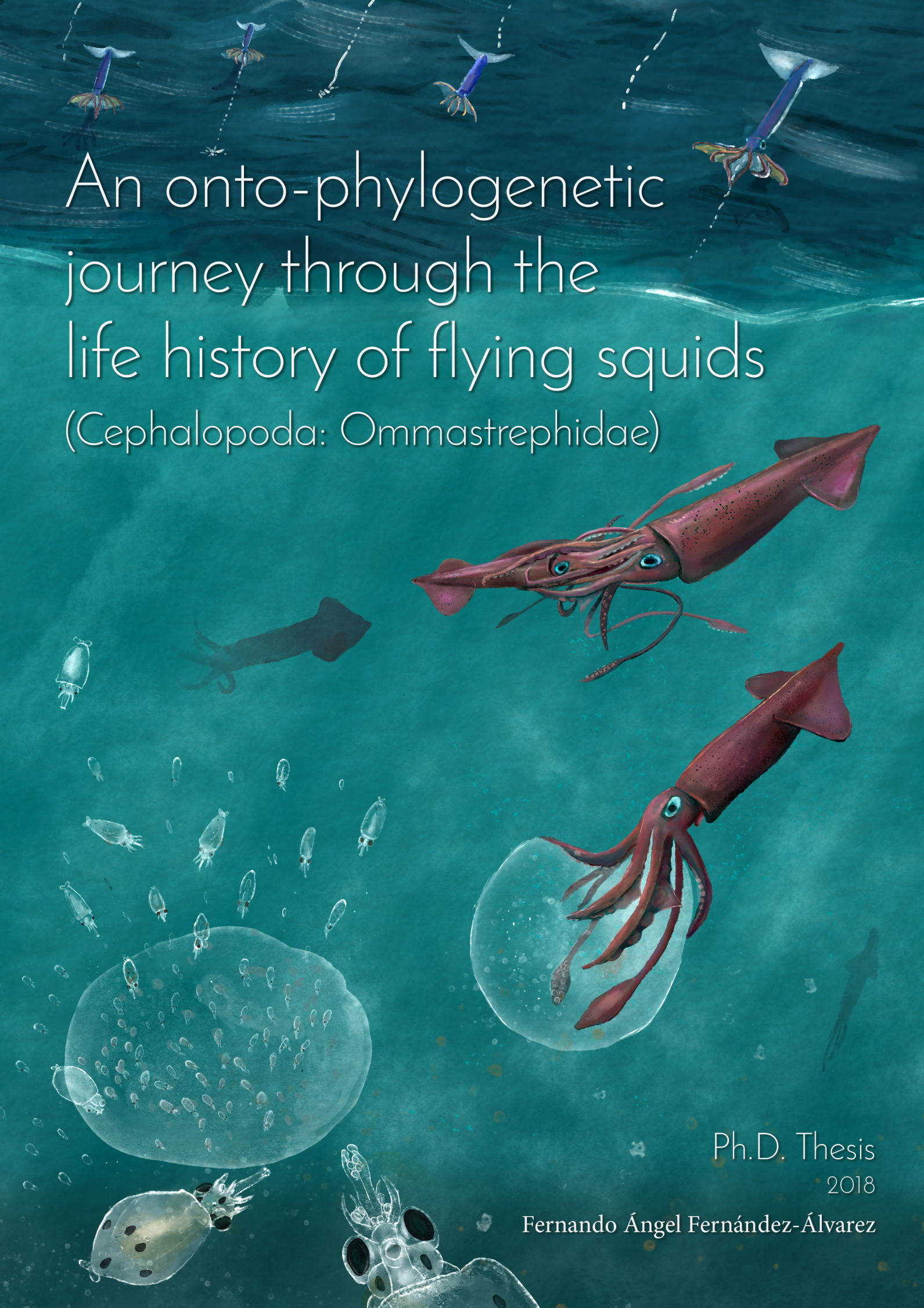
An onto-phylogenetic journey through the life history of flying squids

(Cephalopoda: Ommastrephidae)



Ph.D. Thesis

2018

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UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

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Thesis presented to obtain the Ph. D. Degree by the Polytechnic University of Catalonia (Ph. D. Program in Marine Science)

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"*It is not down in any map; true places never are*"

Melville H. (1851) Moby-Dick

OMNIA VINCIT CONSTANTIA

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me divertí un montón: espero volver a disfrutar de tu compañía algún día. Karmina Arroyo y el pueblo de Santa Rosalía fueron muy acogedores durante mi estancia: ¡viva México!

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Por último, un reconocimiento a todas esas paralarvas, juveniles, subadultos y adultos de numerosas especies de cefalópodos que, en contra de su voluntad, han dado su vida por la ciencia bajo mi Reinado del Terror. ¡Larga Preservación (en colecciones científicas)!

Abstract

Flying squids develop all its life cycle in the water column, as planktonic paralarvae and then as nektonic subadults and adults. In this Ph. D. Thesis, light was shed over several poorly understood aspects of the ontogeny and phylogeny of the Family Ommastrephidae. The mechanism of sperm migration from spermatangia to the female seminal receptacles was studied. Spermatozoa are able to actively migrate between both structures. The morphology of the hatchling of three Mediterranean ommastrephid species was studied based on embryos obtained by *in vitro* fertilization and a dichotomous key was develop to identify NE Atlantic species. The first feeding diet of paralarvae was assessed through laser-capture microdissection and DNA metabarcoding. The results indicate an ontogenetic shift from detritivorism to active predation. Molecular data indicate that the taxonomic name *Ommastrephes bartramii* actually hides four biological species. These advances in scientific knowledge have potential applications for a better understanding of the ecology, physiology, biodiversity and fishery science that will foster a deeper understanding of flying squids.

Keywords: Ommastrephidae, ontogeny, phylogeny, sperm storage, paralarval morphology, first feeding of paralarvae, speciation

Resumen

Las potas desarrollan todo su ciclo vital en la columna de agua, como paralarvas planctónicas y luego como subadultos y adultos nectónicos. En esta Tesis Doctoral, se ha arrojado luz sobre algunos aspectos poco estudiados de la ontogenia y la filogenia de Familia Ommastrephidae. Se estudió el mecanismo de migración espermática desde los espermatangios hasta los receptáculos seminales de la hembra. Los espermatozoides son capaces de migrar activamente entre ambas estructuras. Se estudió la morfología de los recién nacidos de tres especies mediterráneas de pota basándose en embriones generados por fecundación *in vitro* y se desarrolló una clave dicotómica para identificar las especies del Atlántico NE. Se estudió la primera alimentación de las paralarvas mediante una combinación de microdisección láser y DNA metabarcoding: los resultados indican una fase detritívora seguida de un cambio ontogenético hacia la depredación activa. Nuestros datos moleculares muestran que bajo el nombre taxonómico *Ommastrephes bartramii* en realidad se esconden cuatro especies biológicas. Estos avances en el conocimiento científico tienen aplicaciones potenciales para una mejor comprensión de la ecología, fisiología, biodiversidad y ciencia pesquera de las potas.

Palabras clave: Ommastrephidae, ontogenia, filogenia, almacenaje de esperma, morfología de paralarvas, primera dieta de paralarvas, especiación

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Pacific (group 3) and *O. cylindraceus* in South Atlantic (group 2). The closing of the Isthmus of Panama, the Atlantic and Pacific populations got reproductively isolated and the rising of the Gulf Stream and the Pacific Equatorial Current produced the geographic current distribution limits of *Ommastrephes* species in both Atlantic and Pacific waters.

Fig 4.6. Evolutionary scenario 2. (**A**) Between the late Miocene and Early Pliocene the ancestor of *Ommastrephes* spp. distributed in Pacific waters and performed migrations to the Atlantic Ocean. (**B**) The closing of the Isthmus of Panama (~3 mya) reproductively isolated the ancestor of *Ommastrephes caroli* (group 1) from the remaining *Ommastrephes* populations. The oceanic current pattern resulting from the closing of the Isthmus of Panama (the Gulf Stream and the Atlantic and Pacific Equatorial Currents) isolated *Ommastrephes caroli* in North Atlantic and *Ommastrephes bartramii* in North Pacific waters (**C**), respectively. (**C**) Posteriorly, part of the Southwestern Pacific populations migrated into the Indian Ocean and spread across the South and Equatorial Atlantic waters. The East Australian Current and the South Indian currents limited the genetic exchange between Southern Hemisphere groups, producing the last speciation event between *Ommastrephes brevimanus* (group 3) and *Ommastrephes cylindraceus* (group 2). (**D**) The Indian and Atlantic Equatorial Current limited the spreading towards the North of *O. cylindraceus*, creating the current distribution pattern of *Ommastrephes* species.

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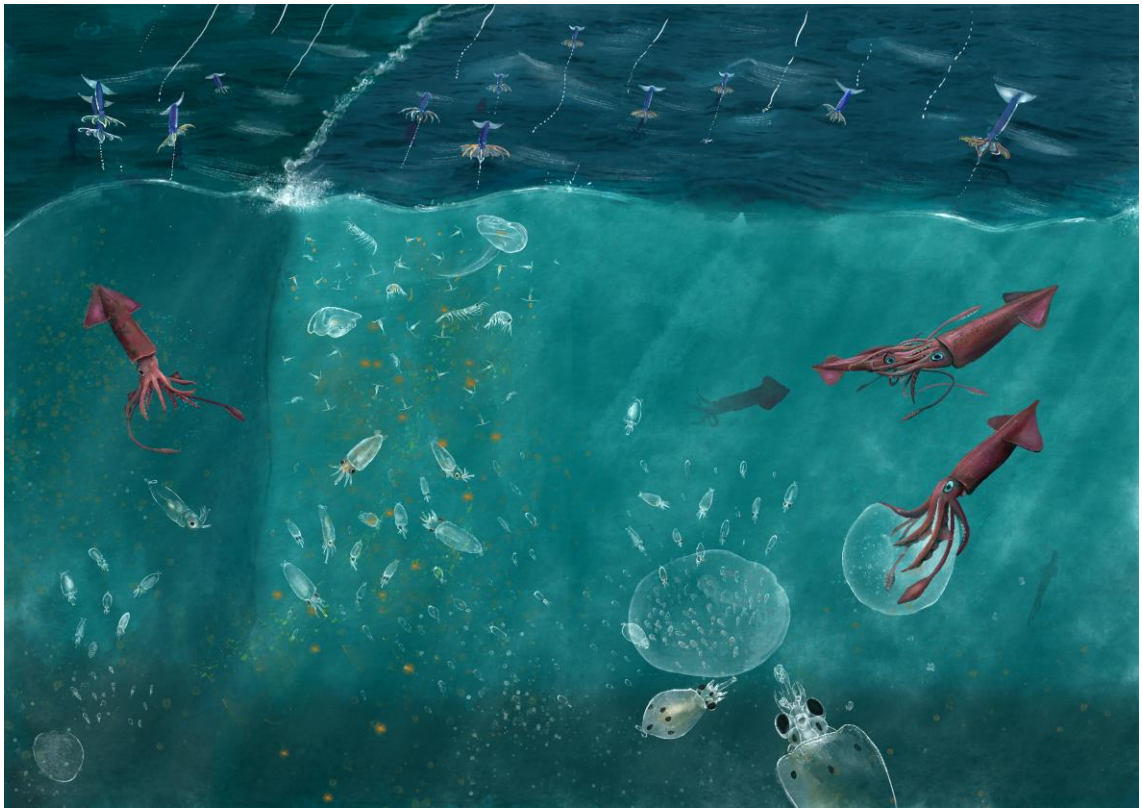
INTRODUCTION



The Family Ommastrephidae Steenstrup, 1857

Ommastrephid squids are distributed in all the world´s oceans and are considered the most economically important family of cephalopods (Jereb & Roper, 2010). Their abundance, rapid growth and massive body size make them one of the most exploited invertebrate fishing resources (Arkhipkin et al., 2015a). Their ability to glide over the sea surface to avoid predators gave them their vernacular name "flying squids" (Fig. 1A). Two taxonomic characters easily identify members of the Family Ommastrephidae: the typical funnel/mantle locking apparatus with an inverted "T" shape (Fig. 1B) and the rhynchoteuthion paralarvae (Fig. 1C). The planktonic rhynchoteuthion paralarvae are unique among cephalopod paralarvae due the fusion of both tentacles in a proboscis with an unknown function (Chapter 2).

Ommastrephids are nektonic organisms with a huge range of maximum size-at-maturity, ranging from the 80 mm of mantle length (ML) of the smallest species, *Hyaloteuthis pelagica* (Bosc, 1802), to more than 1 m of the largest family representative, the jumbo squid *Dosidicus gigas* (d'Orbigny, 1835). They occupy the pelagic habitat, ranging from the sea surface to oceanic depths of nearly 2000 m, but they usually perform diel vertical migrations. Some species have relation with the continental self and even develop resting behaviors over the seafloor (Harrop et al., 2014), but many species have an exclusively nektonic life. They are fast and voracious predators, predating mainly over small to medium size preys, as krill or fishes of the Family Myctophidae Gill, 1893 (e.g., Rosas-Luis et al., 2014). In general terms, they are very abundant in oceanic waters and have wide distribution areas.

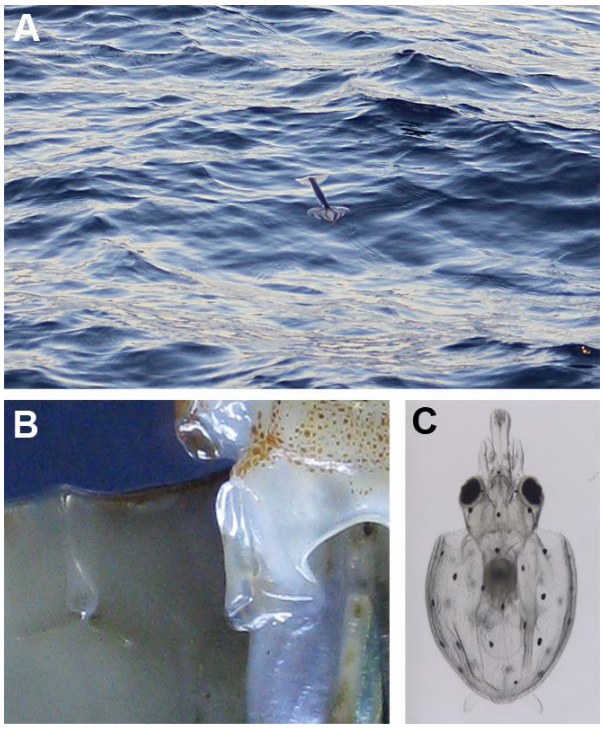


Fig. 1. (**A**) Ommastrephid squid gliding over the sea surface. Photo credit: Rob Leslie. (**B**) Funnel/mantle locking apparatus with an inverted "T" shape of a *Illex coindetii* adult female, the mantle was dissected to show both the mantle component (left) and the funnel component (right). (**C**) *Todaropsis eblanae* paralarva obtained by *in vitro* fertilization (see detailed information in Chapter 2).

In the taxonomic lists, the Family Ommastrephidae is commonly reported as including 22 valid species (e.g., Jereb & Roper, 2010). Traditionally, three subfamilies have been recognized (Roeleveld, 1988; Wormuth, 1998): Illicinae Posselt, 1891, Todarodinae Adam, 1960 and Ommastrephinae Posselt, 1891. This classification was based in the presence/absence of photophores, the

morphology of the funnel grove, the arrangements of the longitudinal lines of suckers in dactylus of the tentacle, the morphology of the horny rings of the manus of the tentacle (Fig. 2) and the hectocotylus morphology (see Roeleveld, 1988 for details). Particularly difficult is the position of *Todaropsis eblanae*, whose placement varies according with the taxonomists´s criteria, being allocated as Illicinae (e.g., Roper et al., 1984; Guerra, 1992) or Todarodinae (e.g., Roeleveld, 1988; Jereb & Roper, 2010). The Subfamily Todaropsinae Nigmatullin, 2000 has been proposed to place this species. The taxonomic position of *Ornithoteuthis* Okada, 1927 is also controversial and another Subfamily has been proposed: the Subfamily Ornithoteuthinae Nigmatullin, 1979. The Figure 2 summarizes the most important morphological characters of each subfamily and the problematic genera *Todaropsis* and *Ornithoteuthis*.

Molecular systematics works (Lindgren et al., 2012, Pardo-Gandarillas et al., 2018) showed a closer relation between *Todaropsis* and *Illex* Steenstrup, 1880 instead that between *T. eblanae* with members of the Subfamily Todarodinae, as traditional taxonomy considered (Roeleveld, 1988; Wormuth, 1998). Escolar Sánchez (2017) studied the *T. eblanae* spermatozoa and embryos and provided morphological support to the clade formed by *T. eblanae* and *Illex* spp., as well as the placement of *T. eblanae* as the single representative of the Subfamily Todaropsinae. The Figure 3 summarized the differences between the cladistic phylogram of Roeleveld (1988) and the molecular phylogeny of Pardo-Gandarillas et al. (2018).

The placement of *Ornithoteuthis* is also controversial: traditional morphological studies placed this genus among Ommastrephinae (e.g., Jereb & Roper, 2010). However, molecular studies place this species between the members of the Subfamily Todarodinae (Wakabayashi et al., 2012; Pardo-Gandarillas et al., 2018). Two solutions are possible to overcome this problem: **a)** the establishment of at least a new Subfamily to place some Todarodinae species (*Nototodarus* spp. and *Todarodes pacificus* (Steenstrup, 1880)) in combination with the recognition of Ornithoteuthinae, or **b)** to include *Ornithoteuthis* inside Todarodinae. Although it might seem taxonomically more straightforward to include the genus *Ornithoteuthis* within the Subfamily Todarodinae, as Wakabayashi et al. (2012) and Pardo-Gandarillas et al. (2018) suggested, this solution might also be complicated from a morphological point of view. To the best of my knowledge, no shared apomorphies are known between *Ornithoteuthis* and members of the Subfamily Todarodinae. Thus, this taxonomy runs the risk of being based in plesiomorphic characters instead in synapomorphies. Although more taxonomic studies are necessary to solve the taxonomy at Subfamily level of the Family Ommastrephidae, I think the best possible solution is the recognizition of the Subfamily Ornithoteuthinae together with the taxonomic revision of the Subfamily Todarodinae, which is beyond the scope of the present Ph. D. Thesis.

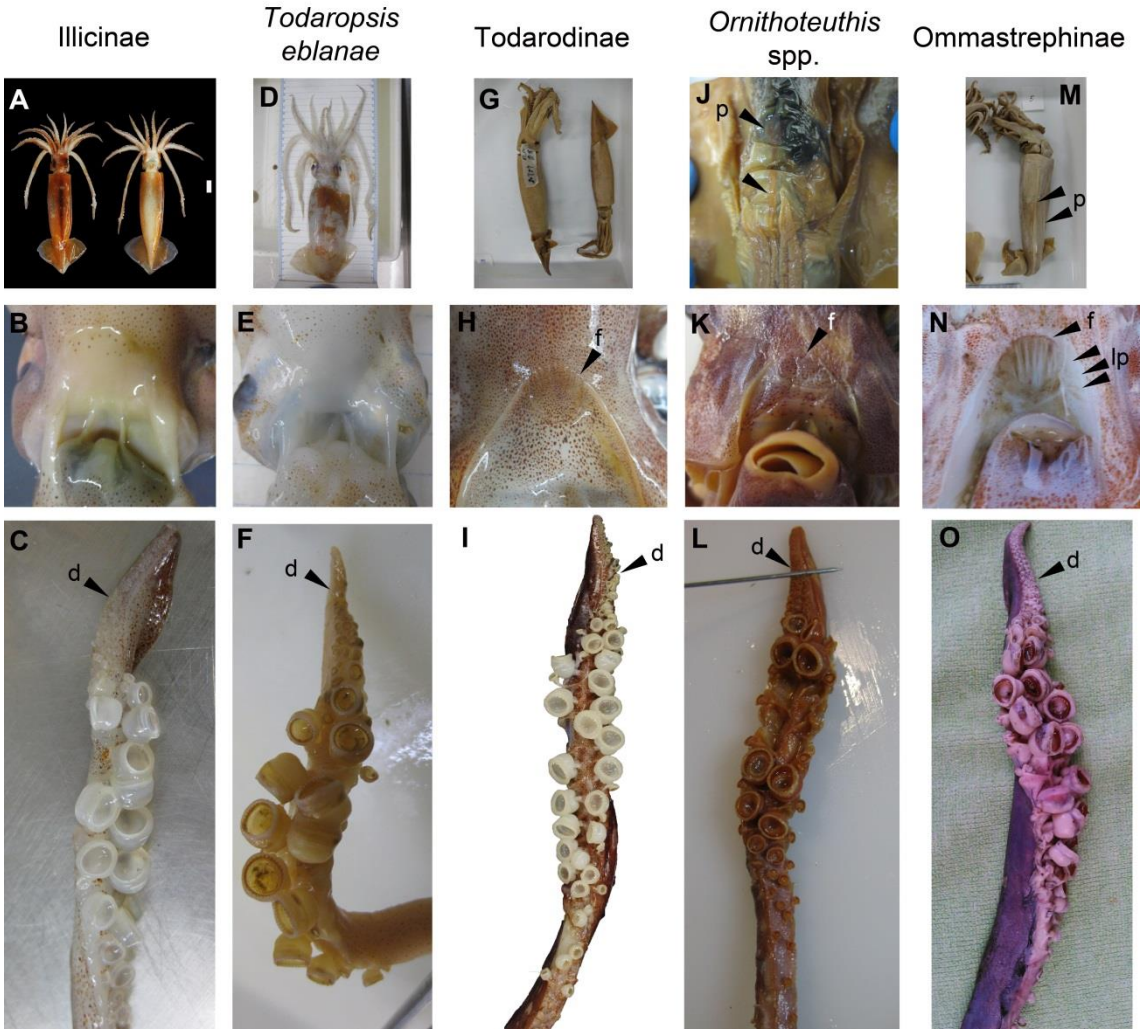


Fig. 2. Most important morphological characters at the subfamily level. (**A-C**) Illicinae. (**A**) General morphology of *Illex coindetii*. Photo credit: Elisabeth Cuesta-Torralvo. (**B**) Funnel grove of *Illex argentinus*. (**C**) Tentacular stalk of *I. coindetii*, with 8 longitudinal rows of dactylar suckers and smooth horny rings in the manus suckers. (**D-F**) *Todaropsis eblanae*. (**D**) General morphology. Photo credit: Oscar Escolar. (**E**) Funnel grove. Photo credit: Oscar Escolar. (**F**) Tentacular stalk, with 4 longitudinal rows of dactilar suckers and regular teeths in the horny rings of the manus suckers. (**G-I**) Todarodinae. (**G**) General morphology of *Todarodes pacificus*, Madoka Sasaki collection (Hokkaido University). (**H**) Funnel grove of *Todarodes sagittatus*, with foveola. (**I**) Tentacular stalk of *T. sagittatus*, with 4 longitudinal rows of dactylar suckers and horny rings of the manus suckers with teeth of regular size. Photo credit: Elisabeth Cuesta-Torralvo. (**J-L**) *Ornithoteuthis volatilis*. (**J**) Dissected specimen (CBR-ICM, ICMC112-2002) showing the presence of visceral photophores. (**H**) Funnel grove of the specimen ICMC112-2002, with foveola. (**L**) Tentacular stalk of the specimen MC113-2002, with 4 longitudinal rows of dactylar suckers and horny rings of the manus suckers with teeth of regular size. (**M-O**) Ommastrephinae. (**M**) General morphology of *Eucleoteuthis luminosa*, type material from the Madoka Sasaki collection (Hokkaido University). (**N**) Funnel grove of *Ommastrephes caroli*, with foveolar and lateral pockets. (**O**) Tentacular stalk of *O. caroli*, with 4 longitudinal rows of dactylar suckers and horny rings of the manus suckers with 4 larger teeth forming a square. Abbreviations: d, dactylus; f, foveola; lp, lateral pockets; p, photophores.

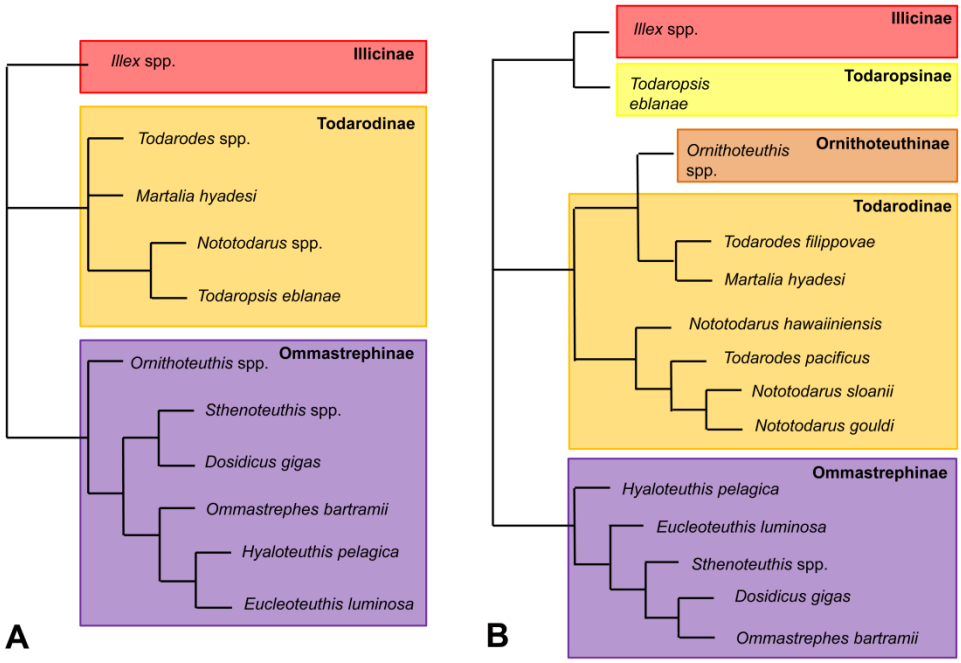


Fig. 3. Brief summary of different systematic treatments for the Family Ommastrephidae. (**A**) The traditional three subfamilies taxonomy based on Roeleveld (1988). (**B**) Systematic treatment adopted in this work based on the published molecular phylogeny of Pardo-Gandarillas et al. (2018). The inconsistency between the two phylogenetic trees of Pardo-Gandarillas et al. (2018) was solved adding a basal polytomy between the clades (Todaropsinae(Illicinae)), (Todarodinae (*Ornithoteuthis* spp.)) and Ommastrephinae. Note that the genus *Todarodes* is diphyletic and the subfamily Todarodinae as considered here is paraphyletic.

Based in the reasons explained above, the taxonomy presented here considers the subfamilies Ornithoteuthidae and Todaropsinae. It must be pointed out that Todarodinae, as considered in this work, is probably paraphyletic and needs to be revised. Additionally, the results of the Chapter 4 of the present Ph. D. Thesis recovered 4 species for the genus *Ommastrephes* D´Orbigny, 1835, instead a single cosmopolitan species recognized in the most recent taxonomic lists (e.g., Jereb & Roper, 2010). In the following taxonomic list all the species of the genus *Ommastrephes* are considered, resulting in 25 flying squids (13 % increase in the number of valid species of the Family Ommastrephidae).

Family Ommastrephidae Steenstrup, 1857

1. Subfamily Illicinae Posselt, 1891

1.1. Genus *Illex* Steenstrup, 1880

1.1.1. *I. illecebrosus* (Lesueur, 1821) [type species]

1.1.2. *I. argentinus* (Castellanos, 1960) (Fig. 2B)

1.1.3. *I. coindetii* (Verany, 1839) (Fig. 2A, C; Fig. 4A)

1.1.4. *I. oxygonius* Roper, Lu & Mangold, 1969

2. Subfamily Ommastrephinae Posselt, 1891

2.1. Genus *Ommastrephes* d'Orbigny, 1834 [in 1834–1847]

2.1.1. *O. bartramii* (Lesueur, 1821) [type species]

2.1.2. *O. brevimanus* (Gould, 1852)

2.1.3. *O. caroli* (Furtado, 1887) (Fig. 2N-O)

2.1.4. *O. cylindraceus* (d'Orbigny, 1835) [in 1834-1847] (Fig. 4E)

2.2. Genus *Dosidicus* Steenstrup, 1857

2.2.1. *D. gigas* (d'Orbigny, 1835) [in 1834–1847]

2.3. Genus *Eucleoteuthis* Berry, 1916

2.3.1. *E. luminosa* (Sasaki, 1915?) (Fig. 2M)

2.4. Genus *Hyaloteuthis* Gray, 1849

2.4.1. *H. pelagica* (Bosc, 1802)

2.5. Genus *Sthenoteuthis* Verrill, 1880

2.5.1. *S. oualaniensis* (Lesson, 1830–1831) [type species]

2.5.2. *S. pteropus* (Steenstrup, 1855)

3. Subfamily Ornithoteuthinae Nigmatullin, 1979

3.1. Genus *Ornithoteuthis* Okada, 1927

3.1.1. *O. volatilis* (Sasaki, 1915) [type species] (Fig. 2J-L)

3.1.2. *O. antillarum* Adam, 1957

4. Subfamily Todarodinae Adam, 1960

4.1. Genus *Todarodes* Steenstrup, 1880

4.1.1. *T. sagittatus* (Lamarck, 1798) [type species] (Fig. 2G, I; Fig. 4C)

4.1.2. *T. angolensis* Adam, 1962

4.1.3. *T. filippovae* Adam, 1975

4.1.4. *T. pacificus* (Steenstrup, 1880) (Fig. 2G)

4.1.5. *T. pusillus* Dunning, 1988

4.2. Genus *Martialia* Rochebrune & Mabille, 1889

4.2.1. *M. hyadesi* Rochebrune & Mabille, 1889

4.3. Genus *Nototodarus* Pfeffer, 1912

4.3.1. *N. sloanii* (Gray, 1849) [type species]

4.3.2. *N. gouldi* (McCoy, 1888)

4.3.3. *N. hawaiiensis* (Berry, 1912)

5. Subfamily Todaropsinae Nigmatullin, 2000

5.1. Genus *Todaropsis* Girard, 1890

5.1.1. *T. eblanae* (Ball, 1841) (Fig. 2D-F; Fig. 4B)

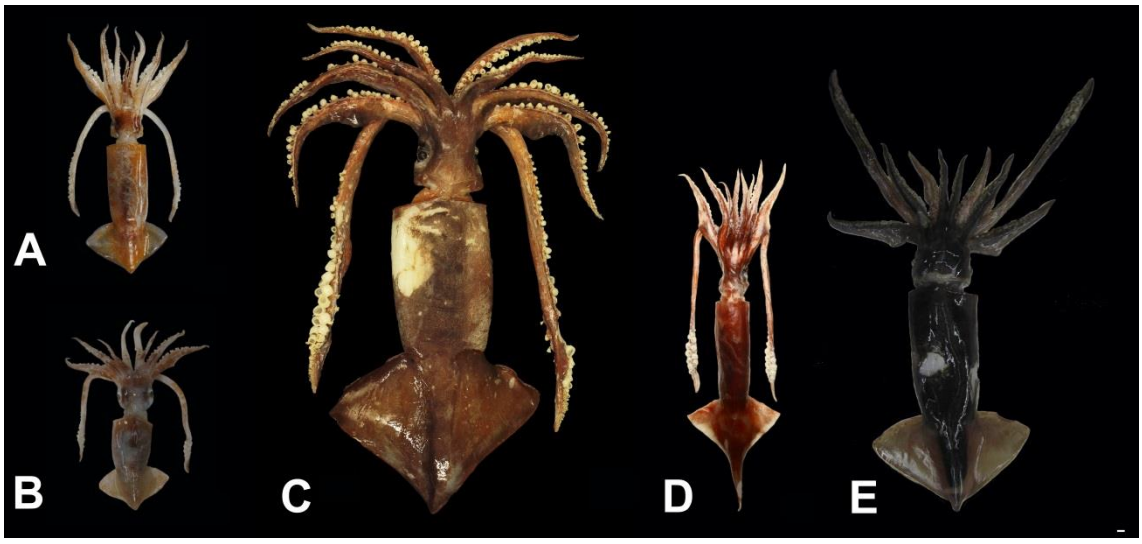


Fig. 4. Some representative species of the Family Ommastrephidae. All the specimens are mature or almost mature in order to give a good idea of the actual size of the species. (**A**) *Illex coindetii*, male, 17 cm mantle length (ML). Photo credit: Elisabeth Cuesta-Torralvo. (**B**) *Todaropsis eblanae*, male, 11 cm ML. Photo credit: Oscar Escolar. (**C**) *Todarodes sagittatus*, female, 39 cm ML. Photo credit: Elisabeth Cuesta-Torralvo. (**D**) *Ornithoteuthis* sp., female, 22 cm ML. Photo credit: Rob Leslie. (**E**) *Ommastrephes cylindraceus*, male, 26 cm ML. Scale bar: 1 cm.

Life cycle of ommastrephid squids

Flying squids develop all its life cycle in the water column, first as planktonic paralarvae (Villanueva et al., 2016) and then as nektonic juveniles, subadults and adults (Jereb & Roper, 2010). A few species, especially members of the genus *Illex* (Harrop et al., 2014) have a closer relationship with the ocean floor, but many others, especially members of the subfamilies Ommastrephinae and Ornithoteuthinae, are truly oceanic and do not interact with benthic environments in normal conditions. The life cycle of ommastrephids is relatively short, ranging from 6 months to 2 years (Rosa et al., 2013; Arkhipkin et al., 2015b) allowing them to quickly respond to environmental changes (Hoving et al., 2013). With the exception of *Vampyroteuthis infernalis* Chun, 1903 (Hoving et al., 2015) and the nautiluses (Jereb & Roper, 2005), the remaining cephalopods are semelparous organisms (Rocha et al., 2001). Flying squids are intermittent spawners, meaning they can produce several egg masses during their unique reproductive cycle situated at the end of their life cycle. It is not totally understood how many egg masses is able to spawn a single ommastrephid female, but the maximum value for *D. gigas* is roughly estimated as 8-12 (Nigmatullin & Markaida, 2009).

Although the fertilization of squids (in broad sense) and cuttlefishes ova occur in the external medium (Hanlon & Messenger, 1996), they do not directly eject their eggs and spermatozoa to the sea, as occurs in many other marine taxa (Brusca & Brusca, 2003), and the process is far more complicated and poorly understood. Representants of 8 different families (Bathyteuthidae Pfeffer, 1900, Idiosepiidae Appellöf, 1898, Loliginidae Lesueur, 1821, Ommastrephidae, Sepiadaridae Fischer, 1882 in 1880–1887, Sepiidae Keferstein, 1866, Spirulidae Owen, 1836 and Thysanoteuthidae Keferstein, 1866) of external fertilizing cephalopods are able to storage sperm in specific organs allocated in their buccal membranes, known as seminal receptacles (SRs from now on) (Marian, 2015). Ommastrephidae and Thysanoteuthidae are the only cephalopods provided with multiple SRs around the buccal membrane (Fig. 5A-B; Chapter 1).

Cephalopod males transfer the sperm to the females through complex spermatophores, which are able to eject a capsule full of sperm after a complex osmotic reaction known as spermatophoric reaction. Once the spermatophore reaction is triggered, the spermatangium (the capsule containing the sperm) is implanted in the female tissues through an injure and the spermatophore is able to automously release the sperm. Although the spermatangia are able to storage and directly fertilize the ova (Marian, 2015), SRs are likely able to storage the spermatozoa for long periods of time, and allows the existence of a delay between mating and fertilization (e.g., Fernández-Álvarez et al., 2018a). In cephalopod with SRs in the oral membrane, as ommastrephids, males usually deploy the spermatangia in the buccal area. The mechanism responsible of the transfer of the spermatophores to the SRs was the subject of the

Chapter 1. While Todarodinae (Fig. 5A), Todaropsinae (Fig. 5B), Ommastrephinae and Ornithoteuthinae hold SRs, members of the subfamily Illicinae are devoid of these structures. Illicinae males insert groups of spermatangia inside the mantle cavity of the females, near the gills and in the vicinity of the oviducts openings (Fig. 5C-D). Fertilization likely occurs when females release the oocytes from the oviducts, but how much time spermatangia are releasing sperm is unknown. Thus, males of this subfamily are more likely to loss a significant part of their sperm and mating energetic investment if mating and egg mass release are too separated in time. A similar placement for spermatangia implantation is known for consort males in some loliginid squids (Hanlon et al., 2002; Iwata et al., 2011), which are known to guard a mated female until spawning takes place, in order to increase the number of eggs they fertilize (Naud et al., 2016). It is unkown if a similar consort behaviour occur in Illicinae.

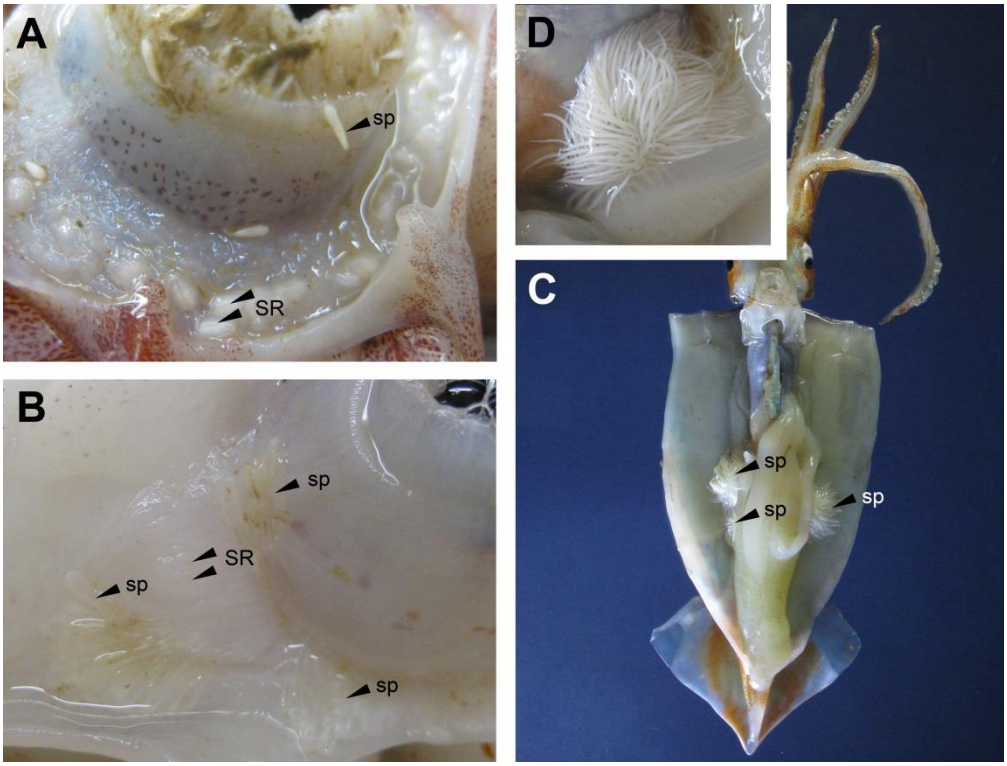


Fig. 5. Sperm storage in ommastrephid squids. (**A**) Buccal area of a mature female of *Todarodes sagittatus*, showing the arrangement of the seminal receptacles (SRs) in the oral membrane and the implanted spermatangia (sp). (**B**) Buccal area of *Todaropsis eblanae*. (**C**) Dissected mature female of *Illex coindetii* showing the spermatangia clusters implanted inside the mantle cavity. (**D**) Close-up of a spermatangia cluster.

Ommastrephid egg masses are large mucous spheres filled with thousands to millions of developing embryos (Staaf et al., 2008; Puneeta et al., 2015; Birk et al., 2017). Since egg masses are spawned in oceanic waters, encounters are scarce (O´Shea et al., 2004) and great part of current scientific knowledge is based in observations from laboratory spawning events (e.g., Boletzky et al., 1973; Staaf et al., 2008; Puneeta et al., 2015). During spawning, the oocytes

allocated inside the oviduct (Fig. 6A) are released (Fig. 6B). With the exception of Illicinae, ommastrephid oocytes are fertilized by the spermatozoa released by the SRs, likely in the buccal area (Vijai, 2016). During spawning, fertilized eggs are embedbed by mucous layers released by oviducal and nidamental glands (Fig. 6C-D). The egg masses apparently rest over the pycnocline (Staaf et al., 2008; Punneta et al., 2015; Birk et al., 2017). Laboratory experiments (e.g., Villanueva et al., 2011) demostrated that temperature critically affect the embryonic development of ommastrephid squids (i.e., embryos developed at higher temperature develop quicker). For instance, *Illex coindetii* embryos hatch at day 10 of development at 17 ºC, while it take only 7 days at 21 ºC (Villanueva et al., 2011). Thus, the length of the embryo development varies according with the water mass temperature, but likely this interval range from one week to two weeks.

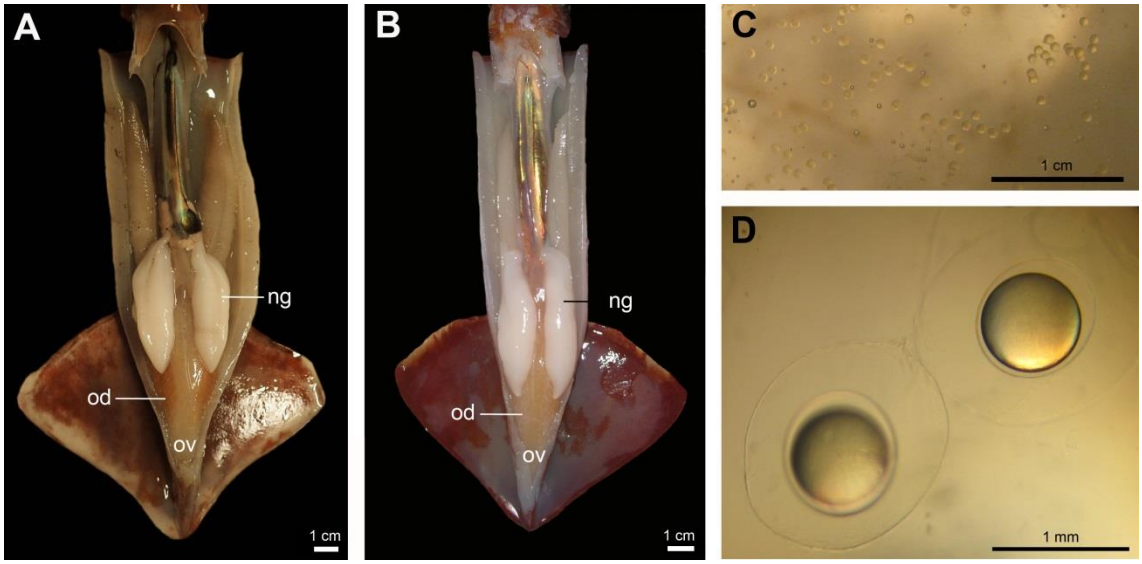


Fig. 6. Humboldt squid (*Dosidicus gigas*) aquarium spawning (Santa Rosalía, México, August 12 2015). Oocytes are generated in the ovary (ov) and finish their maturation in the oviduct (od). The nidamental glands (ng) produce a mucus cover of the egg mass. (**A**) Dissected mature female with their oviducts filled with mature oocytes, as expected just before spawning. This female is unrelated with the spawning event. (**B**) Dissected female just after the release of a egg mass in aquarium, showing empty oviducts and smaller nidamental glands. (**C**) Part of the egg mass produced by the female of the Fig. 6B. (**D**) Close up of two fertilized eggs.

Ommastrephid hatchlings (Fig. 1C) are among the smallest of cephalopods (Villanueva et al., 2016) and also have a minor degree of development than in other cephalopod families (Shigeno et al., 2001a, b). While other cephalopods, such as loliginids (e.g., Fernández-Álvarez et al., 2017a) hatch with the total amount of arms and tentacles, ommastrephid hatchlings usually lack of the arm pair III. They also have an undeveloped nervous, respiratory and digestive systems (Shigeno et al., 2001a, b) and beaks (Uchikawa et al., 2009). Other unique characters are the presence of filamentous buccal papillae (Fernández-Álvarez et al., 2018b) and the fusion of both

tentacles in a proboscis, both features with an unknown function. This characteristic morphology allows the easy recognition of ommastrephids among cephalopod paralarvae. Although morphological differences among ommastrephid paralarvae have been commonly reported in the literature (e.g., Roper & Lu, 1979), the identification at specific level is usually challenging due to the absence of a known link between the paralarval and adult morphology (Villanueva et al., 2012; Fernández-Álvarez et al., 2017b). In the present Ph. D. Thesis (Chapter 2), *in vitro* fertilization of gametes obtained from species-level identified adults of *Illex coindetii* (Fig. 4A), *Todaropsis eblanae* (Fig. 4B) and *Todarodes sagittatus* (Fig. 4C) were performed in order to describe their hatchlings morphologies and provide a reliable identification key of rhynchoteuthion paralarvae for NE Atlantic waters.

