Biology and phylogeography of the black sea urchin *Arbacia lixula* (Echinoidea: Arbacioida)

Biología y filogeografía del erizo de mar negro *Arbacia lixula* (Echinoidea: Arbacioida)

Owen S. Wangensteen Fuentes

Biology and phylogeography of the black sea urchin *Arbacia lixula* (Echinoidea: Arbacioida)

Doctoral Thesis
Owen S. Wangensteen

Programa de Doctorat en Biodiversitat
Departament de Biologia Animal
Universitat de Barcelona

Biología y filogeografía del erizo de mar negro *Arbacia lixula* (Echinoidea: Arbacioida)

Memoria presentada por Owen S. Wangensteen Fuentes para optar al grado de doctor por la Universitat de Barcelona. Año 2013.

El doctorando:

Owen S. Wangensteen Fuentes

Los directores de la tesis:

Francesc Xavier Turon Barrera

Creu Palacín Cabañas

Garota

Sota l'aigua poc fonda de la costa
ancoro la cuirassa. No faig nacre,
ni perles, la bellesa no m'importa:
sóc un guerrer de dol que, amb negres llances,
s'amaga en una escletxa de la roca.
Viatjar és arriscat però, a vegades,
em moc amb les espines fent de crossa
i em rebolquen, maldestre, les onades.
En el mar perillós busco la roca
d'on ja no moure'm mai. Dins l'armadura
sóc el meu propi presoner: la prova
de com fracassa, sense risc, la vida.
A fora hi ha la llum i el cant del mar.
Dins meu, la fosca: la seguretat.

Joan Margarit

Confined to sex, we pressed against the limits of the sea.
I saw there were no oceans left for scavengers like me

Leonard Cohen, *A Thousand Kisses Deep*

AGRADECIMIENTOS / ACKNOWLEDGEMENTS

Aunque, inevitablemente, parezca un tópico, en este caso es absolutamente cierto. Completar esta tesis hubiera sido del todo imposible sin la infinita paciencia y el apoyo incondicional, en todos los sentidos materiales e inmateriales, de mi querida Sandra Garcés. He contraído una enorme deuda contigo, en forma de momentos perdidos y oportunidades aplazadas, que pienso devolverte en cómodos y dulces plazos, durante el resto de mi vida. Con intereses.

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Muchas veces he escuchado que un biólogo marino no se hace en cuatro años, ni en diez, ni en veinte. Yo creo que, de hecho, hacen falta apenas unos segundos para producir un biólogo marino. Es el tiempo que

transcurre entre la primera vez que uno mete la cabeza bajo el agua del mar y el momento en que se da cuenta de que se equivocó de elemento al nacer porque, en el fondo, pertenece a otro mundo. Debo agradecer a mi madre el haberme llevado muchas veces a la playa de un pueblecico almeriense cuyo nombre dicen que trae mala suerte recordar, convenientemente equipado con gafas y tubo (las aletas, ni falta que hacían), y el haber permitido que el mar, la curiosidad por la fantasía y la realidad submarinas, le arrebataran a su hijo pequeño para siempre. No recuerdo cuando fue mi primera vez. Posiblemente fuera antes de aprender a leer y, quizás, casi, casi, antes que a andar; así que, probablemente, uno de mis primeros pensamientos conscientes, mi primer sueño y el primer pasajero de mi cerebro y de mi corazón, fue la biología marina. El resto de mi vida, desde entonces, no ha sido más que un largo viaje, una odisea de retorno a Ítaca, cuyo puerto ya creo comenzar a divisar en el horizonte. Pero mi viaje, aún no exento de rodeos, extravíos, estancamientos e incluso naufragios, está siendo mucho más placentero que el de Ulises. Y es que yo tengo la suerte de disfrutar con cada Escila o cada Caribdis que me voy encontrando por el camino y, aunque estén ya descritas como especie, me conformo con que queden bien en alguna foto submarina. Y, además, ¡siempre podremos tomarles unas muestras para filogeografía!

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Arbacia lixula 4-armed pluteus larvae. Photo: **O. S. Wangensteen**

GENERAL INTRODUCTION

Taxonomy, historical account and phylogeography

The black sea urchin *Arbacia lixula* (Linnaeus, 1758) is currently one of the most conspicuous macroinvertebrates in the Mediterranean shallow rocky reefs. It is a regular sea urchin of uniformly black epithelial colouration, relatively flattened test, with long, hard spines (Fig. I.1). It inhabits shallow coastal ecosystems (from 0 to around 50 m depth) and is more commonly found on vertical rocky surfaces, exposed to a high degree of hydrodynamism, though it may also be found in other habitats such as flat rocky bottoms and even sand bottoms with scattered rock boulders.

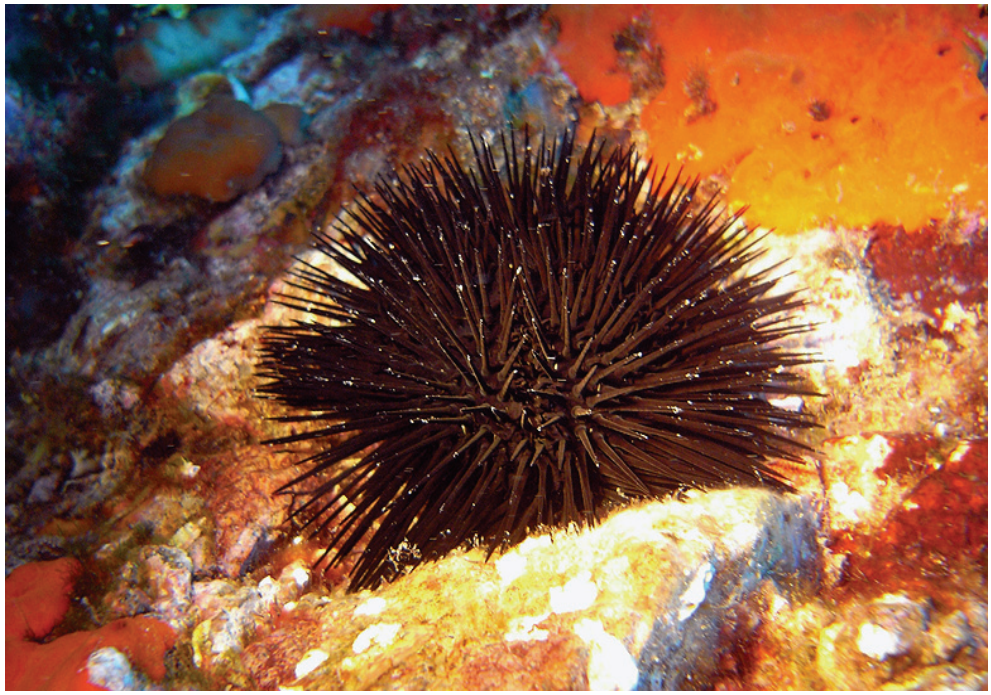

Fig. I.1. *Arbacia lixula*

Linnaeus (1758) described *Echinus lixula* in the tenth edition of his Systema Naturae as a "hemispheric sea urchin with ten ambulacra in proximate pairs, with transversal areas punctuated with short, hard points". Though this description could possibly refer to almost any regular sea urchin, it is commonly accepted that it referred to a species in genus *Arbacia*. Blainville (1825) expressed his conviction that *Echinus lixula* designed his *Echinus aequituberculatus* and this opinion was also adopted by Agassiz (1842-1846) and recognized by Lovén (1887). The name *Arbacia* was coined by John Edward Gray (1835), who removed *E. lixula* from the genus *Echinus* and placed it in the new genus. *Arbacia* just missed being called *Echinocidaris* for just a few weeks. Gray's paper *On the genera distinguisable in Echinus* was published on July 17th of 1835, while Des Moulins (1835) independently published his *Études sur les Échinides* in August 15th of the same year, where he used the name *Echinocidaris aequituberculatus* to designate the same animal. The meaning of the name *Arbacia* is peculiarly puzzling. Agassiz (1842-1846) wrote that it had no special derivation and Mortensen (1935) called it a "nonsensical" name. Harvey (1956) gave the most plausible explanation for the name, considering it a derivation of *Arbaces*, a secondary character in the historical poem *Sardanapalus* by Lord Byron, which was published in 1821, a few years before Gray's work. She also argued that *Salenia*, another sea urchin genus erected by Gray at the same time, could be derived from *Salemenes*, another character in the same poem. The specific name used by Linnaeus, *lixula* probably derives from the name of a flattened round biscuit made of flour and cheese by the ancient Roman, as defined in Matthias Martinius (1655) *Lexicon Philologicum*, derived in turn from the latin word *lixare* (to boil in water).

Synonymy (Kroh and Hansson, 2012)

***Arbacia lixula* (Linnaeus, 1758)**
Arbacia aequituberculata (Blainville, 1825) (subjective junior synonym)
Arbacia australis Lovén, 1887 (subjective junior synonym)
Arbacia grandinosa (Valenciennes, 1846) (subjective junior synonym)
Arbacia pustulosa (Leske, 1778) (subjective junior synonym)
Cidaris pustulosa Leske, 1778 (subjective junior synonym)
Echinocidaris (Agarites) loculatus (Blainville, 1825) (subjective junior synonym)
Echinocidaris (Tetrapygus) aequituberculatus (Blainville, 1825) (subjective junior synonym)
Echinocidaris (Tetrapygus) grandinosa (Valenciennes, 1846) (subjective junior synonym)
Echinocidaris (Tetrapygus) pustulosa (Leske, 1778) (subjective junior synonym)
Echinocidaris aequituberculata (Blainville, 1825) (subjective junior synonym)
Echinocidaris grandinosa (Valenciennes, 1846) (subjective junior synonym)
Echinocidaris loculatua (Blainville, 1825) (subjective junior synonym)
Echinocidaris pustulosa (Leske, 1778) (subjective junior synonym)
Echinus aequituberculatus Blainville, 1825 (subjective junior synonym)
Echinus equituberculatus Blainville, 1825 (misspelling for Echinus aequituberculatus)
Echinus grandinosus Valenciennes, 1846 (subjective junior synonym)
Echinus lixula Linnaeus, 1758 (transferred to *Arbacia*)
Echinus loculatus Blainville, 1825 (subjective junior synonym)
Echinus neapolitanus Delle Chiaje, 1825 (subjective junior synonym)
Echinus pustulosus (Leske, 1778) (subjective junior synonym)

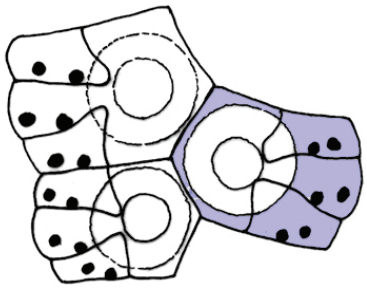

Fig. I. 2. The arbacioid compound ambulacral plates. Three plates are shown. From The Echinoid Directory website (www.nhm.ac.uk/research-curation/research/projects /echinoid-directory/), edited by A.B. Smith and A. Kroh

Arbacioida (Gregory, 1900) is an order within Superorder Echinacea Claus, 1876 (Kroh and Smith, 2010). This superorder includes regular sea urchins which have a rigid test with ten peristomial plates, solid spines and presence of gills (Ruppert et al., 2004). Arbacioida are characterized by the

so called "arbacioid plate" arrangement (Fig. I.2; Duncan, 1885), a compound ambulacral plate composed by three elements, of which the middle element is always the largest and the upper and lower elements do not reach the perradius.

Arbacioida is the sister group of Camarodonta (the most diversified order of regular echinoids, including Echinidae and other ecologically significant sea urchins). The order includes only one extant family, Arbaciidae (Gray, 1855), whose representatives are mostly omnivorous or carnivorous species. The genus *Arbacia* has a Neotropical origin and four different fossil species have been described from the Late Miocene or North America (Fig. I.3), though reasonable doubts have been cast which suggest that they are all synonyms (Kier, 1972). Their morphologies are closely related to the extant *A. punctulata,* which is presently common in the same region.

Fig. I.3. Fossil *Arbacia improcera* from the Pliocene of South Carolina. Resemblance with the extant *A. punctulata* is remarkable. Photo: Aurora Fossil Museum, Aurora, NC, USA

After the recent synonymization of *Arbacia crassispina* within *Arbacia dufresnii* (Lessios et al., 2012), the genus *Arbacia* currently includes five valid

extant species, four of which have exclusively Neotropical distribution (Fig. I.4). All of them inhabit shallow rocky habitats, from the surface to approximately 50 m depth.

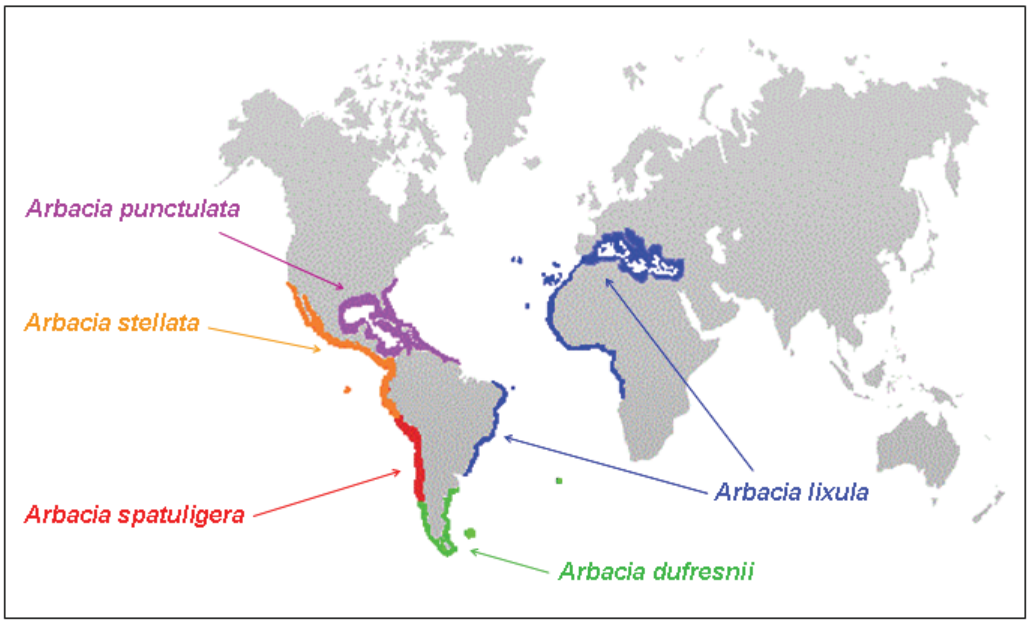

Fig. I.4. Areas of distribution of the five extant species of genus *Arbacia*

The intrageneric phylogeography of *Arbacia* was studied by Metz et al. (1998) and revisited by Lessios et al. (2012). Both studies used the mitochondrial gene COI and nuclear sequences of the gamete recognition protein bindin. The reconstruction of the splitting events sequence suggests that the genus originated in the Pacific coast of South America, from where it expanded into the Atlantic Ocean along the Central American Seaway before the closure of the Isthmus of Panama, which separated the Pacific species *A. stellata* from the Atlantic clade (*A. punctulata* / *A. lixula*). So, the Atlanto-Mediterranean *A. lixula* and the Caribbean *A. punctulata* are sister species, which diverged shortly after the closure of the Isthmus of Panama, some 2.6 Mya (Fig. I.5; Lessios et al., 2012).

