mean temperature was recorded over the study period (2010-2013) (Chi-squared = 2.63; p-value < 0.001) (Table 1). Salinity reached higher values (max = 38.2) during the dry seasons (i.e. winter and summer) and lower values (min = 35.3) in the rainy seasons (i.e. spring and autumn) (Table 1 and Fig. 2). Significant differences were detected between the annual mean salinity values, which such a value being slightly higher in 2012, (Chi-squared < 0.001; p-value < 0.001) (Table 1).

The average DIN concentration in the study area was 7.59 µmol L^{-1} (ranging from 0.24 to 79.68 µmol L^{-1}) (Table 1). Significantly higher DIN values were observed in 2013 (Chi-squared = 39.08; p-value < 0.001). Nitrate was the most dominant N form; however, the interannual variation was mostly due to the significant differences in the ammonium concentrations, which were also higher in 2013 (Chi-squared = 43.97; p-value < 0.001). Interannual significant differences (Chi-squared = 71.02; p-value < 0.001) were also dectected in the case of Si concentration, which varied between 0.01 and 38.1 µmol L^{-1} (Table 1). The concentration of P ranged between 0.002 and 5.21 µmol L^{-1}, and was significantly higher in 2013 at all the sampling stations (Chi-squared = 91.63; p-value < 0.001) (Table 1 and Fig. 2).

Year	Station	Ammonium (µmol L^{-1})	Nitrate + Nitrite (µmol L^{-1})	Phosphate (µmol L^{-1})	Chl-a (µg L^{-1})
2010	AL	0.44 (H)	14.18 (M)	0.10 (H)	1.71 (P)
	MO	0.58 (H)	13.49 (M)	0.13 (H)	1.34 (P)
	BB	0.41 (H)	8.51 (G)	0.09 (H)	1.28 (P)
	RA	0.55 (H)	7.81 (G)	0.08 (H)	0.73 (P)
	MC	0.54 (H)	5.71 (G)	0.09 (H)	0.43 (P)
	RO	0.60 (H)	3.61 (H)	0.05 (H)	0.63 (P)
2011	AL	0.28 (H)	8.78 (G)	0.13 (H)	0.78 (P)
	MO	0.28 (H)	6.81 (G)	0.09 (H)	0.44 (M)
	BB	0.22 (H)	3.93 (H)	0.04 (H)	0.35 (G)
	RA	0.31 (H)	3.51 (H)	0.06 (H)	0.37 (G)
	MC	0.22 (H)	2.52 (H)	0.06 (H)	0.27 (G)
	RO	0.23 (H)	2.51 (H)	0.06 (H)	0.40 (G)
2012	AL	0.60 (H)	16.68 (M)	0.09 (H)	0.51 (M)
	MO	0.52 (H)	5.57 (G)	0.06 (H)	0.48 (M)
	BB	0.42 (H)	2.93 (H)	0.06 (H)	0.50 (M)
	RA	0.42 (H)	2.65 (H)	0.05 (H)	0.30 (G)
	MC	0.64 (H)	3.42 (H)	0.05 (H)	0.30 (G)
	RO	0.51 (H)	3.21 (H)	0.05 (H)	0.36 (G)
2013	AL	7.42 (P)	6.48 (G)	2.00 (P)	0.33 (G)
	MO	5.50 (P)	6.39 (G)	1.97 (P)	0.20 (G)
	BB	9.76 (B)	4.84 (H)	1.61 (P)	0.15 (G)
	RA	10.38 (B)	2.46 (H)	1.04 (M)	0.15 (G)
	MC	5.08 (P)	4.15 (H)	1.28 (M)	0.14 (G)
	RO	6.36 (P)	3.91 (H)	1.41 (P)	0.16 (G)

TABLE 2 Mean Annual values and Ecological quality classes according to the Water Framework Directive (WFD). Stations: Almadrava (AL), Molins (MO), Blay Beach (BB), Raset (RA), Marineta Casiana (MC), Rotes (RO). H, High; G, Good; M, Moderate; P, Poor; B, Bad.

This spike affected the nutrient coefficients DIN:P and Si:P, which decreased in 2013 (Chi-squared = 57.39; p-value < 0.001, and Chi-squared = 78.11; p-value < 0.001, respectively). DIN:P ratios ranged between 0.72 and 3562.2, with a mean value of 118 (Table 1), indicating a global N excess in the system from 2010 to 2012. This situation was reversed in 2013 due to an increase of P, when DIN:P decreased at all the stations, especially between Raset and Marineta Casiana.

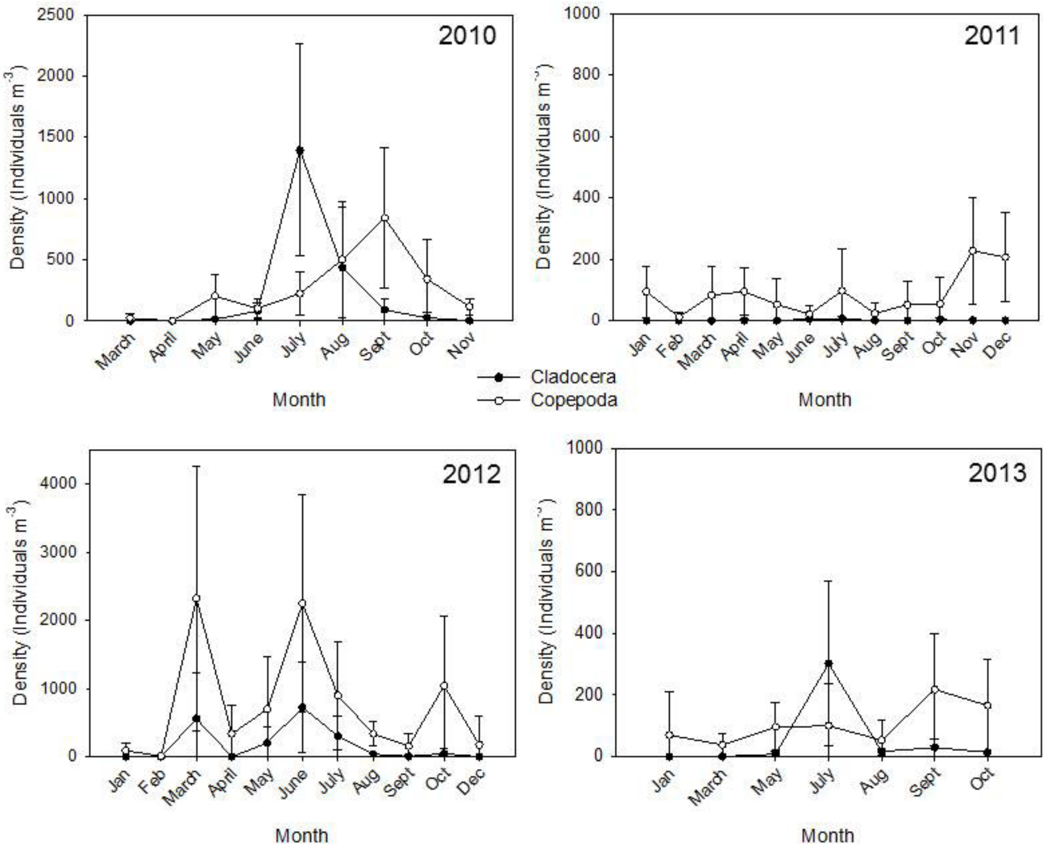

Fig. 3 Interannual comparison in zooplankton abundance (cladocerans and copepods). Each point corresponding to the mean value ± SE (n = 6).

PSM was significantly higher in 2012 and 2013 (Chi-squared = 106.6; p-value < 0.001). Along the coast of Denia, Chl-a concentration ranged from 0.002 to 4.44 µg L^{-1}, with a mean value of 0.52 µg L^{-1} (Table 1). The highest values were observed in spring (March-May) 2010 (Fig. 2). Between 2010 and 2013 the Chl-a concentration decreased in all the beaches (Chi-squared; p-value < 0.001) (Table 1 and Fig. 2). The Chl-a concentration was slightly higher at Almadrava, Molins and Blay Beach (Table 2). Finally, LUSI values ranged between 37.5 and 75% for different locations along the coast, increasing towards the northern stations Almadrava and Molins (Fig. 1), and were significantly lower in 2012 (Chi-squared = 61.35; p-value < 0.001).

Zooplankton community composition

The total abundance of zooplankton varied from one year to another, with higher densities being observed in 2010 and 2012, and lower densities in 2011 and 2013 (Fig. 3). The contribution (%) of the different taxonomic groups to the zooplankton community varied within different seasons of the same year as well as between years (Figs. 3 and 4). Copepods largely dominated the community, representing on average 70-90 % of total abundance (Fig. 4). The highest abundance of copepods was recorded in 2012 (Table 1 and Fig. 3). Cladocerans peaked in July 2010 and 2013 (Fig. 4), when they numerically dominated the zooplankton community (Figs. 3 and 4). Maximum contributions of ichthyoplankton (17-24 %) were observed in the spring season (April-May) of 2010 and 2013 (Fig. 4).

Abundance and growth of *C. marsupialis*

A total of 5315 *C. marsupialis* cubomedusae were collected across the four years: n = 1109 in 2010; n = 1887 in 2011; n = 807 in 2012 and n = 1512 in 2013. A clear seasonality pattern emerged from the monthly histograms (Fig. 5).

