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Stock structure, estimation of the biomass, spawning and nursery areas of the anchovy in the Adriatic Sea

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Abstract

Small pelagic fish are relevant in marine ecosystem, they are able to exert both bottom-up and top-down controls by modulating the abundances of carnivorous fish, seabirds and marine mammals and by foraging on plankton species and therefore limiting primary production rates; through a "wasp-waist" control they manage the abundance of both prey and predator. Stocks are subjected over time and space at biomass fluctuations and, despite constant progress in understanding the processes involved in the variability of pelagic stock biomass, our ability to predict abundance and catches is limited, which in turn, affects our capacity to properly manage the fisheries and ensure sustainable exploitation. The Adriatic Sea, within Mediterranean area, is one of the richest in terms of small pelagic fish, European anchovy (*Engraulis encrasicolus*, Linnaeus, 1758) together with European sardine (*Sardina pilchardus*, Walbaum, 1792) is the main target of the Adriatic fishery. Fishery activities, together with environmental changes, are currently producing a lot of alterations at ecosystem, species and population-level. When enough data on changes of spawning stock biomass, on environmental factors affecting the choice of spawning and nursery areas and on population genetic structure are available, plans for the management of marine stocks can represent effective way to reduce the risks of extreme exploitation. The overall aim of the study was the achievement of knowledge about abundance and spatial distribution of *E. encrasicolus*, as well as the development of information related on the current genetic population structure and on how the genetic diversity has changed between generation (parents and offspring).

The first chapter provide useful information with regard to the spawning stock biomass of anchovy, about fluctuations of biomass and on the potential application of a direct method as Daily Egg production Method (DEPM) in order to integrate the current stock assessment process. Although the method cannot yet be integrated into the stock assessment process by reason of the high uncertainty, however, the application provides valuable information

on the extension and characteristics of spawning habitats and on reproductive parameters of fish stocks.

In the second chapter, the attention was focused on the identification of the spawning and nursery areas updating their boundaries in the south-western Adriatic Sea and on description of environmental and biological factors affecting the choice of spawning areas. Data analysis indicated south-western Adriatic as a favorable area of spawning and nursery, driven by depth, zooplankton abundance and water column stability.

In the last chapter, microsatellite DNA markers were used to resolve the fine-scale genetic structure of anchovy and the potential loss of genetic variability through parents and offspring. The genetic structure of anchovy in two stocks seems to be reconfirmed also in 2015 and moreover, were provided estimates on important parameters in conservation biology as effective population size (N_e) and effective number of breeders (N_b) that can be used to formulate recommendations for the conservation of wild populations.

Sommario

I piccoli pelagici sono costituenti fondamentali dell'ecosistema marino poiché, attraverso un controllo definito a "vita di vespa" (wasp-waist), gestiscono l'abbondanza di prede e di predatori riuscendo così a modulare sia l'abbondanza di pesci, uccelli e mammiferi marini (controllo bottom-up) sia i tassi di produzione primaria (controllo top-down). Gli stock dei piccoli pelagici sono soggetti ad importanti fluttuazioni di biomassa nel tempo e nello spazio, ma, nonostante i costanti progressi nella comprensione dei processi coinvolti in queste fluttuazioni, la nostra capacità di predire l'evolversi dei tassi di abbondanza e di cattura è limitata, il che, a sua volta, influenza la nostra capacità di gestire correttamente le attività di pesca e garantire uno sfruttamento sostenibile delle risorse. Il mare Adriatico è uno dei più ricchi e produttivi dell'intera area mediterranea, l'Acciuga europea (*Engraulis encrasicolus*, Linnaeus, 1758), insieme alla Sardina europea (*Sardina pilchardus*, Walbaum, 1792), è il principale target della pesca adriatica. Le attività di pesca, insieme ai cambiamenti ambientali, stanno attualmente producendo molte alterazioni a livello ecosistemico, di specie e di popolazione. I piani di gestione delle risorse marine possono acquisire maggior efficacia nel ridurre i rischi si sovrasfruttamento grazie all'acquisizione di dati sulle variazioni di biomassa, alla comprensione dei diversi fattori ambientali che influiscono sulla scelta delle aree di deposizione e di nursery e sulla struttura genetica della popolazione. L'obiettivo generale dello studio era il raggiungimento delle conoscenze sull'abbondanza e sulla distribuzione spaziale di *E. encrasicolus* nell'Adriatico sud-occidentale nonché lo sviluppo di informazioni relative all'attuale struttura di popolazione genetica e su come la diversità genetica varia da una generazione all'altra (genitori-prole).

Il primo capitolo fornisce indicazioni sulla possibile applicazione di un metodo diretto di stima della biomassa deponente dell'acciuga (DEPM - Produzione Giornaliera di Uova) al fine di integrare l'attuale processo di valutazione della risorsa, nonché, informazioni utili riguardo alla biomassa deponente dello stock di acciuga e alle sue fluttuazioni.

L'applicazione di questo metodo fornisce preziose informazioni sull'estensione e sulle caratteristiche degli habitat di riproduzione e sui parametri riproduttivi degli stock ittici, ma tuttavia, a causa dell'elevata incertezza di alcuni parametri, non può ancora essere integrato nel processo di valutazione degli stock.

Nel secondo capitolo, l'attenzione è stata focalizzata sull'identificazione delle aree di riproduzione e di nursery aggiornando i loro confini e indicando l'Adriatico sud-occidentale come area favorevole sia per la deposizione delle uova che come nursery per le larve. Sono inoltre stati valutati i principali fattori ambientali e biologici che influenzano la scelta delle aree per la deposizione delle uova evidenziando la profondità, l'abbondanza di zooplancton e la stabilità della colonna d'acqua come i principali fattori che la guidano.

Nell'ultimo capitolo, tramite il DNA microsatellite sono state investigate la struttura genetica della popolazione Adriatica di acciughe e la potenziale perdita di variabilità genetica tra generazioni. La struttura genetica delle acciughe in due stock sembra essere riconfermata anche nel 2015 e, in più, vengono fornite stime su importanti parametri quali la taglia effettiva di popolazione (N_e) e il numero effettivo di riproduttori (N_b) che possono essere utilizzate per formulare raccomandazioni per la conservazione delle popolazioni naturali.

Introduction

Small pelagic fish

Pelagic fish are abundant in all oceans and seas except the Antarctic and are distributed mostly in marine and rarely in freshwater environments (Culley & Kerkut, 1971); anchovies, sardines, pilchards, sprats, shads, wolf-herrings, herrings and mackerels all belong to the Order Clupeiformes, a group of Teleosts encompassing 5 world-wide distributed families, more than 83 genera and over 402 species (Nelson, 1994). There are many definitions of "small pelagic fish", this expression is most commonly refers to gregarious and epipelagic fishes characterized by high horizontal and vertical mobility in coastal areas. They live in the pelagic domain moving in the water-column where they spend most of their time, usually at depths between 0 and 200 m, although the limit of the deeper boundary varies according to species and regions (Freon et al., 2005).

Small pelagic fish are usually slender, streamlined and with a rather non specialized body form, always presenting cycloid scales and a homocercal caudal fin, these characteristics make them good and fast swimmers. Another typical phenotypic trait of coastal pelagic fish is their coloration, ranging from dark gray to silver; the flanks are highly reflective and the dorsal surface is dark in order to render the fish unseen to predators (Blaxter & Hunter, 1982).

Small pelagic fish grow relatively fast, in anchovy-like species sexual maturity is attained at the end of the first year and the average life span typically ranges from 2 to 5 years, whereas, in sardine-like species the life span is 5–8 years, although the average life span can be reduced in stocks with high exploitation rate. Most pelagic fish species are pelagic spawners and fertilization is external, the fecundity is high, varying in duration, from a few weeks to several months in temperate areas, while in sub-tropical and tropical areas spawning occur through all year. Fertilized eggs become embryos after a few days and

hatched larvae have a typical pelagic phase, the yolk sac phase is short and the larvae have to feed quickly to avoid death by starvation (Freon et al., 2005).

Small pelagic fish prey on phytoplankton and/or micro- and meso-zooplankton, several laboratory studies have shown that fish feeding on small particles have 2 different feeding behaviors: filter-feeding, essentially non-selective, and particulate-feeding, which allows the selection of food on a dimensional basis (van der Lingen, 1994; van der Lingen et al., 2006; 2009). Feeding mode essentially depends on the dimensions and density of prey; generally, species showing feeding plasticity filter when high concentrations of small food particles (e.g., phytoplankton or zooplankton of small dimensions) are present and use oriented suction on larger prey. The ability to switch between these feeding modes makes them highly opportunistic and flexible foragers which are able to maximize their energy intake through employing the feeding mode most appropriate to a particular food environment. Because of the resulting ecosystem structuring and their large biomass, small pelagic species have been termed "wasp-waist" (Cury et al., 2000; Bakun, 2006), they are able to exert both bottom-up and top-down controls by modulating the abundances of carnivorous fish, seabirds and marine mammals and by foraging on plankton species and therefore limiting primary production rates.

Small pelagics are globally important not only for their ecological relevance but either socio-economically (Pikitch et al., 2012; 2014); are valuable sources for human consumption throughout the world and several of these species are exclusively harvested from wild populations (Ryman et al., 1995; Christensen, 2014). Economic-wise, despite representing only 4% of all currently-described fish species (Cury et al., 2000), small pelagics on their own compose the greater part of the world's fishery total catch; according to the most recent FAO estimates (FAO, 2016), they represents the 37% of global wild catches accounting approximately to 78.9 million tons and the trade-related value is on the order of several billion US dollars. Small pelagic fish provide a substantial source of

income for many developing countries, in some of these regions, coastal pelagic fish contribute substantially to food security, either directly (human consumption of fresh, smoked, or frozen fish) or indirectly (earnings for part of society).

Seasonal migrations are often observed, the migration distance can vary from a few km to more than 1000 km, and the distance and timing, may vary according to fish age. Migrations are evolutionary adaptations in response to seasonal changes in food abundance or to the need for spawning in a given area where hydrological conditions are favorable for survival. The whole distribution area of a pelagic fish stock can vary from year to year, not only with changes in productivity but also with species abundance. When their abundance decreases, some small pelagic fish species, like sardine or herring, might decrease their area of distribution consequently, in accordance with the "habitat-based hypothesis" (Bertrand et al., 2004). Nevertheless these species may continue to form schools of the same magnitude although there will be fewer of such schools, as a result, the catch rates do not decrease dramatically as soon as the stock declines, until the reduced abundance of schools begins to impact fishing success or the stock even collapses (Freon et al., 2005 and references therein). In fact, small pelagics tend to form monospecific and large schools that are highly adaptive structures. Their sizes, shapes, and dynamics are related to predator avoidance, feeding, migration, energy conservation, and reproduction (Blaxter & Hunter, 1982), as well as to abundance (Freon & Misund, 1999). When sardine, sardinella, or anchovy are abundant tend to form pure schools, whereas when their relative abundance is diminishing they mix with other species. Fish of a species, that are driven to join schools of another more abundant species, must effectively subordinate their specific needs and preferences to the ''corporate volition'' of the school that is largely driven by a different set of needs and preferences; this introduces a mechanism for population instability, the so-called "school trap": a less-abundant species tends to be adversely affected becoming even less abundant, conversely a dominant species tends to become even more dominant (Bakun

& Cury, 1999). Fisheries can generate directional and differential harvest modifying total abundance, but can also alter school dynamics, which in turn affects migration behavior and geographical distribution (Cury et al., 2000). Many examples of small pelagic fishery collapses occurred during the second half of the 20th century and, in most cases, these collapses were clearly associated with an abrupt decline in the corresponding fish stock biomass with delayed recovery, if any (Troadec et al., 1980; Beverton, 1990; Hutchings, 2000).

A short life cycle combined with a fast growth during the first life stages are the main characteristics affecting recruitment success, which was recognized being a crucial step in population dynamics, and in turn strongly depends on environmental conditions (Lasker, 1978; Barange et al., 2009). Biomass fluctuations, to which stocks are subjected over time and space, are one of the most studied issued, whether these biomass variations were mainly driven by natural variability, exploitation or both, has been, and is still, debated at length (Lluch-Belda et al., 1989; Schwartzlose et al., 1999; Lehodey et al., 2006). Physical, biological and behavioral mechanisms as well as interspecific interactions were integrated by MacCall (2009). He described them to be equally important in explaining natural oscillatory patterns: overfishing was among the causes of several collapses; low food availability severely influences larval mortality when larvae switch from endogenous to exogenous feeding; temperature impacts the reproductive cycle as batch fecundity, spawning, hatching time and egg mortality rates (Motos et al., 1996; García Lafuente et al., 2002). Moreover, oxygen concentration, salinity and water turbulence might directly affect reproduction, recruitment, development, physiology, growth and behavior, with a particular emphasis on larval stages (Rice et al., 1987; Fuiman, 1993; Ettahiri et al., 2003; Rätz & Lloret, 2005; Zarrad et al., 2008); finally climate change could indirectly influence small pelagic fish by exerting effects on their preys as van der Lingen, et al., (2009) suggested.

Despite constant progress in understanding the complex processes involved in the variability of pelagic stock abundance, especially at short and medium time scales, our ability to predict abundance and catches is limited, which in turn, limits our capacity to properly manage the fisheries and ensure sustainable exploitation.

Engraulis encrasicolus

Anchovies are included in the Engraulidae family that contains small or moderate-sized fishes usually reaching 10-20 cm in standard length (Whitehead, 1985); characterized by a pointed snout extended beyond the lower jaw, several gill rakers in the lower part of the first branchial arch and a short anal fin posterior to the dorsal fin (Tortonese, 1970).

The Engraulis genus encompass several important species, widespread in all world seas:

- Argentinian anchovy (*Engraulis anchoita*, Hubbs & Marini, 1935), south-west Atlantic Ocean;

- Australian anchovy (*Engraulis australis*, White, 1790), south-east Indian Ocean;

- South-African anchovy (*Engraulis capensis*, Gilchrist, 1913, then named *E. encrasicolus* by Fage in 1920), south-east Atlantic Ocean and south-west Indian Ocean;

- Japanese anchovy (*Engraulis japonicus*, Temminck & Schkegel, 1846), north-west Pacific Ocean;

- Californian anchovy (*Engraulis mordax*, Girard, 1854), north-east Pacific Ocean;

- Peruvian anchovy (*Engraulis ringens*, Jenyns, 1842), south-west Pacific Ocean;

The only Mediterranean representative of the Engraulidae family is the European anchovy (*Engraulis encrasicolus*, Linnaeus, 1758). This species is widely distributed along the eastern part of the Atlantic Ocean, from Norway to South Africa, within the Mediterranean Sea, the Black Sea, the Azov Sea and also in the Gulf of Suez and Suez canal (Whitehead et al., 1988). As an euryhaline species, it tolerates a wide range of salinities (5-41 psu),

often it is found in estuaries and lagoons for feeding and spawning reasons (Palomera et al., 2007; Morello & Arneri, 2009; Zorica et al., 2013). Characterized by large head with big eyes and a prominent maxilla, an elongated and tapered body, a maximum length of 20 cm, with a back colored in blue-green and a silver belly (Fig. 1).

Behavior and migrations Other peculiar features of this species are: short life span (about 6 years), a long spawning period (from April to November) with a peak of spawning in June – July (Regner, 1996) and the tendency to form large monospecific groups, known as "schooling behavior", with the capacity to accomplish diel vertical migrations. In fact, feeding activity of *E. encrasicolus* is mainly diurnal, at daytime anchovies school and swim toward high depths to avoid predation and for feeding reasons (Pitcher & Parrish, 1986); instead, during nighttime and especially in the reproductive season, adult individuals swim above the thermocline in order to spawn their gametes in a more stable environment (Palomera, 1991). *E. encrasicolus* had a zooplanktivorous diet based essentially on copepods and, to a lesser extent, by cladocerans, bivalves, ostracods and decapod larvae, instead phytoplankton is not abundantly present in the diet of European anchovy (Borme et al., 2009). Anchovy, as other small pelagics, can switch from filter-feeding to particulate-feeding; probably filter of planktonic organisms occur only when they encounter an adequate patch of prey, in other words, the fish filters solely when it is energetically convenient to switch from biting on single prey to indiscriminate filtering. Anchovy adults accomplish migration from the deeper overwintering waters to shallower coastal areas for spawning (Sinovčić, 2000), once spawning is completed and temperatures fall, adults return offshore, whilst juveniles generally remain closer to the coast until January or until the following year when first maturity is attained (Sinovčić, 2000; Marano, 2001). Offshore winter migration of the younger portion of the population is therefore only partial and depends on the area considered. Thus, only the larger individuals (releasing larger eggs) are found offshore in spring, when spawning commences, whereas, at this

time, the individuals found inshore are young at their first spawning and release smaller eggs (Regner, 1972).

Figure 1. *Engraulis encrasicolus*

Reproduction and fecundity Anchovy is an indeterminate batch spawner, eggs are continuously recruited for development from a heterogeneous oocyte population and a continuous availability of vitellogenic and mature eggs exists throughout the reproductive season (Scott, 1987). Sexual maturity is reached within or at the end of the first year of life, at a body length that varies between 7.5 and 11 cm (Millán, 1999; Kolitari, 2006). Synchrony between the reproductive cycle and water temperature is likely a strategy that has evolved for allowing eggs to be spawned when stability of water masses is higher, in addition, it seems to be related to biological parameters such as plankton production cycle, increasing availability of food sources that can ensure the survival of anchovy larvae (Basilone et al., 2006). Species fecundity is elevated and, because of its reproductive strategy, not a priori defined; at each reproductive event females are able to produce about 40.000 oocytes. Population fecundity estimates are usually based on the composition of the spawning stock fraction and is correlated to sexual maturity, sex ratio, potential egg production and environmental factors such as nutritional conditions and food availability (Hay et al., 1988; Ma et al., 1998), that have been recognized as crucial variables affecting potential fecundity (Morgan & Brattey, 2005) and egg quality (Brooks et al., 1997). Eggs

are 1.1-1.3 mm-long and elliptic shaped, are released and fertilized in proximity of the water surface from dusk (about 10 pm in Adriatic Sea), are pelagic and get transported by water currents (Regner, 1985). Hatching occurs after 24 to 65 hours, depending on water temperature. Once hatched, the pre-flexion stage is known as "yolk-sac I" (YSI), is a transparent larvae, 2 mm long, presenting the reminiscence of the embryonic developmental structures with posteriorly elongated yolk, unpigmented eyes and without mouth opening . "Yolk-sac II" (YSII) presents a lengthening of the body, a reduction of the yolk and traces of pigment in the eyes. Mouth opening and eye pigmentation occur at 3.50 mm but yolk sac resorption is slightly later, at 3.54 mm (Regner, 1985); when the absorption of the yolk is completed and notochord begins flexioning larval stages begin (D'Ancona, 1956; Somarakis et al., 2002).

Ecological and economic importance The importance of the ecological role of *E. encrasicolus* is amplified in ecosystems dominated by the pelagic compartment, as the Adriatic Sea; *E. encrasicolus*, in fact, has an important ecological role within the marine food web function as forage fish in marine systems, representing an important food resource for numerous top predators such as large pelagic fish, demersal fish, marine birds and mammals (Coll et al., 2007). Although there is some limited evidence for top-down control of forage fish by predator populations, overall many observations suggest bottom-up control of predator populations by forage fish and, consequently, impacts of fishing on the abundance of prey resources can be expected to affect top predators (Cury et al., 2000 and references therein). Top-down control of zooplankton populations by pelagic fish has been described in marine and freshwater ecosystems (Cury et al., 2000 and references therein); anchovies, feeding on zooplankton, transfer energy from the lower trophic levels to the higher ones. Through a "wasp-waist" control (Rice, 1995), intermediate small pelagic fish component exerts top-down control on zooplankton and bottom-up control on

top predators, managing the abundance of both prey and predators (Cury et al., 2000; Coll et al., 2007).

Anchovy is the main target of the Adriatic fishery, in fact, together with European sardine (*Sardina pilchardus*, Walbaum, 1792) it constitutes more than 40% of Mediterranean landings (FAO FishStatJ, 2015) (Fig. 2A). The Italian catches of anchovy represent the majority of the catches, while the Croatian small pelagic fishery concentrate mainly on sardine. Anchovy is fished by purse seiners and pelagic trawlers belonging to Italy, Croatia and, to a much smaller extent, Slovenia, Albania and Montenegro. The fishery takes place all year round; a closure period is observed from the Italian pelagic trawlers in summer and from Croatian purse seiners in winter. The Italian fleet is composed of about 65 pairs of mid-water trawlers and about 40 purse seiners, with the former being predominant on the latter ones (GFCM, 2016). The pelagic fleet is distributed mainly from Trieste in the north to Bari in the south, with most catches coming from the northern and central Adriatic between Trieste and Vieste (Cingolani et al., 1996). Croatia has about 200 active purse seiners targeting small pelagics (mainly sardine) while in Slovenia only 4 purse seiners are currently active. In Montenegro and Albania most of the catches are originated from small-scale fisheries (beach seine fisheries and small purse seiners) (GFCM, 2016). The Croatian fleet is distributed between Umag in the north and Dubrovnik in the south, and the main fishing grounds are between Istria and the mid-Dalmatian islands (Tičina et al., 2000). Important changes in landings (Fig. 2A) and estimated biomasses (Fig. 2B) have been registered within the Adriatic Sea. A pronounced increase between 1970 and 1974 with catches amounted to 42.912 tons was followed by a marked decrease that reached a relative minimum in 1977 and then rose sharply between 1978 and 1979 when an absolute maximum in fisheries production was obtained with 62.492 tons. After that, anchovy catches collapsed, reaching their historical minimum of 7.055 tons in 1987. This minimum was followed by 6 years of stable low catches whose recovery started in 1996. In 2005

overall anchovy catch was reported at 49.301 tons and then showed a descending trend (Cingolani et al., 1996; Azzali et al., 2002; Santojanni et al., 2003, 2005). These demographic fluctuations seem affected by fishing activities, but besides fishing, other factors could adversely affect anchovy biomasses in Adriatic Sea as physical, biological and behavioral mechanisms as well as interspecific interactions (MacCall, 2009).
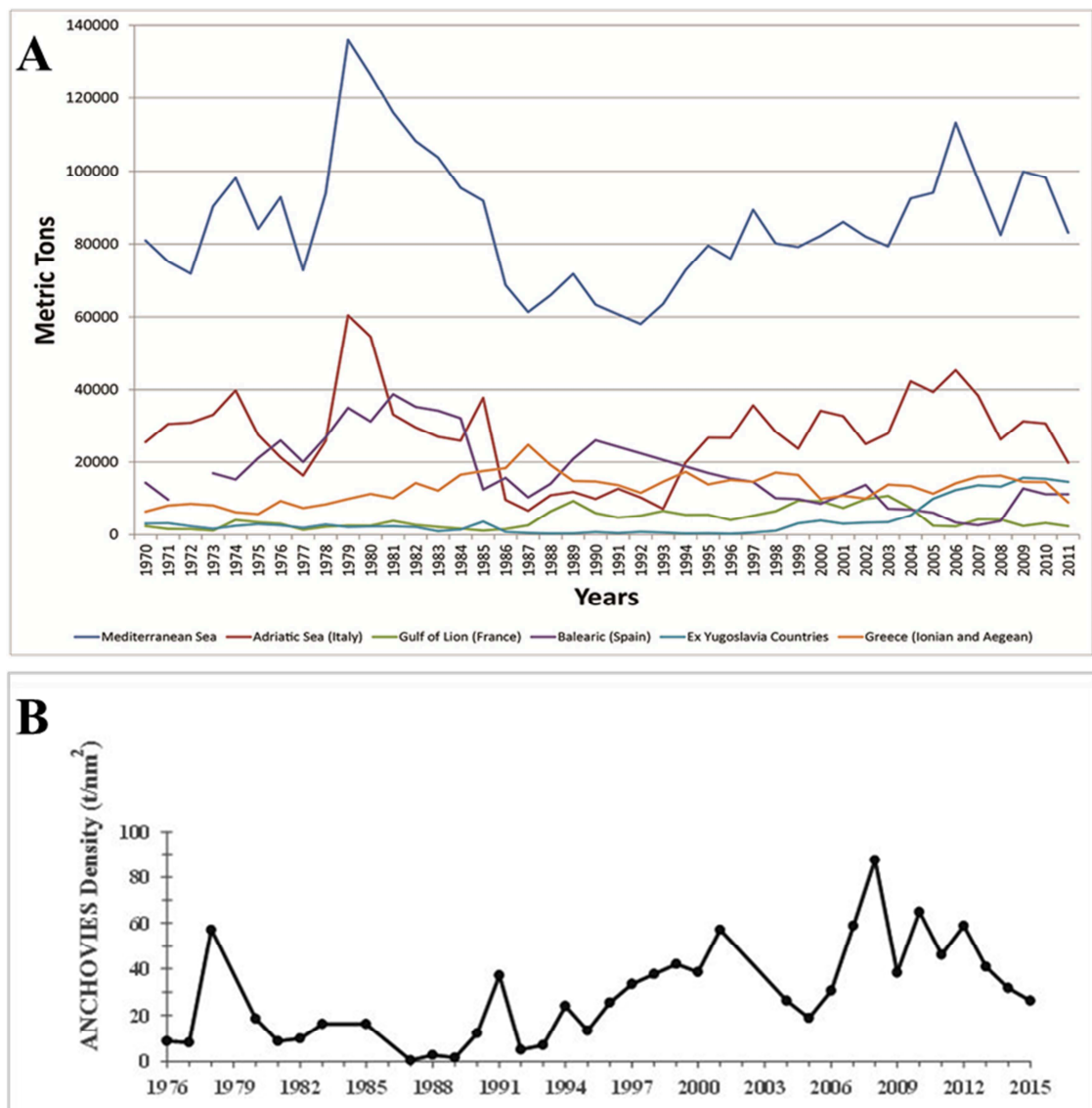

Figure 2. A) Landing fluctuations through the years (FAO, FishStatJ, 2015)
B) Acoustic biomass estimates from MEDIAS acoustic survey (Leonori et al., 2016)

Genetic variability Although it was historically retained that pelagic species should be less structured than benthic related species, water column is, therefore, a very dynamic and complex environment consisting of layers with different biotic and abiotic features that can contribute to genetic structures in several organisms (Riginos & Liggins, 2013). Compared to many other Engraulidae, the European anchovy shows genetic structure revealed by Magoulas et al., (1996, 2006) on the basis of the whole mitochondrial DNA restriction fragment length polymorphism (RFLP) study, in which were discovered 88 mitochondrial haplotypes belonging to two main clades (Clade A and Clade B). This genetic differentiation could be attributable to the interaction of several factors mainly related to cyclical extinctions and recolonization in some areas during the Pleistocene glaciations (Magoulas et al., 1996, 2006; Grant, 2005). The Clade A is the exclusive lineage occurs within the Black Sea, but was observed in high frequencies in the Aegean Sea and in the coastal areas from the Atlantic Ocean. The Clade B was identified at high frequencies in the Adriatic and Ionian Seas, whereas a mixed sharing of both clades were found in the western Mediterranean basin and in the Bay of Biscay (Magoulas et al., 2006). A stock structure was found within the Adriatic Sea on the basis of allozymes electrophoresis by Carvalho et al., (1994) and by Bembo et al., (1996a, b). They found remarkable differences in allelic frequencies, especially within the locus isocitrate dehydrogenase (IDHP-2). Nevertheless, the effective existence of two anchovy stocks within the Adriatic Sea was not additionally confirmed by the RFLP analysis on the mitochondrial NADH-dehydrogenase complex carried out by Carvalho et al., (1994); these analysis, however, supported the existence of genetic differences between the Adriatic-Ionian anchovies and the Aegean ones (Carvalho et al., 1994). A further taxonomic debate concerned the systematics of two distinct phenotypes in the European anchovy, called "silver" and "blue" anchovies (Borsa, 2002; Borsa et al., 2004). Borsa et al., (2004) detected enough differentiation between morphological and morphometric traits of these two anchovy

phenotypes; these authors suggested, on the basis of morphological and genetic variation, that the "silver" anchovy belong to the newly established *Engraulis albidus* species, whereas the "blue" anchovy represents the *E. encrasicolus* species (Borsa et al., 2004). The "silver" anchovy (*Engraulis albidus*) is distributed mainly in shallow and coastal areas with high freshwater input and it was suggested that it could represents a new anchovy species adapted to brackish waters systems; conversely, the "blue" anchovy (*E. encrasicolus*) would represents the open water adapted species (Borsa et al., 2004). The occurrence of *E. albidus* was hypothetically suggested also in the northern Adriatic Sea, where a lagoonal system (the Venetian Lagoon) and high river inputs are available and morphological distinction between color morphs ("blue" and "silver") are associated with mean depth throughout the Adriatic basin. However, a clear distinction of these two anchovy species within the Adriatic Sea was not possible on the basis of nuclear markers (Bouchenak-Khelladi et al., 2008); in addition, recent investigations that availed of nuclear microsatellite loci, pointed out a lack of genetic differences among anchovies caught from coastal and open waters throughout the Adriatic basin, suggesting that Adriatic anchovies belong to a unique species: *E. encrasicolus* (Ruggeri et al., 2016). At the same manner, a lack of statistically significant microsatellite variation was detected among central and southern Adriatic samples and those collected from the Tyrrhenian Sea by Ruggeri et al., 2016. Nevertheless microsatellite DNA and analyses of a fragment of mitochondrial DNA encompassing partial sequences of Cytochrome b and D-Loop revealed a genetic structure in anchovies, especially within the northern Adriatic Sea where north-eastern anchovy samples segregated from those analysed throughout the Adriatic basin (Viñas et al., 2014; Ruggeri et al., 2016).

As mentioned above, fisheries activities together with environmental changes, are currently producing a lot of ecosystem, species and population-level alterations. Most of the effects experienced by marine organisms come from a lack or poor knowledge about the

boundaries of their population or stocks. Effectively, when enough data on population/stock structure is available, plans for the management of marine stocks can represent effective way to reduce the risks of extreme exploitation over the threshold that allows the maintenance of enough evolutionary potential for local population to overcome the pressures derived also from environmental changing (Birkeland & Dayton, 2005; Burgess et al., 2013).

Stock definition and genetic markers

Stock definition Despite "*stock*" is one of the most used terms in fisheries management there is not a universally accepted definition to describe this intraspecific units. Initially, the term stock was attributed only at the part of fish population harvested by anthropogenic activities (Dahl, 1909), but because of the practical nature of this definition, it is better to refer to it as "*harvest stock*". However the increasing knowledge in the field, not only for fisheries management but also for conservation biology, led to the introduction of other stock definitions mostly focused on biological, ecological and genetic aspects of fish populations One of the most accepted definitions was provided by Ihssen et al., (1981), defined a stock as "an intraspecific group of randomly mating individuals with temporal or spatial integrity", while other definitions emphasized better the necessity to distinguish between a "*phenotypic stock*" (Booke, 1981 "a group of fish characterized by phenotypic differences entirely induced by the environment") and a "*genetic stock*", defined as "a reproductively isolated unit, which is genetically different from other such units" (Carvalho & Hauser, 1994). However, for fisheries management the identification of a stock is more important than its accurate definition, different techniques can be used for this purpose: one of the first techniques applied in stock identification was the capture-mark-recapture method which allows, using physical or electronic tags, the tracking of fish movements, bringing also information about homing, schooling behavior and growth rates

(Hall, 2014). Among tagging methods, the use of parasites as natural markers is also very common, this technique is based on the fact that geographically separate populations of the same species, can be infected by distinct parasites or by the same parasite with different levels of infection (Catalano et al., 2014). Another technique to infer the population structure of species is the analysis of phenotypic variation and the chemical analyses of calcified structures (otoliths and scales), the latter indicate if individuals come from distinct stocks because differences in the elemental composition of otoliths and scales are caused by different environmental conditions in which they live (Kerr & Campana, 2014); whereas "*phenotypic stocks*" can be revealed on the basis of meristic or morphological features (Cadrin et al., 2014). Finally, "*genetic stocks*" can be distinguished highlighting differences at genetic level through the use of genetic markers (Begg et al., 1999; Cuéllar-Pinzón et al., 2016). Genetic tools are being extensively used since they can provide additional information on evolutionary issues other than the simple geographical detection of a stock. In fact, the genetic approach to identify marine and freshwater stocks consists in the estimation of the extent of genetic differentiation degree that exists between two or more putative fish stocks.

Molecular markers Molecular markers can be successfully employed to detect genetic structures at both large and fine scales in population of many marine organisms with high potential vagility in adult and larval stages (Kelly & Palumbi, 2010; Selkoe & Robert, 2011; Lamichhaney et al., 2012). A useful molecular marker should be characterized by a high degree of genetic variability, a feature that allows the detection of genetic differentiation between stocks mediated by reproductive isolation degree among them (Feral, 2002); proteins and nucleic acids represent universal molecular tools to investigate the identity of stocks and populations (Carvalho & Hauser, 1994). Within a population genetics perspective, the most adequate molecular markers employable are represented by: i) allozymes (Allendorf et al., 2013), are common biological enzymes that exhibit high

levels of functional evolutionary conservation throughout specific phyla and kingdoms, are protein markers that represents alternative forms of an enzyme which differs structurally but not functionally, encoded by different alleles at the same locus. ii) Mitochondrial genes, are mitochondrial markers used in phylogenetic, systematic and population genetic studies. mtDNA is haploid, maternally inherited without recombination and exhibits a higher rate of mutation than nuclear genes (Brown, 1985), which allows to create differences at species level in a short interval of time. For example, the mitochondrial control region (D-loop) and the Cytochrome-b gene have a high mutation rate and seem useful for studies at species or population level (Brown et al., 1993). The same is for the mitochondrial Cytochrome-c oxidase I gene (CoI) that was selected as a standardize marker for identification of animal species in "the International Barcode of Life project" (iBOL) (Ratnasingham & Hebert, 2007). Between nuclear markers the most common, used in population genetic studies, are iii) variations in a single nucleotide that occurs at specific position in the genome, SNPs (Single Nucleotide Polymorphisms) and iv) microsatellite DNA also known as "*Simple Sequence Repeats*" (SSRs) (Tautz, 1989) or "*Short Tandem Repeats*" (STRs) (Edwards et al., 1991). Microsatellites consist of short, tandemly repeated units, of 1-6 bp in length and are considered as "*neutral markers*" because they are principally found in non-coding regions of nDNA (Selkoe & Toonen, 2006); furthermore, they are Mendelian inherited and show a codominant expression, (allowing the distinction between heterozygote and homozygote individuals). Microsatellites are also characterized by high mutation rates and high polymorphism because mutations lead to changes in the number of repeats and in sequence length, bringing to an increase in number of alleles at a specific locus (Selkoe & Toonen, 2006). The analyses of microsatellites are very easy because they are flanked by conserved sequences of DNA that allow the design of specific primers for their PCR amplification (Selkoe & Toonen, 2006). In addition, distinct alleles that differ in length can be simply revealed by gel electrophoresis or by automated DNA

sizing, if during PCR amplification fluorescent-labeled primers are used. Although working with microsatellites seems very simple and convenient for genetic population studies, many disadvantages can be recognized. Firstly, for some species there are not available primers to amplify microsatellites and the isolation of new microsatellite loci and the design of specific primers could be expansive in term of resources and time (Zane et al., 2002). Secondly, a series of errors can occur during PCR amplification, such as the development of shadow or stutter bands (stuttering), the not amplification of an allele due to mutations in flanking regions that inhibit the primers annealing (null alleles), or the not amplification of longer alleles that are disadvantaged respect to shorter ones during amplification (large allele dropout). In particular, the presence of stutter bands induce to interpret a homozygote as heterozygote, instead the occurrence of null alleles or large allele dropout can lead to an incorrect genotyping of heterozygote individuals as homozygotes (Selkoe & Toonen, 2006).