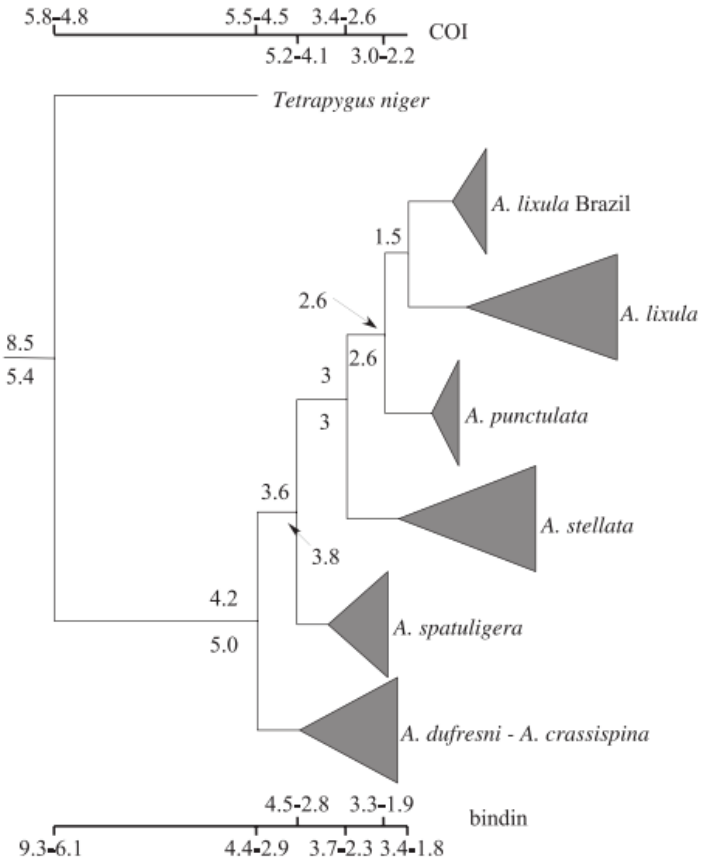

Fig. I.5. Phylogeny of genus *Arbacia* with temporal estimations for the splitting events (in Mya), inferred from the mitochondrial marker COI (upper numbers) and from the nuclear marker bindin (lower numbers). From Lessios et al. (2012), used with permission from H. Lessios

Some controversy exists on the synonymy of *Arbacia lixula* with *Arbaciella elegans* Mortensen, 1910. This small species of arbaciid sea urchin was described by Mortensen from material found in the West coast of Africa, and was considered a different species. It resembles *Arbacia* in many aspects, and has the typical four periproctal plates that form the

characteristic arbacioid cross (Fig. I.6). But it is significantly smaller and flattened, and has a ring of 15 flat, oar-shaped spines around the ambitus. Since its description, *Arbaciella elegans* was also reported from the Canary Islands, Azores, Madeira and the Southern Mediterranean Sea, with considerable abundancy in the Alboran Basin and Sicily.

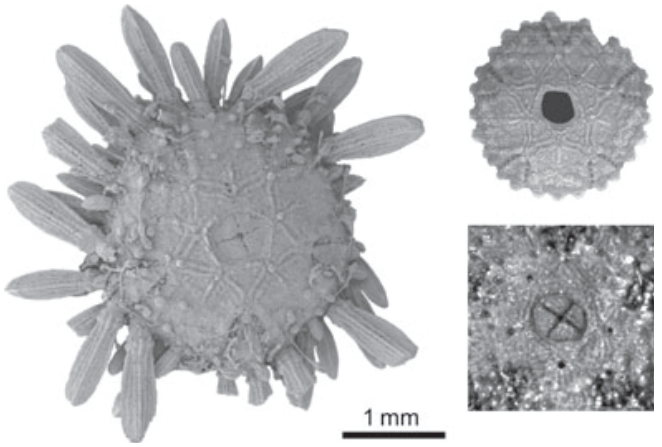

Fig. I.6. *Arbaciella elegans*. Actually, a juvenile *Arbacia lixula* from Azores. The gonopores are already open in individuals as small as 6 mm of diameter (lower right). From Kroh et al., (2011)

Some authors had cast serious doubts about the validity of genus *Arbaciella*. Upon examining juvenile *Arbacia* material from Brazil, Tommasi (1964) suggested that this might be a name given to juvenile specimens of *Arbacia lixula*. These concerns, however, were considered to be clarified by Régis (1982), who compared the microstructure of the spines of *Arbaciella elegans* with those of *Arbacia lixula* and concluded that they were morphologically different species. However, recent works have reopened the issue.

Probably, the most conclusive proof about the nonvalidity of genus *Arbaciella* came from the settlement studies in the Canary Islands by

Hernández et al. (2005). Three species of regular sea urchins completely dominate the shallow rocky reefs in the Canary Islands, namely: *Diadema africanum, Paracentrotus lividus* and *Arbacia lixula*, whereas *Arbaciella elegans* is considered rare. In their year-long study using artificial collectors, Hernández et al. (2005) found great numbers of settlers of *D. africanum*, *P. lividus* and a third sea urchin that was indistinguishable from *A. elegans*. But, despite its dominance in the archipelago, they did not found any trace of other morphologically different settlers which could belong to *A. lixula*. Thus, most probably, the *Arbaciella elegans* settlers were actually settlers of *A. lixula*. Recently, suspicions were confirmed when Kroh et al. (2011) sequenced the mitochondrial gene COI of four specimens from Azores Islands that were morphologically indistinguishable from the *Arbaciella* holotype and the genetic data proved unequivocally that they belonged to *A. lixula*. The same molecular results were obtained by López et al. (2012) with *Arbaciella* individuals from the Canary Islands. The confusion was probably originated by the fact that, contrary to what is usual in other sea urchins, the gonopores develop very early in *A. lixula* juveniles, when they are as small as 6 mm of diameter (Kroh et al., 2011; Fig. I.6). This led Mortensen to erroneously consider that the observed specimens were sexually mature individuals of a different species.

Accepting that *Arbaciella* specimens are, in fact, juveniles of *Arbacia lixula*, a lot of information regarding the ecology of early stages of *A. lixula* can be inferred. Thus, Salas and Hergueta (1994) reported that *Arbaciella elegans* (that is, *A. lixula* juveniles) appeared during late autumn and winter in Southern Spain. Giacobbe and Rinelli (1992) found them associated with *Pinna* shells in the Straits of Messina and Aliani and Meloni (1999) collected them in December on artificial buoys set in the Corsica

Channel. The recent "new records" of *Arbaciella elegans* in Northern Mediterranean waters (Solinas et al., 1990; Merella et al., 1994; Signorelli and Zamboni, 1998; Grubelić and Antolić, 2000) and in the Eastern Mediterranean basin (Koukouras et al., 2007) might be associated with an increase in the frequency of successful *A. lixula* settlement, though a recent increase in the sampling effort, especially during winter months, cannot be ruled out.

Mitochondrial phylogeography in Atlanto-Mediterranean echinoderms

Phylogeography based in mitochondrial markers has been extensively used to study the intraspecific variability of echinoderms and other marine invertebrates (e.g.: Dawson, 2001; Kelly and Palumbi, 2010). The haplotype network approach (Bandelt et al., 1999) allows for the description of the evolutionary history of the different mitochondrial lineages. The analysis of the mismatch distribution (Rogers and Harpending, 1992) allows inferring past demographic history of selected populations. Combined with the use of well calibrated mutation rates, dates for the demographic expansions and other demographic events can be estimated (Rogers, 1995).

In the context of Atlanto-Mediterranean echinoderms, mitochondrial phylogeography has been recently used to study the population structure and demographic history of some of the most representative echinoderms in shallow sublittoral habitats, such as *Paracentrotus lividus* (Calderón et al., 2008; Maltagliati et al., 2010), *Marthasterias glacialis* (Pérez-Portela et al., 2010) or *Holothuria mammata* (Borrero-Pérez et al., 2011). In most cases, these species have been present in the Mediterranean since ancient times

(most of them probably entered the Mediterranean soon after the Zanclean flood which ended the Messinian salinity crisis) and their populations are now genetically well differentiated along the Mediterranean, with the existence of inter-population differences in the haplotype sequences or in their frequencies. The origin of most of this population structure can be attributed to the reduced gene flow between Mediterranean sub-basins. The circulation of surface water between sub-basins is limited by the existence of geographic features and/or oceanic fronts, which largely prevent the mixing of water masses and thus reduce the gene flow of organisms with planktonic larvae among different sub-basins. Some of these fronts may not be geographically obvious, such as the Almeria-Oran front, which separates the Alboran basin from the Western Mediterranean basin. Many marine populations from the Alboran basin, both invertebrates and fishes, are genetically closer to Atlantic stocks than to other Mediterranean populations (Patarnello et al., 2007). In many of these Atlanto-Mediterranean echinoderms, when the species has had enough time to diverge, exclusive Mediterranean lineages have appeared (Calderón et al., 2008; Pérez-Portela et al., 2010; Borrero-Pérez et al., 2011), which in most cases cannot be exported to the Atlantic and remain endemic to the Mediterranean, due to the strong eastward surface water influx through the Strait of Gibraltar, which prevent surface planktonic larvae to go out to the Atlantic Ocean.

Ecological significance

Herbivorous sea urchins are one of the main factors determining the abundance and distribution of algae and seagrasses in shallow marine ecosystems. The crucial importance of sea urchins in shaping benthic

communities (Lawrence, 1975; Lawrence and Sanmarco, 1982; Sala et al., 1998a) has been demonstrated by many ecological experiments along the Mediterranean coasts (e.g.: Benedetti-Cecchi and Cinelli, 1995; Sala and Zabala, 1996; Benedetti-Cecchi et al., 1998; Palacín et al., 1998a; Bulleri et al., 1999; Guidetti et al., 2004; Bonaviri et al., 2011). The ecological role of *Arbacia lixula* cannot be fully understood unless in the context of its interactions with the common European edible sea urchin *Paracentrotus lividus*. Both sea urchins broadly share their habitat and distribution, in such interwoven way that Mediterranean fishermen have traditionally thought of them as the male and female of a single species (Tortonese, 1965; Corcoll, 2012). The common Italian names for *A. lixula* and *P. lividus* (*riccio maschio* and *riccio femina*, respectively) reflect this coexistence. The higher densities usually attained by *P. lividus* compared with coexisting *A. lixula* have often driven marine ecologists to focus ecological research in the former rather than the latter. Thus, *P. lividus* is considered to be a significant structuring force in the Mediterranean ecosystems, not only in barren grounds with high or very high sea urchin densities, but also in communities with low sea urchin densities (Palacín et al., 1998a), more representative of large areas over the geographic range of the species (Boudouresque and Verlaque, 2001). Conversely, *A. lixula* has been traditionally attributed just a secondary ecological role. However, this view may be changing in the last few years, and the ecological importance of this species is being increasingly recognized (Bulleri et al., 1999; Guidetti et al., 2003; Guidetti and Dulcić, 2007; Bonaviri et al., 2011; Gianguzza et al., 2011; Privitera et al., 2011).

Some early ecological works involving Mediterranean littoral sea urchins dealt with the topic of competition between *Paracentrotus lividus*

and *Arbacia lixula*. The trophic studies based in gut contents analyses resulted in the view that *A. lixula* fed mainly in encrusting coralline algae, whereas *P. lividus* tended to feed on fleshy, erect macroalgae and *Posidonia oceanica* leaves and epiphytes (Kempf, 1962; Régis, 1978; Verlaque and Nédélec, 1983; Frantzis et al., 1988; Bulleri et al., 1999; Boudouresque and Verlaque, 2001; Tomas et al., 2006). Thus, the traditional view is that no strong competence for trophic resources was established among both echinoids. Indeed, Privitera et al. (2008) demonstrated that both species occupy different trophic niches in resource-limited (barren) areas, again in the sense that *A. lixula* feeds mainly on encrusting corallines while *P. lividus* feeds on non-encrusting macrophytes.

Though *Arbacia lixula* has been traditionally considered a grazer of encrusting coralline algae, other species in the genus *Arbacia* have been reported to show omnivorous or unambiguously carnivorous behaviour. North American *A. punctulata* feeds on sessile invertebrate species, sand dollars and other *Arbacia* individuals, as well as some algae (Harvey, 1956; Karlson, 1978; Cobb and Lawrence, 2005). The diet of South Atlantic *A. dufresnii* is mainly carnivorous (Penchaszadeh, 1979; Vasquez et al., 1984; Penchaszadeh and Lawrence, 1999; Zaixso, 2004). The Pacific *A. spatuligera* showed preference for animal food over common species of algae from its habitat (Silva et al., 2004). Moreover, some observations indicate omnivorous or carnivorous behaviour of *A. lixula* outside the Mediterranean (Marques, 1984; Oliveira, 1991; Tavares and Borzone, 2005).

Traditional methods based in the analysis of gut contents are subject to the possible bias derived from the different digestibility of diverse food material. The current state-of-the-art method for trophic studies in natural

ecosystems is stable isotopes analysis (Owens, 1987; Peterson and Fry, 1987). This technique analizes and compares tissue samples from the consumers and possible preys, thus testing the assimilated rather than the ingested material. It has been successfully applied to marine ecosystems (e.g.: Hobson and Welch, 1992; Cardona et al., 2007; Jaschinski et al., 2008) and has proved to be very useful to elucidate diet preferences and trophic levels of shallow echinoids of Western Australia (Vanderklift et al., 2006). It has been used in the Mediterranean to investigate the diet of *Paracentrotus lividus* (Tomas et al., 2006), but differences in trophic positions between *P. lividus* and *A. lixula* had not been tested using stable isotopes until the work we present here.

Population outbreaks of both *Arbacia lixula* and *Paracentrotus lividus* have been suggested to be able to create barrens in rocky substrates (Kempf, 1962; Verlaque, 1987; Hereu, 2004; Guidetti and Dulcić, 2007; Bulleri, 2013), affecting both productivity and diversity of benthic assemblages (Bulleri et al., 2002; Sala, 2004; Privitera et al., 2008). Barren zones are not as dominant in northwestern Mediterranean as they are in other temperate or tropical regions (e.g.: the extensive barrens produced by *Diadema africanum* in Madeira and Canary Islands; Tuya et al., 2004; Hernández et al., 2008). But they are becoming more common in the Southern Mediterranean (Guidetti et al., 2003; Gianguzza et al., 2006; Privitera et al., 2008). Many early ecological experiments aimed at testing the relations between sea urchins and barren areas in the Mediterranean did not discriminate between *P. lividus* and *A. lixula* (e.g. Bulleri et al., 2002), and thus the main role in creating barren grounds was attributed to *P. lividus* due to its higher densities. However, some works have found significant associations between the density of *A. lixula* and the extension of

barren grounds (Micheli et al., 2005; Guidetti and Dulcić, 2007), so the role of *A. lixula* in creating or maintaining the barren areas is being increasingly recognized (Gianguzza et al., 2011; Privitera et al., 2011).