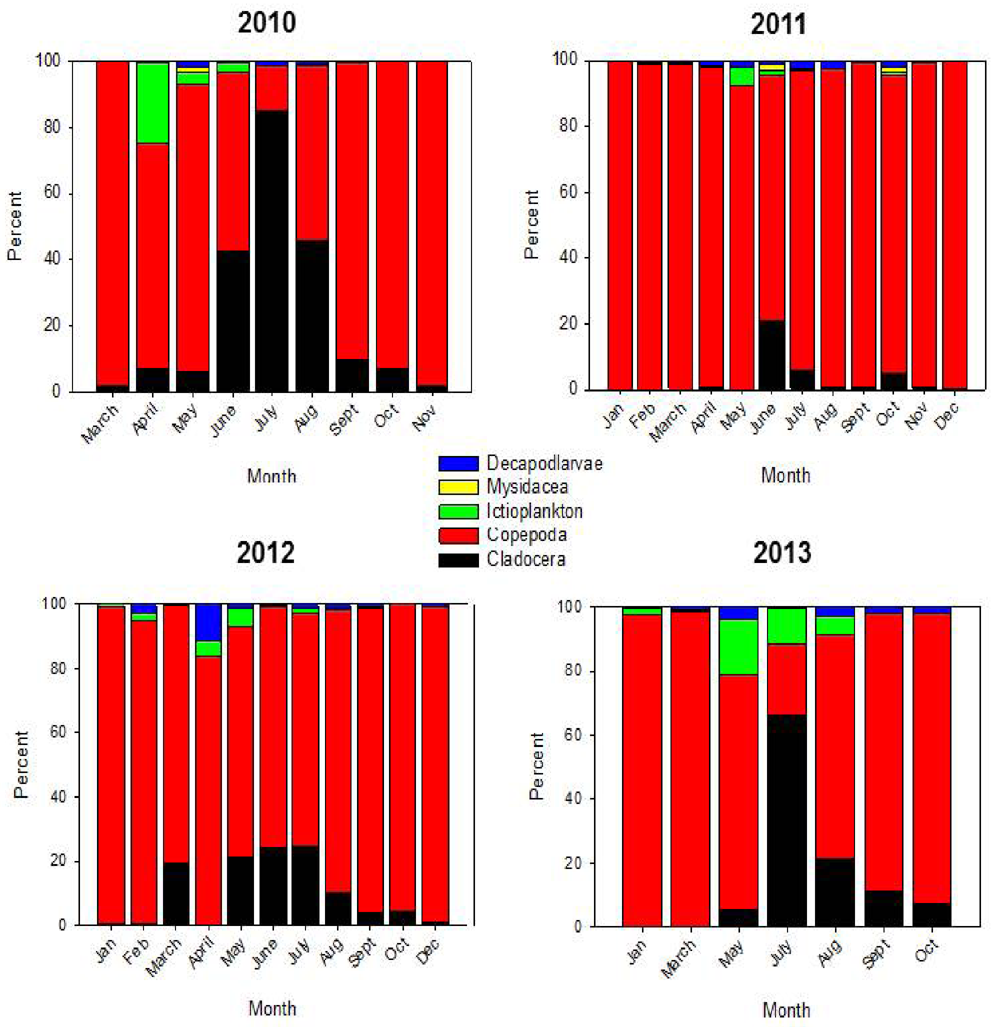

Fig. 4 Seasonality and interannual variability in the composition of the zooplankton community.

Although no significant differences between mean annual cubomedusae density values were observed (Table 1), the results from the cohort analysis (Fisat II) showed inter-annual differences in such values, and a decline of the cubomedusae population throughout the period studied.

In 2010, three pulses (June, July and August) of recent-detached cubomedusae, resulting from metamorphosis of polyps, were detected. The reproduction of the adults was observed in October (Fig. 5). From cohort analysis, two cohorts were identified in June 2010 (SI = 3.73); 2 cohorts also in July 2010 (SI = 4.78); three cohorts in August 2010 (SI = 2.88; 5.68); a maximum of four cohorts in September 2010 (SI = 2.71; 2.87; 6.39); and two cohorts up to the reproduction month of October 2010 (SI = 9.12).

In 2011, three pulses of recent-detached cubomedusae were detected (May, June and July), but the survival of the individuals was low, and few reproductive individuals were registered (Fig. 5). From cohort analysis, one cohort was detected in May 2011; two cohorts in June 2011 (SI = 3.49); three cohorts in July 2011 (SI = 2.44; 4.27); and one cohort in October 2011. Curve adjustment and separation index estimation were not possible in subsequent months, due to the low captures registered in August and September 2011.

In 2012 and 2013, two pulses of recent-detached cubomedusae were recorded in June and July. In both years, the two cohorts presented a very low survival rate. No reproduction was observed in 2012; however, few mature cubomedusae and planulae were registered in 2013 (Fig. 5).

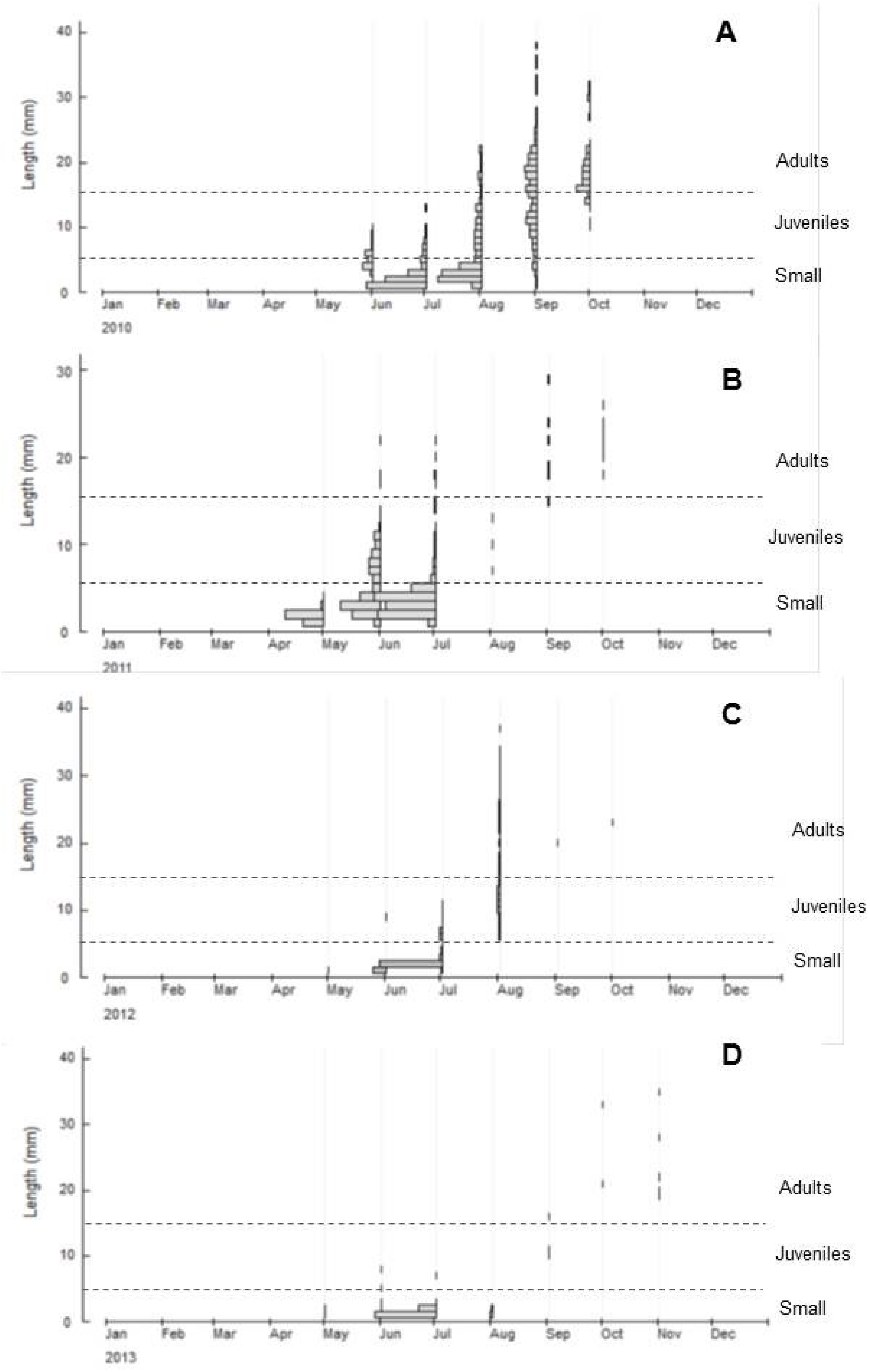

Fig. 5 Monthly length (Diagonal Bell Width) frequency plot of *Carybdea marsupialis* specimens collected: (A) 2010; (B) 2011; (C) 2012; (D) 2013. Dashed lines between different developmental stages: Small (DBW < 5 mm); Juveniles (5 < DBW < 15 mm); Adults (DBW > 15 mm).