During its planktonic life, ommastrephid paralarvae suffer morphological changes, especially regarding the morphology of the arm crown and the buccal area. During their early life, the arm III stalks appear and start its development, the arms elongate and acquire more suckers, the proboscis starts to split in two raptorial appendages, the filamentous buccal papillae disappear and the beaks protrude. When ommastrephid paralarvae reach ~4-6 mm ML, the buccal papillae completely disappear, the arm pair III is functional and the sensory and nervous system reach the same degree of development than in other predatory cephalopod paralarvae (Shigeno et al., 2001a, b). Interestingly, it is in this stage when recognizable preys start to appear inside their guts (e.g., Vidal & Haimovici, 1998; Uchikawa et al., 2009; Camarillo-Coop et al., 2013). The absence of recognizable prey in the gut content of the early paralarvae coupled with their morphology, which is not well suited for hunting and eating live preys, suggesting a different feeding mode that active predation for this ontogenetic phase. Parry (2008) compared the stable isotopic composition of early paralarvae (less than 3mm ML) and adult specimens of the two sympatric Pacific species (*O. bartramii* and *S. oulaniensis*). He did not found significant differences on the trophic level of the paralarvae of both species, while the trophic level of both species increased with their size. The work of Parry (2008) also suggests the existence of an ontogenetic shift in the diet of ommastrephid squids. The Chapter 3 of this Ph. D. Thesis casts light on this long-standing mystery of the ommastrephid life cycle.

Ommastrephids are able to reach massive sizes in a short period of time. For instance, the jumbo squid (*D. gigas*) hatchlings measure 1.1 mm ML (Yatsu et al., 1999) and are able to reach 1000 mm ML and 40-60 kg weight (Rosa et al., 2013) in less than 2.5 years (Arkhipkin et al., 2015b), which the maximum recorded life span of any member of this family. In the Figure 7 the size difference between hatchlings and adults is graphically represented for *Todarodes sagittatus*, a medium-size ommastrephid species. In fact, ommastrephid squids are the fastest growing squids (Jereb & Roper, 2010). Their maximum relative daily growth rates range from 3-4 to 10-12 % of body weight during their juvenile phase, which drop to the still impressive

value of 2-3 % during their subadult phase (Jereb & Roper, 2010). Growth usually decreases or stops with sexual maturity. The tag and recapture experiments of Markaida et al. (2005) demonstrated a growth rate of 1 mm/day for *D. gigas*. Obviously, this impressive growth rate is impossible to attain without eating loads of prey. Thus, late paralarvae, juvenile, subadult and adult ommastrephid squids are voracious predators (e.g., Camarillo-Coop et al., 2013; Rosas-Luis et al., 2014; Merten et al., 2017). The size and taxonomic composition of their prey varies with the size of the squids (Jereb & Roper, 2010). Shchetinnikov (1992) studied the diet of *S. oualaniensis* and revealed three ontogenetic size-groups: group I (40-100 mm ML) feed on micronektonic and epipelagic plankton, group II (100-150 mm ML) feed on planktonic crustaceans, fish larvae and myctophid fishes, and group III (150-365 mm ML), which feed primarily on myctophids and secondarily on squids. This general trend is commonly found in other ommastrephid species (Jereb & Roper, 2010). Cannibalism is also frequent, but studies sometimes struggle to assess if it is a sampling artifact or a natural component of the diet (Markaida & Sosa-Nishizaki, 2003; Ibarra-García et al., 2014). High occurrence of cannibalism behavior has been confirmed by direct observation in some species of other oceanic squid family (e.g., Hoving & Robison, 2016).

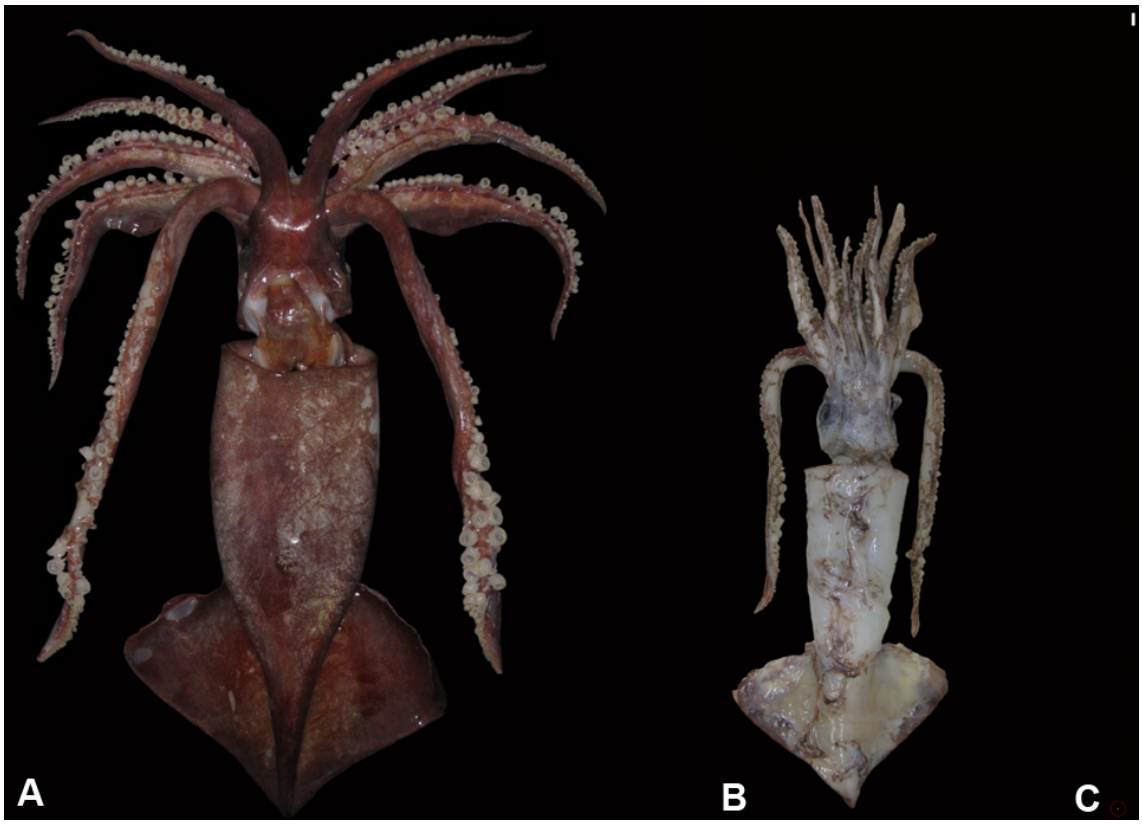


Fig. 7. *Todarodes sagittatus*. Comparative size of a maturing female (**A**, photo credit: Elisabeth Cuesta-Torralvo), a mature male (**B**) and a hatchling (**C**) obtained by *in vitro* fertilization. Scale bar: 1 cm.

Ommastrephid males usually reach smaller size than females (Fig. 7). This is probably due to their earlier sexual maturation and the subsequent decrease of somatic growth (Jereb & Roper, 2010). In some species, this difference of size might be really important and a mature female represents from twice to three times the weight of a mature male (Rosa et al., 2013). Since the mating usually is performed in a head-to-head position and squids sometimes are cannibalistic, a comparatively smaller male might suffer a great risk during mating. However, Fernández-Álvarez et al. (2018a) revealed that immature females usually storage spermatozoa. Mating with younger smaller immature females might be an adaptation to avoid cannibalism risk in ommastrephid squids. Once males had diploid their spermatangia in the buccal area of the female, the cycle starts again…

And again…

And again…

And again [*n* times]

From life history and intraspecific polymorphism to taxonomic decisions: current challenges to understand the ommastrephid biodiversity

Members of the Family Ommastrephidae are nektonic opportunistic predators that occupy a similar bathymetric range, and that is especially true when comparing congeneric species. Thus, they are subject of similar selective pressures that lead to a low level of morphological divergence at the interspecific level. For instance, members of the genus *Illex* have a very similar morphology and the diagnostic differences between the four known species are only present in the hectocotylus of the mature males (Jereb & Roper, 2010). However, the range of variation of these characters overlaps between all species. Although molecular methods proved the presence of 4 species in the genus *Illex*, they also pointed out that species identification is extremely challenging, especially in the NW Atlantic, where three species cohabit (Carlini et al., 2006). If this was not enough, for some species several morphotypes are known, especially regarding the size-at-maturity. Sometimes, these morphotypes are geographically isolated but they also can alternate in different years in the same area, which complicate the taxonomic work (see below). Additionally, for many ommastrephid species the biological knowledge in many fundamental aspects is still fragmentary or vague. This scenario likely has its origin in the combined effect of the huge variability ommastrephids are able to endure in many fundamental biological aspects in combination with similar selective pressures operating over them and driving their morphological evolution. The first effect likely widens the range of morphological variation at intraspecific level, while the second homogenizes the morphology among related species. The combination of both effect sometimes lead to a situation of taxonomic uncertainty with some species occupying wide geographic areas, sometimes even so discontinuous that genetic exchange between individuals are not guaranteed.

Many species are able to modulate their size-at-maturity by reducing their lifespan and mature sooner at a smaller size. For instance, females of the small-size-at-maturity morphotype of *D. gigas* represent 12 % of the ML of the large-size-at-maturity one (Nigmatullin et al., 2001), but this phenomenon is also known for other species, as *I. coindetii* or *S. pteropus* (Jereb & Roper, 2010). The extremely variable size-at-maturity morphotypes of *D. gigas* are related with the latitude; Ecuatorial populations mature at a small size (Nigmatullin et al., 2001), but it might also be a response to changing oceanic environmental conditions of a region. For instance, after the El Niño Modoki in 2009-2010, the size-at-maturity of *D. gigas* in the Gulf of California showed a constant decline (Hoving et al., 2013) with extreme small ML sizes in 2015 (Fernández-Álvarez et al., 2018a). The size-at-maturity has an enormous effect in the fecundity of ommastrephids. In 2006, egg masses of the large-size-at-maturity morphotype of *D. gigas* had up to 3 m of diameter and the potential number of eggs in the entire mass ranges from 0.6 to 2 million (Staaf et al., 2008), while in 2015 the egg masses were up to 1.4 m of diameter and

with 17,000–90,000 potential eggs (Birk et al., 2017). This phenotypic plasticity is another impressive example of variability in a fundamental life history trait that allows ommastrephids to survive periods of food depletion as a small-size-at-maturity population or optimally exploit productive habitats and increase the individual fitness in years with high prey availability as a large-size-at-maturity individual (Rosa et al., 2013). Obviously, this huge polymorphic size can raise suspicious of hidden cryptic biodiversity (i.e., several species been taken as a single one), but the molecular study of Staaf et al. (2010) confirmed that *D. gigas* is a single species with a mild geographic structure across its distribution range.

Members of the genus *Sthenoteuthis* are characterized by the presence of a dorsal lemon-shape photophore patch in the dorsal surface of the mantle. It is recognized the presence of two species: *S. pteropus*, in the tropical and subtropical Atlantic, and *S. oualaniensis*, whose distribution range covers the full extension of tropical and subtropical Indian and Pacific oceanic waters. The main morphological difference between both species is the fusion of the mantle and funnel components of the mantle-funnel locking apparatus in *S. oualaniensis*. The dorsal lemon-patch (Fig. 8A) is absent in specimens of less than 100 mm of mantle length for both species (Fig. 8B-C). *Sthenoteuthis pteropus* has a complex population structure with two intraspecific forms: the small early-maturing equatorial and the large late-maturing mostly peripheral forms (Jereb & Roper, 2010), females mature at 150-320 and 320-820 mm ML respectively. Taking into account that three intraspecific morphotypes are recognized for *D. gigas* (Nigmatullin et al., 2001; Jereb & Roper, 2010), this might seem unimpressive. However, if we look at the other species of the genus, the pattern becomes complicated. *Sthenoteuthis oualaniensis* has a wider discontinuous distribution range and up to 5 "intraspecific" have been recognized (Jereb & Roper, 2010). Four of these morphotypes have the distinctive dorsal photophore, however the smallest equatorial form (also known as the "dwarf morphotype") is able to mature at sizes of 80-150 mm ML and do not develop this important taxonomic feature. Is the dwarf morphotype a neotenic intraspecific form or this morphological difference granted this population a distinctive specific status? Staaf et al. (2010) studied the population structure of *S. oualaniensis* in the Eastern Pacific with mitochondrial markers and found evidence of deep reproductive isolation between three different groups. Fernández-Álvarez et al. (2015) compiled the cytochrome oxydase 1 (COI) sequences from GenBank of ommastrephid squids and performed a DNA barcoding study of the family. They found the three previously recognized clades of Staaf et al. (2010) (Fig. 8D) and the analysis of the uncorrected *p*-distances between each clade revealed values ranging from 4.9 to 8.6 % of divergence, which are more in line with interspecific than intraspecific divergence values comparing with other species of cephalopods (e.g., Gebhardt & Knebelsberger, 2015). These molecular data suggest that some of the recognized morphotypes actually represent cryptic species and under the name *S. oualaniensis*

hidden biodiversity exists. The presence of important differences in the spermatophore morphology (Nigmatullin et al., 2003) also suggests the presence of several species.

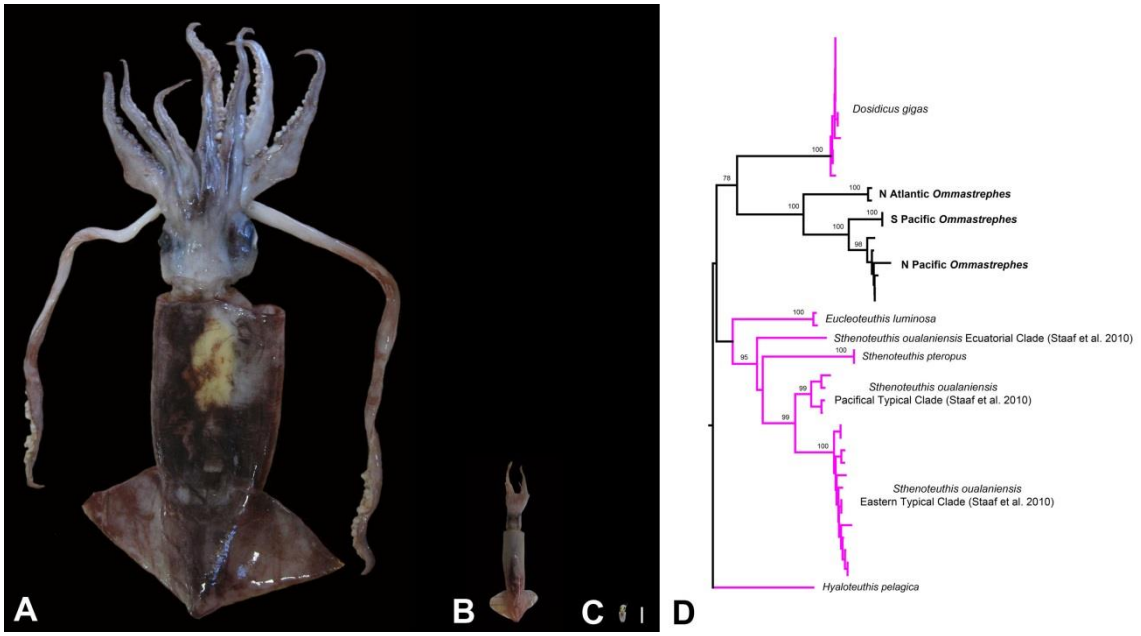


Fig. 8. (A-C) *Sthenoteuthis pteropus*. Immature female with the lemon-shape photophore patch on the dorsal surface of mantle (**A**), while subadults of less than 100 mm ML (**B**) and paralarvae (**C**) lack of this diagnostic character. Scale bar: 1 cm. (**D**) Neighbor joining tree of COI data from Fernández-Álvarez et al. (2015). Values above the branches are bootstrap percentages obtained after 1000 generations.

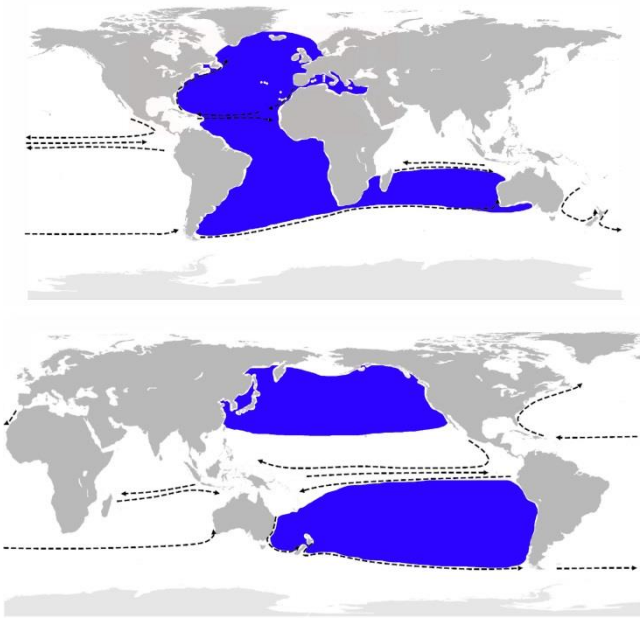


Fig. 9. Distribution área of *Ommastrephes bartramii* based on Jereb & Roper (2010). The main oceanic current that cross its distribution are depicted.

Although the presence of several cryptic species have been previously suggested for *S. oualaniensis* based on morphological and molecular data, the majority of the taxonomic lists still are too conservative and still recognize a single species with a huge morphological variation and an extensive distribution area. *Ommastrephes bartramii* is currently considered as a single species cosmopolitan distribution. Although several species have been recognized for the genus, the most recent taxonomic studies recognized only a single species (e.g., Guerra, 1992; Jereb & Roper, 2010). The Figure 9

represents the current distribution area of this monotypic genus based in the review of Jereb & Roper (2010). Although the species seems to have a continuous distribution in North Atlantic waters, the distribution area is discontinuous in the Pacific Ocean, where it has a subtropical distribution in both hemispheres, and in the Southern hemisphere, with no signals of population connectivity between the Indian and South Pacific populations (Dunning, 1998). The species is morphologically homogeneous throughout all its distribution range, but important differences have been reported in the spermatophore morphology of North Atlantic specimens in comparison with the remaining populations (Nigmatullin et al., 2003). Also, differences in the size-at-maturity of females are known for the different populations (Jereb & Roper, 2010). Importantly, significant differences between populations have been reported for the properties of optic ganglia cholinesterases (Shevtsova et al., 1979; Rozengart & Basova, 2005). Several oceanographic currents cross through the distribution area of the species and potentially might create barriers for paralarval exchange between populations (Fig. 9). The combination of 1) a homogeneous general morphology with some slight differences, 2) the metabolical evidences of reproductive isolation and 3) its vast distribution ranges crossed by several oceanographic features that might act as barriers for genetic exchange, points to *Ommastrephes* as a good model for studying the drivers that produce speciation in oceanic squids. Chapter 4 assessed this taxonomic problem with molecular markers.

Ecological importance of ommastrephid squids

Ommastrephid squids develop an important role as preys and predators, occupying a wide range of trophic levels in marine pelagic food webs (Coll et al., 2013). As predators, ommastrephids are opportunistic generalist predators. Their main preys are small to medium sized planktonic and nektonic animals, primary krill and lanternfish (e.g., Rosas-Luis et al., 2014), but their prey preferences varies with their ontogenetic stage (Schchetinnikov, 1992). Due to their huge growth and fast metabolism, they ingest 6-12 % of their body weight per day (Jereb & Roper, 2010). The abundance and size of ommastrephids make them one of the preferred preys of many marine top predators, as some large fishes (Romeo et al., 2012) or toothed whales (Rosa et al., 2013). In particular, it was suggested that the high abundance of *D. gigas* in the oxygen minimum zone (OMZ) (Rosa & Seibel, 2008, 2010) is the reason of the repetitive diving behavior in the upper layer of the OMZ by many marine top predators (Rosa et al., 2013).

Flying squids are characterized by very high growth rates, complex intraspecific spawning structure, significant intra and interanual fluctuations of abundance and the complete renewal of the population structure. They exhibit typical reproductive traits of a r-strategist, as high fecundities (e.g., Nigmatullin & Markaida, 2009), small hatchlings (Villanueva et al., 2016),

high mortality rates and short life cycle (Arkhipkin et al., 2015b). Although some biota have been associated by egg masses (Birk et al., 2017) no predator is known for ommastrephid during their embryonic development. During their planktonic phase in epipelagic waters, probably they are highly predated and the survival rate of this is critical for recruitment (Jereb & Roper, 2010). They form schools as juveniles and subadults, but as they grow they develop solitary behaviors.

They are capable of performing important migrations (Markaida et al., 2005), usually related with the presence of particular feeding and reproductive grounds (Ichii et al., 2009). It has been suggested that their highly motile nature confer ommastrephids a key role in energy and carbon transfer in oceanic habitats (Jereb & Roper, 2010). Oceanic squids have been reported as an important source of energy and carbon for deep benthic communities when they die (Hoving et al., 2017). Since ommastrephids are very abundant, semelparous, and they group for spawning (Ichii et al., 2009), *en masse* mortality events are expected after they finished their life cycle. Mass mortality of oceanic squids actually means a huge food income for benthic scavengers, which usually depends of decaying organic material from the surface or the water column. Vecchione (2017) observed hundreds of live *Illex* specimens near the seafloor which the author interpreted as a mating or spawning aggregation. Interestingly, he also reported the presence of dozens of dead specimens in the seafloor, some of them being eaten by crabs and others being pulled into burrows.

Interests to fisheries of the Family Ommastrephidae

The most important commercial group of cephalopods is the Family Ommastrephidae. They represent almost 50 % of the total fished cephalopod biomass (FAO, 2016).

Fig. 10. Jumbo squid (*D. gigas*) female of 25 kg of weigh, fished in Guaymas (Baja California Sur, Mexico) in 1996. Photo credit: Unai Markaida.

Among the most important species, the jumbo squid (*D. gigas*) stands as one of the largest invertebrate fisheries in the world (Arkhipkin et al., 2015a). As the reader surely already noted, many biological aspects of the ommastrephid life cycle summarized in this introduction are mainly based on this species. And this is not casual: the fishery interest in this species strengthened the research and this species became the most studied squid species of the century (Markaida & Gilly, 2016). Jumbo squid is exclusively fished by jigging both by the artisanal and industrial fishery fleets of Mexico, Peru, Ecuador and Chile.

The following most important species in base of the volume of captures are the Argentine shortfin squid (*I. argentinus*) and the Japanese flying squid (*T. pacificus*). Both species are also largely fished by the jigging fleet, but some catches came from bottom trawling and, for *T. pacificus*, also by purse siene (Arkhipkin et al., 2015a). Although some species support some minor artisanal fisheries, as *S. pteropus* in Madeira and Canary Islands and *T. sagittatus* in Sicily (Jereb & Roper, 2010), the majority of the ommastrephid catches belong to the industrial jigging and bottom trawling fisheries. For instance, *I. coindetii*, *T. sagittatus* are commonly reported as bycatch by the European bottom trawling fleet, but also *T. eblanae* is commonly fished, but not reported in the fishery statistics. Curiously, *O. bartramii* is object of an intense jigging fishery in North Pacific waters, while rarely fished and not reported in the rest of the world. In Chapter 4 the actual species assemblage of the genus *Ommastrephes* is unraveled, knowledge that should have an important impact in how we understand and assess this fishing resource.

Ph. D. Thesis objectives

The main objective of this Ph. D. Thesis is to ascertain several neglected aspect of the biology of the flying squids. Three of these aspects concern understudied aspects on the ontogeny, such as the transmission of the sperm, the hatchling morphology and paralarval first feeding diet; while the last one solves the complex taxonomy of the type genus of the family (*Ommastrephes*) and tried to explain the evolutionary scenario that created the current species assemblage of the genus from a phylogenetic point of view.

The specific objectives of the Ph. D. Thesis are addressed in the following chapters:

1. To determine the mechanism of sperm transfer from the spermatangia to the seminal receptacles of the female (Chapter 1).

2. To describe and characterize the hatchling morphology and provide a dichotomous key as a tool to identify the ommastrephid paralarvae from NE Atlantic waters (Chapter 2).

3. To ascertain the first feeding diet of ommastrephid paralarvae (Chapter 3).

4. To solve the taxonomy of the circumglobal genus *Ommastrephes* and to identify the speciation drivers that might have created the current distribution of each species (Chapter 4).

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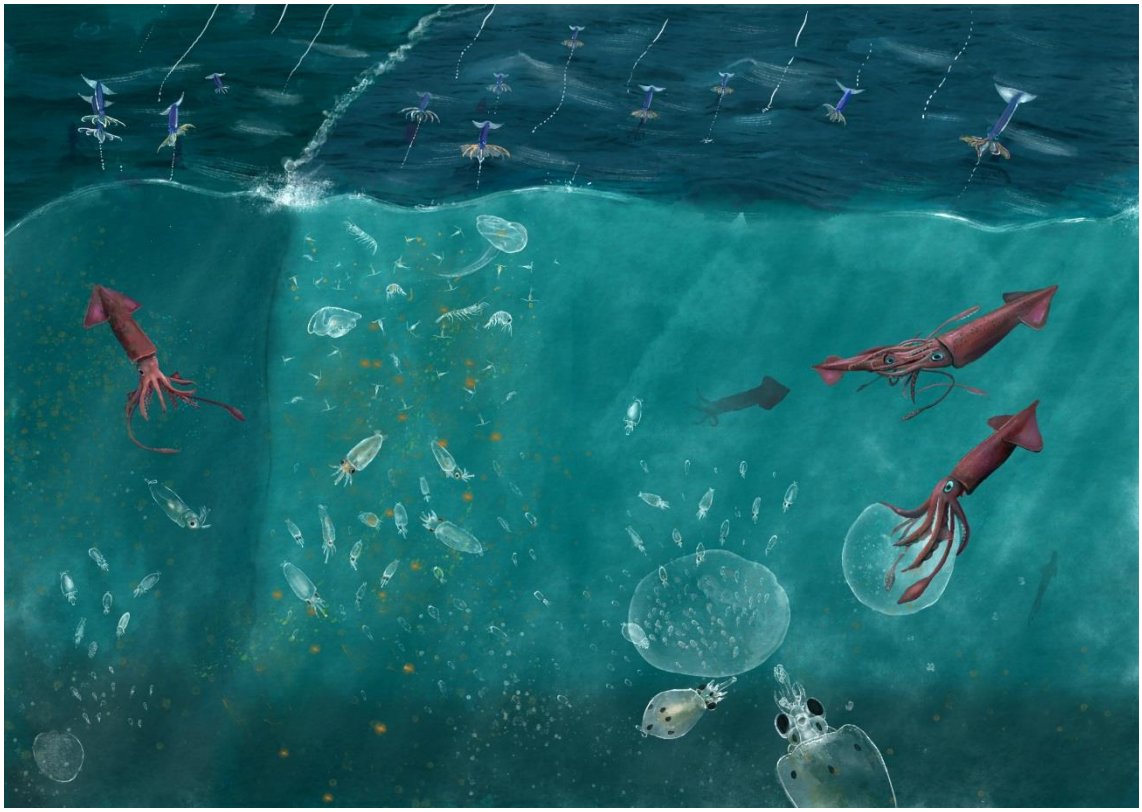
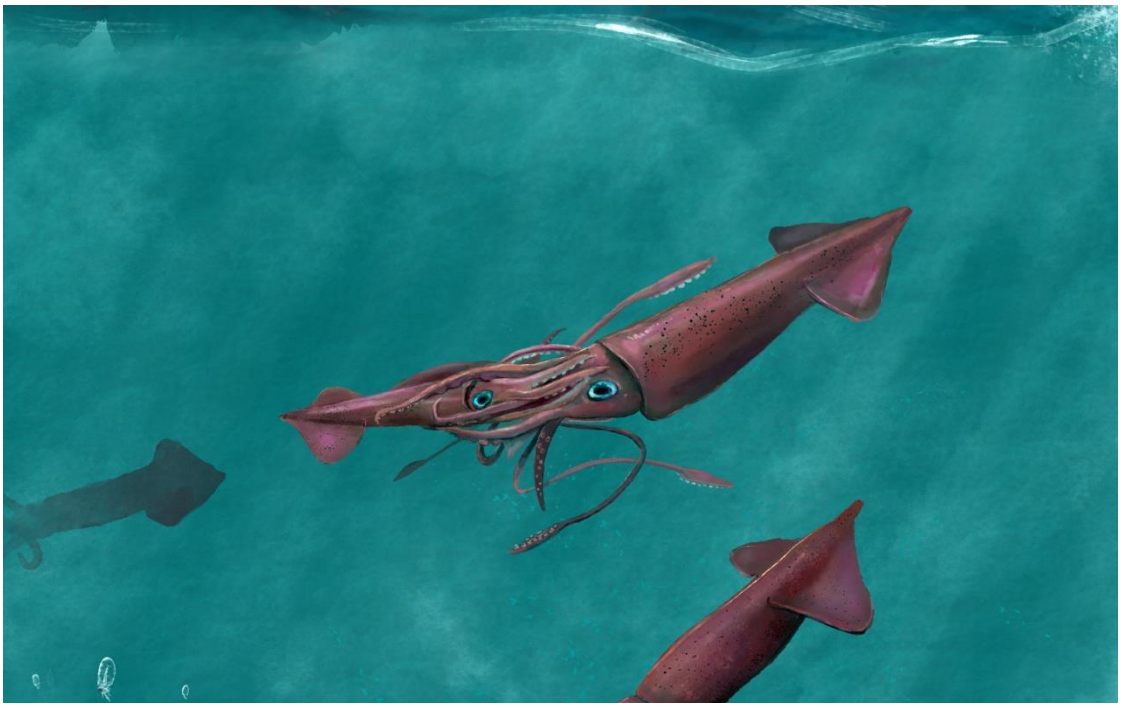
CHAPTERS



CHAPTER 1

The journey of squid sperm



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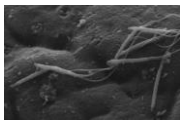


1.1. Abstract

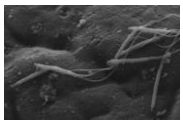
Sperm storage is common in internally fertilizing animals, but is also present in several external fertilizers, such as many cephalopods. Cephalopod males attach sperm packets (spermatangia) to female conspecifics during mating. Females of eight externally fertilizing families comprising 25 % of cephalopod biodiversity have sperm-storage organs (seminal receptacles) in their buccal area, which are not in direct physical contact with the deposited spermatangia. The mechanism of sperm transmission between the implantation site and the storage organ has remained a major mystery in cephalopod reproductive biology. Here, jumbo squid females covering almost the entire life cycle, from immature to a laboratory spawned female, were used to describe the internal structure of the seminal receptacles and the process of sperm storage. Seminal fluid was present between the spermatangia and seminal receptacles, but absent in regions devoid of seminal receptacles. The sperm cellular component was formed by spermatozoa and round cells. Although spermatozoa were tracked over the buccal membrane of the females to the inner chambers of the seminal receptacles, round cells were not found inside the seminal receptacles, suggesting that spermatozoa are not sucked up by the muscular action of the seminal receptacles. This finding supports the hypothesis that spermatozoa are able to actively migrate over the female skin. Although further experimental support is needed to fully confirm this hypothesis, our findings shed light on the elusive process of sperm storage in many cephalopods, a process that is fundamental for understanding sexual selection in the sea.

1.2. Introduction

Sperm storage is widely present in animals, allowing females to maintain viable sperm for an extended period of time and, thus, the existence of a delay between mating and fertilization. This also allows some sexual selection mechanisms, such as sperm competition and cryptic female choice of paternity. Both of these processes have been more extensively studied in vertebrates than in invertebrates (Orr & Brennan, 2015). Among invertebrates, this process is mainly known for internal fertilizers, such as gastropods (Beninger et al., 2016) and insects (Pascini & Martins, 2017). In externally fertilizing animals, however, sperm storage is an extremely rare phenomenon, present in some crustaceans (Waddy & Aiken, 1986; Aiken et al., 2004) and some cephalopods (Orr & Brennan, 2015).

Male cephalopods pack their sperm into complex spermatophores that are transferred to the females during mating. Spermatophores then release a sperm packet, known as a spermatangium, during an osmotic-mediated reaction (Marian, 2015). The spermatangium (the everted spermatophore containing the sperm) attaches to female tissue and either releases the sperm immediately or stays attached to the female, storing it for later use. In at least 8 families of cephalopods (Bathyteuthidae, Idiosepiidae, Loliginidae, Ommastrephidae, Sepiadaridae, Sepiidae, Spirulidae and Thysanoteuthidae) comprising 25 % of the 845 living species described to date (Hoving et al., 2014), females have specialized seminal receptacles (SRs) in their buccal membranes (Marian, 2015), in which spermatozoa released by spermatangia are stored alive for an undetermined period of time before external fertilization and spawning takes place. Buccal membrane surfaces are exposed to the exterior of the cephalopod body (Jereb & Roper, 2010). Since the openings of the spermatangia and SRs are not in physical contact, it is unclear how spermatozoa are translocated to the interior of the SR (Nigmatullin et al., 1995; Sato et al., 2010). At least three hypotheses have been postulated to account for this phenomenon: (I) spermatozoa are sucked up by the muscular action of the SR (van Oordt, 1938); (II) females pick up spermatangia with their arms or beaks and actively transfer the sperm to the SR (Hanlon & Messenger, 1996; Sato et al., 2013); and (III) spermatozoa actively swim from spermatangia to SRs (Tinbergen, 1939; Sato et al., 2010). Although the second hypothesis has been previously refuted (Sato et al., 2013), there is no evidence supporting the first or third hypotheses.

We studied mating, sperm storage and sperm use in females of the jumbo squid, *Dosidicus gigas*, to test the available hypotheses, and collect novel observations to help elucidate reproduction in oceanic squids.



1.3. Material and methods

Immature and mature females of jumbo squid *Dosidicus gigas* were sampled and measured. Their SRs and spermatangia were studied, both by histology and scanning electron microscopy (SEM), to reconstruct the path of the squid spermatozoa from the spermatangia to the SRs.

1.3.1. Study species

Due to its great commercial interest and ecological importance throughout the Humboldt Current, the jumbo squid *D. gigas* is the most studied squid of this century (Markaida & Gilly, 2016). As in other members of the Family Ommastrephidae, its oceanic lifestyle hinders the study of many basic biological aspects, which remain unclear, such as reproduction. During mating, males attach spermatangia to the buccal area (the buccal mass and the buccal membrane) of the females, where 70-150 SRs are located on the buccal membrane (Jereb & Roper, 2010) (Fig. 1.1A). Spermatozoa from spermatangia are stored in SRs until these spermatozoa are mobilized to fertilize the oocytes of the spherical, neutrally buoyant egg masses that contain thousands to millions of eggs (Staaf et al., 2008). To the best of our knowledge, all jumbo squids in the Gulf of California were the small-size-at-maturity morphotype (Jereb & Roper, 2010) during the sampling period (see also Birk et al., 2017).

1.3.2. Squid collection and housing

Squids were sampled at night by jigging in Santa Rosalía, BCS, Mexico (27.3066 °N, 112.1860 °W) during June and July 2015. Most animals used in this study were euthanized by rapid decapitation on board the collecting vessel (Moltschaniwskyj et al., 2007), but some were kept alive in aquaria for other unrelated studies. In all cases, mantle length, weight, maturity stage (after Lipinski & Underhill, 1995), and the number of spermatangia were noted (Table 1.1). The maturity scale of Lipinski & Underhill (1995) consists in six stages for both sexes and can be summarized as follows: immature (stages I and II), in maturation (stage III), mature (stage IV), spawning (stage V), and spent (stage VI). The buccal area was removed by dissection and fixed in 4 % seawater formaline.

Live squids were maintained for unrelated experiments in a 890 l circular tank (1.22 m diameter × 0.76 m depth; RT-430 INS. Frigid Units, Toledo, OH) containing aerated seawater at 15 °C with a salinity of 34 ‰. The holding tank was a closed system with two recirculating loops: one loop provided chilling (AE62B, Pentair Aquatic Eco-Systems, Apopka, FL) and filtering

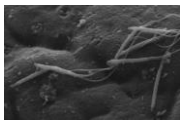
through cartridge filters (50 and 5 μm; VF25, Pentair) and a carbon canister (FCB50H, Pentair), while a second loop provided biological filtration (Trickle 300, Pentair) (Fernández-Álvarez et al., 2017). On August 12, three squids collected the previous night were maintained alive and a single female spawned in this tank, allowing the study of SRs after a spawning event. The spawned female was identified by direct observation of the release of the egg mass. A sample of the egg mass was examined under an inverted microscope to record the fertilization rate. Fertilized eggs were identified following Villanueva et al. (2012). The female was euthanized by rapid decapitation and the buccal area of this female was processed following the protocol described in the preceding paragraph. Laboratory protocols and experimental conditions were in line with current international standards for cephalopod care and welfare reviewed by Fiorito et al. (2015).

Table 1.1. Data from the studied jumbo squid females. Maturity stages after Lipinski & Underhill (1995).

Labcode	Maturity stage	Mantle length (mm)	Weight (g)	Number of spermatangia in the buccal area	Sperm inside the SRs
27-6-2015 2	V	205	210	5	Yes
27-6-2015 3	V	185	150	0	Yes
27-6-2015 4	IV	180	150	11	Yes
27-6-2015 7	V	220	No data	23	Yes
27-6-2015 8	III	180	No data	0	Yes
27-6-2015 9	IV	173	No data	0 (3 in nuchal cartilage and 5 anterior margin of the dorsal mantle)	Yes
29-6-2015 1	IV	195	160	1	Yes
29-6-2015 2	III	185	150	13	Yes
29-6-2015 3	III	165	100	1	Yes
29-6-2015 4	II	162	80	0	No
11-7-2015 captive spawned female	IV	184	No data	Cement body of a single spermatangium	Yes

1.3.3. Experimental procedures

Fixed tissues were washed in filtered seawater followed by dehydration in increasing concentrations of ethanol (20, 30, 40, 50 %) and stored in 70 % ethanol. Each ethanol bath lasted 20 min. Portions of tissues containing SRs and/or spermatangia were extracted from the buccal membrane or the buccal membrane and buccal mass, respectively. Both types of structures were examined by histology and SEM.



Tissues for histology were embedded in paraffin following the Peterfi method (Pantin, 1968), sectioned at 8 µm and stained with haematoxylin-eosin. The histological slides were examined and photographed with a Zeiss Axioskop2 microscope.

Portions of tissues with SRs and/or spermatangia were processed for SEM. The samples were dehydrated in increasing concentrations of ethanol (80, 90, and 96 %) until they were saturated in absolute ethanol. Each ethanol bath lasted 10 min. Some samples were also paraffin-embedded as above, sectioned at 16 µm and, after deparaffinization, processed as the others. After complete dehydration in the ethanol series, samples were dried to critical point in a Bal-Tec CPD 030 Drier using CO_2 as the transition liquid. Samples were then mounted on stubs with double-sided conductive sticky-tape to orient them in the preferred position and sputter coated with gold–palladium in a Quorum Q150RS. Finally, the samples were examined using a scanning electron microscope Hitachi S-3500N with a working voltage of 5 Kv at the Institut de Ciències del Mar (ICM-CSIC) in Barcelona (Spain).

The number of direct observations of each structure or morphological feature are indicated as (*n* = x).

1.4. Results

1.4.1. Seminal receptacle (SR) internal structure

Seminal receptacles of the 11 examined females covered most of the reproductive cycle of jumbo squid, from immature to a recently spawned female (Table 1.1). SRs tower up from the buccal membrane surface (Fig. 1.1A-B) with a characteristic volcano shape (Fig. 1.1B), with the basal portion embedded inside the buccal membrane musculature (Fig. 1.1C-D). Each SR has up to 10 elongate chambers with blind ends (Fig. 1.1C) formed by an outer glandular, cuboidal, ciliated epithelium and circular muscles (Fig. 1.1E). The length of the chambers varies with the reproductive stage of the female, ranging from 275-391 µm in an immature female stage II to 499-828 µm in mature females. The width at the base of the chambers varies with the reproductive stage (up to 74 µm in the immature female II and up to 290 µm in mature females). The width also varies with the contraction stage of the SR circular muscles, showing differences in diameter of up to 125 µm between chambers in a single SR. All of these chambers join in two independent apical openings (10-30 µm wide). This morphology roughly agrees with that of the SR described for the coastal squid *Idiosepius paradoxus* (Sato et al., 2010) and for the deep-sea squid *Bathyteuthis berryi* (Bush et al., 2012).

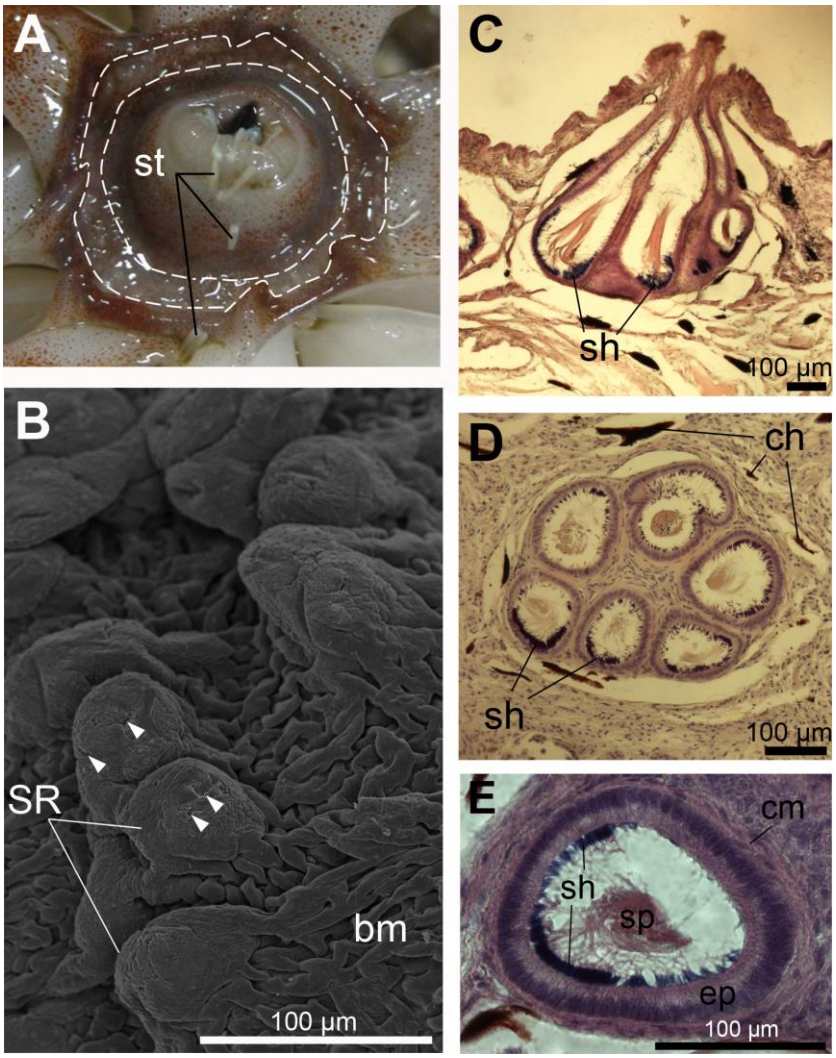
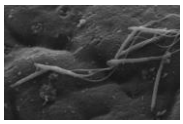


Fig. 1.1. Seminal receptacle (*SR*) structure. (**A**) Oral view of a female buccal area showing some attached spermatangia (*st*); the two dashed concentric lines encircle the area where the SRs are located. (**B**) SEM image of the buccal membrane of a female; *white arrowheads* point to the openings of two SRs. (**C**) Longitudinal section of a SR. (**D**) Transverse section of a single SR showing six different chambers. (**E**) Transverse section of a SR chamber, showing its histological structure. Abbreviations: *bm*, buccal membrane; *ch*, chromatophore; *cm*, circular muscle; *ep*, SR chamber epithelium; *sh*, spermatozoa heads; *sp*, sperm.

1.4.2. Arrangement of the spermatozoa between the spermatangia and the SR

Up to 23 implanted spermatangia (mean 4.9 ± 7.3 SD) were located in the buccal area of the 11 examined females (Table 1.1). Each spermatangium was independently attached through an anchoring in the female skin (Fig. 1.1A, 2A, $n = 62$), and the seminal fluid was released through the apical opening (Fig. 1.2A-B, $n = 8$). Seventeen sperm trails over the buccal membrane skin between the spermatangia and the SRs (Fig. 1.2A-B, C) were observed in 6 females. Released seminal fluid was only present in the direct path between the spermatangium and the SRs ($n = 17$), and no sperm were detected in areas of the buccal membrane that lacked SRs (Fig. 1.2A,



E). Besides the spermatozoa, a large number of round cells (Johanisson et al. 2000) 10 ± 2.4 µm wide (range: 6-16 µm, *n* = 38) were detected in the seminal fluid (Fig. 1.3). Spermatozoa were present over the external surface of the SR and their flagella formed a trail towards the SR openings (Fig. 1.2A, D, *n* = 44 observations in 6 females). The largest trail of spermatozoa from the spermatangium to the farthest SR directly measured by SEM was 1.6 mm. No morphological features (i.e., furrows, slits or grooves) that could drive the movement of sperm released by the spermatangia to the SR openings were found on the buccal membrane. At the bottom of the SR chamber, the spermatozoa heads were attached to the epithelium, while the lumen of the SR chamber was mainly occupied by the flagella (Fig. 1.1C-E, 1.2F, *n* = 135 observations in 9 females), although some dispersed spermatozoa heads were also present in some SR chambers. Round cells were not found inside any of the examined SR chambers (Fig. 1.1C-E, 1.2F). With the exception of one immature female at stage II, all examined females had spermatozoa in every chamber of each sectioned SR (Table 1.1).