There is growing evidence that the northwestern Mediterranean populations of *Arbacia lixula* have experienced a demographic increase over the last decades. Marion (1883) and Koehler (1883) described this species as very rare in the coast of Provence. But Petit et al. (1950) reported that it had become abundant in the same region over a 30-year period. Boudouresque et al. (1989) recorded a 4-fold increase in Corsica from 1980 to 1988. Francour et al. (1994) reported a 12–fold increase at the Scandola Reserve (Corsica) from 1983 to 1992 and speculated that a long term rise in the water temperature could have been the cause for this proliferation. In the same period (1982 to 1995), a 5-fold increase in *A. lixula* densities was reported at the Port-Cros Marine Reserve (France) (Harmelin et al., 1995). On the contrary, in a study comparing densities between 1992 and 2007 along the Catalan Coast, Corbacho et al. (*pers. comm.*) did not find significant differences in the abundances of *A. lixula*. In the longest follow-up registered (from 1995 to 2010), Hereu et al. (2012) did not either find significant differences in the density of *A. lixula* at the Medas Islands Marine Reserve (Catalonia). Unfortunately, there are very few studies comparing abundances of sea urchins in the long term.

Reproduction and development

The reproductive cycle of *Arbacia lixula* in the northwestern Mediterranean was first studied by Fénaux (1968). She studied the gonadosomatic cycle and the occurrence of larvae in the plankton during three years at Villefranche-Sur-Mer (French Riviera). She concluded that the gonadadosomatic cycle peaked every year from May to June. This timing was coherent with the appearance of pluteus larvae in the plankton from July to November, i.e., during the warmest months of the year. Fénaux argued that this behaviour was in agreement with the thermophilous nature of *Arbacia lixula*.

Pedrotti (1993) studied the spatial and temporal distribution patterns of the larvae of several species of echinoderms, including *Arbacia lixula*, in the Ligurian Sea, near Villefranche-Sur-Mer. She found abundant larvae of *A. lixula* during October and November, and a secondary peak in June-July (Fig. I.7). This matches the timing for the presence of *Arbaciella elegans* (putative *A. lixula* settlers) in the coast of Southern Spain (Salas and Hergueta, 1994).

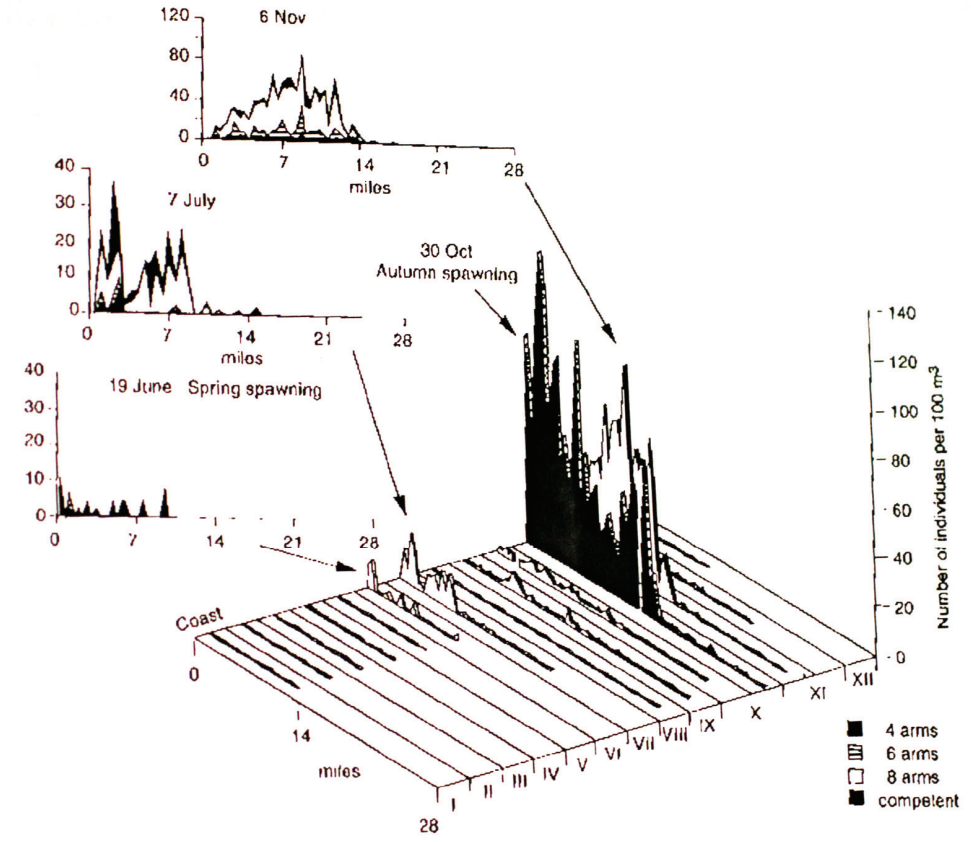

Fig. I.7. Spatial and temporal distribution patterns of *A. lixula* larvae in the Ligurian Sea throughout 1986. Roman numerals represent the months, and the perpendicular axis represents the distance to the coast, in miles. From Pedrotti (1993)

The absence of larvae during the intermediate months (from August to September) in Pedrotti's (1993) results is remarkable, since they coincide with the warmest months and with the spawning predicted by the gonadosomatic index drop in Fénaux (1968). A possible explanation is the low concentration of phytoplankton during these months in the northwestern Mediterranean, which could lead to high larval mortality due to lack of food. Other possible explanation may be the increase in the densities of planktivorous fishes during the same months, which could

predate on the echinoid larvae. Alternatively, interannual variability in reproductive timing could be responsible for the differences between Fénaux's and Pedrotti's results.

Despite the great abundance of adult individuals, which may sometimes form dense patches, *Arbacia lixula* juveniles are very uncommon in the Catalan coast. After studying the size distribution of *A. lixula* at the Medas Islands Marine Protected Area, Sala et al. (1998b) and Hereu et al. (2012) noted the dominance of the 4 - 5 cm size-classes and the scarcity of individuals smaller than 30 mm. Similar size distributions were reported from Marseilles by Kempf (1962) and from the Apulian coast (SE Italy) by Guidetti et al. (2003).

These results suggest that, contrary to the regular yearly pattern observed in *Paracentrotus lividus* settlement (Lozano et al., 1995; López et al., 1998; Tomas et al., 2004), the recruitment process in *A. lixula* seems to be highly irregular in northwestern Mediterranean. It is probable that settlement events are modulated by different physical or biological factors (yet to be studied), and may not happen every year. This fact is possibly related to high mortality rates during the larval development.

Remarkably, Harvey (1956) reported similar results for *Arbacia punctulata* in Massachusetts. Juvenile individuals of this American species were very rare in the surroundings of Woods Hole. Indeed, the majority of the beds were almost exclusively inhabited by big adult sea urchins.

Effects of global change on echinoderm larvae

Although there is considerable uncertainty about the spatial and temporal details, climate change is clearly and fundamentally altering ocean ecosystems (Hoegh-Guldberg and Bruno, 2010). Changes in marine ecosystems come in two different ways. On one side, water warming is an increasingly documented phenomenon affecting every ocean in the World. On the other hand, increased atmospheric CO_2 emissions are inducing changes in seawater carbon chemistry, lowering its pH, a phenomenon known as ocean acidification. The average pH of surface seawater has declined by approximately 0.1 units since the industrial revolution and future reductions are expected to be around 0.3–0.5 units by 2100 and 0.7–0.8 units or more by 2300 (Caldeira and Wickett, 2003, 2005; Intergovernmental Panel on Climate Change, 2007). The result is a decrease in the concentration of the carbonate ion ($CO_3^{=}$) that reduces the saturation state of calcium carbonate minerals (Ω_{CaCO3}) (Feely et al., 2004; Orr et al., 2005). This has been theorized to negatively affect calcifying organisms, such as echinoderm larvae. Nevertheless, recent experimental efforts have demonstrated that echinoderm larvae are more robust to ocean acidification than was initially expected (Dupont et al., 2010c; Dupont and Thorndyke, 2013). Ocean acidification usually produces only sublethal effects on most species of echinoderms, which can be explained in the context of metabolic energy budget (Dupont and Thorndyke, 2013).

Much research effort has been addressed to elucidate the effects of ocean acidification on larval development of echinoderms. The results have been somehow ambiguous. Some species show a clear impairment in the development when exposed to lowered pH conditions (*Ophiothrix fragilis*,

Dupont et al., 2008). In other species, the effects are neutral or undetectable, and the development of a few species may even be improved by moderate levels of acidification (*Crossaster papposus*, Dupont et al., 2010b). Furthermore, the effects of global change on the future populations of thermophilous calcifying organisms are uncertain. On one hand, their larval development is expected to be promoted by increasing temperatures, but it could be impaired by decreasing pH.

Larval development is one of the most vulnerable life-history stages in echinoids, which are usually broadcast spawners, and mortality rates are regularly high during this period (Rumrill, 1990; López et al., 1998). Thus, many echinoid populations may be, at least partially, limited by larval mortality. If the main factors limiting larval survival rate are released, then population blooms of adult echinoids may occur, which have the potential to drastically impact benthic communities. Global change is altering the practical totality of physical and biological processes in marine ecosystems, affecting both planktonic and benthic communities. A plethora of complex interactions are involved in the regulation of life-cycle processes determining population densities of adult echinoids, and virtually all of the physico-chemical and biological modulating factors may be altered by global change. So, the future behaviour of the population dynamics of sea urchins is practically unpredictable. Nevertheless, the effects on the larval development of some critical variables, such as temperature or pH, may be studied through laboratory experimentation, and some insights into the predicted population dynamics of some ecologically significant species may be inferred.

OBJECTIVES

This work aims at studying the factors affecting the phylogeography, trophic ecology and biology of the black sea urchin *Arbacia lixula* in Mediterranean ecosystems, in order to assess the ecological role of this conspicuous species and its possible future impact in benthic communities.

To undertake this general aim, we decided to establish four different specific objectives:

1. To carry out population genetics and phylogeographic studies of *Arbacia lixula* populations throughout its range of distribution, using mitochondrial molecular markers.

2. To assess the trophic relationships between the two sympatric species *Arbacia lixula* and *Paracentrotus lividus* and their possible food sources, using stable isotopes analysis and gut contents analysis.

3. To study the reproductive cycle of *Arbacia lixula* and the factors which regulate it in the northwestern Mediterranean.

4. To investigate the larval development of *Arbacia lixula* in different conditions of temperature and acidification, in order to assess its vulnerability at the organismal level, in the context of current global change.

STRUCTURE OF THE CHAPTERS

This dissertation has been structured in four chapters which correspond to original research articles already published or submitted for publication to different scientific journals of the fields of Marine Biology or Ecology. These articles are presented in a unified format to ease reading, but the original text in the published articles, including captions for images and tables, has been preserved. A translation of the abstract into Spanish has been added at the beginning of each chapter. The numbering of images and tables has been modified to allow easy cross-references, the reference citations have been unified throughout the text and all the bibliographic references have been merged into a common reference list set at the end of the dissertation.

In Chapter 1, the phylogeographic study of twenty-four populations of *Arbacia lixula* sampled throughout most of its area of distribution is addressed, using the mitochondrial marker COI. Some interesting results regarding the genetic structure of its populations and the history of its colonization of the Mediterranean Sea are presented.

In Chapter 2, the results of our study comparing the trophic ecology of *Arbacia lixula* and the sympatric species *Paracentrotus lividus*, using stable isotopes analysis and gut contents analysis are presented. We introduce some unexpected but consistent results for the trophic position of *A. lixula,* which challenge the previously accepted view of this sea urchin as an herbivorous grazer of encrusting coralline algae, suggesting a new ecological role for this abundant species.

Chapter 3 is one of two chapters dealing with the reproductive biology of *Arbacia lixula* and how current global change may affect it in the

near future. A long-term follow up (during four years) of its gonadal cycle was carried out in a locality of the northwestern Mediterranean. Novel methods of circular statistics were used to study the gonad histology. The effects of photoperiod and temperature on the gonadal cycle of this species were assessed and the implications of ocean warming for the reproductive output, and thus for the future population dynamics of this species, are discussed.

The last chapter (Chapter 4) deals with the larval development of *Arbacia lixula*. Laboratory experiments in different conditions of temperature and pH were carried out to check the possible effects of global change on larval development and settlement survival of this species in the near future, in order to assess its vulnerability against the foreseeable changes in temperature and pH.

Finally, an integrative general discussion is presented, where the implications of our findings for the future dynamics of the populations of *Arbacia lixula* in the Mediterranean, and their increased potential to impact the shallow rocky ecosystems, are discussed.

The interested reader will find the published articles, corresponding to Chapters 1 and 2, in their original format, included as an appendix at the end of this volume.

ADVISORS' REPORT

Dr. **Xavier Turon** and Dr. **Creu Palacín,** co-advisers of the PhD thesis entitled "Biology and phylogeography of the black sea urchin *Arbacia lixula* (Echinoidea: Arbacioida)", certify that the dissertation presented here has been carried out by **Owen S. Wangensteen** in its totality and that, as advisers, we have participated in designing, guiding and correcting earlier drafts of the chapters and manuscripts written by the PhD candidate.

Publication status of the chapters of this thesis:

Chapter 1. Natural or naturalized? phylogeography suggests that the abundant sea urchin *Arbacia lixula* is a recent colonizer of the Mediterranean
Owen S. Wangensteen, Xavier Turon, Rocío Pérez-Portela and Creu Palacín
PLoS ONE 7 (9): e45067 (2012)
5-Year Impact Factor (2011): 4.537

Chapter 2. A wolf in sheep's clothing: carnivory in dominant sea urchins in the Mediterranean
Owen S. Wangensteen, Xavier Turon, Alex García-Cisneros, Mireia Recasens, Javier Romero and Creu Palacín
Marine Ecology Progress Series 441: 117-128 (2011)
5-Year Impact Factor (2011): 3.086

Chapter 3. Spiny affairs heating up: photoperiod, temperature and inter-annual variability in the reproductive cycle of the thermophilous sea urchin *Arbacia lixula*
Owen S. Wangensteen, Xavier Turon, Maria Casso and Creu Palacín
Submitted. *Marine Biology*
5-Year Impact Factor (2011): 2.471

Chapter 4. Some like it hot: temperature and acidification modulate larval development and settlement of the sea urchin *Arbacia lixula*
Owen S. Wangensteen, Sam Dupont, Isabel Casties, Xavier Turon and Creu Palacín
Submitted. *Journal of Experimental Marine Biology and Ecology*
5-Year Impact Factor (2011): 2.395

Contributions of the authors:

Chapter 1. Conceived and designed the experiments: XT CP. Performed the experiments: OSW RPP. Analyzed the data: OSW XT RPP. Contributed reagents/materials/analysis tools: OSW XT RPP CP. Wrote the paper: OSW. Revised the paper: XT RPP CP.