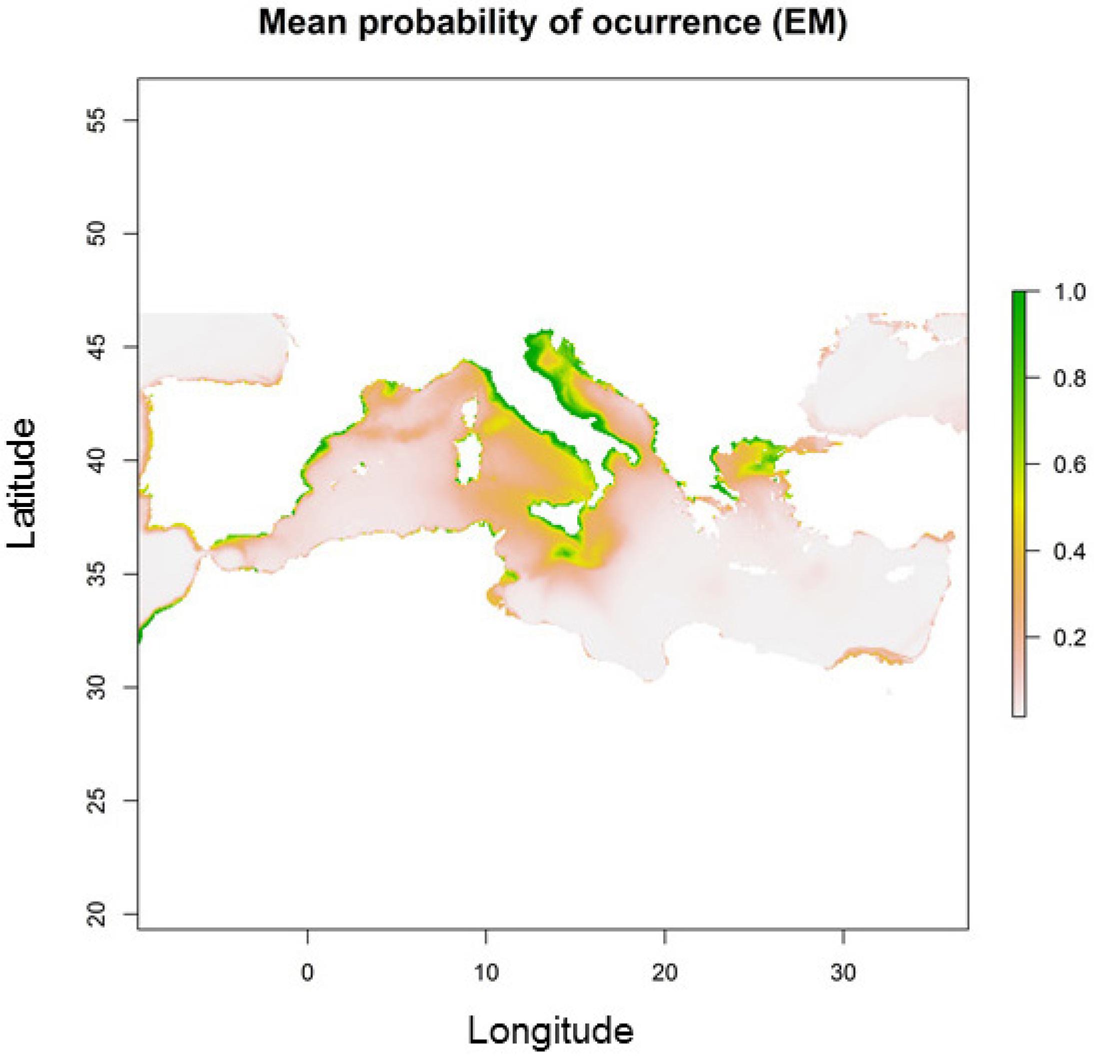

Fig. 6 Ensambled forecasting for the mean probability of occurrence of *Carybdea marsupialis* along the Mediterranean Sea. Legend, mean probability of occurrence from 0 to 1.

Relation with environmental parameters

Collinearity between the explanatory environmental variables was explored and detected before the GLM and GAM analysis. Nitrate presented collinearity with DIN (Supplementary material SM 1), as well as the organic and inorganic fraction of PSM with total PSM (SM 2). Therefore, DIN and PSM fractions were excluded from the analysis. When applied to explore the relation of Chl-a values with different environmental parameters, GLMs presented the most significant positive relation with LUSI ($t = 4.639$; $p\text{-value} < 0.001$) and nitrate concentration

Explanatory variable	GAM	MARS	RF
SST	0.272	0.130	0.182
Salinity	0.242	0.270	0.179
Chl-a	**0.650**	**0.607**	**0.436**
Nitrate	0.109	0.204	0.066
Phosphate	0.082	0.003	0.037

TABLE 3 Output of Species Distribution Model (SPM) for *Carybdea marsupialis* in the Mediterranean. GAM, MARS and RF models; relative importance of explanatory variables. Chl-a concentration was indicated as the most important variable, congruent results among the three models applied

(t = 8.35; p-value < 0.001). In addition, nitrate concentration presented a negative relation with salinity (t=-7.94; p-value < 0.001). Also, the abundance of cladocerans was positive related with Chl-a, LUSI and temperature (t = 3.686; p-value < 0.001).

Significant relationships between *C. marsupialis* density and the interaction of LUSI index, P and cladoceran concentration were observed from the optimal GLM model (t = 6.682; p-value < 0.001). GAM analysis showed the significant influence of Chl-a concentration (F = 2.019; p-value = 0.05), cladocerans (F = 3.97; p-value = 0.01) and ichthyoplankton abundance (F = 27.64; p-value < 0.001) on box jellyfish abundance. The optimal GAM model included salinity, nitrate, phosphate, DIN: P, Chl-a, copepods, cladocerans and ichthyoplankton concentration as response variables, and explained 54.7 % of the observed variability.

Projection along the Mediterranean

A total of 1401 sightings of *C. marsupialis* were recovered from the various citizen science databases, compiled along the Mediterranean coast and used for the development of the Species Distribution Model (SDM): 1252 reports from "Occhio alla medusa" in Italy; 94 from LIFE CUBOMED project; 42 from "Spot the Jellyfish" campaign in Malta; and 13 from ENPI-Medjellyrisk database. In the SDM, known occurrences of species (sightings) were confronted with raster environmental data layers, in order to assemble a predictive model of the distribution of *C. marsupialis* based on its ecological requirements. The probability of occurrence of the species can be then projected across broader areas than the original sampling location, in order to attempt a potential distribution for the species over the entire Mediterranean basin. After the model evaluation (TTS and ROC criteria), the GLM results were discarded due to their low degree of adjustment. In contrast, the GAM, MARS and RF models performed well during the validation exercises (average value for TSS and ROC > 0.7). We also evaluated the consistency among the different models applied (GLM, GAM, MARS, RF) by quantifying the standard deviations among the predictions (SM 3). Although GAM, MARS and RF models differed in some aspects, they displayed largely congruent patterns. The explanatory variable that was identified as having the highest relative importance for the presence of the species was mean Chl-a concentration (Table 3). The ensemble forecasting (Fig. 6) results indicate a high probability of *C. marsupialis* occurrence in some coastal areas where the species has not been reported to date, such as Sicily, Sardinia and the west coast of Africa.

Discussion

Drivers of *C. marsupialis* seasonality and population growth

Water temperature defined the seasonality and the life cycle of *C. marsupialis* populations in Denia. The first small cubomedusae appeared at the end of May, coinciding with the increase in water temperature (from ~12-15 ºC to ~18-20 ºC). The highest values for total abundance of individuals (i.e. considering all stages together) were recorded between July and August, until the last adults disappeared in late October - early November (Fig. 5). Canepa (2014) and Bordehore et al. (2015a) have also reported a similar seasonality along the coast of Denia. However, adult individuals have been sighted in winter in some other Mediterranean localities such as Empuriabrava (Catalan Sea, pers. observation) and Malta (Pulis 2015).

Because of the small size and high concentration of the recently-detached cubomedusae recorded in this study, it was assumed that the species can reproduce in Denia and the polyp stage may be located in the surroundings (Bordehore et al. 2015a). Metamorphosis did not appear to be a single event for *C. marsupialis*. The collection of small individuals (< 5 mm DBW) between May and August indicate that medusae production continued over an extended period, as reported for *C. fleckeri* (Gordon and Seymour 2012). As other authors observed, metamorphosis of cubopolyps is influenced by marked changes in water temperature and salinity (Canepa et al. 2013; Canepa 2014; Kingsford and Mooney 2014) that usually take place during spring. In addition, the mass occurrence of some box jellyfish species have been hypothetically related to rising water temperatures resulting from global warming (Morandini et al. 2014).

Once the metamorphosis is completed, other abiotic and biotic factors may also affect the development, growth and distribution of cubomedusae. For instance, different environmental forcings have been associated with the different developmental stages of *C. marsupialis* cubomedusae (Canepa 2014). Small and juvenile sizes, for instance, were more affected by the dispersion produced by wind and currents; when considering all the different stages, the most important environmental variables were water temperature, salinity and those variables related to primary production (Chl-a) and nutrient concentration (nitrate and phosphate) (Canepa 2014). Chiaverano et al. (2013) also observed a strong positive correlation between box jellyfish (*Alatina moseri*) abundance and primary production, as well as with zooplankton biomass. They proposed that inter-annual changes in box jellyfish abundance were controlled by the zooplankton availability that sustained the population. We observed a similar correlation with environmental parameters in the case of *C. marsupialis*. However, opposite to the tropical *A. moseri*, *C. marsupialis* presents the same seasonality as other medusae species from temperate regions.

We obtained a positive relationship between *C. marsupialis* density and concentration of cladocerans. Generally, the annual seasonal pattern observed in mesozooplankton is mainly influenced by the dynamics of copepods, which normally contributes about 55-65% of the total plankton community and which reaches higher abundances in winter and spring in the western Mediterranean (Saiz et al. 2014). We also observed this pattern in the zooplankton community of Denia, where copepods were the dominating organisms during most of the year, with the exception of the summer season when cladocerans bloom. Water temperature plays an important role in cladoceran population dynamics (Onbé and Ikeda 1995), and other factors such as changes in the size structure of phytoplankton assemblages have been also hypothesized to affect the seasonal dynamics of some zooplankton groups (Atienza et al. 2006). These changes in phytoplanktonic communities are the result of nutrient inputs and imbalances in nutrient ratios (Ludwig et al. 2009; Gadea 2016). When N: P ratio increases (eutrophication), the flagellate-based food path (which ends with jellyfish as consumers) is favoured against the diatom-based path; the flagellate-based food path is characterized by small size zooplankton, which is detrimental to fish because they are visual predators (Pérez-Ruzafa et al. 2002). The phytoplanktonic community of Denia was numerically dominated by nanoflagellates, especially during summer (Padrell 2012). Moreover, the mean Si: DIN ratio in Denia was below the Redfield ratio (<1:1), which may favour the predominance of non-siliceous phytoplankton (Pavlidou and Georgopoulos 2001). When pico and nanoplanktonic autotrophs are abundant, cladocerans feed efficiently and their populations bloom (Atienza et al. 2006). Applying this reasoning, we can explain the inter-annual variability in the abundance of cladocerans, which even influenced the abundance of the cubomedusae. Copepods have been also observed to be important components of the zooplanktonic community of Denia. Although they did not show a significant relation with the abundance of *C. marsupialis* in our analysis (where other groups as cladocerans exerted a major seasonal influence), previous studies demonstrated their importance in the cubomedusae diet (Chapter 3, this thesis). Moreover, ichthyoplankton (mainly fish eggs) presented a significant relationship with cubomedusae abundance, probably due to the marked seasonality with which the former appear. During some years, ichthyoplankton represented about 20-30 % of the zooplankton community in spring, coinciding with the spawning season of most neritic fish species (Sabatés et al. 2007).