Genetic variations Marine species, in most cases as a consequence of their greater dispersal abilities, high fecundity and relatively low physical impediments to connectivity, show less structured population than their respective freshwater phylogenetically related species (DeWoody & Avise, 2000; Hedgecock & Pudovkin, 2011). Water column is, therefore, a very dynamic and complex environment consisting of layers with different biotic and abiotic features that can contribute to genetic structures in several organisms (Riginos & Liggins, 2013). These layers have in many cases different origin and can impose selective controls, that act during both the developmental and adult stages of organisms living therein (Selkoe & Robert, 2011; Riginos & Liggins, 2013). Environmental selection mediated by additional mortality rates on larval and juvenile stages and by variation in individual fitness in adults, together with connectivity processes mediated by oceanic circulations, can be effective ways to promote local ecological adaptation to specific environmental conditions and genetic structuring also in pelagic

species (Pespeni & Palumbi, 2013; Ciannelli et al., 2015). The genetic differentiation and the changes in the genetic composition between one generation and the next one are essentially mediated by two types of evolutionary processes: i) systematic processes (as mutation, migration and selection) and ii) dispersive processes (as inbreeding and genetic drift) (Kapuscinski & Miller, 2007). The major sources of genetic variability in populations are mutations (Allendorf et al., 2013) that occur naturally, in several cases, mutations are lethal because lead to phenotypic changes that have a negative effect on the fitness of individuals. In other cases, positive effects occur and new genetic variations are moved from generation to generation, increasing the genetic variability within populations. In fact, mutations that occur at molecular level modify DNA sequences, creating different alleles and increasing the level of polymorphism at a specific locus. A population can acquire new genetic variants also thanks to the immigration of individuals from other populations of the same species (Allendorf et al., 2013). This migration process, that includes a transfer of new alleles, is known as "*gene flow*" and leads to an increase in the genetic variability within a population. However, a high immigration rate can cause a genetic homogenization of the involved populations bringing to the formation of a new larger single population. An additional evolutionary force is the natural selection (Allendorf et al., 2013) that occurs when some genotypes may bring to a greater fitness than others under certain environmental conditions. These advantageous variants tend to be more widespread in a population and differ between distinct populations subjected to different environmental pressures, increasing the level of genetic differentiation between populations. A stock can be considered endangered when its size is reduced by natural or anthropogenic factors, because usually, small populations may undergo a process known as "*genetic drift*", a change in allele frequencies caused by random sampling of individuals (Allendorf et al., 2013). Special cases of genetic drift can be determined by a drastic reduction in the number of individuals in a population, an event known as "*bottleneck*" or by a "*founder effect*",

that occur when a new population arises from a very limited number of founders (Allendorf et al., 2013). In the long term, genetic drift leads to a loss of variability within populations and to inbreeding. Inbreeding occurs when related individuals randomly mate in a small population; this leads to an increase in the number of homozygotes and to a reduction in genetic variability, sometimes with negative effects on the survival of individuals. In fact disadvantageous alleles, recessive in heterozygote individuals, become more frequently expressed in a population rich of homozygotes, bringing to a reduction in fitness and limiting survival probabilities of the affected population, a process known as "*inbreeding depression*" (Frankham 1999; Charlesworth & Willis 2009).

The Adriatic Sea

The Adriatic Sea is the most continental basin of the Mediterranean, is a semi-enclosed basin located between the Italian peninsula and the Balkans. The Adriatic can be practically divided into two distinct geographical sub-areas (GSAs): the GSA17, namely the northern and the central Adriatic, and the GSA18, representing the southern part (GFCM, 2001). The basin shows clear morphological differences along transversal axis, in fact, the western coast is low and generally sandy while the eastern coast is rocky with islands and coves, and along longitudinal axis, being divided into northern, middle and southern sub-basins.. The northern sub-basin is extremely shallow (with average depth at 35 m) and is characterized by strong river runoff, indeed, the Po and the other northern Italian rivers are believed to contribute about 20% of the whole Mediterranean river runoff (Hopkins, 1992). The middle Adriatic is a transition zone between northern and southern sub-basins with the three Jabuka Depressions reaching 270 m depth. The southern part is characterized by a wide depression about 1200 m in depth (Artegiani et al., 1997a, b; Marini et al., 2002). However this simple subdivision does not take into account the heterogeneity of several ecological parameters driven mainly by oceanographic features

(e.g. circulation pattern of water masses and freshwater input). In fact, several areas can be identifiable on the basis of its levels of primary productivity (PP). The following zonation can be recognizable: i) the *zone A*, located in the central and southern Adriatic Sea and representing its deeper and oligotrophic area, that receives input of warm and high saline water masses from the eastern Mediterranean Sea, ii) the *zone B*, representing the north-western shallow area, with great freshwater input that spreads along all its western side and shows high PP levels and seasonal fluctuations in mean abiotic parameters (e.g., temperature, salinity and dissolved oxygen), iii) *zone C*, indicating the north-eastern area, deeper than the western side, with moderate PP levels, high salinity and quite stable environmental conditions throughout the year; and finally, iv) zone D, represented by lagoons and channels on both eastern and western sides, that display the highest PP levels in the whole Adriatic Sea but representing only 1-2% of its surface (Buljan & Zore-Armanda, 19774; Artegiani et al., 1997a,b) (Fig. 3).

The cyclonic thermohaline circulation is composed of two branches: the East Adriatic Current (EAC) flowing along the eastern coast where warm, high-salinity, modified Levantine Intermediate Water (LIW) is advected northward (Marini et al., 2010); the Western Adriatic Current (WAC) flowing southward along the Italian coast (Artegiani et al., 1997a; Marini et al., 2002), and a bottom current known as Dense Water Outflow Current (DWOC) (Cushman-Roisin et al., 2001). Winds are also important components of the circulation pattern with Bora and Sirocco being the most influential ones (Artegiani et al., 1997b). Bora is a cold and dry wind generally from the north-east; the Bora wind system causes the free sea surface to rise near the coast and this intensifies the coastal current toward the south (WAC). Bora winds can cause the formation of a double gyre structure consisting in a larger cyclone in front of the Po River delta, the NAd Gyre; (Artegiani et al., 1997b) and an anticyclonic gyre along the southern Istrian coast (Poulain et al., 2001).
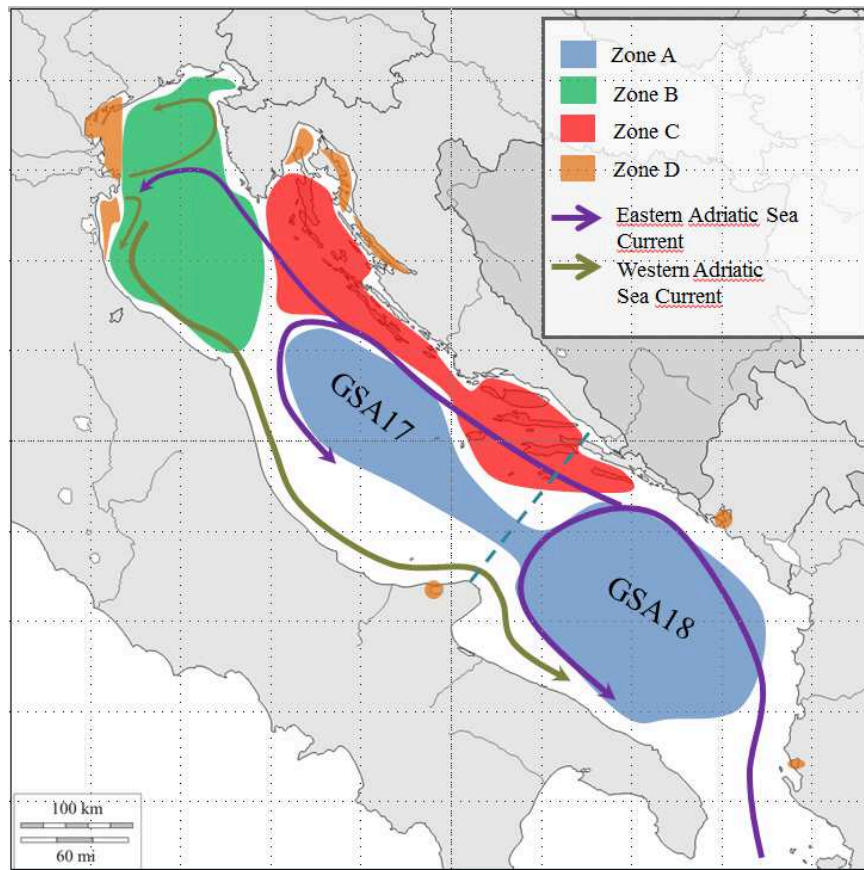
Figure 3.Adriatic Sea

In winter Bora winds cause strong heat loss in the northern Adriatic and formation of the Northern Adriatic Deep Water (NAdDW), another factor influencing the formation of deep water is the water flux, mainly governed by the Po River runoff, that can lower the salinity and hence the density of the NAdDW. The Sirocco wind is a warm and humid wind generally from the south-east and is often associated with flooding events in the shallow lagoons along the Adriatic coast including Venice. In middle and southern Adriatic, two mesoscale eddies are present, the MAd Gyre and the SAd Gyre; this cyclonic gyres are more evident in summer and autumn and enhance water mixing causing water masses exchange between western coast and offshore areas. Due to the wide continental shelf and the environmental conditions, the resulting ecosystem is unique and highly suitable for small pelagic fish species.

Aim of the study

The overall aim of the study performed within my Ph.D. project, which extended from November 2014 to October 2017, was the achievement of knowledge about abundance and spatial distribution of *Engraulis encrasicolus* in south-western Adriatic Sea, as well as the development of information related on the current genetic population structure and on how the genetic diversity has changed between generation (adult and offspring), in collaboration with the 'Italian National Research Council', Institute of Marine Sciences of Ancona.

This work consist of three parts: the first part provide useful information with regard to the spawning stock biomass of European anchovy, about fluctuations of fish biomass over a three-year period (2012, 2013 and 2014) and on the potential application of a direct method as Daily Egg production Method in order to integrate the current stock assessment process. In the second chapter, since the last ichthyoplankton surveys in the Adriatic were conducted in the 1980s and 1990s, the attention was focused on the identification of the spawning and nursery areas updating their boundaries and assessing environmental and biological factors affecting the choice of spawning areas during a four-year period (2012 - 2015). For the last chapter, microsatellite DNA markers were used to resolve the fine-scale genetic structure of anchovy to test whether the stock structure in the basin is consistent with (Ruggeri et al., 2016), and in addition, the potential loss of genetic variability through parents and offspring has been investigated.

The acquisition of information about changes of spawning stock biomass and on environmental factors that could affect the choice of spawning and nursery areas, as well as, information on current genetic population structure and on how the genetic diversity has changed can be useful to develop new management and conservation strategies in

order to prevent the size reduction of populations, their genetic impoverishment and losses in global biodiversity.

References

Allendorf, F.W., Luikart, G., Atiken, S.N., 2013. Conservation and the genetics of populations. *II ed, Hoboken: John Wiley & Sons,* 602 pp.

Artegiani, A., Bregant, D., Paschini, E., Pinardi, N., Raicich, F., Russo, A., 1997a. The Adriatic Sea general circulation. Part I : air – sea interactions and water mass structure. *Journal of Physical Oceanography*, 27, 1492–1514.

Artegiani, A., Bregant, D., Paschini, E., Pinardi, N., Raicich, F., Russo, A., 1997b. The Adriatic Sea general circulation. Part II : baroclinic circulation structure. *Journal of Physical Oceanography*, 27, 1515–1532.

Azzali, M., De Felice, A., Luna, M., Cosimi, G., Parmiggiani, F., 2002. The state of the Adriatic Sea centered on the small pelagic fish populations. *Pubblicazioni della Stazione Zoologica di Napoli: Marine Ecology*, 23 (S1), 78–91.

Bakun, A., and Cury, P., 1999. The ''school trap'': a mechanism promoting large-amplitude out-of-phase population oscillations of small pelagic fish species. *Ecology Letters*, 2 (6), 349–351.

Bakun, A., 2006. Fronts and eddies as key structures in the habitat of marine fish larvae: opportunity, adaptive response and competitive advantage. *Scientia Marina*, 70 (S2), 105–122. DOI: 10.3989/scimar.2006.70s2105.

Barange, M., Bernal, M., Cercole, M.C., Cubillos, L., Cunningham, C.L., Daskalov, G.M., de Oliveira, J.A.A., Dickey-Collas, M., Hill, K., Jacobson, L., Køster, F.W., Masse, J., Nishida, H., Niquen, M., Oozeki, Y., Palomera, I., Saccardo, S.A., Santojanni, A., Serra, R., Somarakis, S., Stratoudakis, Y., van der Lingen, C.D., Uriarte, A., Yatsu, A., 2009. Current trends in the assessment and management of stocks. *In: Climate Change and Small Pelagic Fish Stocks. Checkley, D.M., Roy, C., Oozeki, Y., Alheit, J., (Eds.), Cambridge University Press*, 191–255.

Basilone, G., Guisande, C., Patti, B., Mazzola, S., Cuttitta, A., Bonanno, A., Vergara, A.R., Maneiro, I., 2006. Effect of habitat conditions on reproduction of the European anchovy (*Engraulis encrasicolus*) in the Strait of Sicily. *Fisheries Oceanography*, 15 (4), 271–280. DOI: 10.1111/j.1365-2419.2005.00391.x.

Begg, G.A., Waldman, J.R., 1999. An holistic approach to fish stock identification. *Fisheries research*, 43, 35-44.

Bembo, D.G., Carvalho, G.R., Cingolani, N., Arneri, E., Gianetti, G., Pitcher, T.J., 1996a. Allozymic and morphometric evidence for two stocks of the European anchovy *Engraulis encrasicolus* in Adriatic waters. *Marine Biology*, 126, 529-538.

Bembo, D.G., Carvalho, G.R., Cingolano, N., Pitcher, T.J., 1996b. Electrophoretic analysis of stock structure in northern Mediterranean anchovies, *Engraulis encrasicolus*. *ICES Journal of Marine Science*, 53, 115-128.

Bertrand, A., Segura, M., Gutiérrez, M., Vásquez, L., 2004. From small-scale habitat loopholes to decadal cycles: a habitat-based hypothesis explaining fluctuation in pelagic fish populations off Peru. *Fish and Fisheries,* 5, 296–316. doi:10.1111/j.1467-2679.2004.00165.x.

Beverton, R.J.H., 1990. Small marine pelagic fish and the threat of fishing: are they endangered? *Journal of Fish Biology,* 37, 5–16.

Birkeland, C., Dayton, P.K., 2005. The importance in fishery management of leaving the big ones. *Trends in Ecology and Evolution*, 20 (7), 356–358. DOI: 10.1016/j.tree.2005.03.015.

Blaxter, J.H.S. and Hunter, J.R., 1982. The biology of the clupeoid fishes. *Advances in Marine Biology*, 20, 1–223.

Borme, D., Tirelli, V., Brandt, S.B., Fonda Umani, S., Arneri, E., 2009. Diet of *Engraulis encrasicolus* in the northern Adriatic Sea (Mediterranean): ontogenetic changes and feeding selectivity. *Marine Ecology Progress Series,* 392, 193-209.

Borsa, P., 2002. Allozyme, mitochondrial-DNA, and morphometric variability indicate cryptic

species of anchovy (*Engraulis encrasicolus*). *Biological Journal of the Linnean Society*, 75, 261-269.

Borsa, P., Collet, A., Durand, J.D., 2004. Nuclear-DNA markers confirm the presence of two anchovy species in the Mediterranean. *Comptes Rendus Biologies*, 327, 1113-1123.

Bouchenak-Khelladi, Y., Durand, J.D., Magoulas, A., Borsa, P., 2008. Geographic structure of European anchovy: a nuclear-DNA study. *Journal of Sea Research*, 59: 269-278.

Booke, H.E., 1981. The conundrum of the stock concept - are nature and nurture definable in fishery science? *Canadian Journal of Fisheries and Aquatic Sciences*, 38, 1479-1480.

Brooks, S., Tyler, C.R., Sumpter, J.P., 1997. Egg quality in fish: what makes a good egg? *Reviews in Fish Biology and Fisheries*, 387–416. DOI: 10.1023/A:1018400130692.

Brown, W.M., 1985. The mitochondrial genome of animals. *In: Molecular evolutionary genetics (ed MacIntyre, R.J.), Plenum, New York,* 95-130.

Brown, J.R., Bechenbach, A.T., Smith, M.J., 1993. Intraspecific DNA sequence variation of the mitochondrial control region of white sturgeon (*Acipenser transmontanus*). *Molecular Biology Evolution*, 10, 326-341.

Buljan, M., Zore-Armanda, M., 1974. Oceanographical properties of the Adriatic Sea. *Oceanography and Marine Biology Annual Review*, 14, 11–98.

Burgess, M.G., Polasky, S., Tilman, D., 2013. Predicting overfishing and extinction threats in multispecies fisheries. *Proceedings of the National Academy of Sciences*, 110 (40), 15943–15948. DOI: 10.1073/pnas.1314472110.

Cadrin, S.X., Kerr, L.A., Mariani, S., 2014. Stock identification methods. *II ed. Academic Press, San Diego.*

Carvalho, G.R., Hauser, L., 1994. Molecular genetics and the stock concept in fisheries. *Reviews in Fish Biology and Fisheries*, 4, 326–350.

Carvalho, G.R., Bembo, D.G., Carone, A., Giesbrecht, G., Cingolani, N., Pitcher, T.J., 1994. Stock discrimination in relation to the assessment of Adriatic anchovy and sardine

fisheries. *Final Project Report to the Commission of the European Communities*, EC XIV-1/MED/91001/A.

Catalano, S.R., Whittington, I.D., Donnelan, S.C., Gillanders, B.M., 2014. Parasites as biological tags to assess host population structure: guidelines, recent genetic advances and comments on a holistic approach. *International Journal for Parasitology: Parasites and Wildlife*, 3, 220-226.

Charlesworth, D., Willis, J.H., 2009. The genetics of inbreeding depression. *Nature Reviews Genetics,* 10, 783-796.

Christensen, J., 2014. Unsettled seas: towards a history of marine animal populations in the Central Indo-Pacific. *In: Christensen, J., Tull, M., (eds) Historical Perspectives of Fisheries Exploitation in the Indo-Pacific. MARE Publication Series, Springer, Dordrecht*, 12.

Cingolani, N., Giannetti, G., Arneri, E., 1996. Anchovy fisheries in the Adriatic Sea. *In: Palomera, I., et al. (Ed.) The European anchovy and its environment. Scientia Marina*, 60 (S2), 269-–277.

Coll, M., Santojanni, A., Palomera, I., Tudela, S., Arneri, E., 2007. An ecological model of the Northern and Central Adriatic Sea: analysis of ecosystem structure and fishing impacts. *Journal of Marine Systems*, 67, 119–154. DOI: 10.1016/j.jmarsys.2006.10.002.

Cuéllar-Pinzón, J., Presa, P., Hawkins, S.J., Pita, A., 2016. Genetic markers in marine fisheries: types, tasks and trends. *Fisheries Research*, 173, 194-205.

Culley, M., Kerkut, G.A., 1971. The Pilchard. Biology and exploitation. *Elsevier Ltd,*. DOI: 10.1016/B978-0-08-016523-3.50002-6.

Cury, P., Bakun, A., Crawford, R.J.M., Jarre, A., Quinones, R.A., Shannon, L.J., Verheye, H.M., 2000. Small pelagics in upwelling systems: patterns of interaction and structural changes in "wasp-waist" ecosystems. *ICES Journal of Marine Science*, 57 (3), 603–618. DOI: 10.1006/jmsc.2000.0712.

Cushman-Roisin, B., Gačić, M., Poulain, P-M., Artegiani, A., 2001. Physical oceanography of the Adriatic Sea: Past, Present and Future (*ed Springer Science & Business Media), Netherlands, Dordrecht*, 304 pp.

Dahl, K., 1909. The problem of sea fish hatching. Special Part B, No. 5. *In: Rappurt sur les Travaux de Commision A, Dans La Période 1902-1907, Conseil Permanent International Pour L'Exploration de La Mer. Rapports et Procès-Verbaux, Vol. X (ed Hjort, J.).*

D'Ancona, U., 1956. Uova, larve e stadi giovamili di Teleostei. I*n: Bertolini, F., D'ancona, U., Montalenti, G., Padoa, E., Ranzi, S., Sanzo, L., Spartà, A., Tortonese, E., Vialli, M. Fauna e flora del Golfo di Napoli.*

DeWoody, J.A., Avise, J.C., 2000. Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *Journal of Fish Biology*, 56 (3), 461–473. DOI: 10.1006/jfbi.1999.1210.

Edwards, A.L., Civitello, A., Hammond, H.A., Caskey, C.T., 1991. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *American journal of Human Genetics*, 49, 746-756.

Ettahiri, O., Berraho, A., Vidy, G., Ramdani, M., Do chi, T., 2003. Observation on the spawning of sardina and sardinella off the South Moroccan Atlantic coast (21-26°N). *Fisheries Research*, 60 (2–3), 207–222. DOI: 10.1016/S0165-7836(02)00172-8.

FAO (Food and Agriculture Organization), 2014. World review of fisheries and aquaculture. *Rome: Food and Agriculture Organisation*, 96 pp.

FAO, FishStatJ, 2015. Software for Fishery Statistical Time Series, http://www.fao.org/fishery/statistics/software/fishstatj/en.

FAO (Food and Agriculture Organization), 2016. The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. *Rome: Food and Agriculture Organisation,* 200 pp.

Feral, J.P., 2002. How useful are the genetic markers in attempts to understand and manage

marine biodiversity? *Journal of Experimental Marine Biology and Ecology*, 268, 121–145. DOI: 10.1016/S0022-0981(01)00382-3.

Frankham, R., 1999. Quantitative genetics in conservation biology. *Genetics Research*, 74, 237-244.

Fréon, P., Misund, O.A., 1999. Dynamics of pelagic fish distribution and behaviour : effects on fisheries and stock assessment. *Fishing News Books*.

Fuiman, L.A., 1993. Development of predator evasion in Atlantic herring, *Clupea harengus* L. *Animal Behaviour*, 45 (6), 1101–1116. DOI: 10.1006/anbe.1993.1135.

García Lafuente, J., García, A., Mazzola, L., Quintanilla, J., Delgado, J., Cuttita, A., Patti, B., 2002. Hydrographic phenomena influencing early life stages of the Sicilian Channel anchovy. *Fisheries Oceanography*, 11 (1), 31–44. DOI: 10.1046/j.1365-2419.2002.00186.x.

GFCM (General Fisheries Council for the Mediterranean), 2001. Report of the twenty-six session. Lacco Ameno, Ischia, Italy. *GFCM Report No 26. Rome,* FAO, 27 pp.

GFCM (General Fisheries Council for the Mediterranean), 2016. *Working Group on Stock Assessment of Small Pelagic Species (WGSASP).*

Grant, S.W., 2005. A second look at mitochondrial DNA variability in European anchovy (*Engraulis encrasicolus*): assessing models of population structure and the Black Sea isolation hypothesis. *Genetica*, 125, 293-309. DOI: 10.1007/s10709-005-0717-z.

Hall, D.A, 2014. Conventional and radiofrequency identification (RFID) tags. *In: Stock identification methods, II ed. (eds Cadrin, S.X., Kerr, L.A., Mariani, S.), Academic Press, San Diego*, 365-395.

Hay, D.E., Brett, J.R., Bilinski, E., Smith, D.T., Donaldson, E.M., Hunter, G.A., Solmie, A.V., 1988. Experimental impoundments of prespawning Pacific herring (*Clupea harengus pallasii*): effects of feeding and density on maturation, growth, and proximate analysis. *Canadian Journal of Fisheries and Aquatic Science*, 45, 388–398.

Hedgecock, D., Pudovkin, A.I., 2011. Sweepstakes reproductive success in highly fecund marine fish and shellfish: a review and commentary. *Bulletin of Marine Science*, 87 (4), 971–1002. DOI: 10.5343/bms.2010.1051.

Hopkins, T.S., 1992. The structure of Ionian and Levantine Seas. *Reports in Meteorology and Oceanography*, 41 (II), 35-56.

Hutchings, J.A., 2000. Collapse and recovery of marine fishes. *Nature*, 406, 882–885.

Ihssen, P.E, Booke, H.E., Casselmann, J.M., McGlade, J.M., Paine, N.R., Utter, F.M., 1981. Stock identification: materials and methods. *Canadian Journal of Fisheries and Aquatic Sciences*, 38, 1838-1855.

Kapuscinski, A.R., Miller, L.M., 2007. Genetic guidelines for fisheries management. *University of Minnesota*, (II edition), 116. DOI: 10.1016/0044-8486(88)90386-9.

Kelly, R.P., Palumbi, S.R., 2010. Genetic structure among 50 species of the northeastern pacific rocky intertidal community. *PLoS One*, 5 (1). DOI: 10.1371/journal.pone.0008594.

Kerr, L.A., Campana, S.E., 2014. Chemical composition of fish hard parts as a natural marker of fish stocks. *In: Stock identification methods. II ed. (eds Cadrin, S.X., Kerr, L.A., Mariani, S.), 205-234. Academic Press, San Diego.*

Kolitari, J., 2006. Preliminary results of small pelagic sampling in the context of the project AdriaMed (for Albania). Data presented at *AdriaMed Working Group on Shared Small Pelagic Fisheries Resources*, Ancona 15–19 May 2006.

Lamichhaney, S., Barrio, A.M., Rafati, N., Sundstrom, G., Rubin, C-J., Gilbert, E.R., Berglund, J., Wetterbom, A., Laikre, L., Webster, M.T., Grabherr, M., Ryman, N., Andersson, L., 2012. Population-scale sequencing reveals genetic differentiation due to local adaptation in Atlantic herring. *Proceedings of the National Academy of Sciences*, 109 (47), 19345–19350. DOI: 10.1073/pnas.1216128109.

Lasker, R., 1978. The relation between oceanographic conditions and larval anchovy food in the California Current: identification of factors contributing to recruitment failure. *Rapp.*

Proces-Verbaux des Reun. Cons. Int. pour l'Exploration la Mer, 173, 212–230.

Lehodey, P., Alheit, J., Barange, M., Baumgartner, T., Beaugrand, G., Drinkwater, K., Fromentin, J.M., Hare, S.R., Ottersen, G., Perry, R.I., Roy, C., van der Lingen, C.D., Werner, F., 2006. Climate variability, fish, and fisheries. *Journal of Climate*, 19 (20), 5009–5030. DOI: 10.1175/JCLI3898.1.

Leonori, I., De Felice, A., Biagiotti, I., Canduci, G., Costantini, I., Giuliani, G., Malavolti, S., Gabrielli, G., Miccoli, A., Caccamo, G., Penna, P., Grilli, F., Paschini, E., 2016. Programma Nazionale Italiano per la Raccolta Dati Alieutici 2014-2016 EC-DCR-MIPAAF Anno 2015. Campagna di ricerca in mare sezione G - MEDIAS GSA 17 - *Rapporto Finale. CNR - ISMAR, Ancona, Italia,* 120 pp.

Lleonart, J., Maynou, F., 2003. Fish stock assessments in the Mediterranean: state of the art. *Scientia Marina*, 67 (S1), 37–49. DOI: 10.3989/scimar.2003.67s137.

Lluch-Belda, D., Crawford, R.J.M., Kawasaki, T., MacCall, A.D., Parrish, R.H., Schwartzlose, R.A., Smith, P.E., 1989. World-wide fluctuations of sardine and anchovy stocks: the regime problem. *South African Journal of Marine Science*, 8 (1), 195–205. DOI: 10.2989/02577618909504561.

Ma, Y., Kjesbu, O.S., Jørgensen, T., 1998. Effects of ration on the maturation and fecundity in captive Atlantic herring (*Clupea harengus*). *Canadian Journal of Fisheries and Aquatic Sciences*, 55, 900–908. DOI: 10.1139/cjfas-55-4-900.

MacCall, A.D., 2009. Mechanisms of low-frequency fluctuations in sardine and anchovy populations. *Climate change and small pelagic fish*, 285–299. DOI: 10.1017/CBO9780511596681.014.

Magoulas, A., Tsimenides, N., Zouros, E., 1996. Mitochondrial DNA phylogeny and the reconstruction of the population history of a species: the case of the European anchovy (*Engraulis encrasicolus*). *Molecular, Biology and Evolution*, 13 (1), 178–190.

Magoulas, A., Castilho, R., Caetano, S., Marcato, S., Patarnello, T., 2006. Mitochondrial DNA

reveals a mosaic pattern of phylogeographical structure in Atlantic and Mediterranean populations of anchovy (*Engraulis encrasicolus*). *Molecular Phylogenetics and Evolution*, 39 (3), 734–746. DOI: 10.1016/j.ympev.2006.01.016.

Marano, G., 2001. Small pelagic stock assessment (1984–1996). ADRIAMED Techical Documents 3, 66–77.

Marini, M., Fornasiero, P., Artegiani, A., 2002. Variations of hydrochemical features in the coastal waters of Monte Conero: 1982–1990. *Marine Ecology*, 23, 258–271.

Marini, M., Grilli, F., Guarnieri, A., Jones, B.H., Kljajić, Z., Pinardi, N., Sanxhaku, M., 2010. Is the southeastern Adriatic Sea coastal strip an eutrophic area? *Estuarine Coastal and Shelf Science*, 88, 395–406.

Millán, M., 1999. Reproductive characteristics and condition status of anchovy *Engraulis encrasicolus* L. from the Bay of Cadiz (SW Spain). *Fisheries Research*, 41, 73–86.

Morello, B., Arneri, E., 2009. Anchovy and sardine in the Adriatic Sea - an ecological review. *Oceanography and Marine Biology: an Annual Review*, 47, 209–256.

Morgan, M.J., Brattey, J., 2005. Effect of changes in reproductive potential on perceived productivity of three Northwest Atlantic cod (*Gadus morhua*) stocks. *ICES Journal of Marine Science*, 62 (1), 65–74. DOI: 10.1016/j.icesjms.2004.10.003.

Motos, L., Uriarte, A., Valencia, V., 1996. The spawning environment of the Bay of Biscay anchovy (*Engraulis encrasicolus* L.). *Scientia Marina*, 60 (2), 117–140.

Nelson, J.S., 1994. Fishes of the world. *Journal of the Marine Biological Association of the United Kingdom*, 74 (987). DOI: DOI:10.1017/S0025315400044192.

Palomera, I., 1991. Vertical distribution of eggs and larvae of *Engraulis encrasicolus* in stratified waters of the western Mediterranean. *Marine Biology*, 111, 37–44.

Palomera, I., Olivar, M.P., Salat, J., Sabatés, A., Coll, M., García, A., Morales-Nin, B., 2007. Small pelagic fish in the NW Mediterranean sea: an ecological review. *Progress in Oceanography*, 74, 377–396. DOI: 10.1016/j.pocean.2007.04.012.

Pespeni, M.H., Palumbi, S.R., 2013. Signals of selection in outlier loci in a widely dispersing species across an environmental mosaic. *Molecular Ecology*, 22 (13), 3580–3597. DOI: 10.1111/mec.12337.

Pikitch, E., Boersma, P.D., Boyd, I.L., Conover, D.O., Cury, P., Essington, T., Heppell, S.S., Houde, E.D., Mangel, M., Pauly, D., Plagányi, É., Sainsbury, K., Steneck, R.S., 2012. Little fish, big impact: managing a crucial link in ocean food webs. *A Report from Lenfest Forage Fish Task Force*, 108 pp.

Pikitch, E.K., Rountos, K.J., Essington, T.E., Santora, C., Pauly, D., Watson, R., Sumaila, U.R., Boersma, P.D., Boyd, I.L., Conover, D.O., Cury, P., Heppell, S.S., Houde, E.D., Mangel, M., Plagányi, É., Sainsbury, K., Steneck, R.S., Geers, T.M., Gownaris, N., Munch, S.B., 2014. The global contribution of forage fish to marine fisheries and ecosystems. *Fish and Fisheries*,. DOI: 10.1111/faf.12004.

Pitcher, T.J., Parrish, J.K., 1986. Functions of shoaling behaviour in teleosts. I*n: Pitcher, T.J. (Ed.), Behaviour of teleost fishes. John Hopkins Press, Baltimore.*

Poulain, P.M., Kourafalou, V.H., and Cushman-Roisin, B., 2001. Northern Adriatic Sea. *In Physical Oceanography of the Adriatic Sea: Past, Present, Future. Edited by Cushman-Roisin, B. et al., 2001, Springer Science & Business Media, Netherlands, Dordrecht*, 143-165.

Ratnasingham, S, Hebert, P.D., 2007. BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular ecology notes*, 7, 355-364.

Rätz, H.J., Lloret, J., 2005. Long-term variability of growth and recruitment of cod (*Gadus morhua*) off Greenland. *ICES Journal of Marine Science*, 62 (7), 1310–1321. DOI: 10.1016/j.icesjms.2005.07.009.

Regner, S., 1972. Contribution to the study of the ecology of the planktonic phase in the life history of the anchovy in the Central Adriatic. *Acta Adriatica* 14 (9), 3–43.

Regner, S., 1985. Ecology of planktonic stages of the anchovy *Engraulis encrasicolus*

(Linnaeus, 1758) in the Central Adriatic. *Acta Adriatica* 26 (1), 5–113.

Regner, S., 1996. Effects of environmental changes on early stages and reproduction of anchovy in the Adriatic Sea. *Scientia Marina*, 60 (2), 167–177.

Rice, J.A., Crowder, L.B., Binkowski, F.P., 1987. Evaluating potential sources of mortality for larval bloater (*Coregonus hoyi*): starvation and vulnerability to predation. *Canadian Journal of Fisheries and Aquatic Sciences*, 44, 467–472. DOI: 10.1139/f87-055.

Rice, J., 1995. Food web theory, marine food webs, and what climate change may do to northem marine fish populations. *In: Climate change and northern fish populations, Beamish, R.J., (Ed), Canadian Special Publication of Fisheries and Aquatic Sciences*, 121, 561–568.

Riginos, C., Liggins, L., 2013. Seascape Genetics: populations, individuals, and genes marooned and adrift. *Geography Compass*, 7 (3), 197–216. DOI: 10.1111/gec3.12032.

Ruggeri, P., Splendiani, A., Occhipinti, G., Fioravanti, T., Santojanni, A., Leonori, I., De Felice, A., Arneri, E., Procaccini, G., Catanese, G., Ticina, V., Bonanno, A., Nisi Cerioni, P., Giovannotti, M., Grant, W.S., Caputo Barucchi, V., 2016. Biocomplexity in populations of european anchovy in the Adriatic Sea. *PLoS One*, 11 (4), 1–21. DOI: 10.1371/journal.pone.0153061.

Ryman, N., Utter, F., Laikre, L., 1995. Protection of intraspecific biodiversity of exploited fishes. *Reviwes in Fish Biology and Fisheries*, 5 (4), 417–446. DOI: 10.1007/BF01103814.

Santojanni, A., Arneri, E., Barry, C., Belardinelli, A., Cingolani, N., Giannetti, G., Kirkwood, G., 2003. Trends of anchovy (*Engraulis encrasilocus*, L.) biomass in the northern and central Adriatic Sea. *Scientia Marina*, 67 (3), 327–340. DOI: 10.3989/scimar.2003.67n3327.

Santojanni, A., Cingolani, N., Arneri, E., Kirkwood, G., Belardinelli, A., Giannetti, G., Colella, S., Donato, F., Barry, C., 2005. Stock assessment of sardine (*Sardina pilchardus*, Walb.) in the Adriatic Sea, with an estimate of discards. *Scientia Marina*, 69 (4), 603–617. DOI:

10.3989/scimar.2005.69n4603.

Schwartzlose, R.A., Alheit, J., Bakun, A., Baumgartner, T.R., Cloete, R., Crawford, R.J.M., Fletcher, W.J., Green-Ruiz, Y., Hagen, E., Kawasaki, T., Lluch-Belda, D., Lluch-Cota, S.E., MacCall, A.D., Matsuura, Y., Nevarez-Martinez, M.O., Parrish, R.H., Roy, C., Serra, R., Shust, K.V., Ward, M.N., and Zuzunaga, J.Z, 1999. Worldwide large-scale fluctuations of sardine and anchovy populations. *South African Journal of Marine Science* 21, 289–347.