Chapter 2. Conceived and designed the experiments: OSW XT CP. Performed the experiments: OSW AGC MR. Analyzed the data: OSW AGC MR. Contributed reagents/materials/analysis tools: OSW XT AGC JR CP. Wrote the paper: OSW. Revised the paper: XT JR CP.

Chapter 3. Conceived and designed the experiments: OSW XT CP. Performed the experiments: OSW MC CP. Analyzed the data: OSW MC. Contributed reagents/materials/analysis tools: OSW XT MC CP. Wrote the paper: OSW. Revised the paper: XT CP.

Chapter 4. Conceived and designed the experiments: OSW SD XT CP. Performed the experiments: OSW IC. Analyzed the data: OSW IC. Contributed reagents/materials/analysis tools: OSW SD IC XT CP. Wrote the paper: OSW. Revised the paper: SD XT CP.

From all the authors of the different chapters, AGC, MR, MC and IC have not been awarded a PhD degree. We hereafter guarantee that none of the information contained in the chapters coauthored by them will be used to elaborate part of other PhD theses.

For all of the above, we consider that the contribution of the PhD candidate grants him the right to defend his thesis in front of a scientific committee.

Barcelona, May 8th 2013.

Dr. **Xavier Turon** Dr. **Creu Palacín**

CHAPTER 1 – NATURAL OR NATURALIZED?

Arbacia lixula at Tossa de Mar. Photo: **C. Palacín**

Chapter 1. Natural or naturalized? Phylogeography suggests that the abundant sea urchin *Arbacia lixula* is a recent colonizer of the Mediterranean

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ABSTRACT

We present the global phylogeography of the black sea urchin *Arbacia lixula*, an amphi-Atlantic echinoid with potential to strongly impact shallow rocky ecosystems. Sequences of the mitochondrial cytochrome *c* oxidase gene of 604 specimens from 24 localities were obtained, covering most of the distribution area of the species, including the Mediterranean and both shores of the Atlantic. Genetic diversity measures, phylogeographic patterns, demographic parameters and population differentiation were analysed. We found high haplotype diversity but relatively low nucleotide diversity, with 176 haplotypes grouped within three haplogroups: one is shared between Eastern Atlantic (including Mediterranean) and Brazilian populations, the second is found in Eastern Atlantic and the Mediterranean and the third is exclusive from Brazil. Significant genetic differentiation was found between Brazilian, Eastern Atlantic and Mediterranean regions, but no differentiation was found among Mediterranean sub-basins or among Eastern Atlantic sub-regions. The star-shaped topology of the haplotype network and the unimodal mismatch distributions of Mediterranean and Eastern Atlantic samples suggest that these populations have suffered very recent demographic expansions. These expansions could be dated 94 – 205 kya in the Mediterranean, and 31 – 67 kya in the Eastern Atlantic. In contrast, Brazilian populations did not show any signature of population expansion. Our results indicate that all populations of *A. lixula* constitute a single species. The Brazilian populations probably diverged from an Eastern Atlantic stock. The present-day genetic structure of the species in Eastern Atlantic and the Mediterranean is shaped by very recent demographic processes. Our results support the view (backed by the lack of fossil record) that *A. lixula* is a recent thermophilous colonizer which spread throughout

the Mediterranean during a warm period of the Pleistocene, probably during the last interglacial. Implications for the possible future impact of *A. lixula* on shallow Mediterranean ecosystems in the context of global warming trends must be considered.

KEYWORDS

demographic expansion, gene flow, Pleistocene glaciations, poleward range shift, population genetics, Riss-Würm interglacial, temperate rocky reef

RESUMEN

En el presente trabajo se muestra la filogeografía global del erizo de mar negro *Arbacia lixula*, un equinoideo anfiatlántico con potencial para producir un fuerte impacto en los ecosistemas rocosos someros. Se obtuvieron secuencias del gen mitocondrial citocromo *c* oxidasa de 604 individuos procedentes de 24 localidades, cubriendo la mayor parte del área de distribución de la especie, incluyendo el Mediterráneo y las dos orillas del Atlántico. Se analizaron las medidas de diversidad genética, los patrones filogeográficos, los parámetros demográficos y la diferenciación entre poblaciones. Se encontró una alta diversidad haplotípica, pero una diversidad nucleotídica relativamente baja, con 176 haplotipos agrupados en tres haplogrupos: uno es compartido entre el Atlántico Oriental (incluido el Mediterráneo) y las poblaciones de Brasil, el segundo se encuentra en el Atlántico Oriental y en el Mediterráneo y el tercero es exclusivo de Brasil. Se encontró una diferenciación genética significativa entre Brasil, Atlántico Oriental y Mediterráneo, pero no entre las sub-cuencas del Mediterráneo o entre las sub-regiones del Atlántico Oriental. La topología en forma de

estrella de la red de haplotipos y la distribución de sustituciones por parejas en el Mediterráneo y Atlántico Oriental sugieren que estas poblaciones han sufrido expansiones demográficas muy recientes. Estas expansiones pueden ser datadas entre 94,000 y 205,000 años en el Mediterráneo, y entre 31,000 y 67,000 años en el Atlántico Oriental. En contraste, la población de Brasil no mostró ningún signo de expansión demográfica. Nuestros resultados indican que todas las poblaciones de *A. lixula* constituyen una sola especie. Las poblaciones brasileñas probablemente divergieron de un stock del Atlántico Oriental. La estructura genética actual de la especie en el Atlántico Oriental y en el Mediterráneo está determinada por procesos demográficos muy recientes. Nuestros resultados apoyan la idea (respaldada por la ausencia de registro fósil) de que *A. lixula* es un reciente colonizador termófilo, que se extendió por todo el Mediterráneo durante un período cálido del Pleistoceno, probablemente durante el último interglaciar. Las posibles implicaciones respecto al impacto futuro de *A. lixula* en los ecosistemas mediterráneos costeros, en el contexto del calentamiento global, deben ser tenidas en consideración.

PALABRAS CLAVE

expansión demográfica, flujo genético, glaciaciones del Pleistoceno, expansión de rango hacia los polos, genética de poblaciones, Interglaciar Riss-Würm, arrecifes templados

INTRODUCTION

The European black sea urchin *Arbacia lixula* (Linnaeus, 1758) is currently one of the most abundant echinoids in shallow rocky habitats of the Mediterranean (Palacín et al., 1998b), where it has the potential to greatly influence benthic communities with their grazing activity (Sala et al., 1998a; Palacín et al., 1998a; Bulleri et al., 1999). *A. lixula* has a considerable trophic plasticity, ranging from omnivory to strict carnivory (Wangensteen et al., 2011) and its scraping predatory behaviour can bulldoze the substrate bare of erect and encrusting algae and sessile animals. *A. lixula* broadly overlaps its habitat with the common edible sea urchin *Paracentrotus lividus* (Lamarck, 1816). Both species are traditionally thought to have the ability to trigger the development of subtidal barren zones of reduced benthic productivity and diversity (Verlaque, 1987; Hereu, 2004; Bulleri et al., 2002; Privitera et al., 2008). However, new and increasing evidence suggests that *A. lixula* could actually be playing the principal role in producing and maintaining these barrens (Gianguzza et al., 2011) and that this trend could be worsening in the near future due to foreseeable climatic changes (Privitera et al., 2011).

Arbacia lixula is commonly regarded as a typical native species in the Mediterranean fauna (Riedl, 1983), since it is currently found in shallow rocky shores all along the Mediterranean, often at high densities, and has been so since historical times. However, its tropical affinities have been suggested for a long time. Based on the lack of Mediterranean fossil record, Stefanini (1911) and Mortensen (1935) stated that *A. lixula* (reported as *A. pustulosa*), probably originated at the Tropical Atlantic region, from where it spread into the Mediterranean. Kempf (1962), Tortonese (1965) and Fénaux (1968) also considered that *A. lixula* was a thermophilous species.

In NW Mediterranean, increasing abundances over time have been reported for this species. Petit *et al*. (1950) reported that *Arbacia lixula* had become abundant in Marseilles during the previous 30 years, despite Marion (1883) had described it as rare in the same area in 1883. More recently, Francour *et al.* (1994) reported a 12–fold increase in the abundance of *A. lixula* in Corsica over a period of nine years (1983-1992) and speculated that a long term rise in the water temperature could have been the cause for this proliferation. In the same period (1982 to 1995), a 5-fold increase in *A. lixula* densities was reported at the Port-Cros Marine Reserve (France) (Harmelin et al., 1995). On the other hand, in a recent 5-year follow-up (2003-2008) at Ustica Island (Southern Thyrrenian Basin), a positive correlation was found between the gonado-somatic index of adult *A. lixula* and summer surface water temperature, suggesting increased reproductive potential with temperature (Gianguzza et al., 2011).

Arbacia is an ancient genus with a fossil record that dates back to the Paleocene (Kroh and Smith, 2010) whose distribution is mainly Neotropical. Unlike other sea urchin genera, *Arbacia* has a history of latitudinal shifts (Hart, 2012), and the five extant species inhabit mainly temperate and tropical shallow waters (Metz et al., 1998), being mostly allopatric. Only one species, *A. dufresnii*, is able to live in cold Subantarctic waters. *A. lixula* is the only species in the genus that lives in the Old World. Its present distribution includes Brazil, the African Atlantic coast from Morocco to Angola, the East Atlantic archipelagos of Cape Verde, Canaries, Madeira and Azores, and the whole Mediterranean basin, excluding the Black Sea. It has never been reported from the Atlantic European coast north of Gibraltar (J. Cristobo, X. Troncoso, N. V. Rodrigues; *pers. comms*.), probably due to the low sea surface temperature originated by the southward Portugal

Current (Martins et al., 2002).

Recently, Lessios *et al.* (2012) presented an exhaustive phylogenetic study of genus *Arbacia,* using sequences of the mitochondrial COI (cytochrome *c* oxidase I) and the nuclear gamete recognition protein bindin, which has clarified many interesting questions on inter-specific relationships within this remarkable genus. Notably, the sequence of speciation events was consistently reconstructed and their divergence times were reliably estimated. Thus, the splitting between *A. lixula* and its sister species, the NW Atlantic *A. punctulata,* was estimated to have taken place some 2.2-3.0 Mya (millions years ago) based on COI sequences, or 1.9-3.3 Mya based on bindin sequences. The phylogeny of bindin sequences also allowed these authors to infer that Brazil populations separated from the rest of *A. lixula* some 1.8-3.4 Mya; i.e. very early in the evolution of this species (however, only 5 individuals from Brazil were used in the analysis, and no estimation could be inferred for the same event from mitochondrial sequences, due to the unresolved position of the Brazilian clade within other *A. lixula* haplotypes).

Yet, many questions remain open about the intra-specific relationships of *Arbacia lixula*. Considering its unusually wide present distribution area, which ranges from equatorial waters to temperate Mediterranean, the great colonizing potential shown by this species, including the ability to cross trans-oceanic barriers to gene flow (Lessios et al., 2012), and the massive potential impact of its behaviour on coastal ecosystems, further research on its phylogeography and population genetics is necessary in order to elucidate the history and ongoing processes that shape the distribution of the species. In this work, we present a phylogeographic study using the mitochondrial marker COI, based on a

representative sample of individuals covering most of the distribution area of *Arbacia lixula.* Our goals were to answer relevant questions concerning the history and present-day distribution of the species: What are the relationships between the main geographic areas where the species is found? Do the main geographic barriers to gene flow, that are known to regulate the genetic structure of many other marine organisms, affect the present-day genetic structure of this species? Can recent geographic and/or population expansion events be traced and reconstructed by analysing the signature left in sequence data of this species?

METHODS

Ethics statement

Field sampling required for this work involved only invertebrate species which are neither endangered nor protected. All necessary permits for sampling at localities placed inside protected areas (Cabrera National Park, Columbretes Islands Marine Reserve & Scandola Nature Reserve) were previously obtained from the competent authorities. Non-destructive sampling techniques (external soft tissue biopsy) were used in these localities in order to minimize impact on the ecosystems.

Sampling

Between April 2009 and July 2011, we obtained samples from 24 localities belonging to three predefined regions: West Atlantic, East Atlantic and Mediterranean (see Fig. 1.1 and Table 1.1). For more detailed analyses, we further subdivided the East Atlantic region in two sub-regions (Cape Verde and Macaronesia), while the Mediterranean was divided in three sub-basins (Alboran Sea, West Mediterranean and East Mediterranean). The

sampled localities were: two from Brazil, one from Cape Verde, four from Macaronesian archipelagos, two from the Alboran Sea, twelve from West Mediterranean and three from East Mediterranean. 15 to 30 adult *Arbacia lixula* individuals (average: 25.2) per location were sampled. In all cases, tissue samples were stored in absolute ethanol at -20ºC until processed.

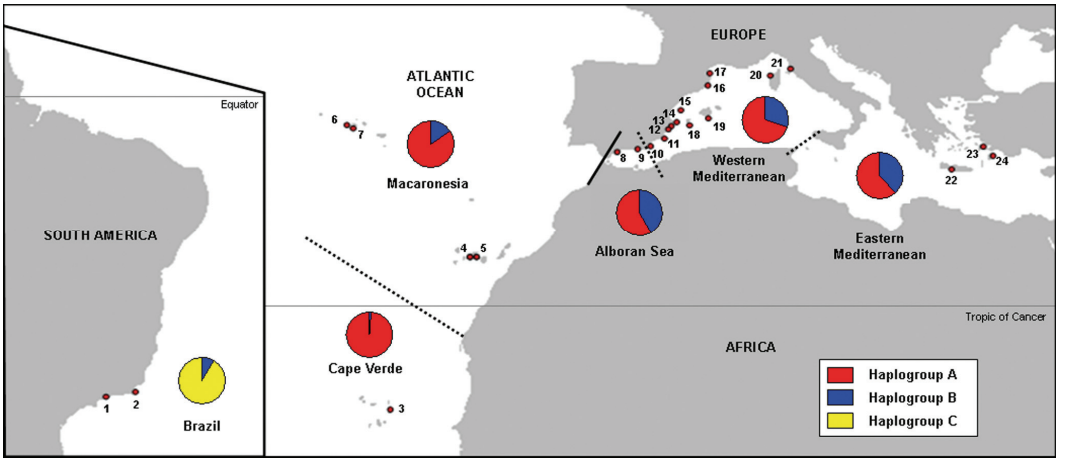

Fig. 1.1. Sampling localities for *Arbacia lixula* populations. See Table 1.1 for locality names and coordinates. Borders between regions are indicated by solid bold lines and borders between sub-regions are represented by dotted lines. Pie charts of haplogroup frequencies are shown for the six sub-regions in which the three studied regions have been subdivided

DNA amplification and sequencing

Total DNA was extracted using REDExtract-N-Amp Tissue kit (Sigma–Aldrich, www.sigma.com) from either one tube foot or a tiny portion (5-10 mg) of gonad. A fragment of the COI gene was amplified and sequenced using specific primers designed using the complete genome sequence of *A. lixula* mitochondrion (De Giorgi et al., 1996) with PRIMER 3.0 (Rozen and Skaletsky, 2000), as follows: COIARB-F: 5'-TTC TCT GCT TCA AGA TGA C-3', COIARB-R: 5'-CTA TAA TCA TAG TCG CTG CT-3', COIAL-R: 5'-GCT CGG GTA TCT AGG TCC AT-3'. Most individuals

were amplified using the COIARB-F/COIARB-R pair, but some individuals belonging to Atlantic populations had to be amplified using COIARB-F/COIAL-R instead.