However, there are no records of small *C. marsupialis* (which is the stage present in the water during spring) feeding on fish eggs, whereas some fish larvae have been recorded from the stomachs of adult jellyfish specimens (Chapter 3, this thesis). Nevertheless, we cannot draw strong conclusions from our results concerning this group, because the sampling methodology used (i.e. hand nets) may have under-estimated fish larval abundance, as they probably increased their avoidance ability with size (Gartz et al. 1999). Overall, an adequate food supply to cubomedusae may increase the survival rate of the population. For instance, in 2010 we observed the coupling between zooplankton and Chl-a pulses. This fact favoured blooming of cladocerans in July and the subsequent peak of copepods in September, and favoured the development and growth of cubomedusae. Conversely, the Chl-a concentration decreased in the following years and the zooplankton dynamics were consequently modified, resulting in a depression in cubomedusae growth.

Camp et al. (2015) established a positive pressure–impact relationship between Chl-a and the coastal pressure index (Land Uses Simplified Index, LUSI). In this study we also obtained a positive relation between Chl-a concentration and LUSI values. However, we observed a discrepancy between high nutrient concentrations and phytoplanktonic biomass (Chl-a) in 2013. This phenomenon has been also reported in previous studies addressing the effects of wastewater discharges from coastal marine outfalls in the Northern Adriatic (Mozetič et al. 2008) and in Gandia (Gadea 2016). Nutrient loadings to coastal systems can alter the Redfield proportion (16:16:1), this being interpreted as a potential limitation for phytoplankton development (Olivos et al. 2002). In general, the high DIN:P ratios recorded during the first years of our sampling may indicate a potential P limitation, as Sebastià and Rodilla (2013) and Gadea (2016) observed in Gandia, and as also Alventosa (2015) reported from Denia. However, the excess of P in 2013 (especially near Rasset and Marineta Cassiana stations, where the WWTP Denia-Ondara-Pedreguer marine outfall is located) caused a change in the nature of the main potential-limiting nutrient. The input of P into the marine system was not translated into an increment of phytoplankton or zooplankton, which may indicate that the excess of P was derived to other trophic pathways (e.g. macrophyte and/or macroalgae production). Interestingly, the model developed by Canepa (2014), with data collected in summer 2010, indicated a decrease in cubomedusae abundance when a threshold of P concentration was exceeded.

The results of this study indicate that *C. marsupialis* was able to grow and develop in highly productive areas. Cubomedusae present a high metabolic demand (Colin et al. 2013); therefore, a high biomass of zooplankton would be needed to support large populations. For instance, the survival of smaller stages of *C. marsupialis* was related to the abundance of cladocerans, showing a clear inter-annual variability in abundance. When conditions are unfavourable, some jellyfish populations are constrained to the benthic polyp stage (Boero et al. 1996). In such situation, the disappearance of active stages in the water column is compensated by the benthic stages, and the population size is considered to be constant (Boero et al. 1996). However, in the case of *Carybdea* spp., a fraction of the cubopolyps (~50 %) may still completely metamorphose into medusae (Straehler-Pohl and Jarms 2005). This will produce reduction in the polyp stock and thus in the number of released cubomedusae in the next season. Therefore, new recruitment of polyps, as result of sexual reproduction, might be necessary every year to maintain the population. The decreasing trend in *C. marsupialis* abundance observed over the 2010-2013 period might be reflecting a progressive decrease in Chl-a concentration and food supply, which would affect the survival of all medusa stages and the reproduction rate of the population. Furthermore, a longer study (> 10 years) might manage to distinguish between natural inter-annual and decadal variability in the species.

Occurrence along Mediterranean coasts

Projection models have been used for other cubozoan species with a variety of applications, including design of sampling strategies and identification of areas vulnerable to species invasions (Bentlage et al. 2009). In general terms, the occurrence of *C. marsupialis* along the Mediterranean coincides with coastal areas influenced by anthropogenic nutrient inputs (nitrate and phosphate) that promote in turn high Chl-a concentrations. The influence of water temperature and salinity at a basin scale was not as clear in this analysis, although a regulation of the species life cycle due to these two parameters is well known, through the regulatory role that water temperature and salinity exert on cubopolyp development, asexual reproduction and metamorphosis (Straehler-Pohl and Jarms 2005; Canepa et al. 2013). Other studies, conducted in Denia (Canepa 2014) and Italy (Zampardi 2016), have observed correlations of these life cycle descriptors with salinity. In contrast, no correlation with salinity has been reported from Malta (Pulis 2015), where fluctuations in other environmental parameters, such as water temperature, exerted more local influence on the life cycle.

According to the species distribution model, the Mediterranean sub-basin with the highest probability of occurrence of the species is the Adriatic Sea (Fig. 6), as reported by other researchers (Boero 2013; Zampardi 2016). Indeed, most of the reports of *C. marsupialis* were compiled through "Occhio alla Medusa" campaign conducted in Italy. The ecosystems in the Adriatic Sea have been greatly affected by coastal eutrophication caused by agricultural, industrial and urban activities emerging in the last 20 years (CIESM 2001; Boero 2013). A high probability of occurrence for the species has also been projected along the Spanish Mediterranean coast. This region bears the brunt of the impact of mass tourism; one important impact is the seasonality of wastewater flow, which is considerably higher during summer due to the increase in coastal population (Sebastiá and Rodilla 2013; Gadea 2016). Moreover, most of the sightings of the species come from areas near harbours and marinas, indicating that these coastal constructions may be suitable settlement substrates for planulae (Bordehore et al. 2011; Boero 2013; Gueroun et al. 2015; Pulis 2015), although some bias could exist since these areas get more attention from observers than others (Duarte et al. 2012). However, up to date the polyps of *C. marsupialis* have been actively sought by researchers, but never located along these areas. In this sense, it is important to mention that cubopolyps are inconspicuous, and have been found only once in the field (Studebaker 1972). Therefore, new sampling methods would be necessary to detect this benthic life stage and to properly evaluate the influence of artificial substrates on *C. marsupialis* proliferations.

The ensemble forecasting also predicted suitable habitats for *C. marsupialis* in Sardinia, Sicily and the W coast of Africa. No reports of the species have been recorded in our database from these locations. However, the SDM can be also applied to other cubozoan species with similar ecological requirements. In fact, two other carybdeid species have been reported from the North Atlantic coast of Africa (Haeckel 1882; Gershwin and Gibbons 2009) and South Atlantic coast of Spain (Pulis 2015). Therefore, it would be interesting to conduct exploratory surveys within these potential occurrence areas so as to clarify species distribution boundaries. Also, citizen science initiatives have been demonstrated to be powerful tools to gather data on species presence that may enable the development of distribution models.

Management recommendations

The Mediterranean is nowadays considered to be one of the most impacted seas in the world (Halpern et al. 2008; Costello et al. 2010; Coll et al. 2012). In fact, the NO_3:PO_4 has increased significantly in the major Mediterranean rivers (e.g. Rhone River) during the last two decades (Ludwig et al. 2009; Alekseenko et al. 2016) triggering local eutrophication events due to river runoff (Saiz et al. 2014). Because of that, the European Marine Strategy Framework Directive (MSFD) aims to reduce the N and P loads in order to minimize human-induced eutrophication. Some eutrophication and water quality degradation indicators within the MSFD included descriptors that refer to the occurrence of nuisance and potentially toxic phytoplankton species that result from increases in nutrient loads, as well as from the increased duration and frequency of blooms (Ferreira et al. 2011). These indicators normally refer to harmful algal blooms (HAB's), but we consider this indicator to be also aplicable to gelatinous zooplankton blooms. Both phenomena are considered as alternative pathways in ecosystem functioning, both arising from the disruption of the "normal" plankton dynamics (GFCM 2011). Therefore, the higher frequency of jellyfish outbreaks in the NW Mediterranean may be indicative of changes in marine ecosystem functioning (Mills 2001; Purcell 2005).

The effects of nutrient loadings are transmitted bottom-up through the food web and affect the organization and dynamics of higher trophic levels (Warry et al. 2016). In relation with this, Bordehore et al. (2015a) compared the effectiveness of two different proposed strategies for the management of *C. marsupialis* overabundance: direct removal of box jellyfish versus indirect reduction in their abundance by reducing prey availability through nutrient control. Their results suggest that reducing prey is a more effective management strategy than removing jellyfish directly, because growth rates were diminished for all life stages (Bordehore et al. 2015a). Overall, choosing the former approach, based on population matrix projection, requires the demonstration of the causative connection between cubomedusae abundance and nutrient availability. In this study, we observed the relation between *C. marsupialis* abundance and the anthropogenic activities developed on the shoreline. Other authors also described evidences of planktonic food web response and a concurrent increase in some jellyfish species, due to changes in nutrient inputs in the Mar Menor coastal lagoon (Spain) (Pérez-Ruzafa et al. 2002).