1.4.3. Spawning in captivity

The female squid that spawned in the laboratory was classified as mature female stage IV and the fertilization rate was high (95 %, *n* = 101).

This squid had no implanted fresh spermatangia (Table 1.1), thus all of the observed sperm came from storage in the SRs. The histological sections of the SRs of this female showed that, in the majority of the SR chambers, some spermatozoa heads were detached from the bottom surface epithelium and occupied the lumen of the chamber (Fig. 1.2G, *n* = 20 chambers out of 24 SR chambers examined). Many spermatozoa did not change their orientation typical for stored sperm (with the heads pointing towards the epithelium of the bottom of the chamber), suggesting that they continue storing spermatozoa for the next spawning event. On the outer SR surface, spermatozoa heads were mainly facing the external medium (Fig. 1.2H, *n* = 34 spermatozoa from 14 different SR openings). No round cells were observed among the sperm expelled by the SRs. The presence of circular muscles in the SR chambers suggests that muscle contractions may assist in the extrusion of spermatozoa.

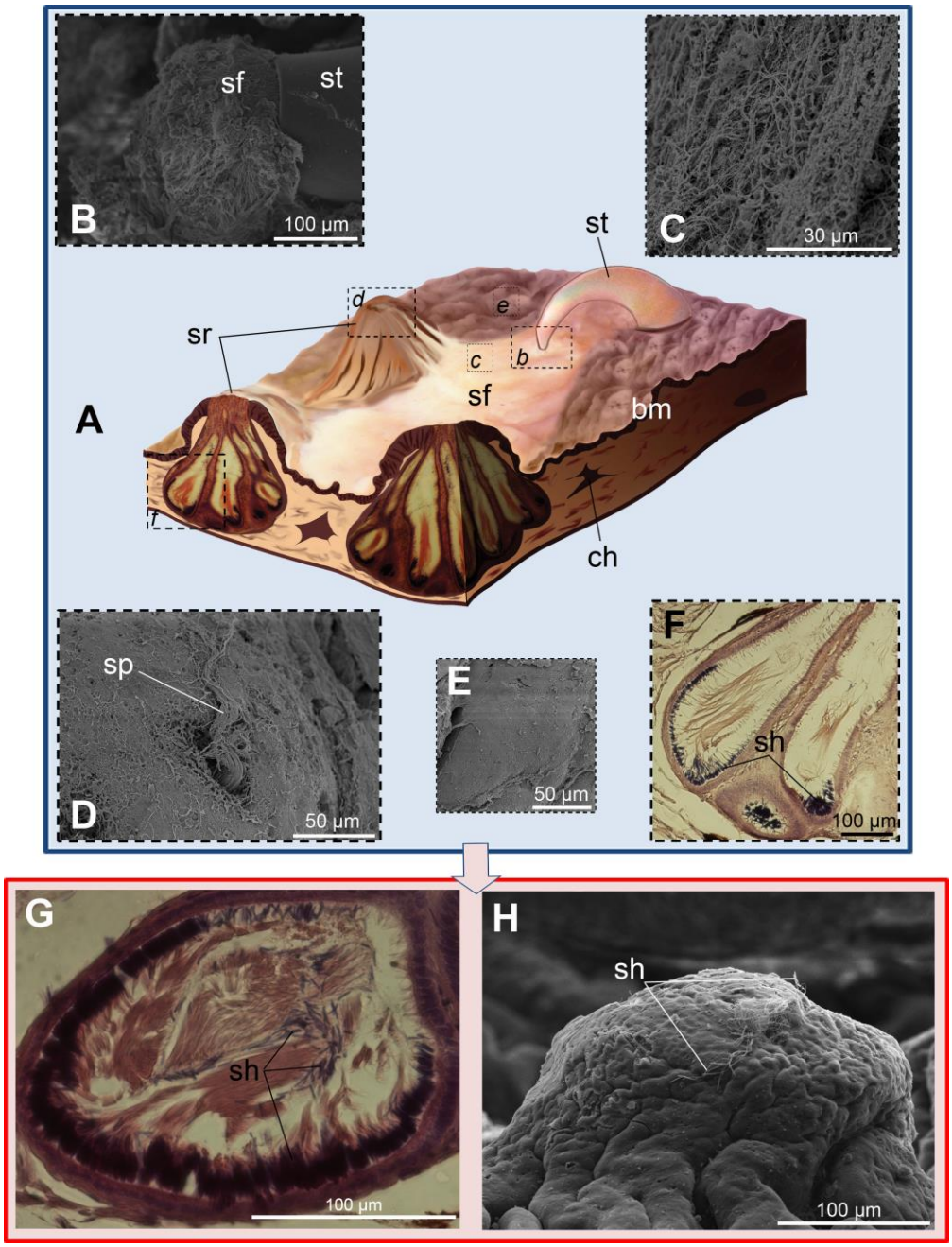


Fig. 1.2. Arrangement of the spermatozoa between the spermatangium and the seminal receptacles (*SRs*) over the female buccal membrane skin (**A-F**) and sperm release during spawning (**G-H**). (**A**) Diagram of a portion of the buccal membrane (*bm*) illustrating the arrangement of the spermatozoa in seminal fluid (*sf*) from the spermatangium (*st*) to SRs; credit diagram: J. M. Anguita. (**B**) SEM image of the apical end of a spermatangium showing released seminal fluid. (**C**) SEM image in the direct path between the spermatangium and the SR, the buccal area is covered with seminal fluid. (**D**) SEM image of the surface of a SR showing a group of spermatozoa (*sp*) at the SR opening. (**E**) SEM image of the buccal membrane surface of an area devoid of SR; spermatozoa are not present. (**F**) Longitudinal section of the basal portion of a SR showing spermatozoa heads (*sh*) facing the epithelium of the SR chamber. (**G**) Histology section of a SR chamber of the spawning female, showing spermatozoa heads attached to the basal epithelium and detached from the basal epithelium and facing the SR chamber lumen. (**H**) SEM image of a lateral view of a SR of the spawning female, showing the spermatozoa released by the SRs. Abbreviation: *ch*, chromatophore.

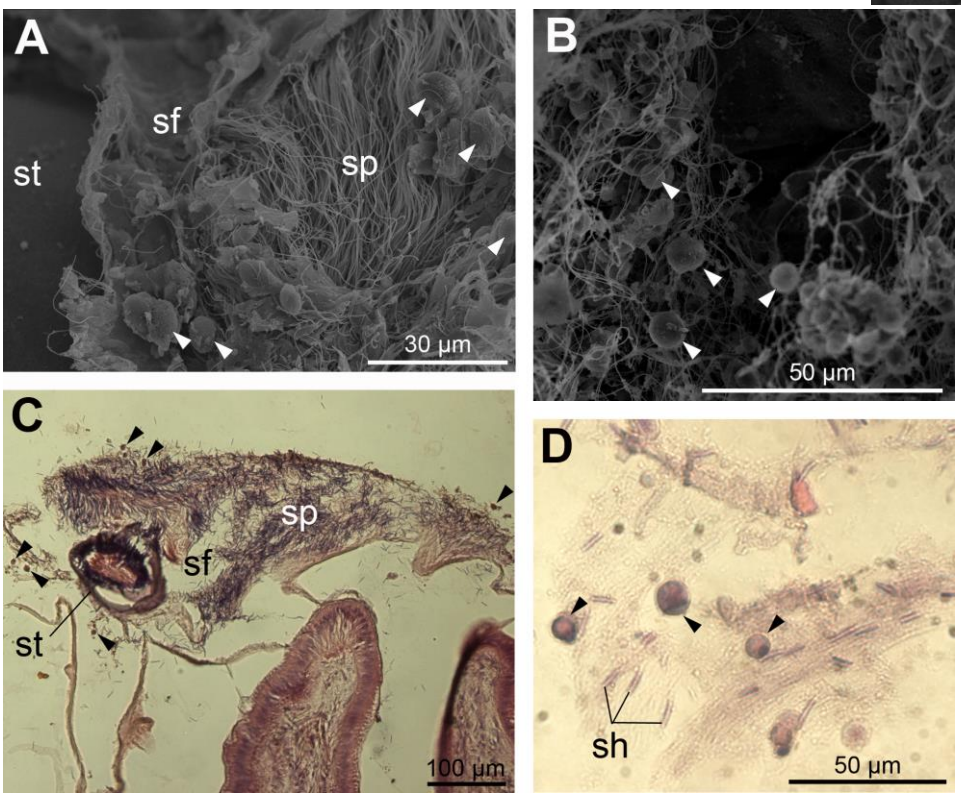


Fig. 1.3. Round cells (*arrowheads*). (**A**) SEM image of the apical end of a spermatangium (*st*) showing released seminal fluid (*sf*) containing spermatozoa (*sp*) and round cells. (**B**) SEM image of the seminal fluid over the buccal membrane of a female showing several round cells. (**C**) Histology section of the apical end of a spermatangium attached to the buccal mass of a female. (**D**) Detail of the seminal fluid showing spermatozoa heads (*sh*) near some round cells.

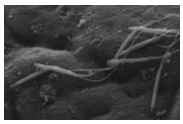
1.5. Discussion

Our results provide direct observations of spermatozoa between the spermatangia and the SRs over the skin of the female squid buccal membrane. This is interpreted as spermatozoa caught during their path from the spermatangia to the SRs of female squids and supports the hypothesis of an active sperm migration. Although females of the squid *Sepioteuthis sepioidea* have been described to manipulate spermatangia and transfer sperm to the SR (Hanlon & Messenger, 1996), studies on other cephalopod species instead support the idea that spermatangia manipulation by females is more likely related to postcopulatory female behaviour, such as spermatangia rejection and feeding (Sato et al., 2013; Wegener et al., 2013), refuting the active role of the female in sperm transmission from spermatangia to the SRs. If the spermatozoa were drawn inside the SR by a muscle-driven suction of these structures, spermatozoa as well as other components of the seminal fluid (i.e., round cells) would be expected inside the SR chambers. Since the size of the round cells (6-16 µm) is smaller than the opening of the SRs (10-30 µm), they would be sucked up by the SRs if this was the mechanism driving the sperm migration. However, only spermatozoa were found inside the SR chambers and no round cells

were expelled by the spawned female. Thus, we considered this hypothesis as refuted. Instead, spermatozoa seem to specifically move towards the SRs (Fig. 1.2A-D), thereby avoiding movement to areas of the buccal membrane without these structures (Fig. 1.2E). It is remarkable that no morphological feature was found on the buccal membrane that could drive the movement of sperm released by the spermatangia to the SR openings. Sperm migration in a targeted manner from the externally-attached spermatophores over the external surface of the female body was also observed in the chaetognath *Sagitta hispida*, although this is an internally fertilizing animal (Reeve & Walter, 1972). Inside the SR, the spermatozoa heads faced the epithelium of the bottom of the chambers (Fig. 1.1C, 2F). These observations strongly suggest the release of sperm-attracting substances by the SRs. In the female buccal areas of jumbo squids larger than those studied in this paper and other large ommastrephids, implanted spermatangia are frequently separated by up to several centimetres from the nearest SR, suggesting that sperm may be able to migrate and survive above the skin long enough to cross this distance. The finding of motile spermatozoa up to 8 hours before its release by spermatangia in the cuttlefish *Sepia apama* (Naud & Havenhand, 2006) supports this idea.

The spermatozoa of *Todarodes pacificus* are able to migrate in swarms (Hirohashi et al., 2016). Since both the jumbo squid and *T. pacificus* belong to the Family Ommastrephidae, both species are provided with seminal receptacles and the placement of spermatangia attachment is the buccal area (Jereb & Roper, 2010). Thus, it is expected that jumbo squid spermatozoa move in the same way. Swarming behaviour of sperm has been suggested to be related to external fertilization in cephalopods (Hirohashi et al., 2016) or as a competitive advantage for sneaker males in sperm competition in species with alternative reproductive systems (Hirohashi et al., 2013). Most cephalopods are promiscuous (Hanlon & Messenger, 1996), and sperm swarming behaviour may therefore be related to sperm competition, an important form of sexual selection. The mechanical force associated with trains of swarming spermatozoa may also result in displacement of previously stored sperm to a less favourable position. This could provide an advantage to spermatozoa from the last male once migration out of the receptacle is triggered. This idea is supported by a previous report of last male sperm precedence in the southern bobtail squid *Euprymna tasmanica* (Squires et al., 2015).

It has been shown that cephalopod females are able to store sperm from different males and affect paternity by cryptic female choice (e.g. Naud et al., 2005; Sato et al., 2017). Each jumbo squid female has dozens of SRs with up to 10 individual chambers storing spermatozoa. The fact that all the females in maturation (maturity stage III) in this study had stored sperm (Table 1.1), as well as the presence of fresh spermatangia in mature (maturity stage IV) and spawning (maturity stage V) females, suggests an expanding mating period with several mating events (i.e., different males) throughout the lifespan. If females are able to control the release of sperm



from each SR or even from the individual chambers, they may bias parentage for a preferred phenotype. Therefore, seminal receptacles should be considered as an active driver of sexual selection in cephalopods.

1.6. Conclusion

We observed spermatozoa over the female buccal membrane skin in the direct path between the male-attached sperm reservoirs (spermatangia) and the female storage organs (SRs), while spermatozoa were absent in areas of the buccal membrane devoid of SRs. This fact, together with the absence of round cells both inside the SRs and in the sperm expelled during spawning, suggests that spermatozoa are able to actively migrate from spermatangia to SRs. Although our findings shed light on the elusive process of sperm storage in squids, future studies with live sperm should focus on collecting direct observations of live sperm actively migrating over the buccal membrane of copulated females. This process is fundamental for understanding sexual selection in the sea, since it may be related to sexual competition in males and cryptic female choice of sperm.

1.7. Acknowledgements

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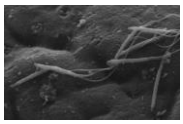

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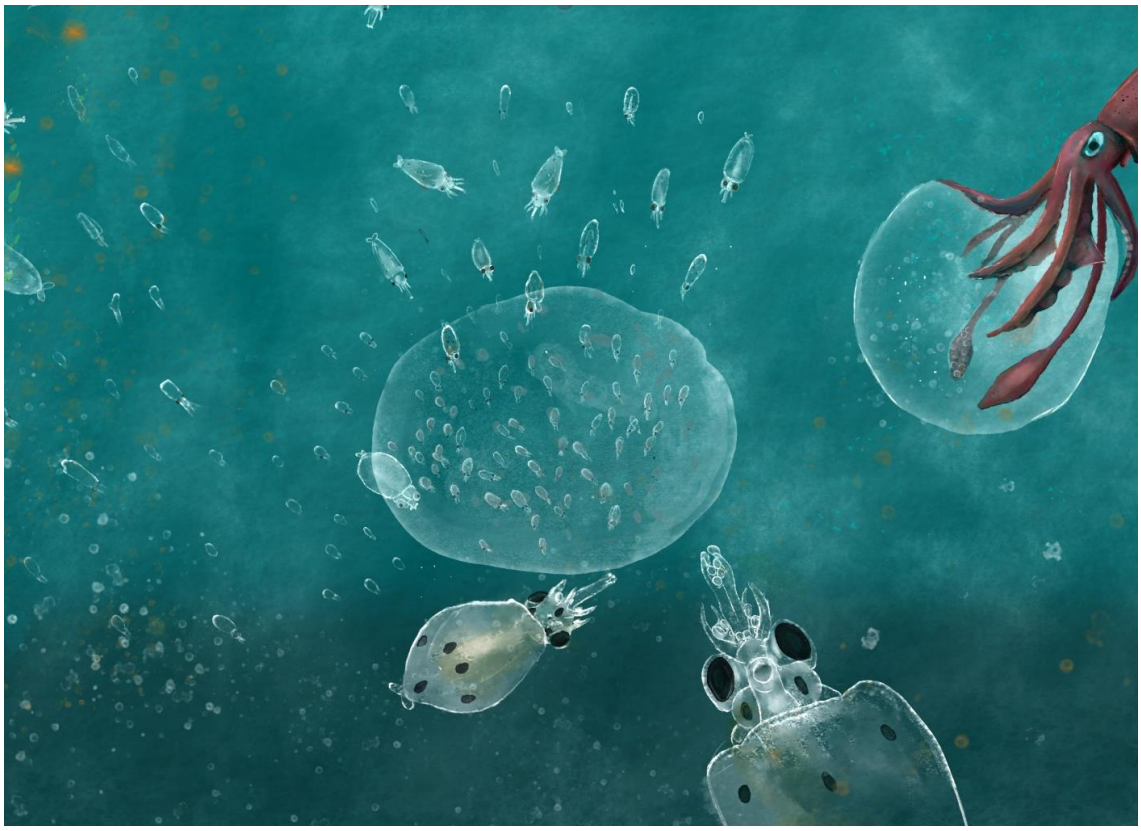
CHAPTER 2

Towards the identification of the ommastrephid squid paralarvae



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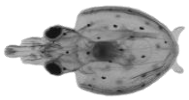


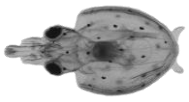
2.1. Abstract

Oceanic squids of the Family Ommastrephidae are an important fishing resource worldwide. Although cumulative knowledge exists on their subadult and adult forms, little is known about their young stages. Their hatchlings are among the smaller cephalopod paralarvae. They are characterized by the fusion of their tentacles into a proboscis and are very difficult to identify to species level, especially in areas where more than one species coexist. Seven species are found in the NE Atlantic. In this study, mature oocytes of *Illex coindetii*, *Todarodes sagittatus* and *Todaropsis eblanae* were fertilized *in vitro* to obtain and describe hatchlings. Full descriptions based on morphometric characters, chromatophore patterns, skin sculpture, and the structure of proboscis suckers are provided based on live specimens. This information was combined with previous descriptions of paralarvae, not necessarily based on DNA or known parentage, from four other ommastrephid species distributed in the same area and a dichotomous key was developed for the identification of paralarvae of the NE Atlantic. The most useful taxonomic characters were: the relative size of the lateral and medial suckers of the proboscis, the presence/absence of photophores, and the arrangement of pegs on the proboscis suckers. This key was successfully used to identify wild collected rhynchoteuthion paralarvae from the NE Atlantic. Reliable identification of wild paralarvae can foster a better understanding of the population dynamics and life cycles of ommastrephid squids.

2.2. Introduction

Ommastrephid squids are distributed in all the world´s oceans and their rapid growth and abundance make them the most important cephalopod fishery resource (Arkhipkin et al., 2015). Knowledge of early life stages is essential for understanding ecology and life cycles as well as for assessment of fisheries. These oceanic squids are important prey and predators, occupying a wide range of trophic levels in marine pelagic food webs (Coll et al., 2013). They are also dominant prey in the diet of many top fish predators (Logan et al., 2013) as well as seabirds and marine mammals (Boyle & Rodhouse, 2005). Despite the cumulative knowledge on the biology of subadult and adult forms of several species of this family, present knowledge of early life stages is fragmentary and limited. Hatchlings of this family are among the smaller of the cephalopods and show a characteristic morphology with tentacles fused into a proboscis. These paralarvae are known as rhynchoteuthion. Identification of the early stages of several species is considerably difficult (Nesis, 1979; Sweeney et al., 1992), especially in areas where more than one species coexists (Gilly et al., 2006; Ramos-Castillejos et al., 2010). Based on wild-collected ommastrephid planktonic stages from around the world, Nesis (1979) stated that it is possible to identify rhynchoteuthion paralarvae to genus, but not species level. Nine of the 22 accepted ommastrephid species (Jereb & Roper, 2010) occur in the N Atlantic Ocean. In the NE Atlantic Ocean, seven ommastrephid species can be found: *Illex coindetii* (Verany, 1839), *Todaropsis eblanae* (Ball, 1841), *Todarodes sagittatus* (Lamarck, 1798), *Ommastrephes bartramii* (Lesueur, 1821), *Sthenoteuthis pteropus* (Steenstrup, 1855), *Hyaloteuthis pelagica* (Bosc, 1802) and *Ornithoteuthis antillarum* Adam, 1957. Besides these, *I. illecebrosus* (Lesueur, 1821) and *I. oxygonius* Roper, Lu & Mangold, 1969 occur in the NW Atlantic. Previous works have dealt with the morphology of the paralarvae of *I. illecebrosus* (e. g. Roper & Lu, 1979; O´Dor et al., 1982). However, the taxonomic status of the NW Atlantic *Illex* spp. populations and the extent of their distribution range are not resolved. Even adult individuals are difficult to identify by morphological characters when more than one species occurs (Carlini, Kunkle & Vecchione, 2006). Thus, NW *I. illecebrosus* and *I. oxygonius* were not included in this work.

The rhynchoteuthion paralarvae of *O. bartramii* (Young & Hirota, 1990; Watanabe et al., 1996; Vijai et al. (2015) and *I. coindetii* (Boletzky et al., 1973; Villanueva et al., 2011) have been studied from hatchlings obtained during laboratory experiments. The morphology of *O. bartramii* paralarvae from the NW Pacific was studied from *in vitro* fertilizations (Watanabe et al., 1996; Vijai et al., 2015) and the morphology of some paralarvae collected in the wild assigned to this species (Young & Hirota, 1990) is consistent with the chromatophore pattern reported in both types of studies. Only descriptions of *H. pelagica*, *O. antillarum* and *S. pteropus* paralarvae collected in the wild are available (Harman & Young, 1985; Sweeney et al.,



1992). Nesis (1979) provided a description of wild-collected individuals of *T. sagittatus*, *T. angolensis* Adam, 1962 and *T. pacificus* (Steenstrup, 1880). However, he merged the characters of the three species into a single generic description and the specific characters of *T. sagittatus* are not available. For *I. coindetii*, complete descriptions of the morphology and chromatophore pattern of the paralarvae are lacking. Moreover, the hatchlings of *T. eblanae* are undescribed. The current situation makes species identification of wild rhynchoteuthion paralarvae from the NE Atlantic nearly impossible using morphological characters (e. g. Zaragoza et al., 2015). This strongly limits the study of the ecology and biology of rhynchoteuthions from the NE Atlantic (e.g. Moreno et al., 2009).

In order to address this problem, we used *in vitro* fertilization methods to obtain live paralarvae of the three most fished NE Atlantic ommastrephid species (*I. coindetii*, *T. sagittatus* and *T. eblanae*) and provide detailed descriptions of their morphology and chromatophore pattern. The available knowledge on the morphology of rhynchoteuthions of the other four NE Atlantic species (*O. bartramii*, *S. pteropus*, *H. pelagica* and *O. antillarum*) was reviewed from the literature. Moreover, a key for the identification of the ommastrephid paralarvae from the NE Atlantic is also provided, aiming to offer a tool for the study of the biology and population dynamics of paralarval stages of ommastrephid squids.

2.3. Material and methods

2.3.1. Obtaining paralarvae through in vitro fertilizations

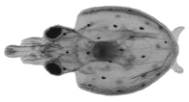
Adult squids of *I. coindetii*, *T. sagittatus* and *T. eblanae* were captured by local bottom trawlers from Barcelona and Vilanova i la Geltrú, NW Mediterranean Sea, between May 2010 and April 2015. *Illex coindetii* and *T. eblanae* were captured from 120 to 350 m depth, and *T. sagittatus* from 300 and 400 m depth. Special care was taken in selecting the freshest squids captured during the latest trawl of the day. Selected individuals were placed on crushed ice covered by a plastic film and transported to the laboratory. Mature females with oocytes in oviducts (stage V-VI according to Brunetti, 1990) were selected for *in vitro* fertilizations following the general methodology described by Villanueva et al. (2012) with minor modifications. In short, for *I. coindetti*, the sperm source used was the bulbs of spermatangia attached to the internal mantle of the female; for *T. eblanae* spermatophores of the Needham´s sac and the *vas deferens* from male individuals were used; for *T. sagittatus*, sperm from spermatophores or seminal receptacles and spermatangia was used depending on availability. The oviducal glands used to make the oviducal jelly were obtained from mature females of the same species collected previously. Freeze-dried oviducal gland powder was stored at -80 ºC until use. The fertilization percentage

of each female used during the experiments was estimated one day after fertilization, by counting the number of fertilized eggs from a sample of 400-500 eggs (Table 2.1). When more than one female was used in an experiment, the mean fertilization rate of the experiment was calculated and is indicated in Table 2.1. Sterile 60-mm diameter polystyrene Petri dishes each containing 20-50 fertilized eggs were maintained in the dark at 15, 17 or 21 ºC using incubators (see Table 2.1). Since vertical distribution of egg masses of the species studied is unknown, temperature conditions during natural embryonic development remain uncertain. Therefore, the temperatures chosen for egg incubation in the present study were based on the temperature ranges of mid-water layers in the Mediterranean Sea (Brasseur et al., 1996), where the egg masses are expected to occur. Throughout the experiment, 25 mg l^{-1} of two antibiotics, ampicillin and streptomycin, were added to the filtered seawater (FSW) (Staaf et al., 2008). The FSW with antibiotics was replaced daily using a binocular microscope and sterile plastic pipettes. Dead embryos and those with abnormalities were removed and counted daily to determine the survival rates until the hatchling stage.

Table 2.1. Data on the material used during the *in vitro* fertilization experiments. * Same information as above.

Species	Females			Sperm source	Fertilized embryos			Temperature (ºC)
	n	DML (mm)	Weight (g)		Fecundation ratio (%)	Initial number	Survival ratio (%)	
Illex coindetii [1]	3	No data	No data	Spermatangia	No data	No data	No data	17
	3	No data	No data	Spermatangia	63.0	2082	81.7	17
	2	No data	150, 165	Spermatangia	85.0	2590	47.2	17
	1	No data	90	Spermatangia	94.1	769	35.5	21
Todarodes sagittatus [2, 3]	1	340	1300	Seminal receptacles, spermatangia	7.2	128	2.3	15
	1	347	1370	Spermatophores from two males	14.3	978	1.6	15
	*	*	*	*	*	282	8.2	17
Todaropsis eblanae [2, 3]	1	181	366	Spermatophores from two males	61.0	550	9.8	15
	*	*	*	*	*	794	0.3	17

[1] In all the experiments, hatching took place during developmental stage XXVII. These paralarvae continued developing in the same Petri dish without apparent problems, and reached the hatchling stage according to the criteria described in the present study.
[2] Hatching took place during developmental stages XXV-XXVI. These paralarvae continued developing in the same Petri dish without apparent problems, and reached the hatchling stage according to the criteria described in the present study.
[3] The embryos of these two species usually suffer a low chorionic expansion in comparison with I. coindetii, which is suspected to be the source of morphological abnormalities and mortality, likely due to the compression of the embryo inside the chorion.



In all the experiments, the paralarvae were cared for and euthanized with the ethical methods in accordance with the European Union Directive 2010/63/EU. Paralarvae were anaesthetized adding drops of 70 % ethanol to Petri dishes containing approximately 12 ml of FSW and overdoses of anaesthesia were used to euthanize them. The initial stages of anaesthesia started very gradually, adding only a few drops of ethanol to the Petri dish containing the paralarvae over a period of 15-30 min, aiming to avoid signals of irritation such as body contractions or ink ejection.

2.3.2. Observations of live paralarvae

For accurate classification of embryonic stages, the scheme and definitions published for *Illex argentinus* (Castellanos, 1960) (Sakai et al., 1998) and *Todarodes pacificus* (Watanabe et al., 1996) were directly applied to *I. coindetii* and *T. sagittatus*, respectively. The *T. pacificus* scale was also adapted for *T. eblanae*. To avoid confusion between supposedly premature hatched individuals and the expected normal hatching, hatchlings are defined here as individuals with a well-developed ink sac, extensible proboscis and functional fins with a fin width nearly equal to the head width. These developmental criteria can be found at stage XXX for the genus *Illex* (Sakai et al., 1998; Villanueva et al., 2011) and stage XXXII for *Todarodes* and *Todaropsis* (see Discussion). The descriptions were based on individuals of these stages.

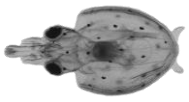
The morphological description was based on measurements of several morphometric characters. These measures were defined according to Roper & Voss (1983) as the ventral mantle length (VML), the dorsal mantle length (DML), the total length (TL), the head length (HL), the head width (HW), the eye diameter (ED), the funnel length (FuL), and the length of the second pair of arms (AIIL). Two other characters were added to the morphometric descriptions. Since the proboscis length was observed to change according to its contraction state, the total length without the proboscis (TL w P) was also measured and was defined as the length of the paralarva from the posterior tip of the mantle to the tip of the arm I. The proboscis width at the base (PW) was defined as the maximum width of the proboscis at its base. The proboscis length has been used as a taxonomic character (e. g., Diekmann et al., 2002). However, this character varies highly with contraction state (Nesis, 1979; Sweeney et al., 1992; Staaf et al., 2008) and usually changes throughout the ontogeny (Shea, 2005). Harman & Young (1985) provided the proboscis length and the proboscis index of *Nototodarus hawaiiensis* (Berry, 1912), *H. pelagica* and *Sthenoteuthis oualaniensis* (Lesson, 1830-1831). Although they considered *N. hawaiiensis* to have a “typically short” proboscis and *S. oualaniensis* often with a “very elongate” proboscis, both the proboscis length (Harman & Young, 1985: Fig. 2) and their proboscis index overlap. Both characters also overlap with *H. pelagica*, as confirmed in other posterior references

(Sweeney et al., 1992; Diekmann et al., 2002). Thus, this character is not very instructive and was not considered in our study. When resting on the Petri dishes, the majority of the embryos or paralarvae lay on the dorsal surface of the body; thus, it was not possible to measure the DML in many specimens. For this reason, the ratio between each morphometric parameter and body size was obtained using the VML instead of the DML. The terminology used for discriminating rhynchoteuthion types was: Type A, the ratio of sucker sizes is greater than 2:1 (lateral suckers 200 % or greater in size than the medial suckers), Type B, this ratio is between 1.1:1 and 1.9:1 (lateral suckers larger than the medial suckers but below 200 %) and Type C, this ratio is 1:1 (there is no size difference among the proboscis suckers). These three categories are consistent with the proboscis sucker proportions of ommastrephid paralarvae A, B, and C treated in Roper & Lu (1979).

For the description of the chromatophore pattern, the dorsal and ventral surfaces were depicted in a schematic drawing of a rhynchoteuthion. Lateral views were only considered for the description when hatchling chromatophores were visible only from the side view. For *I. coindetii* and *T. sagittatus*, the lateral view was not included, since the lateral chromatophore pattern is the sum of the dorsolateral and ventrolateral chromatophores. However, *T. eblanae* has true lateral chromatophores in the midline of the lateral surface of the mantle, which are not visible from dorsal or ventral views. The chromatophores of the head and mantle were assigned to rows in an anteroposterior axis. For example, for the ventral mantle, the pattern 4 + 2 + 3 + 4 + 1 + 2 means: 4 chromatophores in the anterior margin of the mantle, 2 in the second row, 3 in the third row, 4 in the fourth row, 1 in the fifth row and 2 near the posterior tip of the mantle. The mode of the pattern of each row was considered the most representative of the species and a schematic drawing depicting this pattern is provided for each species.

2.3.4. Observations under Scanning Electron Microscopy (SEM)

Paralarvae were euthanized with an overdose of anaesthesia prior to fixation. To avoid body contraction of the paralarvae it is important to add the anaesthetic gradually before killing them, starting anaesthesia with a few drops of ethanol. Once the three hearts stopped beating, the individuals were fixed in 2.5 % glutaraldehyde in seawater for 24-48 h, washed in seawater followed by dehydration in an increasing concentration of ethanol (20, 30 and 50 %) and stored in 70 % ethanol in the dark at 4 °C. At the beginning of SEM preparation, the samples were again dehydrated in an increasing concentration of ethanol (80, 90, and 96 %) until they were saturated in absolute ethanol. Each ethanol bath lasted 10 min. After complete dehydration in the ethanol series, the samples were dried to a critical point using CO_2 as the transition liquid. After the drying stage, samples were mounted on stubs with double-sided conductive sticky tape



to place them in the preferred position. The mounted samples were sputter coated with gold–palladium. Finally, the samples were observed using a scanning electron microscope.

The general morphology and the number and arrangement of pegs in the proboscis and arm suckers were examined. Nomenclature of proboscis suckers was as follows. The suckers were named from 1 to 4 of each hemiproboscis tip, with 1 the most dorsal and 4 the most ventral. Each side of the proboscis tip was named as R (right) or L (left), according to its position on the anteroposterior axis of the body. For example: proboscis sucker R1 is the most dorsal sucker from the right part of the proboscis tip. Suckers 2 corresponded to lateral suckers and suckers 1, 3 and 4 were medial suckers. Newly hatched rhynchoteuthion paralarvae only have one sucker on arms I and II. Thus, no special nomenclature was necessary to designate each sucker.

Characters from both SEM and observations of live paralarvae were used to develop a dichotomous key to facilitate the identification of the seven NE Atlantic rhynchoteuthion paralarvae species. Morphological characters of *O. bartramii*, *H. pelagica*, *S. pteropus* and *O. antillarum* were obtained from the literature (Harman & Young, 1985; Sweeney et al., 1992; Young & Hirota, 1990; Sakurai et al., 1995; Diekmann et al., 2002; Vijai et al., 2015). It should be noted that descriptions of the paralarvae of *H. pelagica*, *S. pteropus* and *O. antillarum* were not confirmed by DNA or known parentage in these works.

2.3.5. Wild rhynchoteuthion paralarvae samples

Wild rhynchoteuthion paralarvae (n = 16) from the study area were collected using zooplankton tows during the oceanic cruise LLUÇ3 (Palomera et al., 2005) in the summer of 1999, NW Mediterranean; four oceanographic cruises conducted in the summers of 2003 and 2004 under the CACO research project (Sabatés et al., 2009), NW Mediterranean; and another cruise near the Canary Islands in the spring of 2015 under the MAFIA research project using the fishing net described by Meillat (2012). An additional paralarva collected during the project FishJelly with a Bongo net with a 40 cm diameter opening and a mesh size of 300 µm in the autumn of 2014 was also analysed from the NW Mediterranean. These zooplankton samples were fixed in 5 % formaldehyde buffered with sodium tetraborate. Ommastrephid paralarvae were distinguished from the other cephalopod paralarvae by the presence of the proboscis. The general morphology of the fixed specimens (chromatophores, size, number of arms, etc.) was examined under the stereomicroscope prior to observation under SEM as described previously. The specimen from the MAFIA cruise was frozen and measurements were taken after defrosting. All of these paralarvae were identified using the dichotomous key developed in the present study.

2.4. Results

2.4.1. Morphological description of the rhynchoteuthion hatchlings from the NE Atlantic.

Morphology of rhynchoteuthion paralarvae is very similar among species: hatchlings usually have only one sucker on both pairs of arms I and II, pair IV is a protuberance without suckers and pair III is totally undeveloped. Morphometric measurements and indices for each species are shown in Table 2.2. Morphometric comparisons between species were performed based on the indices, rather than on the raw measures. A full description of the chromatophore pattern of each species is provided in Table 2.3. The rhynchoteuthion species Type in relation to ratio of proboscis sucker size and the description of the proboscis and arm sucker pegs is summarized in Table 2.4.

Illex coindetii hatchlings

The general morphology of the hatchling (Fig. 2. 1A-D, 2.3A-C) of this species does not diverge from that described for the congeneric *I. argentinus* (Sakai et al., 1998). The mean VML is 1.41 ± 0.15 mm. On the head, there are 2 ventral chromatophores, one below each eye, and dorsally there are two rows of chromatophores, the first formed by a single chromatophore anterior to the eyes and the second formed by 3 chromatophores at the base of the head. In some individuals, small dark brown pigmented dots appear on the tips of the arms and proboscis (Figs. 2.1B, 2.1D and 2.3B). Although uncommon, some individuals showed head chromatophores arranged asymmetrically, generally placed on the lateral sides of the head. On the mantle, there are up to 6 rows ventrally, distributed as follows: 4 + 2 + 4 + 3+ 3+ 2; dorsally, there are up to 5 rows: 2 + 3 + 3 + 0 + 1. The 8 suckers are of similar size (35.1 ± 3.9 µm) (Type C rhynchoteuthion, Fig. 2.2A) and have only one row of pegs (12.9 ± 1.4) (Table 2.4, Fig. 2.2C), but additional asymmetrically distributed pegs can be found outside of this row. The arm suckers measure 40.2 ± 2.3 µm in diameter and bear two rows of pegs (Fig. 2.2B), the internal one with 12.1 ± 1.0 pegs, the external one with 12.2 ± 1.4. A few pegs can appear externally to the external row (Fig. 2.2B). The skin is smooth and does not have any special sculpture (Fig. 2.2D). When compared with the other paralarvae described here (Table 2.2), the FuLI and PWI are larger, which indicates that the funnel is comparatively larger than in the other two species (although the range of this index overlaps with that of *T. eblanae*); and the proboscis is wider, although some overlap exists.

Remarks: Moreno (2008: Fig. 4.18) tentatively identified rhynchoteuthion paralarvae based on the drawings of Salman et al. (2003, see below for more details) and among these, some were identified as *Illex* specimens. Her pictures clearly show a Type C rhynchoteuthion without ocular or intestinal photophores. Thus, these specimens were either *I. coindetii* or *T. eblanae*.

However, the identification of these specimens could not be confirmed from the available description and pictures.

Roura (2013) succeeded in molecularly identifying one *I. coindetii* paralarva out of the 15 barcoded from Cape Silleiro (north-western coast of the Iberian Peninsula). However, he did not provide any morphological description of these paralarvae.

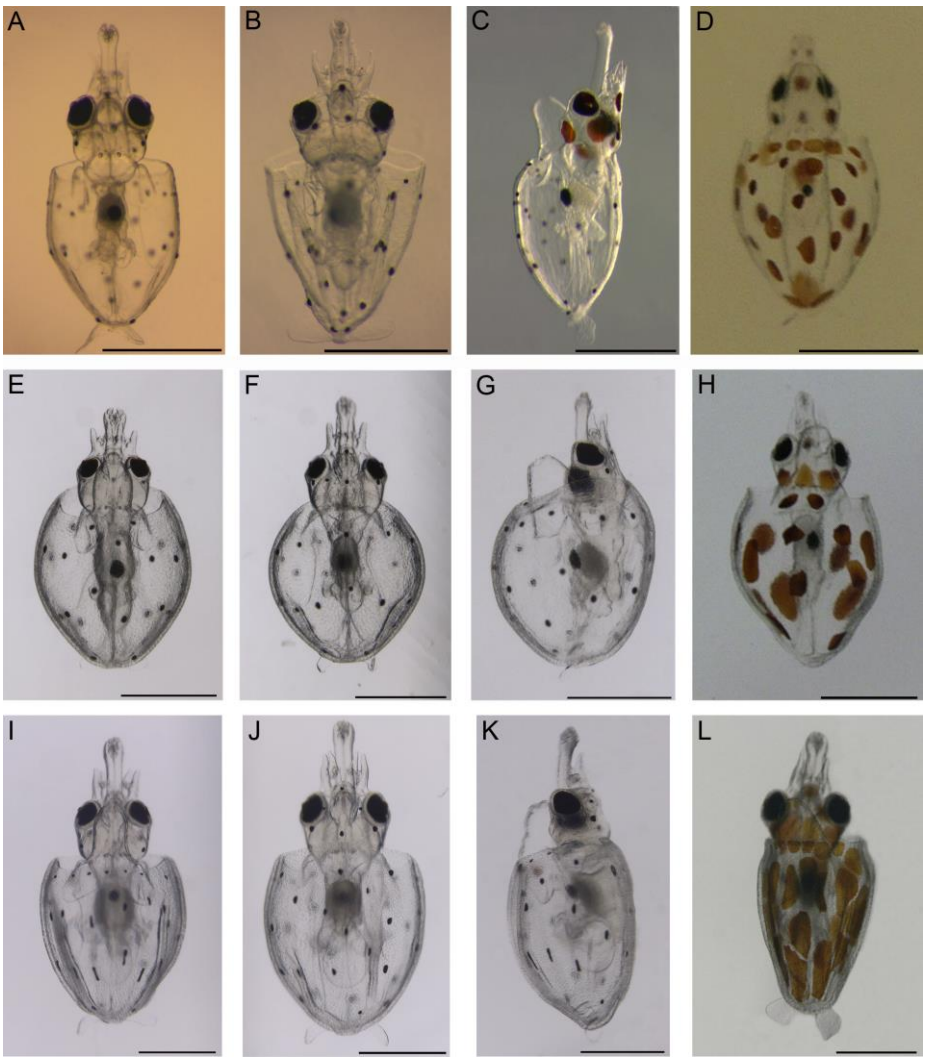


Fig. 2.1. (**A-D**) *Illex coindetii*. (**A**) Ventral view, aged 354 h and incubated at 17 ºC. (**B**) Dorsal view, aged 427 h and incubated at 17 ºC. (**C**) Lateral view, aged 262 h and incubated at 21 ºC. (**D**) Ventral view of an individual with expanded chromatophores, aged 236 h and incubated at 17 ºC. (**E-H**) *Todarodes sagittatus*. (**E**) Ventral view, aged 364 h and incubated at 15 ºC. (**F**) Dorsal view, aged 358 h and incubated at 17 ºC. (**G**) Lateral view, aged 360 h and incubated at 17 ºC. (**H**) Ventral view of an individual with expanded chromatophores, aged 336 h and incubated at 17 ºC. (**I-L**) *Todaropsis eblanae*. (**I**) Ventral view, aged 475 h and incubated at 17 ºC. (**J**) Dorsal view, aged 500 h and incubated at 15 ºC. (**K**) Lateral view, aged 498 h and incubated at 15 ºC. (**L**) Ventral view of an individual with expanded chromatophores, aged 649 h and incubated at 15 ºC. **A-C**, **E-G**, **I-K**, specimens anaesthetized with ethanol, which potentially causes chromatophore contraction. **D**, **H**, **L**, individuals without anaesthesia. Scale bars: 1 mm.

Table 2.2. Morphometric parameters measured (mm) and morphometric parameter ratios in relation to the VML (%). Abbreviations: VML: ventral mantle length, DML: dorsal mantle length, TL: total length, TL w P: total length without the proboscis, HL: head length, HW: head width, ED: eye diameter, FuL: funnel length, PW: proboscis width at the base, AIIL: Arm II length, DMLI: dorsal mantle length index, TLI: total length index, TL w PI: total length without the proboscis index, HLI: head length index, HWI: head width index, EDI: eye diameter index, FuLI: funnel length index, PWI: proboscis width at the base index, AIILI: Arm II Length Index.

Species		VML	DML	TL	TL w P	HL	HW	ED	FuL	PW	AIIL	n
Measures												
Illex coindetii	**Average**	1.41	1.52	2.45	2.24	0.52	0.70	0.22	0.73	0.16	0.18	52
	SD	0.15	0.10	0.21	0.20	0.06	0.06	0.03	0.07	0.02	0.06	
	Range	1.09-1.82	1.40-1.71	1.93-2.93	1.65-2.65	0.39-0.67	0.59-0.87	0.16-0.28	0.55-0.91	0.13-0.19	0.10-0.30	
Todarodes sagittatus	**Average**	1.64	1.80	2.63	2.44	0.61	0.79	0.24	0.47	0.16	0.20	29
	SD	0.12	0.14	0.20	0.19	0.08	0.07	0.03	0.04	0.01	0.04	
	Range	1.25-1.81	1.44-1.96	2.03-2.95	1.90-2.73	0.44-0.75	0.64-0.92	0.16-0.30	0.39-0.55	0.14-0.18	0.11-0.26	
Todaropsis eblanae	**Average**	2.16	2.19	3.60	3.26	0.83	1.00	0.34	0.98	0.21	0.41	36
	SD	0.11	0.14	0.24	0.17	0.05	0.06	0.03	0.10	0.01	0.05	
	Range	1.93-2.43	1.92-2.48	3.03-4.12	2.89-3.55	0.71-0.92	0.89-1.10	0.28-0.41	0.74-1.15	0.19-0.25	0.32-0.52	
Ratios			**DMLI**	**TLI**	**TL w PI**	**HLI**	**HWI**	**EDI**	**FuLI**	**PWI**	**AIILI**	
Illex coindetii	**Average**		107.43	175.53	161.10	37.56	50.46	15.73	53.17	11.75	12.41	42
	SD		6.69	18.68	16.39	5.88	5.77	2.32	6.61	1.57	3.44	
	Range		95.33-116.43	143.86-243.38	131.25-219.05	27.89-55.22	40.36-64.18	11.29-21.20	38.02-65.82	9.21-16.19	6.98-22.60	
Todarodes sagittatus	**Average**		107.93	162.50	149.93	36.90	49.05	15.16	28.45	9.67	12.38	15
	SD		6.98	8.67	7.01	4.55	4.86	2.12	2.90	0.98	2.52	
	Range		95.94-123.27	147.15-182.38	136.93-163.52	28.82-45.73	41.18-61.12	9.41-19.50	23.35-33.54	8.14-11.64	6.47-17.12	
Todaropsis eblanae	**Average**		101.97	168.28	151.72	38.50	46.49	16.08	45.84	9.77	19.00	31
	SD		6.28	10.51	8.40	3.09	3.33	1.42	4.85	0.81	2.50	
	Range		89.78-118.93	142.86-183.58	128.44-166.67	33.78-46.11	39.18-52.24	13.78-19.69	35.48-53.33	8.26-11.65	14.75-24.17	

Table 2.3. Schematic chromatophore pattern of *I. coindetii*, *T. sagittatus* and *T. eblanae*. Numbers indicate rows of chromatophores in an anteroposterior axis (see Material and Methods for further details). N/A, not applicable. Abbreviations: Ven, Ventral; Dor, Dorsal; Lat, Lateral; AI, Arm I; AII, Arm II; Prob, Proboscis.