Table 1.1. *Arbacia lixula.* Sampling localities

Label	*Locality*	*Code*	*Region*	*Sub-region*	*Latitude/Longitude*
1	Itaipu	ITA	W. Atlantic	Brazil	-22.974910/-43.050456
2	Cabo Frio	CFR	W. Atlantic	Brazil	-22.890409/-41.998186
3	Boavista	BOA	E. Atlantic	Cape Verde	16.136858/-22.941055
4	Los Gigantes	GIG	E. Atlantic	Macaronesia	28.200925/-16.8294084
5	Tenerife (East)	TEN	E. Atlantic	Macaronesia	28.100823/-16.478088
6	Faial	FAI	E. Atlantic	Macaronesia	38.522720/-28.620937
7	Pico	PIC	E. Atlantic	Macaronesia	38.423336/-28.415823
8	Torremuelle	TOR	Mediterranean	Alboran Sea	36.577369/-4.565396
9	La Herradura	HER	Mediterranean	Alboran Sea	36.721044/-3.728487
10	Carboneras	CAR	Mediterranean	W. Medit.	36.993869/-1.890274
11	Palos	PAL	Mediterranean	W. Medit.	37.634580/-0.693749
12	Villajoyosa	VIL	Mediterranean	W. Medit.	38.509007/-0.212885
13	Benidorm	BEN	Mediterranean	W. Medit.	38.502530/-0.128329
14	Xabia	XAB	Mediterranean	W. Medit.	38.752880/0.224511
15	Columbretes	CLM	Mediterranean	W. Medit.	39.898115/0.685179
16	Tossa	TOS	Mediterranean	W. Medit.	41.722109/2.939914
17	Colera	COL	Mediterranean	W. Medit.	42.391077/3.155390
18	Formentera	FOR	Mediterranean	W. Medit.	38.693415/1.376867
19	Cabrera	CAB	Mediterranean	W. Medit.	39.155689/2.944236
20	Scandola	SCA	Mediterranean	W. Medit.	42.361842/8.549023
21	Populonia	POP	Mediterranean	W. Medit.	42.993752/10.498702
22	Crete	CRE	Mediterranean	E. Medit.	35.171626/24.400875
23	Kos	KOS	Mediterranean	E. Medit.	36.888477/27.308822
24	Rhodes	ROD	Mediterranean	E. Medit.	36.319364/28.207868

PCR amplification reactions were performed in a 20 µl total-reaction volume with 10 µl of REDExtract-N-Amp PCR reaction mix (Sigma–Aldrich), 0.8 µl of each primer (10 µM), 6.4 µl of ultrapure water (Sigma–Aldrich) and 2 µl of template DNA. A single denaturing step at 94 ºC for 5 min was followed by 40 cycles (denaturation at 94 ºC for 40 s, annealing at 43 ºC for 45 s and extension at 72 ºC for 45 s) and a final extension at 72 ºC for 5 min in a S1000 dual thermal cycler (BioRad, www.bio-rad.com). The

PCR products were purified and both strands sequenced in Macrogen (www.macrogen.com) using the same primers for the sequencing reaction.

Genetic diversity analyses

All the sequences were edited in BIOEDIT (Hall, 1999) and aligned using CLUSTALW as implemented in MEGA 5 (Tamura et al., 2011). The single nucleotide mutations found were double-checked by contrasting the agreement and quality of forward and reverse sequencing chromatograms. The Nei and Gojobori (1986) procedure with the Jukes and Cantor (1969) correction implemented in MEGA 5 was used for detecting positive natural selection. Sequences of the haplotypes found have been deposited in GenBank (accession numbers from JQ745096 to JQ745256).

Number of haplotypes (N_h), haplotype diversity (H_d) and nucleotide diversity (π) were computed with DNASP v. 5.10 (Librado and Rozas, 2009). Haplotype richness was calculated with CONTRIB v. 1.02 (Petit et al., 2008) using a rarefaction size equal to the smallest sample size (n = 15) and Student's *t*-test was used for comparing its values between regions having more than two sampled locations (i.e., Eastern Atlantic and Mediterranean).

We used BAPS v. 5.2 (Bayesian Analysis of Population Structure) (Corander and Tang, 2007; Corander et al., 2008) for clustering the sampled haplotypes into monophyletic clusters of haplotypes (haplogroups). We ran five replicates for every value of the maximum number of clusters (k) up to k = 10. Haplotypes were assigned to one of the clusters by admixture analysis, performing 50 simulations from posterior haplotype frequencies. The assigned haplotype names reflect the haplogroup they belong.

Phylogeography and phylogeny

Relationships and geographical distribution of the haplotypes were

analysed in a haplotype network constructed with NETWORK v. 4.6.0.0 (http://www.fluxus-engineering.com/sharenet.htm), which implements the median-joining method, in the absence of recombination (Bandelt et al., 1999). The network was optimized using maximum parsimony criterion and the obtained loops were solved using criteria derived from coalescent theory (Templeton et al., 1987; Templeton and Sing, 1993). In order to determine the putative ancestral haplotypes, the outgroup weights based on haplotype frequency and connectivity (Castelloe and Templeton, 1994) were calculated for each haplotype using the TCS v. 1.21 program (Clement et al., 2000).

For phylogenetic analysis of the haplotypes obtained, we included a sequence of *Strongylocentrotus purpuratus* from GenBank (Acc. number NC_001453; Jacobs et al., 1988). Though the use of an outgroup sequence for rooting intraspecific genealogies has been shown to have little resolution (Crandall et al., 1994), we nevertheless used it since the resulting tree is coherent with the outgroup weights calculated using TCS. We used JMODELTEST v. 0.1.1 (Posada, 2008), based on a hierarchical series of likelihood ratio tests (Guindon and Gascuel, 2003) and the Bayesian Information Criterion (BIC), to assess the most appropriate nucleotide substitution model for our data. This condition was satisfied by the Tamura and Nei (1993) model with a gamma correction (α= 0.240) (TrN + G). This evolution model was fed into MRBAYES software v. 3.1.2 (Huelsenbeck and Ronquist, 2001) and the haplotype tree was estimated under the BIC after 1 million generations of 8 MCMC chains with a sample frequency of 100 (10,000 final trees). After verifying that stationarity had been reached, the first 2,000 trees were discarded, an independent majority-rule consensus tree was generated from the remaining (8,000 trees), and it was drawn using

MESQUITE v. 2.75 (Maddison and Maddison, 2011).

Population structure analyses

Pairwise genetic distances between populations (F_{st}) were calculated with ARLEQUIN v. 3.1 (Excoffier et al., 2005) considering the genetic distance between haplotypes, and their significances were tested by performing 40,000 permutations. The level of significance for these multiple tests was corrected by applying the B-Y false discovery rate (FRD) procedure (Benjamini and Yekutieli, 2001; Narum, 2006). Kruskal's non-metric multidimensional scaling (MDS; Cox and Cox, 1994) of F_{st} values was performed with RSTUDIO (Racine, 2012) to graphically visualise these results. In order to have a different differentiation measure based only on haplotype frequencies, Jost's (2008) *D* was calculated using SPADE (Chao and Shen, 2010). Negative values for *D* were corrected to zero. We calculated a confidence interval around the obtained values by 1,000 bootstrap replicates. We set this confidence interval, using the normal approximation, at the appropriate *P*-value following the B-Y correction as explained above. Significant differentiation was inferred when this confidence interval excluded zero.

Analyses of molecular variance (AMOVA) were performed to assess population structure, using conventional *F*-statistics (i.e. only with haplotype frequencies), and their significances were tested running 90,000 permutations in ARLEQUIN (Excoffier et al., 1992). AMOVAs were performed using different population sets in order to test the significance of population structure among regions, or among sub-basins within regions. These AMOVAs were repeated also considering genetic distances between haplotypes, in order to check the robustness of the results.

The effect of isolation by geographical distance was assessed, for the whole dataset or separately for different populations sets, by the correlation of linearized genetic distances (F_{st}/1-F_{st}) (Slatkin, 1995) with geographical distances between localities. Though ideally the oceanic current patterns should be included in the geographical distances calculation, currently we do not know of any reliable method for accurately quantifying this, so we used the shortest distance by sea on GOOGLE EARTH 6 (http://www.google.com/earth). The significance of the correlation was tested by the Mantel test procedure (Rousset, 1997), implemented in ARLEQUIN, with 20,000 permutations for each analysis.

Demographic history inference

Demographic history was inferred for the three studied regions and for each sub-basin by analysing the mismatch distributions. Populations that have recently experienced a sudden demographic growth show unimodal distributions, whereas those at demographic equilibrium show multimodal distributions (Rogers and Harpending, 1992). The expected mismatch distributions under a sudden expansion model were computed in ARLEQUIN using Monte Carlo simulations with 10,000 random samples. The sum of squared deviations (SSD) between observed and expected distributions was used as a measure of fit, and the probability of obtaining a simulated SSD greater than or equal to the expected was computed by randomisation. If this probability was >0.05, the expansion model was accepted, and its parameters θ_0, θ_1 and τ were calculated. For those populations showing large values for the final effective population size θ_1, this method does not usually converge and flawed results could be obtained. In this case, we kept the value of τ calculated by this method, which is consistently robust (Schneider

and Excoffier, 1999), and used DNASP to calculate the value of θ_0 which minimized the SSD, letting θ_1 have an arbitrary large value of 1000 (Rogers, 1995). In the case that the mismatch distribution was not unimodal, the data were fitted to a constant population size model (Watterson, 1975; Slatkin and Hudson, 1991) for graphical representation.

To estimate the approximate time of a demographic expansion (t) from coalescence methods, the relationship $\tau = 2\mu kt$ was used (Rogers and Harpending, 1992) where τ is the mode of the mismatch distribution, μ is the mutation rate per nucleotide and k is the number of nucleotides of the analysed fragment. A range of mutation rates from 1.6% to 3.5% per million years was used for the COI gene, as calculated previously for echinoids (McCartney et al., 2000; Lessios et al., 2001).

In order to add more statistical support for population expansions, Tajima's (1989) *D* test of neutrality, Fu's (1997) F_s, and Ramos-Onsins and Rozas' (2002) R_2 indices of population expansion were calculated using DNASP. The confidence limits of Tajima's *D* were obtained assuming that it follows the beta distribution (Tajima, 1989), while statistical tests and confidence intervals for F_s and R_2 were based on a coalescent simulation algorithm implemented in DNASP, with 20,000 simulations. Harpending's (1994) raggedness index *r* was calculated using ARLEQUIN and its significance was tested using parametric bootstrapping (10,000 replicates). These indices were calculated for the three regions and the six predefined sub-regions.

RESULTS

Genetic diversity

We sequenced 635 bp of the mitochondrial gene COI from 604 *Arbacia lixula* individuals from 24 localities (Fig. 1 and Table 1). We found 135 polymorphic sites (21%), with a total of 144 mutations. All differences between haplotypes were substitutions, 42 of which were non-synonymous. The Nei-Gojobori Z-test did not detect any significant positive selection ($P > 0.95$). A total of 161 haplotypes were obtained from all the sequences (Table S1). Of them, 126 (78.3%) were private haplotypes (found in only one locality) and 117 (72.7%) were represented by only one sampled individual. The number of haplotypes per locality ranged between 4 and 18. Haplotype diversity (H_d) and nucleotide diversity (π) calculated for the whole geographical range were 0.912 (±0.007 SD) and 0.00658 (±0.00026 SD), respectively (Table 1.2).

All diversity measures were remarkably uniform among localities within each East Atlantic or Mediterranean regions, but were quite different in the case of the two sampled localities in Brazil, having the smallest values in Itaipu (the westernmost and southernmost locality in our study). The haplotype richness in the Eastern Atlantic samples was higher than in the Mediterranean ($t = 3.336$, 20 d.f.; $P = 0.0033$), indicating that the Eastern Atlantic populations are more genetically diverse than their Mediterranean counterparts. The small number of samples available from Brazil prevented us from performing any diversity comparison of this area with other regions.

The analysis of haplotype relationships using BAPS clustered the sampled haplotypes into three haplogroups (henceforth named A, B & C). Haplogroup A is the most abundant in all Eastern Atlantic and

Mediterranean populations, but it is absent from Brazil, haplogroup B can be found in all three regions and haplogroup C is exclusive from Brazilian populations (Fig. 1.1).

Table 1.2. *Arbacia lixula.* Estimates of genetic diversity for all locations and regions sampled.