The coastal land use in the study area, mainly dedicated for agricultural and urban use, determines the nutrient inputs to the coast through channels, submarine outfalls and runoff. The agricultural activity developed in the region is the main source of nitrates in coastal waters (Sebastiá and Rodilla 2013). The excess of dissolved N that is not assimilated by agricultural plants is leached to the aquifer or to the superficial channels that drain the wetland (Sebastiá and Rodilla 2013; Gadea 2016). Furthermore, maximum P values were recorded in summer, as observed in Gandia in coincidence with the increase in the waste waters discharge volumes (Gadea 2016). As a result, the water quality of Denia has deteriorated in the last decades. In fact, these waters are categorized as poor, taking into account the annual mean Chl-a concentrations registered in 2010 (Table 2). In addition, the water quality was also considered poor on the basis of the DIN and phosphates concentrations registered in 2013 (Table 2). According to these observations, the coastal area of Denia can be defined as nutrient-sensitive, as has been proposed for the adjacent coastal location of Gandia (Sebastiá and Rodilla 2013), where outbreaks of the species *C. marsupialis* have been also reported. A strategy combining reduction in both N and P loads will be the best practice to prevent marine ecosystem degradation, and reduce the causes triggering the outbreak of *C. marsupialis* along the coast of Denia in particular, and in the Mediterranean in general. On one hand, the seepage of nitrates into marine systems from agricultural sources should be reduced by promoting the use of good farming practices (Council European Union 1991a). On the other hand, a reduction in phosphate seepage due to a better treatment through sewage treatment plants or by substituting P sources in detergents (Jickells 2005) would be another feasible mitigation measure to protect the marine environment from the adverse effects of eutrophication due to wastewater discharges (Council European Union 1991b), particularly during the touristic months. Regular monitoring to prevent and control deterioration of the marine environment and the adoption of effective management strategies are recommended.

Finally, studies on the polyp stage are essential to completely understand the life cycle of *C. marsupialis* and the forcing parameters that drive its seasonal occurrence. The increase in shipping, aquaculture and other coastal defense structures, could be driving the rise and prevalence of jellyfish blooms, including *C. marsupialis* outbreaks, in some Mediterranean areas (Bordehore et al. 2011; Duarte et al. 2012; Boero 2013). The potential for artificial substrates to serve as a substrate for jellyfish polyps, and concretely for *C. marsupialis* cubopolyps, should be also considered in coastal planning (Bordehore et al. 2011; Duarte et al. 2012; Boero 2013; Canepa 2014), which should include specific monitoring strategies in the environmental impact assessment (EIA) procedure.

Conclusions

Nutrient inputs have a significant impact on coastal ecosystems, altering their biodiversity and functioning, and in some cases even leading to outbreaks of "nuisance" organisms as the box jellyfish *C. marsupialis*. A reduction in continental nutrient inputs to the marine system would control the abundance of primary and secondary producers, and thus prevent outbreaks of *C. marsupialis*. This and previous studies (Sebastiá and Rodilla 2013; Canepa 2014; Bordehore et al. 2015a; Gadea 2016) may provide a good baseline reference for the long-term environmental management and planning in Denia and adjacent coastal areas.

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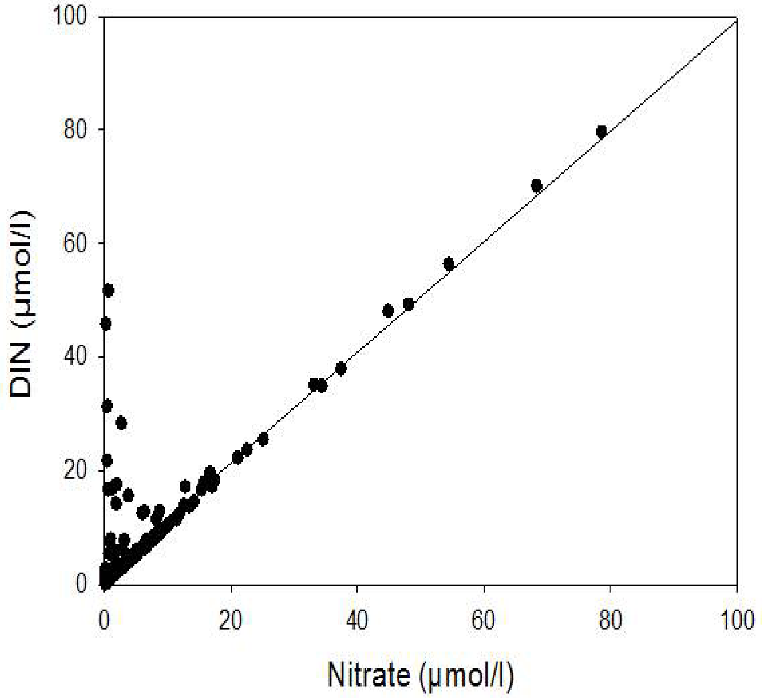
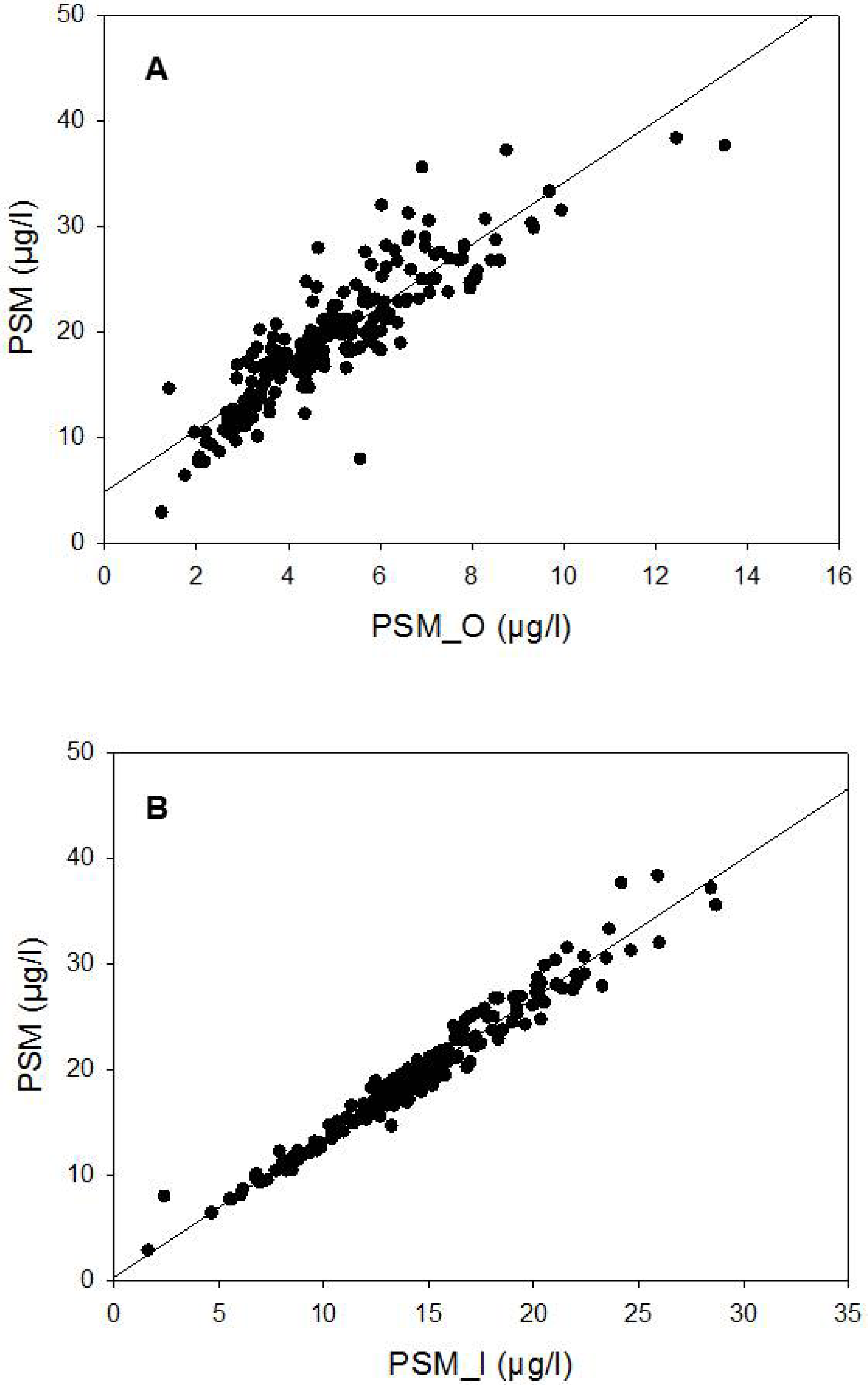
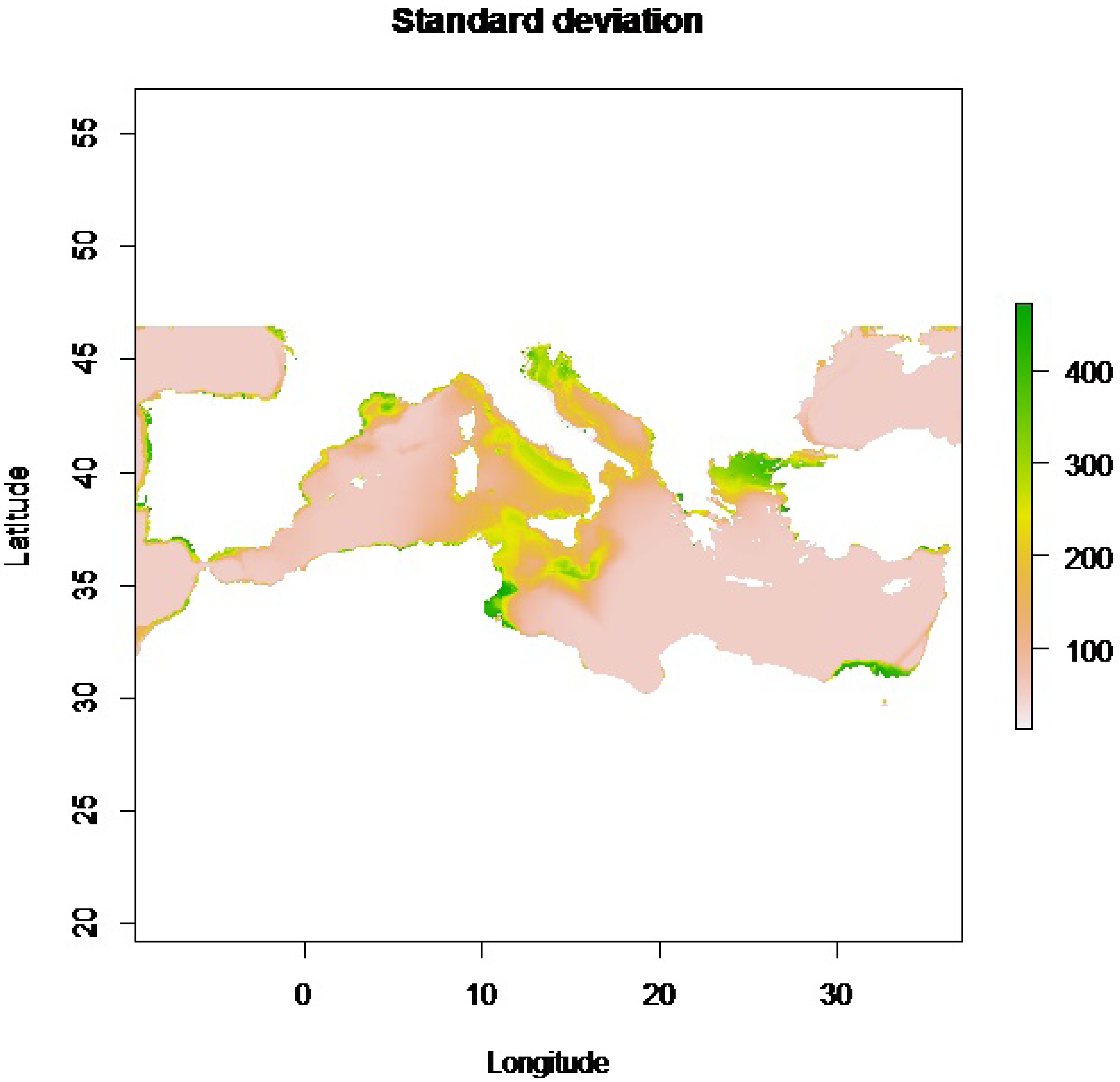
Supplementary material