		Head					Arm Crown			Mantle														
Species		Ven	Dor			Lat	AI	AII	Prob	Ven						Dor					Lat			n
		1	1	2	3	N/A	N/A	N/A	N/A	1	2	3	4	5	6	1	2	3	4	5	1	2	3	
Illex coindetii	**Average**	1.9	1.0	3.0	N/A	0.2	1.8	0.2	3.4	4.3	2.4	3.7	3.2	1.8	2.0	1.0	2.6	2.4	0.9	1.0	3.1	N/A	3.8	52
	SD	0.4	0.1	0.3	N/A	0.5	2.5	0.4	3.7	0.8	1.4	1.2	1.3	1.3	0.1	1.0	1.1	0.9	1.3	0.0	0.5	N/A	0.7	
	Range	0-2	1.0	2-4	N/A	0-2	0-10	0-1	0-16	3-6	0-6	0-6	0-5	0-4	1-2	0-3	0-5	1-4	0-4	1	2-4	N/A	3-5	
	Mode	**2**	**1**	**3**	**N/A**	**0**	**3**	**0**	**2**	**4**	**2**	**4**	**3**	**3**	**2**	**2**	**3**	**3**	**0**	**1**	**3**	**N/A**	**4**	
Todarodes sagittatus	**Average**	0	1.0	3.0	N/A	0.0	0.0	0.0	0.0	2.6	5.1	3.9	1.8	N/A	N/A	3.1	1.0	3.1	0.2	0.0	1.9	N/A	2.5	29
	SD	0	0.2	0.5	N/A	0.0	0.0	0.0	0.0	0.8	1.1	1.1	0.6	N/A	N/A	0.9	0.9	1.0	0.5	0.2	0.2	N/A	0.6	
	Range	0	0-1	1-4	N/A	0	0	0	0	2-5	3-6	2-5	0-3	N/A	N/A	2-5	0-4	2-5	0-2	0-1	1-2	N/A	1-3	
	Mode	**0**	**1**	**3**	**N/A**	**0**	**0**	**0**	**0**	**2**	**6**	**5**	**2**	**N/A**	**N/A**	**3**	**1**	**4**	**0**	**0**	**2**	**N/A**	**3**	
Todaropsis eblanae	**Average**	1.8	1.0	1.9	2.7	0.0	0.0	0.0	0.0	6.6	4.5	1.3	0.8	4.2	0.8	3.6	1.7	2.7	2.3	1.9	3.0	2.3	3.2	43
	SD	0.5	0.0	0.5	0.7	0.0	0.0	0.0	0.0	0.8	1.4	1.5	1.4	1.4	0.5	1.2	1.5	1.7	1.7	0.6	0.5	0.8	0.7	
	Range	0-2	1	0-2	0-3	0	0	0	0	5-9	1-6	0-5	0-5	0-6	0-2	1-5	0-5	0-5	0-5	1-3	2-4	1-4	2-4	
	Mode	**2**	**1**	**2**	**3**	**0**	**0**	**0**	**0**	**7**	**5**	**0**	**0**	**5**	**2**	**3**	**0**	**5**	**0**	**2**	**3**	**2**	**3**	

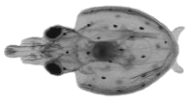
Table 2.4. Rhynchoteuthion paralarvae species according to the ratio of sucker sizes, number and arrangement of pegs of the proboscis and arm suckers. The ratio of sucker sizes is indicated only when greater than 1.

Species		Paralarval type	Ratio	Proboscis suckers			Arm suckers			n
				Internal pegs	External pegs	Free pegs	Internal pegs	External pegs	Free pegs	
Illex coindetii	Average	C	-	12.9	N/A	6.4	12.1	12.2	0.5	11
	SD			1.4	N/A	2.8	1.0	1.4	0.5	
	Range			10-14	N/A	1-12	10-14	10-14	0-1	
	Mode			14	N/A	8	12	11	0	
Todarodes sagittatus	Average	B	1.2:1	18.0	17.0	0.0	16.2	14.0	0.0	7
	SD			3.0	3.0	0.0	1.7	2.1	0.0	
	Range			12-24	12-22	0	14-19	9-17	0	
	Mode			18	14	0	16	14	0	
Todaropsis eblanae	Average	C	-	18.2	22.0	0.5	17	20.6	1.6	11
	SD			1.0	2.2	1.0	0.8	1.5	2.3	
	Range			16-24	17-27	0-3	15-18	18-23	0-8	
	Mode			18	22	0	17	20	0	

Todarodes sagittatus hatchlings

The general morphology of this hatchling (Fig. 2.1E-H, 2.3D-F) is similar to the congeneric *T. pacificus* (Watanabe et al., 1996; Puneeta et al., 2015). The mean VML is 1.64 ± 0.12 mm. On the head, there are no ventral chromatophores. Dorsally there are two rows of chromatophores, the first formed by a single chromatophore anterior to the eyes and the second formed by 3 chromatophores at the base of the head. On the mantle, there are up to 4 rows ventrally, distributed as follows: 2 + 6 + 5 + 2; dorsally, there are up to 5 rows, with the following configuration: 3 + 1 + 4 + 0 + 0. The lateral proboscis suckers are larger in size (41.2 ± 8.0 µm) than the medial suckers (35.0 ± 6.7 µm) (Type B rhynchoteuthion, Fig. 2.2E). The ratio between the size of the sucker diameter of the lateral suckers and the medial suckers is 1.2:1. Two rows of pegs are found (Fig. 2.2G), the internal row with 18.0 ± 3 and the external with 17.0 ± 3 pegs. There are no differences in the number or arrangement of the sucker pegs between lateral and medial suckers. All pegs belonged to either of these two rows. The skin of this species shows a hexagon-like structure under the stereomicroscope (Fig. 2.2H). The FuLI (Table 2.2) is comparatively smaller than in *I. coindetii* and *T. eblanae*, showing a shorter funnel.

Remarks: Salman et al. (2003: Fig. 7) identified 2 rhynchoteuthions from the Aegean Sea as *I. coindetii*. However, the drawing of the proboscis tip clearly shows a type B rhynchoteuthion paralarva. The value of the ratio of sucker sizes is 1.4:1. No photophores were drawn and *H. pelagica* and *O. antillarum* have never been found in Mediterranean waters (Jereb & Roper,



2010), excluding the other Type B paralarva from the NE Atlantic area. Although the ratio of sucker sizes does not fit accurately with the value obtained here for *T. sagittatus* (1.2:1), we considered these two paralarvae as members of this species, rather than *I. coindetii*. Possible sources of this variation could be: a) differences related to the developmental state, since those drawn for Salman et al. (2003) show more than one sucker on each arm and are of a larger size (2.5-3.5 mm ML); b) intraspecific or regional variation; c) differences related to the fixation procedure, since plankton samples usually are not anaesthetized before fixation and some contraction is expected to occur; d) lower levels of accuracy in the measurements from the drawings.

Moreno (2008) described a rhynchoteuthion paralarva with lateral suckers larger than the medial ones in Atlantic Iberian waters. It is not possible to take measurements from the pictures (Moreno, 2008: Fig. 4.22c) due to the orientation of the animal. No evidence of eye photophores is found based on the pictures. However, the chromatophore pattern of both dorsal and ventral views of the head and mantle is visible. The chromatophore pattern of the ventral surface of the mantle is 4 + 4 + 4 + 1 + 1 and for the dorsal surface 3 + 4 + 3 + 2. The dorsal chromatophore pattern of the head is not fully visible, but in the ventral view no chromatophores appear. The chromatophore pattern of this individual is consistent with that described for *T. sagittatus* in this work, especially regarding the absence of ventral chromatophores on the head in the hatchlings of this species, which is of high taxonomic significance.

An 18 mm TL juvenile individual assigned to *T. sagittatus* sampled in Sicily (Central Mediterranean Sea) has been documented (Piatkowski et al., 2015:Fig. 16.4). The absence of chromatophores on the ventral surface of the head is remarkable. Although this observation is based only on one specimen and more observations are necessary, this fact stresses this character as highly diagnostic for this species, possibly throughout its life as a rhynchoteuthion.

The rhynchoteuthion paralarvae of the congeneric species *T. pacificus* differ from *T. sagitattus* in their smaller size (up to 1.4 mm ML, Puneeta et al., 2015: Fig. 9), the chromatophore pattern (see Discussion) and the proboscis suckers, which are of equal size in *T. pacificus* (Puneeta et al., 2015: Fig. 8). Therefore, this constitutes a Type C rhynchoteuthion.

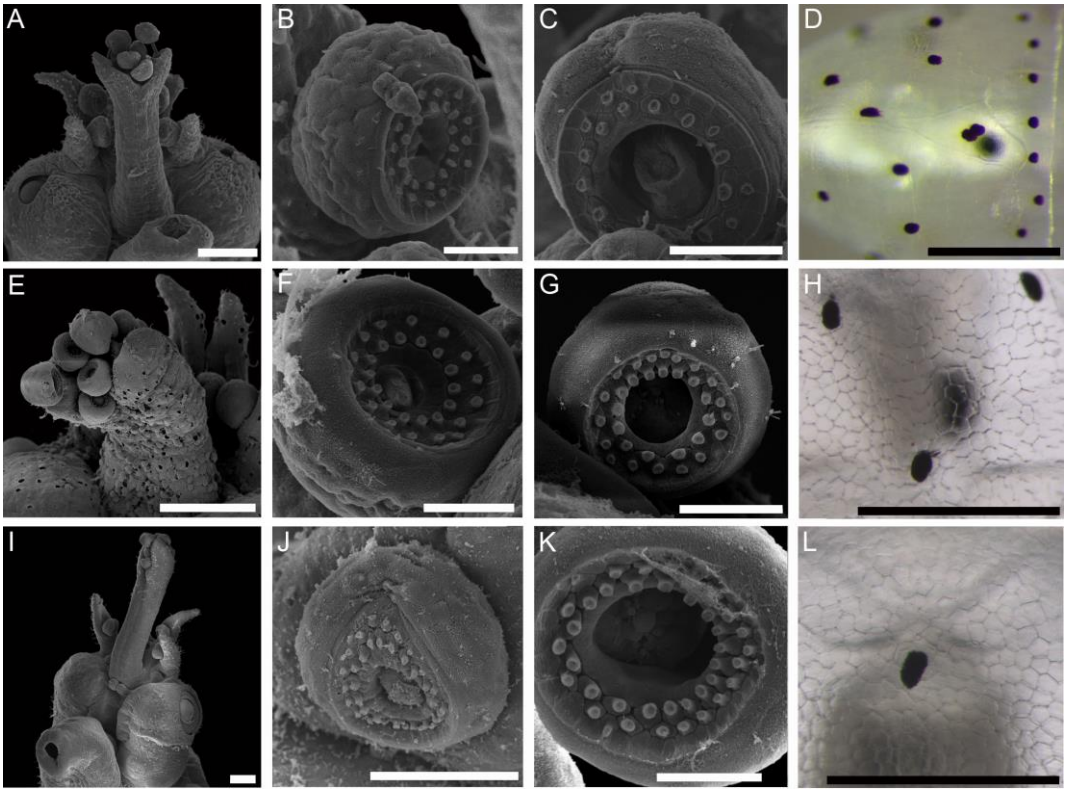
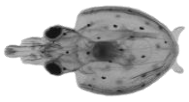


Fig. 2.2. (**A-D**) *Illex coindetii*. (**A**) SEM image of the ventral view of the head, aged 270 h and incubated at 17 ºC. (**B**) SEM image of the arm I sucker, aged 270 h and incubated at 17 ºC. (**C**) SEM image of a proboscis sucker, aged 454 h and incubated at 17 ºC. (**D**) Detail of the ventral skin of an anaesthetized specimen, aged 329 h and incubated at 21 ºC. (**E-H**) *Todarodes sagittatus*. (**E**) SEM image of proboscis tip, showing the differences between the lateral and medial suckers, aged 361 h and incubated at 15 ºC. (**F**) SEM image of the left arm I sucker, aged 361 h and incubated at 15 ºC. (**G**) SEM image of a proboscis sucker, aged 361 h and incubated at 15 ºC. (**H**) Detail of the ventral skin of an anaesthetized specimen, aged 383 h and incubated at 15 ºC. (**I-L**) *Todaropsis eblanae*. (**I**) SEM image of the ventrolateral view of the head of a paralarva, aged 477 h and incubated at 15 ºC, the III and IV pairs of arm stumps are visible. (**J**) SEM image of the sucker of the left arm I, aged 475 h and incubated at 15 ºC. (**K**) SEM image of a proboscis sucker, aged 477 h and incubated at 15 ºC. (**L**) Detail of the ventral skin of an anaesthetized specimen, aged 498 h and incubated at 15 ºC. Scale bars: **A**, **E**, **I**, 100 µm; **B**, **C**, **F**, **G**, **K**, 20 µm; **J**, 50 µm; **D**, **H**, **L**, 0.5 mm.

Todaropsis eblanae hatchlings

This species is the largest rhynchoteuthion hatchling (Table 2.2) described to date and exhibits more advanced development at the time of hatching than other previously described species (Fig. 2.1I-L, 2.2I, 2.3G-I). In addition to the arm pairs I, II and IV, the pair III arm primordia are present as well (Fig. 2.2I). While arm pairs I and II possess one sucker, pairs III and IV have no suckers. The mean VML is 2.16 ± 0.11 mm. On the head, there are 2 chromatophores ventrally, one below each eye; dorsally there are three rows of chromatophores, the first formed by a single chromatophore anterior to the eyes, the second formed by two chromatophores at the level of the eyes and the third formed by 3 chromatophores at the base of the head. On the mantle, there are up to 6 rows ventrally, distributed as follows: 7 + 5 + 0 + 0 + 5 + 2; dorsally, there are up to 5 rows: 3 + 0 + 5 + 0 + 2. Between 1 and 4 true lateral chromatophores are



present on the mantle (Fig. 2.3I, Table 2.3). The 8 proboscis suckers are of similar size (54.3 ± 5.0 µm) (Type C rhynchoteuthion) and have two rows of pegs, the internal one with 18.1 ± 1.0 and the external one with 22.0 ± 2.2 pegs (Fig. 2.3K), with up to 3 additional pegs asymmetrically distributed outside of the external row. The arm suckers measure 63.0 ± 5.5 µm and bear two rows of pegs (Fig. 2.2B), the internal one formed by 17.0 ± 0.8 and the external by 20.6 ± 1.5 pegs. Between 0 and 8 pegs arranged externally to the external row can be found asymmetrically scattered. The skin of the species shows a hexagon-like structure under the stereomicroscope (Fig. 2.2L). DMLI is smaller than in the other two species, which indicates a similar length between DML and VML. Although there is some overlap with the other two species, HWI is smaller, and thus the head is narrower. The AIILI shows a larger pair of arms II than *I. coindetii* and *T. sagittatus*.

Remarks: Roura (2013) sequenced two wild rhynchoteuthion paralarvae which did not produce a species-level match with any previously sequenced ommastrephid. He hypothesized that those collected in the oceanic realm should be assigned to *Todarodes sagitattus* and those from the shelf should be assigned to *Todaropsis eblanae*. The recent work of Gebhardt & Knebelsberger (2015) provided available barcodes for these two species, which could be used to positively identify wild rhynchoteuthions by DNA barcoding (Hebert et al., 2003). A BLAST (Altschul et al., 1990) search shows that the sequence with the GenBank accession number LN614712, uploaded and identified as *T. eblanae* by Roura, does represent an individual of this species.

Ommastrephes bartramii paralarvae (Fig. 2.3J-K)

The following description is based on Sweeney et al. (1992), Young & Hirota (1990) Sakurai et al. (1995) and Vijai et al. (2015). The hatchling bears the pairs of arms I, II and IV, the latter devoid of suckers. On the head, there is a row of two ventral chromatophores; dorsally, there are two rows formed by 1 and 2 chromatophores, respectively. On the mantle, the ventral surface has up to 3 rows with the following formula: (3-4) + (0-1) + 1; dorsally there are up to 2 rows: (4-5) + (0-1). Later (~3 mm DML), two ventrally centred chromatophores appear on the edge of the mantle. The dorsal pattern becomes scattered-like at size ~4 mm mantle length and ventrally at ~6 mm mantle length. The ratio between the lateral and medial proboscis suckers is 2:1 (Type A rhynchoteuthion). There are two rows of pegs on the proboscis suckers. There are more pegs on the lateral suckers (~20 internal, ~27 external) than on the medial ones (~11 internal, ~14 external). The skin shows a hexagon-like structure under the stereomicroscope (Vijai et al., 2015: Fig 7j-m).

Sthenoteuthis pteropus paralarvae (Fig. 2.3N)

The following descriptions are based on Sweeney et al. (1992). This species is characterized by the presence of two equally-sized intestinal photophores and another single photophore on the ventral surface of each eye. The chromatophore pattern has not been described for this species. There are no size differences among the proboscis suckers (Type C rhynchoteuthion). The pegs of the proboscis suckers are unknown.

Hyaloteuthis pelagica paralarvae (Fig. 2.3L-M)

The following descriptions are based on Harman & Young (1985), Sweeney et al. (1992) and Diekmann et al. (2002). This paralarva bears one central intestinal photophore and another on the ventral surface of each eye. According to Diekmann et al. (2002: Table 5), the intestinal photophore is visible in individuals of 1.5 mm ML or larger. The chromatophore pattern is formed by highly scattered units on both the mantle and head. On the head, one chromatophore is located on the ventral surface of each eye and another one is located on the dorsal surface of the eye. On the head, dorsally there are four rows of chromatophores (excluding the ocular chromatophores): 1 + 2 + 1. Individuals smaller than 2 mm of DML lack chromatophores on the ventral surface of the mantle; later a single row of chromatophores on the anterior edge and four chromatophores forming a diamond-shape pattern between the fins appear. Dorsally on the mantle, a single large chromatophore is present on the first third of the mantle surface during most of its life as a rhynchoteuthion. The ratio of the proboscis suckers is between 1.25:1 and 1.5:1 (Type B rhynchoteuthion). There are two rows of pegs on the proboscis suckers. There are more pegs on the lateral suckers (14-16 internal, 18-19 external) than on the medial ones (8-11 internal, 8-15 external).

Ornithoteuthis antillarum paralarvae (Fig. 2.3O)

The following description is based on Sweeney et al. (1992) and Diekmann et al. (2002). This paralarva has single and round ventral photophores on the ventral surface of each eye and two unequally-sized intestinal photophores: the anterior one appears first (Sweeney et al., 1992), but the posterior one grows larger (Diekmann et al., 2002: Table 5). The chromatophore pattern is not known. The ratio of the proboscis suckers is up to 1.5:1 (Type B rhynchoteuthion). The pegs of the proboscis suckers are not known.

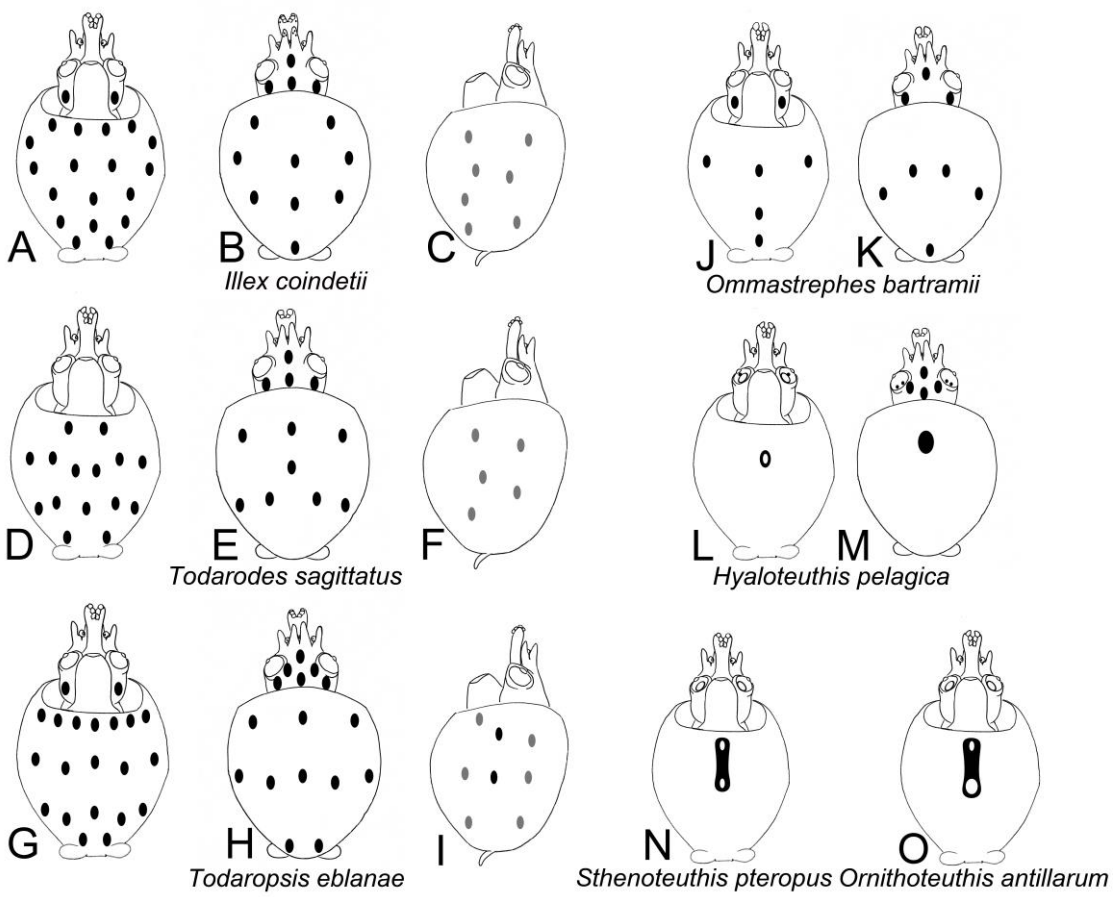


Fig. 2.3. Schematic drawing of the chromatophore and photophore pattern of the seven North-eastern Atlantic rhynchoteuthions. (**A-C**) *Illex coindetii*. (**A**) Ventral view. (**B**) Dorsal view. (**C**) Lateral view. (**D-F**) *Todarodes sagittatus*. (**D**) Ventral view. (**E**) Dorsal view. (**F**) Lateral view. (**G-I**) *Todaropsis eblanae*. (**G**) Ventral view. (**H**) Dorsal view. (**I**) Lateral view. (**J-K**) *Ommastrephes bartramii*. (**J**) Ventral view. (**K**) Dorsal view. (**L-M**) *Hyaloteuthis pelagica*.(**L**) Ventral view. (**M**) Dorsal view. (**N**) *Sthenoteuthis pteropus*, ventral view. (**O**) *Ornithoteuthis antillarum*, ventral view. Grey chromatophores of **A-F** depict those seen in both dorsal and ventral views. Concentric black and white circles on l-n depict ocular and intestinal photophores. Chromatophore pattern of a-f is based on the mode of the chromatophore pattern (see Table 2.3); **J-K** based on Sweeney et al. (1992), Young & Hirota, (1990), Sakurai et al. (1995) and Vijai et al. (2015). Photophore and chromatophore pattern of **L-M** based on Harman & Young (1985) and Sweeney et al. (1992). Photophore pattern of **N** based on Sweeney et al. (1992), of **O** based on Sweeney et al. (1992) and Diekmann et al. (2002). The chromatophore pattern of *S. pteropus* (**N**) and *O. antillarum* (**O**) are not known.

2.4.2. Key for the NE Atlantic rhynchoteuthion paralarvae

The primary characters of this key are the size of the proboscis suckers, the presence/absence of photophores and the arrangement of the proboscis sucker pegs. Although the first two characters are observable under a standard stereomicroscope, the proboscis pegs should be observed by specialized microscopy methods, such as SEM. Since the number and arrangement of the chromatophores are variable during the ontogeny of the rhynchoteuthions, we only use these characters when they are taxonomically significant (key step 3a).

1a. Ratio of sucker sizes of 2:1 (Type A rhynchoteuthion), two rows of pegs in proboscis suckers. More pegs in the lateral proboscis suckers (~20 internal, ~27 external) than in the medial ones (~11 internal, ~14 external). Photophores absent. *Ommastrephes bartramii*

1b. Ratio of sucker sizes lower than 2:1 **2**

2a. Ratio of sucker sizes between 1.1:1 and 1.9:1 (Type B rhynchoteuthion) **3**

2b. Ratio of sucker sizes of 1:1 (Type C rhynchoteuthion) **5**

3a. Absence of intestinal or ocular photophores, two rows of pegs in proboscis suckers (12-24 internal, 12-22 external), no differences in the number of pegs between lateral and medial proboscis suckers. No ventral head chromatophores. *Todarodes sagittatus*

3b. With ocular and intestinal photophores. **4**

4a. Presence of one central intestinal photophore. More pegs in the lateral proboscis suckers (14-16 internal, 18-19 external) than in the medial ones (8-11 internal, 8-15 external).

Hyaloteuthis pelagica

4b. Presence of two unequal intestinal photophores, the posterior one larger than the first one, proboscis sucker pegs unknown. *Ornithoteuthis antillarum*

5a. Presence of two equally-sized intestinal photophores and another single photophore on the ventral surface of each eye, proboscis sucker pegs unknown. *Sthenoteuthis pteropus*

5b. Without intestinal or ocular photophores **6**

6a. Only one row of pegs on the proboscis suckers (10-14, 1-12 free ones), skin without a hexagon-like pattern. *Illex coindetii*

6b. Two rows of pegs (16-24 internal, 17-27 external plus 0-3 free ones) on the proboscis suckers, presence of the 3rd pair of arms at the time of hatching, skin with a hexagon-like pattern.

Todaropsis eblanae

2.4.3. Test of the dichotomous key using wild rhynchoteuthion paralarvae

Fourteen of the 16 rhynchoteuthions examined were successfully identified with the aid of the key: 9 belonged to *I. coindetii*, 4 to *T. sagittatus* and 1 to *S. pteropus* (Table 2.5, Fig. 2.4). Among those not identified to the species level with the key there is a Type C paralarva with 2 rows of dorsal head chromatophores (Fig. 2.4B), compatible with the pattern of *I. coindetii* and not with *T. eblanae*. Thus, this emphasizes the utility of the chromatophore pattern for the identification of hatchlings when other characters are not available. The second unidentified rhynchoteuthion is a Type C paralarva without photophores. It was not possible to determine whether it was *I. coindetii* or *T. eblanae*. Fifteen of the specimens were obtained in the Mediterranean Sea: this is the first time that identification of Mediterranean ommastrephid paralarvae from formalin-preserved plankton samples is possible with certainty. In general, the most useful characters for the identification of the paralarvae are: 1) the size of the proboscis suckers, 2) the presence/absence of ocular or intestinal photophores and 3) the proboscis pegs.

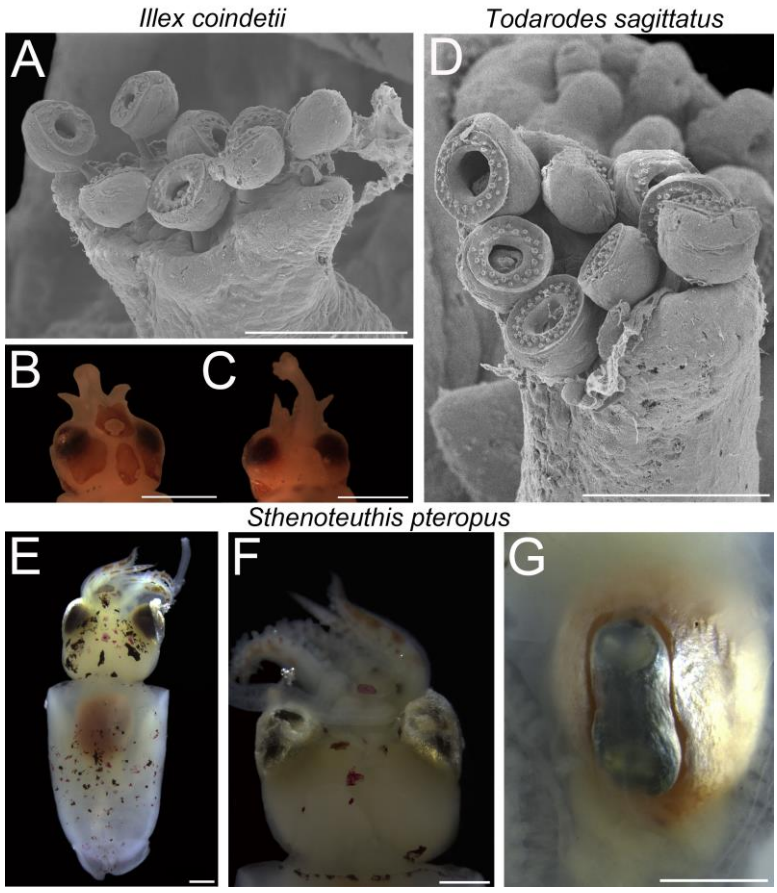
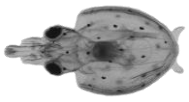


Fig. 2.4. Main taxonomic characters used to identify wild-collected rhynchoteuthions by the dichotomous key provided here. (**A-C**) *Illex coindetii*. (**A**) 2.4 mm DML. SEM image of the proboscis suckers, showing a single row of pegs. (**B-C**) 1.03 mm DML. (**B**) Dorsal view of the head showing the chromatophore pattern 1 + 3. (**C**) Ventral view of the head showing one row of two chromatophores. (**D**) *Todarodes sagittatus*, 2.20 mm DML. SEM image of the proboscis suckers showing lateral suckers larger than the medial sucker. (**E-G**) *Sthenoteuthis pteropus*, 7.71 mm DML. (**E**) Dorsal view of the specimen. (**F**) Ventral view of the head showing the ocular photophores. (**G**) Ventral view of the specimen with the mantle opened to show the two equally-sized intestinal photophores. Scale bars: **A**, **D**: 0.1 mm; **B-C**: 0.5 mm; **E-G**: 1 mm.

Table 2.5. Wild rhynchoteuthion from the NE Atlantic (most from the NW Mediterranean) identified to the species level using the identification key described here. The collecting information and the specific diagnostic characters used in the identification are shown. The measurements were performed on preserved paralarvae, so an undetermined shrinkage is expected in relation to live paralarvae. * Measurements taken from a defrosted specimen.

Species	Cruise, station and geographic coordinates	Depth (m)	Seafloor depth (m)	Date and hour	n	DML (mm)	VML (mm)	Diagnostic characters
Illex coindetii	CACO 1, st. 9, 40.42 ° N 1.05 E	0-81	88	7-19-2003, 12:31	1	No data	0.97	Type C rhynchoteuthion, no photophores, only one row of pegs in proboscis suckers.
Illex coindetii	CACO 2, st. 5, 40.23º N 1.16º E	0-120	125	9-12-2003, 2:35	1	2.43	2.18	Type C rhynchoteuthion, no photophores, only one row of pegs in proboscis suckers (Fig. 4a).
Illex coindetii	CACO 2, st. 9, 40.43º N 1.06º E	0-80	88	9-12-2003, 13:10	1	2.15	2.13	Type C rhynchoteuthion, no photophores, only one row of pegs in proboscis suckers.
Illex coindetii	CACO 3, st. 4, 40.27º N 1.02º E	0-85	95	6-24-2004, 0:47	1	1.29	1.21	Type C rhynchoteuthion, no photophores, only one row of pegs in proboscis suckers.
Illex coindetii	CACO 3, st. 8, 40.38º N 1.21º E	0-100	107	6-24-2004, 12:03	2	1.06, 1.16	1.02, no data	Type C rhynchoteuthion, no photophores, only one row of pegs in proboscis suckers.
Illex coindetii	CACO 3, st. 23, 40.95º N, 1.12º E	0-65	75	6-26-2004, 00:48	1	2.02	2.06	Type C rhynchoteuthion, no photophores, only one row of pegs in proboscis suckers.
Illex coindetii	CACO 3, st. 24, 40.90º N 1.26º E	0-100	110	6-26-2004, 05:38	1	1.33	1.24	Type C rhynchoteuthion, no photophores, only one row of pegs in proboscis suckers
Todarodes sagittatus	CACO 3, st. 43, 41.49º N, 2.70º E	0-125	135	6-28-2004, 03:40	2	1.68, 2.20	1.60, 2.09	Type B rhynchoteuthion, no photophores, two rows of pegs in proboscis suckers (Fig. 4d).
Illex coindetii	CACO 3, st. 55, 41.75º N 3.50º E	0-200	765	6-29-2004, 13:47	1	2.84	3.06	Type C rhynchoteuthion, no photophores, only one row of pegs in proboscis suckers.
Todarodes sagittatus	CACO 4, st. 34, 41.11º N 2.20º E	0-200	625	7-25-2004	2	0.77, 1.03	0.94, 0.93	Type B rhynchoteuthion, no photophores, two rows of pegs in proboscis suckers.
Illex coindetii or *Todaropsis eblanae*	LLUÇ3, 4.3, 41.00 °N 1.42° E	0-186	No data	4-06-1999, 21:00	1	No data	1.97	Type C rhynchoteuthion, no photophores, proboscis suckers not visible.
Illex coindetii	FishJelly, 41.38´ N 2.21´ E	0-10	No data	10-21-2014, 11:00	1	1.03	0.94	Type C rhynchoteuthion, no photophores. Chromatophore pattern visible in this specimen and compatible with *I. coindetii* and not with *T. eblanae* (Fig. 4b-c).
Stenoteuthis pteropus	MAFIA, PEL07, 7.15 ºN 23.97 ºW	0-100	4245	4-17-2015, 23:28	1	7.71*	7.11*	Type C rhynchoteuthion, two equal-sized intestinal photophores (Fig. 4g) and an ocular photophore in each eye (Fig. 4f).



2.5. Discussion

Previous studies have stressed the difficulties of identifying ommastrephid paralarvae from plankton samples (Collins et al., 2002; Gilly et al., 2006; Moreno, 2008; Moreno et al., 2009; Roura, 2013; Zaragoza et al., 2015). Morphological clusters could sometimes be identified based only on the paralarvae morphology, but it was not possible to confirm the species (e. g. Roper & Lu, 1979; Vecchione et al., 2001; Moreno, 2008). Other times, species have been identified based on the adult characters that could be seen in the paralarvae, such as photophores (e. g. Sweeney et al., 1992). Three different sources of paralarval species confirmation could be used, namely: a) aquarium spawning, b) *in vitro* fertilization and c) molecular methods. The first method is limited by the difficulties in catching, housing and maintaining broodstock and inducing spawning (Durward et al., 1980; Bower & Sakurai, 1996). The last method is constrained by the need for previous molecular data obtained from properly identified adults, which may not exist for some species (Roura, 2013). Thus, the main advantage of using *in vitro* fertilization is that the species identification of the paralarvae produced is ensured. Here, this method was successfully used to describe the paralarvae morphology of three ommastrephid species.

2.5.1. Towards the reliable morphological identification of rhynchoteuthion paralarvae: which are the most useful characters?

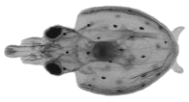
The morphological description of *I. coindetii*, *T. sagittatus* and *T. eblanae* revealed new taxonomic characters that permit the identification of rhynchoteuthion paralarvae collected in plankton samples from the NE Atlantic. In the case of *I. coindetii*, when alive, they can be easily identified by the absence of any special skin sculpture (Fig. 2.2D), a character shared with the congeneric *I. argentinus* (Sakai et al., 1998:Fig. 4). Skin sculpture is present in fresh *T. sagittatus* and *T. eblanae* (Fig. 2.2H, 2.2L, respectively), *T. pacificus* (Watanabe et al., 1996: Fig. 7; Puneeta et al., 2015:Fig. 8), *O. bartramii* (Vijai et al., 2015:Fig 7j-m) and *Dosidicus gigas* (d'Orbigny, 1835: 50 [in 1834–1847]) (D. Staaf, pers. comm.). This skin sculpture is an optical effect created by light that crosses vertical expansions of the basal membrane of the outer epithelium between the mucous cells (F. Á. Fernández-Álvarez, unpubl. observation). Thus, the absence of this skin sculpture in hatchlings seems to be a synapomorphy of the genus *Illex*. When dealing with fixed specimens, the presence of only one row of pegs in the proboscis suckers allows *I. coindetti* to be differentiated from other sympatric rhynchoteuthions described to date.

Todarodes sagittatus has a type B paralarva, a condition that differentiates this species from sympatric ommastrephids, except for *H. pelagica* and *O. antillarum*, which bear ocular and intestinal photophores, with the former bearing a peculiar chromatophore pattern. When we compare *T. sagittatus* hatchlings with the congeneric species *T. pacificus* (Watanabe et al., 1996), the main difference is the absence of ventral head chromatophores in *T. sagittatus*. Based on the chromatophore pattern depicted by Watanabe et al. (1996:Fig. 8), *T. pacificus* bears 5 rows on the ventral surface of the mantle, whereas *T. sagittatus* hatchlings have 3-4 (Table 2.3). The specimens of *T. pacificus* depicted by Puneeta et al. (2015) are Type C rhynchoteuthions, however more accurate information on the structure of the proboscis suckers, such as the arrangement of pegs of the suckers, is lacking for comparison against *T. sagittatus*.

Todaropsis eblanae is the largest rhynchoteuthion hatchling described so far and the presence of the third arm buds at hatching is a diagnostic character that differs from other described rhynchoteuthion hatchlings. It can be easily differentiated from *S. pteropus* (also Type C rhynchoteuthion) because it has no photophores. It can also be differentiated from *I. coindetii* because *T. eblanae* has two rows of pegs in the proboscis suckers and a hexagon-like skin sculpture. The morphological descriptions provided here for *I. coindetii*, *T. sagittatus* and *T. eblanae* paralarvae were based on hatchlings and some characters are known to change during their development (see below), such as morphometrics (Table 2.2) and the chromatophore pattern (Table 2.3). However, the structure of the eight proboscis suckers (Table 2.4) is unlikely to change during the rhynchoteuthion phase (see below).

Ommastrephes bartramii is unlikely to be confused with other rhynchoteuthions present in the NE Atlantic, as it is the only Type A rhynchoteuthion in these waters. However, in other areas of its known distribution, this species overlaps with other species with this type of paralarva, such as one of the species of the genus *Ornithoteuthis* Okada, 1927 (Wakabayashi et al., 2002): *Ornithoteuthis volatilis* (Sasaki, 1915). This species is sympatric with *O. bartramii* in both the Pacific and Indian Oceans, but its rhynchoteuthion has fewer pegs on the lateral proboscis suckers (Wakabayashi et al., 2002:Table 6). Moreover, *Ornithoteuthis* rhynchoteuthions have two intestinal photophores (Sweeney et al., 1992; Diekmann et al., 2002: Table 5). It is not clear if *Nototodarus* Pfeffer, 1912 species are Type B or A rhynchoteuthions or both (Sweeney et al., 1992). Since these species also lack photophores, they are the only species that may be misidentified as *O. bartramii* in areas where their distribution overlaps. More research is needed to clarify the morphology of *Nototodarus* paralarvae.

The presence of two intestinal photophores in *S. pteropus* can easily differentiate this species from *H. pelagica* (which has a single round intestinal photophore) in the NE Atlantic, in addition to the proboscis suckers (Type C in the first species, Type B in the second one).



Ornithoteuthis antillarum also has two intestinal photophores, however the presence of large lateral suckers and the unequal size of the intestinal photophores in *O. antillarum* distinguishes the two species.

The presence of a single intestinal photophore differentiates *H. pelagica* from the other rhynchoteuthions from the NE Atlantic. The proboscis sucker pegs of *H. pelagica* were studied by Harman & Young (1985), showing a pattern with ~14 internal, ~17 external pegs in the lateral suckers and ~9 internal, ~10 external in the medial ones. However, another type B species with a single intestinal photophore, *Eucleoteuthis luminosa* (Sasaki, 1915), is sympatric throughout the distribution area of *H. pelagica* with the exception of the entire North Atlantic. For both species, the proboscis sucker pegs have been described (Harman & Young, 1985 for *H. pelagica* and Wakabayashi et al., 2002 and Granados-Amores et al., 2013 for *E. luminosa*), and the number of pegs in the proboscis suckers seems to be higher than in *H. pelagica*, although some overlap exists (Wakabayashi et al., 2002:Table 2.5). Wakabayashi et al. (2006) and Granados-Amores et al. (2013) molecularly identified paralarvae of *E. luminosa*, validating its description.

Ornithoteuthis antillarum can be easily differentiated from other Type B rhynchoteuthions by its two unequally-sized photophores. This character also differentiates this species from *E. luminosa* in the S Atlantic. The congeneric *O. volatilis* is a Type A rhynchoteuthion (Wakabayashi et al., 2002), while *O. antillarum* is a Type B rhynchoteuthion (Diekmann et al, 2002).

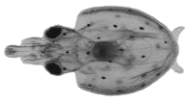
Based on the information presented above, it is possible to evaluate the reliability of identifications of rhynchoteuthions based on each taxonomic character. Although the chromatophore pattern led to the correct identification of a *T. sagittatus* specimen from the literature and an *I. coindetii* specimen from our wild collected paralarvae (see above and Table 2.5, Fig. 2.4B-C), it should be noted that this pattern changes during the ontogeny (e.g. Young & Hirota, 1990). The same occurs with morphometrics: the shape of the body of rhynchoteuthion paralarvae changes with the size of the animal (e.g. Young & Hirota, 1990, Vidal, 1994 or Gilly et al., 2006) and the different morphometric indices change during ontogeny (Ramos-Castillejos et al., 2010). Although differences between the number of pegs on arm suckers do exist between species (Table 2.4), comparisons are only possible between paralarvae of similar sizes (in the case of the three rhynchoteuthion species described here, hatchlings and the immediately following stages). Moreover, while the paralarva grows, more suckers are added to the arms, making it difficult to identify each single sucker. As can be seen in Ramos-Castillejos et al. (2010) and Wakabayashi et al. (2002), the number of pegs per row on arm suckers increases while the paralarva grows. Thus, we do not recommend relying only

on chromatophore pattern, morphometrics and arm sucker pegs, without the support of others characters for identification purposes. A combination of both proboscis suckers and photophores seems to be the most reliable combination. Photophores are easy to find under the stereomicroscope, both in fresh and fixed specimens. However, no member of the Subfamilies Illicinae Posselt, 1891, Todarodinae Adam, 1960 or Todaropsinae Nigmatullin, 2000 is known to possess photophores. Together these subfamilies represent 64 % of the species biodiversity among the Ommastrephidae. Moreover, this character should be considered with caution since it is not known when photophores appear in some species (Sweeney et al., 1992; Diekmann et al., 2002) and at least in *D. gigas* some variation for photophore appearance during ontogenetic development is suspected (Gilly et al., 2006 found paralarvae of this species with the typical photophores present in the juvenile). Again, by using a binocular microscope it is easy to differentiate between rhynchoteuthion Types A, B and C. When species of the same Type coexist (*I. coindetii* and *T. eblanae* in NE Atlantic), the best approach is to study the proboscis pegs by SEM (Table 2.5, Fig. 2.4A). While an individual is a rhynchoteuthion, they have the same 8 proboscis suckers until the proboscis splits and the animal becomes a juvenile (Shea, 2005; Wakabayashi et al., 2002). As Ramos-Castillejos et al. (2010:Fig. 9) have shown for *D. gigas*, the number of internal and external pegs in the 8 proboscis suckers remains the same throughout the rhynchoteuthion stage.

2.5.2. The search for the hatchling stage in ommastrephids

An unresolved discussion remains regarding which of the embryological stages described for ommastrephid squids represents the actual hatchling. This question was raised by Watanabe et al. (1996), who observed a two-stage delay between hatching from aquaria-spawning eggs and those from *in vitro* fertilization in *T. pacificus*. They hypothesize that the hatching stage should be addressed between stages XXV and XXVII, because Hoyle´s organ was visible during these stages. A similar observation was reported by Staaf et al. (2008) for *D. gigas*. Recently, Puneeta et al. (2015) observed hatchlings of stage XXXI from aquarium-spawned *T. pacificus*. In short, although several experiments with eggs obtained by *in vitro* fertilization and by aquaria-spawning in ommastrephids have been performed, the question is unresolved.

In naturally-hatched individuals the development of the characteristics that allow them to swim and avoid predators is expected. However, individuals at stages XXIX or younger never have a fully developed ink sac, fins or statoliths. Thus, correct swimming is not possible at this time and these individuals can only perform horizontal movements on the bottom of the Petri dish, probably by using cilia on the skin (R. Villanueva, unpubl. observation). Additionally, in the case of *T. pacificus* (Watanabe et al., 1996), *T. sagittatus* and *T. eblanae*, fins are not fully



developed until stage XXXII. In the present study, the criteria to select a stage as hatchling was a paralarva capable of swimming, with a fully developed ink sac, statoliths and fins as wide as the head. This definition corresponds to stages beyond XXX (*sensu* Sakai et al., 1998) for *Illex* and XXXII (*sensu* Watanabe et al., 1996) for *Todarodes* and *Todaropsis*. Taking into account these criteria, any stage below XXXII should not be addressed as the hatchling for *O. bartramii*, based on the description by Vijai et al. (2015). In addition to these arguments, individuals less developed than those considered here are never reported from plankton samples (for instance, a paralarva without fins). The presence of these prematurely hatched individuals, in our opinion, does not indicate the natural hatchling stage and is a probable consequence of suboptimal artificial conditions in the laboratory (Villanueva et al., 2011). Better culture conditions would likely produce hatchlings that are morphologically more similar to those produced in the wild as the results reported by Puneeta et al. (2015) suggest.