Locality or region	**N**	**N_h (N_{priv})**	**r_{hap}**	**H ± SD**	**π ± SD**
Itaipu	20	4 (3)	3.491	0.432 ± 0.126	0.00074 ± 0.00024
Cabo Frio	15	8 (7)	8.000	0.790 ± 0.105	0.00594 ± 0.00156
Total W. Atlantic	**35**	**11 (11)**	**5.935**	**0.605 ± 0.096**	**0.00317 ± 0.00098**
Boavista	27	15 (10)	10.172	0.920 ± 0.038	0.00358 ± 0.00067
Los Gigantes	24	12 (5)	8.698	0.851 ± 0.064	0.00389 ± 0.00092
Tenerife (East)	24	18 (10)	11.869	0.942 ± 0.040	0.00577 ± 0.00089
Faial	24	15 (7)	10.572	0.928 ± 0.039	0.00444 ± 0.00095
Pico	24	14 (5)	10.299	0.938 ± 0.028	0.00528 ± 0.00069
Total E. Atlantic	**123**	**56 (41)**	**10.924**	**0.921 ± 0.019**	**0.00461 ± 0.00040**
Torremuelle	27	14 (5)	8.638	0.826 ± 0.069	0.00480 ± 0.00065
La Herradura	26	15 (6)	9.999	0.917 ± 0.037	0.00517 ± 0.00040
Carboneras	26	15 (6)	9.750	0.905 ± 0.041	0.00451 ± 0.00051
Palos	28	12 (5)	8.031	0.860 ± 0.047	0.00530 ± 0.00062
Villajoyosa	30	16 (5)	9.596	0.894 ± 0.044	0.00542 ± 0.00058
Benidorm	29	12 (4)	7.808	0.842 ± 0.051	0.00410 ± 0.00033
Xabia	27	15 (5)	10.028	0.917 ± 0.038	0.00544 ± 0.00051
Columbretes	25	13 (7)	8.943	0.887 ± 0.045	0.00549 ± 0.00068
Tossa	29	15 (5)	8.980	0.877 ± 0.044	0.00588 ± 0.00068
Colera	25	14 (4)	9.433	0.883 ± 0.052	0.00534 ± 0.00069
Formentera	27	14 (4)	9.032	0.889 ± 0.041	0.00511 ± 0.00041
Cabrera	16	8 (3)	7.625	0.825 ± 0.076	0.00493 ± 0.00067
Scandola	21	10 (3)	8.199	0.886 ± 0.043	0.00589 ± 0.00069
Populonia	27	11 (1)	8.179	0.889 ± 0.035	0.00529 ± 0.00057
Crete	29	14 (4)	9.400	0.916 ± 0.029	0.00492 ± 0.00068
Kos	27	13 (5)	8.517	0.875 ± 0.044	0.00503 ± 0.00063
Rhodes	27	14 (7)	9.026	0.883 ± 0.045	0.00550 ± 0.00053
Total Mediterranean	**446**	**109 (94)**	**8.930**	**0.881 ± 0.010**	**0.00519 ± 0.00014**
TOTAL	604	161	9.954	0.912 ± 0.007	0.00658 ± 0.00026

N: sample size, N_h: number of haplotypes, N_{priv}: number of private haplotypes, r_{hap}: haplotype richness after rarefaction to a sample size of 15, *H*: haplotype diversity, π: nucleotide diversity, *SD*: standard deviation

Haplotype network and phylogenetic inference

The haplotype network (Fig. 1.2) showed a strikingly star-shaped topology with a high ratio of singletons (81.4 % of all haplotypes), which is typical of populations that have suffered a recent demographic expansion. The three most abundant haplotypes (A2, A17, B6) occupy central positions.

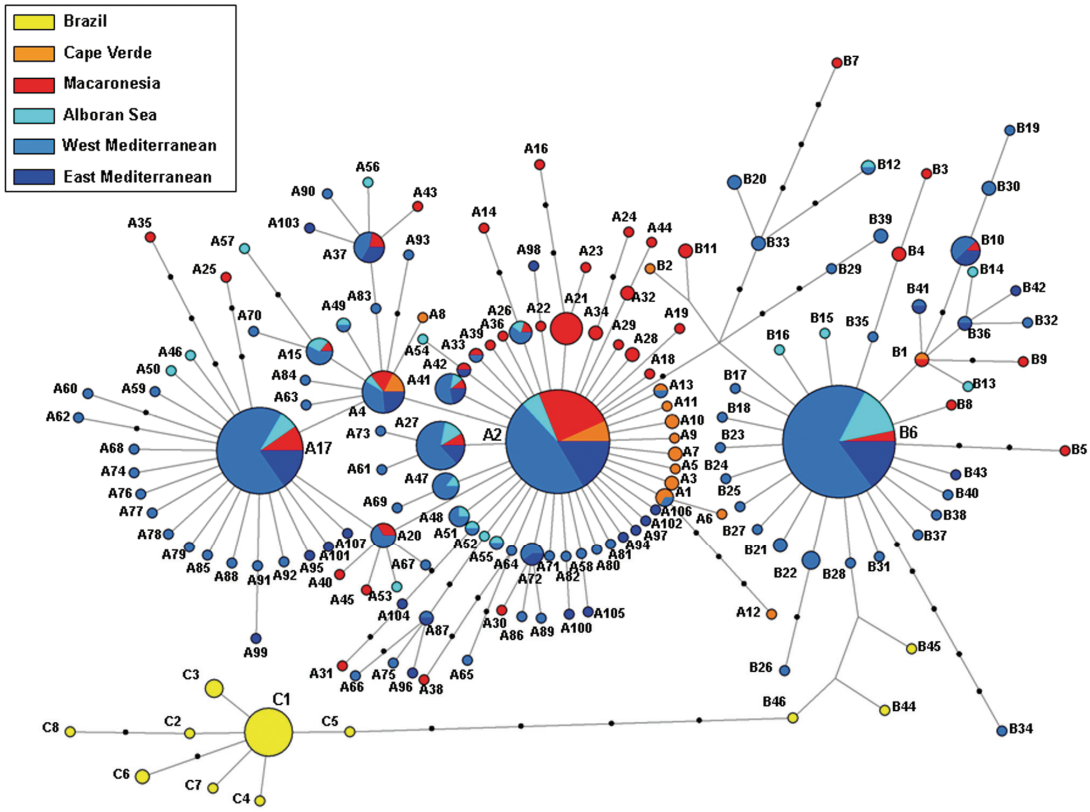

Fig. 1.2. Median-joining haplotype network for *Arbacia lixula* COI. Haplotype numbers are preceded by a letter indicating the haplogroup they belong, A, B or C. Each haplotype is depicted by a circle coloured after the sub-region where it has been sampled. Areas are proportional to haplotype frequency. Each line represents a single nucleotide substitution step and additional mutations are represented by black bullets. The four haplotypes occupying central positions in each haplogroup, A2, A17, B6 and C1 are labelled in bigger font size

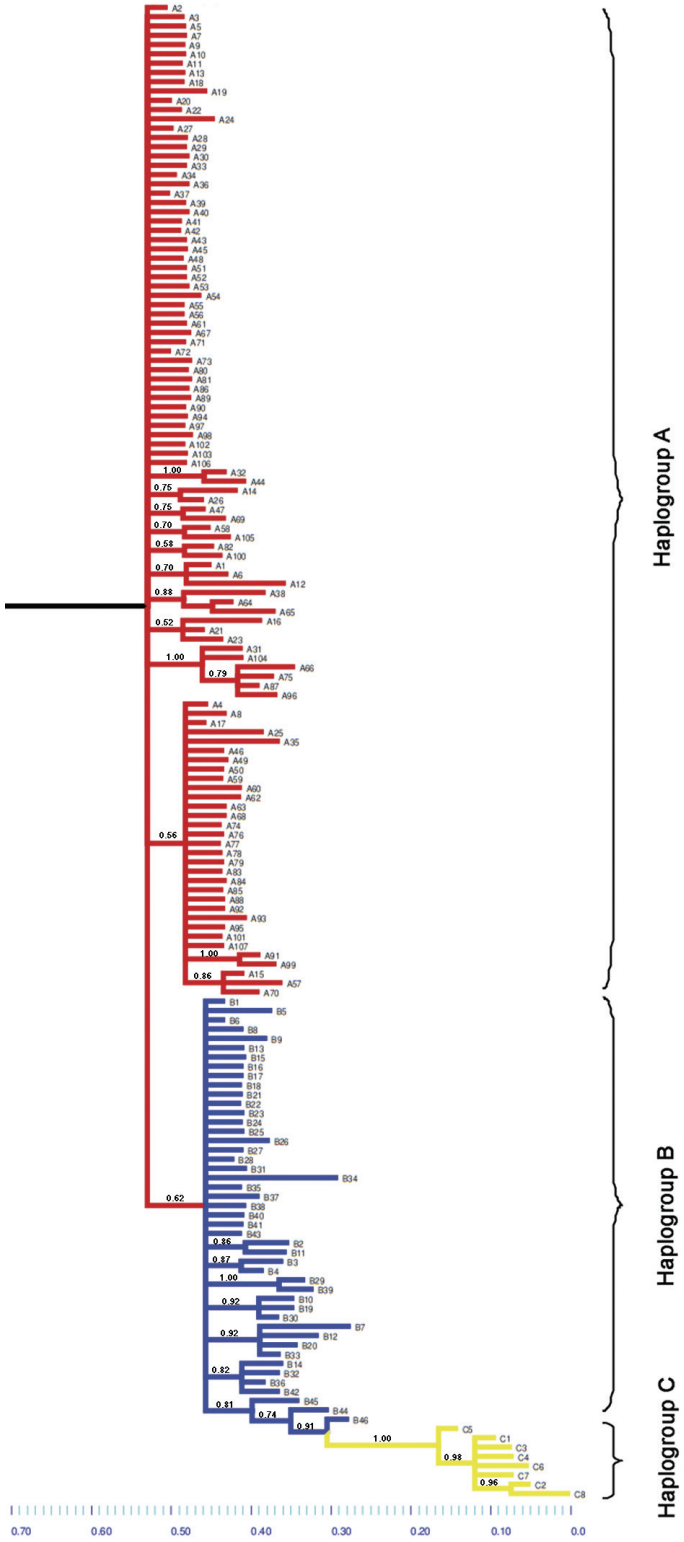

Fig. 1.3. Bayesian inference consensus tree for haplotypes of *Arbacia lixula* COI. The tree is rooted using *Strongylocentrotus purpuratus* as outgroup (not shown); values for posterior probabilities > 0.5, supporting non-collapsed clades, are indicated

All initial loops obtained by the MP criterion could be resolved using coalescent theory, except one, comprising 2 of the most frequent haplotypes (A2, A17), plus haplotypes, A4 & A20, which is therefore left unresolved in the figure. The outgroup weights calculated by the TCS program identified A2 as the ancestral haplotype (Table S1, Appendix II). This is the second most frequent haplotype and the only which is present in all localities except in the Brazilian ones. Haplotypes of groups A & B, widely shared among Eastern Atlantic and Mediterranean populations, appear close together in the network. Conversely, the Brazilian private haplogroup C is separated by six mutation steps from haplogroup B. The three haplotypes belonging to group B that are present in Brazilian populations are the most closely related to haplogroup C.

The consensus phylogenetic tree obtained by Bayesian Inference (Fig 1.3) is coherent with the topology of the haplotype network. Haplotypes belonging to haplogroup A were collapsed at the base of the phylogram, indicating that this group is paraphyletic and ancestral, in accordance with the results of the outgroup weights analysis. Haplotypes of group B form a homogenous clade from which haplogroup C derives. The collapsed comb-like shape of haplogroups A and B suggests a recent demographic expansion. Interestingly, Brazilian haplotypes B44, B45 & B46 formed a monophyletic clade with haplogroup C, supported by a PP value of 0.81. This is consistent with previous results by Lessios *et al.* (2012) which found that the samples from Brazil included in their analysis formed a clade nested within Eastern Atlantic (and Mediterranean) sequences.

Population structure

The analyses of population pairwise genetic differentiation (F_{st} and Jost's D, Table 1.3) reflected a lack of population structure within both Eastern Atlantic and Mediterranean regions, but a clear differentiation between them and a complete differentiation (no alleles shared) of both regions from the Brazilian samples. Results from F_{st} and D were largely consistent. No significant differences could be found between any pair of localities from Cape Verde and Macaronesia, suggesting a high level of genetic flow among these Eastern Atlantic sub-regions. Likewise, no significant differences were found between any pair of Mediterranean localities (out of 136 possible pairs), with the exception of Torremuelle (the westernmost Mediterranean locality) where F_{st} analysis showed significant differences with two other Mediterranean localities, though these differences were not significant when D measures were analysed. Between Eastern Atlantic and Mediterranean, however, 38 (D) and 31 (F_{st}) comparisons (out of 85) were significant. Remarkably, the localities of Carboneras (Western Mediterranean), Crete and Kos (Eastern Mediterranean) did not show any significant difference to any other Eastern Atlantic or Mediterranean population, despite the large geographical distances involved in the case of the two latter localities.

Table 1.3. Genetic differentiation between *Arbacia lixula* populations, F_{st} (below the diagonal) and Jost's *D* (above the diagonal).