SM 1 Collinearity between Nitrate and DIN (Dissolved Inorganic Nitrogen).



SM 2 Collinearity between Particulate Supended Material (PSM) and (A) organic (PSM_O) and (B) inorganic (PSM_I) fractions.



SM 3 Consistency among different models applied (GLM, GAM, MARS, RF), quantified using standard deviation among them.

General discussion

The results obtained in this thesis enlighten the research on the possible causes and consequences of *Carybdea marsupialis* overabundance. As shown in the Introduction, blooms of *C. marsupialis* appear to be increasing in both intensity and frequency in many coastal areas along the Mediterranean Sea. This increase in records could be considered a consequence of the establishment of different jellyfish spotting web platforms and projects. However, many reports were accompanied by observations of people ensuring they saw this species for the first time in the area at least for the past 20-25 years, which can be considered and indicator of the abnormal condition of this proliferations. Therefore, we hypothesized coastal eutrophication and other human impacts – as translocation, climate change and habitat modification – might be related with them.

Translocation and invasion

The possible introduction of the species *C. marsupialis* in the Mediterranean due to translocation and subsequent invasion was initially considered as one of the causes of its increase. However, based on the literature, as well as on morphological and genetic analysis conducted in the frame of this thesis (Chapter 1), we concluded that *C. marsupialis* is a native species in the Mediterranean, and not an exotic species as initially considered. Therefore, the apparent recent increase in the abundance of this species along the Mediterranean coast cannot be attributed to a recent introduction in the basin.

Nevertheless, a biological invasion can occur not only following the introduction of a non-native species, but also following a major modification in the environment (e.g. eutrophication, loss/addition of top predators, etc.) that changes the biological interactions (mainly competition) (Valéry et al. 2008). In this sense, human alteration of ecosystems may drive native species to an invader status (Valéry et al. 2009), which can be a plausible situation for *C. marsupialis* in the Mediterranean. In such scenario, native species can dominate the community by exploiting new niche opportunities resulting from the decline of other species (Valéry et al. 2009). Increased food supply, derived from anthropogenic sources, and the absence of predators may be also drivers of invasive species.

Aquatic invasive animals present some physiological adaptations (e.g. rapid growth, early maturity, short life spans and elevated fecundity) allowing rapid population increase (McMahon 2002). In fact, jellyfish (including cubozoans) have some characteristics that enable them to survive in disturbed marine ecosystems: they present fast growth rates, they can shrink when starved, and they are able to tolerate hypoxia (Richardson et al. 2009). In addition, the polyps own the capacity to conduct asexual reproduction, which also enables a rapid growth of the population. These are characteristics of opportunistic species, and may give jellyfish an advantage over fish in environments stressed by different factors such as climate change and eutrophication (Richardson et al. 2009).

Climate change

We cannot evaluate the influence of climate change on *C. marsupialis* proliferation with the results obtained here, because a longer study would be required for that purpose. However, we expect that an increase in temperature would affect the asexual reproduction rate of polyps, extent the period for cubomedusae production, and increase the metabolic rates of the species. Our results demonstrate that temperature defined the seasonality and the life cycle of *C. marsupialis* population in Denia (Chapter 4). The seasonality patterns observed for *C. marsupialis* in the Mediterranean differ from that reported for *C. xaymacana*, which have been reported to be present all year round in Puerto Rico (Studebaker and Cutress 1972). The recent-detached cubomedusae of *C. marsupialis* were detected in the coastal waters at the end of May, coinciding with the increase in temperature (from ~12-15 ºC to ~18-20 ºC). The highest densities of total individuals (i.e. considering all stages together) were recorded between July and August, until the last adults disappeared in late October - early November. Also from laboratory experiments (Chapter 2), an association between cubomedusae survival rate and temperature was obtained. A higher mortality rate was related to the decrease in temperature (< 18ºC), but specimens maintained at warmer temperatures (21ºC) were able to survive for 3 more weeks (Acevedo et al. 2013). In addition, other environmental variables also related with the climate change, as precipitation, may influence seasonal and interannual variability in the abundance of *C. marsupialis*. On one hand, changes in coastal salinity have been observed to promote carybdeid polyps to metamorphose (Canepa et al. 2013). On the other hand, interannual variation in salinity, in combination with temperature oscillation, may determine the composition of the plankton community,

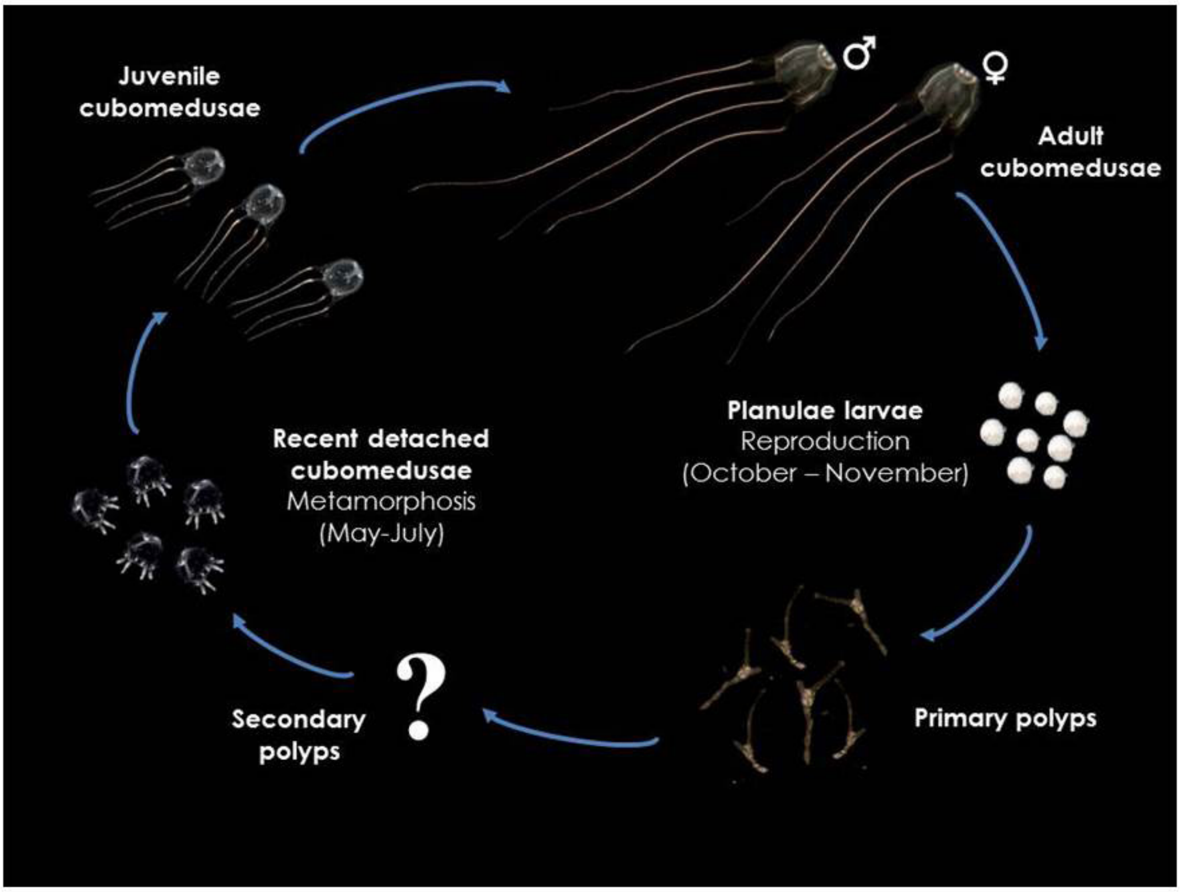


Fig. 1 Life cycle of *Carybdea marsupialis* in the Mediterranean; the seocndary polyp stage remains undescribed.

finally influencing *C. marsupialis* abundance (Chapter 4). Nevertheless, further investigations would be needed to clarify the effects of climate change in the population dynamics.