Scott, A.P., 1987. Reproductive endocrinology of fish. *In: Fundamentals of comparative vertebrate endocrinology. Springer US, Boston*, 223–256 pp. DOI: 10.1007/978- 1-4899-3617-2_7.

Selkoe, K.A., Toonen, R.J., 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, 9 (5), 615–629. DOI: 10.1111/j.1461-0248.2006.00889.x.

Selkoe, K.A., Robert, J.T., 2011. Marine connectivity: a new look at pelagic larval duration and genetic metrics of dispersal. *Marine Ecology Progress Series*, 436, 291–305.

Sinovčić, G., 2000. Anchovy, *Engraulis encrasicolus* (Linnaeus, 1758): biology, population dynamics and fisheries case study. *Acta Adriatica,* 41 (1), 3–53.

Somarakis, S., Koutsikopoulos, C., Machias, A., Tsimenides, N., 2002. Applying the daily egg production method (DEPM) to small stocks in highly heterogeneous seas. *Fisheries Research*, 55 (1–3), 193–204. DOI: 10.1016/S0165-7836(01)00284-3.

Tautz, D., 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic acids research*, 17, 6463-6471.

Tičina, V., Ivančić, I., and Emrić, V., 2000. Relation between the hydrographic properties of the northern Adriatic Sea water and sardine (*Sardina pilchardus*) population schools. *Periodicum Biologorum,* 102, 181–192.

Tortonese, E., 1970. Fauna d'Italia. Osteichthyes (pesci ossei). Vol X. *In: Calderini E (ed)*

Bologna.

Troadec, J-P., Clark, W.G., and Gulland, J.A., 1980. A review of some pelagic fish stocks in other areas. *Rapports et Procès Verbaux des Réunions du Conseil International pour l'Exploration de la Mer,* 177, 252–277.

van der Lingen, C.D., 1994. Effect of particle size and concentration on the feeding behaviour of adult pilchard *Sardinops sagax*. *Marine Ecology Progress Series*, 109, 1–13.

van der Lingen, C.D., 2002. Diet of sardine *Sardinops sagax* in the southern Benguela upwelling ecosystem. *South African Journal of Marine Science*, 24, 301–316.

van der Lingen, C.D., Hutchings, L., Field, J.G., 2006. Comparative trophodynamics of anchovy *Engraulis encrasicolus* and sardine *Sardinops sagax* in the southern Benguela: are species alternations between small pelagic fish trophodynamically mediated? *African Journal of Marine Science,* 28, 465–477.

van der Lingen, C.D., Bertrand, A., Bode, A., Brodeur, R., Cubillos, L., Espinoza, P., Friedland, K., Garrido, S., Irigoien, X., Miller, T., Mollman, C., Sanchéz, Rodriguez, R. Tanaka, H., Temming, A., 2009. Trophic dynamics. *In: Checkley, D., Alheit, J., Oozeki, Y., Roy, C. (Eds.), Climate change and small pelagic fish. Cambridge University Press*.

Viñas, J., Sanz, N., Penarrubia, L., Araguas, R-M., Garcia-Marin, J-L., Roldan, M-I., Pla, C., 2014. Genetic population structure of European anchovy in the Mediterranean Sea and the northeast Atlantic Ocean using sequence analysis of the mitochondrial DNA control region. *ICES Journal of Marine Sciences*, 71(2), 391-397. DOI: 10.1093/icesjms/fst132.

White, C., Selkoe, K.A., Watson, J., Siegel, D.A., Zacherl, D.C., Toonen, R.J., 2010. Ocean currents help explain population genetic structure. *Proceedings of the Royal Society B: Biological Sciences*, 277 (1688), 1685–1694. DOI: 10.1098/rspb.2009.2214.

Whitehead, P.J.P., Nelson, G.J., Wongratana, T., 1988. An annotated and illustrated catalogue of the herrings, sardines, pilchards, sprats, shads, anchovies and wolf-herrings. Part 1. Chirocentridae, Clupeidae & Pristigasteridae. *In: FAO species catalogue, No 125, Vol. 7.*

Clupeoid fishes of the world (Suborder Clupeoidei).

Zane, L., Bargelloni, L., Patarnello, T., 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology*, 11 (1), 1–16. DOI: 10.1046/j.0962-1083.2001.01418.x.

Zarrad, R., Missaoui, H., Alemany, F., Hamza, A., Romdhane, MS., García, A., 2008. Distribution and abundance of early life stages of *Sardina pilchardus* in the Gulf of Tunis (Central Mediterranean Sea) in relation to environmental and biological factors. *Scientia Marina*, 72 (2), 299–309.

Zorica, B., Vilibić, I., Keč, V.Č., Šepić, J., 2013. Environmental conditions conducive to anchovy (*Engraulis encrasicolus)* spawning in the Adriatic Sea. *Fisheries Oceanography*, 22 (1), 32–40. DOI: 10.1111/fog.12002.

Chapter 1

Evaluation of spawning stock biomass of *Engraulis encrasicolus* using a direct method like Daily Egg Production Method (DEPM)

1.1   Introduction

The Daily Egg Production Method (DEPM) is an ichthyoplankton-based method and, together with acoustic surveys (Simmonds & MacLennan, 2005), has been the preferred fishery-independent method of assessing spawning-stock biomass (SSB). The spawning stock biomass of an exploited species is an important variable in fisheries management; DEPM surveys provide a method of estimating it independently of any commercial catch data, and have become important because of heightened demands for fishery-independent information (Hunter & Lo, 1993). Egg production survey methods have the advantage of providing SSB estimates for spatially discrete spawning sites, and avoid the bias in fishery-dependent SSB estimates associated with assumptions regarding the accuracy of fishery data. However there are many steps leading to the final SSB estimates from egg production methods, each with potential biases. The underlying principle of this method is that the abundance of an early life stage (eggs and yolk-sac larvae) can be used to estimate the reproductive outcome of the population over a time period. This outcome, divided by the mean contribution per unit of fish body weight (fecundity rate) over the same period, provides an estimate of spawning biomass (Gunderson, 1993). The DEPM is applicable to batch-spawning species with pelagic eggs and indeterminate fecundity, and was developed in the late 1970s at the Coastal Division of the Southwest Fisheries Science Center in La Jolla, California (Parker, 1980) as a response to the growing need to devise a suitable direct method for the assessment of northern anchovy (*Engraulis mordax*). Lasker (1985) compiled the work developed in California into the first, and most thorough to date, report defining the underlying principles of the method and describing the parameters, data and techniques required to obtain a DEPM estimate of spawning biomass. There are three main ways DEPMs are used in the assessment process: i) direct use in assessment, as an index of biomass, ii) monitoring trends or status of the target stocks, without directly incorporating

the information into the assessment, iii) providing information about spatial or temporal shifts in distribution. The DEPM received immediate attention and its applications rapidly extended to several important anchovy (*Engraulis mordax, Engraulis japonicus* and *Engraulis encrasicolus*) stocks worldwide, like in Peru, Korea, South Africa and the Bay of Biscay. In the 1990s, applications for anchovy were more frequent in Atlantic and Mediterranean waters of Europe, but also extended to Chile. Towards the end of the decade, applications to sardines (*Sardinops sagax* and *Sardina pilchardus*) appeared first in California and then in the Iberian Peninsula. Sardine DEPMs were also regularly performed in Australia and the method was extended to other pelagic species, like sprat (*Sprattus sprattus*), mackerel (*Scomber scombrus*), horse mackerel (*Trachurus trachurus*), and, for the first time to a demersal species, snapper (*Pagrus auratus*) in Australia and New Zealand (Stratoudakis et al., 2006). Concerning the Mediterranean Sea three major anchovy stocks exist, supporting the largest anchovy fisheries in the basin (Barange et al., 2009). These stocks are genetically distinct, with reduced gene exchanges (Magoulas et al., 1996, 2006; Ruggeri et al., 2016) and inhabit the north-western Mediterranean (Catalan Sea and Gulf of Lions), the Adriatic Sea and the northern Aegean Sea (Somarakis et al., 2004). Within Mediterranean Sea the DEPM has been used to evaluate the anchovy spawning biomass of the Catalan Sea (Palomera & Pertierra, 1993), Catalan Sea-Gulf of Lions (García & Palomera, 1996; Olivar et al., 2001), Ligurian-north Tyrrhenian Sea (García & Palomera, 1996), Aegean Sea (Somarakis et al., 2012), Ionian Sea (Somarakis et al., 2002), south-western Adriatic Sea (Marano et al., 1998), and Sicilian Channel (Quintanilla & García, 2001). Spawning biomass and DEPM parameters estimates in the Mediterranean show high variability both within (inter-seasonal and inter-annual variations) and between regions. Different methodologies together with environmental variability could partially explain these variations. For instance, the temperature range during the peak spawning period may vary from 16 to 25°C in different Mediterranean

areas; egg development duration and post-ovulatory follicle degeneration can present great differences within this temperature range, thus affecting these parameter estimates. Overall, the parameters with the highest variance are the daily egg production (P) and the spawning fraction (S), while the large variation in egg mortality rates should also be noted (Table 1.1). Considering the Adriatic Sea, Marano et al. (1998), during 10 years of DEPM application in the south-western Adriatic, found fluctuations on biomass with an increasing trend in the early '90s respect to the late '80s where the stock was really impoverished. Moreover they reported highest biomass of eggs and larvae in the gulf of Manfredonia describing this area as a nursery zone.

Besides biomass estimation, the application of DEPM provides regional time series on the extension and characteristics of spawning habitats and on important reproductive parameters of fish stocks which can help in improving our understanding of the mechanisms by which environmental changes can modulate the reproductive biology of small pelagic fish (e.g., Somarakis et al., 2004, 2006). Since the last attempt to apply DEPM in Adriatic has been done in 1980s and 1990s, the object of this chapter is to present the results of trials to apply DEPM method during three years of sampling (2012, 2013 and 2014) in the south-western Adriatic Sea.

1.2 Material and methods

1.2.1 The DEPM model The spawning stock biomass was estimated according to the model described by Parker (1980) and subsequently modified by (Stauffer & Picquelle, 1980):

$$B = \frac{kPAW}{RFS}$$

where B is the spawning stock biomass in metric tons, k the conversion factor from grams to metric tons, P the daily egg production (number of eggs per sampling unit, m^2), A the total survey area (in sampling units, m^2), W the average weight of mature females (g), R the sex ratio (fraction of mature females by weight), F the batch fecundity (mean number of eggs per mature females per spawning) and S the fraction of mature females per spawning night (spawning frequency). Based on the delta method, the approximate variance of the biomass estimate is a function of sample variances and covariances (Stauffer & Picquelle, 1980):

$$Var\ B \cong \hat{B}^2\ (CV\ (P)^2 + CV\ (W)^2 + CV\ (F)^2 + CV\ (S)^2 + 2COVS)$$

where CV denotes coefficient of variation and COVS the sum of terms involving co-variances. It has been suggested that the minimum precision for a tuning index to be useful for assessment will be coefficients of variation (CV) ≈30% (De Oliveira et al., 2006). Different assessment models cope in different ways with imprecise indices, typical CV's for DEPMs lie between 20 and 40% (Jacobson et al., 1994; Armstrong et al., 2001), suggesting that high precision for some stocks does not achieve. The life history strategy of the exploited fish (short lived, long lived, etc.), the available catch information and the

assessment method all interact to determine the precision and bias appropriate from the biomass estimates.

1.2.2 Sampling method and data collection Plankton samples were collected during the Italian acoustic survey on small pelagic fish (MEDIAS project; Leonori et al. 2016) in FAO Geographical Sub Areas 18 during three years (2012, 2013 and 2014). Vertical tows have been performed using a WP2 (mouth opening: 0.255 m^2; mesh-size: 0.200 mm) until 3 m above the bottom (max depth sampled: 100 m). Sampling design was based on a grid of stations along parallel transect perpendicular to the coast, spaced approximately 5 nautical miles apart, if at least one egg was found in the last station of the transect, the sampling was extended for another 5 nm, the sampling remained the same for the three years (Fig. 1.1). A total of 58 stations per year was sampled, representing an area of 2510 nm^2 (square nautical miles). Ichthyoplankton samples were fixed immediately after collection in 4% formaldehyde-seawater solution. Hydrographic sampling (temperature and salinity profiles of the water column) was performed at each station. Adult individuals were caught by pelagic trawls in different conditions of sunlight and depth, a total of 39 trawl samples of anchovy were collected (Fig. 1.2). Processing of an adult sample consisted of measuring length and weight (both total and gonad free weight) and sexing all or, at least, 100 fish per sample; gonads of all processed females were weighed (0.01 mg) and subsequently the gonads of 20 females per sample were randomly selected and preserved in 10% buffered formalin for following histological analysis. All macroscopically detected hydrated or running females were measured and their gonads weighed and preserved in formalin for subsequent histological and batch fecundity analysis. The total weight of hydrated females was corrected for the increase in weight due to hydration of the ovaries (Somarakis, S., Tsimenides, 1997) and a correction factors were applied to convert formalin weight to wet weight (Hunter et al., 1985).

1.2.3 Laboratory analysis Anchovy eggs and larvae were sorted from the plankton samples, the eggs were staged using the 10-stage defined on the basis of morphological and structural changes that occur during embryogenesis (Regner, 1985) (Fig. 1.3). The newly hatched larva is called yolk-sac larva; it is transparent, 2 mm long, with posteriorly elongated yolk, unpigmented eyes and without mouth opening (YSI) (Somarakis et al., 2002). In the first two days there is a significant lengthening of the body, a clear reduction of the yolk and traces of brown pigment or brownish eyes (YSII) (Somarakis et al., 2002) (Fig. 1.3). On the fourth or fifth day the yolk is completely resorbed, the body length is increased and the eyes are pigmented, hence the stage of larva begins (D'Ancona, 1956). Eggs and yolk-sac larvae at each developmental stage were counted and their abundance were standardized to the number per square meter. The hydrated oocyte method was used for batch fecundity (F) measurements (Hunter et al., 1985), gonads with fully hydrated oocytes of one of the two ovaries were used, since anchovy gonads are homogenous and there are no significant differences in the number of hydrated oocytes per unit weight between left or right ovary (Sanz & Uriarte, 1989; Somarakis, 1999). Oocytes were counted in 40–60 mg subsamples (containing 100–200 eggs) taken from the anterior, middle and posterior part of the gonad, only fully hydrated gonads were used, namely, gonads without post-ovulatory follicles (POFs) which had previously been tested through histological analysis. Estimations of spawning frequency (S) is based on the post-ovulatory follicle method described by Hunter & Macewicz (1985), each hydrated oocyte is surrounded by a thinly stretched follicle of an inner, epithelial layer of granulosa cells and a single, outer connective tissue layer of theca cells with some blood capillaries. At ovulation, the fully hydrated oocytes are released from their encompassing follicles. The follicle does not fragment and pass out of the ovary with the hydrated oocyte but retains its integrity, the follicle collapses away from the opening formed for the release of the hydrated oocyte into the lumen and remains in the ovary as an evacuated follicle, or

postovulatory follicle (POF). Application of the post-ovulatory follicle method for the estimation of S require understanding the degeneration process of POFs through time, as POFs have to be aged, i.e. assigned to a daily spawning cohort (daily class) according to their degeneration state, histological scoring included the incidence of immature females, migratory nucleus oocytes, hydrated oocytes, POF-0, POF-1 and POF-2, (Hunter & Macewicz, 1985) (Fig. 1.4). The ageing, according to the oocytes and the follicles (pre and postovulatory), are defined as follows (Motos, 1996):

Day_-1: Females showed gonads with oocytes in the nuclear migration stage, which evidenced that spawning will take place the following night, this correspond to pre-spawning females.

Day_0: females that will spawn, are spawning or have spawned the day of capture, which typically showed: at the beginning oocytes with early or advanced nuclear migration, later on hydration, and finishing with POF-0 (relatively large and irregular in shape, with folds and loops and irregular lumen. Granulosa cells are columnar or cuboidal and are arranged orderly along the edge of the lumen, well defined theca cell layer noticeable separated from the granulosa).

Day_1: follicles of females that spawned the night before capture with POF-1 (greatly shrunken with fewer folds and reduced lumen. Granulosa cells no longer orderly arranged with pycnotic nuclei and vacuoles, the layer of theca cells is still present but less distinct).

Day_2: follicles of females that spawned 2 nights before capture with POF-2 (from 1/2-to 1/4 the size of mature POFs with reduced or absent lumen, granulosa cells almost disappeared, few vacuoles and pycnotic nulcei may be seen, indistinct theca cells incorporated with ovarian connective tissue stroma).

Day_3+: follicles of females that spawned 3 nights or more before capture, however, in Adriatic by 3 days after spawning all post-ovulatory follicles have been resorbed due to the high water temperatures).

Actively spawning anchovy (Day_0 females) are not generally used for spawning frequency estimates since are oversampled prior to, or during, the hours of spawning (Picquelle and Stauffer, 1985; Ganias, 2003), thus only Day_1 and Day_2 females are used.

1.2.4 Daily egg production estimation Parameter estimation generally followed procedures described in (Picquelle & Stauffer, 1985). Age of eggs was calculated based on a temperature dependent model of European anchovy developmental rate (Regner, 1985), the station surface temperature (5 m), peak spawning time (10 p.m. in Adriatic; Regner 1985), and time of tow (Lo, 1985). Due to the high surface temperatures characterizing most stations, the duration of the egg stage was generally very short (<2 days). Thus, eggs could easily be grouped into ''spawning nights''; distribution of eggs over the different developmental stages formed distinct groups (one or two) with unrepresented stages separating each group. Eggs younger than 2 h and older than 90% of the expected hatching time were excluded to avoid possible biases caused by incomplete recruitment of eggs to the plankton or hatching. The estimation of the daily egg production (P) generally involves the fit of an exponential mortality model to the abundance at age egg data set (Picquelle & Stauffer, 1985). To increase the number of age categories for constructing the mortality curves, we assumed that the mortality rate (Z) was the same for eggs and yolk-sac larvae, and we included both in single embryonic mortality curves (Lo et al., 1996; Hunter & Lo, 1997). The estimate of daily production of eggs was derived by regressing the counts of embryos (eggs and yolk-sac larvae) on their age using the exponential mortality model:

$$P_t = Pe^{-Zt}$$

where P_t is the number of embryos (eggs or yolk-sac larvae) at age t produced per day per m^2, t the age in days, P the daily egg production per m^2 and Z the daily rate of instantaneous embryonic mortality. We used both yolk-sac larvae stages (YSI and YSII) and calculated their duration and their age from fertilization, from temperature-dependent

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curves given in Regner (1985), assuming station surface temperatures (5 m) as the yolk-sac larvae incubation temperatures. Stage durations were used to calculate the daily production of YSI and YSII larvae (Lo et al., 1996). The technique to estimate P and Z was weighted non-linear least squares regression. We used the ratio estimator (Picquelle and Stauffer, 1985) for adult parameters like: average weight of mature females (W), weight specific sex ratio (R), batch fecundity (F) and spawning frequency (S):

$$\bar{\bar{y}} = \frac{\sum_{i=1}^{n} m_i \bar{y}_i}{\sum_{i=1}^{n} m_i} \qquad (1)$$

with sample variance

$$\widehat{Var}(\bar{\bar{y}}) = \frac{\sum_{i=1}^{n} m_i^2 (\bar{y}_i - \bar{\bar{y}})^2}{[\sum_{i=1}^{n} m_i/n]^2 n(n-1)}$$

where $\bar{\bar{y}}$ is the estimate of the population mean, n the number of stations, $\bar{y}_i = \sum_{j=1}^{m_i} y_{ij}/m_i$ the mean of the ith station, m_i the number of fish sampled from the ith catch and y_{ij} the value for the jth female in the ith sample. Data on the number of eggs per batch (F_{ij}) and the ovary free weight (W^*_{ij}) recorded for the hydrated females were used to fit a linear model:

$$F_{ij} = a + bW^*_{ij} \qquad (2)$$

The variance estimator of the batch fecundity was (Draper & Smith, 1966):

$$\widehat{Var}(\bar{\bar{F}}) = \frac{\sum_{i=1}^{n} m_i^2 \left[\frac{(\bar{F}_i - \bar{\bar{F}})^2}{n-1} + \frac{s_h^2}{n_h} + (\overline{W_i^*} - \overline{W_h^*})\widehat{Var}(\hat{b}) \right]}{\left[\sum_{i=1}^{n} \frac{m_i}{n} \right]^2 n}$$

Where $(\bar{\bar{F}})$ is the estimate of batch fecundity for the whole population of mature females, $\bar{F}_i$ the average batch fecundity of the ith sample, $\bar{F}_i = \sum_{j=1}^{m_i} \hat{F}_{ij}/m_i$, where $\hat{F}_{ij}$ is the estimated batch fecundity for the jth female in the ith sample, s_h^2 the variance about the regression (Eq. (2)), n_h the number of hydrated females used to fit the regression (Eq. (2)), $\overline{W_i^*}$ the

average ovary-free weight of the ith sample, $\overline{W_h^*}$ the average ovary-free weight of the n_h hydrated females and $\widehat{Var}(\hat{b})$ the variance of the slope of the regression (Eq. (2)). Actively spawning anchovy (hydrated and Day_0 females) are oversampled during the hours of spawning (Picquelle and Stauffer, 1985). To compensate for the bias in sampling Day_0 spawners, the m_i in Eq. (1) was replaced by (Picquelle and Stauffer, 1985):

$$m_i^* = 2m_i^1 + m_i^2$$

where m_i^1 is the Day_1 spawners and m_i^2 is the Day_2 spawners (females with POF-2 or without POFs in their gonads).

1.3 Results

The distribution and abundance of anchovy eggs and yolk-sac larvae is shown in Fig. 1.5; concerning eggs, there were very few negative stations in the periphery of the survey area and within the Gulf od Manfredonia, instead regarding yolk-sac larvae, there were more negative stations throughout the area of sampling, but taking into account eggs and yolk-sac larvae together, the entire area has been considered positive with regard to anchovy embryos.

1.3.1 Daily egg production Daily egg production was estimated using both eggs and yolk-sac larvae. To estimate the daily egg production (P) and the mortality rate (Z) have been sorted and staged 960 eggs in 2012, 511 in 2013 and 459 eggs in 2014; in 2012 have been found 278 yolk-sac larvae, 186 in 2013 and 214 in 2014. Values of P ranged between 64.48 eggs m^{-2} in 2012 and 30.45 eggs m^{-2} in 2014, the mortality rate displayed the same trend with values of 0.47 in 2012 and 0.19 in 2014 (Fig. 1.6A and Table 1.2); thus daily egg production and mortality rate decreased during the three years of sampling. The precision of the estimates of P and Z are in line with the results of the previous surveys in the Mediterranean Sea, with the exception of the coefficient of variation (CV) of mortality

rate in 2014 which is very high (for comparison with values of previous surveys see Table 1.1).

1.3.2 Adult parameters Summarized data on adult samples (values and coefficients of variation for mean weight of mature females, sex ratio by weight, spawning frequency and batch fecundity) are given in Table 1.3. The weight of mature females remained stable throughout years (Fig. 1.6B). The sex ratio varied between 0.48 in 2013 and 0.60 in 2014 and, in this year, the ratio is shift towards females (Fig. 1.6C) To find out the spawning frequency three types of POFs were detected in the histological sections analysing 97 gonads, 100 and 113 during the three years of sampling. The spawning frequency decrease during the three years, in fact, in 2012 anchovies spawned every 2.4 days, in 2013 every 2.9 day and in 2014 every 3 days (Fig. 1.6D).

Batch fecundity was measured on all the running females that have been found during the sampling, a total of 16 fishes have been examined in 2012, 25 in 2013 and only 9 in 2014; batch fecundity ranged between 2817 eggs per spawning batch in 2012 and 3343 in 2013, with and increasing trend through years but, due to the very low number of hydrated females found, this values had high uncertainty (Fig. 1.6E and Table 1.3).

1.4 Discussion and conclusion

The characteristic of the estimated DEPM parameters for anchovy in the Mediterranean Sea displayed high inter-regional and inter-annual variability (Tables 1.1), sometimes due to differences in methodological procedure and sometimes due to large spatial and inter-annual variations in biological productivity characteristic of the Mediterranean (Somarakis et al., 2004; Somarakis, 2005). Some marked inter-regional or inter-annual differences in anchovy reproductive parameters were observed in the more southern Mediterranean areas, such as the central Aegean and Ionian Seas or the Sicilian channel, where small and heterogeneous stocks of anchovy exhibit patchy distributions (Somarakis et al., 2004). In this work sampling procedures and subsequent laboratory and analytical methods have been identical during the three years of analysis, thus the between-year comparison of the estimated parameters is allowed to assume that the observed heterogeneity could be caused by natural variability of the estimated factors. Estimates of daily egg production (P) displayed a decreasing trend from 2012 to 2014 being double in 2012 respect 2014 and the mortality rate (Z) showed an opposite trend with higher value in 2012 and very low rate in 2014 (Table 1.2, Fig. 1.6A). This data agreed with those reported by García & Palomera (1996) who found, in the western Mediterranean, a relationship between egg production and mortality rate which indicated high mortality values associated with high egg production. Values of P of the present study were similar to those of the previous surveys in Mediterranean Sea, instead, Z values were lower than the average, especially in 2014, where value is the lowest respect all the previous surveys. The mortality rate of the present study show high uncertainty (CV = 0.47 in 2012, CV = 0.41 in 2013, CV = 1.32 in 2014) probably due to a not adequate number of egg data. Despite the addition of yolk-sac larvae substantially improved the precision of the estimate of daily egg production and allowed a sufficient estimation of P and Z, the effect of high average sub-surface temperatures

characterizing the south western Adriatic Sea (Table 1.1) greatly ''reduces'' available data points in the egg data set; indeed egg developmental times are very short (generally <2 days at peak spawning season) with the occurrence of only one or two daily cohorts of eggs in the samples. Estimates of spawning frequency (S) displayed slight decrease from 2012 to 2014 (Fig. 1.6D), specifically female anchovies spawned with lower frequency (large inter-spawning interval), contrarily, batch fecundity (F) exhibited an increasing trend during the years (Table 1.3, Fig. 1.6E). Comparing this parameters in relation to food supply we observed that adult food availability (zooplankton abundance) was higher in 2012 and lower in 2014 (Fig. 1.6F). In many small pelagic fishes energy is derived primarily from feeding rather than from energy reserves; planktivorous short-lived small pelagic species, such anchovies, are 'income breeder', namely, they spawning soon after energy for egg production becomes available (Somarakis et al., 2000; Mcbride et al., 2015). In this species, it has been shown that spawning frequency is very sensitive to variations in food supply, instead, batch fecundity is less, so we can hypothesize that spawning frequency respond more rapidly to fluctuations in food availability than batch fecundity. However, the effects of extrinsic factors such as feeding conditions on batch fecundity of indeterminate spawners have not been extensively studied, probably due to the difficulties in conducting controlled studies on small pelagic fish in aquaria over the necessary time periods (Armstrong & Witthames, 2012). The pretty high uncertainty of the estimate batch fecundity (CV = 0.38 in 2012, CV = 0.29 in 2013. CV = 0.57 in 2014) was probably due to the small numbers of running females that have been found during the sampling. Lastly, coupled with a decline in daily egg production and spawning frequency, we observed an overall decrease of spawning stock biomass from 2012 to 2014 that seemed to be associated with a corresponding changes and decline of food availability. The estimated SSB have been relatively high in 2012 (9072 tons), but it decreased to 4325 tons in 2014 (Table 1.3). According to the basin hypothesis of MacCall (1990), in period of

high abundances the distribution of stocks expands, contrarily, with low abundances, the distribution of stocks shrinks and spawning would be practically restricted to the more favorable spawning sites. However the decline of spawning biomass, in this study, have not been coupled with a contraction of the spawning area or vice versa (Fig. 1.5). The same situation has been found by Somarakis et al., (2004) in Mediterranean waters, suggesting that these suitable anchovy spawning habitats are spatially restricted and separated from each other by deep and extremely oligotrophic basins, which would not be likely to support anchovy feeding and reproduction; given this heterogeneity of adjacent Mediterranean basins, the expansion of spawning areas at high stock densities would not be beneficial for Mediterranean stocks.

The ability to use egg production surveys to obtain SSB estimates for spatially discrete spawning grounds occupied by sub-stocks or metapopulations provides a powerful tool to support ecosystem-based fishery management. The high CV of spawning stock biomass (CV = 0.42 in 2012, CV = 0.34 in 2013. CV = 0.64 in 2014) has been due mostly to an uncertainty about mortality rate and batch fecundity, therefore, because of these uncertainties, the estimates from the application of DEPM should not use directly in assessment as an index of biomass nowadays. However, extending the use of the survey results, besides biomass estimation, can provide valuable time series on the extension and characteristics of spawning habitats and on important reproductive parameters of fish stocks like knowledge of fecundity and its relationship with fish size and age. The present trends towards maximizing the information provided by expensive large-scale surveys means that the arguments for egg production survey are strengthened due to their ability to provide major insights into fish productivity in contrasting areas of productivity and environmental conditions (Somarakis et al., 2004; Armstrong & Witthames, 2012) and to provide direct evidence of how environmental change could affect the distribution and productivity of fish stocks.

References

Agostini, V.N., Bakun, A., 2002. " Ocean triads " in the Mediterranean sea : physical mechanisms potentially structuring reproductive habitat suitability (with example application to European anchovy, *Engraulis encrasicolus*). *Fisheries Oceanography*, 11 (3), 129–142.

Armstrong, M.J., Connolly, P., Nash, R.D.M., Pawson, M.G., Alesworth, E., Coulahan, P.J., Dickey-Collas, M., Milligan, S.P., O'Neill, M.F., Witthames, P.R., Woolner, L., 2001. An application of the annual egg production method to estimate the spawning biomass of cod (*Gadus morhua* L.), plaice (*Pleuronectes platessa* L.) and sole (*Solea solea* L.) in the Irish sea. *ICES Journal of Marine Science*, 58 (1), 183–203. DOI: 10.1006/jmsc.2000.1001.

Armstrong, M.J., Witthames, P.R., 2012. Developments in understanding of fecundity of fish stocks in relation to egg production methods for estimating spawning stock biomass. *Fisheries Research*, 117–118, 35–47. DOI: 10.1016/j.fishres.2010.12.028.

Barange, M., Bernal, M., Cercole, M.C., Cubillos, L.A., Daskalov, G.M., de Moor (formerly Cunningham), C.L., De Oliveira, J.A.A., Dickey-Collas, M., Gaughan, D.J., Hill, K., Jacobson, L.D., Köster, F.W., Massé, J., Ñiquen, M., Nishida, H., Oozeki, Y., Palomera, I., Saccardo, S.A., Santojanni, A., Serra, R., Somarakis, S., Stratoudakis, Y., Uriarte, A., van der Lingen, C.D., Yatsu, A., 2009. Current trends in the assessment and management of stocks. *In: Climate Change and Small Pelagic Fish. Checkley, D.M, Oozeki, Y., Roy, C., Alheit, J. (Eds.), Cambridge University Press,* 191–255.

D'Ancona, U., 1956. Uova, larve e stadi giovamili di teleostei. *In: Fauna e flora del Golfo di Napoli. Bertolini, F., D'ancona, U., Montalenti, G., Padoa, E., Ranzi, S., Sanzo, L., Spartà, A., Tortonese, E., Vialli, M.*

Draper, N.R., Smith, H., 1966. Applied Regression Analysis. *Wiley Online Library, New York*.

Ganias, K., Somarakis, S., Machias, A., Theodorou, A.J., 2003. Evaluation of spawning frequency in a Mediterranean sardine population (*Sardina pilchardus sardina*). *Marine*

Biology, 142, 1169–1179.

García, A., Palomera, I., 1996. Anchovy early life history and its relation to its surrounding environment in the Western Mediterranean basin. *Scientia Marina*, 60, 155–166.

Gunderson, D.R., 1993. *Surveys of Fisheries Resources*, 248 pp.

Hunter, J.R., Lo, N.C.H., Leong, R.J.H., 1985. Batch fecundity in multiple spawning fishes. *In: An Egg Production Method for estimating spawning biomass of pelagic fish: application to the Northern Anchovy, Engraulis mordax. Lasker, R. (Ed), NOAA Technical Report*, 36, 67–77.

Hunter, J.R., Macewicz, B.J., 1985. Rates of atresia in the ovary of captive and wild Northern anchovy; *Engraulis Mordax. Fishery Bulletin*, 83 (2), 119–136.

Hunter, J.R., Lo, N.C.H., 1993. Ichthyoplankton methods for estimating fish biomass. Introduction and terminology. *Bulletin of Marine Science*, 53, 723–727.

Hunter, J.R., Lo, N.C.H., 1997. The daily egg production method of biomass estimation: some problems and potential improvements. *In: Improvements of Daily Egg Production Method techniques. Motos, L. (Ed.), Ozeanografika*, 2, 41–69.

Jacobson, L.D., Lo, N.C.H., Barnes, J.T., 1994. A biomass-based assessment model for Northern anchovy, *Engraulis mordax. Fishery Bulletin US*, 92 (4), 711–724.

Lasker, R., 1985. An Egg Production Method for estimating spawning biomass of pelagic fish : application to the Northern anchovy, *Engraulis mordax. NOAA Technical Report, 36*.

Leonori, I., De Felice, A., Biagiotti, I., Canduci, G., Costantini, I., Giuliani, G., Malavolti, S., Gabrielli, G., Miccoli, A., Caccamo, G., Penna, P., Grilli, F., Paschini, E., 2016. Programma Nazionale Italiano per la Raccolta Dati Alieutici 2014-2016 EC-DCR– MIPAAF Anno 2015. Campagna di ricerca in mare Sezione G - MEDIAS GSA 18 - *Rapporto Finale. CNR - ISMAR, Ancona, Italia,* 75 pp.

Lo, N.C.H., 1985. A model for temperature-dependent northern anchovy egg development and an automated procedure for the assignment of age to staged eggs. *In: An Egg Production*

Method for estimating spawning biomass of pelagic fish: application to the Northern Anchovy, Engraulis mordax. Lasker, R. (Ed). NOAA Technical Report, 36, 43–50.

Lo, N.C.H., Green-Ruiz, Y.A., Cervantes, M.J., Moser, H.G., Lynn, R.J., 1996. Egg production and spawning biomass of Pacific sardine (*Sardinops sagax*) in 1994, determined by the Daily Egg Production Method. *CalCOFI Report*, 37, 160–174.

MacCall, A.D., 1990. *Dynamic geography of marine fish populations.* 163 pp.

Magoulas, A., Tsimenides, N., Zouros, E., 1996. Mitochondrial DNA phylogeny and the reconstruction of the population history of a species: the case of the European anchovy (*Engraulis encrasicolus*). *Molecular, Biology and Evolution*, 13, 178–190.

Magoulas, A., Castilho, R., Caetano, S., Marcato, S., Patarnello, T., 2006. Mitochondrial DNA reveals a mosaic pattern of phylogeographical structure in Atlantic and Mediterranean populations of anchovy (*Engraulis encrasicolus*). *Molecular Phylogenetics and Evolution*, 39 (3), 734–746. DOI: 10.1016/j.ympev.2006.01.016.

Marano, G., Casavola, N., Rizzi, E., De Ruggeri, P., Lo Caputo, S., 1998. Valutazione delle risorse pelagiche, consistenza dello stock di sardine e alici nell'Adriatico Meridionale. Anni 1984-1996. *Biologia Marina Mediterranea*, 5 (3), 313–320.

Mcbride, R.S., Somarakis, S., Fitzhugh, G.R., Albert, A., Yaragina, N.A., Wuenschel, M.J., Alonso-Fernández, A., Basilone, G., 2015. Energy acquisition and allocation to egg production in relation to fish reproductive strategies. *Fish and Fisheries*, 16 (1), 23–57. DOI: 10.1111/faf.12043.

Motos, L., 1996. Reproductive biology and fecundity of the Bay of Biscay anchovy (*Engraulis Encrasicholus* L.). *Scientia Marina*, 60, 195-207.