	Brazil		Eastern Atlantic					Mediterranean															
	ITA	CFR	BOA	GIG	TEN	FAI	PIC	TOR	HER	CAR	PAL	VIL	BEN	XAB	CLM	TOS	COL	FOR	CAB	SCA	POP	CRE	KOS	ROD
ITA		0.115	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*
CFR	0.132*		1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*	1*
BOA	**0.876***	**0.740***		0.170	0.012	0.064	0.413	**0.671***	0.763*	0.275	**0.553***	**0.598***	**0.557***	**0.656***	0.225	**0.889***	**0.751***	**0.664***	0.510	**0.632***	**0.608***	0.181	0.270	**0.715***
GIG	**0.865***	**0.712***	0.030		0.039	0.024	0.215	0.445	0.555*	0.129	0.343	0.380	0.290	0.427	0.093	0.720*	**0.669***	0.512*	0.252	0.443	0.407	0.203	0.096	0.500*
TEN	**0.825***	**0.676***	0.015	0.002		0	0.037	**0.662***	0.499*	0.216	0.431	0.524*	**0.454***	0.453*	0.081	0.677*	0.448	0.453*	0.444	0.382	0.295	0.142	0.087	0.522*
FAI	**0.861***	**0.719***	0.007	0.022	0.000		0.038	**0.670***	0.623*	0.226	**0.482***	**0.531***	**0.500***	**0.559***	0.185	**0.772***	**0.608***	**0.544***	0.496	**0.510***	**0.495***	0.130	0.197	**0.605***
PIC	**0.832***	**0.679***	0.035	0.010	-0.006	0.013		**0.501***	0.150	0.214	0.226	0.309	0.272	0.178	0.081	0.230	0.097	0.102	0.326	0.092	0.067	0.124	0.040	0.146
TOR	**0.830***	**0.674***	**0.228***	0.136*	**0.143***	**0.222***	**0.135***		0.164	0.015	0	0	0	0.015	0.150	0.205	0.430	0.121	0	0.187	0.187	0.153	0.216	0.064
HER	**0.833***	**0.687***	0.053	0.043	0.002	0.043	0.001	0.113*		0.084	0	0	0.018	0	0.103	0	0	0	0.122	0	0	0.136	0.121	0
CAR	**0.846***	**0.697***	0.063	0.015	0.019	0.056	0.017	0.053	0.002		0	0	0	0	0	0.241	0.322	0.059	0	0.071	0.073	0	0	0.045
PAL	**0.820***	**0.670***	**0.101***	0.047	0.039	**0.092***	0.028	0.024	0.003	-0.014		0	0	0	0	0.008	0.118	0	0	0	0	0.020	0.013	0
VIL	**0.810***	**0.662***	**0.100***	0.038	0.043	**0.095***	0.036	0.015	0.013	-0.017	-0.020		0	0	0.037	0.074	0.227	0	0	0.019	0.042	0	0.110	0
BEN	**0.849***	**0.701***	**0.177***	0.090	**0.097***	**0.172***	0.083	-0.010	0.061	0.011	-0.006	-0.012		0	0	0.083	0.231	0	0	0.010	0.039	0.082	0.015	0
XAB	**0.814***	**0.659***	**0.148***	0.072	0.075	**0.142***	0.061	-0.006	0.039	0.002	-0.012	-0.020	-0.020		0.030	0	0.089	0	0	0	0	0	0.074	0
CLM	**0.822***	**0.667***	0.093*	0.057	0.027	0.084*	0.019	0.051	-0.012	-0.002	-0.019	-0.011	0.015	0.003		0.224	0.201	0.022	0	0.011	0.022	0	0	0.045
TOS	**0.805***	**0.659***	**0.123***	0.077	0.051	**0.115***	0.035	0.028	0.008	0.005	-0.018	-0.007	-0.000	-0.007	-0.021		0	0	0.174	0	0	0.269	0.194	0
COL	**0.831***	**0.682***	**0.092***	**0.084***	0.025	**0.077***	0.020	0.113*	-0.020	0.024	0.005	0.025	0.073	0.046	-0.017	0.001		0	0.338	0	0	0.236	0.175	0.009
FOR	**0.829***	**0.681***	**0.085***	0.053	0.020	**0.074***	0.013	0.065	-0.018	-0.009	-0.019	-0.006	0.024	0.010	-0.026	-0.017	-0.020		0.062	0	0	0.054	0.047	0
CAB	**0.858***	**0.676***	0.099*	0.037	0.026	0.088*	0.020	0.022	-0.001	-0.023	-0.028	-0.029	-0.015	-0.022	-0.029	-0.022	0.010	-0.023		0.051	0.093	0.069	0.001	0.003
SCA	**0.819***	**0.651***	**0.122***	0.069	0.044	**0.111***	0.026	0.037	0.002	0.002	-0.018	-0.007	0.006	-0.014	-0.022	-0.020	0.000	-0.017	-0.025		0	0.101	0	0
POP	**0.826***	**0.680***	**0.096***	0.067	0.027	**0.087***	0.018	0.063	-0.014	0.001	-0.014	0.000	0.028	0.011	-0.023	-0.021	-0.019	-0.020	-0.015	-0.017		0.105	0	0
CRE	**0.833***	**0.693***	0.037	0.016	0.003	0.030	0.004	0.088	-0.008	-0.011	0.001	0.002	0.047	0.027	-0.003	0.016	0.007	-0.005	-0.014	0.014	-0.001		0	0.105
KOS	**0.833***	**0.686***	0.064	0.025	0.013	0.058	0.005	0.053	-0.004	-0.016	-0.013	-0.008	0.013	0.007	-0.016	-0.011	0.008	-0.014	-0.029	-0.005	-0.016	-0.019		0.047
ROD	**0.817***	**0.666***	**0.100***	0.055	0.034	**0.091***	0.014	0.037	-0.004	-0.008	-0.021	-0.011	0.003	-0.009	-0.023	-0.024	-0.004	-0.023	-0.026	-0.024	-0.023	-0.000	-0.020	

Consistently significant differences obtained by both methods after false discovery rate correction are represented in bold.
Significant *P* values for F_{st} obtained from randomization. *: significant after false discovery rate correction ($P < 0.0085$).
Significant *P* values for *D* indicate that confidence interval obtained by bootstrapping excludes 0. *: significant after FDRcorrection ($P < 0.0085$)

The MDS analysis (Fig. 1.4) graphically expresses the relationships among populations obtained from F_{st} measures. Brazilian localities are widely separated in the first dimension from Eastern Atlantic and Mediterranean populations, whereas the Mediterranean and Eastern Atlantic populations were separated along the second axis. The lack of structure between sub-regions within the Eastern Atlantic and the Mediterranean is also apparent in the graphical arrangement. The same analysis using *D* measures (not shown) reflected the same overall structure.

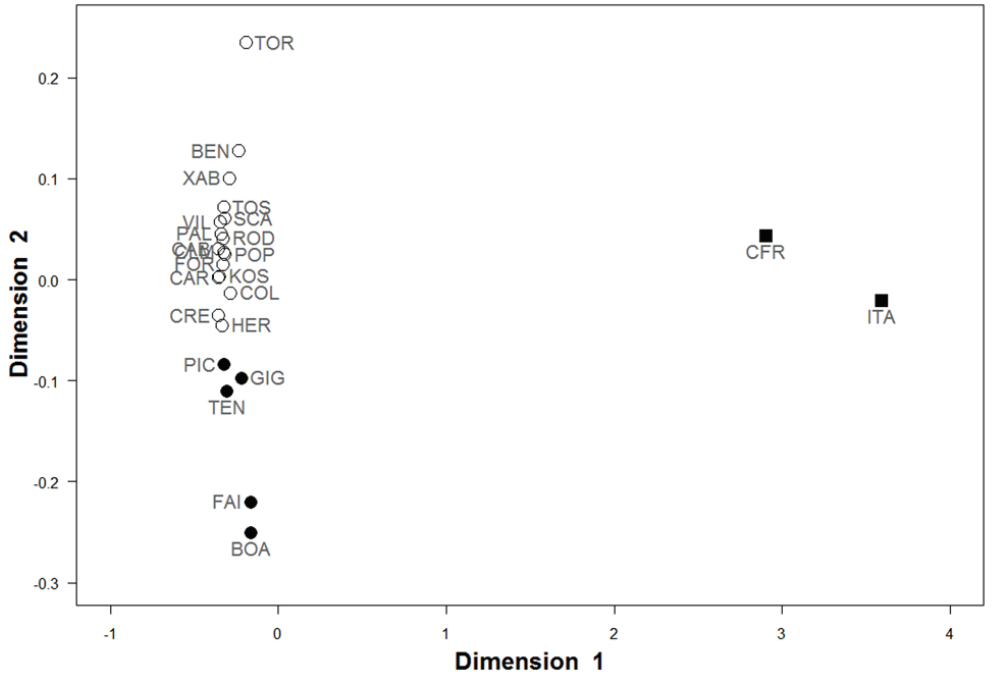

Fig. 1.4. Multidimensional scaling (MDS) for F_{st} differentiation of *Arbacia lixula* COI haplotypes. Filled squares (■) represent Brazilian populations, whereas filled circles (●) represent Eastern Atlantic populations and open circles (○) correspond to Mediterranean populations

Consistent with the pairwise differentiation analysis, the AMOVA found significant differences between the three regions (Table 1.4), which remained significant when only Eastern Atlantic *vs.* Mediterranean regions were compared (Table 1.5). Conversely, and again in agreement with the pairwise differentiation analyses, no significant differences within regions between Eastern Atlantic sub-regions (Table 1.6) or among the three Mediterranean sub-basins (Table 1.7) were detected by AMOVA. The same results were obtained when these AMOVAs were repeated considering genetic distances between haplotypes (data not shown).

Table 1.4. Analysis of molecular variance (AMOVA) among regions using COI haplotype frequencies. Brazil vs. East Atlantic vs. Mediterranean

Source of variation	***df***	***Sum of squares***	***Variance components***	***Variation %***	**P *value***	***Fixation index***
Among groups	2	12.530	0.04690	9.69	**< 0.0001*** **	0.09692
Among populations within groups	21	9.728	0.00107	0.22	0.2583	0.00245
Within populations	580	252.833	0.43592	90.09	**< 0.0001*** **	0.09913
Total	603	275.091	0.48389			

Table 1.5. Analysis of molecular variance (AMOVA) among regions using COI haplotype frequencies. East Atlantic vs. Mediterranean

Source of variation	***df***	***Sum of squares***	***Variance components***	***Variation %***	**P *value***	***Fixation index***
Between groups	1	4.104	0.01893	4.08	**< 0.0001*** **	0.04081
Among populations within groups	20	9.075	0.00035	0.08	0.3916	0.00080
Within populations	547	243.200	0.44461	95.84	**0.0002*** **	0.04157
Total	568	256.380	0.46389			

Table 1.6. Analysis of molecular variance (AMOVA) among sub-regions within Eastern Atlantic region, using COI haplotype frequencies: Macaronesia vs. Cape Verde

Source of variation	*df*	*Sum of squares*	*Variance components*	*Variation %*	P *value*	*Fixation index*
Between groups	1	0.681	0.00483	1.04	0.400	0.01042
Among populations within groups	3	1.427	0.00074	0.16	0.385	0.00161
Within populations	118	54.046	0.45802	98.80	0.179	0.01201
Total	122	56.154	0.46359			

Table 1.7. Analysis of molecular variance (AMOVA) among sub-regions within the Mediterranean, using COI haplotype frequencies: Alboran vs. Western Mediterranean vs. Eastern Mediterranean

Source of variation	*df*	*Sum of squares*	*Variance components*	*Variation %*	P *value*	*Fixation index*
Among groups	2	0.829	-0.00023	-0.05	0.495	-0.00052
Among populations within groups	14	6.138	-0.00009	-0.02	0.482	-0.00021
Within populations	429	189.154	0.44092	100.07	0.514	-0.00073
Total	445	196.121	0.44059			

The Mantel test showed significant isolation by distance when the whole dataset was analyzed (Fig 1.5A). This result remained significant when populations from Brazil were excluded (Fig 1.5B). Contrarily, no significant correlation between genetic differentiation and geographical distance was found when populations within just one region, either East Atlantic or Mediterranean, were analyzed (Fig 1.5C & 1.5D).

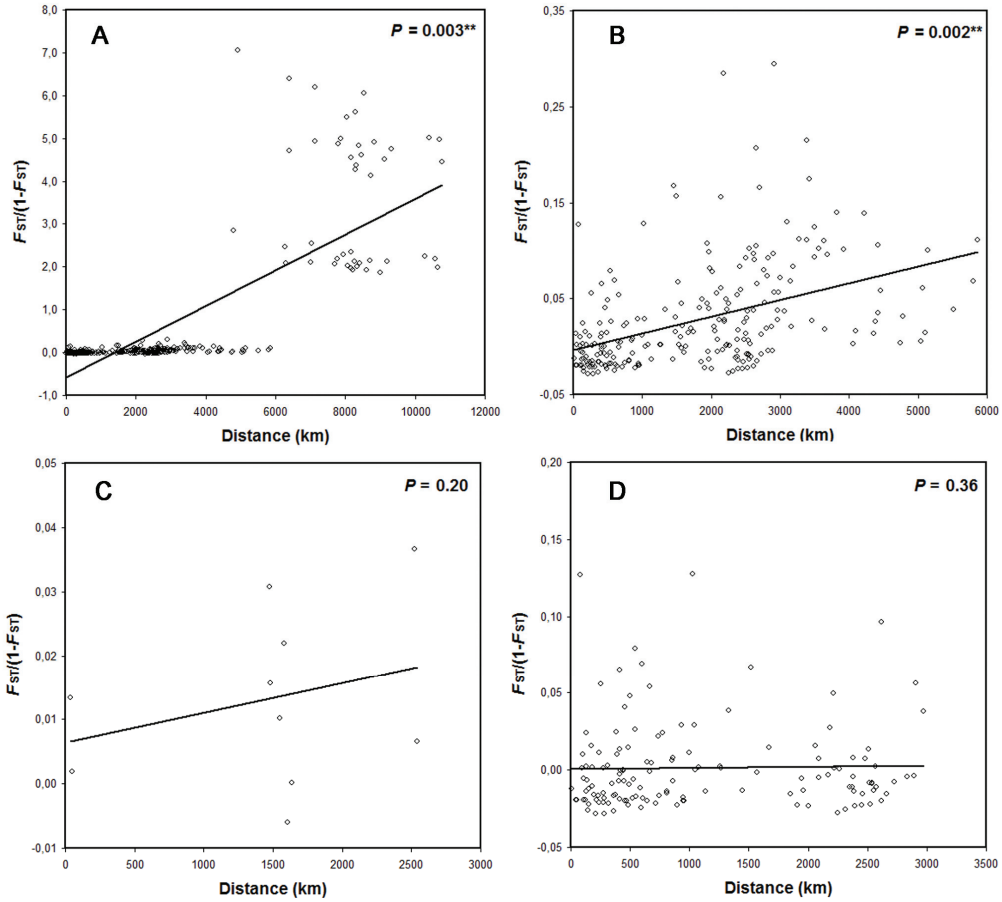

Fig. 1.5. Relationships between genetic and geographic distances for different datasets of *Arbacia lixula* populations. Results of the Mantel test for isolation by distance are indicated

Historical demography

The mismatch distribution of *Arbacia lixula* populations from the Brazilian region (Fig. 1.6A) did not fit the sudden expansion model (Table 1.8). Conversely, the mismatch distribution for the Eastern Atlantic region (Fig. 1.6B) was remarkably unimodal. This indicates that a recent demographic expansion has occurred in this population. Similar results were obtained when only the Macaronesian sub-region was analyzed (Table 1.8). However,

the distribution for the Cape Verde sub-basin did not fit the sudden expansion model, as reflected by a high SSD (Table 1.8). Nevertheless, this result may be an artefact due to small sample size (n=27). The demographic expansion in the Eastern Atlantic populations could be dated, from the value of τ and the known mutation rate for the COI of Echinoidea, between 30.6 – 66.9 kya (thousand years ago), which is a surprisingly recent time.