Habitat modification and polyp stage

As explained above, the life cycle traits of *C. marsupialis* are key aspects determining the invasiveness or spread potential. Polyps multiply asexually through the production of buds and they are potentially resistant to stressful conditions such as low food supply and hypoxia, as observed in other jellyfish species (Arai 2009). Although the life cycle of *C. marsupialis* has been not completely resolved and described (the polyp stage remains unknown; Fig. 1), those characteristics seem to be also attributable to the species due to the similarity with other cubozoans. For instance, the polyps of a similar carybdeid species can contract and encapsulate under unfavourable conditions (Straehler-Pohl 2001; Fischer and Hofmann 2004; Straehler-Pohl and Jarms 2011; Kingsford and Mooney 2014). Therefore, future efforts should be addressed to fill this gap in the biological knowledge. This is particularly important because high abundances of *C. marsupialis* have been observed in correspondence with coastal defences (Bordehore et al. 2011; Boero 2013).

The habitat modification and construction of coastal defences could have increased the substrate availability for the polyp stage of *C. marsupialis*. The asexual reproduction of polyps is a key driver of medusae outbreaks, and artificial structures may be acting as nurseries and reservoirs of polyps (Duarte et al. 2012). The availability of new hard bottom habitats in some areas normally dominated by soft bottoms might have favoured the persistence and spread of *C. marsupialis* (Boero 2013, Fig. 2).

Fig. 2 Bloom of *C. marsupialis* in correspondence of coastal defences along the Adriatic coast of Italy (FAO 2013).

Unfortunately, the benthic habitat of the polyps remains undiscovered for many species, including *C. marsupialis*, limiting the understanding of the drivers of jellyfish blooms (Duarte et al. 2012).

In the frame of LIFE CUBOMED project, we have unsuccessfully been searching for the polyps of *C. marsupialis* on different substrates (i.e. both natural and artificial) along the coast of Denia for 5 years. We hypothesized that polyps might be near the sampling stations where we have detected the small cubomedusae (supposed recent-detached from the polyp) year after year. But, cubopolyps are inconspicuous and difficult to detect; carybdeid polyps have been found only once in the sea on dead bivalve shells (Studebaker and Cutress 1972). Nevertheless, some evidences may indicate the importance of artificial substrates for *C. marsupialis* proliferation. For instance, cubomedusae have been often reported from harbours or in beaches with abundant artificial substrates (Bordehore et al. 2011; The Mendeley Support Team 2011; Boero 2013; Gueroun et al. 2015; Pulis 2015). Other jellyfish species have been also reported in harbours and coastal lagoons along the Mediterranean, as *Aurelia aurita* (Marques et al. 2015). In fact, ship wrecks have been reported in the Adriatic to be ideal substrate for the polyps of *A. aurita* (Di Camillo et al. 2010), and in the Italian coast a positive correlation between *A. aurita* and *C. marsupialis* has been observed (Zampardi 2016). Equally, also in the northern Catalan coast, we have registered *C. marsupialis* to share the waters in the channels of Empuriabrava with *A. aurita* (personal observation). In this case, we confirmed the growth of *A. aurita* polyps on artificial substrates inside the harbour, and a similar behaviour was hypothesized for cubopolyps. The possible effect of ballast waters or spread on boat structures should be also taken in consideration. Moreover, there is also a succinct reference indicating that during the polyp phase,

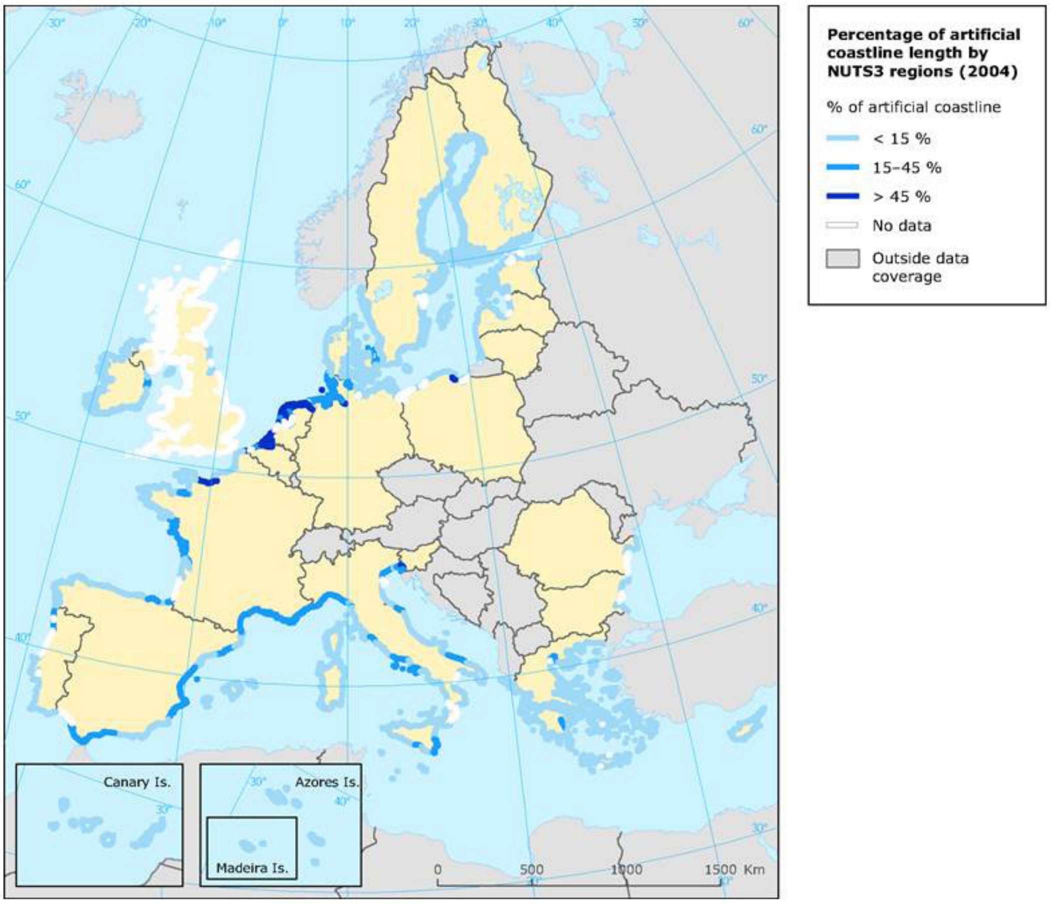


Fig. 3 Percentage of artificial coastline length by NUTS3 regions (2004). Source: ETC-ET (EEA).

many cnidarians as cubozoans may remain in the meiobenthos (Giere 1993).

According to the European Environmental Agency (EEA) and EUROSION project (2005), artificial shorelines prevail over the natural ones in 21 European regions (Fig. 3). In some areas, the level of coastal construction is really high (e.g. 20.9% in Malta), and it is mostly related to harbour, recreation or road development (EUROSION project 2005). A part from providing artificial substrates, the waters in ports and harbours are often characterised by high levels of nutrients, organic matter, turbidity and pollutants, which may increase polyp and medusa food supply (Duarte et al. 2012). Moreover, many of these environments may have hypoxic waters, where jellyfish polyps are particularly resistant (Purcell and Arai 2001; Duarte et al. 2012).

Nutrient enrichment and coastal eutrophication

European coastal zones are under multiple pressures facing several economic, social and environmental problems (EEA 2010). The human population in the coastal regions is around 10 % higher than inland, but in some countries this could be up to 50 % (EEA, 2006). This increase in the human pressure along the Mediterranean is one of the major causes producing changes in the coastal ecosystems, leading to eutrophication and the degradation of water quality, especially in those areas with high nutrient loads (EEA 1999; Duarte et al. 2000). In the urban areas, the wastewaters are especially rich in phosphorus and nitrogen, but poor in silica (Artioli et al. 2008). This can change the nutrients dynamics affecting the relations among organisms, and finally producing disequilibrium in the ecosystem. Under these conditions, the non-siliceous phytoplankton may proliferate and replace diatoms, resulting in a reduction in the size of the zooplankton (Richardson et al. 2009). It has been hypothesised that this scenario is more favourable for jellyfish than for fish (Richardson et al. 2009). In addition, if jellyfish produce a pulse synchronized with that of crustaceans, they can overtake fish, and their bloom could be reinforced (GFCM 2011; Boero 2013).

Particularly, the trophic role of *C. marsupialis* varies along the life cycle, as observed both in the laboratory (Chapter 2) and in the field (Chapter 3). But, the adult cubomedusae occupy a similar position

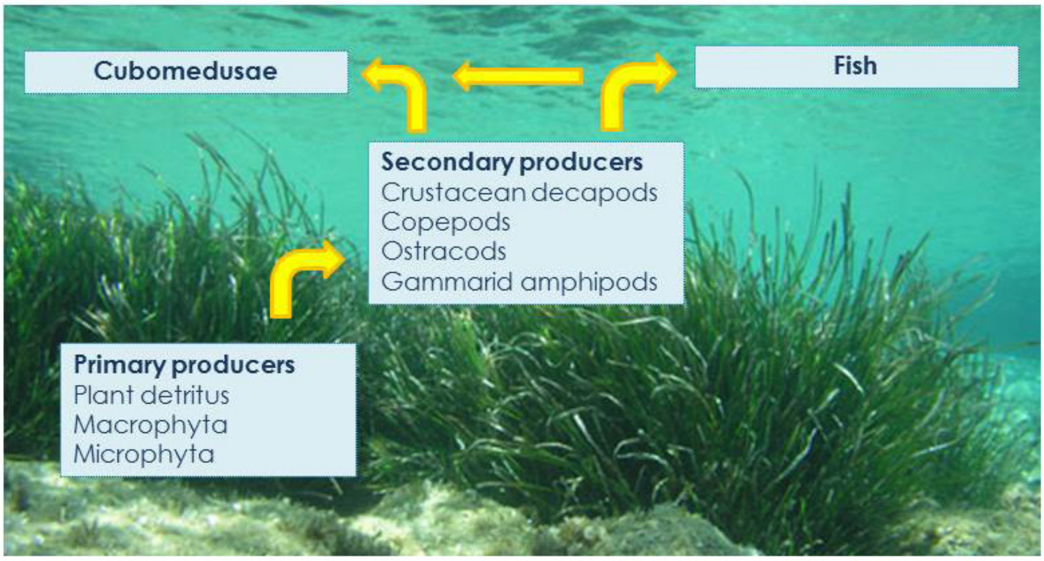


Fig. 4 Ecosystem functioning: alternate trophic pathways in the seagrass meadows, role of cubomedusae in benthic-pelagic coupling.

in the food chain as fish larvae and juveniles (Chapter 3). The increase in abundance of cubomedusae in the coast of Denia may be reflecting disequilibrium in the ecosystem, in the sense that more energy (i.e. food supply) would be available and derived towards gelatinous organisms (Fig. 4). The feeding rates of *C. marsupialis* estimated from our experiments (Chapter 2) increased with prey availability. There was no evidence of saturation, even at the highest concentrations, which were greater than maximum values observed in the field. Although results in vitro should be interpreted with caution, these may indicate that an increase in food supply will favour the growth of *C. marsupialis* population. Outbreaks of cubomedusae may significantly affect amphipods and copepods (by predation) and fish larvae (by competition and predation). The predation impact of *C. marsupialis* seems to be intense, especially during night (Chapter 3). But, due to the microscale distribution of the cubomedusae, this impact would not significantly affect the dynamics of the prey population at a larger scale. However, we observed a close diet overlap and potential competition between *C. marsupialis* and fish larvae and juvenile (Chapter 3).