Olivar, M.P., Jordi, S., Palomera, I., 2001. Comparative study of spatial distribution patterns of the early stages of anchovy and pilchard in the NW Mediterranean Sea. *Marine Ecology Progress Series*, 217, 111–120.

De Oliveira, J.A.A., Roel, B.A., Dickey-Collas, M., 2006. Investigating the use of proxies for

fecundities to improve managment advice for western horse makerel *Trachurus trachurus*. *ICES Journal of Marine Science*, 63, 25–35. DOI: 10.1016/j.icesjms.2005.07.006.

Palomera, I., Pertierra, P., 1993. Anchovy biomass estimate by daily egg production method in 1990 in the western mediterranean Sea. *Scientia Marina*, 57 (2–3), 243–251.

Parker, K., 1980. A direct method for estimating Northern anchovy, *Engraulis mordax*, spawning biomass. *Fishery Bulletin US*, 78, 541–544.

Picquelle, S.J., Stauffer, G., 1985. Parameter estimation for an egg production method of anchovy biomass assessment. *In: An Egg Production Method for estimating spawning biomass of pelagic fish: application to the Northern anchovy, Engraulis mordax. Lasker, R. (Ed.), NOAA Technical Report, 36, 7–16.*

Quintanilla, L., García, A., 2001. Daily egg production method for estimating Sicilian Channel anchovy spawning biomass in 1998 and 1999. *Rapports de la Communité Internationalle du mer Mediterranée*, 36, 7–15.

Regner, S., 1985. Ecology of planktonic stages of the anchovy *Engraulis encrasicolus* in the Central Adriatic. 5-113 pp.

Ruggeri, P., Splendiani, A., Occhipinti, G., Fioravanti, T., Santojanni, A., Leonori, I., De Felice, A., Arneri, E., Procaccini, G., Catanese, G., Ticina, V., Bonanno, A., Nisi Cerioni, P., Giovannotti, M., Grant, WS., Caputo Barucchi, V., 2016. Biocomplexity in populations of european anchovy in the Adriatic Sea. *PLoS One*, 11 (4), 1–21. DOI: 10.1371/journal.pone.0153061.

Sanz, A., Uriarte, A., 1989. Reproductive cycle and batch fecundity of the bay of Biscay anchovy (*Engraulis encrasicolus*) in 1987. *CalCOFI Rep.*, 30, 127–135.

Simmonds, E.J., MacLennan, D.N., 2005. *Fisheries Acoustics: Theory and Practice, II ed.* 429 pp.

Somarakis, S., 1999. Ichthyoplankton of the NE Aegean Sea with emphasis on anchovy, *Engraulis encrasicolus* (L., 1758) (June 1993, 1994, 1995, 1996). PhD thesis, *University of*

Crete (in Greek).

Somarakis, S., Tsimenides, N., 1997. A daily egg production biomass estimate of the northern Aegean Sea anchovy stock. *In: Improvements of Daily Egg Production Method techniques. Motos, L. (Ed.), Ozeanografika*, 2, 133–148.

Somarakis, S., Maraveya, E., Tsimenides, N., 2000. Multispecies Ichthyoplankton associations in epipelagic species: is there any intrinsic adaptive function? *Belgian Journal of Zoology*, 130 (SUPPL. 1), 125–129.

Somarakis, S., Koutsikopoulos, C., Machias, A., Tsimenides, N., 2002. Applying the daily egg production method (DEPM) to small stocks in highly heterogeneous seas. *Fisheries Research*, 55 (1–3), 193–204. DOI: 10.1016/S0165-7836(01)00284-3.

Somarakis, S., Palomera, I., Garcia, A., Quintanilla, L., Koutsikopoulos, C., Uriarte, A., Motos, L., 2004. Daily egg production of anchovy in European waters. *ICES Journal of Marine Science*, 61 (6), 944–958. DOI: 10.1016/j.icesjms.2004.07.018.

Somarakis, S., 2005. Marked interannual differences in reproductive parameters and daily egg production of anchovy in the northern Aegean Sea. *Belgian Journal of Zoology*, 135 (2), 247–252.

Somarakis, S., Ganias, K., Siapatis, A., Koutsikopoulos, C., Machias, A., Papacostantinou, C., 2006. Spawning habitat and daily egg production of sardine (*Sardina pilchardus*) in the eastern Mediterranean. *Fisheries Oceanography*, 15 (4), 281–292. DOI: 10.1111/j.1365-2419.2005.00387.x.

Somarakis, S., Schismenou, E., Siapatis, A., Giannoulaki, M., Kallianiotis, A., Machias, A., 2012. High variability in the Daily Egg Production Method parameters of an eastern Mediterranean anchovy stock : influence of environmental factors, fish condition and population density. *Fisheries Research*, 117–118, 12–21. DOI: 10.1016/j.fishres.2010.11.027.

Stauffer, G.D., Picquelle, S.J., 1980. Estimates of the 1980 spawning biomass of Northern

anchovy. *National Marine Fisheries Service, Southwest Fisheries Science Center, La Jolla, CA*, Admin. Rep.

Stratoudakis, Y., Bernal, M., Uriarte, A., 2004. The DEPM estimation of spawning-stock biomass for sardine and anchovy. *ICES Cooperative Research Report*, 268, 91.

Stratoudakis, Y., Bernal, M., Ganias, K., Uriarte, A., 2006. The daily egg production method: recent advances, current applications and future challenges. *Fish and Fisheries*, 7 (1), 35–57. DOI: 10.1111/j.1467-2979.2006.00206.x.

Figures and tables
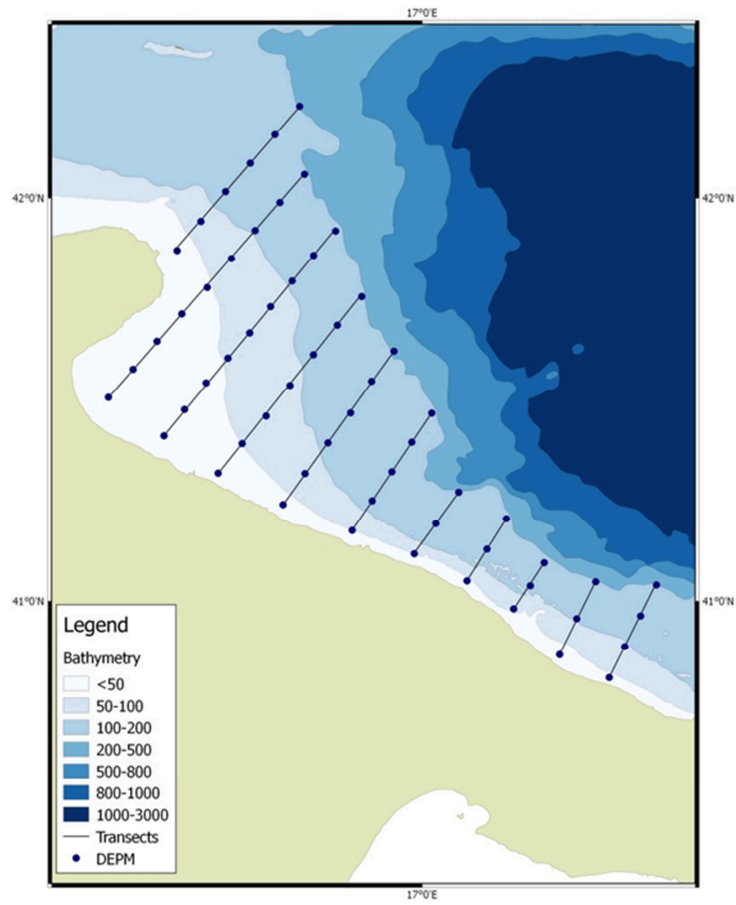
Figure 1.1. Ichthyoplankton sampling design (the same for the three years)
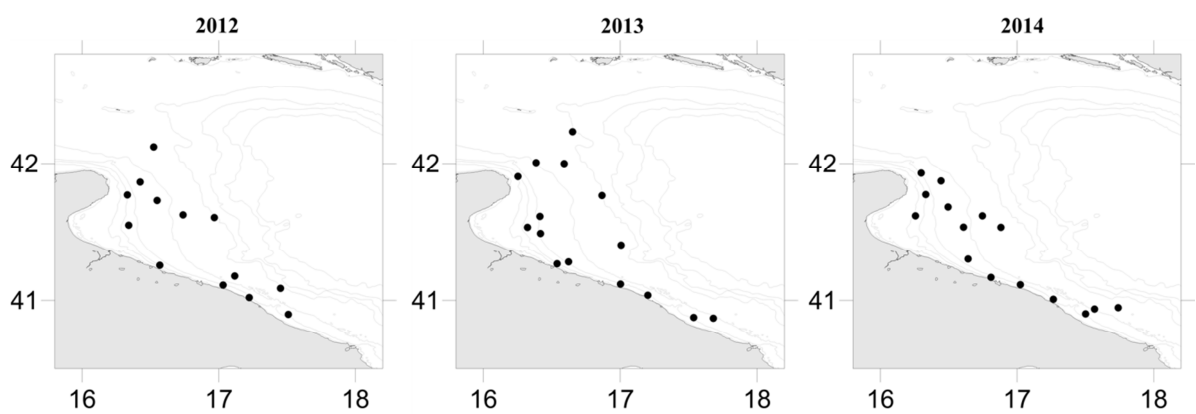
Figure 1.2. Pelagic trawl sampling
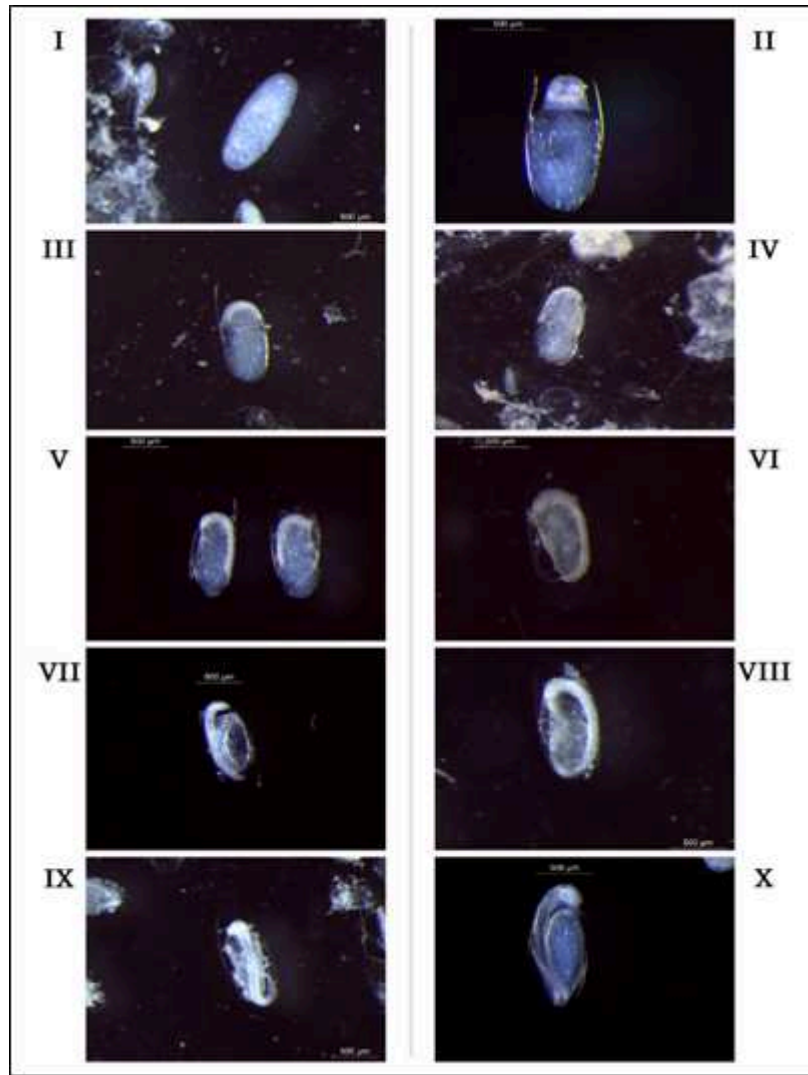
Figure 1.3. Upper panel) Stages of anchovy eggs. Lower panel) Yolk-sac I, yolk-sac II and larval stages
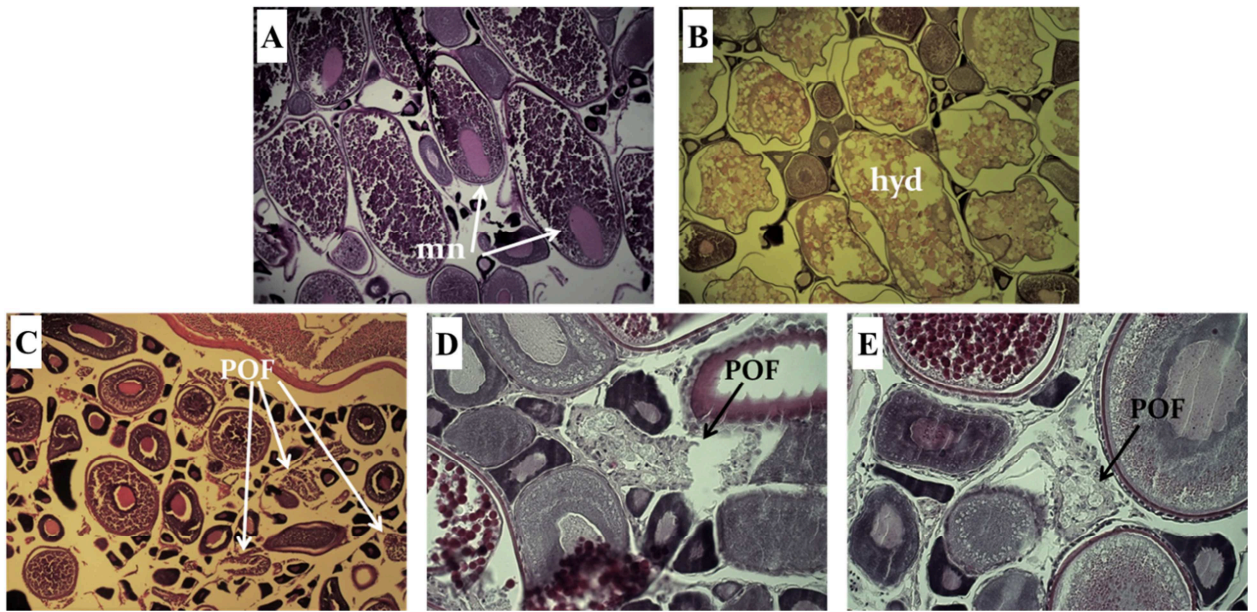
Figure 1.4. Histological sections of females gonads: A) migratory nucleo (mn), B) hydrated female, C) POF-0, D) POF-1, E) POF-2

Figure 1.5. Bubble plots with abundances of anchovy eggs and yolk-sac larvae

Figure 1.6. Trends of DEPM parameters during the three years of sampling and zooplankton abundance

Table 1.1. Spawning Biomass and DEPM parameters estimates for anchovy in the Mediterranean (CVs: coefficient of variation in parentheses) (Stratoudakis et al., 2004)

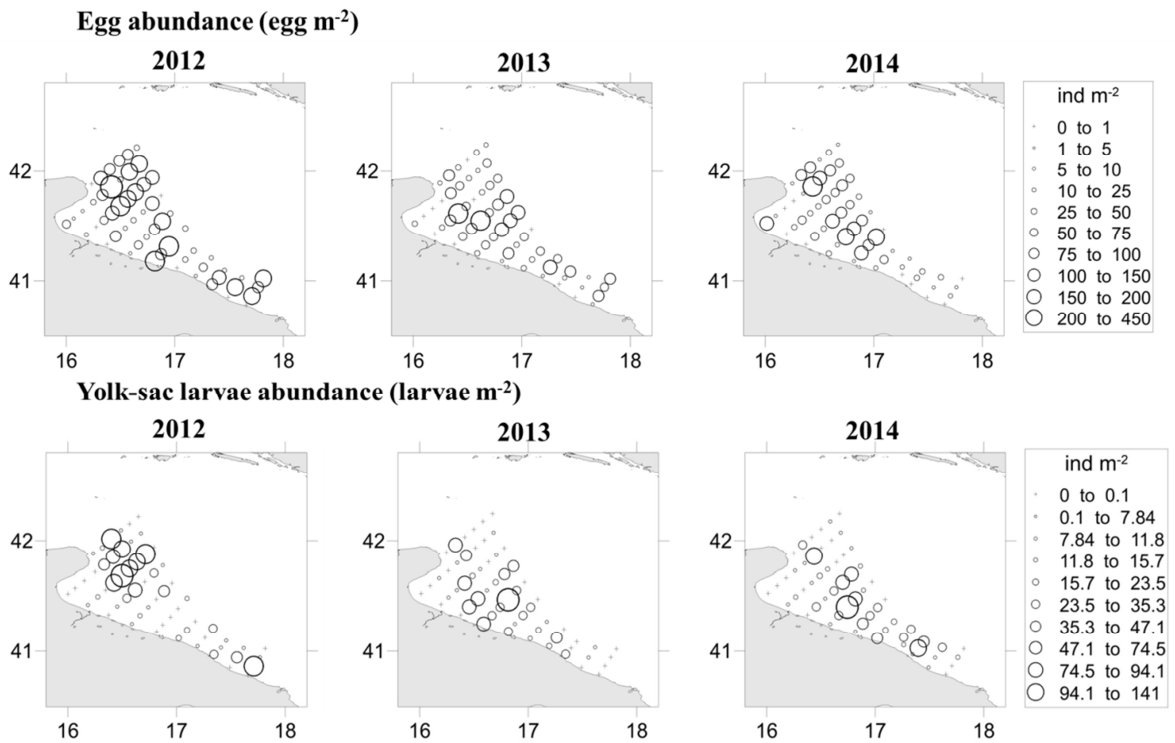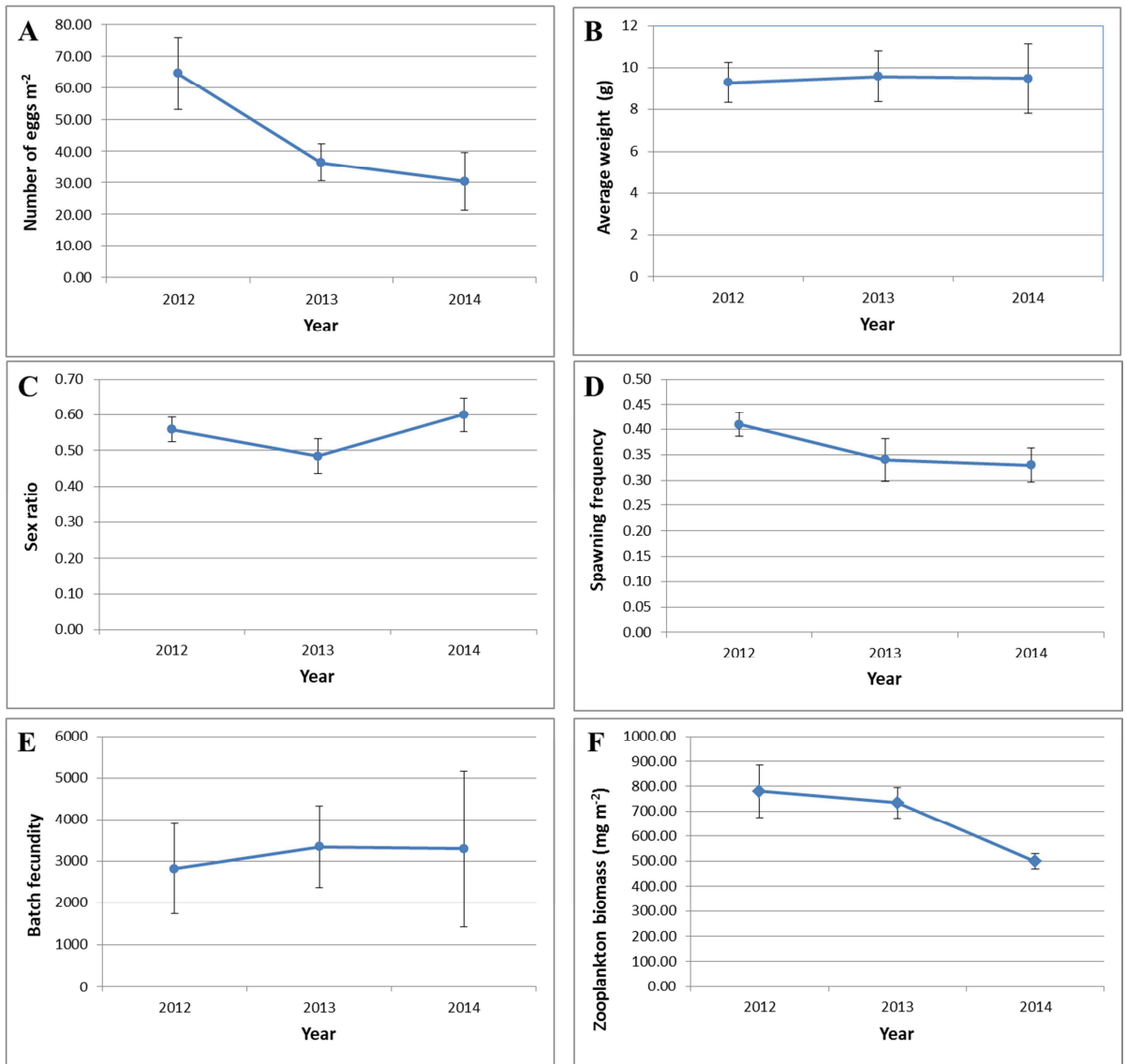|				Egg Parameters						Adult Parameters						
		T^a	A	A_1	P_1	P	Z	P_t	F	S	W	R	RF	DSF	SF	B
SICILIAN CHANNEL	Jun-Jul 1998	18.5 22.5	13295	5329	65.55 (0.21)	26.27 (0.33)	1.63 (0.33)	0.14 (0.33)	4835 (0.16)	0.14 (0.12)	15.18 (0.07)	0.59 (0.12)	319	26	7	13224 (0.22)
	Jun 1999	18.4 22.7	5878	2692	45.86 (0.22)	21.00 (0.32)	1.25 (0.33)	0.05 (0.32)	5871 (0.11)	0.17 (0.10)	14.08 (0.08)	0.55 (0.10)	417	39	6	3138 (0.31)
	Jun-Jul 2000	16.3 25.8	11812	4505	34.98 (0.15)	13.34 (0.24)	2.07 (0.20)	0.06 (0.24)	8379 (0.06)	0.20 (0.28)	18.90 (0.04)	0.62 (0.08)	443	55	5	2850 (0.46)
CATALAN SEA	May 1990	17.6 19.6	17081	8095	120.61 (0.15)	57.16 (0.29)	0.56 (0.44)	0.46 (0.22)	8006 (0.02)	0.36 (0.10)	14.25 (0.04)	0.54 (0.09)	562	110	3	4199 (0.26)
	Jul 1990								7283 (0.12)	0.31 (0.16)	12.79 (0.10)	0.56 (0.10)	569	99	3	
CATALAN SEA & GULF OF LIONS	July 1993	13.3 22.5	44554	33012	86.67 (0.15)	64.22 (0.17)	1.09 (0.26)	2.12 (0.17)	4958 (0.11)	0.31 (0.13)	14.31 (0.07)	0.64 (0.05)	346	69	3	30849 (0.30)
	May-Jun 1994	15 22.0	42085	31692	81.71 (0.18)	61.53 (0.21)	0.47 (0.26)	1.95 (0.21)	7039 (0.02)	0.21 (0.20)	22.92 (0.06)	0.59 (0.19)	307	38	5	52557 (0.36)
LIGURIAN & TYRRHENIAN	July 1993	18.9 22.5	15424	8221	93.57 (0.28)	49.87 (0.32)	0.86 (0.34)	0.41 (0.32)	4894 (0.10)	0.32 (0.11)	14.17 (0.07)	0.63 (0.05)	345	70	3	5829 (0.36)
AEGEAN SEA	Jun 1993	16.7 25.0	17396	17396	259.49 (0.32)	259.49 (0.32)	1.04 (0.46)	4.51 (0.32)	11542 (0.04)	0.28 (0.15)	22.73 (0.02)	0.55 (0.04)	508	78	4	58988 (0.35)
	Jun 1999	18.0 25.0	8604	8604	13.29 (0.39)	13.29 (0.39)	0.53 (0.48)	0.11 (0.39)	4725 (0.05)	0.13 (0.21)	15.77 (0.03)	0.47 (0.09)	300	18	8	6273 (0.43)
IONIAN SEA	May-Jun 1999	18.0 25.0	12362	12362	8.88 (0.24)	8.88 (0.24)	0.52 (0.36)	0.10 (0.24)	9428 (0.08)	0.06 (0.26)	15.60 (0.05)	0.53 (0.07)	604	19	17	5588 (0.33)
SW ADRIATIC	Jul-Aug 1994		14790	9244	50.11 (0.16)	31.32 (0.10)	0.55 (0.12)	0.29 (0.10)	11866 (0.03)	0.16 (0.08)	18.57 (0.03)	0.55 (0.05)	639	56	6	8129 (0.24)

T^a	Temperature range (ºC)		F	Batch fecundity
A	Total survey area (km^2)		S	Spawning fraction
A_1	Positive stratum area (km^2)		W	Mean females weight
P_1	Daily egg production per m^2 registered in the positive stratum		R	Sex ratio
P	Daily egg production per m^2 registered in the whole sampled area		RF	Relative fecundity, ratio between F and the W
Z	Daily rate of instantaneous mortality		SF	Spawning frequency
P_T	Daily egg production for the whole sampled area (eggs/day $*10^{-12}$)		DSF	Daily specific fecundity (DSF = FSR/W)
			B	Spawning biomass

Table 1.2. Average temperature at 5 meters, survey area, egg and yolk-sac parameters, mortality estimates (CVs in parentheses)

	2012	2013	2014
Average temperature (°C)	25.80	25.13	24.63
Survey area (km^2)	9776.7	9776.7	9776.7
Plankton samples	58	58	58
Nember of eggs	960	511	459
Number of yolk-sac larvae	278	186	214
Daily egg production (P, egg m^{-2})	64.48 (0.18)	36.41 (0.16)	30.45 (0.30)
Mortality (Z)	0.47 (0.43)	0.42 (0.41)	0.19 (1.32)

Table 1.3. DEPM adult parameters and spawning biomass estimates (CVs in parentheses)

	2012	2013	2014
Average weight of mature females (W, g)	9.28 (0.11)	9.58 (0.13)	9.47 (017)
Weight specific ratio (R)	0.56 (0.06)	0.48 (0.10)	0.60 (0.08)
Spawning fraction of mature females (S)	0.41 (0.06)	0.34 (0.13)	0.33 (0.10)
Batch fecundity (F, number of eggs)	2817 (0.38)	3343 (0.29)	3292 (0.57)
Spawning stock biomass (SSB, tons)	9072.4 (0.42)	6201.6 (0.34)	4325.3 (0.64)

Distribution of *Engraulis encrasicolus* eggs and larvae in relation to coastal oceanographic conditions (a south-western Adriatic Sea case study)

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2.1 Introduction

Small pelagic fishes such as anchovies and sardines (Clupeiformes) play a significant role in shaping the structure of marine ecosystems and constitute an intermediate trophic level in the marine food web (Coll et al., 2007). Since they act as "wasp-waist" species, exerting top-down control on zooplankton and bottom-up control on top predators, strong fluctuations in their populations have the ability to modify ecosystem structure and functioning (Cury et al., 2000).

Small pelagic fish species are characterized by a short lifespan and a reproductive strategy that involves production of large quantities of pelagic eggs over an extended season; such features make them susceptible to environmental variability, food availability, and physical processes (Bakun, 1996; Cushing, 1996; Giannoulaki et al., 2013).

These species also have an important economic role, since they are target species worldwide; for instance, in 2009 anchovies accounted for 22% of total marine catches (FAO, 2014). Most of these catches come from upwelling zone areas in the eastern Atlantic and eastern Pacific Ocean, where the landing of small pelagic fishes is dominated by a limited number of species (Fréon et al., 2005).

The Adriatic Sea is rich in small pelagic fish. It is one of the richest basins in the Mediterranean and by far the most important fishing ground among the Italian seas, providing more than 66% of the landings of small pelagic species of Italian fisheries (IREPA Onlus 2012). The chief species are European anchovy (*Engraulis encrasicolus*), European sardine (*Sardina pilchardus*), horse mackerel (*Trachurus* spp.), Atlantic mackerel (*Scomber scombrus*), and Chub mackerel (*Scomber japonicus*). Anchovy and sardine account for than 40% of the total catch of the Adriatic Sea (FAO, 2016). *E. encrasicolus* is a coastal, pelagic and euryhaline fish with a broad distribution in the Mediterranean and adjacent seas (Black Sea and Sea of Azov) and in the eastern Atlantic,

from Scandinavia to the Gulf of Guinea and South Africa (Whitehead et al., 1988; Bellido et al., 2000). It is characterized by high growth rates and early maturity, which is attained at the end of the first year of life (Sinovčić & Zorica, 2006). The species is a serial batch spawner with indeterminate fecundity; in the Adriatic Sea, it reproduces in coastal areas from April to October, egg production peaking in June-July (Regner, 1996; Morello & Arneri, 2009).

Data on anchovy egg and larval distribution have been reported by several studies in a number of areas. In the Bay of Biscay spawning occurs close to the coast, in highly stratified river plumes, or at the shelf break, and seems to be constrained by temperature, especially bottom temperature (Planque et al., 2007). In the Benguela ecosystem, where the species shows a spatially structured spawning pattern, habitat selection is also driven by environmental conditions, most often temperature (van der Lingen et al., 2001). In the north-western Mediterranean Sea anchovy prefer areas under the influence of the continental water runoff; the eggs and larvae show relatively low abundance in shallow waters close to the coast (< 50 m) and are densest near the edge of the shelf (ca. 200 m) (García & Palomera, 1996; Palomera et al., 2007). In the Strait of Sicily egg abundance was maximum within a depth of 100 m, where shallow waters with upwelling (low temperature, high water density, high chlorophyll-a concentration) and moderate current speed appear to be preferred for spawning (Basilone et al., 2013). In the Gulf of Tunis spawning areas and larval abundance seem to be related to the central part of the area beyond the 75 m isobaths, where productivity is enhanced by rivers, and the main factor controlling spawning intensity seems to be sea surface temperature (Zarad et al., 2006). In the eastern Mediterranean anchovy spawning occurs in river plumes and/or upwelling areas, which may be less saline and more productive than other areas and eggs and larvae are associated with a broad range of salinity, temperature, and water density (Somarakis & Nikolioudakis, 2007).

The Adriatic Sea is characterized by a cyclonic circulation with two branches, the East Adriatic Current (EAC) flowing northward along the eastern coast (Marini et al., 2010) and the West Adriatic Current (WAC) flowing southward along the western coast (Artegiani et al., 1997a; Marini et al., 2002), which flushes nutrient-rich water out of the northern Adriatic (Hopkins et al., 1999; Marini et al., 2008; Grilli et al., 2013). Other important features are local gyres like the South Adriatic Gyre (SAG), which may enhance water mixing and foster its enrichment by concentrating nutrients (Marini et al., 2015); the gyres may act as inshore "retention traps" for egg and larvae, limiting their dispersal and making this area highly suitable for spawning (Specchiulli et al., 2016) (Fig. 2.1). These oceanographic features would also explain the genetic subdivision of local populations along the Croatian coast and at the Po river estuary (Ruggeri et al., 2016b).

Anchovy eggs in the Adriatic have been described at temperatures between 11.6 and 28 °C and at salinity values ranging from 9.1 to 38.7; spawning peaks between 18 and 28 °C, and egg abundance is inversely proportional to salinity (Morello & Arneri, 2009 and references therein). Vucetic (1975) found that variations in egg abundance in the central Adriatic closely correlated with food availability. These findings were confirmed by Gamulin & Hure (1983), who found that egg distribution throughout the Adriatic correlated closely with zooplankton distribution. Regner (1985) suggested that temperature and salinity were proxies for hydrographic conditions favouring production, and that food availability governs the spawning dynamics of anchovy in spatial and temporal terms. During the spawning season, eggs have been detected throughout the Adriatic within a depth of 200 m, but not at greater depths (Gamulin & Hure, 1983; Regner, 1985). The main spawning areas have been identified in coastal waters along the western coast, from the Gulf of Trieste to the Gargano Promontory (Casavola & Marano, 1985; Regner, 1996). In the south Adriatic Casavola (1998) reported that egg abundance ranged from 0 to 100 eggs m^{-2}, with a greater abundance between Bari and Brindisi (50 - 100 eggs m^{-2}); in the Gulf of

Manfredonia abundance ranged between 0 - 20 eggs m^{-2}, and was greater between 50 and 100 m. Melia et al. (2002) described an egg abundance between 0 - 300 m^{-3}, with peaks (>100 m^{-3}) off Bari between 100 and 200 m, and a lower abundance in the Gulf of Manfredonia (0 – 100 m^{-3}), peaking at a depth of 100 m. In the Gulf of Manfredonia Marano et al. (1998) reported a large number of larvae, post-larvae, and juveniles but relatively low egg abundances. Other spawning areas have been identified in the eastern Adriatic, between Susak Island and the Jabuka pit as well as around Palagruža Island, even though spawning intensity in these areas was lower (Sinovčić, 1978, 2000; Piccinetti et al., 1979; Regner, 1996).

Since the last ichthyoplankton surveys in the Adriatic were conducted in the 1980s and 1990s, this study was performed to identify the spawning and nursery areas of *E. encrasicolus* in the southern Adriatic, update their boundaries, and assess by single parameter quotient analysis the environmental and biological factors affecting the choice of spawning areas during a four-year period (2012 - 2015).

2.2 Materials and methods

2.2.1 Sampling method and data collection Ichthyoplankton samples and oceanographic data were collected on board R/V "G. Dallaporta" during four acoustic surveys carried out in June and July 2012-2015 in the framework of project MEDIAS (pan MEDIterranean Acoustic Surveys, Leonori et al., 2016) and Flag Project RITMARE. The area covered by the survey was along the western continental shelf of Geographical Sub-Area (GSA) 18 and measured about 2510 nm^2 (square nautical miles). The ichthyoplankton sampling design was the same for all surveys. Sampling was performed at intervals of 5 nm along parallel transects perpendicular to the coastline up to the 200 m bathymetry; the distance between transects was 7-10 nm (Fig. 2.2). If at least one egg was found in the last station of the transect, the sampling was extended for another 5 nm. A total number of 232 samples were collected at 58 stations. Ichthyoplankton samples were collected using a 200-μm mesh size WP2 net. Vertical tows were performed from 3 m above the bottom to the surface; maximum sampling depth was 100 m. Samples were immediately placed in 4% formaldehyde-seawater solution. In the laboratory, anchovy eggs and larvae were identified and sorted under a stereomicroscope; they were counted and their abundance was standardized as number per square meter.

Samples collected in 2014 and 2015 were also used to assess the zooplankton biomass. After subtracting anchovy eggs and larvae, samples were dried in an oven for 24 h at 60 °C and weighed; the procedure was repeated until a constant weight was reached (Postel et al., 2000). Possible loss of material due to formalin fixation was corrected by applying a conversion factor obtained by averaging several conversion factors used for different zooplankton taxa (Giguère et al., 1989). The mass loss after drying was estimated to be 31.32% of the total original mass.

In 2012 and 2013, ichthyoplankton and zooplankton samples were collected in parallel; samples were frozen on board and dried in the laboratory (Postel et al., 2000). Then, the estimated dry mass of anchovy eggs and larvae was subtracted from the total biomass, assuming that an equal number of eggs and larvae was found in the zooplankton and ichthyoplankton samples.

Conductivity, temperature, and depth data were collected with a CTD probe (SeaBird Electronics Model 9, Bellevue, WA, USA) at all 58 stations in 2014 and 2015 and at 39 stations in 2012 and 2013. The missing CTD data were integrated by applying Inverse Distance Weighting (IDW) to the spatial interpolation made with SURFER 12 software (Golden Software, Golden, CO, USA). Sea surface chlorophyll-a satellite data (CHLA 8-day composite version, resolution, 4 km) were extracted based on the sampling grid and dates (https://oceancolor.gsfc.nasa.gov/) using SeaDAS software (v.7.4).