2.6. Conclusions

Comprehensive knowledge on the life cycle of ommastrephid squids is hindered by the fact that many aspects of the first stages of their life cycle remain a mystery, especially regarding the paralarvae. For instance, we still do not know the main diet of early rhynchoteuthions as their stomachs are usually empty or contain unrecognized food (e.g. Uchikawa et al., 2009; Camarillo-Coop et al., 2013). Vidal & Haimovici, (1998) have found microorganisms (ciliates, flagellates and bacteria) with mucus inside the digestive tract of early rhynchoteuthions and copepod appendages on the digestive tracts of paralarvae > 3.7 mm ML; together these observations indicate that ommastrephid paralarvae certainly adopt different feeding strategies as they grow (Uchikawa et al., 2009; Shea, 2005). This lack of knowledge inevitably leads to a total failure when any culture experiment is attempted (e. g. Yatsu, Tafur & Maravi, 1999; Staaf et al., 2008). Thus, the little knowledge available on paralarvae beyond yolk consumption proceeds from paralarvae collected in the wild, including the morphology of post-hatchlings (e. g., Young & Hirota, 1990), the bathymetric layers suitable for their development (e. g., Roper & Lu, 1979), their distribution (e.g., Staaf et al., 2013) and the tentative length of the paralarval period (e. g., Uchikawa et al., 2009). Clearly, if our knowledge in all these matters relies on wild collected paralarvae, a well-established taxonomic knowledge of each species for reliable identification is necessary. Here, we fill a gap in the knowledge of NE Atlantic rhynchoteuthions by describing the morphology of three of the seven species and used this new knowledge in conjunction with the literature to develop the first dichotomous key covering all the ommastrephid squid paralarvae present in a wide area. Moreover, this methodology was applied to identify 16 plankton sampled ommastrephid paralarvae and, for the first time, data on

properly identified paralarvae was provided for the Mediterranean Sea. This key will permit the identification of rhynchoteuthions from plankton samples collected in the NE Atlantic, thus providing a useful tool for future studies on the planktonic life and population dynamics of ommastrephid squids.

2.7. Acknowledgements

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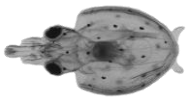

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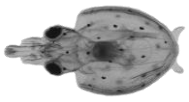

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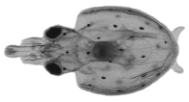

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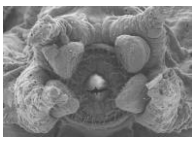
CHAPTER 3

Predatory flying squids are detritivores during their early planktonic life

Fernández-Álvarez FÁ, Machordom A, García-Jiménez R, Salinas-Zavala CA & Villanueva R. (2018) Predatory flying squids are detritivores during their early planktonic life. *Scientific Reports*, doi: 10.1038/s41598-018-21501-y [in press].



3.1. Abstract

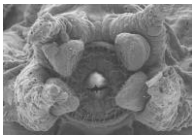
Cephalopods are primarily active predators throughout life. Flying squids (Family Ommastrephidae) represents the most widely distributed and ecologically important family of cephalopods. Due to their huge biomass in the oceanic realm, they support some of the largest invertebrate fisheries. While the diets of subadult and adult flying squids have been extensively studied, the first feeding diet of early paralarvae remains a mystery. The morphology of this ontogenetic stage notably differs from other cephalopod paralarvae, suggesting a different feeding strategy. Here, a combination of Laser Capture Microdissection (LCM) and DNA metabarcoding of wild-collected paralarvae gut contents for eukaryotic 18S v9 and prokaryotic 16S rRNA, covering almost every life domain. The gut contents were mainly composed by fungus, plants, algae and animals of marine and terrestrial origin, as well as eukaryotic and prokaryotic microorganisms commonly found in fecal pellets and particulate organic matter. This assemblage of gut contents is consistent with a diet based on detritus. The ontogenetic shift of diet from detritivore suspension feeding to active predation is a unique life strategy feature among cephalopods and allows ommastrephid squids to take advantage of an almost ubiquitous and accessible food resource during their early stages, which may explain the ecological success of these squids in the oceanic realm and, thus, the high biomass that sustains successful fisheries. LCM was successfully applied for the first time to tiny, wild-collected marine organisms, proving its utility in combination with DNA metabarcoding for dietary studies.

3.2. Introduction

Cephalopods are active carnivorous predators, with only a few exceptions: *Nautilus* spp. are mainly scavengers and opportunistic predators (e.g., Saunders, 1984; Dunstan et al., 2011), the vampire squid *Vampyroteuthis infernalis* is a detritivore (Hoving & Robison, 2012), and the mesopelagic Ram's horn squid *Spirula spirula* feeds mainly on detritus and zooplankton (Ohkouchi et al., 2013). The remaining 845 species described to date (Hoving et al., 2014) are active predators (Villanueva et al., 2017) and their diets are mainly known from studies on their subadult and adult forms. Cephalopods can hatch as large juveniles similar to the adult in morphology, habitat and feeding habits, or may have a less developed planktonic form, known as paralarvae, usually with a different lifestyle than the adults (Young & Harman, 1988). The behavior and diet of cephalopod hatchlings reported to date has demonstrated their active predatory habits from hatching (e.g., Chen et al., 1996; Sugimoto & Ikeda, 2013), however, this knowledge is mainly based on coastal shallow-water species, due to availability for sampling and laboratory maintenance (Iglesias et al., 2014).

The squid Family Ommastrephidae is currently formed by 22 oceanic species, and represents the most widely distributed and ecologically important family of cephalopods (Jereb & Roper, 2010). Due to their huge biomass in the oceanic realm, they support some of the largest invertebrate fisheries (Arkhipkin et al., 2015a) and represent nearly 50 % of the total cephalopod biomass fished worldwide (FAO, 2016). The life cycle of ommastrephids is relatively short, ranging from six months to two years (Rosa et al., 2013; Arkhipkin et al., 2015b), allowing them to quickly respond to environmental changes, such as depletions in prey availability driven by climate change (Hoving et al., 2013). The characteristic paralarva of ommastrephids, known as rhynchoteuthion, is characterized by the fusion of both tentacles into a proboscis (Fig. 3.1A), the function of which is unknown (Fernández-Álvarez et al., 2017). Along the ontogeny of the squid, the proboscis starts to split (Fig. 3.1B; Shea, 2005) and eventually becomes two independent raptorial tentacles (Fig. 3.1C), used for prey capture. Newly hatched paralarvae are provided with numerous filamentous buccal papillae around the mouth (Fig. 3.1D; Shigeno et al., 2001a), which become less abundant as the paralarvae grow until they totally disappear (Fig. 3.1E; Shigeno et al., 2001a), coinciding with the split of the proboscis into raptorial tentacles (Fig. 3.1B, E; Shea, 2005). The function of these papillae is also unknown. For clarity, throughout the current work, the paralarvae prior to losing the buccal papilla are referred to as "early paralarvae" and after as "late paralarvae".

The diet of both subadult and adult ommastrephids has been extensively studied (e. g., Markaida et al., 2008, Rosas-Luis et al., 2014), however, the diet of the early paralarvae remains unknown (Uchikawa et al., 2009; Camarillo-Coop et al., 2013). All attempts of ommastrephid paralarval rearing have been unsuccessful as paralarvae would not ingest any offered prey (Yatsu et al., 1999; Staaf et al., 2008).



Studies on wild caught ommastrephid paralarvae did not provide recognizable prey (Uchikawa et al., 2009; Camarillo-Coop et al., 2013) until the proboscis began to split and the remains of crustaceans and cephalopods appeared in their stomach contents (Vidal & Haimovici, 1998; Uchikawa et al., 2009). Interestingly, Vidal & Haimovici (1998) observed a great diversity of microorganisms (dinoflagellates, flagellates, ciliates, cysts and bacteria) on the paralarva mucus cover, on the proboscis suckers and in the digestive tracts of the early ommastrephid paralarvae. They suggested that this mucus may act as a substrate for microbial growth that paralarvae may use this as food and ingest it with the aid of the proboscis. Other authors suggested that ommastrephid paralarvae feed on suspended particles by using the mucus cover of the body (O´Dor et al., 1985), but they did not provide further evidence.

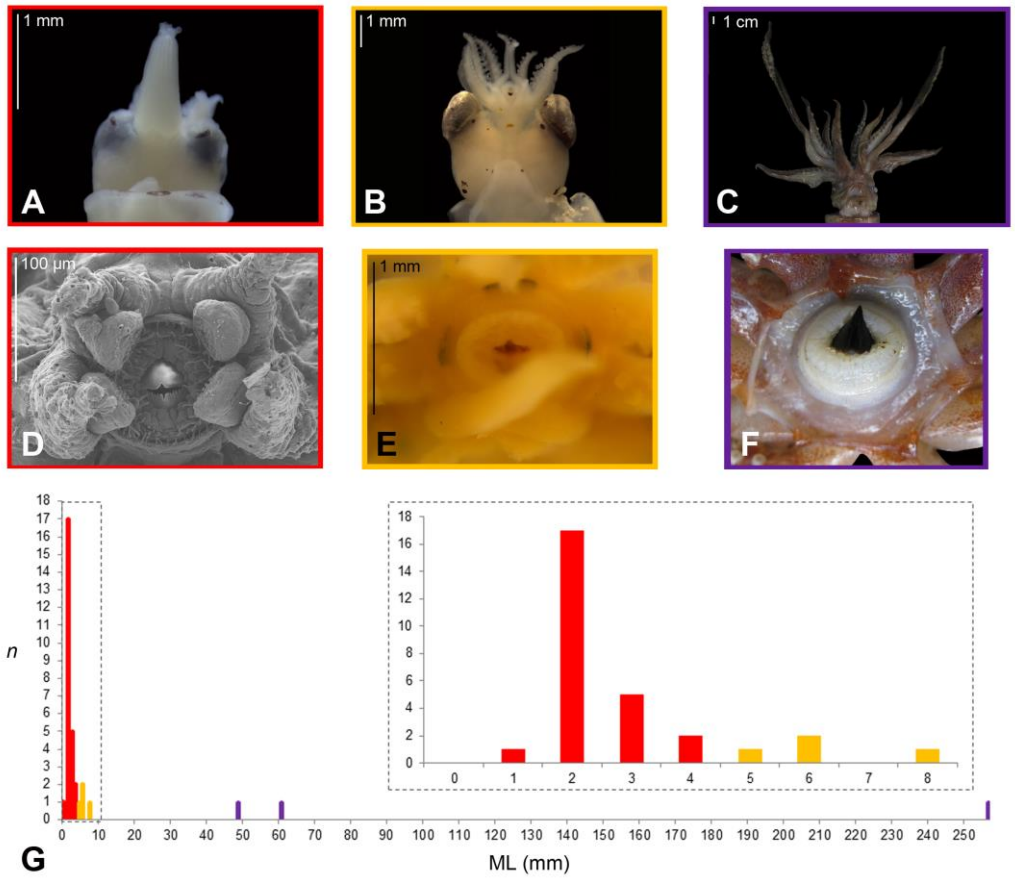
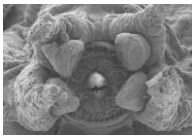


Fig. 3.1. (**A-F**) Morphology of ommastrephid squids. (**A**) Early paralarva (individual E100) showing an unsplit proboscis. (**B**) *Todarodes sagittatus* late paralarva (individual E5) with the proboscis beginning to split. (**C**) Adult *Ommastrephes cylindraceus* individual E3 with the two raptorial tentacles. (**D**) SEM frontal photomicrograph of a *Illex coindetii* early paralarva obtained by *in vitro* fertilization (after Fernández-Álvarez et al., 2017), showing the buccal papillae around the mouth. (**E**) Buccal area of a *Todarodes sagittatus* late paralarva (individual E7). (**F**) Buccal area of a *T. sagittatus* subadult. (**G**) Histogram representing the size classes used in this study, vertical axis represents the number of individuals, the horizontal axis represents the mantle length (mm); the colors of each size cathegory (red, early paralarvae; yellow, late paralarvae; violet, subadults and adult) are consistent with the subfigures A-E color margins.

Assessing the diet of marine animals is considered a critical issue in marine ecology (Nielsen et al., 2017). However, recognizing partially or highly digested remains is a difficult task, even when highly skilled taxonomists are involved. Thus, identification based on visual analysis is usually restricted to animals with hard structures that resist digestion, such as fish otoliths and bones, crustacean exoskeletons or cephalopod beaks. In recent years, studies using molecular tools for identifying gut contents have become more common (O´Rorke et al., 2012a), especially since Next-Generation Sequencing (NGS) methods became more affordable. Based on this approach, DNA metabarcoding of gut samples is a powerful approach to identify prey remains (Piñol et al., 2014; Albaina et al., 2016; Olmos-Pérez et al., 2017). Particularly, the high number of reads that NGS platforms produce allows the detection of DNA traces or underrepresented prey, highly improving the understanding of the diet of the focal species (Albaina et al., 2016).

Despite these advantages, co-amplification of the target species (self-contamination, hereafter) is an important problem. Unless the primers do not hybridize with the target species (which is rare when using universal primers, see Deagle et al., 2007), self-contamination is expected to occur. The key factor is to avoid amplification of the target species, which may be the major (Piñol et al., 2014; Olmos-Pérez et al., 2017) or only component of the gut content reads (Falk & Reed, 2015). A number of methods have been selected to overcome this problem, such as the use of primers specific to the prey species (Jarman et al., 2004). However, this method may serve to increase the previously extant bias in our knowledge (or belief) about the predator diet (see O´Rorke et al., 2012a) and it cannot be applied when no previous knowledge is available, as in the case of ommastrephid paralarvae. PCR enrichment methods are based on a combination of amplified products with restriction enzymes (Dunshea, 2009), DNA blockers (Vestheim & Jarman, 2008) or peptide nucleic acid clamps (O´Rorke et al., 2012b, 2014). Nevertheless, Piñol et al. (2014) stressed the fact that such blocking molecules are not necessary given the huge number of sequence reads obtained by NGS platforms, which are sufficient to study the diet of focal species even if its DNA co-amplifies.

A critical step that can help diminish self-contamination of the target species is to decrease the amount of predator tissue as much as possible during gut content dissections or extractions. Although this step seems straightforward in large animals, it is not easy to achieve in some tiny organisms, such as small larvae or juveniles of marine animals, which may measure from <1 mm to a few centimeters. Until now, the best dissection method applied to tiny wild-collected marine organisms is syringing of the gut contents (O'Rorke et al., 2013). However, the Laser-Capture Microdissection (LCM) method allows the selection of particular tissues or cells from histological sections (Bonner et al., 1997) and thus, it is a promising method for gut content extraction from tiny animals. Nevertheless, for dietary studies LCM has only tentatively been applied for aquaria-reared cod larvae with a previously known diet,



and specific prey primers were applied (Maloy et al., 2011). Here, we applied LCM gut content dissections in combination with DNA metabarcoding for the first time to assess the first feeding diet of wild-collected ommastrephid squid paralarvae.

3.3. Material and Methods

3.3.1. Sample collection

In total, 32 individuals were analyzed. Wild-collected ommastrephid paralarvae (n = 25) from the northeast Pacific were sampled using a Bongo net (500 µm) during four oceanic cruises: under the IMECOCAL-CICESE Program on the west coast of the Baja California Peninsula in January-February 2005 and July-August 2005; in the Pacific central region of Mexico under the PCM-University of Colima Program in January 2010; and under the DGGOLCA Program-UNAM in June 2008 in the Gulf of California. The samples of these four oceanic cruises were directly fixed in 70-96 % ethanol. The remaining individuals were collected in the central Atlantic under the MAFIA cruise during April 2015 using the methodology described by Olivar et al. (2017). Additionally, a nearly mature *Ommastrephes cylindraceus* was fished by jigging during the MAFIA cruise. All the samples from the MAFIA cruise were frozen on board at -20 ºC and until they reached the lab. The mantle or the gut contents were then directly fixed in 96 % ethanol. Table 3.1 summarizes the information for each individual and the sampling location coordinates are available in Table S3.1.

Each individual was identified at the finest taxonomic level possible by morphological characters and this identification was molecularly corroborated by amplifying a region of the cytochrome c oxidase subunit I (COI), as a DNA barcode (Hebert et al., 2003). The squids sampled included almost every ontogenetic phase of the ommastrephid life cycle after hatching, from a putative hatchling (individual with the labcode E666, Table 3.1), bearing only 1 sucker on arms I and II (see Fernández-Álvarez et al., 2017), to an almost mature male (individual E3, Table 3.1). The size of the individuals used for this study is depicted in Fig. 3.1G. The paralarvae stages were classified according to the criteria proposed by Shea (2005) (Table 3.1). For the dietary analyses, three different size classes were considered to cluster the samples: early paralarvae (0.6-3.8 mm ML, n = 25, paralarvae stages 1-2 *sensu* Shea, 2005), late paralarvae (4.8-7.7 mm ML, n = 4, paralarvae stage 3 *sensu* Shea, 2005) and subadults and adults (49-257 mm ML, n = 3). Cephalopod paralarvae are usually fixed in formalin in most oceanographic surveys (e.g., Zaragoza et al., 2015; Fernández-Álvarez et al., 2017). Thus, available paralarvae suitable for DNA extraction are scarce. In order to overcome this problem and represent the entire ommastrephid life cycle as much as possible, we sampled available specimens, which belonged to different species and had different origins (Table 3.1, Table S3.1). All

ommastrephid paralarvae share the same specialized morphological characters of the mouth, tentacles and arm crown and all the previously examined species also share the same ontogenetic shift in their diet (see Vidal & Haimovici, 1998; Uchikawa et al., 2009). Thus, it is expected that the diet is similar to the same ontogenetic stage in each ommastrephid species.

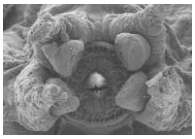
Table 3.1. Individuals studied ordered by mantle length (ML). LCM, Laser Capture Microdissection; N/A, not applicable. Paralarvae stages after Shea (2005).

Labcode	ML (mm)	Species	LCM dissected area (μm^2)	Approximate weight of dissected gut content (g)	Observations
Early paralarvae					
E666	0.69	*Dosidicus gigas*†	315,441	N/A	Paralarva stage 1
E41	1.02	*Dosidicus gigas*†	1,375,292	N/A	Paralarva stage 1
E126	1.13	SD complex‡	846,826	N/A	Paralarva stage 1
E138	1.14	SD complex‡	55,824	N/A	Paralarva stage 1
E142	1.21	*Sthenoteuthis oualaniensis*†	1,978,216	N/A	Paralarva stage 1
E147	1.29	SD complex‡	453,431	N/A	Paralarva stage 1
E130	1.39	*Sthenoteuthis oualaniensis*†	219,731	N/A	Paralarva stage 1
E95	1.4	SD complex‡	2,509,496	N/A	Paralarva stage 1
E90	1.49	*Dosidicus gigas*†	3,033,288	N/A	Paralarva stage 1
E112	1.55	SD complex‡	1,893,110	N/A	Paralarva stage 1
E115	1.59	SD complex‡	1,181,529	N/A	Paralarva stage 1
E103	1.64	*Sthenoteuthis oualaniensis*†	6,503,474	N/A	Paralarva stage 1
E99	1.67	SD complex‡	328,239	N/A	Paralarva stage 1
E107	1.74	SD complex‡	831,532	N/A	Paralarva stage 1
E625	1.88	*Dosidicus gigas*†	3,161,446	N/A	Paralarva stage 1
E108	1.9	SD complex‡	2,166,047	N/A	Paralarva stage 1
E88	1.91	SD complex‡	1,328,333	N/A	Paralarva stage 1
E97	1.91	SD complex‡	483,834	N/A	Paralarva stage 1
E89	2.06	SD complex‡	3,263,717	N/A	Paralarva stage 1
E626	2.15	*Dosidicus gigas*†	970,475	N/A	Paralarva stage 1
E92	2.17	SD complex‡	919,236	N/A	Paralarva stage 1
E100	2.29	SD complex‡	2,432,780	N/A	Paralarva stage 1
E654	2.75	SD complex‡	4,625,858	N/A	Paralarva stage 2
E153	3.23	SD complex‡	3,310,476	N/A	Paralarva stage 1
E510	3.75	*Dosidicus gigas*†	6,975,999	N/A	Paralarva stage 1
Late paralarvae					
E6	4.8	*Todarodes sagittatus*†	N/A	No data	Paralarva stage 3
E7	5.2	*Todarodes sagittatus*†	N/A	No data	Paralarva stage 3
E5	5.9	*Todarodes sagittatus*†	N/A	No data	Paralarva stage 3
E0	7.7	*Sthenoteuthis pteropus*†	3,300,000	N/A	Paralarva stage 3
Subadults and adult					
E1	49	*Sthenoteuthis pteropus*†	N/A	0.009	Subadult
E2	61	*Sthenoteuthis pteropus*†	N/A	0.045	Subadult
E3	257	*Ommastrephes cylindraceus* †§	N/A	1.643	Adult male
Extraction blanks					
B1	N/A	N/A	5,415,922	N/A	Extraction blank 1
B2	N/A	N/A	6,343,695	N/A	Extraction blank 2

† DNA barcoded individual.

‡ *Sthenoteuthis*/*Dosidicus* species complex: there are no known morphological differences between the two species until *S. oualaniensis* paralarvae develop their photophores (ca. 4 mm ML).

§ The genus *Ommastrephes* is a species complex according to Fernández-Álvarez et al. (2015) although the genus is currently considered monotypic (Jereb & Roper, 2010). For more detailed information of the identification of this particular specimen, please see Chapter 4 of the present Ph. D. Thesis.



3.3.2. Gut contents extraction

Two methods were developed to extract the gut contents of the individuals according to their size (Fig. 3.2). The gut contents of the paralarvae were isolated by LCM using a Leica LMD 6000. The whole paralarva or its mantle (depending on the size) was placed on standard histological cassettes and embedded in paraffin following the Peterfi method (Pantin, 1968). The paraffin blocks were stored at -20 ºC until histological processing. Each sample was serially sectioned at 10 µm and sections were mounted on Leica nuclease-free polyethylene naphthalate (PEN) membrane slides (PEN slides, hereafter). After mounting, slides were air-dried at room temperature and stored at -20 ºC until staining with hematoxylin-eosin. Several measures were performed to avoid any possible ambient contamination: (1) each reagent was new; (2) every lab tool (microtome, blades, histological hand tools, gloves and nuisance face mask) was cleaned and UV-sterilized at the beginning of each lab session and between each sample; (3) the slides were covered with a UV-sterilized box during drying; (4) a nuisance face mask was used during sectioning to prevent contamination from breathing; (5) the staining protocol was carried out in a laminar flow hood; (6) no additional people were working in the same lab during the histology procedures. Moreover, two paraffin blocks containing no sample were processed as controls following the same methodology as the samples (B1-B2, Table 3.1) in order to identify any possible ambient contamination during lab sessions.

After the drying stage, the PEN slides were stored at -20 ºC until the LCM sessions. The caecum sac of the paralarvae is lined with a short epithelium (Fig. 3.2B) and is usually full of contents, simplifying the LCM gut content extraction. Thus, the caecum sac was the structure selected to extract the gut contents during the LCM sessions. Another advantage is that this part of the digestive system occupies a medial position, posterior to the esophagus, digestive gland and stomach (Fig. 3.2A-B, G-H), preventing possible bias due to food ingestion inside the fishing net. All laser excisions were performed at 10x magnification and catapulted into sterile 0.2 ml microcentrifuge tubes. For each paralarva, the excised area (Fig. 3.2E) was annotated (Table 3.1) as a proxy for the amount of gut contents used in each DNA extraction. The samples were stored at -20 ºC until the DNA extraction. The PEN slides with the remaining tissues of the paralarvae (Fig. 3.2D) were also stored at -20 ºC for their molecular identification. Portions of the extraction blanks were LCM-excised (Fig. 3.2F) following the same protocol.

Samples of the subadult and adult individuals were directly dissected (Fig. 3.2G-I). The caecum and caecum sac were carefully dissected (Fig. 3.2H) and the gut contents (Fig. 3.2I) transferred to a pre-tared sterile Petri dish and weighed. Approximately one-third of the gut content was fixed in 96 % ethanol for DNA extraction (Table 3.1). Of the other two-thirds, one was used for DNA extraction probes and the other was fixed in 4 % seawater formalin as a morphological voucher of the gut

contents. A small portion of the mantle was preserved in 96 % ethanol for molecular corroboration of the morphological identification. The remaining whole body of the squids was fixed in 4 % buffered formalin and transferred to the Biological Reference Collections of the Institut de Ciències del Mar (CBR-ICM, Barcelona, Spain) as morphological vouchers under the accession numbers ICMC000057-ICMC000059 (individuals E1 to E3, respectively, Table 3.1). In order to test the effect of the efficiency of the LCM, the late paralarvae labeled with the codes E5 to E7 (Table 3.1) were directly dissected instead of LCM-processed, and the whole digestive system of each individual was dissected and used for the DNA extraction.

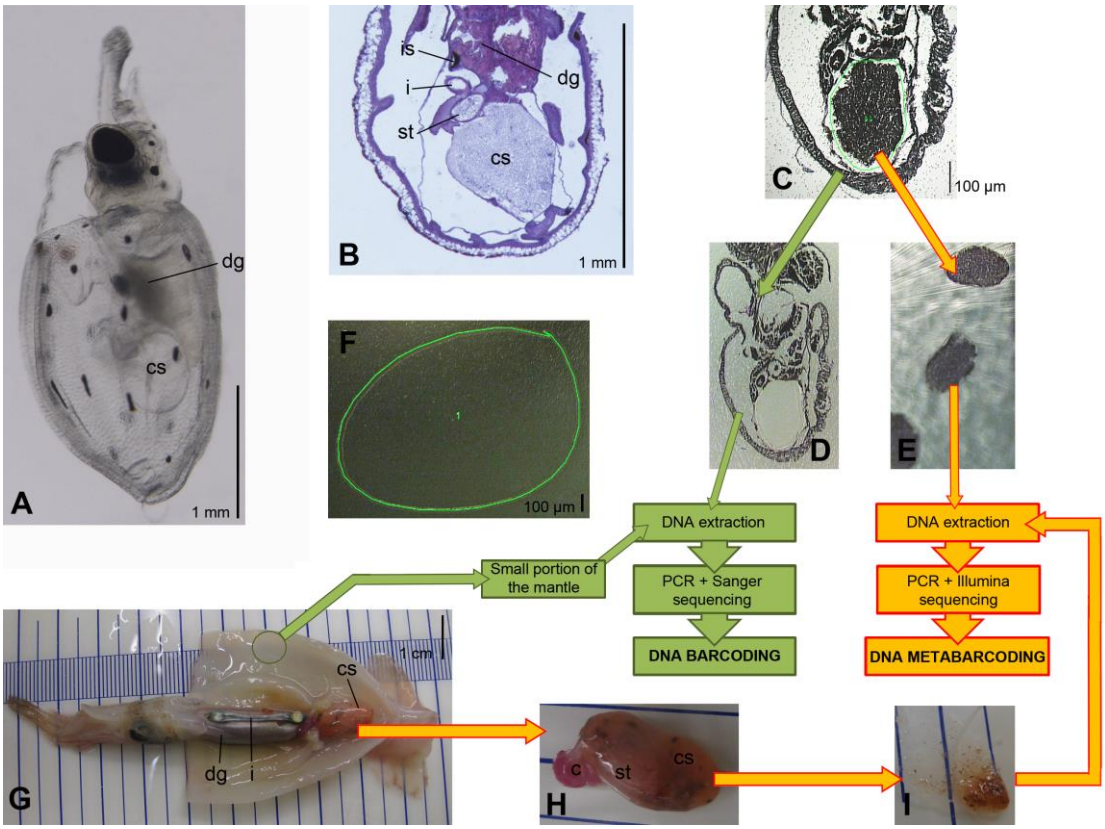
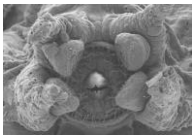


Fig. 3.2. Diagram of the lab workflow. (**A-F**) LCM gut content extraction (late paralarva E0 and early paralarvae, Table 3.1). (**G-I**) Direct dissection of gut contents (subadult and adult individuals E1 to E3 and late paralarvae E5 to E7, Table 3.1). (**A**) Lateral view of a live hatchling of the ommastrephid squid *Todaropsis eblanae*, obtained by *in vitro* fertilization (after Fernández-Álvarez et al., 2017). (**B**) Histological sagittal section of a *T. eblanae* paralarvae, showing the structure of the digestive system. (**C**) Sagittal section of the early paralarva E41 (*Dosidicus gigas*) mounted on the PEN slide during a LCM session; the green line encircles the area selected for laser cutting. (**D**) Same section as in subfigure C with the caecum sac contents LCM-excised. (**E**) Cuts of LCM-isolated gut contents of several sections of the paralarva E41. (**F**) PEN slide without tissues (blank), the green line shows the portion selected for laser cutting. (**G**) Subadult individual E2 (*Sthenoteuthis pteropus*) with the mantle opened to show the internal organs. (**H**) Caecum sac and caecum of individual E2. (**I**) Isolated gut contents by direct dissection. Abbreviations: c, caecum; cs, caecum sac; dg, digestive gland; I, intestine; is, ink sac; st, stomach.



3.3.3. DNA extraction

The DNA from the gut samples obtained by LCM dissections was extracted using the QIAamp DNA Investigator Kit (Qiagen) following the corresponding manufacturer´s protocols, and "Isolation of Total DNA from Tissues" for the remaining samples (Table 3.1). The samples were eluted twice in 30 µl and the second elution was stored at -20 ºC as a back-up. Ambient contamination was avoided as much as possible working in the "Ancient DNA lab" of the Museo Nacional de Ciencias Naturales (MNCN-CSIC, Madrid, Spain), isolated from the other rooms and provided with UV light sterilization. Beyond the usual measures to avoid contamination in a molecular systematics lab, additional measures to avoid ambient contamination were: (1) the whole laboratory was cleaned and UV-sterilized before starting the work, and (2) no additional people were working in the same lab during the DNA extraction session.

For molecular identification of the individuals, the remaining tissues on the PEN slides (Fig. 3.2D) of the LCM-dissected paralarvae and a small portion of the mantle (Fig. 3.2G) of the remaining squids were dissected with a sterile blade. DNA was extracted using the BioSprint 15 DNA kit (Qiagen), following the manufacturer's protocol.

3.3.4. DNA barcoding for squid identification

We amplified sequences from the partial COI gene, using the primer pair LCO1490 (Folmer et al., 1994) and COI-H (Machordom et al., 2003). All PCRs were performed in a total volume of 25 µl that included 0.2 µl of MyTaq polymerase (5 U/µl, Bioline), 5 µl of MyTaq reaction buffer, 0.5 µl of each primer (10 µM), and 2 µl of template DNA. PCRs consisted of an initial denaturation at 95 ºC (1 min), followed by 5 cycles of denaturation at 95 ºC (15 s), annealing at 40 ºC (90 s) and extension at 72 ºC (1 min) and 35 cycles with the same conditions, but with 44 ºC as annealing temperature. The post-PCR products were purified with ExoSAP-IT (Thermo Fisher) prior to sequencing both strands on an ABI Prism 3730 (Applied Biosystems).

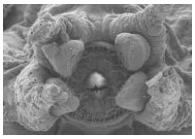
3.3.5. DNA metabarcoding of gut contents

DNA extractions of gut contents were used to construct two libraries, for eukaryotic and prokaryotic DNA identification, covering almost all life domains. This strategy was selected due to of the absence of reliable knowledge of the actual diet of ommastrephid paralarvae.

For eukaryotic DNA, the universal primers Euk-B (Medlin et al., 1988) and 18s_v9_Con (O´Rorke et al., 2012b), which amplify a ~105-165 bp fragment of the hypervariable v9 region of 18S rRNA, were selected. These primers have the advantage of amplifying almost every eukaryotic organism (i.e., animals, plants, fungi, algae, etc.) and the amplicons obtained are small enough to amplify highly digested DNA. In addition, 18S RNA is a multicopy gene, which also increases the possibility of amplifying prey. However, this primer versatility comes at the price of taxonomic resolution (Leray & Knowlton, 2016) and identification to the species level is frequently not possible (O´Rorke et al., 2014; Albaina et al., 2016). For prokaryotic DNA, a ~200-210 bp region of the 16S rRNA was amplified with the universal primers 16S-F and 16S-R (Reddy et al., 2012). The PCRs for both fragments were performed in a total volume of 25 µl, adding 1 µl of template DNA, 0.5 µM of the selected primers, and 12.5 µl of Phusion DNA polymerase mix (Thermo Scientific). The reaction conditions were 98 ºC for 30 s, followed by 35 cycles of 98 ºC for 10 s, 60 ºC for 20 s, 72 ºC for 20 s, and a final extension step at 72 ºC for 10 minutes. Strict precautions were taken to avoid environmental contamination during the PCRs, including: 1) the use of a laminar flow hood previously treated with UV light, 2) only filter pipette tips were used, and 3) all surfaces were periodically wiped with bleach. A second PCR round with identical conditions and only 5 cycles were performed for attaching the index sequences. A negative control without DNA was added to check for contamination during library preparation. Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek) following the manufacturer´s protocol. They were then quantified with the Qubit dsDNA HS Assay (Thermo Fisher) and pooled in equimolar amounts (10 nM). The pool was sequenced in ½ of a MiSeq paired-end 300 bp run (Illumina).

3.3.6. Bioinformatic analysis

The quality of the FASTQ files was checked using the software FastQC (Andrews, 2010) and the Illumina-specific adapters were trimmed by running the cut adapter tool implemented in Trimmomatic (Bolger et al., 2014). The sequences were quality-filtered (minimum Phred quality score of 20) and labeled using Qiime 1.9.0 (Caporaso et al., 2010). The paired-end assembly of forward (R1) and reverse (R2) reads was executed in FLASH (Magoč & Salzberg, 2011) implemented in Qiime. The mismatch resolution in the overlapping region was accomplished by keeping the base with the higher quality score. Artifacts such as point mutations and chimaeras were detected and deleted using the UCHIME algorithm (Edgar et al., 2011) implemented in VSEARCH (Rognes et al., 2016). Using the final list of representative sequences, each molecular operational taxonomic unit (MOTU) was searched against the reference database SILVA (Quast et al., 2013) v. 128 (September, 2016) and the last available version (May, 2013) of Greengenes (DeSantis et al., 2006) for the 18S v9 and 16S



databases, respectively. The 18S v9 reads were clustered into MOTUs using the closed-reference approach with the UCLUST algorithm (Edgar, 2010) in Qiime with a similarity threshold of 100 %. Sequences that did not provide a 100 % match were discarded. The 16S reads were clustered using the open-reference approach with a similarity threshold of 97 % and reads that did not hit the reference sequence collection were subsequently clustered de novo. After this step, singletons and sequences representing less than 0.005 % of the total number of sequences of each dataset were excluded. Sequences were compared with the GenBank database by BLAST (Altschul et al., 1990). For 16S, 30 % of the reads remain unidentified using only Greengenes as a database. Thus, BLAST hits were applied to identify at the lowest taxonomic level possible following the same criteria: 1) sequences with <90 % for identity or coverage were not considered; 2) 97 % similarity is considered the species-level threshold; 3) when more than one sequence has the same identity value, the one identified to the lowest taxonomic level was selected; 4) if the GenBank identification differs at the genus level to that of Greengenes, the latter is applied. Before applying the BLAST identifications, only 0.6 % of the sequences were unidentified.

In DNA metabarcoding studies, the mistagging phenomenon has been reported (Esling et al., 2015, Bartram et al., 2016), in which a low percentage of the reads of a sample can be assigned to another as the result of the misassignment of the indices during library preparation, sequencing, and/or demultiplexing steps. To correct for this phenomenon, for 18S v9 the low abundance MOTUs of each sample were removed by applying a threshold based on the presence of mistagging in the PCR negative control (i.e., the higher number of reads for a particular sequence in the PCR blank), resulting in a particular threshold for each sequence. As a result, for 18S v9 no sequences were assigned to three of the late paralarvae (individuals E5 to E7) and the adult (individual E3). For 16S, the 0.005 % threshold was selected according to the presence of low abundance MOTUs in the whole dataset. Although extraction measures for avoiding ambient contamination were applied, some MOTUs were present in the extraction blanks. Any sequence present in at least one of these blank samples was taken as ambient contamination and removed from the study. For 18S v9, the identifications were performed at the Class level, since some Orders of some of the Classes (e.g., Mammalia and Actinopterigii) could not be reliably assessed with this region. It should also be noted that several related species may share the same metabarcode (Leray & Knowlton, 2016) and thus, the number of actual eukaryotic species inside the gut may be larger than the number of detected MOTUs. Taxonomic assignments of 16S reads were considered as species-level identifications. Rarefaction plots of each sample were constructed showing the rarefied number of MOTUs defined at 100 and 97 % similarity thresholds for 18S v9 and 16S, respectively (Fig. S3.1).

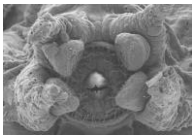
3.3.7. Evaluation of self-contamination for 18S v9

The selected primers for 18S v9 can amplify ommastrephid squid DNA. Thus, although the dissection methodology was performed carefully, DNA of the paralarvae or the squids may be present among the gut contents and must be considered as self-contamination. In a first step, all the 18S v9 ommastrephid sequences available in GenBank were downloaded. An additional *Todarodes sagittatus* sequence obtained with the primers Euk-B and 18_v9_Con following the PCR conditions explained above and Sanger sequenced (GenBank accession number MF980452) was added. All of these sequences were aligned and the *p*-distance percentages were calculated to determine if there were sufficient differences to distinguish these species with this molecular marker (Table 3.2). The *p*-distance percentages ranged from 1.4 to 4.1 %, although sequences of *Dosidicus gigas* and *Eucleoteuthis luminosa* were identical and, therefore, identification of the two species with this molecular marker was not possible. For those paralarvae successfully identified with DNA barcoding (Table 3.1), the reads that matched their identification at a genus level were regarded as self-contamination and the others as a component of the diet. For paralarvae that were not molecularly identified and had reads for only one ommastrephid species, the reads were considered as self-contamination reads. If an unidentified paralarva had sequences for two genera, the most represented was regarded as self-contamination and the other as gut contents. The self-contamination component of the reads is expressed as a percentage of the individual reads in relation to the total number of reads obtained for the sample.

Table 3.2. Uncorrected *p*-distances (%) of 18S v9 sequences of ommastrephid squids.

Sthenoteuthis oualaniensis (AY557511)					
Eucleoteuthis luminosa (EU735294)	1.4				
Dosidicus gigas (KY387931)	1.4	0			
Ommastrephes bartramii (AY557510)	2.1	2.1	2.1		
Todarodes sagittatus (MF980452)	4.1	2.8	2.8	2.1	
Illex coindetii (AY557509)	2.8	2.8	2.8	3.4	2.8

Once the self-contamination reads were identified, they were discarded and the remaining reads were analyzed. The percentage of each gut content item was calculated in relation to the number of total sequences of each sample without the self-contamination reads.



3.3.8. Data Accessibility

DNA sequences from ommastrephid 18S v9 and 16S gut content and COI sequences of barcoded ommastrephids were deposited in GenBank with the accession numbers MF980393-MF980451, MF980453-MF980593 and MF980594-MF980608, respectively.

Tables S3.2 and S3.3 can be accessed through the following link:

https://www.dropbox.com/sh/s329efd3h77ay77/AACnQ_n1oBhzRgvfp1BLktzza?dl=0

3.4. Results

3.4.1. Identification of the individuals

The taxonomic identification of the individuals is indicated in Table 3.1. The morphological identification of the *Sthenoteuthis pteropus* late paralarva coded as E0, the *S. pteropus* subadults E1 and E2, and the *Ommastrephes cylindraceus* adult individual E3 were molecularly confirmed. The late paralarvae E5 to E7 were successfully molecularly identified as *T. sagittatus*. Regarding the early paralarvae, six individuals (labcodes E41, E90, E510, E625, E626 and E666) were identified as *Dosidicus gigas* and another three (E103, E130 and E142) as *Sthenoteuthis oualaniensis*. The remaining paralarvae were preliminarily identified after Fernández-Álvarez et al. (2017) on the basis of the proboscis suckers as Type C paralarvae, which has proboscis suckers all of the same size. In north Pacific waters, the Type C paralarvae can belong to *S. oualaniensis* or *D. gigas*. Until *S. oualaniensis* develops ocular and visceral photophores at 4 mm mantle length (ML), there are no known morphological differences between *D. gigas* and *S. oualaniensis* (Vecchione, 1999) and these paralarvae are commonly referred to in the literature as the SD complex (e. g., Aceves-Medina et al., 2017). Thus, 16 of the studied paralarvae were classified as members of the SD complex.

3.4.2. Self-contamination reads

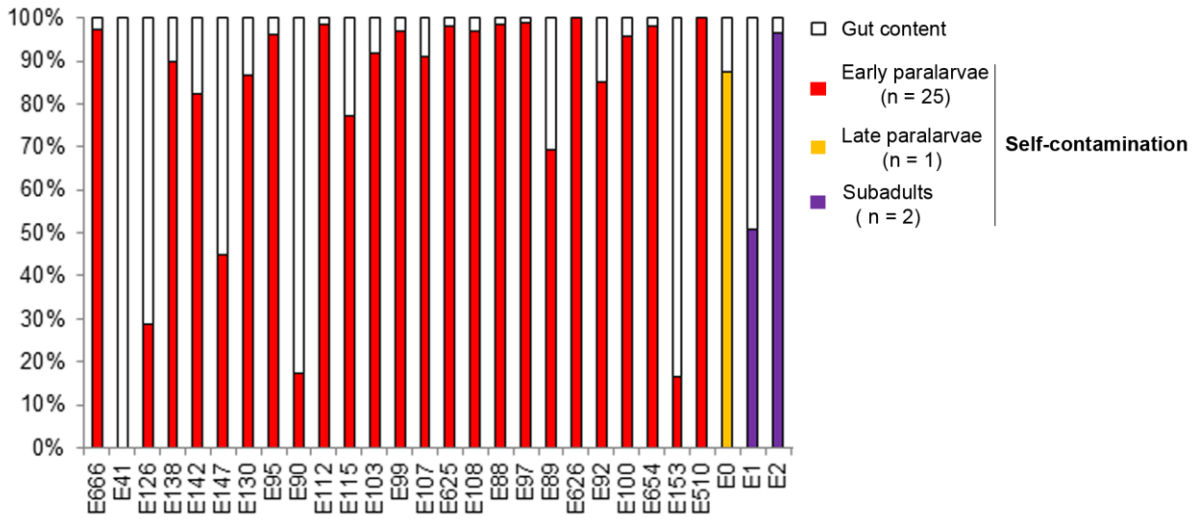
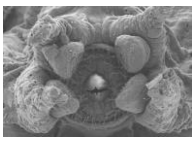


Fig. 3.3. Percentage of self-contamination (solid color) found in the 18S v9 metabarcoding gut content analysis. Individuals are ordered by mantle length.

Self-contamination reads represented 88.5 % of the 2,587,082 reads obtained for 18S v9. Fig. 3.3 represents the percentage of self-contamination and gut contents of each sample successfully sequenced for this molecular marker. For the early paralarvae (n = 25), self-contamination was 78 ± 30 % of the reads (range: 0-100 %). Four gut samples (E3 to E7) failed to provide any 18S v9 reads matching the SILVA database by the closed-reference approach in Qiime. Whereas the adult individual E3 did not provide any read, the late *T. sagittatus* paralarvae E5 to E7 provided 4,814-208,655 identical sequences that were discarded by the software because they did not match any sequences in the database. A subsequent analysis revealed that these reads matched the 18S v9 Sanger sequenced *T. sagittatus* sequence MF980452, resulting in a self-contamination value of 100%. The LCM-dissected individual E0 was the only late paralarva whose gut contents were successfully sequenced for 18S v9, with a self-contamination percentage of 87.6 %. The subadult individuals E1 and E2 were successfully amplified for 18S v9 and showed self-contamination values of 51.0 and 96.7 %, respectively.