Cubomedusae may have an important role in benthic-pelagic coupling, as observed in the seagrass meadows studied in Denia (NW Mediterranean). Substantial predation on epizooplankton, including amphipods, might contribute to the transfer of C from benthic to pelagic food webs (Fig. 4). Amphipods are key organisms in the food webs associated to *P. oceanica* because of their importance in the transfer of organic matter from primary producers and detritus to higher trophic levels (Michel et al. 2014). In this sense, nutrient loads have promoted the sea grass decline and the growth of algal epiphytes in many regions (Hughes et al. 2013), which in consequence may have favoured the growth

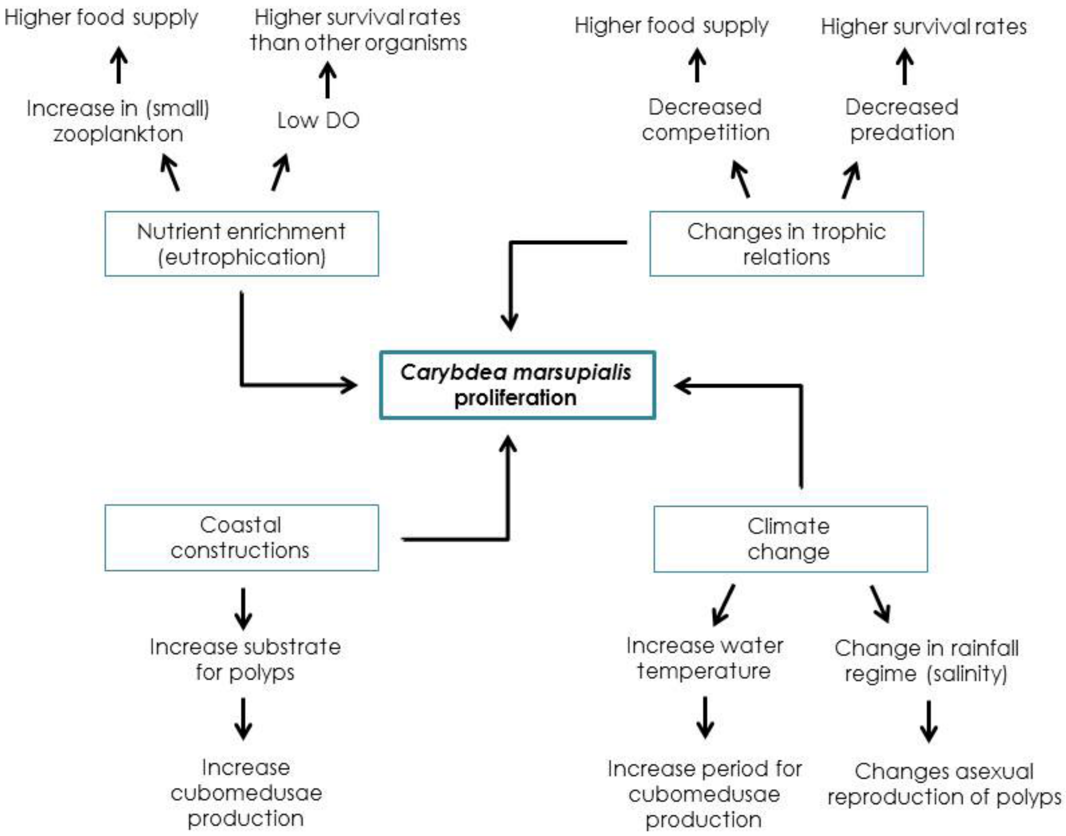


Fig. 5 Diagram of factors promoting blooms of *Carybdea marsupialis* box jellyfish (adapted from Fujii et al. 2011).

of gammarid amphipods, since their diet rely on epiphytes and seagrass detritus (Michel et al. 2014). These kind of changes in nearshore-ecosystems functioning due to eutrophication may be enhancing *C. marsupialis* outbreaks along the Mediterranean Sea by means of food availability. Moreover, direct predation control (e.g. exerted by organisms as turtles) has not been observed. Some attempts to feed marine turtles *Caretta caretta* with *C. marsupialis* have been conducted in the frame of LIFE CUBOMED project, but negative results were obtained (Alonso et al. 2016). As we have previously seen, increased food supplies from anthropogenic sources and the absence of predators are common drivers of native invaders.

Preventive coastal management

Overall, coastal blooms of *C. marsupialis* may be attributed not only to a combination of environmental parameters which regulate the life cycle, development and distribution of the species (e.g. temperature, salinity and currents), but also as a result of anthropogenic causes (e.g. nutrient enrichment and availability of artificial substrates), which interact and may favour the proliferation (Fig. 5). Evidences of relation between *C. marsupialis* outbreaks and anthropogenic impacts on the sea shore may determine whether box jellyfish proliferations are normal events or an environmental status indicator. We observed the higher probability of cubomedusae presence in highly productive areas along the Mediterranean (i.e. elevated Chl-a and nutrients concentration) (Chapter 4). Moreover, we obtained a positive relationship between *C. marsupialis* abundance and human impacts in the coast of Denia (Chapter 4). This was observed by the application of the Land Use Simplified Index (LUSI) which estimates the influence of different activities (i.e. agricultural, urban, and industrial) on the marine coastal areas (Flo et al. 2011; Camp et al. 2015). LUSI indicated between 37.5 and 75% of anthropogenic impact along different beaches in Denia (Chapter 4). In the light of these results, we recommend a precautionary approach, which suggests prevention as the basis of management actions to be developed (Richardson et al. 2009). Some proposals for jellyfish blooms mitigation are the same as suggested for HABs, therefore probable synergies are expected (Brotz and Pauly 2012). In conclusion, reducing nutrient loads, overfishing and artificial constructions would be positive measures in order to reduce the consequences of jellyfish blooms and other environmental problems. Remediation of anthropogenic nutrient enrichment can be achieved through concrete actions as the implementation of efficient sewage treatment (Cloern 2003). Reducing nutrient loading would attenuate eutrophication, algal blooms, and other stressors on marine systems, favouring the recovery of jellyfish competitors and predators, and ecosystem resilience (Richardson et al. 2009; Boero 2013). Blooms of *C. marsupialis* could be sustained and even spread the Mediterranean coasts in the absence of environmental quality improvement, indicating disequilibrium in the marine ecosystems.

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Conclusions

The main conclusions and goals extracted from the investigations conducted in this PhD thesis are the following:

Chapter 1:

1. Cubozoans specimens from Denia (Spain, NW Mediterranean) studied in this work belong to the species *C. marsupialis*.

2. *C. marsupialis* should be considered as a native species in the Mediterranean, and this fact should be taken into account when control measures or protocols against population outbreaks are developed and applied in that region.

Chapter 2:

3. Optimal conditions for the growth and maintenance of this cubomedusa species in the laboratory have been identified (i.e. aquarium design, environmental parameters and prey type). Cubomedusae were maintained for 140 days, and they reached the subadult condition; during that time a progressive shift in their preferred prey was observed.

4. We observed an association between survival rate of cubomedusae and water temperature; the mortality rate increased with the decrease in temperature.

5. Experimentation under controlled conditions enabled the estimation of some the metabolic rates of the species (i.e. feeding, growth and mortality rates).

Chapter 3:

6. Gut contents analysis and prey selectivity indices showed a preference for nocturnal, epibenthic zooplankton, highlighting the nocturnal feeding behaviour of *C. marsupialis* and its contribution to the bentho-pelagic coupling.

7. Trophic level and trophic niche estimates, based on stable isotope analysis, varied significantly along the development and growth of the *C. marsupialis*.

8. The potential predation impact of *C. marsupialis* can be intense, particularly during its patchy population outbreaks, but it remains unclear whether this would be important enough to regulate prey stocks over large spatial scales.

9. Potential competition between *C. marsupialis* and juvenile fish would exist, since they occupy a similar trophic level in *P. oceanica* meadows, and their diets strongly overlap.

Chapter 4:

10. The abundance of *C. marsupialis* in the coast of Denia was related to the nutrient inputs derived from human activities along the coast, which finally increased the food availability to the species.

11. The abundance of prey (i.e. cladocerans) is especially important for the survival of the smaller cubomedusae stages, and this fact will determine the survival and development of the *C. marsupialis* population.

12. The forecasting of the probability of occurrence of *C. marsupialis* along the Mediterranean region indicated that this species inhabits in coastal areas characterized by high chlorophyll a concentration.

13. Nutrient concentrations control abundance and composition of primary and secondary producers in coastal waters, therefore the reduction of continental nutrient inputs may prevent outbreaks of *C. marsupialis*.

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