2.2.2 Data analysis A modified version of single parameter quotient analysis was used to characterize anchovy spawning and nursery areas, using the Shachar package in R (v. 2.15.3, R Core Team 2013) (Bernal et al., 2007). A quotient curve was considered as an explanatory tool describing the relationship between abundance of eggs or larvae and environmental variables, to identify ranges of variables where fish "prefer" or "avoid" spawning (Lluch-Belda et al., 1991; Drapeau, 2005). The variables included in the study - salinity, temperature, depth, chlorophyll-a and zooplankton biomass - were divided into categories to ensure that maximum occurrence per category did not exceed 25% of all measurements. The quotient value (Q) for each category was estimated as:

$$Q(c) = \frac{\% \text{ eggs or larvae } (c)}{\% \text{ environmental variable } (c)}$$

where (c) is each category of the environmental variable considered; % eggs or larvae is the sum of eggs or larvae per m^2 that were counted in the stations falling into each category, divided by the total number of eggs or larvae per m^2 counted at all the stations

sampled in the year considered; and % environmental variable is the ratio of the number of stations falling within a category to the total number of stations sampled. A bootstrap (n = 999 times, with replacement) was used to calculate the confidence intervals (0.025 and 0.975 percentiles) with the simulated random pseudo-surveys. Q values exceeding the upper confidence limit indicated positive habitat selection (preference) and those under the lower confidence interval were considered as negative selection (avoidance). The Q values between the lower and the upper confidence limit was considered as tolerated habitats (Mhlongo et al., 2015).

Mean egg and larval abundance was calculated as the geometric mean number of eggs and larvae per m^2:

$$\text{MG} = 10^{\frac{1}{n}\sum_{i=1}^{n} log Y_i}$$

where *n* is the number of stations where at least one egg or larva was retrieved and Y*i* is the number of eggs and larvae per m^2.

The 95% confidence interval (CI) was calculated as follows:

$$\text{CL}_{\pm} = \text{MG} \pm \sqrt{\frac{s^2}{n}}$$

where s^2 is the variance and *n* the number of stations with at least one egg or larva.

To test the relationships between each environmental variable and the abundance of anchovy eggs and larvae, the physical data were averaged for the first 20 m, assuming that the eggs and larvae were concentrated in this water layer, then non-parametric Spearman's rank correlation test was applied. Principal Component Analysis (PCA) was used to test the presence of structure in the environmental dataset taking into account all the environmental variables. Differences in egg and larval abundance, water salinity, temperature, and depth, and chlorophyll-a and zooplankton biomass in the four sampling

years were subjected to non-parametric Kruskal-Wallis test. A post-hoc test (Dunn's test) was performed to detect differences between years.

2.3 Results

The spatial distribution of eggs showed different patterns in the four years of sampling. Their abundance ranged between $0 - 454.9$ egg m^{-2}. The percentage of negative stations ranged from 12 to 34%; it was lowest in 2012 and highest in 2015.

In 2012 the geometric mean of the eggs showed a peak (39.49 egg m^{-2}; CI 27.66 – 51.32). Their distribution was wide, with abundance peaks in front of the Gulf of Manfredonia, in front of Bari, and in the southern portion of the study area. The most frequent abundance classes were those with 10 - 25 eggs m^{-2} and 50 - 75 eggs m^{-2}, which together accounted for 31.6% of total abundance (Fig. 2.3A). In 2013 the geometric mean was 26.99 eggs m^{-2} (CI 21.52 – 32.46); most of the eggs were retrieved in the central portion of the study area, and the most frequent class of abundance was 25 - 50 eggs m^{-2}, which accounted for 22.4% of total abundance (Fig. 2.3B). In 2014 the value of the geometric mean was lowest (20.51 egg m^{-2}; CI 14.92 – 26.10), and the eggs were found only in the Gulf of Manfredonia and in the central part of the study area. The most frequent class of abundance was $1 - 5$ eggs m^{-2} (Fig. 2.3C). In 2015 the geometric mean was 25.61 eggs m^{-2} (CI 15.73 – 35.49) and the eggs were mostly concentrated in the northern portion of the study area and in the Gulf of Manfredonia; the class with $10 - 25$ eggs m^{-2} accounted for 20.7% of the total abundance (Fig. 2.3D).

The abundance of larvae ranged between $0 - 1054.9$ individuals (ind) m^{-2}. Negative stations ranged from 6% in 2012 to 27% in 2013. In 2012 the value of the geometric mean was highest (52.78 ind m^{-2}; CI 43.37 – 62.20) and larvae were found throughout the study area; 20.7% of the total abundance was concentrated in the class with $50 - 75$ ind m^{-2} (Fig. 2.4A). In 2013 the geometric mean was 40.04 ind m^{-2} (CI 27.52 – 52.56), the larvae were mostly concentrated in front of the Gargano Promontory and in the central part of the study area, and the most frequent abundance class was 25 - 50 ind m^{-2} (13.8%) (Fig. 2.4B). In

2014 the data showed the lowest value of the geometric mean (24.47 ind m^{-2}; CI 20.04 – 28.89) with no particular abundance hotspots; the most frequent abundance class was the one with 25 - 50 ind m^{-2} (22.4%) (Fig. 2.4C). In 2015 the geometric mean was 44.66 ind m^{-2} (CI 21.08 – 68.24), the larvae were concentrated in the northern and southern part of the study areas, and the most frequent abundance class was the one with 25 - 50 ind m^{-2} (13%) (Fig. 2.4D).

Data analysis demonstrated significantly different abundances of eggs and larvae, different environmental conditions, and different chlorophyll-a and zooplankton biomass values in the different years (Table 2.1). Dunn's post-hoc test highlighted that 2012 was the year when salinity was highest and chlorophyll-a concentrations were lowest and that 2015 was the year with the lowest water temperature. Moreover, 2012 was the year when the abundance of eggs and larvae was highest, with significant differences with 2014 and 2015 for eggs and with 2013 and 2014 for larvae. As regards the biomass of zooplankton, 2014 and 2015 were the years with the lower abundances and were significantly different from the 2013 data. A clear water stratification was detected throughout the study period, with a thermocline between 20 and 40 m (Fig. 2.5). Spearman's correlation analysis identified significant and positive correlations among egg abundance ($\rho = 0.38$, P < 0.001), larval abundance ($\rho = 0.52$, P < 0.001), and zooplankton biomass and between depth and salinity ($\rho = 0.46$, P < 0.001), and negative correlations between chlorophyll-a and salinity ($\rho = -0.72$, P < 0.001), between chlorophyll-a and depth ($\rho = -0.80$, P < 0.001), and between temperature and depth ($\rho = -0.41$, P < 0.001) (Table 2.2).

PCA demonstrated that the environmental parameters accounted for 75% of the variability captured by the first two axes. The first axis explained 58% of the variance and correlated highly and positively with chlorophyll-a and negatively with depth. The second axis, which captured 17% of the variability, showed a strong correlation with the zooplankton biomass.

Quotient analysis of egg abundance data indicated that anchovy prefer spawning areas found at intermediate depth (91 - 120 m) and that they avoid shallower waters (11 – 30 m) with high chlorophyll-a concentrations (> 0.52 mg m^{-3}) and low zooplankton biomass (14.82 – 199 mg m^{-2}). Quotient analysis of larval abundance highlighted a preference for depths between 11 and 60 m with high zooplankton biomass (> 1000 mg m^{-2}) and avoidance of deeper water (> 150 m) with low zooplankton biomass (14.82 – 199 mg m^{-2}) (Table 2.3, Fig. 2.6).

2.4 Discussion and conclusion

A considerable effort has been made to understand the factors that determine habitat selection by anchovy in several ecosystems worldwide (e.g., Somarakis & Nikolioudakis, 2007; Palomera et al., 2007; Bonanno et al., 2014; Mhlongo et al., 2015; Zarrad et al., 2017). As regards the Adriatic Sea, although it is a key area for small pelagic stocks, the available information is limited and dated. In a study of the Mediterranean Sea, Giannoulaki et al. (2013) suggested that the south-western Adriatic was a potentially favourable habitat for adults and juveniles. The present study was directed at identifying suitable anchovy spawning and nursery areas.

The major finding of this work is the significant correlation found between egg and larval abundance and the zooplankton biomass. These data agree with the preference ranges of eggs and larvae for high zooplankton biomass and by the avoidance for low zooplankton biomass values of eggs, highlighted by quotient analysis. Similar findings have been reported in other studies (Regner, 1996; Twatwa et al., 2005; Somarakis & Nikolioudakis, 2007; Zarrad et al., 2012), where the hypothesis was advanced that spawning could occur in areas with high zooplankton concentrations, which provided better conditions for egg and larval survival. Anchovy spawning areas are known to be characterized by a stratified and stable water column, which allows the formation and maintenance of food aggregations. According to Agostini & Bakun (2002), processes that favour retention in optimal areas for egg and larval survival are present in the southern Adriatic. Here, fronts are created by the clash between riverine inputs and the more saline waters of the Mediterranean sea; these factors, combined with limited mixing of the water column and with a high degree of stratification (due to mild wind action in summer), create an area where nutrients and zooplankton are densely concentrated. The seasonal cyclonic gyre

(SAG) also exercises a strong influence, promoting retention mechanisms for zooplankton and ichthyoplankton (Artegiani et al., 1997b; Bakun, 2006; Specchiulli et al., 2016).

Quotient analysis also highlighted an avoidance range of egg deposition in correspondence with high levels of chlorophyll-a, even though several studies have reported a positive correlation between the abundance of different anchovy life stages and chlorophyll-a concentrations, both in highly productive systems like the Humboldt Current ecosystem (Bertrand et al., 2008) and in less productive ecosystems like the Strait of Sicily and the north Aegean Sea (Bonanno et al., 2014). However, our chlorophyll-a data come from satellite measurements, which explore only a few centimetres from the surface, where chlorophyll-a concentrations are lower than in the deeper layers. Indeed, it is well established that sub-surface maxima of phytoplankton productivity and biomass occur especially during summer stratification (Ninčević et al., 2002). The Deep Chlorophyll-a Maximum (DCM) is found at the lowest depth where the light makes it possible for phototrophic populations to exploit nutrient advection from sub-euphotic depths. Since in the middle and southern Adriatic a DCM is consistently detected between 50 and 75 m (Ninčević et al., 2002; Alcaraz et al., 2016), it is conceivable that avoidance of egg deposition in presence of high chlorophyll-a concentrations is an artefact due to the collinearity between chlorophyll-a and depth.

Quotient analysis of depth values highlighted a preference range for egg deposition between 91 - 120 m and avoidance at shallower depths (11 - 30 m); the larvae showed a preference for coastal waters (11 – 60 m) and avoided deeper waters (> 150 m). Several studies have reported that the selection of spawning and nursery areas is driven by depth (García & Palomera, 1996; Regner, 1996; Somarakis et al., 2004; Somarakis & Nikolioudakis, 2007; Basilone et al., 2013; Giannoulaki et al., 2013; Bonanno et al., 2014). According to these findings the spawning habitat is confined to the continental shelf within the 200 m isobaths and the nursery area is near the coast, where photosynthetic activity can

support abundant zooplankton growth and conditions for larval survival are optimal. These data agree with those reported by Giannoulaki et al. (2013), who found in the Mediterranean Sea a preference for spawning areas at depths of 40 - 150 m, and with those of Saraux et al. (2014), who identified two recurrent areas further from the coast (isobaths of ∼70 to 100 m) and unfavourable areas close to the coast in the Gulf of Lion for adult anchovy. The results of the present study show significantly different distributions of eggs and larvae over the years, in line with the notion that the biomass of small pelagic species undergoes wide fluctuations over time throughout the world (Brander, 2007; Leonori et al., 2009, 2011; Ruggeri et al., 2016a). The PCA data explained the environmental variability mainly in terms of depth and chlorophyll-a, but also stressed the importance of zooplankton abundance. In 2012, the high abundance of eggs and larvae was paralleled by peak values of salinity and by trough concentrations of chlorophyll-a, whereas the years when the abundance of eggs (2014 and 2015) and larvae (2013 and 2014) was lower were also cooler (2015) and characterized by lower zooplankton biomass (2014 and 2015). Thus, there emerged a clear trend relating the environmental parameters to egg and larval distribution; in particular, egg and larval abundance were higher at intermediate depths in areas characterized by low concentrations of chlorophyll-a and high zooplankton abundance.

Anchovy spawning is believed to be related to certain temperature and salinity ranges; for example, in the southern Mediterranean Sea temperatures > 25°C, depth and water stratification are major factors controlling spawning intensity (Zarrad et al., 2006, 2017). In the Benguela ecosystem the spawning temperature ranges between 16 – 21 °C (van der Lingen et al., 2001; Twatwa et al., 2005), whereas in the north-western Mediterranean anchovy eggs are mainly found between 17 - 23 °C (Palomera et al., 2007). However, a number of studies have failed to identify a clear relationship; for example, the spawning distribution of Californian anchovy has been reported to be related to temperature in some

studies but not in others (Fréon et al., 2005 and references therein). In the Mediterranean Sea, different ranges of preference have been described in relation to salinity. In the western Mediterranean Palomera et al. (2007) reported two peaks of preference, one at 35.8 and another at 37.6 PSU, whereas in the eastern Mediterranean a broad range of values have been described, spawning being most intense in areas with lower salinity (Somarakis & Nikolioudakis, 2007). These studies have stressed the flexibility of anchovy preferences for spawning habitats, suggesting that they change according to the prevailing ecosystem conditions (Basilone et al., 2013). The data analysed in the present work demonstrate a rising gradient from the coast to the open sea for salinity and an opposite gradient for temperature. However, these data correlated neither with the abundance of eggs or larvae nor with preference/avoidance behaviors according to quotient analysis, suggesting that salinity and temperature are not the oceanographic variables driving egg and larval distribution in the southern Adriatic.

Earlier surveys in the northern Adriatic have found that anchovy eggs were consistently more abundant near the shore (25 – 50 m), that their number declined seawards and southwards with a N-S and W-E gradient, and that abundance was highest (> 400 eggs m^{-2}) in the low salinity area of the Po River estuary and in the Gulf of Trieste. The larvae were distributed farther offshore, but the area around the Po estuary is known to favour their survival, due to water column stability conferred by the low salinity of the estuary surface water. These conditions help to maintain the water column stratification, hence the vertical aggregation of food particles (Coombs et al., 2003). A study conducted by Specchi et al. (1998) in the northern and central Adriatic found a strong correlation between anchovy egg abundance and mesozooplankton standing crop, whereas no correlation was detected between egg abundance and environmental variables, except a correlation - limited to the Gulf of Trieste - between egg abundance and temperature. Marano et al. (1998) reported that in the southern Adriatic most of the eggs were concentrated between

Bari and Brindisi at depths ranging between 50 and 100 m; abundance values were between 0 - 100 eggs m^{-2}, the lowest values being measured in the Gulf of Manfredonia, which the authors described as an exclusively nursery area. In all past survey conducted in the central and southern Adriatic food availability and bathymetry were described as the key factors affecting spawning dynamics in spatial and temporal terms (Vucetic, 1975; Gamulin & Hure, 1983; Regner, 1985). In the present study, data analysis indicated depth, zooplankton abundance, and water column stability as the factors characterizing favourable spawning and nursery areas in the south-western Adriatic. In conclusion, the present data do not outline a clear spatial distribution pattern of anchovy eggs and larvae in the area; nonetheless, their high abundance found throughout the study area in four successive years, suggests that the entire area can be considered as a favourable spawning and nursery area for anchovy.

References

Agostini, V.N., Bakun, A., 2002. " Ocean triads " in the Mediterranean Sea : physical mechanisms potentially structuring reproductive habitat suitability (with example application to European anchovy , Engraulis encrasicolus). *Fisheries Oceanography*, 11 (3), 129–142.

Alcaraz, M., Calbet, A., Isari, S., Irigoien, X., Trepat, I., Saiz, E., 2016. Variability of mesozooplankton biomass and individual size in a coast-offshore transect in the Catalan Sea: relationships with chlorophyll a and hydrographic features. *Scientia Marina*, 80 (S1), 79–87. DOI: 10.3989/scimar.04353.15A.

Artegiani, A., Bregant, D., Paschini, E., Pinardi, N., Raicich, F., Russo, A., 1997a. The Adriatic Sea General Circulation. Part I : Air – Sea Interactions and Water Mass Structure. *Journal of Physical Oceanography*, 27, 1492–1514.

Artegiani, A., Bregant, D., Paschini, E., Pinardi, N., Raicich, F., Russo, A., 1997b. The Adriatic Sea General Circulation. Part II : Baroclinic Circulation Structure. *Journal of Physical Oceanography*, 27, 1515–1532.

Bakun, A., 1996. Patterns in the ocean: ocean processes and marine population dynamics. *California Sea Grant, in cooperation with Centro de Investigaciones Biologicas del Noroeste, La Paz, Mexico.*

Bakun, A., 2006. Fronts and eddies as key structures in the habitat of marine fish larvae: opportunity, adaptive response and competitive advantage. *Scientia Marina*, 70 (S2), 105–122. DOI: 10.3989/scimar.2006.70s2105.

Basilone, G., Bonanno, A., Patti, B., Mazzola, S., Barra, M., Cuttitta, A., McBride, R., 2013. Spawning site selection by European anchovy (*Engraulis encrasicolus*) in relation to oceanographic conditions in the Strait of Sicily. *Fisheries Oceanography*, 22 (4), 309–323.

DOI: 10.1111/fog.12024.

Bellido, J., Pierce, G., Romero, J., Millán, M., 2000. Use of frequency analysis methods to estimate growth of anchovy (*Engraulis encrasicolus* L. 1758) in the Gulf of Cádiz (SW Spain). *Fisheries Research*, 48 (2), 107–115. DOI: 10.1016/S0165-7836(00)00183-1.

Bernal, M., Stratoudakis, Y., Coombs, S., Angelico, M.M., Lago de Lanzós, A., Porteiro, C., Sagarminaga, Y., Santos, M., Uriarte, A., Cunha, E., Valdés, L., Borchers, D., 2007. Sardine spawning off the European Atlantic coast: characterization of and spatio-temporal variability in spawning habitat. *Progress in Oceanography*, 74, 210–227. DOI: 10.1016/j.pocean.2007.04.018.

Bertrand, A., Gerlotto, F., Bertrand, S., Gutiérrez, M., Alza, L., Chipollini, A., Díaz, E., Espinoza, P., Ledesma, J., Quesquén, R., Peraltilla, S., Chavez, F., 2008. Schooling behaviour and environmental forcing in relation to anchoveta distribution: an analysis across multiple spatial scales. *Progress in Oceanography*, 79, 264–277. DOI: 10.1016/j.pocean.2008.10.018.

Bonanno, A., Giannoulaki, M., Barra, M., Basilone, G., Machias, A., Genovese, S., Goncharov, S., Popov, S., Rumolo, P., Di Bitetto, M., Aronica, S., Patti, B., Fontana, I., Giacalone, G., Ferreri, R., Buscaino, G., Somarakis, S., Pyrounaki, M-M., Tsoukali, S., Mazzola, S., 2014. Habitat selection response of small pelagic fish in different environments. Two examples from the oligotrophic Mediterranean Sea. *PLoS One*, 9 (7), 1–17. DOI: 10.1371/journal.pone.0101498.

Brander, K.M., 2007. Global fish production and climate change. *Proceedings of the National Academy of Sciences of the United States of America*, 104 (50), 19709–19714. DOI: 10.1073/pnas.0702059104.

Casavola, N., 1998. Daily egg production method for spawning biomass estimates of anchovy in the south-western Adriatic during 1994. *Rapp. Comm. int. Mer Médit*, 35, 394–395.

Casavola, N., Marano, G., 1985. Considérations sur la distribution des clupeiformes *Engraulis encrasicolus* et *Sardina pilchardus* en Adriatique. *FAO Fisheries Report*, 345, 153–155.

Coll, M., Santojanni, A., Palomera, I., Tudela, S., Arneri, E., 2007. An ecological model of the northern and central Adriatic Sea: analysis of ecosystem structure and fishing impacts. *Journal of Marine Systems*, 67, 119–154. DOI: 10.1016/j.jmarsys.2006.10.002.

Coombs, S.H., Giovanardi, O., Halliday, N.C., Franceschini, G., Conway, D.V.P., Manzueto, L., Barrett, C.D., McFadzen, I. R.B., 2003. Wind mixing , food availability and mortality of anchovy larvae *Engraulis encrasicolus* in the northern Adriatic Sea. *Marine Ecology Progress Series*, 248, 221–235.

Cury, P., Bakun, A., Crawford, R.J.M., Jarre, A., Quinones, R.A., Shannon, L.J., Verheye, H.M., 2000. Small pelagics in upwelling systems: patterns of interaction and structural changes in "wasp-waist" ecosystems. *ICES Journal of Marine Science*, 57 (3), 603–618. DOI: 10.1006/jmsc.2000.0712.

Cushing, D.H., 1996. Towards a science of recruitment in fish populations. *Kinne, O. Ed. Excellence in Ecology*, 7, 175 pp.

Drapeau, L., 2005. Introduction to the use of quotient curves for characterizing spawning habitat of small, pelagic fish. *SPACC Workshop*, (January), 5–6.

FAO (Food and Agriculture Organization), 2014. World review of fisheries and aquaculture. *Food and Agriculture Organisation*, 1–96.

FAO (Food and Agriculture Organization), 2016. The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. *Rome: Food and Agriculture Organisation,* 200 pp.

Fréon, P., Cury, P., Shannon, L., Roy, C., 2005. Sustainable exploitation of small pelagic fish stocks challenged by environmental and ecosystem changes: a review. *Bulletin of marine*

science, 76 (2), 385–462.

Gamulin, T., Hure, J., 1983. The spawning and spawning areas of pelagic fishes (*Sardina plichardus, Engraulis encrasicolus, Scomber scombrus, Sardinella aurita and Sprattus sprattus sprattus*) in the Adriatic Sea. *Acta Adriatica*, 24, 91–131.

García, A., Palomera, I., 1996. Anchovy early life history and its relation to its surrounding environment in the Western Mediterranean basin. *Scientia Marina*, 60, 155–166.

Giannoulaki, M., Iglesias, M., Tugores, M.P., Bonanno, A., Patti, B., De Felice, A., Leonori, I., Bigot, J.L., Tičina, V., Pyrounaki, M.M., Tsagarakis, K., Machias, A., Somarakis, S., Schismenou, E., Quinci, E., Basilone, G., Cuttitta, A., Campanella, F., Miquel, J., Oñate, D., Roos, D., Valavanis, V., 2013. Characterizing the potential habitat of European anchovy *Engraulis encrasicolus* in the Mediterranean sea, at different life stages. *Fisheries Oceanography*, 22 (2), 69–89. DOI: 10.1111/fog.12005.

Giguère, L.A., St-Pierre, J.F., Bernier, B., Vézina, A., Rondeau, J.G., 1989. Can we estimate the true weight of zooplankton samples after chemical preservation? *Canadian Journal of Fisheries and Aquatic Science*, 46 (3), 522–527. DOI: https://doi.org/10.1139/f89-070.

Grilli, F., Marini, M., Book, J.W., Campanelli, A., Paschini, E., Russo, A., 2013. Flux of nutrients between the middle and southern Adriatic Sea (Gargano-Split section). *Marine Chemistry*, 153, 1–14. DOI: doi:10.1016/j.marchem.2013.04.005.

Hopkins, T.S., Artegiani, A., Bignami, F., Russo, A., 1999. Water-mass modification in the northern Adriatic: A preliminary assessment from the ELNA data set, in the Adriatic Sea. *Ecosyst. Res. Rep*, 32, 3–23.

Leonori, I., Azzali, M., De Felice, A., Parmiggiani, F., Marini, M., Grilli, F., Gramolini, R., 2009. Small pelagic fish biomass in relation to environmental parameters in the Adriatic Sea. p. 213–217. *In: Proceedings of the Joint AIOL-SItE Meeting, 17-20 September 2007,*

Ancona, Italy.

Leonori, I., De Felice, A., Campanella, F., Biagiotti, I., Canduci, G., 2011. Assessment of small pelagic fish biomass in the Adriatic Sea by means of acoustic methodology. p. 2019–2029. *In: Fishery and sea resources. Marine research at CNR. (Eds) Brugnoli E., Cavaretta, G., Mazzola, S., Trincardi, F., Ravaioli, M., and Santoleri, R.*

Leonori, I., Ticina, V., De Felice, A., Vidjak, O., Grubišić, L., Pallaoro, A., 2012. Comparisons of two research vessels' properties in the acoustic surveys of small pelagic fish. *Acta Adriatica*, 53, 389–398.

van der Lingen, C., Kruse, G.H., Bez, N., Booth, A.J., Dorn, M.W., Hills, S., Lipcius, R.N., Pelletier, D., Roy, C., Smith, S.J., Witherell, D., 2001. Comparative spawning habitats of anchovy (*Engraulis capensis*) and sardine (*Sardinops sagax*) in the Southern Benguela Upwelling Ecosystem. p. 185–209 *In: Spatial processes and management of marine populations. Kruse, G.H., Bez, N., Booth, T., Dorn, M., Hills, S., Lipcius, R.N., Pelletier, D., Roy, C., Smith, S.J., Witherell D. (Eds). Fairbanks, USA; University of Alaska Sea Grant, AK-SG-01-02.*

Lluch-Belda, D., Lluch-Cota, D.B., Hernandez-Vazquez, S., Salinas-Zavala, C.A., Schwartzlose, R.A., 1991. Sardine and anchovy spawning as related to temperature and upwelling in the California Current System. *CalCOFI Rep.*, 32, 105–111.

Marano, G., Casavola, N., Rizzi, E., De Ruggeri, P., Lo Caputo, S., 1998. Valutazione delle risorse pelagiche, consistenza dello stock di sardine e alici nell'Adriatico meridionale. Anni 1984-1996. *Biologia Marina Mediterranea*, 5 (3), 313–320.

Marini, M., Campanelli, A., Sanxhaku, M., Kljajić, Z., Betti, M., Grilli, F., 2015. Late spring characterization of different coastal areas of the Adriatic Sea. *Acta Adriatica*, 56 (1), 27–46.

Marini, M., Fornasiero, P., Artegiani, A., 2002. Variations of hydrochemical features in the coastal waters of Monte Conero: 1982–1990. *Marine Ecology*, 23, 258–271.

Marini, M., Grilli, F., Guarnieri, A., Jones, B.H., Kljajić, Z., Pinardi, N., Sanxhaku, M., 2010. Is the south-eastern Adriatic Sea coastal strip an eutrophic area? *Estuarine Coastal and Shelf Science*, 88, 395–406.

Marini, M., Jones, B.H., Campanelli, A., Grilli, F., Lee, C.M., 2008. Seasonal variability and Po River plume influence on biochemical properties along western Adriatic coast. *Journal of Geophysical Research*, 113.

Melia, P., Casavola, N., Gatto, M., 2002. Estimating Daily Egg Production of European anchovy in the Adriatic Sea: a Critical appraisal. *Marine Ecology*, 23 (S1), 272–279. DOI: 10.1111/j.1439-0485.2002.tb00025.x.

Mhlongo, N., Dawit, Y., Hendricks, M., van der Lingen, D., 2015. Have the spawning habitat preferences of anchovy (*Engraulis encrasicolus*) and sardine (*Sardinops sagax*) in the southern Benguela changed in recent years ? *Fisheries Oceanography*, 24, 1–14. DOI: 10.1111/fog.12061.

Morello, B., Arneri, E., 2009. Anchovy and sardine in the Adriatic Sea - an ecological review. *Oceanography and Marine Biology: an annual review*, 47, 209–256.

Ninčević, Z., Marasović, I., Kušpilić, G., 2002. Deep chlorophyll-a maximum at one station in the middle Adriatic Sea. *Journal of the Marine Biological Association of the United Kingdom*, 82 (1), 9–19.

Palomera, I., Olivar, M.P., Salat, J., Sabatés, A., Coll, M., García, A., Morales-Nin, B., 2007. Small pelagic fish in the NW Mediterranean sea: an ecological review. *Progress In Oceanography*, 74, 377–396. DOI: 10.1016/j.pocean.2007.04.012.

Piccinetti, C., Regner, S., Specchi, M., 1979. Estimation du stock d'anchois (*Engraulis*

encrasicolus L.) de la haute et moyenne Adriatique. *Investigation Pesquera*, 43 (1), 69–81.

Planque, B., Bellier, E., Lazure, P., 2007. Modelling potential spawning habitat of sardine (*Sardina pilchardus*) and anchovy (*Engraulis encrasicolus*) in the Bay of Biscay. *Fisheries Oceanography*, 16 (1), 16–30. DOI: 10.1111/j.1365-2419.2006.00411.x.

Postel, L., Fock, H., Hagen, W., 2000. Biomass and abundance. *ICES zooplankton methodology manual*,, 83–174. DOI: 10.13140/2.1.4985.3765.

R Core Team., 2013. R: a language and environment for statistical computing. *R Foundation for Statistical Computing*. Vienna, Austria. version 2.15.3.

Regner, S., 1985. Ecology of planktonic stages of the anchovy *Engraulis encrasicolus* in the central Adriatic. 5-113 pp.

Regner, S., 1996. Effects of environmental changes on early stages and reproduction of anchovy in the Adriatic Sea. *Scientia Marina*, 60 (2), 167–177.

Ruggeri, P., Splendiani, A., Di Muri, C., Fioravanti, T., Santojanni, A., Leonori, I., De Felice, A., Biagiotti, I., Carpi, P., Arneri, E., Nisi Cerioni, P., Giovannotti, M., Caputo Barucchi, V., 2016a. Coupling demographic and genetic variability from archived collections of European anchovy (*Engraulis encrasicolus*). *PLoS One*, 11 (3), 1–18. DOI: 10.1371/journal.pone.0151507.

Ruggeri, P., Splendiani, A., Occhipinti, G., Fioravanti, T., Santojanni, A., Leonori, I., De Felice, A., Arneri, E., Procaccini, G., Catanese, G., Ticina, V., Bonanno, A., Nisi Cerioni, P., Giovannotti, M., Grant, W.S., Caputo Barucchi, V., 2016b. Biocomplexity in populations of European anchovy in the Adriatic Sea. *PLoS One*, 11 (4), 1–21. DOI: 10.1371/journal.pone.0153061.

Saraux, C., Fromentin, J.-M., Bigot, J.-L., Bourdeix, J.-H., Morfin, M., Roos, D., Van Beveren, E., Bez, N., 2014. Spatial structure and distribution of small pelagic fish in the

Northwestern Mediterranean Sea. *PLoS One*, 9 (11). DOI: https://doi.org/10.1371/journal.pone.0111211.

Sinovčić, G., 1978. On the ecology of anchovy, *Engraulis encrasicolus* (L.), in the central Adriatic. *Acta Adriatica*, 19 (2), 3–32.

Sinovčić, G., 2000. Anchovy, *Engraulis encrasicolus* (Linnaeus, 1758): biology, population dynamics and fisheries case study . *Acta Adriatica*, 41 (1), 3–53.

Sinovčić, G., Zorica, B., 2006. Reproductive cycle and minimal length at sexual maturity of *Engraulis encrasicolus* (L.) in the Zrmanja River estuary (Adriatic Sea, Croatia). *Estuarine, Coastal and Shelf Science*, 69 (3–4), 439–448. DOI: 10.1016/j.ecss.2006.04.003.

Somarakis, S., Nikolioudakis, N., 2007. Oceanographic habitat, growth and mortality of larval anchovy (*Engraulis encrasicolus*) in the northern Aegean Sea (eastern Mediterranean). *Marine Biology*, 152 (5), 1143–1158. DOI: 10.1007/s00227-007-0761-6.

Somarakis, S., Palomera, I., Garcia, A., Quintanilla, L., Koutsikopoulos, C., Uriarte, A., Motos, L., 2004. Daily egg production of anchovy in European waters. *ICES Journal of Marine Science*, 61 (6), 944–958. DOI: 10.1016/j.icesjms.2004.07.018.

Specchi, M., Casetti, P., Orlandi, C., 1998. Osservazioni ecologiche su *Sardina pilchardus* (Walb) ed *Engraulis encrasicolus* (L.) dell'Adriatico e del Golfo di Tireste. *Biologia Marina Mediterranea*, 5 (3), 339–348.

Specchiulli, A., Bignami, F., Marini, M., Fabbrocini, A., Scirocco, T., Campanelli, A., Penna, P., Santucci, A., D'Adamo, R., 2016. The role of forcing agents on biogeochemical variability along the south-western Adriatic coast: The Gulf of Manfredonia case study. *Estuarine, Coastal and Shelf Science*, 183, 136–149.

Twatwa, N.M., van der Lingen, C.D., Drapeau, L., Moloney, C.L., Field, JG., 2005.

Characterising and comparing the spawning habitats of anchovy *Engraulis encrasicolus* and sardine *Sardinops sagax* in the southern Benguela upwelling ecosystem. *Africal Journal of Marine Science*, 27 (2), 487–499. DOI: 10.2989/18142320509504107.

Vučetić, T., 1975. Synchronism of the spawning season of some pelagic fishes (sardine, anchovy) and the timing of the maximal food (zooplankton) production in the central Adriatic. *Pubblicazioni della Stazione Zoologica di Napoli*, 39 (1), 347–365.

Whitehead, P.J.P., Nelson, G.J., Wongratana, T., 1988. An annotated and illustrated catalogue of the herrings, sardines, pilchards, sprats, shads, anchovies and wolf-herrings. Part 2 Engraulididae. p. 305–579 *In: FAO species catalogue. No 125, Vol. 7. Clupeoid fishes of the world (Suborder Clupeoidei)*.

Zarrad, R., Alemany, F., Jarboui, O., Garcia, A., Akrout, F., 2012. Comparative characterization of the spawning environments of European anchovy, *Engraulis encrasicolus*, and round sardinella, *Sardinella aurita* (Actinopterygii: Clupeiformes) in the eastern coast of Tunisia. *Acta Ichthyologica et Piscatoria*, 42 (1), 9–19. DOI: 10.3750/AIP2011.42.1.02.

Zarrad, R., Alemany, F., Jarboui, O., Missaoui, H., Garcia, A., 2017. Spawning habitats of two small pelagic species (*Engraulis encrasicolus* and *Sardinella aurita*) in the Gulf of Gabès (Central Mediterranean). *Hydrobiologia,*, 1–14. DOI: 10.1007/s10750-017-3301-5.

Zarrad, R., Missaoui, H., Alemany, F., Salah, R.M., García, A., Ridha, MR., Othman, J., Amor, EA., 2006. Spawning areas and larval distributions of anchovy *Engraulis encrasicolus* in relation to environmental conditions in the Gulf of Tunis (Central Mediterranean sea). *Scientia Marina*, 70 (S2), 137–146. DOI: 10.3354/meps09475.