3.4.3. Taxonomic assignment of eukaryotic reads

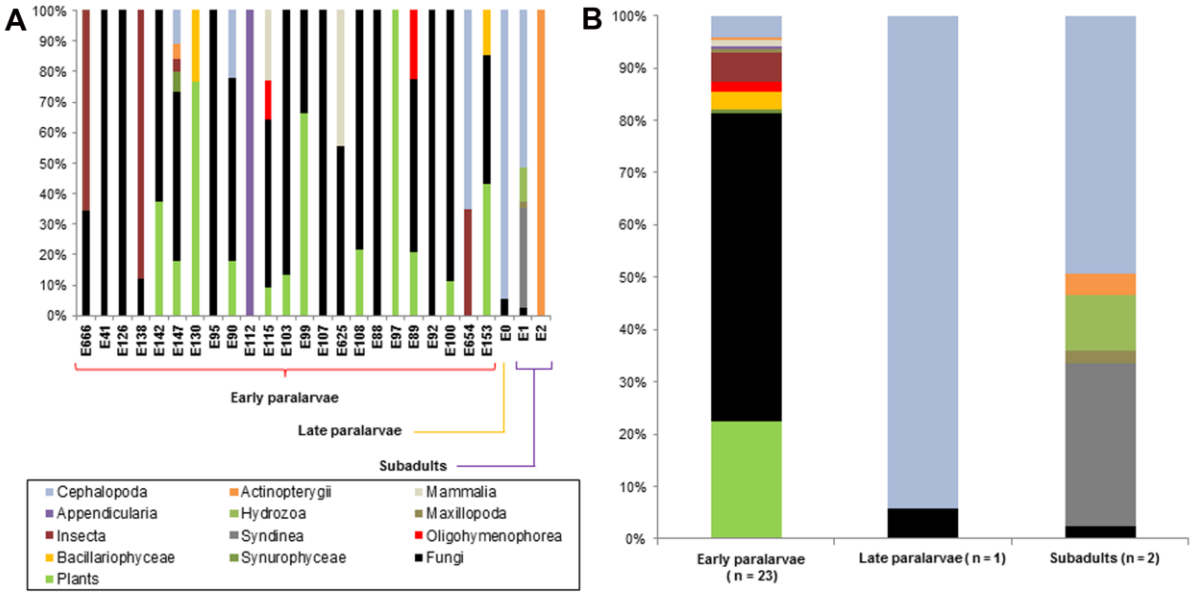


Fig. 3.4. Percentage (%) of eukaryotic 18S v9 reads in the gut contents of each sample (**A**) and grouped by size class (**B**). The taxonomic assignments are at the Class level except plants and fungi, which were collapsed. Self-contamination reads were excluded. Individuals are ordered by mantle length.

After cleaning the self-contamination reads, 299,509 total reads of eukaryotes remained, resulting in 11,519 ± 9,331 (range: 1,089-31,566) reads per sample. A total of 59 molecular operational taxonomic units (MOTUs) were identified in the gut contents of all the samples. The percentages of each gut content item of each sample and size class are represented in Fig. 3.4A and 3.4B, respectively, and are summarized by size class in Table 3.3. The raw gut content reads are available in Table S3.2. Early paralarvae shows 3 ± 2.2 (range 1-11, n = 23) MOTUs, the late paralarva E0 showed 3 MOTUs, and the 2 subadults, 1 and 9 different MOTUs. For the early paralarvae, 22.3 % of the gut content reads was composed of plants and 59 % was fungi. Animals accounted for 12.6 % of the reads, with insects (5.5 %) and cephalopods (4.2 %) being the most represented groups. The protist groups Chromista and Ciliophora were also present in this size class. The most represented group for the late paralarva and subadults was Metazoa, representing 94 and 66 %, respectively. In both the late paralarva and subadults, cephalopods were the most represented group (87.8 and 49.3 %, respectively). Parasitic dinoflagellates of the Class Syndinea were only present in the subadult squid E1, representing 31 % of the reads of this size class.

Table 3.3. 18S v9 eukaryotic MOTUs detected in the gut contents of ommastrephid squids as a percentage (%) and clustered by size categories. Taxonomic assignments are at a Class level, with the exception of Cephalopoda, which are identified at a genus level. The count number indicates the number of individuals of each class size category with reads for the gut content item. N/A, not applicable.

Kingdom	Phyllum	Class	Early paralarvae (n = 23)		Late paralarva (n = 1)	Subadults (n =2)	
			Reads (%)	Count	Reads (%)	Reads (%)	Count
Plantae	**Magnoliophyta**	N/A	3.38	2			
		Eudicotyledoneae	0.69	1			
		Magnoliopsida sp. 1	0.25	1			
		Magnoliopsida sp. 2	0.82	2			
		Magnoliopsida sp. 3	9.95	3			
		Magnoliopsida sp. 4	1.97	2			
		Magnoliopsida sp. 5	1.01	1			
		Magnoliopsida sp. 6	0.47	1			
		Monocotyledoneae sp. 1	0.71	1			
		Monocotyledoneae sp. 2	1.40	1			
		Rosopsida	1.69	3			
Fungi	N/A	N/A	2.71	1			
	Ascomycota	Dothideomycetes sp. 1	5.10	1			
		Dothideomycetes sp. 2	0.33	1			
		Eurotiomycetes sp. 1	4.89	1			
		Eurotiomycetes sp. 2	1.05	1			
		Eurotiomycetes sp. 3	0.29	1			
		Eurotiomycetes sp. 4	2.33	1		2.52	1
		Pezizomycetes	0.33	1			
		Pleosporomycetidae sp. 1	0.42	1			
		Pleosporomycetidae sp. 2	0.64	1			
		Saccharomycetes	12.59	2			
	Basidiomycota	Agaricomycetes sp. 1	4.55	2			
		Agaricomycetes sp. 2	0.27	1			
		Agaricomycetes sp. 3	0.23	1			
		Agaricomycetes sp. 4	1.05	1			
		Basidiomycetes	1.30	1			
		Microbotryomycetes sp. 1	0.49	1			
		Microbotryomycetes sp. 2	2.42	1	5.68		
		Microbotryomycetes sp. 3	0.64	1			
		Microbotryomycetes sp. 4	3.05	4			
		Tremellomycetes sp. 1	8.60	5			
		Tremellomycetes sp. 2	0.56	1			
		Tremellomycetes sp. 3	2.04	1			
		Tremellomycetes sp. 4	0.64	1			
	Entomophthoromycota	Entomophthoraceae	2.51	1			
Chromista	**Ochrophyta**	Synurophyceae	0.62	1			
		Bacillariophyceae sp. 1	1.78	1			
		Bacillariophyceae sp. 2	1.73	1			
Protista	**Ciliophora**	Oligohymenophorea sp. 1	1.50	1			
		Oligohymenophorea sp. 2	0.42	1			
	Dinoflagellata	Syndinea sp. 1				2.53	1
		Syndinea sp. 2				4.17	1
		Syndinea sp. 3				3.14	1
		Syndinea sp. 4				21.28	1
Metazoa	**Arthropoda**	Insecta sp. 1	3.83	1			
		Insecta sp. 2	0.41	1			
		Insecta sp. 3	1.21	2			
		Maxillopoda sp. 1	0.71	1			
		Maxillopoda sp. 2				2.32	1
	Chordata	Appendicularia	0.55	1			
		Actinopterygii sp. 1				4.13	1
		Actinopterygii sp. 2	0.46	1			
		Mammalia	1.16	2			
	Cnidaria	Hydrozoa				10.64	1
	Mollusca	Cephalopoda sp. 1 (*Ommastrephes* sp.)			6.53	6.59	1
		Cephalopoda sp. 2 (*Illex* sp.)				42.68	1
		Cephalopoda sp. 3 (*Sthenoteuthis* sp.)	3.27	2			
		Cephalopoda sp. 4 (*Eucleoteuthis luminosa* / *Dosidicus gigas*)	1.00	2	87.78		
Total reads			261,611		11,541	26,357	

3.4.4. Taxonomic assignment of prokaryotic reads

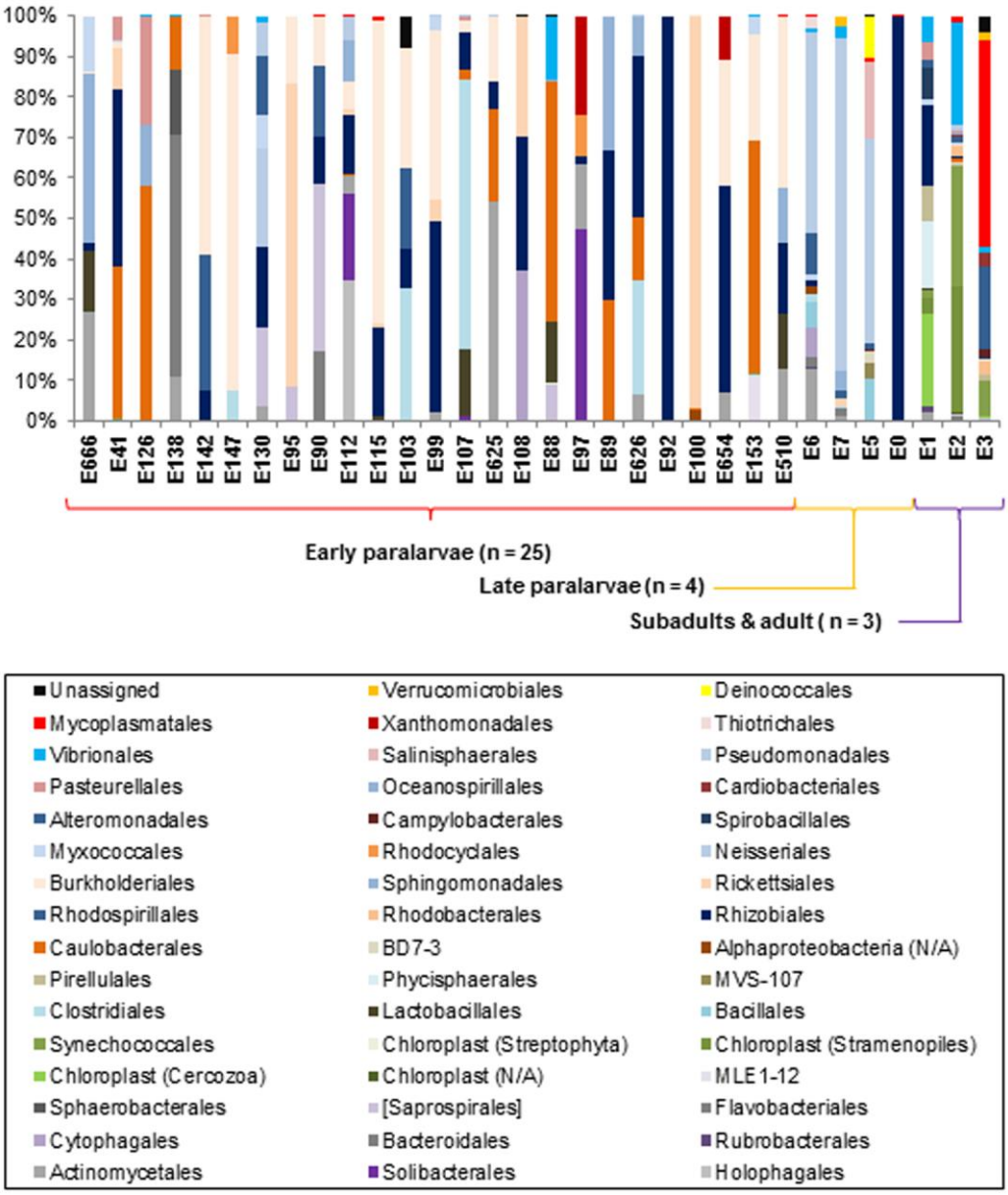


Fig. 3.5. Percentage (%) of the prokaryotic 16S reads in the gut contents of each sample. The taxonomic assignments are at the Order level. Individuals are ordered by mantle length. Chloroplast sequences are eukaryotic chloroplasts amplified with the 16S primers. N/A, not applicable (the finest identification was at the Class level).

A total of 453,883 prokaryotic reads were obtained from the gut contents resulting in 14,183 ± 28,280 (range 12-124,004) reads per sample. Interestingly, an effect of the gut content dissection method was identified. LCM samples (n = 26, Table 3.1) represented 207,070 of the total reads resulting in 7,964 ± 23,325 (range 12-124,004) reads per sample, while the directly dissected individuals (n = 6) represented 246,813 of the total reads resulting in 41,135 ± 31,838 (range 12,405-107,096) reads per

sample. A total of 141 different MOTUs were identified, with three of them unassigned to any taxonomic level (2,608 reads in total). The percentages of each gut content item at the Order level are represented in Fig. 3.5 for each sample. Percentages of each bacterial Order grouped by size class are represented in Fig. 3.6 and in Table 3.4. The raw data are available in Table S3.3. For early paralarvae (n = 25), the most represented group was the Class Proteobacteria (86 % of the reads). The Proteobacteria Order Rickettsiales represented 60 % of the reads. However, it should be noted that the paralarva E100 accounted for 97 % of the sequences of this bacterial Order and two sequences with 90% identity with the candidatus *Hepatobacter* GenBank sequence JX981946 were the only contributors for this Order for this paralarva (Table S3.3). The autotrophic component (Cyanobacteria and chloroplasts) represented 0.3 % of the reads of early paralarvae. The Phylum Acidobacteria was only present in early paralarvae (2.6 %), while Planctomycetes, present in the other two size categories, was absent in early paralarvae. For late paralarvae (n = 4), the Class Proteobacteria was the most represented group (80 %) and the autotrophic component represented 0.05 % of the reads. For subadults and the adult (n = 3), Cyanobacteria and chloroplasts were the most represented groups (42 %), while Proteobacteria accounted for 35 % of the reads and the parasitic Mycoplasmatales for 14 %.

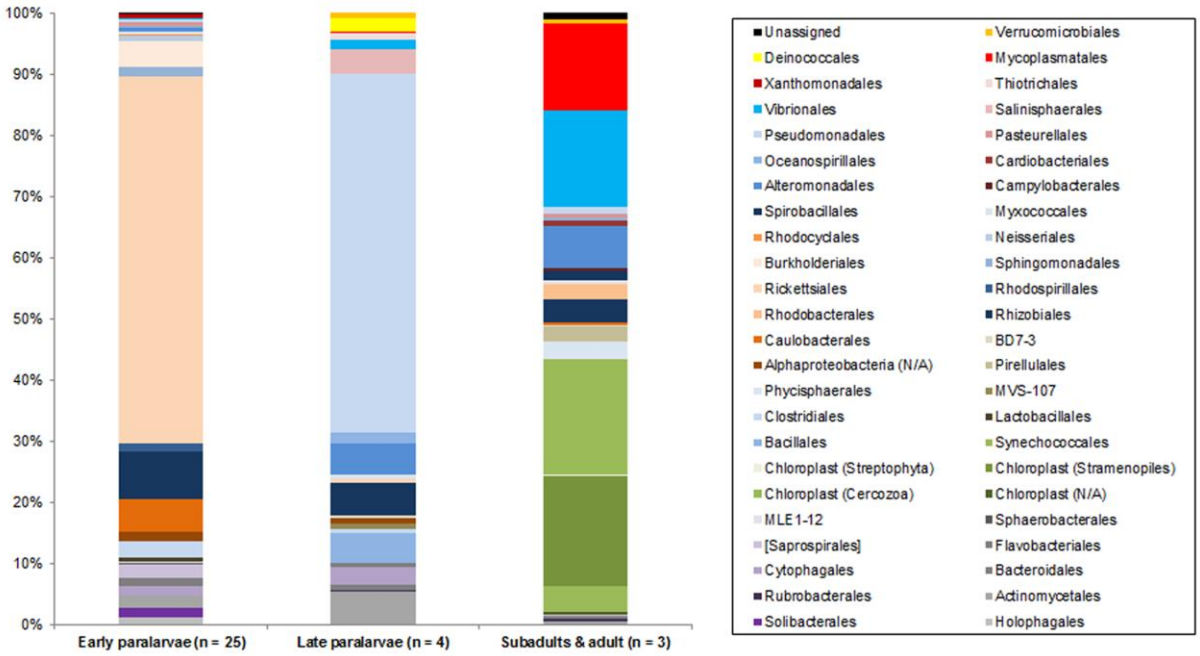


Fig. 3.6. Percentage (%) of the prokaryotic 16S reads in the gut contents grouped by size class. The taxonomic assignments are at the Order level. Chloroplast sequences are eukaryotic chloroplasts amplified with this molecular marker. N/A, not applicable (the finest identification was at the Class level).

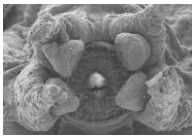


Table 3.4. 16S prokaryotic MOTUs detected in the gut contents of ommastrephid squids as a percentage (%) and sorted by size category. Taxonomic assignments are at the Order level. N/A, not applicable.

Kingdom	Phyllum	Class	Order	Early paralarvae (n = 25) reads (%)	Late paralarvae (n = 4) reads (%)	Subadults & adult (n = 3) reads (%)
Bacteria	**Acidobacteria**	**Holophagae**	Holophagales	1.0354		
		Solibacteres	Solibacterales	1.6018		
	Actinobacteria	**Actinobacteria**	Actinomycetales	2.0923	5.3699	0.5777
		Rubrobacteria	Rubrobacterales		0.2250	0.2582
	Bacteroidetes	**Bacteroidia**	Bacteroidales	0.0005	0.9424	
		Cytophagia	Cytophagales	1.4985	2.7949	
		Flavobacteriia	Flavobacteriales	1.3095	0.7464	0.4268
		[Saprospirae]	[Saprospirales]	2.2642	0.0017	0.3750
	Chloroflexi	**Sphaerobacteridae**	Sphaerobacterales	0.1870		
	Cyanobacteria	**4C0d-2**	MLE1-12	0.2908		
		Chloroplast	N/A			0.3389
			Cercozoa	0.0005	0.0051	4.1795
			Stramenopiles	0.0020	0.0119	18.0005
			Streptophyta	0.0010		0.2289
		Synechococcophycideae	Synechococcales	0.0039	0.0375	18.9647
	Firmicutes	**Bacilli**	Bacillales		4.7189	
			Lactobacillales	0.6359		0.1409
		Clostridia	Clostridiales	2.7018	0.6919	
	Planctomycetes	**C6**	MVS-107		0.8316	
		Phycisphaerae	Phycisphaerales			2.8827
		Planctomycetia	Pirellulales		0.0034	2.3244
	Proteobacteria	**Alphaproteobacteria**	N/A	1.5773	0.8862	
			BD7-3	0.0005	0.6374	0.1713
			Caulobacterales	5.2989	0.0017	0.6002
			Rhizobiales	7.6903	5.1518	3.6583
			Rhodobacterales	0.0005	0.0136	2.4391
			Rhodospirillales	1.3247		
			Rickettsiales	60.1709	0.7601	0.1990
			Sphingomonadales	1.5759		
		Betaproteobacteria	Burkholderiales	4.1440	0.0068	0.1461
			Neisseriales	0.8959		
			Rhodocyclales	0.2286		
		Deltaproteobacteria	Myxococcales	0.5189	0.7004	0.3734
			Spirobacillales			1.4712
		Epsilonproteobacteria	Campylobacterales		0.0017	0.5651
		Gammaproteobacteria	Alteromonadales	0.5488	4.9660	6.8196
			Cardiobacteriales	0.0005	0.0034	0.9438
			Oceanospirillales	0.3099	1.7860	0.4667
			Pasteurellales	0.6124	0.0205	0.7228
			Pseudomonadales	0.4773	58.7110	0.9014
			Salinisphaerales		4.0406	
			Vibrionales	0.1601	1.4997	15.8191
			Thiotrichales		1.2066	
			Xanthomonadales	0.6271		
	Tenericutes	**Mollicutes**	Mycoplasmatales	0.0029	0.1432	14.3777

Table 3.4 (continuación). 16S prokaryotic MOTUs detected in the gut contents of ommastrephid squids as a percentage (%) and sorted by size category. Taxonomic assignments are at the Order level. N/A, not applicable.

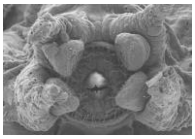
	[Thermi]	**Deinococci**	Deinococcales		2.2206	
	Verrucomicrobia	**Verrucomicrobiae**	Verrucomicrobiales		0.8453	0.4907
Unassigned	N/A	N/A	N/A	0.2095	0.0170	1.1365
Total reads				204270	58679	190934

3.5. Discussion

The assemblage of gut contents found inside the early paralarvae was unexpected, showing a mixture of components that is not consistent with predation on zooplankton, as all the previous studies on cephalopod paralarvae under laboratory conditions had shown (Iglesias et al., 2014), or even herbivory. The presence of fungal MOTUs along with animals of marine and terrestrial origin, as well as plants, algae and eukaryotic and prokaryotic microorganisms commonly found in fecal pellets and particulate organic matter (POM) leads to the conclusion that ommastrephid early paralarvae are detritivores.

3.5.1. The first feeding diet of ommastrephid squid

The gut contents of the early ommastrephid paralarvae notably differed from other stages of their life cycle (Tables 3.3 and 3.4, Fig. 3.4-6), as well as from the rest of the cephalopod paralarvae whose diet is known (Iglesias et al., 2014; Olmos-Perez et al., 2017). This is especially true for some taxonomic groups among the gut content reads. For instance, some taxa are present in early paralarvae, and are absent (the algae Ochrophyta, the protists Ciliophora and plants) or barely present (Fungi) in late paralarvae or subadults. Interestingly, these gut content items have not been previously reported in the literature for any other cephalopod paralarvae (Vidal & Haimovici, 1998; Camarillo-Coop et al., 2013; Iglesias et al., 2014; Olmos-Perez et al., 2017). Even the ingested animals belong to unexpected taxonomic groups: insects represented an important part of the diet of early paralarvae, and are absent in the late paralarva and subadults and have not been previously recorded in the diet of any other cephalopod (Villanueva et al., 2017). Since there are no insect species living in oceanic waters, they are not expected to be part of the diet of oceanic squids. Other animal DNA present in the gut contents of early paralarvae had a very likely marine origin (the crustaceans of the Class Maxillopoda), while other MOTUs are exclusively marine (appendicularians and cephalopods). Since we took special



measures to prevent and detect ambient contamination of the gut contents and considering the extraction blank results, the MOTUs with an unequivocal continental origin are also part of the diet of the paralarvae and not lab artifacts.

Magnoliophyta (plants) was the second most represented taxonomic group among the early paralarvae (22.3 %). Twelve of the examined early paralarvae had plants in their gut and four of them had reads of two (specimens E99 and E130) or three (E142 and E147) different plant MOTUs (Table S3.2). Plant DNA is not unusual in open ocean studies (O´Rorke et al., 2014; Riemann et al., 2010; Suzuki et al., 2007). For instance, pollen may form part of the diet of some filter organisms (Borme et al., 2009; Morote et al., 2010) and, thus, it must also be represented in their feces. Due to the very small size of the single cell free-living organisms, such as cyanobacteria (Table 3.4, Table S3.3), diatoms and ciliophorans (Table 3.3, Table S3.2), it is difficult to explain their presence in the gut contents of the early paralarvae. A possible origin for these MOTUs is organic material aggregations enriched with this type of material, such as fecal pellets of zooplankton (Govoni, 2010). Supporting this view is the fact that diatoms and fecal pellets have been reported in the digestive system of the detritivore cephalopod *V. infernalis* (Hoving & Robison, 2012), while algae have been reported in *S. spirula* gut contents and its origin was explained as components of the marine snow consumed by this squid (Ohkouchi et al., 2013).

Fungal DNA was found in 21 of the 23 successfully analyzed early paralarvae and represents more than half of the reads of the gut contents for this size class. Due to their predominantly saprobiontic nature, fungi are widespread among rotting material, such as POM. Similar assemblages of fungi DNA were found in the gut contents of the suspension feeding leptocephalii eel larvae (Riemann et al., 2010) and spiny lobsters (O´Rorke et al., 2012b; 2014), supporting the idea that early ommastrephid paralarvae are detritivores. The presence of some bacterial groups commonly found in POM, such as Cytophagia, Deltaproteobacteria, Flavobacteria and Firmicutes (e.g., Mestre et al., 2017) (Table 3.4) in early paralarvae supports the hypothesis of a detritus-based first feeding diet.

At hatching, ommastrephid paralarvae usually measure ~1-2 mm in ML (Villanueva et al., 2016) and are only provided with short arms I and II with a single sucker each and the arm stumps of the pairs IV (Fernández-Álvarez et al., 2017). Moreover, early ommastrephid paralarvae have very fragile beaks (Uchikawa et al., 2009), thus they do not seem well-suited for grabbing, killing and eating zooplankton prey as in posterior ontogenetic stages. Supporting this view, the suspension feeding eel larvae also has a buccal feeding apparatus functionally constrained to feed on soft material (Bouilliart et al., 2015). Furthermore, the central nervous system of early ommastrephid paralarvae is undeveloped relative to hatchlings of other cephalopod families (Shigeno et al., 2001b), suggesting that they are not able to perform the complex behaviors that active hunting demands. Since the present

results show that ommastrephid paralarvae feed on detritus, the function of the buccal papillae might be the manipulation of soft bunches of POM and fecal pellets, although a possible sensorial function should also be considered.

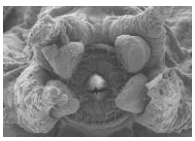
3.5.2. The transition to predation

When ommastrephid paralarvae reach ~4 mm ML, some important morphological changes take place (Shigeno et al., 2001b): the brain develops to a level similar to that found in juveniles and adults, the pair of arms III rapidly develops, the buccal papillae disappear, the beaks protrude (Uchikawa et al., 2009) and they experience an important increase in the development of both sense organs and the digestive system (Shigeno et al., 2001a). Shortly before this important morphological transformation, the paralarvae are more likely to develop hunting behavior and, interestingly, in this ontogenetic stage zooplankton items begin to appear in the gut contents of wild collected paralarvae (Vidal & Haimovici, 1998, Uchikawa et al., 2009). The late paralarva E0 showed 18S v9 reads of ommastrephids (~94 %) and fungi (~6 %) (Table 3.3). A possible explanation for this content may be predation on early ommastrephid paralarvae, which feed on detritus enriched with fungi. Interestingly, the gut contents of subadults and adults have a high component of Cyanobacteria and chloroplast reads (Table 3.4, Table S3.3). Since the small size of unicellular Cyanobacteria prevent its selected ingestion by subadults and adults, these sequences can only be explained by the ingestion of food items enriched with these organisms, suggesting predation over herbivores. Although some early paralarvae (E88, E153 and E510) showed prokaryotic autotrophic sequences, they did not show any animal DNA reads (Table S3.2; paralarvae E510 provided 100 % self-contamination reads and thus is not represented in this table), suggesting that these reads have a detritus origin rather than predation on herbivores.

It has been reported in diet-based studies of ommastrephid juveniles and subadults that they feed on pelagic crustaceans, fishes, other cephalopods, pteropods, bivalves and polychaetes (e.g., Camarillo-Coop et al., 2013), while adults mainly feed on fishes, crustaceans and squids (e.g., Field et al., 2007; Rosas-Luis et al., 2014). Here, the 18S v9 reads of the subadults recovered prey of cephalopods, cnidarians, fishes and crustaceans (Table 3.3), which is consistent with the previous literature.

3.5.3. Does detritus feeding help explain the ecological success of ommastrephid squids?

Although detritus (such as POM or fecal pellets) is widely distributed throughout the water column (Hagen et al., 2012), only two lineages of cephalopods independently developed a suspension feeding diet (*V. infernalis* and *S. spirula*), without any known evidence of ontogenetic shifts in their feeding



habits. The detritus-based diet of early ommastrephid paralarvae is an unexpected finding, since posterior ontogenetic stages are voracious predators (e. g., Jereb & Roper, 2010). It constitutes a unique feature among living cephalopods.

Although it is a poorly nutritious resource (Anderson et al., 2016), detritus has several advantages beyond its practical ubiquity in the water column. Since marine detritus has multiple origins (e. g., fecal pellets, body remains, gelatinous plankton houses and continental contributions) and usually circulates among many consumers increasing the length of time it is available for consumption (Polis & Strong, 1996), important depletions in its availability are not expected to occur. A common feature of food webs is that the majority of the primary productivity is consumed as detritus (Moore et al., 2004; Polis & Strong, 1996) and detritivore biomass usually exceeds that of herbivores or carnivores (Hagen et al., 2012; Polis & Strong, 1996). Saprobiont organisms, such as fungi and bacteria, grow on detritus and transform dead organic matter into living microbial biomass increasing the trophic level of this type of food (Steffan et al., 2017). Thus, although ommastrephid paralarvae feed on nutrient-poor detritus (sometimes formed by not edible plant or algae remains; Fig. 3.4, Table 3.3), it is enriched by microorganisms that provide them with usable amino acids and other biomolecules (Anderson et al., 2016). Furthermore, small ommastrephid paralarvae of 1-2 mm ML are equipped with a well-developed digestive system with high proteolytic activity (Boucaud-Camou & Roper, 1995; 1998), which may potentially help in the extraction of nutrients.

Finally, the process of harvesting detritus does not come with the energy costs that hunting behaviors demand, allowing paralarvae to save energy and invest in growth. Since ommastrephid paralarvae may not need highly developed sensory organs for detecting prey and muscular power to chase their prey, a detritus diet allows ommastrephids to produce small undeveloped paralarvae (Shigeno et al., 2001a, 2001b) and allocate more energy to producing large quantities of offspring. Ommastrephid paralarvae are among the smallest of cephalopods (Villanueva et al., 2016). Since cephalopod hatchling size is negatively correlated with their distribution range (Villanueva et al., 2016), the production of small hatchlings has an additive positive effect of increasing the dispersion range of ommastrephids to explore new suitable habitats in a changing oceanic realm.

3.5.4. Reads of self-contamination in LCM-dissected paralarvae

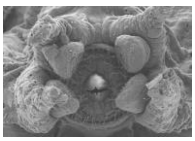
The LCM-dissected paralarvae showed a relatively low self-contamination percentage (79 ± 30 %, n = 26) and a value of 0 % self-contamination was even obtained in the molecularly identified *D. gigas* early paralarva E41. Three late paralarvae were not LCM-processed (E5-E7, Table 3.1) and the whole digestive system was used in the DNA extraction. Despite the fact that these paralarvae revealed

conspicuous gut contents in the digestive system during dissection, no reads were retrieved from the first bioinformatic analysis. A posterior bioinformatic analysis showed 100 % self-contamination of *T. sagittatus*, a species not present in the database used (SILVA) (see in Material and Methods). Thus, the only possible explanation is that paralarvae tissues strongly prevail in the PCR product producing 100 % of the reads during NGS sequencing. This indicates the importance of avoiding the inclusion of gut tissues from the focal species when performing dietary analyses. The low self-contamination reads obtained in this study for LCM-dissected paralarvae are unusual in the literature of dietary metabarcoding studies of tiny organisms with universal primers and without PCR enrichment methods, which usually show self-contamination values above 90 % (e. g., Piñol et al., 2014; Olmos-Pérez et al., 2017).

3.6. Conclusions

The mixture of continental (insects, plants and freshwater algae) and exclusively marine animal DNA (appendicularians and cephalopods) in combination with single cell organisms (cyanobacteria, diatoms and ciliophorans), other organisms usually associated with organic material degradation (fungi) and bacteria typically associated with POM, strongly suggest that ommastrephid squids are detritivores during their early planktonic life. Similar assemblages of general gut content composition and protists taxa have been reported in other marine suspension feeders during their larval life, such as eel and spiny lobsters larvae (Chow et al., 2010; Govoni, 2010; O'Rorke et al., 2012a; 2014). A first feeding feeding diet based on detritus is a unique life strategy among predatory cephalopods and is potentially one of the reasons for the ecological success of the Family Ommastrephidae in the oceanic realm. This ontogenetic shift in the diet allows ommastrephid squids to take advantage of an almost ubiquitous and accessible food resource during their early stages, such that they do not directly compete with conspecifics of later ontogenetic stages for the same prey (even if they do predate on different ontogenetic stages of a particular species) or with other cephalopod paralarvae. Since detritus is almost ubiquitous, competition for trophic resources between early ommastrephid paralarvae should also be minimal. The new knowledge provided in this work can be applied in the future to the development of experimental culture protocols for ommastrephid hatchlings obtained by *in vitro* fertilization (Villanueva et al., 2012) or aquaria spawning (Puneeta et al., 2015).

Identifying the diet of wild cephalopod paralarvae by DNA sequencing is a poorly studied topic. Only two previous studies exist, both working with coastal species whose paralarvae have a very different morphology and ecology, the common octopus *Octopus vulgaris* and the midsize squid *Alloteuthis media* (Roura et al., 2012; Olmos-Perez et al., 2017). As far as we know, no previous attempts to study



the diet of ommastrephid squids by NGS sequencing have been made. Since no reliable knowledge on the diet of early ommastrephid paralarvae was available, a mixed approach based on sequencing the hypervariable eukaryotic 18S v9 and prokaryotic 16S rRNA was performed here, covering almost every life domain. This combination provided a good snapshot of the diet of early ommastrephid paralarvae. Although more specific eukaryotic metabarcodes are available, the spectrum of taxonomic groups they are able to amplify is usually narrower (Leray & Knowlton, 2016). Thus, if one of these molecular markers was selected, many eukaryotic MOTUs would not be detected and the study may be critically biased, providing very different results and possibly misleading conclusions.

It should be noted that the bacteria found did not only come from the diet, since gut microbiomes of marine animals are formed by an enormous diversity of bacteria (Nayak, 2010). The cephalopod gut microbiome is poorly understood at present, but has recently gained attention in efforts to overcome mortality problems in laboratory reared paralarvae (Roura et al., 2017). To the best of our knowledge, there is no previous work dealing with the gut microbiome of ommastrephid squids, either for paralarvae or later ontogenetic stages. The absence of this information precluded us from reliably distinguishing the bacteria that came from the diet from those that are common residents in the gut microbiome of squids. Similarly, some prokaryotic MOTUs may represent parasites, such as seven MOTUs of Mycoplasmataceae, which represented an important part of the subadult and adult reads (14 %, Table S3.4, Table S3.3), or the 2 Rickettsiales MOTUs that produced a 90 % match with the pathogenic enteric bacterium *Hepatobacter penaei* (Nunan et al., 2013) (Table S3.3). Although the prokaryotic data generated here (Table S3.4, Table S3.3; Fig. 3.5-6) are in the context of a dietary study, the results provided may aid in the understanding of the gut microbiome of ommastrephids when more directed studies are carried out and may bring to light the possible pathogens that infect these oceanic cephalopods.

In this study, gut contents were successfully LCM-isolated from histological paralarvae sections (Fig. 3.2) and NGS sequencing was carried out with small portions of gut contents (Table 3.1) obtaining low values of self-contamination (Fig. 3.3). This is the first time LCM has been applied on wild-collected samples in a dietary study and our results are promising for applying this methodology to other tiny animals, even when universal primers are used without PCR enrichment methods.

3.7. Acknowledgements

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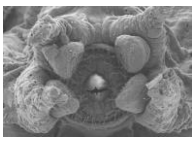

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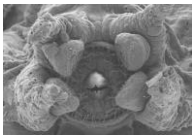

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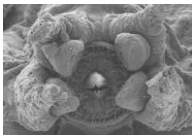

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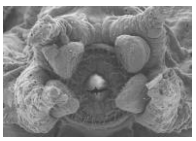


3.9. Supporting information

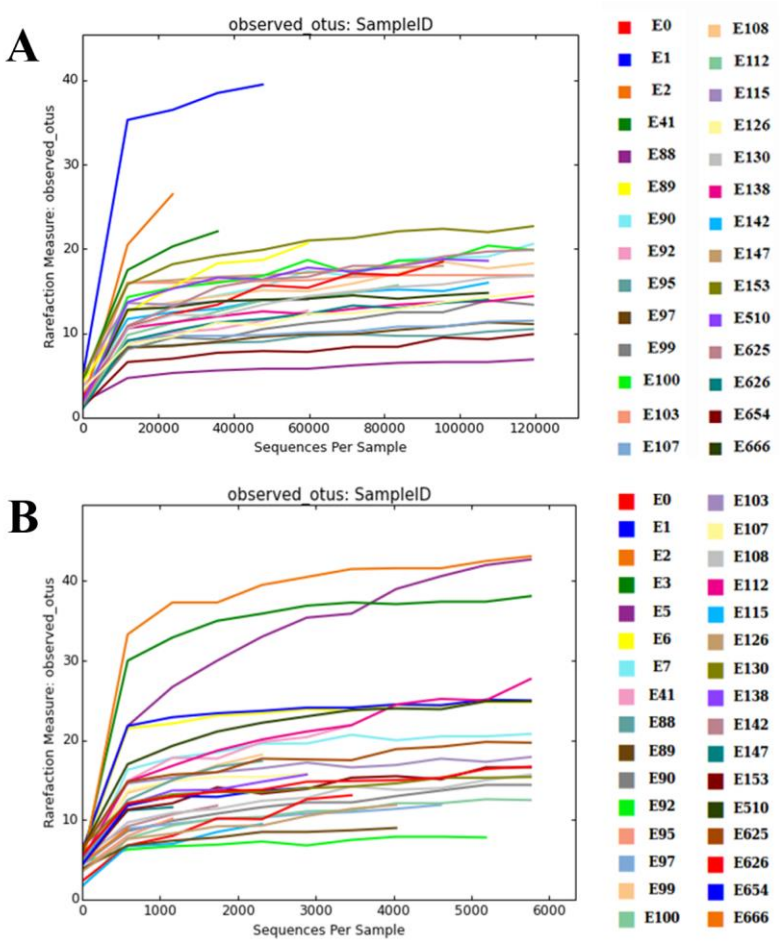


Fig. S3.1. (**A**) Rarefaction plot of 18S v9 eukaryotic reads of each individual at a 100 % similarity threshold. (**B**) Rarefaction plot of 16S prokaryotic reads of each individual at a 97 % similarity threshold.

Table S3.1. Collection data of the individuals used in this work. Individuals are ordered by their ML (more information in Table 3.1).

Labcode	Geographical coordinates	Depth (m)	Seafloor depth (m)	Date	Hour	Cruise
Early paralarvae						
E666	25º 45´N, 113º 27´W	0-300	3347	9.2.2005	17:14	IMECOCAL-CICESE, Pacific cruise
E41	27º 51´N, 112º 17´W	0-150	684.5	26.6.2008	22:33	DGGOLCA, Pacific cruise
E126	18º 28´N, 106º 9´W	0-150	>1500	18.1.2010	11:38	PCM, Pacific cruise
E138	18º 0´N, 105º 26´W	0-150	>1500	18.1.2010	4:08	PCM, Pacific cruise
E142	18º 0´N, 105º 26´W	0-150	>1500	18.1.2010	4:08	PCM, Pacific cruise
E147	18º 0´N, 105º 26´W	0-150	>1500	18.1.2010	4:08	PCM, Pacific cruise
E130	18º 0´N, 105º 26´W	0-150	>1500	18.1.2010	4:08	PCM, Pacific cruise
E95	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	PCM, Pacific cruise
E90	18º 52´N, 105º 5´W	0-150	>1500	15.1.2010	22:44	PCM, Pacific cruise
E112	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	PCM, Pacific cruise
E115	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	PCM, Pacific cruise
E103	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	PCM, Pacific cruise
E99	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	PCM, Pacific cruise
E107	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	IMECOCAL-CICESE, Pacific cruise
E625	24º 39´N, 114º 2´W	0-300	No data	3.8.2005	3:32	IMECOCAL-CICESE, Pacific cruise
E108	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	PCM, Pacific cruise
E88	18º 59´N, 104º 28´W	0-150	259	21.1.2010	19:19	PCM, Pacific cruise
E97	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	PCM, Pacific cruise
E89	18º 52´N, 105º 5´W	0-150	>1500	15.1.2010	22:44	PCM, Pacific cruise
E626	24º 39´N, 114º 2´W	0-300	No data	3.8.2005	3:32	IMECOCAL-CICESE, Pacific cruise
E92	19º 18´N, 107º 18´W	0-150	>1500	19.1.2010	6:25	PCM, Pacific cruise
E100	17º 31´N, 104º 54´W	0-150	>1500	17.1.2010	20:56	PCM, Pacific cruise
E654	25º 45´N, 113º 27´W	0-300	878	9.2.2005	0:11	IMECOCAL-CICESE, Pacific cruise
E153	17º 31´N, 103º 44´W	0-150	>1500	16.1.2010	17:45	PCM, Pacific cruise
E510	26º 9´N, 114º 7´W	0-300	No data	31.7.2005	16:53	IMECOCAL-CICESE, Pacific cruise
Late paralarvae						
E6	21º 36´N, 18º 55´W	0-50	2989	25.4.2015	4:56	MAFIA, Atlantic cruise
E7	21º 36´N, 18º 55´W	0-50	2989	25.4.2015	4:56	MAFIA, Atlantic cruise
E5	21º 36´N, 18º 55´W	0-50	2989	25.4.2015	4:56	MAFIA, Atlantic cruise
E0	7° 9´N, 23° 58´W	0–100	4245	17.4.2015	23:28	MAFIA, Atlantic cruise
Subadults and adult						
E1	3º 45´N, 25º 15´W	0-800	4170	15.4.2015	21:47	MAFIA, Atlantic cruise
E2	3º 45´N, 25º 15´W	0-800	4170	15.4.2015	21:47	MAFIA, Atlantic cruise
E3	18º 7´N, 20º 11´W	No data	3174	23.4.2015	23:05	MAFIA, Atlantic cruise

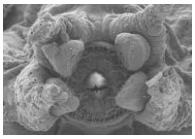


Table S3.2. Raw data of eukaryotic 18S v9 gut contents. Self-contamination reads were discarded. The OTU ID number includes the GenBank Accession number of the closest match (100 % similarity) in the SILVA database. The paralarvae E626 and E510 produced 100% self-contamination and late paralarvae E5-E7 and the adult E3 did not provide any gut content reads based on the database. Therefore, these specimens are not included in this table.

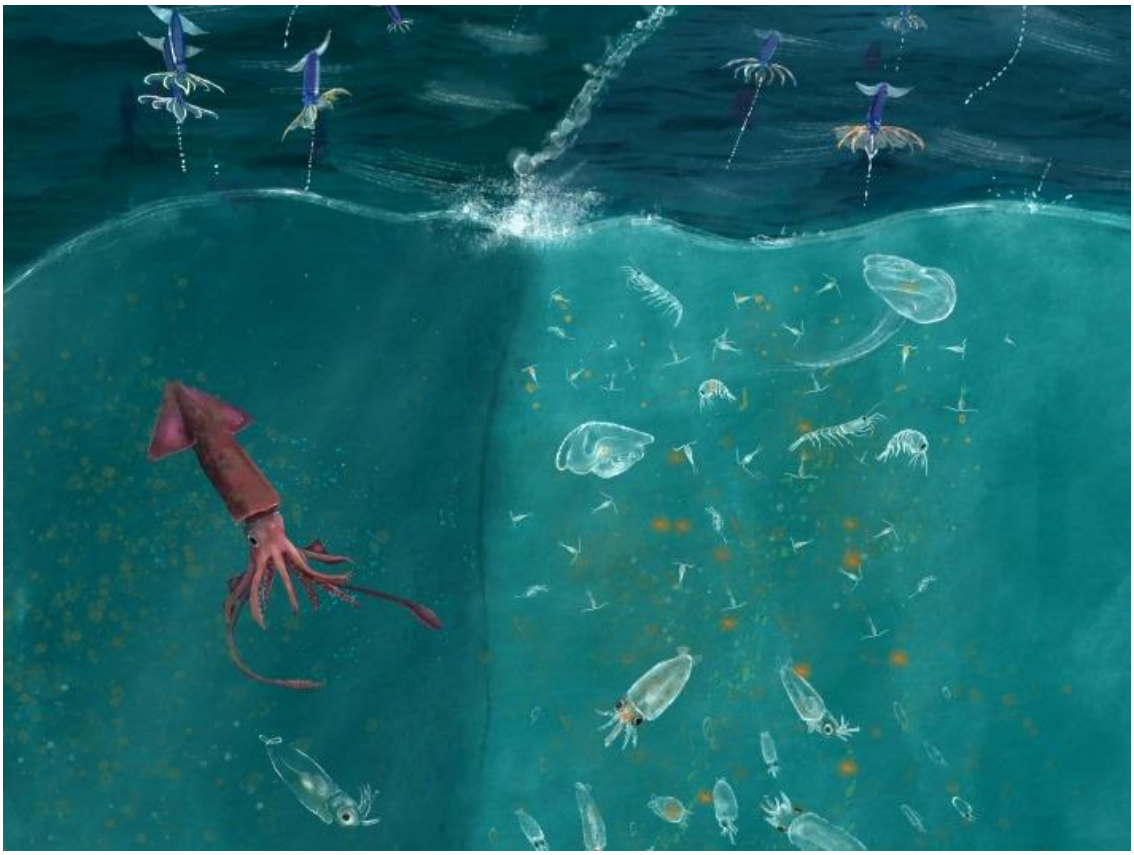
Table S3.3. Raw data of prokaryotic 16S gut contents. The OTU ID number is the Greengenes identifier. New.ReferenceOTU dessignates a MOTU not included in Greengenes when the analysis was performed. Those sequences not identified with Greengenes were subsequently identified by a BLAST search in GenBank following the criteria explained in the text. The taxonomy of GenBank is applied when it was not provided by Greengenes; if it was provided by Greengenes it is indicated with "N/A". For chloroplast and mitochondria the taxonomy of GenBank was applied for eukaryotic organisms. N/A, not applicable.

Tables S3.2 and S3.3 can be accessed through the following link:

https://www.dropbox.com/sh/s329efd3h77ay77/AACnQ_n1oBhzRgvfp1BLktzza?dl=0

CHAPTER 4

Global biodiversity of the genus *Ommastrephes* d'Orbigny, 1834 (Ommastrephidae: Cephalopoda): a cosmopolitan monotypic genus or a cryptic species complex?