Figures and tables
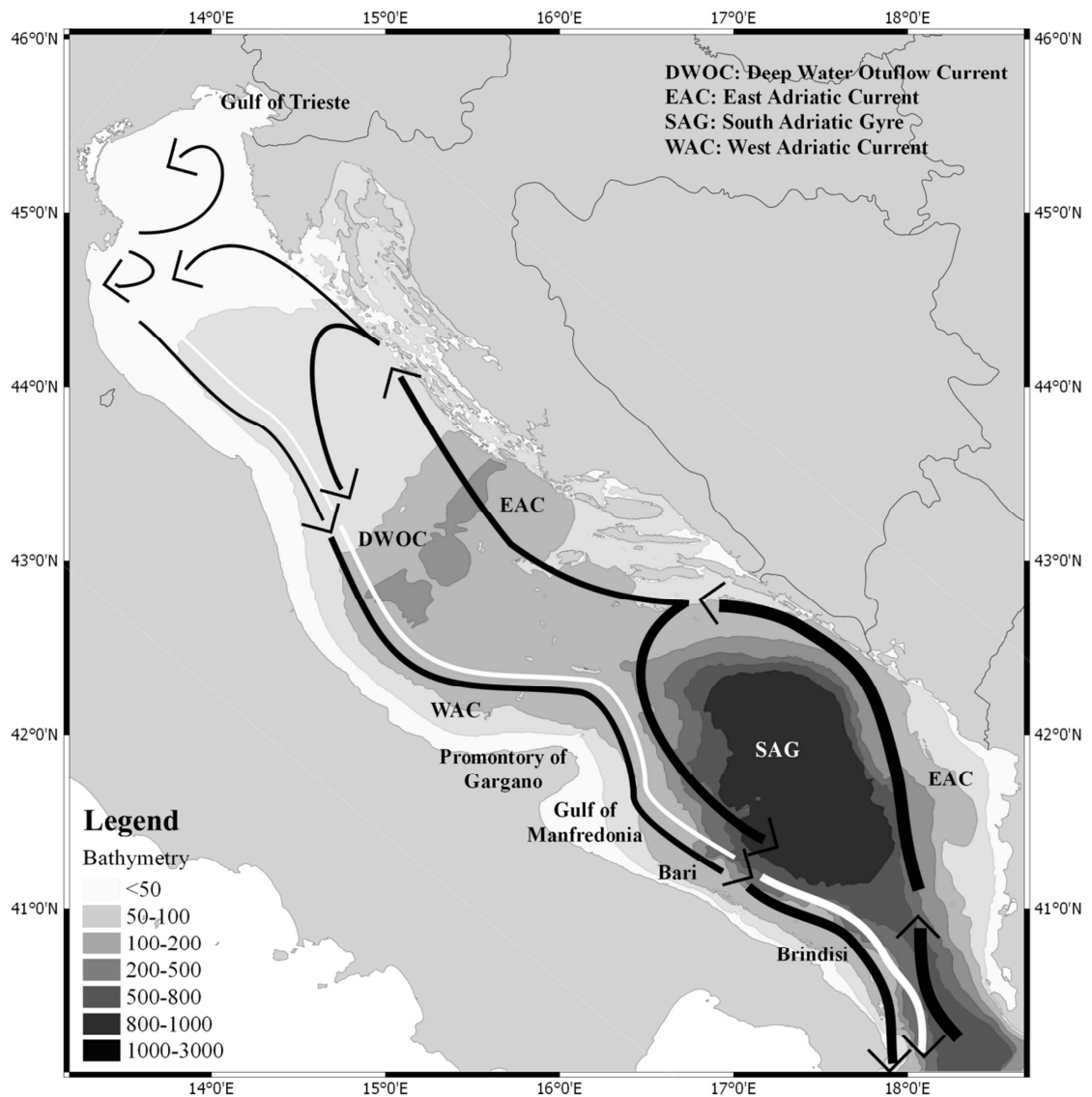
Figure 2.1. Adriatic Sea surface circulation (redrawn from https://commons.wikimedia.org/wiki/File:Adriatic_Sea_Currents_2.svg)
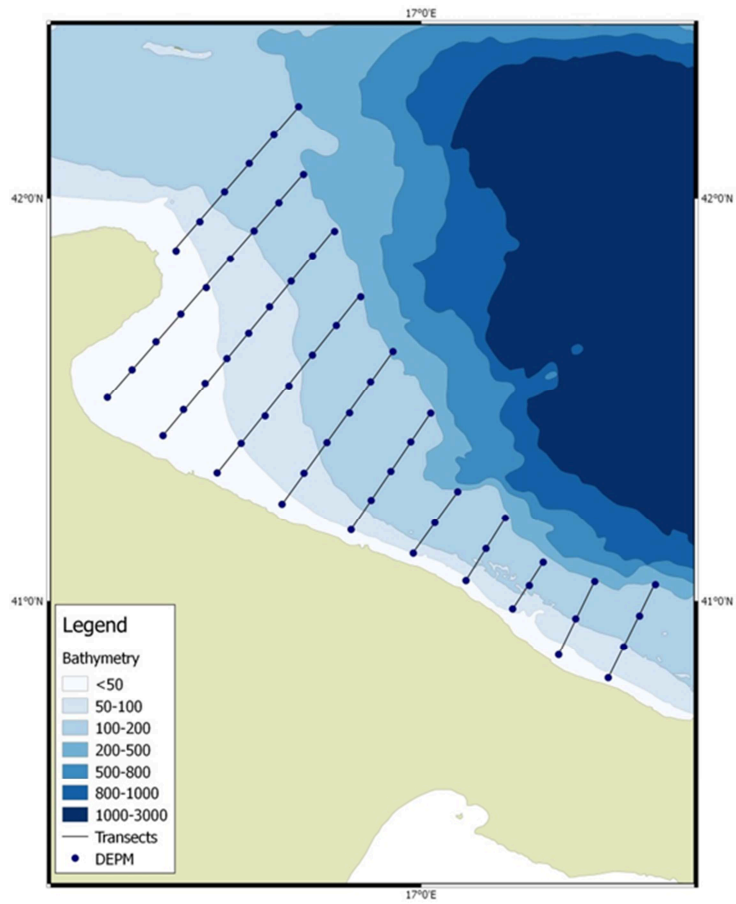
Figure 2.2. Sampling area
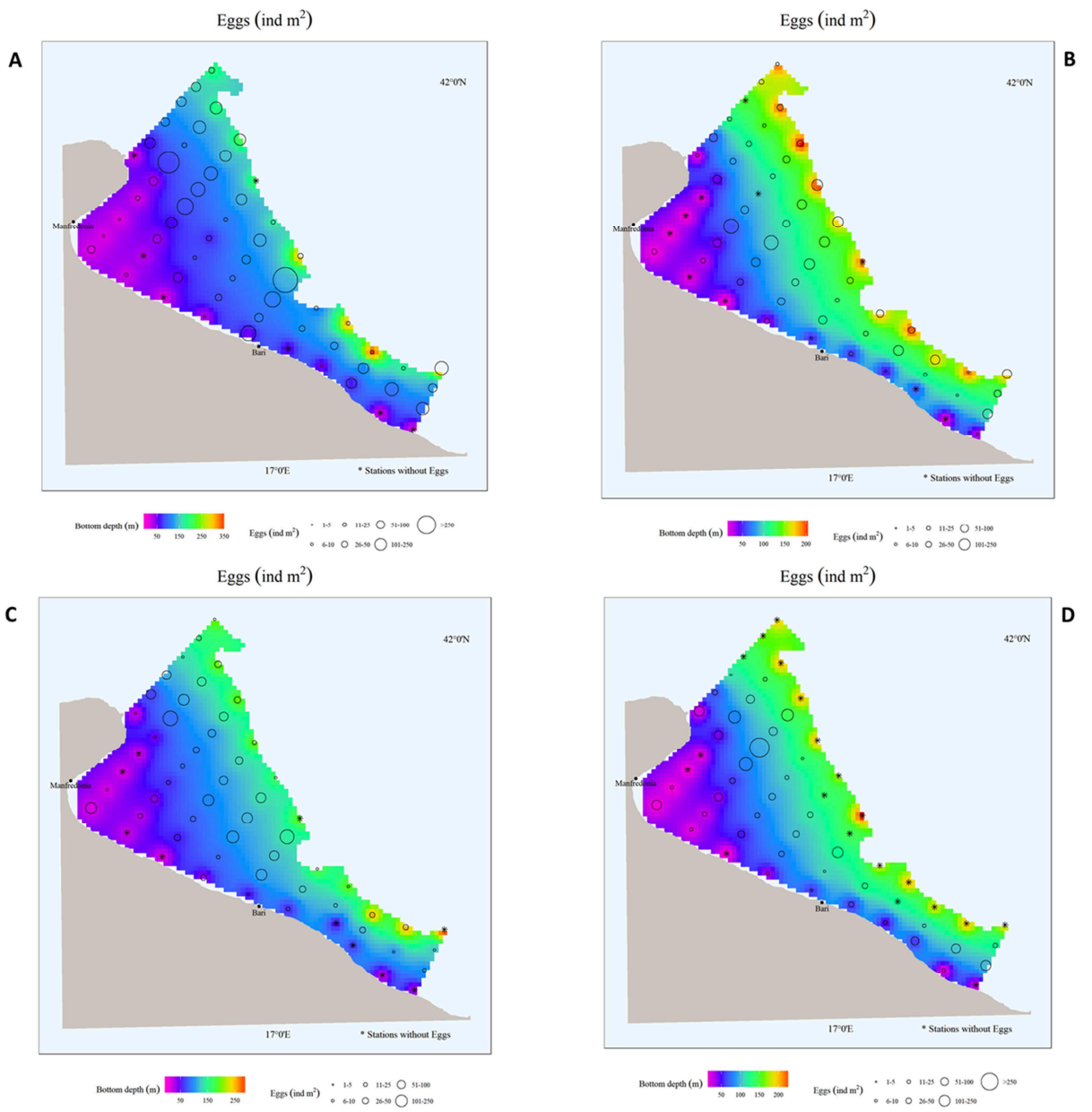
Figure 2.3. Spatial distribution of egg abundance in 2012 (A), 2013 (B), 2014 (C) and 2015 (D) in relation to bottom depth * stations where no eggs were retrieved
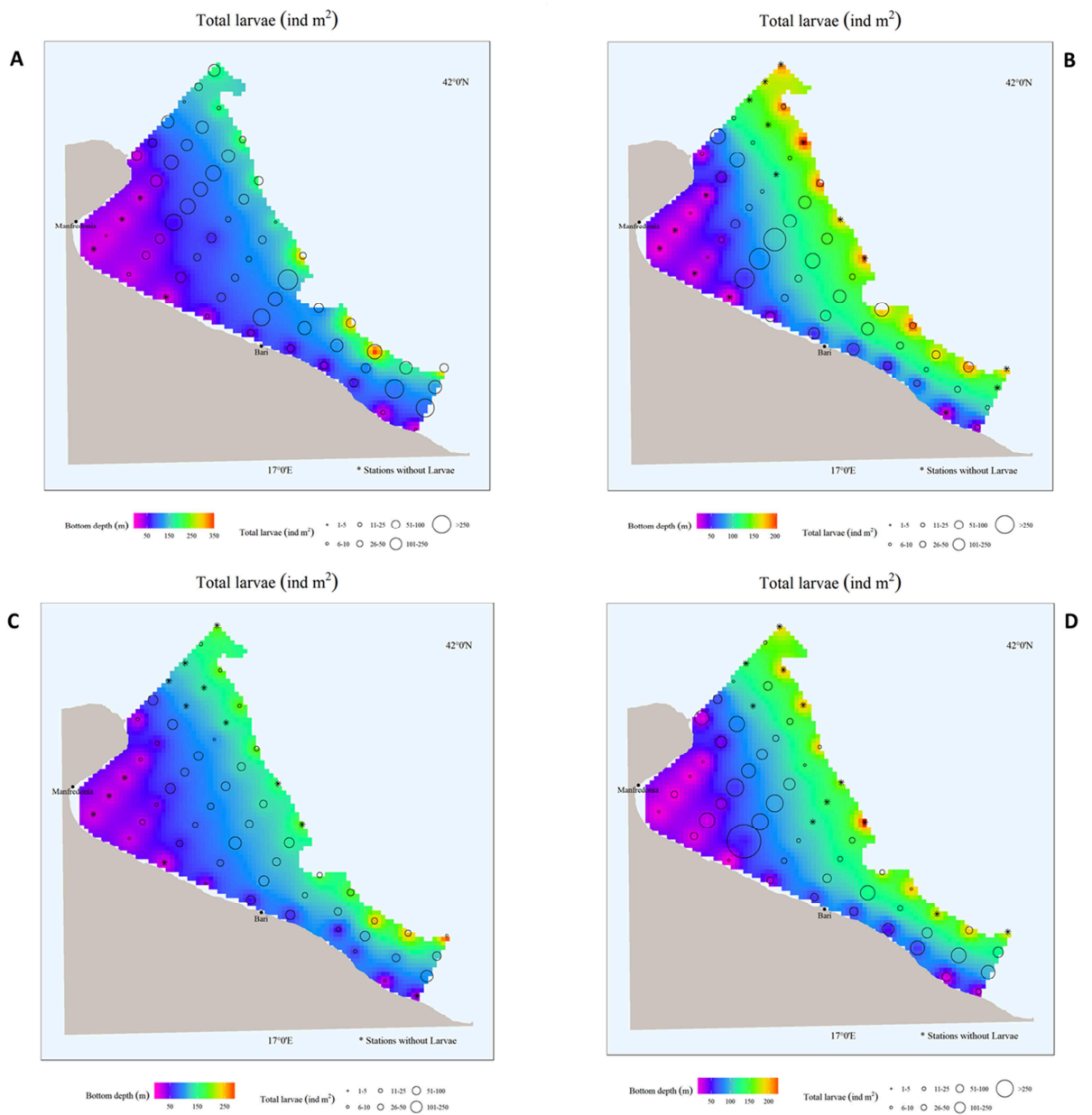
Figure 2.4. Spatial distribution of larval abundance in 2012 (A), 2013 (B), 2014 (C) and 2015 (D) in relation to bottom depth * stations where no eggs were retrieved
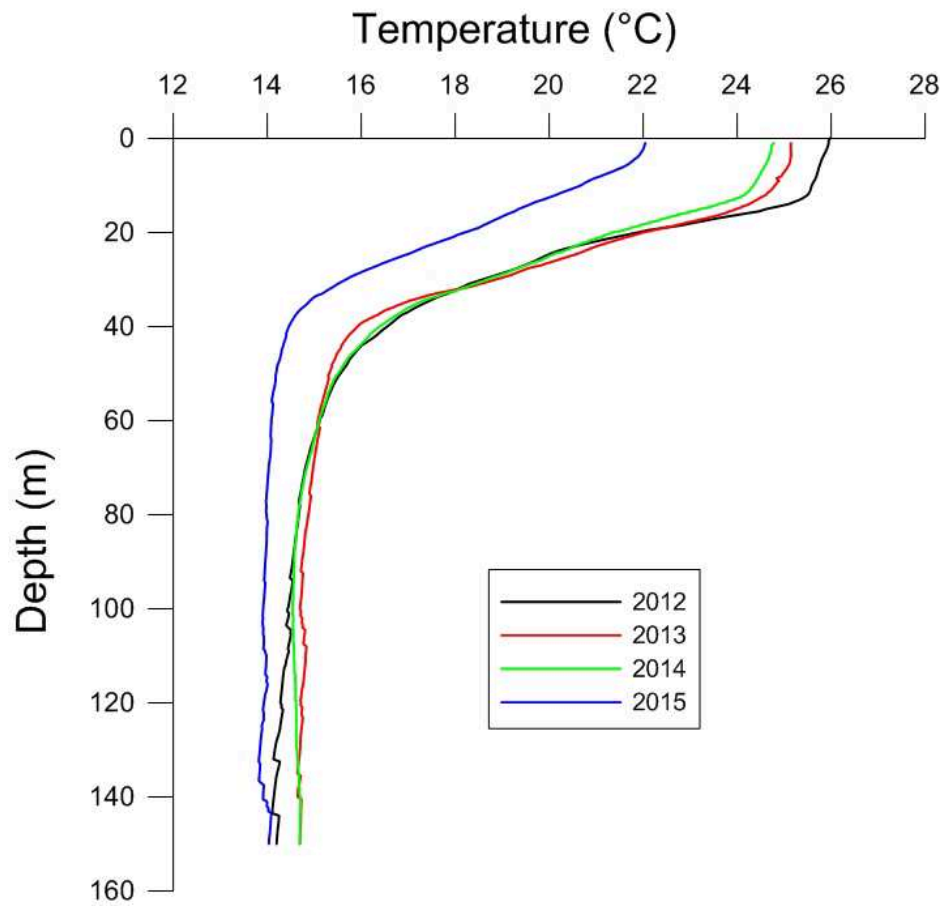
Figure 2.5. Mean profiles of temperature for the 4 years of sampling
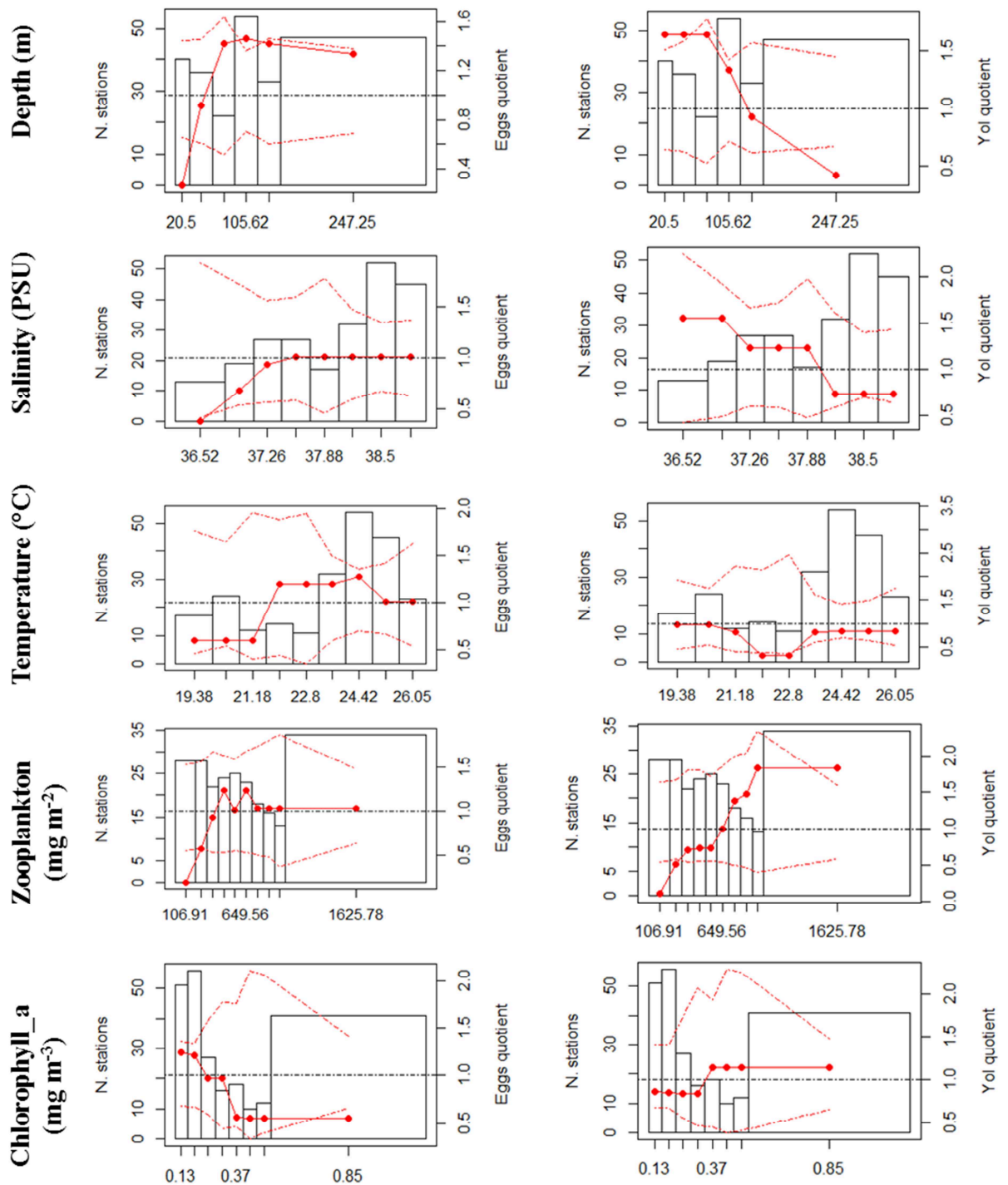
Figure 2.6. Results of quotient analysis. Histograms represent the number of stations comprised in each category of the environmental variables. The continuous line represents egg (left) and larval (right) quotient values, the dashed lines represent the upper and lower confidence intervals. The horizontal line represents the null hypothesis (uniformly distributed eggs or larvae), with quotient value=1

Table 2.1. Temperature and salinity values (averaged for the upper water column, 0-20 m); bottom depth (average), satellite chlorophyll-a, zooplankton biomass, and abundance of eggs and larvae with median values, interquartile ranges (IQR) and Kruskal-Wallis ($\chi2$) test results

|	2012		2013		2014		2015		χ^2	d.f
	Average	Median (IQR)	Average	Median (IQR)	Average	Median (IQR)	Average	Median (IQR)		
Temperature (°C)	25.01	25.17 -1.91	24.52	24.75 -1	23.88	24.33 -1.44	20.49	20.37 -1	145.7***	3
Salinity (PSU)	38.74	38.74 -0.22	37.67	37.65 -1.14	37.67	37.77 -0.91	37.9	37.95 -1.32	118.72***	3
Depth (m)	105.6	101.75 -87.13	99.87	103.5 -93.25	103.34	101.75 -91.25	101.3	103 -91.75	ns	3
Chlorophyll-a (mg m^{-3})	0.21	0.16 -0.12	0.36	0.27 -0.22	0.33	0.25 -0.28	0.46	0.35 -0.48	36.32***	3
Zooplankton (mg m^{-2})	780.07	537.57 -692.68	734.82	680.25 -654.96	498.87	486.79 -370.17	534.34	490.29 -516.87	10.11*	3
Eggs (egg m^{-2})	64.91	43.14 -90.2	34.55	23.53 -50.98	30.9	15.69 -48.04	30.76	9.8 -42.16	12.84**	3
Larvae (ind m^{-2})	77.35	54.9 -93.14	52.81	21.57 -79.41	28.74	19.61 -43.14	82.55	33.33 -106.86	17.47***	3

*P < 0.05; ** P < 0.01; *** P < 0.001

ns Not significant

Table 2.2. Spearman correlation illustrating the possible relationships among abundance of eggs and larvae and environmental variables

	Salinity (PSU)	Temperature (°C)	Depth (m)	Chlorophyll-a (mg m^{-3})	Zooplankton (mg m^{-2})	Eggs (egg m^{-2})	Larvae (ind m^{-2})
Salinity	1.00	-0.004	0.46***	-0.72***	0.03	0.09	-0.04
Temperature		1.00	-0.41***	0.14	0.08	0.04	0-06
Depth			1.00	-0.80***	0.15	0.12	-0.04
Chlorophyll-a				1.00	-0.09	-0.15	0.04
Zooplankton					1.00	0.38***	0.52***
Eggs						1.00	0.49***
Larvae							1.00

*P < 0.05; ** P < 0.01; *** P < 0.001

Table 2.3. Results of single parameter quotient analysis for anchovy eggs and larvae

		Parameter range	Preference range	Tolerance range	Avoidance range
Eggs	Temperature (°C)	18.80-26.47		18.80-26.47	
	Salinity (PSU)	36.24-39.00		36.24-39.00	
	Depth (m)	11-399	91-120	31-90 and > 120	11-30
	Chlorophyll-a (mg m^{-3})	0.11-1.65		0.11-0.52	> 0.52
	Zooplankton (mg m^{-2})	14.82-5410.37		199-5410.37	14.82-199
Larvae	Temperature (°C)	18.80-26.47		18.80-26.47	
	Salinity (PSU)	36.24-39.00		36.24-39.00	
	Depth (m)	11-399	11-60	61-150	> 150
	Chlorophyll-a (mg m^{-3})	0.11-1.65		0.11-1.65	
	Zooplankton (mg m^{-2})	14.82-5410.37	> 1000	299-999	14.82-299

Chapter 3

Stock structure, genetic diversity and estimation of effective number of breeders in *Engraulis encrasicolus*

3.1 Introduction

Many aquatic species are valuable sources for human consumption throughout the world and several of these species are exclusively harvested from wild populations (Ryman et al., 1995; Christensen, 2014). Small pelagic fish hold a relevant role in the economy of fisheries representing the 37% of global wild catches. *E. encrasicolus* is the main target of the Adriatic Sea fishery, in fact, together with *Sardina pilchardus* it constitutes more than 40% of landings within the Adriatic Sea (FAO, 2016). Most of the effects experienced by marine organisms, as a consequence of over-harvesting, come from a poor knowledge about the boundaries of their populations. Growing body of evidence indicates that marine organisms, with potentially dispersive planktonic larvae, can show genetically patchy distributions (Selkoe et al., 2008; Hedgecock & Pudovkin, 2011). This structure has been attributed to natural selection on life-history stages (Kelly & Palumbi, 2010; Selkoe & Robert, 2011), on isolation by hydrographic barriers to gene flow (White et al., 2010) and on adaptation to local environments (Gaggiotti et al., 2009) indicating that local-scale processes are important in the ecology of these populations. Small-scale spatial and temporal heterogeneity can impose selective constraints on the development and survival of larvae and juveniles (White et al., 2010; Riginos & Liggins, 2013) and hence on the distributions of adults (Lamichhaney et al., 2012; Riccioni et al., 2013; Milano et al., 2014).

A genetic structure in anchovies was found within the Adriatic Sea, the recent use of microsatellite DNA and the analyses of a fragment of mitochondrial DNA encompassing partial sequences of Cytochrome b and D-Loop revealed that samples from north-eastern Adriatic segregated from those analysed throughout the entire basin (Vinas et al., 2013; Ruggeri et al., 2016b). The genetic structure observed within the Adriatic seems to be related to both adaptation to habitats in the numerous coves and embayments and around

offshore isles of the north-eastern Adriatic and adaptive selection to local environmental conditions (Artegiani et al., 1997a,b; Giannoulaki et al., 2013; Ruggeri et al., 2016b).

Anchovy is a serial batch spawner that exhibits indeterminate and high fecundity (about 40.000 eggs per spawning season) and produces multiple batches of pelagic eggs over a protracted spawning period. Marine fishes with large population size, like anchovies, are thought to be immune from rapid collapse but genetics studies underline that species with high fecundity and high early mortality are susceptible to large variance in reproductive success (Ruggeri et al., 2012; O'Leary et al., 2013). Pelagic eggs and larvae are very vulnerable to predation and have strict requirements in terms of feeding and environmental conditions, they are faced with a continuous series of "survival windows" with low probability of success (Bakun, 1996). On average, only one or two of the many eggs spawned by each female survive, this means that the normal fate of an egg or larva, prior to metamorphosis from the larval stage, is death (Fréon et al., 2005). Only individuals that spawned "at the right place and at the right time" are involved in reproduction and can contribute genetically to the next generation. Due to the large inter-annual variability of environmental conditions (transport, temperature, abundance of prey, etc.) the "right place and right time" are likely to vary from year to year (Fréon et al., 2005). As a consequence of this unpredictable scenario, the reproduction may be viewed as a sweepstakes, with chance events determining which adults are successful each spawning season, the so called "Sweepstake Reproductive Success" (SRS) (Hedgecock, 1994). Variance in the reproductive success influences genetic diversity through its effects on effective population size (N_e) and reducing the ratio between effective population size and the census size (N_e/N_c). N_c is the census size of a population and governs ecological processes such as competition, predation, and population growth rate. N_e is a key conservation parameter, representing the "genetic currency" of evolutionary potential of a population and, generally, is lower than N_c because of disparities among individuals in their genetic

contribution to the next generation (Frankham, 1995; Nielsen & Hansen, 2008; Hare et al., 2011), long-term evolutionary processes (including the rates of genetic drift and loss of genetic variability and the effectiveness of selection and gene flow) depend primarily on the effective size of the population (N_e). Another important factor is the effective number of breeders (N_b), that statistic gives an estimate of the actual number of adults in a populations who contribute offspring to the next generation. Many eco-evolutionary process in age structured species play out in a theater defined by seasonal events of reproduction for which N_b is more relevant than N_e (Waples & Antao, 2014), being samples comprised individual belonging to the same age group and issued from a single spawning season, N_b could be calculated estimating the N_e statistic obtained from each sample (Hare et al., 2011; St-Onge et al., 2015). Recent genetic studies of small pelagic fishes, including anchovies, show that temporal variation in N_e and genetic diversity is often lower than previously believed, these studies suggest that both fishing and other factors, such as environment shifts and interactions with other species, can lead to the loss of genetic diversity (Pinsky & Palumbi, 2014; Ruggeri et al., 2016a).

In this work we used microsatellite DNA markers to resolve the fine-scale genetic structure of anchovy and to assess genetic variation between adults and larvae of three populations in the Adriatic Sea. We had the following objectives: i) test whether the stock structure in the Adriatic basin is consistent with the one postulate by Ruggeri et al., 2016b, ii) estimate genetic diversity among larvae and adults samples, iii) investigate sibship relations within samples iv) estimate the effective number of breeders (N_b) and the ratio N_b/N_c.

3.2 Material and methods

3.2.1 Sampling method and data collection Samples were collected during the Italian acoustic survey on small pelagic fish in 2015 (MEDIAS project; Leonori et al. 2016) in FAO Geographical Sub Areas 17 and 18 and during Croatian acoustic survey (MEDIAS 2015) in FAO Geographical Sub Areas 17. To achieve the first objective 60 adults anchovies have been collected during winter 2015 from two Croatian sampling sites in the north-eastern Adriatic Sea: a sampling site was off southern part of the Murter Island (UZO) and the second one, was caught from the open water south off Solta Island (SOL) (Fig. 3.1 and Tab. 3.1).

For the three remaining objectives a total of 144 adult individuals and 144 larvae were collected during the spawning season, in the south-western Adriatic Sea (ASA = adults and LSA= larvae) in the central Adriatic Sea (ACA = adults and LCA = larvae) and in the north-eastern Adriatic Sea (ACR = adults and LCR = larvae) (Fig. 3.1 and Tab. 3.1).

The ichthyoplankton was sampled using a WP2 plankton net constructed of 200 micron mesh, instead adult individuals were caught by pelagic trawls net. The collected samples was directly stored in a 70% ethanol solution at room temperature, larval sorting was carried out under stereo microscope in laboratory, larvae were stored individually in separate Eppendorf tubes and were preserved in absolute ethanol maintained at -20°C.

3.2.2 DNA extraction The DNA was extracted automatically using MagCore® Genomic DNA Tissue Kit and manually using the protocol described by Taggart et al. (1992). Quantity and quality of DNA recovered were evaluated by means of a spectrophotometer assay (Desjardins & Conklin, 2011). Eleven loci were labeled with fluorescent dyes and multiplexed in three separated reactions, the remaining two loci (Ee2-508 and Ee2-165b), due to their moderate level of polymorphism, were amplified individually (Tab. 3.2). All samples were screened at 13 polymorphic microsatellite loci previously described (Landi

et al., 2005; Pakaki et al., 2009; Lin et al., 2011) and conditions for the PCR amplification were optimized as in (Ruggeri et al., 2013). PCR products were separated on a 2% (w/v) agarose gel and stained with GelRed™ (Biotium, Inc.) to check for size and PCR specificity.

3.2.3 Genotyping After amplification, PCR products in multiplex were genotyped by automatic sequencing using an ABI-PRISM 3130xl genetic analyser (Applied Biosystems) and the program Peak Scanner (freely available from Applied Biosystems, http://www.appliedbiosystems.com/peakscanner.html) was used for the peaks identification of emission products by single fluorescent dye labels. PCR products in simplex were genotyped by vertical electrophoresis on 5% denaturing polyacrylamide gel and visualized by a silver staining protocol (Bassam et al., 1991; Benbouza et al., 2006). Additional information for spectrophotometer assay, PCR amplification protocols, amplification profiles and genotyping are provided in Supplementary Material file.

3.2.4 Statistical analysis After the genotyping, a series of statistical analyses were carried out. The overall quality of raw data (e.g., misidentification of alleles), the occurrence of null alleles and the incidence of other genotyping errors (allele dropout and stutter peaks) were assessed with MICROCHECKER 2.2.1 (Van Oosterhout et al., 2004). In SOL and UZO populations loci affected by null alleles were corrected following the Brookfield algorithm (Brookfield, 1996), instead for adult and larvae, the software FreeNa (Chapuis & Estoup, 2007) was used to detected null alleles and to compare pairwise F_{ST} between row microsatellite data and a corrected version, after applying the excluding null alleles correction method (ENA) with 10000 bootstrap repetition. Genetic diversity parameters as mean number of alleles (N_A), expected (H_E) and observed (H_O) heterozygosities and inbreeding coefficient (F_{IS}), were provided by the use of Microsatellite toolkit (Park, 2001) and FSTAT 2.9.3.2 (Goudet, 2001).

Genepop 4.0.11 (http://genepop.curtin.edu.au) (Rousset, 2008; Raymond & Russet, 1995) was first employed to test departure from Hardy-Weinberg equilibrium (HWE) with the exact test implemented in using a Markov Chain Monte Carlo (MCMC) method with 100 batches of 10000 iterations each, with a burnin of 1000 iterations (the first 1000 iterations discarded before sampling), and to determine whether any locus pair were in linkage disequilibrium by a MCMC chain executed with 100 batches of 1000 iterations each. A sequential Bonferroni adjustment of P-values was used to account for an increase in type-I error for multiple comparisons ($0.05/\alpha$; α = pairwise test) (Rice, 1989).

Population structure were assayed by STRUCTURE v.2.3.2.1 (Pritchard et al., 2000; Falush et al., 2003). This software use a Bayesian calculation of probability for assess the number of population genetically differentiated within the data analysed. In addition, it provides the individual proportion of multi-locus genetic variability that belong to the genetic population detected for all the specimen analysed. Runs in STRUCTURE were repeated 10 times per K (number of expected clusters/stocks) and imposing an admixture model with correlated allele frequencies. Each K value was replicated with ten independent runs of 100000 MCMC iterations, after a burnin of 10000 iterations. A set of STRUCTURE simulations were made using all loci with Prior information (LocPrior mode). The Evanno method (Evanno et al., 2005) in STRUCTURE HARVESTER (Earl & VonHoldt, 2012) was used to determine the most likely K value from all simulations and graphical representation of multiple runs per K values were produced by the CLUMPAK Server (http://clumpak.tau.ac.il) (Kopelman et al., 2015).

The number of breeders (N_b) statistic gives an estimate of the actual number of adults responsible for producing all larvae in a given sample. Because samples comprised individuals belonging to the same age group and issued from a single breeding cycle and/or spawning season, N_b could be calculated by estimating the effective population size (N_e) statistic obtained from each sample (Hare et al., 2011). This was achieved using a

maximum-likelihood assignment method assuming random mating (Wang, 2009; Wang & Santure, 2009) as implemented in the software COLONY version 2.0.2.2 (Jones & Wang, 2010). For comparison purposes, estimates of N_e were also obtained with the N_e Estimator version 2.0. software (Do et al., 2014) using the linkage disequilibrium method (Waples & Do, 2008). Estimates of N_c were obtained from annual acoustic surveys (MEDIAS Project, http://www.medias-project.eu) (Leonori et al., 2011) for stocks in the northern and middle-southern Adriatic Sea.

To further infer the possible origin of the parents and offspring, we used a maximum-likelihood sibship assignment procedure implemented in COLONY using multi-locus genotype data (Wang and Santure, 2009). This analysis infers sibship by assuming that all individuals present within each sample of larvae populations (LSA, LCA, LCR) were offspring issued from unknown sets of unrelated candidate mothers and fathers from adults populations (CSA, ACA, ACR). Offspring pairs can either be full-sibs (sharing both parents), half-sibs (sharing only one of two parents) or unrelated to each other (sharing no parents). We assumed polygamous mating system, only relationships with probability higher than 0.95 were considered.

CERVUS 3.0 (Kalinowski et al., 2007) is a paternity/maternity allocation program calculates the log-likelihood of each candidate parent being the true parent relative to an arbitrary individual and then calculates the difference between the two most likely parents. The program derives likelihood ratios for paternity/maternity for each offspring which, taken with population allele frequency data, is used to define a statistic for allocating, with confidence, the most likely parent. The program allocates one parent at a time. In studies where both parents need to be resolved, two consecutive allocations need to be performed. The first allocation attempts to find the most likely (and statistically robust) parent in the entire population (male or female). A second allocation can then be performed to assign the second parent (informed by the now known genotype of the allocated first parent).

Simulation parameters were set at 10000 offspring, with 100% of candidate parents sampled and a total proportion of loci typed over all individuals of 0.92, mistyping error rates = 0.01 and likelihood calculation error rates = 0.01. Strict confidence was set to 95%, while the relaxed confidence level was 80%.

3.3 Results

3.3.1 Genetic diversity and stock structure The SOL sample was completely PCR amplifiable and genotyped, whereas a single individual from UZO sample was un-amplifiable at almost all loci and consequently the UZO sample was represented by 29 typed individuals. The missing data accounted for 13.9% of the total raw genotyped SOL and UZO data. Most of the missing genotypes come from the locus Enja83, since it was more prone to PCR failure. The simulation of statistical procedures including or excluding this locus seemed to not affect particularly any results, therefore the locus Enja83 was maintained in all further statistical analysis. The MICROCHECKER analysis revealed a lack of PCR artifacts (allele dropout and stuttering) at all the microsatellite loci analysed. However, signals for null alleles were identified in 10 out of 28 tests. The use of Brookfield algorithm allowed to reduce the incidence of null alleles to 4 out of 28 tests.

Globally, genetic variability was similar between SOL and UZO, the most polymorphic loci were Ee2-507 and Ej27.1 with 21 alleles in SOL and Ej2 in UZO with 19 alleles. The less polymorphic locus was Ee2-165b both in SOL and UZO, respectively displaying 3 and 4 alleles. Expected heterozygosity ranged between 0.575 and 0.954 in SOL and between 0.531 and 0.949 in UZO. Observed heterozygosity ranged between 0.591 and 0.964 in SOL and between 0.296 and 0.962 in UZO (Tab. 3.3). The Bayesian analysis for population structure carried out by STRUCTURE software confirmed the existence of two genetic stocks within the Adriatic basin (Fig. 3.2) and included SOL and UZO samples together with north-eastern Adriatic samples (EM38, EM05 and EM07) (Fig. 3.3 and Fig. 3.4).

3.3.2 Genetic diversity in adult and larval populations, parentage analysis, N_b and N_e
The PCR success rate was high for the 13 microsatellite loci, only one individual, belonging to the larval population LSA, was not PCR-amplifiable. Since all the marker

utilized were specifically designed on *Engraulis* species, it was conceivable that these larva was not anchovy, the remaining 287 individuals were easily amplifiable. Missing data consisted of 12 out of 3731 genotypes over all the 13 loci analysed (0.32% of missing data). Albeit the data showed a lack of allele dropout and stuttering, signals of null alleles were detected in 40 of 78 global tests. Globally null alleles have been observed in all the analysed loci, with the exception of Ee2-91b, Ej35 and Ee2-165b. The major incidence of the phenomenon was observed in Ej-41.1, Ej-27.1 and Enja83, where null alleles have been detected in all the populations. Between larval populations, LCR is the population with the higher incidence of null alleles (7 loci on 13) whereas, between adults, ACA and ACR are the most affected populations (8 loci on 13). However pairwise F_{ST} did not show values with completely different order of magnitudes, either when estimated from raw allele frequencies or from allele frequencies corrected for null alleles (Tab. SM1). This suggest that null alleles have very little influence on our analysis; therefore all further tests were performed with uncorrected allele frequencies. The observed number of alleles across samples varied from 9 at locus Ee2-165b to 39 at locus Ee2-507. Concerning larval populations the mean number of alleles (N_A) per sample over all loci varied from 14.15 alleles in LCA to 15.62 alleles in LSA. The smallest values of expected (H_E) and observed (H_O) heterozygosities were in samples LCA, whereas the largest values were in samples LSA (Tab. 3.4). Concerning adults N_A per sample over all loci varied from 14.46 alleles in ACA to 14.62 alleles in ASA. The smallest values of H_E and H_O were in samples ACA and ACR respectively, whereas the largest values were in samples ACR and ASA (Tab. 3.4). The overall genetic variability do not display significant differences neither between adults and larvae nor between different localities (Fig 3.5). F_{IS} value are significant for all the populations with the highest value in LCR and ACR (Tab. 3.4). Significant departures from the Hardy-Weinberg (HW) proportions over all loci were found in 8 loci (Ee2-91b, Ej-41.1, Ej-27.1, Ej35, Enja83, Ee2-507, Ej-2, Ee2-508). Of 468 tests, 16 showed

significant linkage disequilibrium but, with the Bonferroni correction, the significant pairwise test become only 2, which occurred between Ej-41.1 x Ee2-135 in the LCR sample and Ee2-91b x Enja83 in the ACR sample.

We used COLONY to infer the possible sibships between offsprings and results highlight 5 half-sib pairwise and 2 full-sib, albeit the latter cannot take into account due to the low percentage of probability (Tab. 3.5). The parentage analysis with CERVUS assessed 18 offspring to a parents with relaxed confidence level (80%), dropping to 5 offspring assigned parentage at strict confidence level (95%) (Tab 3.5). Moreover COLONY was used to assess the mean number of breeders (N_b) obtained with the maximum-likelihood method. Results ranged between 98 (LCA and LCR) and 114 (LSA), whereas the census size (N_c) obtained from MEDIAS acoustic survey data were really high, 4.40E+09 for the LSA population and 2.64E+10 for LCA causing a very low ratio between N_b/ N_c. The method of linkage disequilibrium with the software N_e Estimator provided higher estimates of effective population size (N_e), this method however seemed to be less accurate for dataset with small sample size, since it provide confidence intervals with infinite values. We reported estimated N_b and N_e with 95% confidence intervals (CI) (Tab. 3.6).

3.4 Discussion and conclusion

3.4.1 Stock structure within Adriatic Sea The main objective of the genetic characterization of SOL and UZO samples was the identification of the genetic origin of these two samples on the basis of what evidenced in Ruggeri et al., 2016b. Particularly, the results from genetic analysis of SOL and UZO samples can reveal the geographical extension of the north-eastern Adriatic population/stock identified in the aforementioned work. An important result of this study indicates that anchovies throughout the Adriatic belong to a single species, *Engraulis encrasicolus*. Nevertheless, the individuals collected in the study area, which covered the entire Adriatic basin and part of the Tyrrhenian Sea, were partitioned into two major populations. As in many marine species, gene flow in Adriatic anchovies occurs by larval dispersal in currents or by adult movement and prevents the accumulation of genetic differences among populations (Waples, 1998), albeit, significant differences between some samples indicate that Adriatic anchovies are not entirely panmictic. The analysis of neutral makers with STRUCTURE resolved two major population groups: populations in north-eastern coastal areas (EM38, EM05 and EM07) and the remaining populations in the Adriatic and Tyrrhenian Seas. The oceanic front between the Istrian Peninsula and Mid-Adriatic Pit may explain, in part, the north-south genetic discontinuity by limiting gene flow. The anticlockwise gyre off the Po River delta may also act as a barrier to gene flow; although both the front and the gyre are ephemeral, they are strongest in summer and autumn (Artegiani et al., 1997a,b) during the anchovy spawning season when larvae and juveniles are most abundant (Morello & Arneri, 2009; Giannoulaki et al., 2013). Larval retention by ocean fronts and gyres is a well-known isolating mechanism in other pelagic fishes (Iles & Sinclair, 1982; Warner & Cowen, 2002). Hence, these oceanic mechanisms may limit the movement of larvae and juveniles and prevent mixing between larvae and juveniles from neighboring spawning sites.

Moreover genetic discontinuities among populations of anchovies may be related to the effects of environmental variables on the growth and survival of early developmental stages, as well as on adult reproductive success. The complex genetic patterns among populations of marine organisms have been correlated only recently with the influence of environmental variables on the genomes of several marine species (Riginos & Liggins, 2013). Adriatic anchovy sub-populations show a patchy distribution of genetic variability that appears to reflect selective processes mediated by several environmental variables and the genetic uniqueness of north-eastern anchovies may be due to adaptation to habitats in the numerous coves and embayments and around offshore isles (Ruggeri et al., 2016b). The analysis of SOL and UZO samples add further information about the geographical extent of north-eastern Adriatic anchovy stock, suggesting that it typically occurs in marine environment dominated by embayment and islands along the coastline with distinct environmental variables and it reach the southern Dalmatia.

3.4.2 Genetic diversity, parentage analysis, N_b and N_e The main goals of this section were: i) estimate genetic diversity among samples of larvae and adults; ii) investigate sibship relations within samples; iii) estimate the effective number of breeders and the ratio N_b/N_c. Microsatellite markers represent ideal molecular tools to achieve this goals because are easily amplifiable, hypervariable and co-dominant and can provide information about genetic variability between populations and infer family relations between individuals providing information at single independent loci (Selkoe & Toonen, 2006; Jones et al., 2010); our results highlight a wide range of polymorphism of microsatellite DNA, in agreement with previous study on small pelagic fishes (DeWoody & Avise, 2000) and on anchovy (Yu et al., 2002; Zarraonaindia et al., 2009). Anchovy, as many marine species, have extremely high fecundities and typically suffer high juvenile mortality as a consequence of unpredictable environmental variation. If the unpredictability is associated with reproduction, resulting in a small fraction of adult population contributing to each

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annual cohort, low levels of genetic diversity in each cohort are expected. In this case the reproduction may be viewed as a sweepstake, in which chance events determine which adults are successful each spawning season (Hedgecock, 1994). Sweepstake reproductive success is expected to leave a diagnostic signature on the genetic composition of marine larvae (Hedgecock, 1994). First, the cohort are predicted to exhibit reduced levels of genetic variation relative to the parental population, estimation of genetic diversity (as expected (H_E) and observed (H_o) heterozygosities) of this work are consistent with other results on anchovy and on other marine species (DeWoody & Avise, 2000; Ruzzante et al., 2006; Borrell et al., 2012; Ruggeri et al., 2013, 2016a), moreover, genetic variability in offspring populations are consistently high and are not reduced relative to estimates of diversity from the adult populations. Comparison between offspring and adult samples revealed no genetic differentiation and no reduction in genetic variation of larvae; similar findings have been recorded by Flowers et al., (2002) in sea urchin and Gilbert-Horvath et al., (2006) in kelp rockfish, this can be indicates that sweepstakes reproduction is likely not occurring in anchovy populations within Adriatic Sea. However significant inbreeding coefficient (F_{IS}) have been found in all populations, with the highest values in adults and larvae of the north-eastern Adriatic; this can be due to natural selection and genetic drift causing a decline in genetic diversity due to loss of alleles, which leads to an increase in homozygosity. A second prediction of the SRS is that individual within cohorts should be significantly more related to each other than in groups of randomly selected individuals originating from the same populations. To test this prediction a parentage analysis have been performed using the maximum-likelihood method in two different programs (COLONY and CERVUS), the biggest problem of this analysis, when is carried out an a very large natural population with high mortality rate, is certainly the risk of underestimating the result obtained due to limitations in the number of markers and to the low number of analysed samples that could affect the assignments or generate false parent-

offspring pairs (Jerry et al., 2004; Christie, 2010). The maximum-likelihood method used in this study is predicted to be approximately 65% accurate, although this method seems to be the more accurate, more powerful and more conservative than other commonly used methods (St-Onge et al., 2015). However sibships in larval pools or high relatedness between offspring and adult have not been found, in fact using COLONY, only 5 significant half-sib pairwise have been detected with 4 related larvae from eastern, 4 from southern and 2 from middle Adriatic, on the other hand, CERVUS assessed 18 offspring to a parents with relaxed confidence level (80%), dropping to 5 offspring assigned parentage at strict confidence level (95%) with most of the assignments between the south and the east Adriatic. From these abovementioned results, one would normally conclude that sweepstake lottery did not govern the observed pattern of recruitment, but, assessment of small effective number of breeders (N_b) estimated with the effective population size (N_e) in COLONY, and the extremely low ratio between number of breeders and census size, may suggested otherwise. In fact, our estimates of N_b for Adriatic anchovy indicated values between 98 to 114, moreover, estimates of N_b/N_c was between 10^{-8} and 10^{-9} orders of magnitude and are much smaller than those typical of many marine species ($N_b/N_c \geq 10^{-5}$) (Hare et al., 2011). Small number of breeders in anchovies suggest that genetic drift, together with repeated selective sweeps, can mold the anchovy genome, resulting in local adaptation to major environmental drivers. In addition, small ratios indicate that the large variance in reproductive success can lead to demographic instability, challenging the idea that marine populations are invulnerable and inexhaustible resources by virtue of their huge census sizes (Gaggiotti & Vetter, 1999; Pinsky & Palumbi, 2014). Although the estimation of N_e with N_e estimator seemed to be less accurate, it is nevertheless important to compare our estimates of N_e with conservation guidelines provided by the 50/500 rule (Frankham et al., 2002). The 50/500 rule recommends that populations be maintained at sizes larger than 50 individuals over the short term and at least 500 individuals over the

long term to avoid inbreeding and the loss of genetic diversity, also this rule has recently been revised and raised at 500/1000 for indefinitely retain evolutionary potential (Frankham et al., 2014). Our estimates of N_e ranged from hundreds to low thousands, such values, are comparable to those recorded in a study conducted on the European sardine population from the Bay of Biscay (Laurent & Planes, 2007) and in other studies carried out on highly exploited marine species (Hauser et al., 2002; Hutchinson et al., 2003; Cuveliers et al., 2011). The lowest N_e value (562) was detected within Croatian larval population and it may be somewhat consistent with the risk of inbreeding in places with reduced gene flow (Allendorf, 1986; Ruggeri et al., 2016a,b). The vast majority of studies reporting an absence of sweepstake reproduction have based their conclusions on statistical analysis solely focused on the level of differentiation between samples so, it seems somewhat paradoxical to have observed an absence of sweepstake due to temporal variations in allelic frequencies and weak relatedness patterns but, on the other hand, have been detected low level of N_e and N_b and very low levels of N_b/N_c ratio; however, it is plausible that the decreasing of genetic variability could be hidden by the effects of overlapping generations acting as a reservoir of diversity, (Gilbert-Horvath et al., 2006). Finally N_e or N_b estimates can used to formulate recommendations for the conservation of wild populations for mitigating loss of genetic diversity, preserving evolutionary potential and decreasing extinction risks (Frankham et al., 2010, 2014).

References

Allendorf, F.W., 1986. Genetic drift and the loss of alleles versus heterozygosity. *ZOObiology*, 5 (2), 181–190. DOI: 10.1002/zoo.1430050212.

Artegiani, A., Bregant, D., Paschini, E., Pinardi, N., Raicich, F., Russo, A., 1997a. The Adriatic Sea General Circulation. Part I : Air – Sea Interactions and Water Mass Structure. *Journal of Physical Oceanography*, 27, 1492–1514.

Artegiani, A., Bregant, D., Paschini, E., Pinardi, N., Raicich, F., Russo, A., 1997b. The Adriatic Sea General Circulation. Part II : Baroclinic Circulation Structure. *Journal of Physical Oceanography*, 27, 1515–1532.

Bakun, A., 1996. Patterns in the ocean: ocean processes and marine population dynamics. *California Sea Grant, in cooperation with Centro de Investigaciones Biologicas del Noroeste, La Paz, Mexico*.

Bassam, B.J., Caetano-Anollés, G., Gresshoff, P.M., 1991. Fast and sensitive silver staining of DNA in Polyacrylamide gels. *Analytical Biochemistry*, 196 (1), 80–83. DOI: 10.1016/0003-2697(91)90120-I.

Benbouza, H., Jacquemin, J., Baudoin, J., Mergeai, G., 2006. Optimization of a reliable , fast , cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. *Biotechnology, Agronomy, Society and Environment*, 10 (2), 77–81. DOI: 10.1067/mod.2002.129195.

Borrell, Y.J., Pinera, J.A., Sanchez Prado, J.A., Blanco, G., 2012. Mitochondrial DNA and microsatellite genetic differentiation in the European anchovy *Engraulis encrasicolus* L. *ICES Journal of Marine Science*, 69 (8), 1357–1371. DOI: doi:10.1093/icesjms/fss129.

Chapuis, M.P., Estoup, A., 2007. Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*, 24 (3), 621–631. DOI: 10.1093/molbev/msl191.

Christensen, J., 2014. Unsettled Seas: Towards a History of Marine Animal Populations in the Central Indo-Pacific. *In: Christensen J., Tull M. (eds) Historical Perspectives of Fisheries Exploitation in the Indo-Pacific. MARE Publication Series, Springer, Dordrecht*, 12.

Christie, M.R., 2010. Parentage in natural populations: novel methods to detect parent-offspring pairs in large data sets. *Molecular Ecology Resources*, 10 (1), 115–128. DOI: 10.1111/j.1755-0998.2009.02687.x.

Cuveliers, E.L., Volckaert, F.A.M., Rijnsdorp, A.D., Larmuseau, M.H.D., Maes, G.E., 2011. Temporal genetic stability and high effective population size despite fisheries-induced life-history trait evolution in the North Sea sole. *Molecular Ecology*, 20 (17), 3555–3568. DOI: 10.1111/j.1365-294X.2011.05196.x.

Desjardins, P.R., Conklin, D.S., 2011. Microvolume Quantitation of Nucleic Acids. *Current Protocols in Molecular Biology*.

DeWoody, J.A., Avise, J.C., 2000. Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *Journal of Fish Biology*, 56 (3), 461–473. DOI: 10.1006/jfbi.1999.1210.

Do, C., Waples, R.S., Peel, D., Macbeth, G.M., Tillett, B.J., Ovenden, J.R., 2014. NeEstimator v2: Re-implementation of software for the estimation of contemporary effective population size (N_e) from genetic data. *Molecular Ecology Resources*, 14, 209–214. DOI: 10.1111/1755-0998.12157.

Earl, D.A., VonHoldt, B.M., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, 4 (2), 359–361. DOI: 10.1007/s12686-011-9548-7.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology*, 14 (8), 2611–2620. DOI: 10.1111/j.1365-294X.2005.02553.x.

FAO (Food and Agriculture Organization), 2014. World review of fisheries and aquaculture.

Food and Agriculture Organisation, 1–96.

FAO (Food and Agriculture Organization), 2016. The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. *Rome: Food and Agriculture Organisation,* 200 pp.

Flowers, J.M., Schroeter, S.C., Burton, R.S., 2002. The recruitment sweepstakes has many winners: genetic evidence form the Sea Urchin *Strongylocentrotus purpuratus*. *Evolution*, 56 (7), 1445–1453.

Frankham, R., 1995. Effective population size/adult population size ratios in wildlife: a review. *Genetical Research*, 66 (2), pp: 95.

Frankham, R., Briscoe, D.A., Ballou, J.D., 2002. Introduction to conservation genetics. I edition, *Cambridge Univeristy Press*.

Frankham, R., Ballou, J.D., Briscoe, D.A., 2010. Introduction to Conservation Genetics. II edition, *Cambridge Univeristy Press*.

Frankham, R., Bradshaw, C.J.A., Brook, B.W., 2014. Genetics in conservation management: revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. *Biological Conservation*,. DOI: 10.1016/j.biocon.2013.12.036.

Fréon, P., Cury, P., Shannon, L., Roy, C., 2005. Sustainable exploitation of small pelagic fish stockschallenged by environmental and ecosystem changes: a review. *Bulletin of marine science*, 76 (2), 385–462.

Gaggiotti, O.E., Bekkevold, D., Jørgensen, H.B.H., Foll, M., Carvalho, G.R., Andre, C., Ruzzante, D.E., 2009. Disentangling the effects of evolutionary, demographic, and environmental factors influencing genetic structure of natural populations: Atlantic herring as a case study. *Evolution*, 63 (11), 2939–2951. DOI: 10.1111/j.1558-5646.2009.00779.x.

Gaggiotti, O.E., Vetter, R.D., 1999. Effect of life history strategy, environmental variability, and overexploitation on the genetic diversity of pelagic fish populations. *Canadian Journal of Fisheries and Aquatic Sciences*, 56 (8), 1376–1388. DOI: 10.1139/f99-060.

Giannoulaki, M., Iglesias, M., Tugores, M.P., Bonanno, A., Patti, B., De Felice, A., Leonori, I., Bigot, J.L., Tičina, V., Pyrounaki, M.M., Tsagarakis, K., Machias, A., Somarakis, S., Schismenou, E., Quinci, E., Basilone, G., Cuttitta, A., Campanella, F., Miquel, J., Oñate, D., Roos, D., Valavanis, V., 2013. Characterizing the potential habitat of European anchovy *Engraulis encrasicolus* in the Mediterranean sea, at different life stages. *Fisheries Oceanography*, 22 (2), 69–89. DOI: 10.1111/fog.12005.

Gilbert-Horvath, E.A., Larson, R.J., Garza, J.C., 2006. Temporal recruitment patterns and gene flow in kelp rockfish (*Sebastes atrovirens*). *Molecular Ecology*, 15 (12), 3801–3815. DOI: 10.1111/j.1365-294X.2006.03033.x.

Hare, M.P., Nunney, L., Schwartz, M.K., Ruzzante, D.E., Burford, M., Waples, R.S., Ruegg, K., Palstra, F., 2011. Understanding and estimating effective population size for practical application in marine species management. *Conservation Biology*, 25 (3), 438–449. DOI: 10.1111/j.1523-1739.2010.01637.x.

Hauser, L., Adcock, G.J., Smith, P.J., Bernal Ramirez, J.H., Carvalho, G.R., 2002. Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus*). *Proceedings of the National Academy of Sciences*, 99 (18), 11742–11747. DOI: 10.1073/pnas.172242899.

Hedgecock, D., 1994. Does variance in reproductive success limit effective population sizes of marine organisms? *Genetics and Evolution of Aquatic Organisms*, (October), 122–134.

Hedgecock, D., Pudovkin, A.I., 2011. Sweepstakes reproductive success in highly fecund marine fish and shellfish: a review and commentary. *Bulletin of Marine Science*, 87 (4), 971–1002. DOI: 10.5343/bms.2010.1051.

Hutchinson, W.F., van Oosterhout, C., Rogers, S.I., Carvalho, G.R., 2003. Temporal analysis of archived samples indicates marked genetic changes in declining North Sea cod (*Gadus morhua*). *Proceedings of the Royal Society B*, 270 (1529). DOI: 10.1098/rspb.2003.2493.

Iles, T.D., Sinclair, M., 1982. Atlantic herring: stock discreteness and abundance. *Science*, 215

(4533), 627–633. DOI: 10.1126/science.215.4533.627.

Jerry, D.R., Preston, N.P., Crocos, P.J., Keys, S., Meadows, J.R.S., Li, Y., 2004. Parentage determination of Kuruma shrimp *Penaeus (Marsupenaeus) japonicus* using microsatellite markers (Bate). *Aquaculture*, 235 (1–4), 237–247. DOI: 10.1016/j.aquaculture.2004.01.019.

Jones, A.G., Small, C.M., Paczolt, K.A., Ratterman, N.L., 2010. A practical guide to methods of parentage analysis. *Molecular Ecology Resources*, 10 (1), 6–30. DOI: 10.1111/j.1755-0998.2009.02778.x.

Jones, O.R., Wang, J., 2010. COLONY: A program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, 10 (3), 551–555. DOI: 10.1111/j.1755-0998.2009.02787.x.

Kalinowski, S.T., Taper, M.L., Marshall, T.C., 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, 16, 1099–1106. DOI: 10.1111/j.1365-294X.2007.03089.x.

Kelly, R.P., Palumbi, S.R., 2010. Genetic structure among 50 species of the northeastern pacific rocky intertidal community. *PLoS One*, 5 (1). DOI: 10.1371/journal.pone.0008594.

Kopelman, N.M., Mayzel, J., Jakobsson, M., Rosenberg, N.A., Mayrose, I., 2015. CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, 15 (5), 1179–1191. DOI: 10.1111/1755-0998.12387.

Lamichhaney, S., Barrio, A.M., Rafati, N., Sundstrom, G., Rubin, C-J., Gilbert, E.R., Berglund, J., Wetterbom, A., Laikre, L., Webster, M.T., Grabherr, M., Ryman, N., Andersson, L., 2012. Population-scale sequencing reveals genetic differentiation due to local adaptation in Atlantic herring. *Proceedings of the National Academy of Sciences*, 109 (47), 19345–19350. DOI: 10.1073/pnas.1216128109.

Landi, M., Garoia, F., Piccinetti, C., Tinti, F., 2005. Isolation of polymorphic microsatellite loci

from the European anchovy, *Engraulis encrasicolus*. *Molecular Ecology Notes*, 5 (2), 266–268. DOI: 10.1111/j.1471-8286.2005.00892.x.

Laurent, V., Planes, S., 2007. Effective population size estimation on *Sardina pilchardus* in the Bay of Biscay using a temporal genetic approach. *Biological Journal of the Linnean Society*, 90, 591–602. DOI: 10.1111/j.1095-8312.2007.00747.x.

Leonori, I., De Felice, A., Campanella, F., Biagiotti, I., Canduci, G., 2011. Assessment of small pelagic fish biomass in the Adriatic Sea by means of acoustic methodology. p. 2019–2029. *In: Fishery and Sea Resources. Marine Research at CNR, Eds Brugnoli, E., Cavaretta, G., Mazzola, S., Trincardi, F., Ravaioli, M., and Santoleri, R.*

Lin, L., Zhu, L., Liu, S.F., Su, Y.Q., Zhuang, Z.M., 2011. Polymorphic microsatellite loci for the japanese anchovy *Engraulis japonicus* (engraulidae). *Genetics and Molecular Research*, 10 (1), 764–768. DOI: 10.4238/vol10-2gmr1085.

Milano, I., Babbucci, M., Cariani, A., Atanassova, M., Bekkevold, D., Carvalho, G.R., Espiñeira, M., Fiorentino, F., Garofalo, G., Geffen, A.J., Hansen, J.H., Helyar, S.J., Nielsen, E.E., Ogden, R., Patarnello, T., Stagioni, M., Tinti, F., Bargelloni, L., 2014. Outlier SNP markers reveal fine-scale genetic structuring across European hake populations (*Merluccius merluccius*). *Molecular Ecology*, 23 (1), 118–135. DOI: 10.1111/mec.12568.

Morello, B., Arneri, E., 2009. Anchovy and sardine in the Adriatic Sea - an ecological review. *Oceanography and Marine Biology: An Annual Review*, 47, 209–256.

Nielsen, E.E., Hansen, M.M., 2008. Waking the dead: the value of population genetic analyses of historical samples. *Fish and Fisheries*, 9 (4), 450–461. DOI: 10.1111/j.1467-2979.2008.00304.x.

O'Leary, S.J., Hice, L.A., Feldheim, K.A., Frisk, M.G., McElroy, A.E., Fast, M.D., Chapman, D.D., 2013. Severe inbreeding and small effective number of breeders in a formerly abundant marine fish. *PLoS One*, 8 (6), 2–9. DOI: 10.1371/journal.pone.0066126.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P., 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4 (3), 535–538. DOI: 10.1111/j.1471-8286.2004.00684.x.

Pakaki, V., Magoulas, A., Kasapidis, P., 2009. New polymorphic microsatellite loci for population studies in the European anchovy, *Engraulis encrasicolus* (L.). *Molecular Ecology Resources*, 9 (5), 1406–1409. DOI: 10.1111/j.1755-0998.2009.02678.x.

Pinsky, M.L., Palumbi, S.R., 2014. Meta-analysis reveals lower genetic diversity in overfished populations. *Molecular Ecology*, 23 (1), 29–39. DOI: 10.1111/mec.12509.

Riccioni, G., Stagioni, M., Landi, M., Ferrara, G., Barbujani, G., Tinti, F., 2013. Genetic structure of bluefin tuna in the mediterranean sea correlates with environmental variables. *PLoS One*, 8 (11). DOI: 10.1371/journal.pone.0080105.

Rice, W.R., 1989. Analyzing tables of statistical tests. *Evolution*, 43 (1), 223–225.

Riginos, C., Liggins, L., 2013. Seascape Genetics: populations, individuals, and genes marooned and adrift. *Geography Compass*, 7 (3), 197–216. DOI: 10.1111/gec3.12032.

Rousset, F., 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, 8, 103–106. DOI: 10.1111/j.1471-8286.2007.01931.x.

Ruggeri, P., Splendiani, A., Bonanomi, S., Arneri, E., Cingolani, N., Santojanni, A., Belardinelli, A., Giovannotti, M, Caputo, V., Moran, P., 2012. Temporal genetic variation as revealed by a microsatellite analysis of European sardine (*Sardina pilchardus*) archived samples. *Canadian Journal of Fisheries and Aquatic Sciences*, 69 (10), 1698–1709. DOI: 10.1139/f2012-092.

Ruggeri, P., Splendiani, A., Bonanomi, S., Arneri, E., Cingolani, N., Santojanni, A., Colella, S., Donato, F., Giovannotti, M., Caputo Barucchi, V., 2013. Searching for a stock structure in *Sardina pilchardus* from the Adriatic and Ionian Seas using a microsatellite DNA-based approach. *Scientia Marina*, 77 (4), 565–574. DOI: 10.3989/scimar.03843.26A.

Ruggeri, P., Splendiani, A., Di Muri, C., Fioravanti, T., Santojanni, A., Leonori, I., De Felice, A., Biagiotti, I., Carpi, P., Arneri, E., Nisi Cerioni, P., Giovannotti, M., Caputo Barucchi, V., 2016a. Coupling demographic and genetic variability from archived collections of European anchovy (*Engraulis encrasicolus*). *PLoS One*, 11 (3), 1–18. DOI: 10.1371/journal.pone.0151507.

Ruggeri, P., Splendiani, A., Occhipinti, G., Fioravanti, T., Santojanni, A., Leonori, I., De Felice, A., Arneri, E., Procaccini, G., Catanese, G., Ticina, V., Bonanno, A., Nisi Cerioni, P., Giovannotti, M., Grant, W.S., Caputo Barucchi, V., 2016b. Biocomplexity in populations of European anchovy in the Adriatic Sea. *PLoS One*, 11 (4), 1–21. DOI: 10.1371/journal.pone.0153061.

Ruzzante, D.E., Mariani, S., Bekkevold, D., André, C., Mosegaard, H., Clausen, L.A.W., Dahlgren, T.G., Hutchinson, W.F., Hatfield, E.M., Torstensen, E., Brigham, J., Simmonds, EJ., Laikre, L., Larsson, L.C., Stet, R.J.M., Ryman, N., Carvalho, G.R., 2006. Biocomplexity in a highly migratory pelagic marine fish, Atlantic herring. *Proceedings of the Royal Society B*, 273 (1593). DOI: 10.1098/rspb.2005.3463.

Ryman, N., Utter, F., Laikre, L., 1995. Protection of intraspecific biodiversity of exploited fishes. *Reviwes in Fish Biology and Fisheries*, 5 (4), 417–446. DOI: 10.1007/BF01103814.

Selkoe, K.A., Henzler, C.M., Gaines, S.D., 2008. Seascape genetics and the spatial ecology of marine populations. *Fish and Fisheries*, 9 (4), 363–377. DOI: DOI 10.1111/j.1467-2979.2008.00300.x.

Selkoe, K.A., Robert, J.T., 2011. Marine Connectivity: a new look at pelagic larval duration and genetic metrics of dispersal. *Marine Ecology Progress Series*, 436, 291–305.

Selkoe, K.A., Toonen, R.J., 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, 9 (5), 615–629. DOI: 10.1111/j.1461-0248.2006.00889.x.

St-Onge, P., Tremblay, R., Sévigny, J.M., 2015. Tracking larvae with molecular markers

reveals high relatedness and early seasonal recruitment success in a partially spawning marine bivalve. *Oecologia*, 178 (3), 733–746. DOI: 10.1007/s00442-015-3245-2.

Taggart, J.B., Hynes, R.A., Prodöuhl, P.A., Ferguson, A., 1992. A simplified protocol for routine total DNA isolation from salmonid fishes. *Journal of Fish Biology*, 40, 963–965. DOI: doi: 10.1111/j.1095-8649.1992.tb02641.x.

Vinas, J., Sanz, N., Penarrubia, L., Araguas, R-M., Garcìa-Marìn, J-L., Roldan, M-I., Pla, C., 2013. Genetic population structure of European anchovy in the Mediterranean sea and the Northeast Atlantic cean using sequence analysis of the mitochondrial DNA control region. *ICES Journal of Marine Science*, 1–7.

Wang, J., 2009. A new method for estimating effective population sizes from a single sample of multilocus genotypes. *Molecular Ecology*, 18 (10), 2148–2164. DOI: 10.1111/j.1365-294X.2009.04175.x.

Wang, J., Santure, A.W., 2009. Parentage and sibship inference from multilocus genotype data under polygamy. *Genetics*, 181 (4), 1579–1594. DOI: 10.1534/genetics.108.100214.

Waples, R.S., 1998. Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. *Journal of Heredity*, 89 (5), 438–450. DOI: 10.1093/jhered/89.5.438.

Waples, R.S., Antao, T., 2014. Intermittent breeding and constraints on litter size: Consequences for effective population size per generation (N_e) and per reproductive cycle (N_b). *Evolution*, 68 (6), 1722–1734. DOI: 10.1111/evo.12384.

Waples, R.S., Do, C., 2008. LDNE: a program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources*, 8, 753–756. DOI: 10.1111/j.1755-0998.2007.02061.x.

Warner, R.R., Cowen, R.K., 2002. Local retenion of production in marine populations: evidence, mechanisms, and consequences. *Bulletin of Marine Science*, 70 (1), 245–249.

White, C., Selkoe, K.A., Watson, J., Siegel, D.A., Zacherl, D.C., Toonen, R.J., 2010. Ocean

currents help explain population genetic structure. *Proceedings of the Royal Society B: Biological Sciences*, 277 (1688), 1685–1694. DOI: 10.1098/rspb.2009.2214.

Yu, H-T., Lee, Y-J., Huang, S-W., Chiu, T-S., 2002. Genetic analysis of the populations of Japanese anchovy (Engraulidae: *Engraulis japonicus*) using microsatellite DNA. *Marine Biotechnology*, 4 (5), 471–479. DOI: 10.1007/s10126-002-0035-8.