Fernández-Álvarez FÁ, Bolstad KSR, Braid HE, Haimovici M, Nigmatullin CM, Sánchez P & Villanueva R. Global biodiversity of the genus *Ommastrephes* d'Orbigny, 1834 (Ommastrephidae: Cephalopoda): a cosmopolitan monotypic genus or a cryptic species complex? [In preparation].

4.1. Abstract

Cryptic speciation among morphologically homogeneous species is a phenomenon increasingly frequently reported among marine invertebrates. This situation usually leads to a new scenario in which each of the newly discovered species actually represents a small fraction of the original distribution range. Since the unit of action for conservation and natural resource management politics is the species, solving the taxonomic status of species-complexes is a highly important task. *Ommastrephes bartramii* is considered until now a monotypic species of flying squid with a cosmopolitan and discontinuous distribution range. However, some morphological and metabolic difference between individuals from different regions raised some doubts about its taxonomic status. Here, mitochondrial sequences of individuals from almost all the distribution range of this oceanic genus were studied in order to solve this problem. The following five molecular species delimitation methods provided consistent results and identified four species: haplotype networks analysis, *p*-distance analysis, Automatic Barcode Gap Discovery (ABGD), Poisson Tree Processes (PTP) and the Generalized Mixed Yule Coalescent (GMYC). This information was combined with the previous knowledge from the literature to resurrect three previously synonymized names and propose the actual distribution range of each species. In addition, diagnostic characters were extracted from the molecular sequences and incorporated to the species description. The possible role of the Isthmus of Panama as a trigger of the allopatric speciation of the genus *Ommastrephes* is discussed. Only one of the four newly recognized species is commercially exploited by fisheries, but it is important to note that the actual distribution range of the species is far more reduced than previously thought and this is important for a proper fishery management. This earns even more importance if other commercial fisheries developed in the future over other *Ommastrephes* species.

4.2. Introduction

Most species descriptions are based on morphological characters. Sometimes, two related species evolve and develop different genetic barriers (habitat, life history or recognition systems) without a parallel morphological differentiation (Knowlton, 1993), cheating taxonomic work based on morphology. Species-complexes might be formed by cryptic (i.e., there are not morphological differences) or pseudocryptic (i.e., there are morphological differences, which might be overlooked or considered as intraspecific variation due to mistaken or overconservative taxonomic practices). For marine animals, this situation is quite common due to the long-standing general thinking that the oceanic realm is a continuum devoid of barriers for genetic exchange. As a consequence, many populations of marine groups with more or less homogeneous morphology have been assigned to previously described species from a distant place (e.g., Carrera-Parra et al., 2011) or several similar biological species from distant areas have been synonymized under a single cosmopolitan morphospecies (e.g., Nesis, 1987). However, in the last years and usually with the aid of molecular systematics, it was revealed that many of this "cosmopolitan" species actually represent many species (e.g., Kawauchi & Giribet, 2010; Valdés et al., 2017). This is a direct consequence that the marine environment is not devoid of physical barriers to dispersal and several biological and physiological factors might affect the distance that species are able to disperse.

Cryptic biodiversity might critically hinder the conservation or natural resource management of a particular taxon. In fact, it is becoming increasingly frequent the recognition of cryptic species in marine invertebrates, which usually leads to an scenario that each species actually represents a small portion of the original distribution area described for the original species (e.g., Bickford et al., 2007; Calvo et al., 2009; Amor et al., 2017). Since the accepted unit of action for conservation and resource management politics is the species level, it is especially important to ascertain how many cryptic species are involved in problematic taxa and describe and name the newly discovered species of the species-complex (Templado et al., 2016). However, both for practical reasons or journal requirements (Jörger & Schrödl, 2013), many researchers postpone formal description of detected cryptic species and they remain unnamed and, therefore, exempt of an appropriate treatment for conservation and natural resource management.

Squids of the Family Ommastrephidae Steenstrup, 1857 are considered the most economically and commercially important cephalopods worldwide (Jereb & Roper, 2010). Their abundance, rapid growth and massive body size make them one of the most exploited invertebrate fishing resources (Arkhipkin et al., 2015a). Ecologically, these oceanic squids are both important prey (Romeo et al., 2012) and predators (Villanueva et al., 2017), occupying a wide range of trophic levels in marine pelagic food webs (Coll et al., 2013). They are characterized by a short life

span, usually less than two years (Arkhipkin, 2015b), and by a unique paralarval phase known as rhynchoteuthion (Jereb & Roper, 2010), for which many fundamental life history are starting to be unraveled (Fernández-Álvarez et al.; 2017, 2018a). The neon flying squid *Ommastrephes bartramii* (Lesueur, 1821) is the second biggest representative of the family, only exceeded in size by the Humboldt flying squid *Dosidicus gigas* (d'Orbigny, 1835). The neon flying squid may reach a maximum size of 1200 mm in dorsal mantle length and 35 kg in weight (Guerra et al., 2010), but usually reach smaller sizes. Although during decades the taxonomic status of one of their synonyms, *Ommastrephes caroli* (Furtado, 1887), was matter of discussion (Young, 1972; Zuev et al., 1975 and references therein), current taxonomic criteria consider the genus *Ommastrephes* d'Orbigny, 1834 as monotypic and the single recognized species as cosmopolitan (Zuev et al., 1975), with three undescribed units (Nesis, 1987; Jereb & Roper, 2010): the North Atlantic, the Southern Hemisphere and the North Pacific groups. These undescribed groups were defined according with differences in the size structure of the different populations (Zalygalin et al., 1983), the spermatophore structure (Nigmatullin et al., 2003) and by substrate-inhibitory traits of optic ganglia cholinesterases (Shevtsova et al., 1979; Rozengart & Basova, 2005). Young (1972) also found slight differences between immature male specimens from Guadalupe Island (North Pacific) and Florida (North Atlantic) in morphometrics and beak morphology. Here, molecular analyses of two mitochondrial markers are carried out in order to solve this long standing debate among the cephalopod research community.

4.3. Material and methods

4.3.1. Sample collection

Newly collected *Ommastrephes* samples were obtained from the local markets, from land strandings or collected during the Atlantic research cruise MAFIA (Olivar et al., 2017). After collection, a small piece of the mantle was preserved for molecular analysis. In some cases, the full body of the animal was preserved in 4 % buffered seawater formalin and deposited as morphological vouchers in the following collections: the Biological Reference Collections of the Institut de Ciències del Mar (Barcelona, Spain) (CBR-ICM), Museu Oceanográfico of the Rio Grande Federal University (MORG) and the National Museum of New Zealand Te Papa Tongarewa (NMNZ). Information on the locality and GenBank and collection accession numbers is summarized in Table 4.1. In order to increase the geographical range of the sampling, formalin-fixed specimens from SE Atlantic (Namibia) (Villanueva and Sánchez, 1993) and SE Pacific (Chile) (Guerra et al., 2010) were added to the DNA extractions, without success.

Table 4.1. Sample data of the ommastrephid squid individuals studied, including their accession numbers for GenBank and the morphological vouchers. N/A, not available.

Species	Locality	N	Voucher accession number	GenBank accesion number		Reference
				COI	16S	
Ommastrephes group 1	Arguineguín, Las Palmas de Gran Canaria, Spain. **NE Atlantic**.	1	ICMC000070[1]	N/A[2]		This work
	Gijón, Asturias, Spain. **NE Atlantic**.	1	N/A[3]	N/A[2]	N/A[2]	This work
	Colunga, Asturias, Spain. **NE Atlantic**.	1	N/A[3]	N/A[2]		This work
	Luka Šipanska, Island of Šipan, Croatia. **Mediterranean Sea**.	1		KF212462		Franjevic et al., 2015
	Selvagem Grande island, Portugal. **NE Atlantic**.	8			KC603479, KC603482-KC603484, KC603486-KC603489	Alonso et al. (2014)
Ommastrephes group 2	Cabo Verde. 18.11ºN, 20.20ºW. **Equatorial E Atlantic**.	1	ICMC000059[1]	MF980596	N/A[2]	Fernández-Álvarez et al. (2018a),
	Parana State, Brasil. 25.86ºS, 45.75ºW. **SW Atlantic**.	4	MORG 51418[4]	N/A[2]	N/A[2]	this work
	S Atlantic.	6			AB635411-AB635416	Wakabayashi et al. (2012b)
	34-36ºN, 40-50ºE. **SW Indian Ocean**.	2			AB635465-AB635466	Kurosaka et al. (2012)
Ommastrephes group 3	Mangaia, Cook Islands (William F. Gilly, pers. comm.). **Central S Pacific**.	2		HQ829183, HQ829184	HQ829182	Unpublished
	Wellington, New Zealand. **SW Pacific**.	1	M318162[5]	N/A[2]	N/A[2]	This work
	W of Auckland, New Zealand. **SW Pacific**.	1	M318203[5]	N/A[2]	N/A[2]	This work
Ommastrephes group 4	Cruise Hokusei-Maru, **NW Pacific**.	1		AF000057		Carlini & Graves (1999)
	Northern Haiwaiian waters, **Central N Pacific**.	2		AB199549, AB199551		Wakabayashi et al. (2006)
	Cruise Shunyo-Maru, Northern Hawaiian waters, **Central N Pacific**.	1		AB270941		Wakabayashi et al. (2012a)
	N Pacific.	7			AB635404-AB635410	Wakabayashi et al. (2012b)
	39-46ºN, 163-173ºW. **NW Pacific** and **Central N Pacific**.	30			AB509422-AB509451	Kurosaka et al. (2012)
Dosidicus gigas (Orbigny, 1835)		1		AB270944	AB270959	Wakabayashi et al. (2012a)

[1] Biological Reference Collections of the Institut de Ciencies del Mar (CBR-ICM), Barcelona, Spain.
[2] To be submitted to GenBank after article acceptance.
[3] Parque de la Vida (La Mata, Asturias, Spain, http://www.parquedelavida.org). Morphological voucher accession number not available.
[4] Museu Oceanográfico of the Rio Grande Federal University (MORG), Brazil.
[5] National Museum of New Zealand Te Papa Tongarewa (NMNZ), New Zealand.

4.3.2. DNA extraction, amplification and sequencing

Tissues for molecular analysis were fixed in 96 % ethanol. Total genomic DNA was extracted from ethanol-fixed piece of the mantle using the NZY Tissue gDNA isolation kit (NZYTech, Lisbon, Portugal), following the manufacturers' protocol and resuspended in a final volume of 100 µL. A negative control that contained no sample was included in every isolation round to check for contamination during the experiments. Sequences from the partial mitochondrial cytochrome *c* oxidase I (COI) gene were amplified, using the primer pair LCO1490 and HCO2198 (Folmer et al., 1994). For the partial mitochondrial 16S rRNA (16S) fragment, the primer pair 16sbr-H-myt and 16sar-L-myt (Lydeard et al., 1996) was used. Standard PCR reactions were performed using the NZYTaq Green PCR Master Mix (NZYTech, Portugal) following the manufacturer's protocol in a total volume of 25 mL, which included 0.5 µM of each primer, 25 ng of template DNA and PCR-grade water up to 25 µL. PCRs consisted of an initial denaturation at 95 ºC for 5 min, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 50 ºC for COI and 45 ºC for 16S for 30 s and extension at 72 ºC for 45 s, with a final extension of 5 min at 72 ºC. The amplified products were sequenced using both forward and reverse PCR primers on an ABI 3730xl. DNA sequence data were edited and aligned with Geneious 8.1.5 (http://www.geneious.com). Ommastrephid squids have duplicated regions of the mitochondrial genome, including the gene COI (Yokobori et al., 2004; Staaf et al., 2010). Although it is assumed that concerted evolution mechanisms apply in ommastrephid mitochondria (Allcock et al., 2015), several variable positions were found. The IUPAC ambiguity codes were used to codify these nucleotide variable positions as in Fernández-Álvarez et al. (2015a).

4.3.3. Phylogenetic analysis

Newly obtained and available sequences for both COI and 16S in GenBank were downloaded and used for the development of each databases (Table 4.1), namely *Ommastrephes*-COI and *Ommastrephes*-16S from now on, respectively. Sequences whose geographical origin was not possible to obtain from the available literature as well as COI sequences with alterations in the protein reading pattern or stop signals were discharged. The final alignment included 17 individuals for COI and 62 individuals for 16S. Sequences were manually aligned using the software Bioedit v. 7.0.1 (Hall, 1999). Since sequences of different lengths were included in the alignment, the extremes of the sequences were trimmed by both extremes to the nucleotide (nt) number of the shorter sequence, resulting in a 612 and 467 nt alignment for *Ommastrephes*-COI and *Ommastrephes*-16S, respectively. For the 16S alignment, a single gap was needed to be added to the sequence KC603489 due to the presence of a single nucleotide deletion. The DNA

sequence evolution model that better fits both dataset was Tamura-Nei gamma according with the Akaike Information Criterion, through the FindModel web (http://hiv.lanl.gov/content/sequence/findmodel/findmodel.html).

As a preliminary species delimitation analysis, the software TCS v. 1.21 (Clement et al., 2000) was used to construct the haplotype networks with a maximum connectivity limit of 95 % with the COI database. This analysis resulted in four separate networks which were named as *Ommastrephes* groups 1 to 4 to simplify the nomenclature (Table 4.1). The TCS function of the software PopART (Leigh & Bryant, 2015) was used to represent the statistical parsimony networks. Uncorrected *p*-distances within each *Ommastrephes* group and between groups were calculated with MEGA6.06 (Tamura et al., 2013) for both molecular markers. The online version of software Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012) was employed to check the distribution and size of a potential barcoding gap for both databases.

The phylogenetic relationships of Ommastrephinae are not yet fully resolved. According with different works the sibling group of *Ommastrephes* might be either *Dosidicus gigas* or *Sthenoteuthis* spp. (Zuev et al., 1975; Nigmatullin, 2007; Lindgren et al., 2012, Strugnell et al., 2017; Pardo-Gandarillas et al., 2018). Uncorrected *p*-distances of both COI and 16S showed a closer relation between *Ommastrephes* and *D. gigas* (Fernández-Álvarez, pers. obs.). Thus, this species was selected as outgroup for the phylogenetic analyses. A concatenated dataset with COI and 16S sequences were constructed with the specimens with both markers available. For the N Pacific individuals, both molecular markers were not sequenced for the same individual in any case. Thus, we randomly combined *Ommastrephes* group 4 COI and 16S sequences for this matrix, arranged as follows: AF000057-AB635404, AB1999549-AB509422, AB199551-AB635410 and AB270941-AB509430. This database is named *Ommastrephes*-COMBI.

As alternative species delimitation methods, the Poisson Tree Processes (PTP) (Zhang et al., 2013) and the Generalized Mixed Yule Coalescent (GMYC) (Fujisawa & Barraclough, 2013) were applied for all the *Ommastrephes* databases. For PTP, the starting Maximum Likelihood (ML) trees were constructed with MEGA6.06 using the Tamura 3-parameter gamma model with bootstrap values calculated from 1,000 replicates. In the PTP portal (http://species.h-its.org/ptp/) the default parameters and 300,000 MCMC generations were used. For the GMYC, Bayesian analysis under a lognormal relaxed clock was performed with Beast v1.5.4 (Drummond & Rambaut, 2007) under the TN93 gamma model for 100 million generations. Tracer v1.6 (Rambaut & Drummond, 2003–2009) was used to check whether the parameter had reached values of effective sample size over 100 and a burn-in of 10 % was used through TreeAnnotator v.1.5.4 (Drummond & Rambaut, 2007). The obtained tree was visualized and converted to Newick format using FigTree v1.4.3 (Rambaut, 2006–2009) and submitted to the GMYC web

server (http://species.h-its.org/gmyc/) by both the single and multi threshold methods. Preliminar analyses showed that the results of both PTP and GMYC methods improved when the outgroup was removed, and therefore it was not included in the analyses. This might mean that *Dosidicus gigas* is too distantly related with each *Ommastrephes* group for these analyses.

In this work, the biological species concept (Mayr, 1942) was applied, i.e.: a group of individuals that can breed together but cannot breed with other related groups. The concordance of results of many molecular species delimitation methods was considered a confirmation of the species status of a particular *Ommastrephes* group.

4.3.4. DNA diagnostic characters

DNA sequences contain valuable diagnostic characters to be added to the species descriptions, especially when cryptic species-complexes are involved. Here, the DNA matrices *Ommastrephes*-COI and *Ommastrephes*-16S were examined by eye to recover diagnostic characters. Diagnostic characters are defined as characteristic nucleotides that respectively occur in all investigated specimens of a single species but are absent in all the members of the remaining species (i.e., autoapomorphies). Only homogeneous characters were considered, i.e., the diagnostic characters provided here are not variable among specimens of the same group. Special care was taken to avoid the inclusion of plesiomorphic characters as diagnostic characters by the inclusion of the putative sister species of the genus (*Dosidicus gigas*, see above) as outgroup. For the presentation of the results and to ensure the reproducibility of this work, the general recommendations of Jörger & Schröld (2013, 2014) were followed. Positions refer to the position of the diagnostic nucleotide within the respective alignment, which are provided as supplementary material in order to ensure the maximum traceability of the information. FASTA files of *Ommastrephes*-COI and *Ommastrephes*-16S can be accessed through the link:

https://www.dropbox.com/sh/s329efd3h77ay77/AACnQ_n1oBhzRgvfp1BLktzza?dl=0

4.4. Results

The haplotype parsimony statistical network analysis of *Ommastrephes*-COI identified 4 groups with the conspicuous geographical pattern (Fig. 4.1A). *Ommastrephes* group 1 included exclusively North Atlantic individuals and 46 mutations separated it with the closest group. *Ommastrephes* groups 2-4 were more closely related, with 15-20 mutations between them.

Ommastrephes group 2 was formed by Equatorial and South Atlantic individuals, while groups 3 and 4 comprise exclusively South and North Pacific individuals, respectively. The COI intragroup *p*-distances ranged from 0 to 0.8 % (Table 4.2), while intergroup *p*-distances were 2.5-9.1 % (Table 4.3). The ABGD analysis of *Ommastrephes* COI identified 4 groups (prior maximal distance P <0.005). The histogram of distances (Fig. 4.2) show two gaps in the distribution of distances, the first one coincides with the gap between the maximum intragroup (0.8 %, Table 4.2) and the minimum intergroup (2.5 %, Table 4.3) distances. Although there is a tendency to group the sequences by geographic origin, the statistical parsimony network analysis of the *Ommastrephes*-16S database (Fig. 4.1B) revealed a pattern not as clear as *Ommastrephes*-COI. *Ommastrephes* group networks were separated by 2-6 mutations and it also was *Ommastrephes* group 1 the more isolated from the remaining ones. Intragroup and intergroup distances were 0-0.9 and 0.7-1.9 %, respectively (Tables 4.2-3). Thus, a slight overlap between the maximum distance among sequences of a group and the minimum distance between groups exists. The ABGD analysis did not identify groups consistent with the COI analysis.

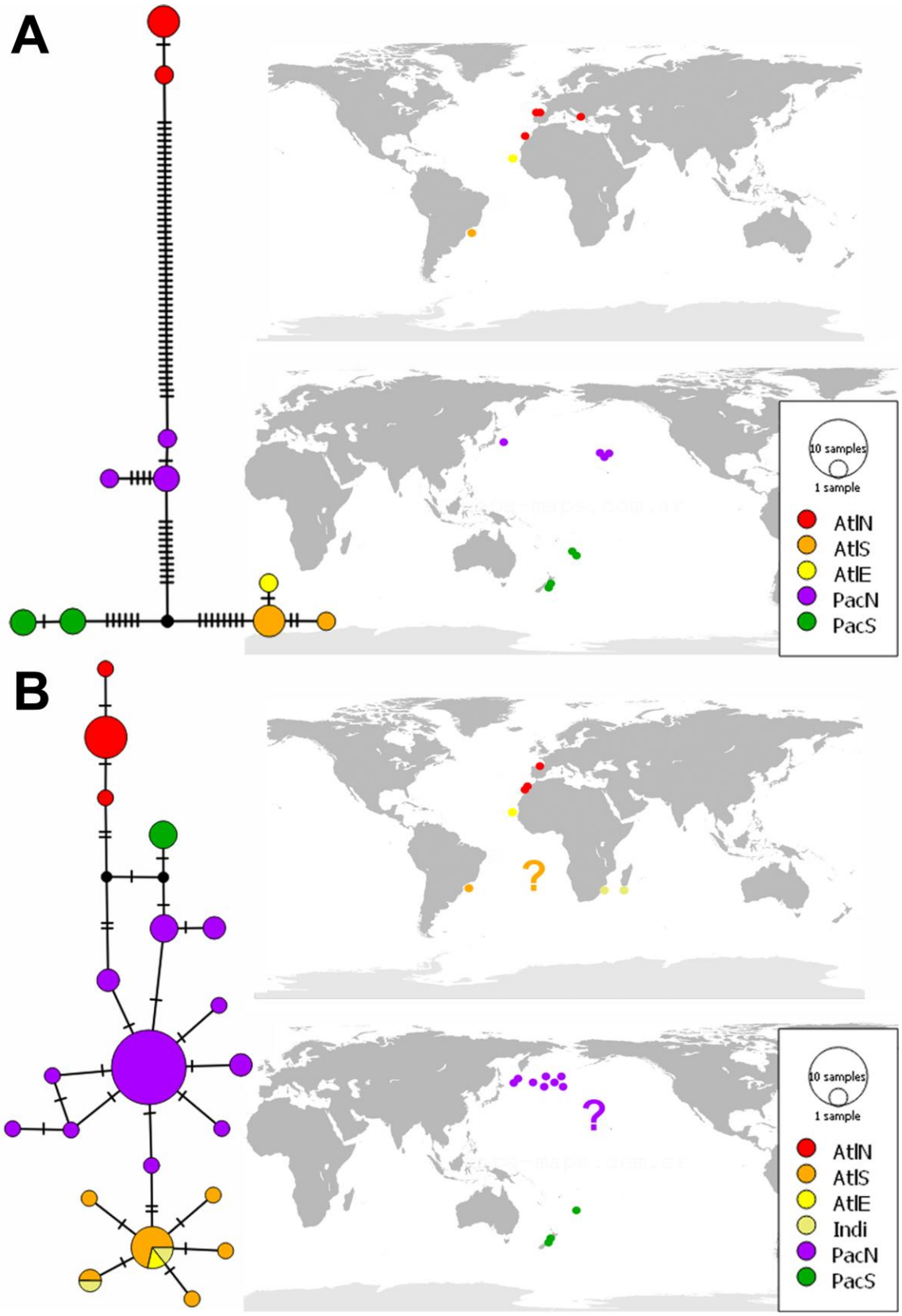


Fig. 4.1. Haplotype statistical parsimony networks constructed by the TCS function of PopART. The geographic point of the samples is indicated in the map. For GenBank records, the approximate geographic position was inferred when necessary, if the reference was vague, the point is represented by a question mark. (**A**) COI. (**B**) 16S. Abbreviations: AtlN, North Atlantic; AtlS, South Atlantic; AtlE, Equatorial Atlantic; Indi, Indian; PacN, North Pacific; PacS, South Pacific.

Table 4.2. Intraclade *p*-distances (%) among *Ommastrephes* groups.

COI	Mean	Range	n
Ommastrephes group 1	0.1	0-0.3	4
Ommastrephes group 2	0.2	0-0.6	5
Ommastrephes group 3	0.1	0-0.2	4
Ommastrephes group 4	0.4	0-0.8	4
16S			
Ommastrephes group 1	0.1	0-0.4	9
Ommastrephes group 2	0.2	0-0.4	13
Ommastrephes group 3	0	0	3
Ommastrephes group 4	0.7	0-0.9	37

Table 4.3. Interclade mean *p*-distances (%) between *Ommastrephes* groups.

COI			
Ommastrephes group 1			
Ommastrephes group 2	9.1		
Ommastrephes group 3	8.9	2.5	
Ommastrephes group 4	7.9	3.1	3.4
16S			
Ommastrephes group 1			
Ommastrephes group 2	1.9		
Ommastrephes group 3	1.1	1.4	
Ommastrephes group 4	1.3	0.8	0.7

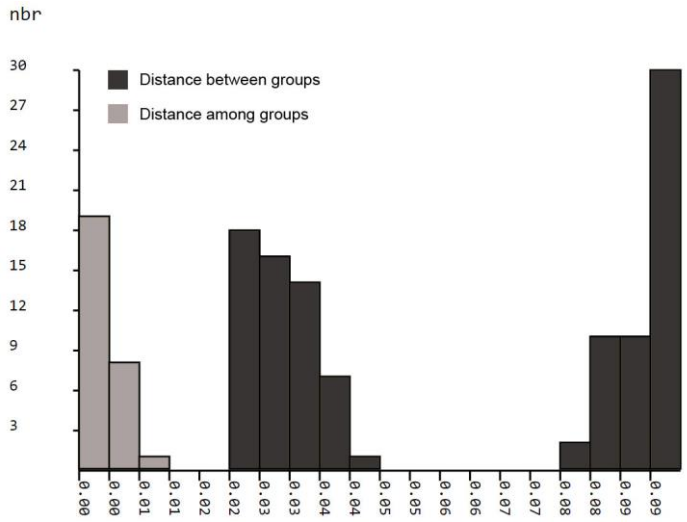


Fig. 4.2. Results from the ABGD analysis for *Ommastrephes* COI. Note that the gap approximately between 4 and 8 % of distance is formed by distances between *Ommastrephes* groups.

ML analyses of *Ommastrephes*-COI and *Ommastrephes*-COMBI revealed four well-supported clades consistent with *Ommastrephes* groups 1-4 (Fig. 4.3). *Ommastrephes* group 1 was basal respecting to the clade composed by the remaining *Ommastrephes* groups, which was formed by a basal branch covering the group 4 and a distal clade formed by the groups 2 and 3. The results of the PTP for both matrices delimited 4 species consistent with the four clades revealed

in the ML analyses. The GMYC result for *Ommastrephes*-COI slightly differed with the single and the multi threshold approaches. The single threshold revealed 4 (confidence interval 3-4) clusters and 7 (confidence interval 4-8) entities, with a Likelihood Ratio (LR) test below 0.05. The multi threshold revealed 4 clusters and 4 entities with the same confidence intervals (3-4) (LR test <0.05). Both methodologies showed clusters consistent with the *Ommastrephes* groups, but the single threshold tends to oversplit the *Ommastrephes* groups 1, 2 and 4 in two entities each one. The *Ommastrephes*-COMBI GMYC analysis identified 3 clusters (confidence interval 2-4) and 4 entities (confidence interval 2-7) with the single threshold, but the LR test was not significant (0.06). The multi threshold method revealed 3 clusters (confidence interval 2-3) and 5 entities (confidence interval 2-7) with a LR test <0.05. The difference in the number of entities between both methodologies is due to that the multiple threshold oversplitted *Ommastrephes* group 3 in two entities. The topology of the *Ommastrephes*-16S ML analysis differed in the fact that neither clade but *Ommastrephes* group 3 (bootstrap value = 85 %) received enough support, *Ommastrephes* group 4 was paraphyletic, and *Ommastrephes* groups 2 and 3 did not formed a distal clade. This indicates that 16S might be too much conserved to apply it for this group and maybe incomplete lineage sorting is operating between *Ommastrephes* groups 2-4 due to recent speciation events. The PTP analyses revealed an extreme scenario of 54 species: almost each sequence represent a different species. The GMYC revealed 10 clusters (confidence interval 1-19) and 11 entities (confidence interval 1-22) with the single threshold method (LR test = 0.2), while the multi threshold method revealed 10 clusters (confidence interval 8-14) and 11 entities (confidence interval 9-18) (LR test <0.01). Figure 4.3 compiled the results of all the species molecular delimitation methods for *Ommastrephes*-COI (TCS, *p*-distance, ABGD, PTP and GMYC) and for *Ommastrephes*-COMBI (PTP and GMYC). For PTP, the topology represented was the one obtained by the multi threshold method for COI and the single threshold for the combined matrix, since both were coincident between them and with the results of the remaining tests.

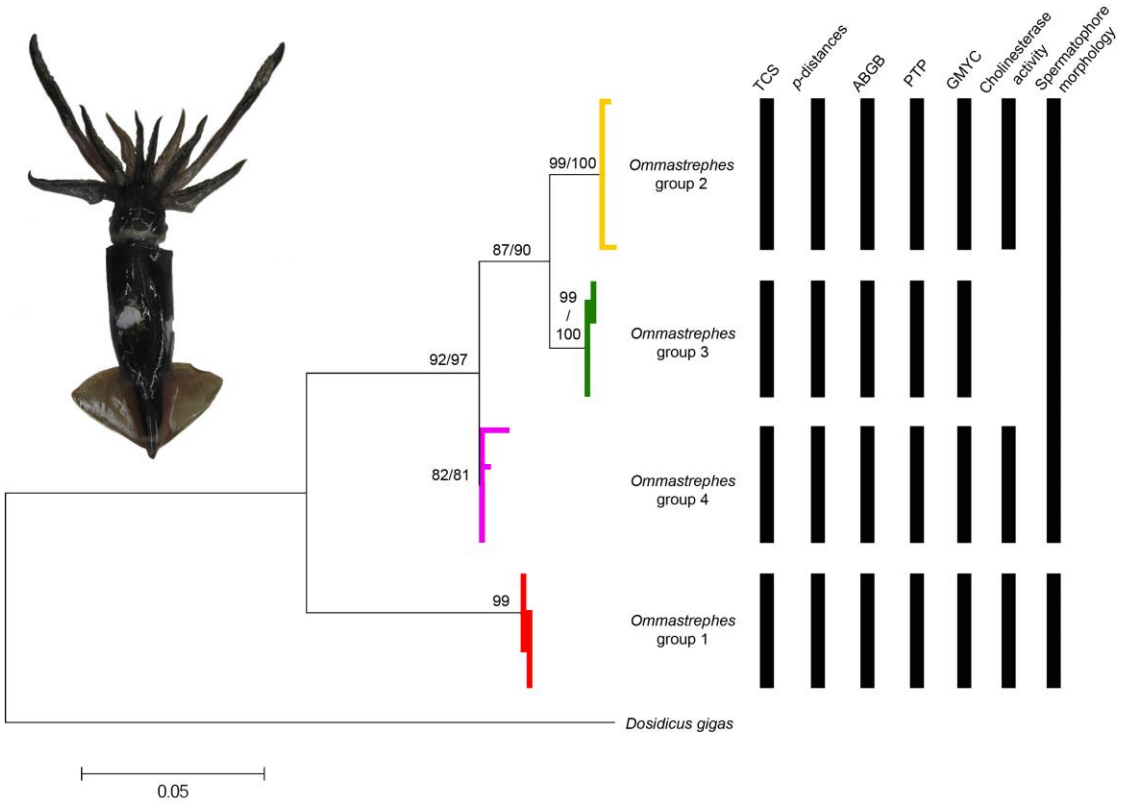


Fig. 4.3. Summarized results from the molecular species delimitation analyses and the enzymatic and spermatophore information, represented on a maximum likelihood tree resulted from the analysis of the *Ommastrephes* COI dataset. Values on the nodes represent the bootstrap values of the COI and the combined matrices, respectively. Black bars to the right represent the hypothesized species groupings based on the molecular delimitation results of TCS, *p*-distances, ABGD, PTP and GMYC analyses for COI and PTP and GMYC for the combined matrix, as well as the previously identified groups from the literature based on the differences in substrate and inhibitor specificity of cholinesterase activities of the optical ganglia (after Shevtsova et al., 1979 and Rozengart & Basova, 2005) and the spermatophore morphology (after Nigmatullin et al., 2003). It is important to note that Shevtsova et al. (1979) and Rozengart & Basova (2005) did not include any South Pacific individual in their studies. The *Ommastrephes* specimen of the left is the specimen ICMC000059, which belong to *Ommastrephes* group 2.

For COI, a total of 32 diagnostic positions were identified (Table 4.4). *Ommastrephes* group 1 had 24 diagnostic positions, while *Ommastrephes* group 2, 3 and 4 had 5, 3 and 1 diagnostic positions, respectively. 16S did not reveal any diagnostic position for *Ommastrephes* groups 1 and 4, but provided a two diagnostic positions for groups 2 (123, C; 434, G) and one for group 3 (17, C) (Table 4.5). It is important to note that diagnostic characters (as considered here) are not the only variable positions among species, since only homogeneous autoapomorphies were considered.

Table 4.4. Molecular diagnostic characters obtained from *Ommastrephes*-COI. Diagnostic characters are shaded.

	Ommastrephes				Outgroup
Position	**Group 1**	**Group 2**	**Group 3**	**Group 4**	*Dosidicus gigas*
6	**T**	A	A	A	A
30	A	**G**	A	A	A
36	A	A	**G**	A	A
48	**G**	T	T	T	A
61	T	T	**C**	T	T
81	**A**	T	T	T	C
113	A	**G**	A	A	A
153	**G**	A	A	A	A
159	**G**	C	C	C	C
177	**C**	A	A	A	A
180	T	**C**	T	T	T
198	**G**	A	A	A	A
207	**A**	G	G	G	G
228	**G**	C	C	C	T
258	**C**	G	G	G	T
273	**G**	A	A	A	A
288	**G**	A	A	A	T
306	**C**	**A**	T	T	T
327	**T**	C	C	C/A	C
334	**G**	A	A	A	A
366	**C**	T	T	T	T
429	**G**	A	A	A	A
432	**C**	G	G	G	T
450	A	A	**G**	A	A
454	T	T	T	**C**	T
456	**A**	G	G	G	G
493	C	**T**	C	C	C
495	**C**	A	A	A	A
534	**G**	A	A	A	C
573	**G**	T	T	T	A
597	**G**	A	A	A	A

Table 4.5. Molecular diagnostic characters obtained from *Ommastrephes*-16S. Diagnostic characters are shaded.

	Ommastrephes				Outgroup
Position	**Group 1**	**Group 2**	**Group 3**	**Group 4**	*Dosidicus gigas*
17	T	T	**C**	T	T
123	T	**C**	T	T	T
434	A	**G**	A	A	A

4.5. Discussion

Previous empirical studies showed that individuals assigned to a statistical parsimony network with a 95 % probability for COI correspond to species (Pons et al., 2006; Hart & Sunday, 2007; Bond & Stockman, 2008; Kang et al., 2015). Statistical parsimony network analysis of the *Ommastrephes* COI dabase with 95 % of confidence intervals identified four isolated groups with a recognizable geographic pattern for both markers: NE Atlantic samples belong to group 1; W Equatorial, S Atlantic samples belong to group 2; SW and Central S Pacific samples are

group 3 and NW and Central N Pacific individuals form group 4 (Fig. 4.1). The maximum intragroup COI *p*-distance was 0.8 % (Table 4.2), while intergroup distances ranged from 2.5 to 9.1 % (Table 4.3) suggesting the existence of a shallow barcoding gap (Fig. 4.2). This is supported by the recognition of four different groups with the ABGD analysis. While distances between *Ommastrephes* group 1 (N Atlantic) with the other groups ranged from 7.9 to 9.1 %, the distances between the remaining groups ranged from 2.5 to 3.4 %. Similar levels of minimum interspecific distances between related species have been reported between several other invertebrate related species, such as octopuses (Allcock et al., 2011), bobtail squids (Gebhardt & Knebelsberger, 2015), land planarians (Lago-Barcia et al., 2015), crustaceans (Robles et al., 2007) and annelids (Tomioka et al., 2016), and even minimal interspecific distances of ~1 % have been found between closely related freshwater mites (García-Jiménez et al., 2017). The 16S *p*-distances were smaller between *Ommastrephes* groups (Table 4.3), a phenomenon frequently reported in the literature (e.g., Hebert et al., 2003; Rodríguez-Flores et al., 2017). The Indian Ocean 16S sequences came from specimens not sequenced for COI and grouped with S and Equatorial Atlantic sequenced animals for both markers and thus should be considered members of *Ommastrephes* group 2. Although a small overlap between intra- and intergroup distances was found between 16S sequences (Tables 4.2-3), it is important to note that no haplotype from a particular geographic group were found in another one (Fig. 4.1B), which also supports that an allopatric reproductive isolation exists and specimens from one region do not interbreed with others from other region. According with the biological species concept (Mayr, 1942), reproductive isolation is considered the boundary between species. This combined evidence suggests that each *Ommastrephes* group should be considered a single geographically isolated species. This vision is reinforced by the fact that the phylogenetic analysis and all the molecular species delimitation molecular methods recognized four groups consistent with the haplotype network analyses (Fig. 4.3).

While no important morphological taxonomic differences exist among specimens from different regions (Jereb & Roper, 2010), differences in their size structure (Zalygalin et al., 1983; Nigmatullin et al., 2003), their spermatophores (Nigmatullin et al., 2003) and substrate-inhibitory traits of optic ganglia cholinesterases (Shevtsova et al., 1979, Rozengart & Basova, 2005) have been identified. Maximum size-at-maturity isolates the N Pacific *Ommastrephes* species from its remaining distribution area, reaching a smaller maximum size-at-maturity, especially for females (Jereb & Roper, 2010). It is important to indicate that maximum size-at-maturity does not represent a good taxonomic character for ommastrephid squids, since it is hugely variable at intraspecific level. For instance, in the related species *D. gigas*, the smaller mature females represent only 12 % of the mantle length of the largest ones (Nigmatullin et al., 2001) and this difference might occur inter-annually in members of the same geographic area

(Hoving et al., 2013; Fernández-Álvarez et al., 2018b). North Atlantic *Ommastrephes* have larger spermatophores (up to 53 mm, up to 41 mm for the remaining *Ommastrephes* groups) and the morphology in the cement body, sperm reservoir and posterior empty part of the spermatophore has important differences with specimens from other parts of the genus range (Nigmatullin et al., 2003). It is noteworthy that these differences are even bigger among *Ommastrephes* group 1 (N Atlantic *Ommastrephes*) and the other *Ommastrephes* species than between other ommastrephid genera (Nigmatullin et al., 2003). This important morphological difference is consistent with the fact that *Ommastrephes* group 1 is the most divergent of the analyzed groups in our phylogenetic analyses (Figs. 4.1 and 4.3, Table 4.3) and holds more diagnostic molecular characters (Table 4.4). Thus, this species had a longer evolutionary history isolated from the remaining congeneric species and was the first group to speciate. Since morphological differences are known at least in the spermatophore between *Ommastrephes* group 1 and the remaining ones, it could be considered a pseudocryptic species, while the absence of morphological differences between the remaining species means they are cryptic species. Shevtsova et al. (1979) and Rozengart & Basova (2005) compared the differences in substrate and inhibitor specific cholinesterase activities of optical ganglia between *Ommastrephes* individuals from 4 geographic locations: the North Atlantic, the South Atlantic, the Great Australian Bight (Southeastern Indian Ocean) and the North Pacific. They found significant differences for these metabolic traits between specimens from all the studied geographic areas with the exception of South Atlantic and Southwestern Indian *Ommastrephes* specimens. None of their specimens came from South Pacific waters, were *Ommastrephes* group 3 occurs (and thus this character was not represented in Fig. 4.3). These metabolic traits differences are interpreted as another source of evidence to support the specific status of each *Ommastrephes* group.

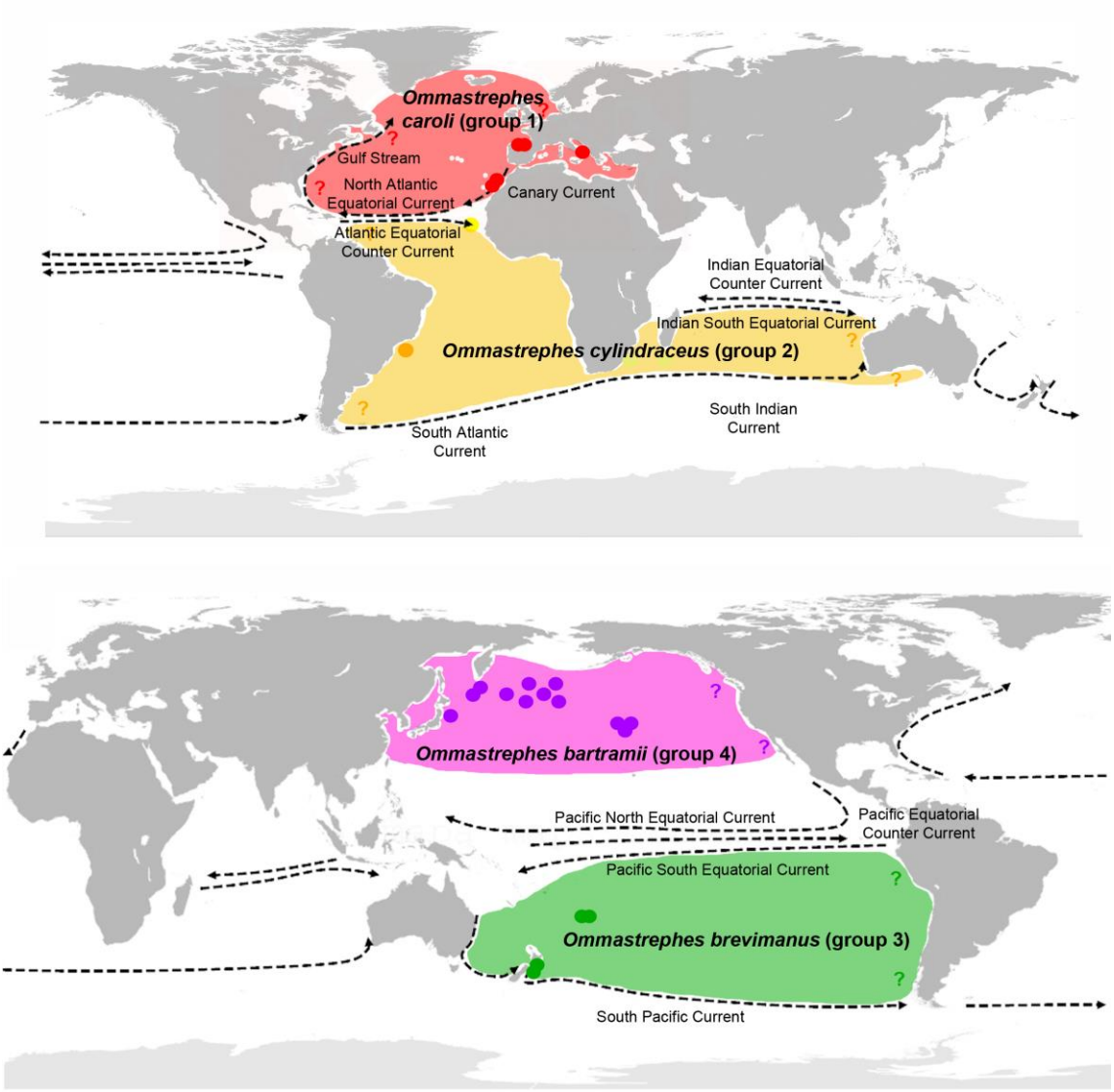


Fig. 4.4. Schematic map of the distribution range of each species of the genus *Ommastrephes*, based on the distribution depicted by Jereb & Roper (2010) and the results of this work. Sampled points for both molecular markers are depicted on the map. The oceanic currents that probably contribute to the reproductive isolation of each species are depicted. Parts of the distribution range of each species that still are not molecularly confirmed are indicated with a question mark.

Subadult and adult ommastrephid specimens are nektonic organisms able to perform large-scale horizontal oceanic migrations (Markaida et al., 2005). Migrations related with feeding and reproductive grounds have been described for *Ommastrephes* group 4 (Ichii et al., 2009). However, the first ontogenetic stages of ommastrephids are planktonic detritivores (Fernández-Álvarez et al., 2018a), representing one of the smallest cephalopod hatchling recorded (1-2 mm ML, Villanueva et al., 2016), and paralarvae dispersion is very likely limited by oceanic features, as the main oceanic gyres and currents. In fact, it is known that hatching size is inversely related with cephalopod distribution ranges (Villanueva et al., 2016). Consequently, the distribution range of *Ommastrephes* spp. seems to be mainly shaped by the Equatorial

oceanic currents (Fig. 4.4). The boundary between *Ommastrephes* spp. 3 and 4 can be related with the Pacific Equatorial currents. The distribution boundary between Atlantic *Ommastrephes* groups 1 and 2 seems to be related with the Canary and Atlantic Equatorial currents, since Canary and Salvagem Grande Island specimens belong to group 1 while the specimen caught near Cabo Verde belongs to the group 2. These sampling localities are only ~1600 Km away, while larger geographic distances (~3500-11400 Km) between conspecific have been molecularly confirmed for both *Ommastrephes* species. Thus, isolation of paralarvae by the Canary and Atlantic Equatorial currents seems to be the most reliable boundary between both groups. In the particular case of *Ommastrephes* group 1, other important oceanographic barriers (as the Strait of Gibraltar and the Sicily Channel (Pascual et al., 2017)) seem to not produce a further effect on *Ommastrephes* geographic structure at this evolutionary scale. However, the impact of these barriers over the population structure of *Ommastrephes* group 1 should be tested with polymorphic markers (microsatellites or SNPs) and it is beyond the scope of this work.