Zarraonaindia, I., Pardo, M.A., Iriondo, M., Manzano, C., Estonba, A., 2009. Microsatellite variability in European anchovy (*Engraulis encrasicolus*) calls for further investigation of its genetic structure and biogeography. *ICES Journal of Marine Science*, 66 (10), 2176–2182. DOI: 10.1093/icesjms/fsp187.
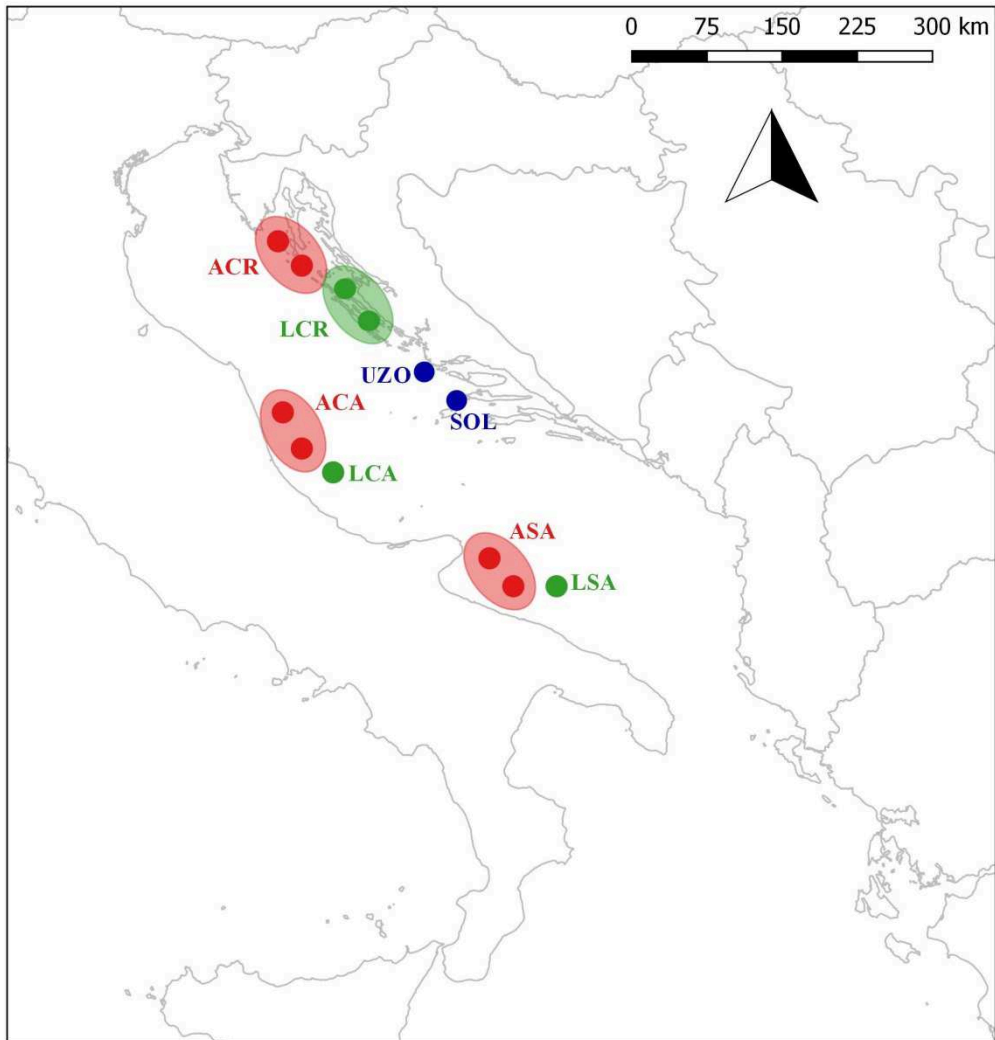
Figures and tables

Figure 3.1. Map of sampling locations. LSA = larvae south Adriatic Sea; ASA = adults south Adriatic Sea; LCA = larvae central Adriatic Sea; ACA = adults central Adriatic Sea; LCR = larvae eastern Adriatic Sea; ACR = adults eastern Adriatic Sea; SOL and UZO = adults eastern Adriatic Sea
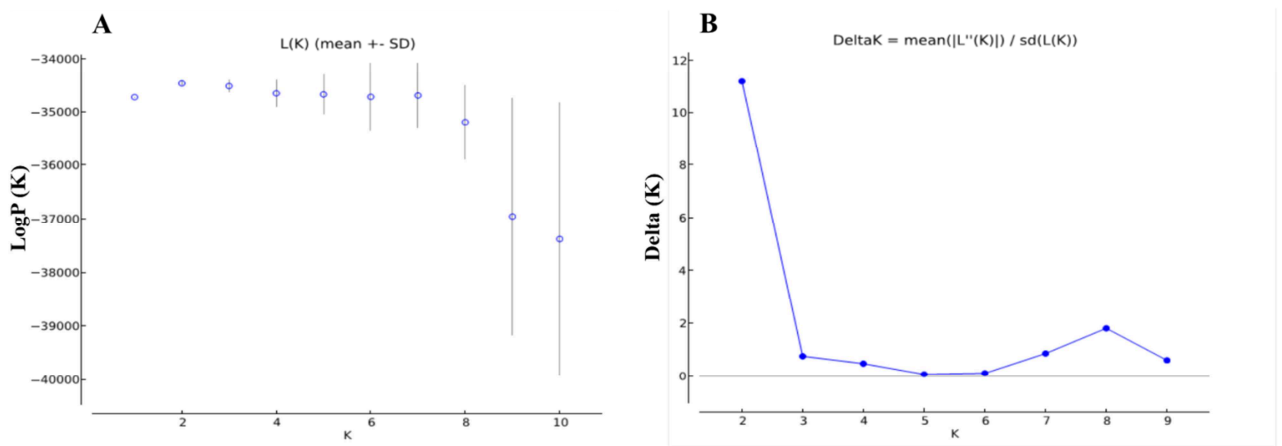
Figure 3.2. A) Graphical plot of logarithmic probability [LogP(K)] obtained from each K (number of clusters) simulated using STRUCTURE. Blue points refer to mean LogP(K) values obtained and bars on them explain the standard deviation (SD) among 10 simulation per K performed in this analysis. B) Plot of best K based on Evanno method
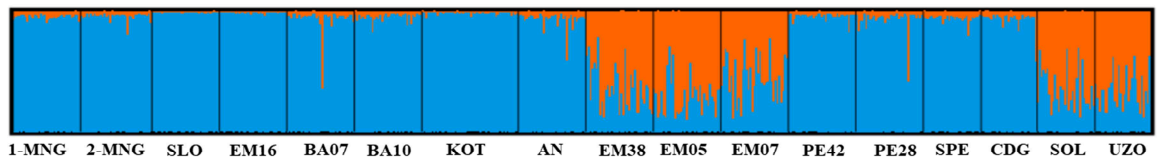
Figure 3.3. Graphical bar plot outcomes of STRUCTURE Bayesian clustering with K = 2 using all loci and the LocPrior function. Vertical bars display for each individual show the estimate membership proportions in two clusters colored in blue and orange. Populations are separated by black vertical lines and described by site abbreviation as in Fig. 3.4
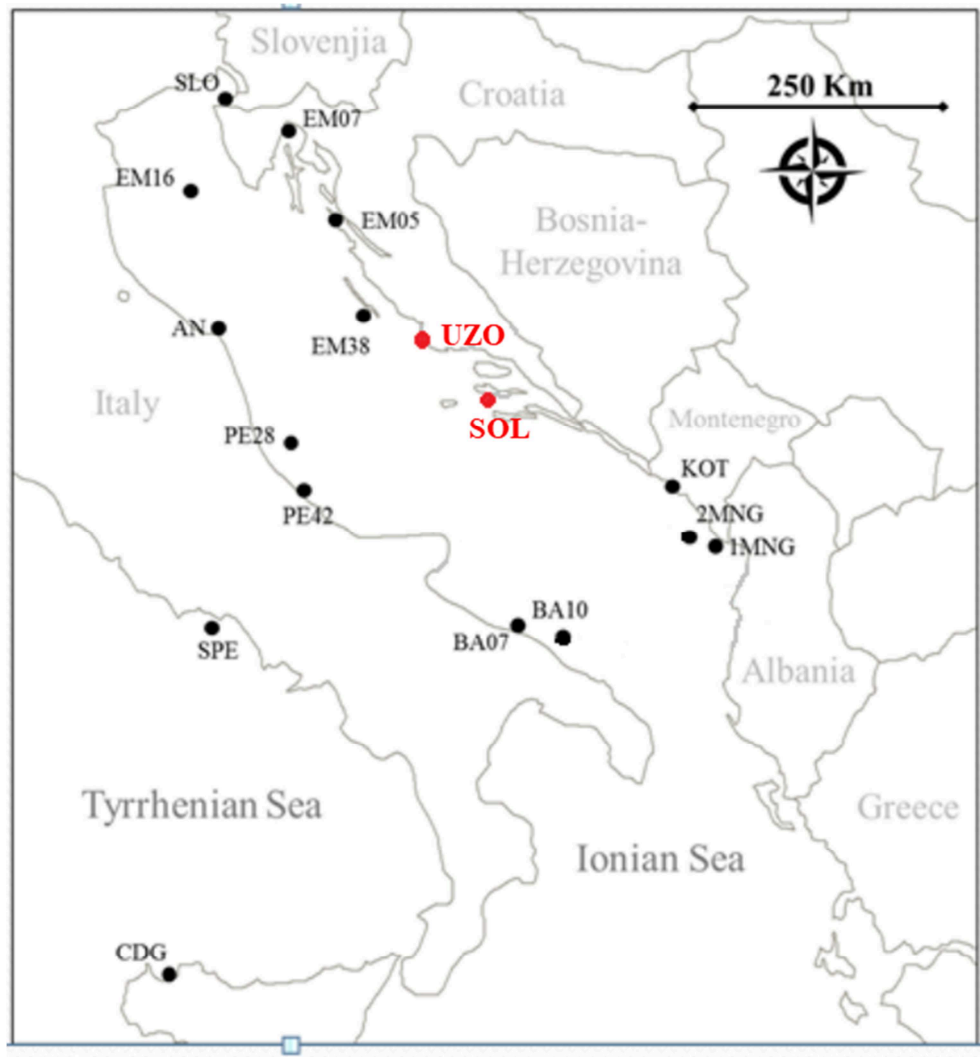
Figure 3.4. Locations denoted by the black dots are referring to adults sampling in 2012 and analysed in Ruggeri et al., 2016b. Localities denoted by red dots are referring to anchovies sampling sites caught in 2015

Figure 3.5. Change in adults and larvae of mean number of alleles (N_a; **upper panel**), mean expected (H_E; **middle panel**) and observed (H_O; **lower panel**) heterozygosities, with standard deviations, in southern Adriatic Sea (SA); central Adriatic Sea (CA); eastern Adriatic Sea (CR)

Table 3.1. Summary of sampling localities and samples; SA = southern Adriatic Sea; CA = central Adriatic Sea; CR = eastern Adriatic Sea; A= adults stage; L= larval stage

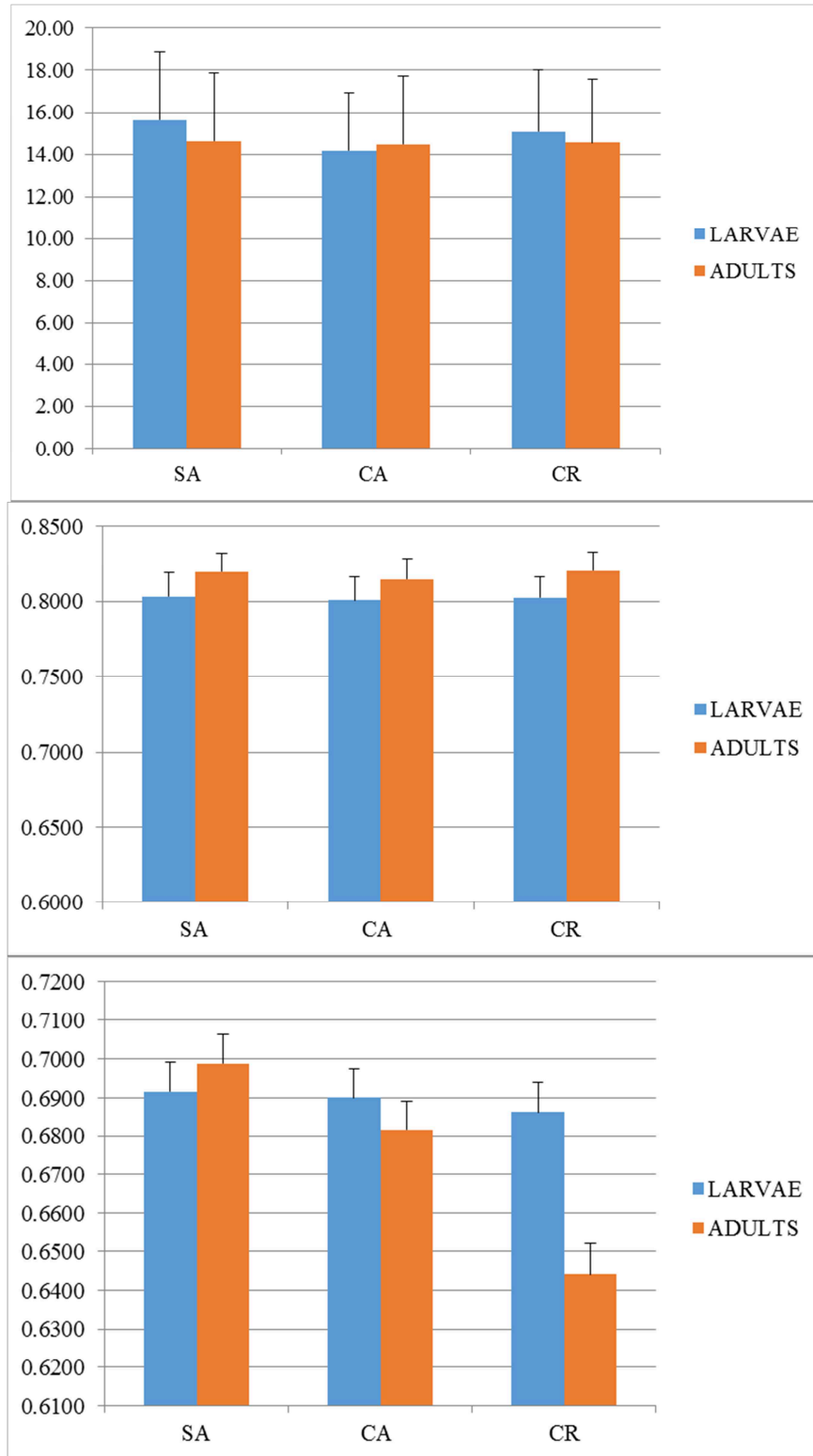|	Station	Sample label	Stage	N° of individuals	Sampling coordinates	Sampling depth (m)	Bottom depth (m)	Sampling date (month/year)
SA	LIT 3	LSA	L	48	41°32.910'N; 16°37.152'E	71	76	06/2015
CA	LIT 12	LCA	L	48	42°37.482'N; 14°28.698'E	100	109	06/2015
CR	LEM 3	LCR	L	26	44°28.302'N; 14°40.608'E	59	/	09/2015
	LEM 4			22	44°41.148'N; 14°32.268'E	83		
SA	AIT 3	ASA	A	30	41°26.850'N; 16°29.808'E	9	61	06/2015
	AIT 4			18	41°43.128'N; 16°16.470'E	14	19	
CA	AIT 11	ACA	A	24	43°12.252'N; 14°30.348'E	9	54	06/2015
	AIT 12			24	42°35.796'N; 14°30.198'E	13	108	
CR	AEM 4	ACR	A	24	44°41.148'N; 14°32.268'E	83	/	09/2015
	AEM 8			24	44°58.218'N; 14°18.150'E	51		
CR	SOL	SOL	A	30	43°18.14'N; 16°22.78'E	20	30	02/2015
CR	UZO	UZO	A	30	43°44.17'N; 15°38.57'E	20	24	02/2015

Table 3.2. Microsatellite loci and primers for their PCR amplification used in the present study. T_A = annealing temperature; Fluor. = 5' Fluorescent labels; 1 = Pakaki et al., 2009; 2 = Chiu et al., 2002; 3 = Landi et al., 2005; 4 = Lin et al., 2010

Locus Name	Primers Sequences	Repeat motifs	T_A	Fluor.	Multiplex	Species	Authors
*Ee*2-91b	F: GGTCTTGAGCTTGGCATAGG	$(CCGCA)_7$	60	6-FAM	A	*E. encrasicolus*	*1*
	R: CCGGAAGACACTCTGCACAC						
*Ee*2-407	F: AGGAATCTCCTTCCCGTCTC	$(CA)_{13}$	60	VIC	A	*E. encrasicolus*	*1*
	R: GTGGGTCTGTGGGTGTTTTG						
*Ej*41.1	F: TCTACCCCTGGAGGACACAC	$(CACAA)_8$	55	NED	A	*E. japonicus*	*2*
	R: ACAGGGGGTTGAGAAAGAGG						
*Ee*10	F: GGTGGATGAAGTGGCAATCT	$[(GT)_9CT]_2$	54	PET	A	*E. encrasicolus*	*3*
	R: CTGGGGTGGCATAACTGAAG	$[(GT)_2CT]_3$					
*Ej*27.1	F: GACTGTGAAGGAACGCTGGT	$(GA)_{36}$	58	6-FAM	B1	*E. japonicus*	*2*
	R: AATAGGATTAGTCATCACAGGG						
*Ej*35	F: AGTGAGAGGACTCGCAAAGC	$(TG)_{15}$	60	PET	B1	*E. japonicus*	*2*
	R: CACACGAAGACAGACAAGCAA						
*Enja*83	F: AAGGGACATCGGGTAGTGA	$(AC)_6(TG)_7$	55	NED	B1	*E. japonicus*	*4*
	R: AAGGCAAGTTCTCAGACGAG						
*Ee*2-507	F: GGAAGGGACCTAGATGGAGTG	$(GAAA)_n$	60	VIC	B1	*E. encrasicolus*	*1*
	R: ATCCCATTGATGTCCTGAGC						
*Eja*17	F: CCATTCAACTCCTCCCCAAGC	$(CA)_7$	55	6-FAM	B2	*E. japonicus*	*4*
	R: GGCTCTTCAGCTCCCTGAGAC						
*Ej*2	F: AGCAAGGGAGCAAACAATC	$(CT)_{43}$	58	NED	B2	*E. japonicus*	*2*
	R: TGCAATTTGACAGAAACCACA						
*Ee*2-135	F:AGGGCAGTGACAGGAGAGTC	$(ATTAG)_{10}$	55	VIC	B2	*E. encrasicolus*	*1*
	R: TCGTTACCCTGCGTTTATACTG						
*Ee*2-165b	F: GGGTGGGTTAAAGATGAAGC	$(CCT)_7$	59			*E. encrasicolus*	*1*
	R: AGGGATCTTCAGGGAACCAG						
*Ee*2-508	F: CACATGCTCGCTAAACATTG	$(AGG)_8$	55			*E. encrasicolus*	*1*
	R: ACCTGATGCTGCTTGGTAGC						

Table 3.3. Summary of genetic variability observed at 13 microsatellite loci in SOL and UZO samples. N = number of individuals correctly genotyped; N_a = number of alleles observed; H_e = expected heterozygosity; H_o = observed heterozygosity; F_{IS} = inbreeding coefficient

Ee2-91b	N	N_a	H_o	H_e	F_{IS}	*Ee2-507*	N	N_a	H_o	H_e	F_{IS}
SOL	22	6	0.591	0.728	0.192	SOL	23	21	0.609	0.954	0.367
UZO	25	6	0.625	0.797	0.219	UZO	21	15	0.524	0.927	0.441
Ee2-407	N	N_a	H_o	H_e	F_{IS}	*Eja17*	N	N_a	H_o	H_e	F_{IS}
SOL	30	13	0.733	0.857	0.146	SOL	29	8	0.643	0.750	0.145
UZO	28	13	0.724	0.881	0.181	UZO	25	6	0.500	0.754	0.341
Ej41.1	N	N_a	H_o	H_e	F_{IS}	*Ej2*	N	N_a	H_o	H_e	F_{IS}
SOL	30	10	0.700	0.777	0.101	SOL	28	19	0.964	0.945	-0.020
UZO	28	7	0.679	0.775	0.126	UZO	26	19	0.962	0.937	-0.026
Ee10	N	N_a	H_o	H_e	F_{IS}	*Ee135*	N	N_a	H_o	H_e	F_{IS}
SOL	29	16	0.690	0.878	0.218	SOL	29	11	0.897	0.856	-0.048
UZO	25	15	0.600	0.867	0.312	UZO	27	13	0.741	0.905	0.184
Ej27.1	N	N_a	H_o	H_e	F_{IS}	*Ee2-508*	N	N_a	H_o	H_e	F_{IS}
SOL	28	21	0.643	0.947	0.325	SOL	28	6	0.607	0.692	0.124
UZO	23	17	0.304	0.949	0.684	UZO	27	5	0.296	0.531	0.447
Ej35	N	N_a	H_o	H_e	F_{IS}	*Ee2-165b*	N	N_a	H_o	H_e	F_{IS}
SOL	29	13	0.655	0.909	0.283	SOL	29	3	0.621	0.575	-0.080
UZO	27	12	0.889	0.905	0.018	UZO	29	4	0.517	0.533	0.030
Enja83	N	N_a	H_o	H_e	F_{IS}	*Average*	N	N_a	H_o	H_e	F_{IS}
SOL	11	7	0.727	0.723	-0.006	SOL	30	11.93±5.74	0.694±0.024	0.820±0.030	0.155
UZO	6	5	0.500	0.667	0.268	UZO	29	10.57±5.00	0.609±0.027	0.807±0.038	0.250

Table 3.4. Summary of genetic variability observed at 13 microsatellite loci in offspring and adult samples. N = number of individuals correctly genotyped; N_a = number of alleles observed; H_e = expected heterozygosity; H_o = observed heterozygosity; SD = standard deviation; SE = standard error; F_{IS} = inbreeding coefficient, bold F_{IS} values are significant (<0.0003) after a sequential Bonferroni correction

Pop_ID	Adults/Recruits	Year	N	N_a	$SD(N_a)$	$SE(N_a)$	H_e	$SD(H_e)$	$SE(He)$	H_o	$SD(H_o)$	$SE(H_o)$	F_{IS}
LSA	recruits	2015	47	15.62	7.98	3.26	0.8035	0.0384	0.0157	0.6916	0.0187	0.0076	**0.141**
LCA	recruits	2015	48	14.15	6.80	2.78	0.8006	0.0385	0.0157	0.6900	0.0185	0.0076	**0.139**
LCR	recruits	2015	48	15.08	7.17	2.93	0.8026	0.0335	0.0137	0.6862	0.0186	0.0076	**0.146**
ASA	adults	2015	48	14.62	7.94	3.24	0.8200	0.0299	0.0122	0.6987	0.0184	0.0075	**0.149**
ACA	adults	2015	48	14.46	7.98	3.26	0.8148	0.0334	0.0137	0.6815	0.0187	0.0076	**0.165**
ACR	adults	2015	48	14.54	7.47	3.05	0.8206	0.0307	0.0125	0.6441	0.0192	0.0078	**0.217**

Table 3.5. A) Parentage analysis test with COLONY to infer the possible sibships between offsprings
B) Parentage analysis with CERVUS, in bold parentage with strict confidence level (95%), others are with relaxed confidence level (80%)

A

Offspring 1	Offspring 2	Sibship	Probability
South Adriatic	East Adriatic	Full-sib	50%
South Adriatic	South Adriatic	Full-sib	76%
South Adriatic	East Adriatic	Half-sib	98%
South Adriatic	Central Adriatic	Half-sib	98%
Central Adriatic	East Adriatic	Half-sib	97%
East Adriatic	East Adriatic	Half-sib	96%
South Adriatic	South Adriatic	Half-sib	95%

B

Offsprings	Adults
South Adriatic	**South Adriatic**
Central Adriatic	**Central Adriatic**
Central Adriatic	**South Adriatic**
Central Adriatic	**East Adriatic**
East Adriatic	**South Adriatic**
South Adriatic	South Adriatic
East Adriatic	East Adriatic
Central Adriatic	Central Adriatic
Central Adriatic	Central Adriatic
South Adriatic	East Adriatic
South Adriatic	East Adriatic
South Adriatic	East Adriatic
East Adriatic	South Adriatic
Central Adriatic	South Adriatic
Central Adriatic	South Adriatic
South Adriatic	Central Adriatic
East Adriatic	Central Adriatic
Central Adriatic	East Adriatic

Table 3.6. Pop_ID = codes of larvae analysed; Lower/Upper 95% CI = lower and upper Confidence Interval (CI). **Upper panel**: N_b = number of breeders, estimated with maximum-likelihood sibship method (COLONY); N_c = census size, obtained from MEDIAS acoustic survey data; N_b/N_c ratio = ratio between effective population size and census size; \ = data not available. **Lower panel**: N_e = effective population size, estimate with linkage disequilibrium method ($N_{e\_}$estimator)

Pop_ID	N_b	N_c	Lower 95% CI	Upper 95% CI	N_b/N_c
LSA	114	4.40E+09	77	179	2.59E-08
LCA	98	2.64E+10	66	152	3.71E-09
LCR	98	\	65	152	\

Pop_ID	N_e	Lower 95% CI	Upper 95% CI
LSA	1689.2	242.2	INF
LCA	3781.3	348.6	INF
LCR	562.2	192.6	INF

Management and final remarks

The Common Fisheries Policy (CFP) fixed the rules and directions for a sustainable exploitation of marine resources for north-east Atlantic and the Mediterranean Sea fisheries, the main objective is the restoration and maintenance of harvested populations species above Maximum Sustainable Yield (MSY) levels. This approach would ensure that fisheries are sustainable and profitable in the long term, and that comply with European Union (EU) environmental legislation, as well as, with international law principles. Nevertheless, a large discrepancy in management performance occurs, with considerable improvement of stock status in the north-east Atlantic and a rapidly deteriorating situation in the Mediterranean Sea (Cardinale & Scarcella, 2017). Notwithstanding the enforcement in the early 2000s of the EU-Data Collection Regulation (EU, 2000) by all EU members, and the rapid increase in the number of assessed stocks by the General Fisheries Commission for the Mediterranean (GFCM) and the European Scientific Technical and Economic Committee for Fisheries (STECF), Mediterranean marine resources are still exploited above the MS and no signs of recovery are evident (Vasilakopoulos et al., 2014). Results of the stock assessments carried out in the last 10 years clearly show that the fishing pressure is determining a generalized overfishing status of stocks; it is straightforward that the current level of fishing pressure in the Mediterranean basin has spoiled the productivity of stocks, increased the extinction risks for sensible species and contributed to disrupt the productivity and functions of the ecosystem.

National management plans and measures to protect the environment have been already implemented, Mediterranean Sea countries are limited mainly to control fishing effort and fishing capacity together with specific technical measures, such as gear regulation (mainly mesh size and net configuration), establishment of a minimum conservation reference size and closures of areas and seasons for fishing (EU, 2006). Unfortunately all these measures have not been successful in improving the situation of stocks and there are not yet clear

signs of an inversion on the trend. In this context the development of a more effective management for Mediterranean fisheries is extremely urgent to prevent that unregulated fishing and climate forcing might disrupt the productivity of the ecosystem with impacts on the goods and services provided.

Summarizing the results of this Ph.D. thesis, DEPM method nowadays cannot be used for stock assessment tuning by reason of the high uncertainty, but, the application provides valuable information on the extension and characteristics of spawning habitats and on reproductive parameters of fish stocks, which can help in improving our understanding of the mechanisms by which environmental changes, together with fishing pressure, can modulate the abundances of small pelagic fish. Would be reasonable an intensification of research efforts and stock assessments with the possibility of applying Total Allowable Catches (TACs) and quotas (e.g. a maximum catch per day and vessel for some target species in monospecific capture system). Data analysis of the present study indicated south-western Adriatic as a favorable area of spawning and nursery, driven by depth, zooplankton abundance and water column stability. The CFP is, inter alia, oriented towards spatio-temporal closures tailored to protect spawning areas and periods, promoting the establishment of biologically sensitive protected areas, including nursery and spawning grounds of exploited stocks, in which, all or certain fishing activities are temporarily or permanently banned or restricted in order to improve the exploitation and conservation of living aquatic resources and marine ecosystems. The current overfishing of most anchovy stocks could well lead not only to commercial collapse but also to the biological extinction of some populations due to a decreasing genetic variability. However, anchovy in Adriatic seems not undergo to sweepstake reproduction which, on the other hand, can be hide by the presence of overlapping generations. The genetic structure of anchovy in two stocks seems to be reconfirmed also in 2015, finally, are provided estimates of N_e and N_b that can be used to formulate recommendations for the conservation of wild populations; in fact,

given the ecological and commercial value of anchovy, genetic monitoring needs to be implemented so that accurate, geographic, and temporal assessments of the state of anchovy stocks, at least those considered outside safe biological limits, can be obtained.

Bibliographical support

Cardinale, M., & Scarcella, G., 2017. Mediterranean Sea: a failure of the European fisheries management system. *Frontiers in Marine Science*, 4 (72), 1-14. DOI: 10.3389/fmars.2017.00072.

EU, 2000. Council Regulation (EC) N. 1543/2000 of 29 June 2000. Establishing a community framework for the Collection and Management of the Data needed to conduct the Common Fisheries Policy, EU.

EU, 2006. Council Regulation (EC) N. 1967/2006 of 21 December 2006. Concerning management measures for the Sustainable Exploitation of Fishery Resources in the Mediterranean Sea. Amending Regulation (EEC) N. 2847/93 and Repealing Regulation (EC) N. 1626/94, EU.

Vasilakopoulos, P., Maravelias, C.D., Tserpes, G., 2014. The Alarming Decline of Mediterranean Fish Stocks. *Current Biology* 24 (14), 1643–1648. DOI: 10.1016/j.cub.2014.05.070.

Supplementary Materials

The "generalized" DNA extraction protocol

The "generalized" DNA extraction protocol followed suggestions included in Taggart et al. (1992). Piece of caudal fin from SOL and UZO stations were individually incubated in 400 µL of sterile homogenizing buffer composed by 1X TE (Tris-EDTA salt-solution), 50 µL of 10% SDS and 5 µL of Proteinase K (20 mg/ml), samples were individually homogenized for 1.5 hours at 55°C. After incubation, the homogenized solution was treated with 60 µL of 5M Sodium Perchlorate, laying samples in ice for at least 15 minutes. A volume of 500 µL of Chloroform (absolute) was added to each sample and the DNA extraction phase was carried out putting the tubes horizontally on an orbital shaker for 30 minutes. A centrifugation of tubes at 7500 rpm for 15 minutes allowed to separate solvent-protein-DNA phases into each tubes. The supernatants phase contains the DNA in solution and a volume of at least 300 µL of it was recovered and transferred in new sterile tubes. The DNA precipitation was achieved through the addition of 800-1000 µL of isopropanol and a subsequent gentle mixing of solution. The DNA pellet was recovered by spinning tubes in a microcentrifuge at 13000 rpm for 10 minutes. Liquid solution in each tubes was discarded and two sequential washing with 70% ethanol were provided. Dried DNAs were resuspended in 70 µL of sterile water.

The "automated" DNA extraction protocol

The "automated" DNA extraction protocol was applied to anchovy larvae and adults. The procedure was performed on a MagCore ® Nucleic Acid Extractor (MagCore ® HF16) and using the MagCore ® Genomic DNA Tissue Kit (Cartridge code 401). The kit is designed for extraction and purification of total DNA (both nuclear and mitochondrial genomic DNA plus viral DNA). The method uses pre-filled cartridge contains proteinase K

and a chaotropic salt to lysis cells and degrade proteins from the tissue. This the first stage of automated DNA extraction and each sample is treated in 400 μL of GT Buffer, which is added 20 μL of proteinase K (10mg/ml) and incubated at 55°C for 90 minutes or overnight, adult samples were filtered due to the remaining residual. A volume of 400 μL of homogenized tissues are then processed by automated DNA extractor, that bind DNA molecules using cellulose coated magnetic beads. DNA captured by beads are then subjected to repeated washing from all contaminants. The automated procedure requires about 30 minutes and allow to obtain 20-30 kb DNA molecules that can be eluted in sterile water.

Spectrophotometer assay

Quantity and quality of DNA recovered were evaluated by means of a spectrophotometer assay carried out on a ©NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific) and using a volume of 2 μL per sample (Desjardins and Conklin, 2011). The following wavelength spectrum was taken into consideration in spectrophotometer analysis of genomic DNA extracted: i) 260nm (referable to nucleic acid absorbance wavelength); ii) 280nm (referable to protein absorbance wavelength); iii) 230nm (referable to organic compounds absorbance wavelength). The quality of DNA recovered was spectrophotometrically estimated with A260/A230, the absorbance (A) ratio for nucleic acid vs organic compounds (optimum value of ratio > 1.5) and A260/A280, the absorbance (A) ratio for nucleic acid vs proteins (optimum value of ratio = 1.8) (Desjardins and Conklin, 2011).

Microsatellite PCR protocols

PCR conditions were optimized for the 13 microsatellite loci using a touchdown amplification profile. The PCR profile included an initial denaturation step at 92°C for 5 min, followed by 10 cycles of 92°C/30 s denaturation, 40 s annealing (see Table 3.2 for

starting annealing temperature) and 72°C/50 s extension. The starting annealing temperature was reduced of 0.5°C/cycle. Additional 25 cycles were performed with annealing temperature fixed at 5°C less than the starting annealing temperature. A step of final elongation at 72°C for 5 min was performed.

Three multiplex PCR mixes were optimized as follows:

- the multiplex A contained loci Ee2-91b, Ee2-407, EJ41.1 and Ee10 and was performed in a total volume of 20 μL.

- the multiplex B1 contained loci EJ27.1, EJ35, Enja83 and Ee2-507 and was performed in a total volume of 20 μL.

- the multiplex B2 contained loci EJ2, Ee2-135 and Eja17 and was performed in a total volume of 15 μL.

The PCR reactions for non-labelled loci (Ee2-508 and Ee2-165b) were performed individually in a total volume of 10 μL, each.

PCR reaction mixture contained approximately 1-5 ng·μL^{-1} of genomic DNA, 0.025 U·μL^{-1} of Taq DNA polymerase (MyTaq DNA Polymerase, Bioline), 0.25 μM of each primer, 1× MyTaq Reaction Buffer (5mM dNTPs, 15 mM MgCl$_2$, stabilizers and enhancers).

	92°C x 30 sec	90°C x 30 sec	
92° x 5 min	Ta°C x 50 sec	Ta-5°C x 50 sec	72°C x 5 min
	72°C x 40 sec	72°C x 40 sec	
	(10 cycle)	(25 cycle)	
	-0.5°C/cycle		

Ta = Temperature of annealing are reported in Table. 3.2

Microsatellite Genotyping Procedures

Labelled loci were analysed by means of an Automated sequencer ABI-PRISM 3130xl Genetic Analyzer (Applied Biosystems) using LIZ500 as internal standard. The genotypes

were obtained using the software PeakScanner^{TM} v.1.0 (freely available from Applied Biosystems, http://www.appliedbiosystems.com/peakscanner.html). Raw genotyped data were refined using the binning procedure implemented in Flexibin (Amos et al., 2007). Non-labelled loci were genotyped using a 5% Polyacrylamide (Acrylamide-Bis-Acrylamide 19:1) denaturing sequencing gel and visualized by the silver staining protocol proposed by Bassam et al. (1991) modified by Benbouza et al. (2006). Genotyping error rates for non-labelled loci were limited by applying the allele ladder method as proposed by LaHood et al. (2002), the allele ladder is a pool of PCR products from multiple individuals and is representative of all or many of the alleles encountered in a given species for a particular locus (Moran et al. 2006). This method reduces the risks of misaligning alleles and consequently increases the accuracy of genotyping.

Table SM1. Pairwise estimations of F_{ST}: above the diagonal (without correction) and below the diagonal (with ENA correction)

	LSA	LCA	LCR	ASA	ACA	ACR
LSA		-0.00126	0.00130	-0.00012	0.00155	0.00155
LCA	-0.00111		-0.00215	0.00051	0.00106	0.00613
LCR	0.002052	-0.00024		-0.00008	-0.00139	0.00670
ASA	0.000465	0.001055	0.000314		-0.00166	0.00032
ACA	0.001685	0.001597	-0.00051	-0.00096		0.00091
ACR	0.002405	0.00596	0.007667	0.001365	0.001517	

Bibliographical support to Supplementary Materials

Amos, W., Hoffman, J.I., Frodsham, A., Zhang, L., Best, S., Hill, A.V.S., 2007. Automated binning of microsatellite alleles: problems and solutions. *Molecular Ecology Notes*, 7, 10-14. DOI: 10.1111/j.1471-8286.2006.01560.x.

Bassam, B.J., Caetano-Anollés, G., Gresshoff, P.M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Analytical Biochemistry*, 196 (1), 80-83. DOI:10.1016/0003-2697(91)90120-I.

Benbouza, H., Jacquemin, J.M., Baudoin, J.P., Mergeai, G., 2006. Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. *Biotechnologie, Agronomie, Société et Environnement,* 17, 77-81.

Desjardins, P.R., Conklin, D.S., 2011. APPENDIX 3J Microvolume quantitation of nucleic acids. *Current protocols in molecular biology*, 93: A.3J.1-A.3J.16. DOI: 10.1002/0471142727.mba03js93.

LaHood, E.S., Moran, P., Olsen, J., Grant, W.S., Park, L.K., 2002. Microsatellite allele ladders in two species of Pacific salmon: preparation and field-test results. *Molecular Ecology Notes*, 2(2), 187–190. DOI:10.1046/j.1471-8286.2002.00174.x.

Moran, P., Teel, D.J., LaHood, E.S., Drake, J., Kalinowski, S., 2006. Standardizing multi-laboratory microsatellite data in Pacific salmon: an historical view of the future. *Ecology of Freshwater Fish*, 15 (4), 597–605. DOI:10.1111/j.1600-0633.2006.00201.x.

Taggart, J.B., Hynes, R.A., Prodöuhl, P.A., Ferguson, A., 1992. A simplified protocol for routine total DNA isolation from salmonid fishes. *Journal of Fish Biology*, 40 (6), 963-965.