The consequences directly derived from the results of this work go beyond natural history or taxonomists´ interests. Since *Ommastrephes* is an important fishing resource commercially exploited in the North Pacific (Arkhipkin et al., 2015a), it is important to define which biological species within this complex is being impacted by fishery activities. As Figures 4.1 and 4.3 show, the single cryptic species distributed on North Pacific waters is *Ommastrephes* group 4. Although the genus is widely distributed around the world, it is not commercially exploited in other parts of their distribution and only is fished as bycatch in some fisheries (Battaglia et al., 2010). However, this situation might change due to the reported increase of consumers´ demand of cephalopod products (Markaida & Gilly, 2016) and the reported global increase in cephalopod abundance (Doubleday et al., 2016). If in the future other commercial fisheries develop over other *Ommastrephes* species, managers should consider the presence of four species instead of a single cosmopolitan species for a proper assessment of the state of the resource. Since the operational unit for conservation and nature resource management is the species, identifying, defining and naming each biological species from taxonomically complex groups as an increasingly important task.

4.5.1. Untidying a Gordian Knot: *Ommastrephes* spp. taxonomy

Since it is increasingly frequent the discovery of new cryptic species which are morphologically impossible to distinguish (e.g., Jörger & Schrold, 2013), currently many taxonomists agree that future taxonomic descriptions should be integrative, covering many aspects for species delimitation (morphology, molecular sequences, biogeography, behavior and others). Traditional cephalopod taxonomy usually took advance of many body measures and indexes

(Roper & Voss, 1983) that in this case did not produce any relevant taxonomic characters (Young, 1972; Jereb & Roper, 2010). For *Ommastrephes*, previous morphological works only revealed important morphological differences in the spermatophore morphology of North Atlantic specimens in comparison with the remaining ones (Nigmatullin et al., 2003). Here, molecular diagnostic characters are integrated in the description and the geographical range of each species is defined in base of the available molecular information. Although adding molecular characters is gaining support among researchers working in other animal groups (e.g., Jörger & Schrödl 2013; 2014; Sundberg et al., 2016), as far as we know this is the first time that molecular characters are integrated as diagnostic characters in cephalopod taxonomic descriptions, although it has commonly used for detecting new species or solving other taxonomic problems (Allcock et al., 2015).

The phylogenetic analyses (Figs. 4.1-3; Tables 4.1-3) showed molecular evidence of four different biological species under the genus *Ommastrephes*, supported by the molecular diagnostic characters (Tables 4.4 and 4.5). Thus, we recommend split the species *O. bartramii* into four nominal species (see below) corresponding to each identified *Ommastrephes* group. Several names of different squids have been considered as junior synonyms of *O. bartramii*. As primary source of *O. bartramii* synonyms, the compilation of Sweeney & Young (2003) was used as a starting point, followed by a bibliographic review. Morphological and molecular characters are used here in an integrative approach to obtain the diagnostic characters of each species and three of the previously synonymized names are resurrected to name three of the *Ommastrephes* species (*Ommastrephes* groups 1-3).

Genus *Ommastrephes* d'Orbigny, 1834 in 1834-1847:45.

Type Species: *Loligo bartramii* Lesueur, 1821.

Synomyms:

(?) *Cycria* Leach in Gray, 1849:58. Listed as synonym of *Ommastrephes* [fide Hoyle (1910:408)]. Type species: No type given [fide Hoyle (1910:408)]

Lolimnites Risso, 1854:41. [fide Adam (1942:17)]. Type species: *Lolimnites meridionalis* Risso, 1854 by monotypy.

Ommatostrephes Loven, 1845:122. Emendation of *Ommastrephes* D'Orbigny 1834 in 1834-1847 [fide Hoyle (1910:411)].

Diagnosis. The following diagnosis was adapted from the morphological description of Jereb & Roper (2010) but additional paralarval characters were added following the descriptions of Sweeney et al. (1992), Young & Hirota (1990), Sakurai et al. (1995) and Vijai et al. (2015), reviewed by Fernández-Álvarez et al. (2017):

Maximum mantle length 1020 mm; mantle wide, posterior end without pronounced pointed tail; fins rhomboidal, slightly attenuate posteriorly, fin length 40 to 50 % and width 60 to 85 % of mantle length, fin angle 46° to 65°; funnel groove with a foveola with 5 to 8, occasionally 9, longitudinal folds and 2 to 5 (mainly 3 or 4) distinct side pockets; small, scattered, subcutaneous photogenic tissue embedded in the tissue of the mantle, head and ventral arms, without large dorsal mantle photophore nor ocular or intestinal photophores; long wide silvery or golden opalescent strip along the ventral midline from the anterior edge of the fin to the mantle opening and similar ventral strip on the ventral surface of the head and ventral arms, relatively dense aggregations of small subcutaneous photogenic tissue under the opalescent tissue; tentacle suckers covering ~60 % of the tentacle length, dactylus of the tentacular club with 4 series of small suckers, carpal-locking apparatus on the tentacular stalk with 2 to 5 knobs and 2 to 4 smooth-ringed suckers; 4 to 7 suckers with denticulate rings occur on the carpus proximal to the first knob, largest medial suckers on the manus of the tentacular club have 4 large pointed teeth, 1 at each quadrant; arm tips not attenuate, 24 to 35 pairs of arm suckers; tips of the trabeculae of the protective membranes do not project beyond the edge of the membrane; ventral protective membranes of arms III are very wide and in adult females expanded into a large, triangular, membranous lobe; right or left ventral arm is hectocotylized with the tip lacking suckers; cone flags of the gladius are short, rhomboidal, with distinct radial creases, greatest width of the cone flags is about 56 % of the width of the rachis, marginal rigidity ribs of the rachis are doubled; axial rigidity rib of the rachis is wide rounded-rectangular in cross-section, lateral plates of the gladius not adhered to the dorsal surface of the rachis but form wide free folds over the rachis, stem of the rachis short, width of the stem is slightly greater than its thickness, cone short and laterally flattened, rostrum absent, thick alveola covered with tiny ribs and thorns; monoflagellate spermatozoon; hatchlings provided with skin sculpture, two rows of pegs in proboscis suckers, lateral proboscis suckers twice the length of the central ones and with unequal number of pegs and 3 leaflets in the gills, and devoid of ocular or visceral photophores.

***Ommastrephes bartramii* (Lesueur, 1821)** [nomem protectum]

Loligo bartramii Lesueur, 1821:90, pl 7.

Type material: Academy of Natural Sciences (ANSP). Not extant [fide Voss, 1962: 1; Lu et al., 1995: 312]

Type locality: Not designated in the original description. Here it is designated as the distribution area of *Ommastrephes* group 4 in North Pacific waters (Table 4.1, Fig. 4.4) (see *Remarks*).

Diagnosis:

Ommastrephes with a maximum mantle length of 600 mm and weight of 6 Kg; maximum spermatophore length of 41 mm, cement body of the spermatophore 11 %, sperm reservoir 44.7 % and posterior empty end 22 % of the spermatophore length; cytochrome *c* oxidase I diagnostic character: 454, C.

Name of the species in the phylogenetic analyses: *Ommastrephes* group 4.

Distribution: Temperate North Pacific, from the coasts of China (25ºN) to Russia (60ºN) by the west to probably from Alaska (55ºN) to the Gulf of California (20ºN) by the East. Molecularly confirmed distribution through almost its distribution range, excluding Northeastern Pacific waters (Fig. 4.4).

Remarks: *Ommastrephes bartramii* was described as *Loligo bartramii* by Lesueur (1821: 90-92, pl. VII) and later was transferred to the genus *Ommastrephes* by D'Orbigny (1834-1848). In his description, Lesueur did not provide any specific locality for the species and the type specimen no longer exist (Voss, 1962:1; Lu et al., 1995:312). The only reference to the origin of the material he examined is that they came "*from the collection of the academy, and that from the Philadelphia Museum*" (Lesueur, 1821:89). Without any further accurate reference, it is likely that the material came from the Philadelphia shores and adjacent waters (i.e., probably *Ommastrephes* group 1). However, Lesueur also participated in the Baudin Expedition (1800-1803, see Péron & Freycinet, 1816) from Le Havre (France) to Australia and he might have collected specimens during this cruise, covering the distribution area of *Ommastrephes* groups 1, 2 and 3. With the available knowledge, we cannot objectively exclude any other precedence of the specimen via a donation to the Philadelphia Museum from any other distant place. Thus, the geographic collection of *O. bartramii* type material is unresolved and uncertain.

Regarding the recent usage of the name, another problem arose: *Ommastrephes* squids are only commercially exploited in North Pacific waters. Therefore, the majority of the literature focused in this taxon is based in material collected from this area. The International Code of Zoological

Nomenclature (ICZN from now on) precludes the substitution of long-accepted name in its accustomed meaning in order to increase taxonomic stability (ICZN Article 32.2) (International Commission on Zoological Nomenclature, 1999). A Web of Science search revealed than in the last four years (period 2015-2018, search performed 8-2-2018), 49 different works referred to "*Ommastrephes bartramii*". Only two of them (Franjevic et al., 2015 and Tsiamis et al., 2015) referred to *Ommastrephes* group 1, while the remaining ones referred to *Ommastrephes* group 4. The conditions described in the ICZN Article 23.9.1.2 for the prevalence of the stability criterion over the Principle of Priority stablished that a name should be referred in a particular meaning in more than 25 works authored by more than 10 authors in the last 50 years. Although *O. bartramii* has been commonly used to name the remaining species in other parts of the distribution range of the genus (recent examples: Franjevic et al., 2015 and Tsiamis et al., 2015 for *Ommastrephes* group 1; Villanueva & Sánchez, 1993 for *Ommastrephes* group 2; and Guerra et al., 2010 for *Ommastrephes* group 3), these species are far less studied and consequently the name is less commonly applied for them. Thus, either considering the name *O. bartramii* invalid, or using it to designate any other species than *Ommastrephes* group 4, would generate further taxonomic instability and problems to track much of the current biological knowledge on the species and to interpret the most recent literature, instead of solving the taxonomy of the genus.

***Ommastrephes brevimanus* (Gould, 1852) comb. nov.**

Onychoteuthis brevimanus Gould, 1852:483, Fig. 596.

Type material: Not extant [fide Johnson (1964:32)]

Type locality: 120 miles west of Tutuila, Samoa Islands (Southwestern Pacific).

Synonyms:

Ommastrephes caroli stenodactyla Rancurel, 1976:81. [fide Dunning (1998:426)] Type: Museum National d'Histoire Naturelle (MNHN), Laboratoire Biologie Invertebres Marins et Malacologie, syntypes 1974, 1975, 1976, 1977 [fide Lu et al. (1995:325)]. Type Locality: Auameo, Ile des Pins, New Caledonia (Southwestern Pacific).

Diagnosis:

Ommastrephes with a maximum mantle length of 1020 mm and 35 kg of weight; maximum spermatophore length of 41 mm, cement body of the spermatophore 11 %, sperm reservoir 44.7

% and posterior empty end 22 % of the spermatophore length; cytochrome *c* oxidase I diagnostic characters: 36, G; 61, C; 450, G; 16S rRNA diagnostic character: 17, C.

Name of the species in the phylogenetic analyses: *Ommastrephes* group 3.

Distribution: Equatorial and Temperate Southwestern Pacific. Molecularly confirmed in the western part of its range (Fig. 4.4), probably it reaches Chilean waters in the east. The eastern distribution range of this species as defined here should be taken with caution. Although we tried to sequence the Chilean specimen collected by Guerra et al. (2010), it was impossible to sequence it and confirm if the distribution range of the species actually covers all the South Pacific.

***Ommastrephes caroli* (Furtado, 1887)** [nomem protectum]

Ommatostrephes caroli Furtado, 1887:5, pls. 1-2.

Type material: Kobenhavns Universitet, Zoologisk Museum (ZMUC), syntypes (suckers only) [fide Kristensen & Knudsen (1983:221)].

Type Locality: "Barre de Lisbonne", Portugal (North Atlantic Ocean).

Synonyms:

Loligo pironneauii Souleyet, 1852:20, pl. 2 Figs. 1-5 [fide Pfeffer (1912:466)] MNHN Syntype 2-4-402 (gladius only) [fide Lu et al. (1995:322)]. Type locality: 48ºN, 22ºW of Paris, France (North Atlantic Ocean). [nomem oblitum]

Lolimnites meridionalis Risso, 1854:41, pl. 19, Figs. 1-3 [fide Adam (1942:18)]. Locality: "regions profondes", (?) Nice, France (Northwestern Mediterranean Sea). [nomem oblitum]

(?) *Ommatostrephes bartrami sinuosus* Lonnberg, 1896:701. Zoologiska Museet, Uppsala Universitets (ZMUU), holotype 126 [fide Wallin (1991:66)]. Locality: Teneriffe, Canary Islands, Spain (North Atlantic). [junior synonym]

Diagnosis:

Ommastrephes with a maximum mantle length of 900 mm and weight of 25 kg; maximum spermatophore length of 53 mm, cement body of the spermatophore 9.8 %, sperm reservoir 33 % and posterior empty end 30.3 % of the spermatophore length; cytochrome *c* oxidase I diagnostic characters: 6, T; 48, G; 81, A; 153, G; 159, G; 177, C; 198, G; 207, A; 228, G; 258,

C; 273, G; 288, G; 306, C; 327, T; 334, G; 366, C; 429, G; 432, C; 456, A; 495, C; 534, G; 573, G; 597, G; 612, C.

Name of the species in the phylogenetic analyses: *Ommastrephes* group 1.

Distribution: North Atlantic, molecularly confirmed from the Bay of Biscay (43ºN) to Canary Islands (27ºN) and the Mediterranean Sea (Fig. 4.4); not molecularly tested in Northeastern Atlantic from the Bay of Biscay from Scandinavia (60ºN) and in Northwestern Atlantic from the Gulf of Mexico (24ºN) to Nova Scotia (45ºN).

Remarks: The name *O. caroli* has been marginally used to refer some North Atlantic *Ommastrephes* specimens during part of the XX Century (e.g., Rees, 1950; Lozano Soldevilla & Franquet Santaella, 1986). As far as we know, the remaining names that might be resurrected to name to *Ommastrephes* group 1 have not been further used before its publication. In accordance with the ICZN Article 23.9.1.1, junior synonyms should be applied when the senior synonym or homonym has not been used as a valid name after 1899. Thus, *O. caroli* is here designated as *nomem protectum* to name *Ommastrephes* group 1, while *L. pironneauii* and *L. meridionalis* are both considered senior synonyms [nomina oblita].

***Ommastrephes cylindraceus* D'Orbigny, 1835** *In* 1834-1847:54, pl. 3 figs 3-4 [plate as *Loligo cylindracea*].

Type material: MNHN Type; specimen not extant [fide Lu et al. (1995:314)].

Type locality: Austral Atlantic, 35ºS, 40ºW of Paris, slightly South of Buenos Aires parallel of latitude, Argentina (South Atlantic).

Synonyms:

Loligo cylindricus D'Orbigny, 1835 *In* 1834-1847:pl 3, figs 3-4.

Loligo vitreus Rang, 1837:71, pl. 96. MNHN Type; specimen not extant [fide Lu et al. (1995:327)]. Type locality: Equatorial coast of Africa (Equatorial Atlantic). [junior synonym]

Diagnosis:

Ommastrephes with a maximum mantle length of 900 mm and weight of 25 kg; maximum spermatophore length of 41 mm, cement body of the spermatophore 11 %, sperm reservoir 44.7 % and posterior empty end 22 % of the spermatophore length; cytochrome oxidase I diagnostic

characters: 30, G; 113, G; 180, C; 306, A; 493, T; 16S rRNA diagnostic characters: 123, C; 434, G.

Name of the species in the phylogenetic analyses: *Ommastrephes* group 2.

Distribution: Equatorial and South Atlantic (from 18ºN to ~50ºS) and Equatorial and South Indian (~10 to ~35ºS) waters. A great part of the distribution range is molecularly confirmed (Fig. 4.4), but the eastern part of Indian and southernmost part of Atlantic waters lack molecular confirmation at present. The absence of differences in substrate and inhibitor specific cholinesterase activities of optical ganglia between specimens sampled in South Atlantic and Southeastern Indian waters reported by Shevtsova et al. (1979) and Rozengart & Basova (2005) supports the conspecificity of all the *Ommastrephes* specimens of the distribution range depicted for *O. cylindraceus* in Figure 4.4. It is also noteworthy that Nesis (1979) and Dunning (1998) described a discontinuous distribution range of *Ommastrephes* spp. at the tip of South America and the southeastern tip of Australia and considered both populations reproductively isolated. The results provided here (Figs. 4.1, 4.3-4; Tables 4.2-5) support this point of view and the application of the biological species concept ensure the recognition of *O. cylindraceus* and *O. brevimanus* as different species.

Unavailable *Ommastrephes* names:

Loligo touchardii Souleyet, 1852:22, pl. 2 Figs. 6-13 [fide Pfeffer (1912:466)] MNHN Syntype 7-3-724 [fide Lu et al. (1995:326)]. Locality: Pacific Ocean [nomem dubium]

Remarks: The type locality refers to the Pacific Ocean, without a further more accurate location. Since two species occur in the Pacific Ocean isolated by the Pacific Equatorial currents, this name cannot be reliably applied to any of them.

Ommastrephes ayresii Gabb *In* Carpenter, 1864:613, 664 [nomen nudum]

Ommastrephes californica Heath, 1908:582 [nomen nudum]

Ommastrephes crassus Lafont, 1871:275, pl 16. No designed Type Locality [nomem dubium]

Ommastrephes ensifer Owen, 1881:144, pl 28. Systematic status undetermined. Type repository unresolved; Holotype [Royal College of Surgeon, London?] Type locality not designated [species inquirenda]

4.5.2. Phylogeography of *Ommastrephes* spp.

Zuev et al. (1975) outlined an evolutionary scenario in which sister group of *Ommastrephes* spp. was *Sthenoteuthis* spp. They also postulated that members of each genus of this putative clade diverged during the Pliocene (3.6-5.3 mya). Although molecular data still did not solve the relationships between *Dosidicus*, *Sthenoteuthis* and *Ommastrephes* (Lindgren et al., 2012; Strugnell et al., 2017; Pardo-Gandarillas et al., 2018), the minor genetic divergence between *Dosidicus* and *Ommastrephes* for both molecular markers studied here suggest a closest relationship between both genera. Thus, both genera are considered sibling groups in this work. Moreover, Pardo-Gandarillas et al. (2018:Fig. 2) found a clade formed by this two genera in their coalescent analysis. They estimated the divergence time between *Dosidicus* and *Ommastrephes* was 33 mya (High Posterior Density 95 % of 30-47 mya).

The closing of the Isthmus of Panama (~3 mya) is one of the most significant geological events in the near past regarding ocean circulation, since it created the physical separation of the Atlantic and Pacific Oceans, diverted the Equatorial currents of both oceans and caused the creation of the Gulf Stream, which subsequently affected the current pattern of Equatorial oceanic circulation in both oceans (O´Dea et al., 2016). Due to its distribution in both Atlantic and Pacific basins, it is likely that the closing of the Isthmus of Panama had an important effect in triggering the allopatric speciation and shaping the distribution range of each species of the genus *Ommastrephes*, as occurred in other marine organisms with a transisthmian distribution (e.g., Lessios & Cunningham, 1990; Knowlton et al., 1993; Lessios, 2008). The putative sibling group of the genus, *D. gigas*, is endemic

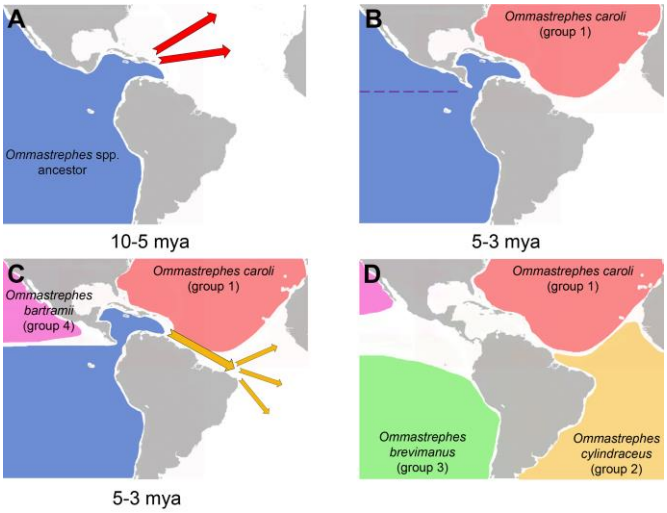


Fig. 4.5. Evolutionary scenario 1. (**A**) In the late Miocene the ancestor of *Ommastrephes* spp. distributed by Pacific Ocean and performed migrations to the Atlantic Ocean. (**B**) At least one of these migrations was successful and led to speciation of *Ommastrephes caroli* (group 1) in North Atlantic waters while the northernmost part of the distribution of the Pacific populations of *Ommastrephes* started its reproductive isolation. (**C**) The northernmost *Ommastrephes* population speciated to *Ommastrephes bartramii* and a second migration occurs to South Atlantic. (**D**) South Atlantic population suffered reproductive isolation from South Pacific populations due to the closing of the Isthmus of Panama (~3 mya). Two new species emerged: *O. brevimanus* in South Pacific (group 3) and *O. cylindraceus* in South Atlantic (group 2). The closing of the Isthmus of Panama, the Atlantic and Pacific populations got reproductively isolated and the rising of the Gulf Stream and the Pacific Equatorial Current produced the geographic current distribution limits of *Ommastrephes* species in both Atlantic and Pacific waters.

of the Humboldt Current system. Thus, a Pacific origin of the ancestor of both groups is likely. Here, two evolutionary scenarios are proposed to explain the current distribution pattern of each species. Figures 4.5-6 depicts both evolutionary scenarios. The color of the distribution areas is consistent with Fig. 4.4, but blue areas depict the hypothetical distribution area of the ancestor of a particular group. For instance, in Figs. 4.5B and 4.6B, the blue area depict the hypothetical distribution area of the clade (*O. bartramii*(*O. cylindraceus*(*O. brevimanus*))), while the red color depicts the distribution area of *O. caroli*.

The evolutionary scenario 1 dates the ancestor of *Ommastrephes* spp. in late Miocene (10-5 mya). Specimens of this putative Pacific ancestor might migrate to Atlantic waters (Fig. 4.5A) and at least one of them was successful, giving rise to *O. caroli* (group 1) in North Atlantic. Before this, the Northernmost part of the Pacific *Ommastrephes* spp. started its isolation process from the remaining populations, probably by vicariance or parapatric speciation (Fig. 4.5B). The isolated Northern Pacific population speciated to *O. bartamii* (Fig. 4.5C). A second migration occurred before the closing of the Isthmus and *Ommastrephes* occupied Equatorial and Southern Atlantic waters and migrated to the Southern Indian waters (Fig. 4.5C). Finally, the closing of the Isthmus permanently closed the reproductive continuity of Atlantic and Pacific *Ommastrephes* populations and produced the reproductive isolation between *O. brevimanus* (group 3) in Southern Pacific and *O. cylindraceus* (group 2) in Equatorial and Southern Atlantic and Indian Oceans (Fig. 4.5D). After the closing of the Isthmus the oceanographic pattern created by the Gulf Stream and the Equatorial currents isolated and delimited the distribution area of each Atlantic and Pacific group.

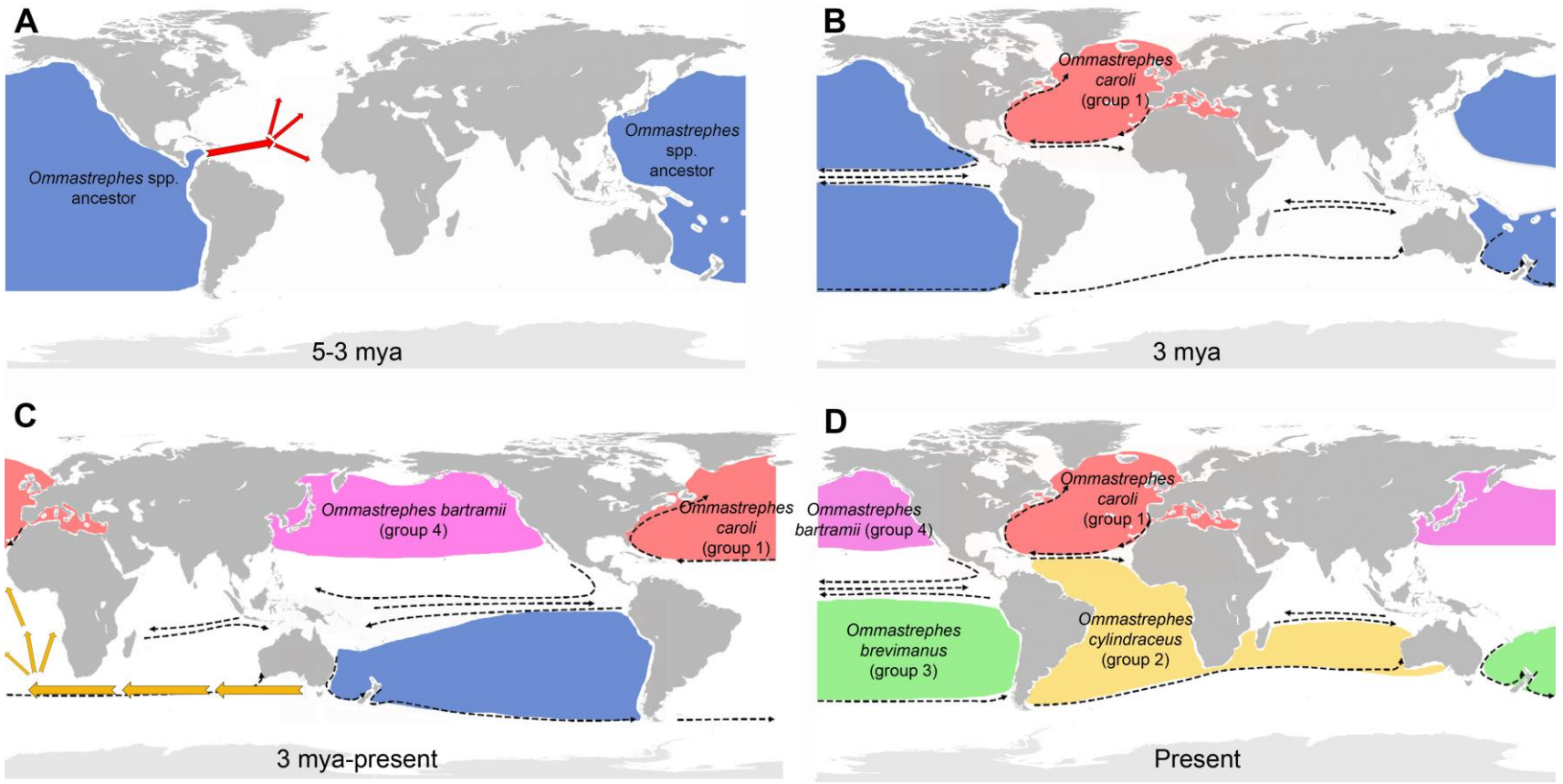


Fig 4.6. Evolutionary scenario 2. (**A**) Between the late Miocene and Early Pliocene the ancestor of *Ommastrephes* spp. distributed in Pacific waters and performed migrations to the Atlantic Ocean. (**B**) The closing of the Isthmus of Panama (~3 mya) reproductively isolated the ancestor of *Ommastrephes caroli* (group 1) from the remaining *Ommastrephes* populations. The oceanic current pattern resulting from the closing of the Isthmus of Panama (the Gulf Stream and the Atlantic and Pacific Equatorial Currents) isolated *Ommastrephes caroli* in North Atlantic and *Ommastrephes bartramii* in North Pacific waters (**C**), respectively. (**C**) Posteriorly, part of the Southwestern Pacific populations migrated into the Indian Ocean and spread across the South and Equatorial Atlantic waters. The East Australian Current and the South Indian currents limited the genetic exchange between Southern Hemisphere groups, producing the last speciation event between *Ommastrephes brevimanus* (group 3) and *Ommastrephes cylindraceus* (group 2). (**D**) The Indian and Atlantic Equatorial Current limited the spreading towards the North of *O. cylindraceus*, creating the current distribution pattern of *Ommastrephes* species.

The evolutionary scenario 2 places the Pacific ancestor of *Ommastrephes* spp. between late Miocene and Early Pliocene. Before the closing of the Isthmus of Panama, a population migrated to Atlantic waters (Fig. 4.6A). With the closing of the Isthmus, they suffered reproductive isolation from the remaining Pacific *Ommastrephes* specimens and the Gulf Stream confined its distribution to Northern Atlantic, giving rise to *O. caroli* (group 1) (Fig. 4.6B). Meanwhile, the resulting oceanic circulation pattern progressively created the reproductive isolation of northern *O. bartramii* (group 4) and Southern Pacific *Ommastrephes* individuals (Fig. 4.6B-C). Later, Southwestern Pacific *Ommastrephes* performed a migration to Southern Indian Ocean, and then to Southern Atlantic and northwards (Fig. 4.6C). This migration was restrained by the Canary Current and the Atlantic Equatorial Counter Current (Fig. 4.6D). The last event leaded to the reproductive isolation and speciation of Southern Hemisphere *Ommastrephes* populations and gave rise to *O. brevimanus* (group 3) and *O. cylindraceus* (group 2) and the current distribution pattern of the last two species.

4.6. Conclusion

The long-standing problem of *Ommastrephes* taxonomy (Jereb & Roper, 2010) is here reevaluated using two molecular markers and multiple molecular species-delimitation methods in combination with metabolic and morphological knowledge in an integrative taxonomic approach. The analyses revealed that genus *Ommastrephes* is not formed by a single cosmopolitan species and in fact under the taxonomic name *O. bartramii* had been hidden four different biological species with a more restricted geographic area. Furthermore, we went beyond the mere recognition of three cryptic species (*Ommastrephes* groups 2-4) and 1 pseudocryptic species (*Ommastrephes* group 1) and used all the available morphological, metabolic and molecular information in a integrative approach to resurrect three previously synonymized names (*O. brevimanus*, *O. caroli* and *O. cylindraceus*) and define the diagnostic characters as well as the expected distribution of each species. It must be underlined that some areas are currently not molecularly sampled and thus the distribution area of each species should be taken with caution until more detailed molecular information is available. We also used this information to hypothesize the oceanographic features that delimit the distribution of each species and the possible evolutionary history of each lineage. Although morphology has previously studied and only spermatophores showed important taxonomic differences for a single one of the recognized species (*Ommastrephes* group 1, defined here as *O. caroli*), molecular characters were especially important for the recognition of diagnostic differences where other methods failed. The single species of the genus commercially exploited at present is *O. bartramii* (group 4), but it is important to note that the real distribution range of the species is

far more reduced than previously considered in previous publications (e.g., Jereb & Roper, 2010; Guerra et al., 2010). Thus, this knowledge is important for the proper fishery management of the species. This earns even more importance if other commercial fisheries developed in the future over other *Ommastrephes* species. Although considerable efforts were carried out to obtain samples covering the maximum geographic representation of *Ommastrephes* specimens and by asking international collaboration in the 2015 Cephalopod International Advisory Council Conference (Hakodate, Japan) (Fernández-Álvarez et al., 2015b), not all the distribution area of each *Ommastrephes* species was covered. This is a direct consequence of the oceanic lifestyle of this genus coupled with the absence of directed fisheries in most of its distribution range, which makes in some cases its collection a fortuitous phenomenon. However, a great part of the distribution range is molecularly represented for *O. bartramii* and *O. cylindraceus*. The metabolic results of Shevtsova et al. (1979) and Rozengart & Basova (2005) suggest conspecifity of South Atlantic and Southeastern Indian specimens and it is here used as a complement to delimit all the distribution area of *O. cylindraceus*. This study increase the number of accepted species of the Family Ommastrephidae from 22 (Jereb & Roper, 2010) to 25, which represents a 13 % increase of the known biodiversity of the family.

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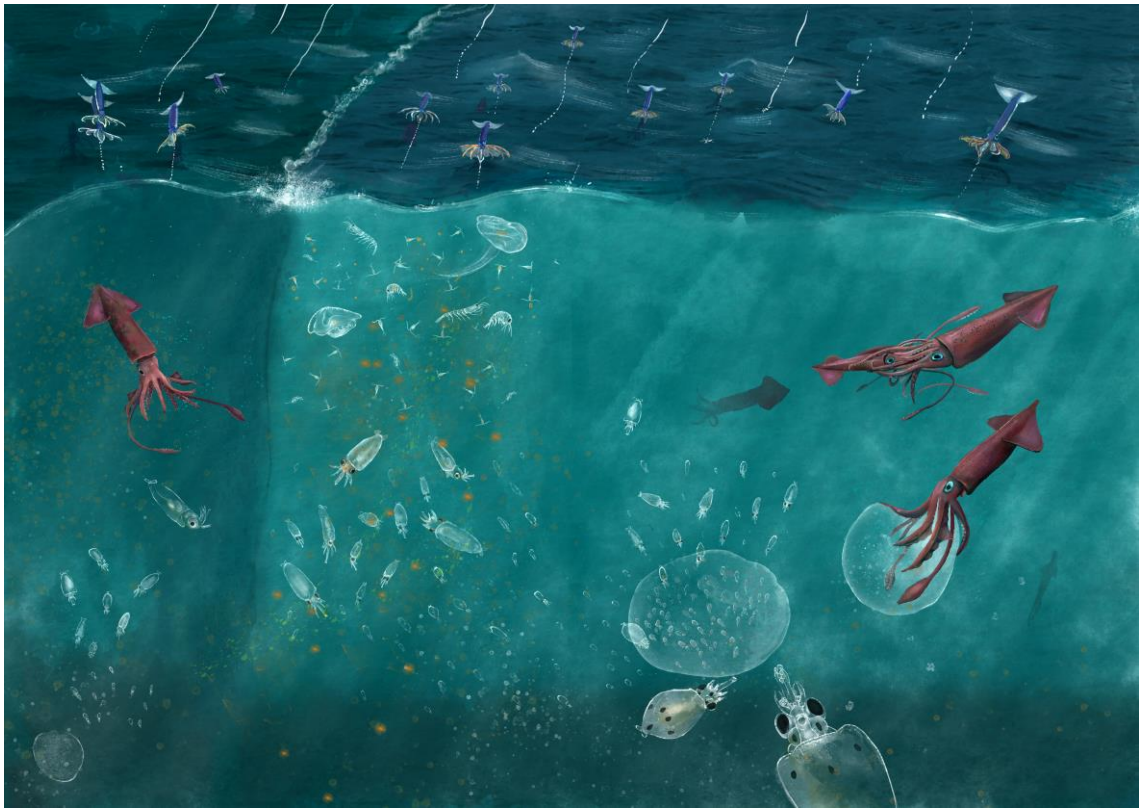
GENERAL DISCUSSION

and

CONCLUSIONS



Although their huge economic importance, the oceanic lifestyle of flying squids hinders the study of many fundamental aspects of their biology. In this Ph. D. Thesis, light was shed over several neglected aspects of the ontogeny and phylogeny of the Family Ommastrephidae. During this onto-phylogenetic journey through their life history, we gained knowledge in how sperm is transferred between sexes, the general morphology and the first feeding diet of the hatchlings once they abandon the egg mass and the actual biodiversity of a genus of cosmopolitan oceanic squids. These advances in scientific knowledge have potential applications for a better understanding of the ecology, physiology, biodiversity and fishery science that will foster a better understanding of this economically important family of cephalopods.

Chapter 1 provided an explanation for the long-standing mystery of the mechanism that drives the sperm transfer between the spermatangia and the seminal receptacles over the buccal area of ommastrephid squids as well as the first description of the jumbo squid (*Dosidicus gigas*) seminal receptacles. Our results suggest that spermatozoa are able to actively migrate from the spermatangia to the seminal receptacles in a targeted way. Several open questions remain, as which is the sperm-attracting substance that draws sperm motion in the right way, the time spermatozoa are able to survive their storage inside the seminal receptacles, and if females are able to bias the offspring to a preferred phenotype (cryptic female choice). These questions might have a great impact in how we understand sexual selection in oceanic squids.

Chapter 2 addressed the study of the morphology of three hatchlings of the Family Ommastrephidae. This knowledge was combined with the previous literature in the development of a new dichotomous key that allow the identification of the seven NE Atlantic species. The most useful characters were the relative size of the lateral and medial suckers of the proboscis, the presence/absence of photophores, and the arrangement of pegs on the proboscis suckers. Also, the embryonic stage for hatching was set as stages XXX for *Illex coindetii* and XXXII for the remaining studied ommastrephids. Also, important morphological characters that might impact the taxonomy of the family can be obtained from the hatchlings descriptions, as the absence of skin sculpture in the genus *Illex* (possible autoapomophy of the genus) and the larger size and higher degree of development of the arm crown of *Todaropsis eblanae* (possible two autoapomorphies of the species). The dichotomous key developed in this study was tested over wild-collected samples and constitute a powerful resource for identification of NE Atlantic wild-collected paralarvae from oceanographic cruises and, thus, it can be used to assess several fundamental life history traits of ommastrephid paralarvae (e.g., growth and ecology). Despite its taxonomic importance, the function of the proboscis remains as an unresolved mystery.

The first feeding diet of ommastrephid paralarvae was assessed in **Chapter 3** using Laser-Capture Microdissection and DNA metabarcoding. The gut content of wild paralarvae was composed by fungus, plants, animals with continental and marine origin, algae and prokaryotic and eukaryotic single-cell microorganisms. This taxonomic assemblage is in line with a detritivore suspension feeding, while posterior ontogenetic stages are voracious predators. This ontogenetic shift is a unique life strategy among cephalopods and probably allows ommastrephid squids to produce a massive number of undeveloped small hatchlings that take advantage of an almost ubiquitous food resource that is devoid of the energetic costs that hunting demands.

Chapter 4 ascertained the actual biodiversity among the genus *Ommastrephes*. Instead a single cosmopolitan species, four biological species with a smaller distribution range each were hidden under the name *Ommastrephes bartramii*. A literature review was done in order to resurrect three previously synonymized names to solve the complex taxonomy of the genus. Since morphology was unable to distinguish between three of the four species, molecular diagnostic characters were considered in the description of each species. The only morphological difference between *Ommastrephes caroli* and the remaining species was the spermatophore morphology, while the three other cryptic species (*Ommastrephes bartramii*, *Ommastrephes brevimanus* and *Ommastrephes cylindraceus*) were only molecularly defined. The distribution range of each species was tentatively drawn based in the available molecular data and putative oceanographic features that are acting as reproductive barriers. Moreover, two different evolutionary scenarios were proposed. It seems likely that the arising of the Isthmus of Panama together with the subsequent development of the Gulf Stream had a key role in the speciation of the genus. The current species assemblage of the genus should be considered to take decisions on the assessment and management of this fishing resource. As an open question remains the definition of the concrete boundary of each species, to answer this question new samples covering a wider distribution of the genus are necessary.

Conclusions

1. Squid spermatozoa are able to actively migrate in targeted way over the female skin from the spermatangia to the seminal receptacles of the female buccal membrane.

2. Morphological identification of ommastrephid paralarvae is reliable. The most useful taxonomic characters were: the relative size of the lateral and medial suckers of the proboscis, the presence/absence of photophores, and the arrangement of pegs on the proboscis suckers. A dichotomous identification key for NE Atlantic ommastrephid paralarvae was developed.

3. The absence of skin sculpture in the genus *Illex* likely represents an apomophy of the genus. The comparatively larger size and degree of development in the arm crown of *Todaropsis eblanae* likely represent apomorphies of the species. These characters potentially might impact the taxonomy of both genera.

4. The hatching stage is the embryonic stage XXX for *Illex coindetii* and XXXII for the remaining studied ommastrephids. This finding constitutes the baseline for future comparative morphological descriptions of ommastrephid hatchlings obtained in the lab.

5. Ommastrephid first feeding was composed of fungus, plants, algae and animals of marine and terrestrial origin, as well as eukaryotic and prokaryotic microorganisms commonly found in fecal pellets and particulate organic matter. This assemblage of gut contents is consistent with a diet based on detritus.

6. The ontogenetic shift of diet from detritivore suspension feeding to active predation is a unique life strategy feature among cephalopods and allows ommastrephid squids to take advantage of an almost ubiquitous and accessible food resource during their early stages, which may explain the ecological success of these squids in the oceanic realm.

7. Molecular data suggest that the genus *Ommastrephes* is formed by four species, instead of a cosmopolitan single species. *O. bartramii* distributes in North Pacific; *O. brevimanus*, in South Pacific; *O. caroli* in North Atlantic; and *O. cylindraceus*, in Equatorial and South Atlantic and South Indian waters.

8. The main oceanographic currents might represent the boundaries of the distribution of each species, probably affecting paralarval exchange between regions. The Isthmus of Panama might have had a key role as trigger of the speciation of the genus.

ANNEX 1

Published works of this Ph. D. Thesis

Attention¡
Pages 154 to 194 of the thesis,
containing the texts mentioned below, should be consulted on the web pages of the respective publishers

A1.1. Fernández-Álvarez FÁ, Villanueva R, Hoving HJT & Gilly WF. (2018) The journey of squid sperm. *Reviews in Fish Biology and* Fisheries, 28, 191-199. doi.org/10.1007/s11160-017-9498-6
https://link.springer.com/article/10.1007/s11160-017-9498-6

A1.2. Fernández-Álvarez FÁ, Martins CPP, Vidal EAG & Villanueva R. (2017) Towards the identification of the ommastrephid squid paralarvae (Mollusca: Cephalopoda): morphological description of three species and a key to the north-east Atlantic species. *Zoological Journal of the Linnean Society* , 180 , 268-287. doi.org/10.1111/zoj.12496
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A.1.3. Fernández-Álvarez FÁ, Machordom A, García-Jiménez R, Salinas- Zavala CA & Villanueva R. (2018) Predatory flying squids are detritivores during their early planktonic life. *Scientific Reports*,
doi: 10.1038/s41598-018-21501-y [in press].
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ANNEX 2
Supporting papers and posters

This section includes papers and a poster of the author, which are related with cephalopods but not included as chapters in the Ph. D. Thesis

Attention¡
Pages 194 to 266 of the thesis,
containing the texts mentioned below, should be consulted on the web pages of the respective publishers

A2.1. Fernández-Álvarez FÁ, Li DH, Portner E, Villanueva R & Gilly WF. (2017) Morphological description of egg masses and hatchlings of *Lolliguncula diomedeae* (Cephalopoda: Loliginidae). *Journal of Molluscan Studies*, **83,** 194-199. doi: doi.org/10.1093/mollus/eyx008
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A2.2. Tsiamis K, Aydogan Ö, Bailly N, Balistreri P, Bariche M, Carden-Noad S, Corsini-Foka M, Crocetta F, Davidov B, Dimitriadis C, Dragičević B, Drakulić M, Dulčić J, Escánez A, Fernández-Álvarez FÁ, Gerakaris V, Gerovasileiou V, Hoffman R, Izquierdo-Gómez D, Izquierdo-Muñoz A, Kondylatos G, Latsoudis P, Lipej L, Madiraca F, Mavrič B, Parasporo M, Sourbès L, Taşkin E, Tűrker A & Yapici S. (2015) New Mediterranean Biodiversity Records (July 2015). *Mediterranean Marine Science*, **16**, 472-488.
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A2.3. Villanueva R, Vidal EAG, Fernández-Álvarez FÁ & Nabhitabhata J. (2016). Early mode of life and hatchling size in cephalopod molluscs: influence on the species distributional ranges. *PLoS ONE*, **11**, e0165334.
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A2.4. Martins CPP, Fernández-Álvarez FÁ & Villanueva R. (2018) Invertebrate predation on egg masses of the European cuttlefish, *Sepia officinalis*: an experimental approach. *Estuarine, Coastal and Shelf Science*, **200**, 437-448. doi.org/10.1016/j.ecss.2017.11.016
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A2.5. Fernández-Álvarez FÁ, Sánchez P, Martins CPP, Cuesta-Torralvo E & Villanueva R. (2015) Biodiversity assessment of Mediterranean Sepiolidae by DNA barcoding. CIAC 2015, Book of Abstracts p. 235, Hakodate, Hokkaido, Japan, 11-2015 [poster]

ANNEX 3
Outreach contributions

Here, my outreach contributions regarding ommastrephid squids are compiled. The format has been adapted for this Ph. D. Thesis, but the links of the original sources are provided. This material is only available in Spanish.

Attention¡
Pages 267 to 288 of the thesis,
containing the texts mentioned below, should be consulted on the web pages of the respective publishers

A3.1. Fernández-Álvarez FÁ, Cuesta-Torralvo E, Roig L, Valls G, Martins CPP, Mirabel JV, Quintana D, Sánchez P, Vidal EAG & Villanueva R. (2015) Las primeras etapas de vida de los calamares oceánicos y su estudio a través de fecundación *in vitro*. Available at the outreach blog *La Biothèque* : http://www.labiotheque.org/2015/09/calamares-oceanicos.html [accessed on 6-2-2018] [in Spanish].

A3.2. Cuesta-Torralvo E & Fernández-Álvarez FÁ. (2014) *Todarodes sagittatus* (Lamarck, 1798). *Asturnatura*, **497**. Available at https://www.asturnatura.com/especie/todarodes-sagittatus.html [accessed on 6-2-2018] [in Spanish].

A3.3. Fernández-Álvarez FÁ. (2016) *Sthenoteuthis pteropus* (Steenstrup, 1855). *Asturnatura*, **602**. Available at https://www.asturnatura.com/especie/sthenoteuthis-pteropus.html [accessed on 6-2-2018] [in Spanish].

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