Table 1.8. Mismatch distribution parameters for *Arbacia lixula* populations

Region	**SSD**	**τ**	**θ_0**	**θ_1**	***Estimated expansion time (kya)***
Brazil	0.3525 **	N.A.	N.A.	N.A.	N.A.
Cape Verde	0.0265 *	N.A.	N.A.	N.A.	N.A.
Macaronesia	0.0004 ^{ns}	1.39	1.850	1000	31.3 – 68.4
Pooled East Atlantic	0.0014 ^{ns}	1.36	1.286	1000	30.6 – 66.9
Alboran Sea	0.0067 ^{ns}	4.24	0.000	12.54	95.4 – 208.7
West Mediterranean	0.0028 ^{ns}	4.20	0.000	9.75	94.5 – 206.7
East Mediterranean	0.0024 ^{ns}	3.90	0.001	10.86	87.7 – 191.9
Pooled Mediterranean	0.0026 ^{ns}	4.17	0.000	10.20	93.8 – 205.2
Whole Dataset	0.0030 ^{ns}	2.91	1.376	13.13	65.5 – 143.2

SSD values and their significances are presented along with sudden expansion model parameters and estimated time for the expansion (where applicable), for the studied regions and sub-regions and for the whole dataset. *: Significant at $P < 0.05$. **: Significant at $P < 0.01$. ^{ns}: Not significant. N.A.: Not applicable (sudden expansion model rejected)

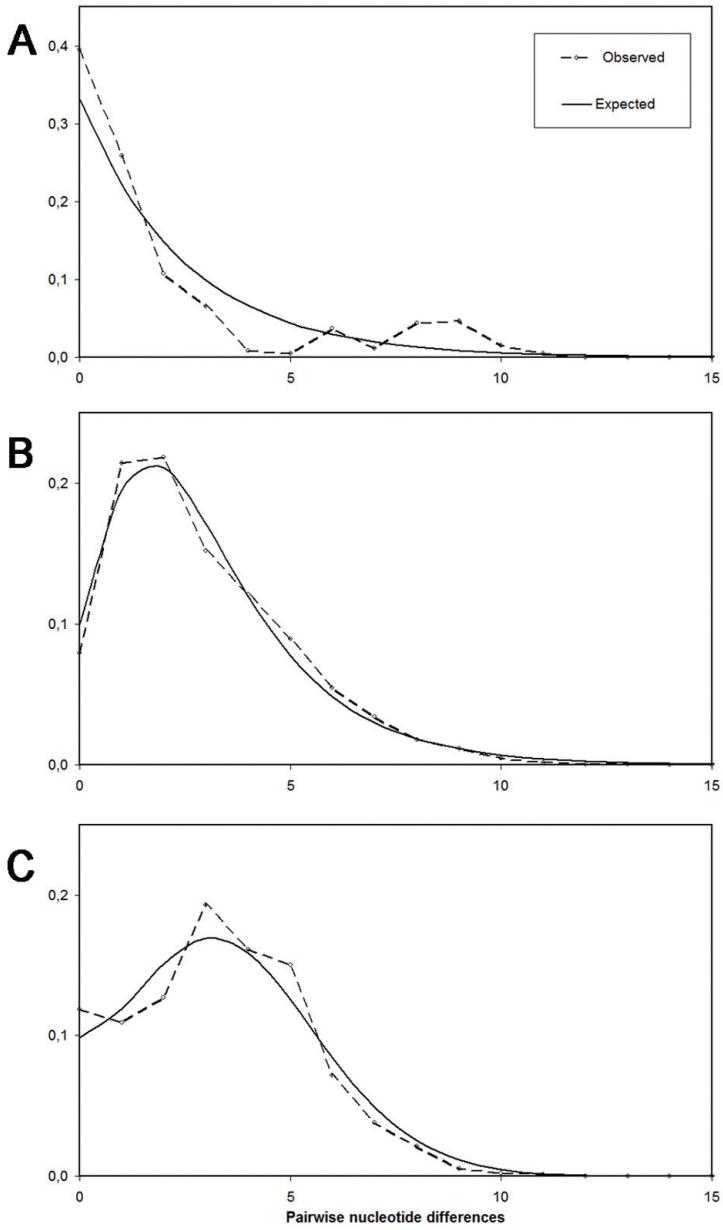

Fig. 1.6. Mismatch distributions of *Arbacia lixula* populations in the three studied regions. Observed data and theoretical expected distributions are represented by discontinuous and solid lines, respectively. For Brazil (A), the theoretical expected distribution shown is that of a population of constant size. In the case of the East Atlantic (B) and the Mediterranean (C), data were fitted to a sudden expansion model

The mismatch distribution obtained for the Mediterranean region (Fig 1.6C) was also typically unimodal. The parameters of the theoretical curves calculated individually for each Mediterranean sub-basin had all similar values, comparable to those of the whole Mediterranean region (Table 1.8), reinforcing the idea that all the Mediterranean *Arbacia lixula* populations belong to the same genetic pool. The demographic expansion in the Mediterranean could be dated between 93.8 – 205.2 kya. This estimation is a little older than that obtained for the Eastern Atlantic expansion, but is still a recent time.

Table 1.9. Neutrality and population expansion tests for *Arbacia lixula* in the studied regions or sub-regions and for the whole dataset

Region	**N**	**D**	**F_s**	**R_2**	**r**
Brazil	35	-1.80405 ns	-3.712 *	0.0566 **	0.0503 *
Cape Verde	27	-2.08319 *	-9.809 ***	0.0527 ***	0.1254 *
Macaronesia	96	-2.40571 **	-45.988 ***	0.0234 ***	0.0167 ns
Pooled East Atlantic	123	-2.51677 ***	-70.825 ***	0.0185 ***	0.0265 ns
Alboran Sea	53	-1.83549 *	-15.648 ***	0.0421 **	0.0221 ns
West Mediterranean	310	-2.25417 **	-98.101 ***	0.0187 **	0.0137 ns
East Mediterranean	83	-1.49411 ns	-17.677 ***	0.0494 ns	0.0160 ns
Pooled Mediterranean	446	-2.28043 **	-155.806 ***	0.0162 ***	0.0137 ns
Whole Dataset	604	-2.32451 **	-256.026 ***	0.0150 **	0.0094 ns

Tajima's *D*, Fu's F_s statistic, Ramos-Onsins & Rozas' statistic (R_2), and raggedness index (*r*).
*: Significant at $P < 0.05$; **: Significant at $P < 0.01$; ***: Significant at $P < 0.001$; ns: Not significant.

The neutrality and population expansion tests calculated for the different regions and sub-basins (Table 1.9) were largely coherent with the results inferred from the mismatch distributions.

Tajima's *D* detected significant differences from neutrality in all cases, except for Brazil and the Eastern Mediterranean sub-basin. Fu's F_s test for demographic expansion was significant in all cases (though just marginally so in the case of Brazil). Ramos-Onsins & Rozas' R_2 was significant for all cases except the Eastern Mediterranean sub-basin, and the raggedness value *r* was consistent with unimodal distributions, except for Brazilian and Cape Verdean populations.

DISCUSSION

COI and other mitochondrial markers have proven to be the most useful tool for tracing both intraspecific and intrageneric genealogies of many echinoid species (Lessios et al., 1999, 2001, 2003, 2012; McCartney et al., 2000; Zigler and Lessios, 2004; Calderón et al., 2008) and usually yield easily interpretable results which are consistent with those of other nuclear markers. Nevertheless, our analyses are based on a single mitochondrial marker (COI). Thus, these results must be taken with caution, and further analyses using nuclear markers would be desirable. On the other hand, previous works in Echinoidea have shown that other nuclear markers were mainly used only to confirm the evolutionary history depicted by mtDNA (Lessios et al., 2012; Zigler and Lessios, 2004) or else displayed too much diversity to produce interpretable results (Calderón et al., 2008).

The *Arbacia lixula* populations sampled showed high values of haplotype diversity and haplotype richness, but relatively low

values of nucleotide diversity. The lowest diversity was found in Brazilian populations and, specifically, in the westernmost locality (Itaipu), which is close to the distribution limit of the species and separated from the other Brazilian locality by the Cabo Frio upwelling. In contrast, the highest diversity was found in the East Atlantic, as expected if this region is the geographical origin of the species (Tortonese, 1965; Lessios et al., 2012). We detected three haplogroups in *A. lixula*. One of them (Group A) seems to be ancestral and is found only in Eastern Atlantic and Mediterranean populations, while another (Group B) is present at both sides of the Atlantic. The third one (Group C) is derived from Group B and found only in Brazil.

In a recent work, Lessios *et al.* (2012) concluded that *Arbacia lixula* split from a common ancestor with *A. punctulata* ca. 2.6 Mya, and attributed this split to the mid-Atlantic barrier, separating the western *A. punctulata* from the eastern *A. lixula*, which would later have crossed back this barrier to establish itself, as an isolated clade, in the coast of Brazil. A problem with this view is that the mid-Atlantic barrier was fully in place long before the estimated date of the split, so the separation of the two species could not be a vicariance event but a range expansion event (on the part of the lineage that would become *A. lixula*), and two crossings of the barrier are required to fully explain the present-day distribution of the species (though the second crossing could be facilitated by the South Equatorial Current system; Lumpkin and Garzoli, 2005). An alternative scenario would be that the two Atlantic species diverged in Western Atlantic, after the rise of the

Panama Isthmus isolated their ancestor from the eastern Pacific region (the possible origin of the genus *Arbacia;* Lessios *et al.,* 2012), and that *A. lixula* crossed the Atlantic ridge only once to colonize the Eastern Atlantic. Our results favour the first (Lessios') view, as the haplotypes from Brazil formed a derived monophyletic group nested within the amphi-Atlantic Group B, rather than the opposite. This indicates a derived lineage in Western Atlantic, old enough to have had time to evolve forming the haplotype Group C. A more thorough sampling of the whole range of the Western Atlantic distribution and the inclusion of more data from Western Africa, are necessary before firm evidence can be obtained about the historical whereabouts of the main lineages of *A. lixula*.

Overall, the pattern of distribution of genetic variability (as shown in F_{st}, Jost's *D*, MDS and AMOVA analyses) showed three groups of populations that differed significantly from each other (Brazilian, Eastern Atlantic and Mediterranean), while little structure could be found within these groups. It is remarkable that the F_{st} measures based on sequence distance metrics and the differentiation measure *D* based on haplotype frequencies yielded essentially the same results. This is attributable to the prevalence of close haplotypes separated by small number of mutations (hence the low nucleotide diversity in general) that are widespread among populations. Thus, haplotype genetic differences had relatively little weight and most population structure derives from haplotype frequency differences.

Another striking pattern resulting from our molecular analyses is that recent demographic phenomena have shaped the

present-day genetic structure of *Arbacia lixula* populations in the Eastern Atlantic and the Mediterranean. This does not seem to be the case of the Brazilian population but, given the small sample size, it is unclear if the resulting mismatch distribution (Fig. 1.6A) is either multimodal or L-shaped in this population. Multimodal curves are typical of populations at demographic equilibrium, but L-shaped distributions may result from very recent demographic bottlenecks (Marjoram and Donnelly, 1994). More extensive sampling would be required to get the full picture of the demographic processes that have shaped the Brazilian populations of *A. lixula*.

The lack of an exclusively Mediterranean mitochondrial lineage of *Arbacia lixula* is remarkable. Other Atlanto-Mediterranean echinoderms such as *Marthasterias glacialis* (Pérez-Portela et al., 2010), *Holothuria mammata* (Borrero-Pérez et al., 2011) or *Paracentrotus lividus* (Calderón et al., 2008; Maltagliati et al., 2010) do have lineages exclusive of the Mediterranean. These species have been probably present in the Mediterranean for several million years and their populations may have suffered several episodes of impaired gene flow during the Pleistocene glaciations. The genetic structure shown by *A. lixula* probably reflects a different demographic history from these other species.

Even if there is no phylogenetic break in the Mediterranean (as also found by Lessios *et al.*, 2012) and alleles are widely shared at both sides of the Gibraltar boundary, this barrier seems nevertheless to restrict gene flow in *Arbacia lixula*, so as to establish significant differences in terms of haplotype frequencies

between Mediterranean and Eastern Atlantic populations. The AMOVA (and pairwise comparisons) detected significant genetic differentiation between these groups of populations (Table 1.5), suggesting a reduced gene flow through the Strait of Gibraltar. Differently to what can be found in other marine organisms (Patarnello et al., 2007), the Strait itself, and not the Almeria-Oran Front (some 350 Km east of Gibraltar), is the place of the phylogeographic break, as the populations from the Alboran Sea are undistinguishable from other Mediterranean populations, but are significantly differentiated from most Atlantic populations (Fig 1.4, Tables 1.3 and 1.7). Thus, *A. lixula* does not show any genetic differentiation among populations throughout the whole Mediterranean Sea. This could be due to recurrent gene flow, but oceanographic barriers such as the Almeria-Oran Front or the Siculo-Tunisian Strait (Patarnello et al., 2007) are strong enough to maintain genetic differentiation among different sub-basins in the case of other echinoderms of similar larval dispersive capacity (Calderón et al., 2008; Maltagliati et al., 2010; Borrero-Pérez et al., 2011). We favour the alternative explanation (for the lack of genetic structure) that the colonization of the Mediterranean by *A. lixula* is so recent (see below) that populations in the different Mediterranean sub-basins have not had yet enough time to diverge from each other.

In the case of Macaronesian and Cape Verdean populations (Table 1.6), it seems likely that the present-day genetic similarity could be the result of a recent demographic expansion (see below), which could have swamped any trace of previous differentiated

lineages potentially formed during periods of restricted gene flow among archipelagos.

Brazilian populations of *Arbacia lixula* are completely differentiated from Eastern Atlantic and Mediterranean populations (Tables 1.3 & 1.4). In addition, they showed the lowest genetic diversity and did not show any signature of demographic expansion. Nevertheless, our sample size is small, and Northern and Central Brazilian populations of *A. lixula* have never been sampled for phylogeographic studies. More extensive sampling along the Brazilian coast would be required for a full understanding of factors shaping the genetic structure of the West Atlantic populations of *A. lixula*.

The almost complete lack of fossil record for *Arbacia lixula* in the Mediterranean is most revealing. At present, the species is highly abundant and occurs in areas that have been thoroughly sampled by palaeontologists. Other Mediterranean echinoids currently co-occurring in the same habitats are commonly found in assemblages of the Pleistocene and have been abundantly reported in the paleontological literature (Cuerda et al., 1986; Villalba-Currás et al., 2007; Néraudeau and Masrour, 2008; Scicchitano et al., 2011). In contrast, only one fossil individual of *A. lixula* from the Mediterranean has ever been reported in the literature (Stefanini, 1911). It was found in very young deposits from Livorno (Italy) whose recency led Stefanini (1911) to speculate that *A. lixula* had an exotic origin and had entered the Mediterranean in recent times. *A. lixula* is consistently absent from fossil assemblages of the so-called "Senegalese fauna" that characterize the warmer periods

from the Tyrrhenian stage (ca. 260 - 11.4 kya), which have been extensively sampled and thoroughly described (Cuerda, 1957; Hillaire-Marcel et al., 1986; Lillo-Carpio, 1988; Lario et al., 1993; Belluomini et al., 2002; Bardají et al., 2009).

As for the Atlantic archipelagos, recent work on the fossil echinoid fauna of Azores Islands (Madeira et al., 2011) has revealed the presence of *A. lixula*, providing several tens of pieces of individuals, including the oldest known record of this species. These deposits are currently dated to 130-120 kya (Ávila et al., 2008), which corresponds to the last interglacial or Riss-Würm (also called MIS 5e, ca. 130-114 kya). These specimens add up to the only other Atlantic *A. lixula* fossil specimen known from the Pleistocene of Madeira (Stefanini, 1911), whose dating is more uncertain.

Thus, there is scarce paleontological evidence of the occurrence of *Arbacia lixula* in the Mediterranean, and somewhat more, but still scarce, evidence of the colonization of the Atlantic archipelagos of Azores and Madeira, which probably occurred during the last interglacial period of the Pleistocene (MIS 5e). These observations are in agreement with the genetic signatures we observed in the mismatch distributions, which clearly show that recent sudden expansions have occurred in the Mediterranean and Macaronesian populations (Fig. 1.6). This is also supported by the strikingly star-shaped topologies of the haplotype network (Fig. 1.2) and by the comb-like clades in the BI phylogenetic tree (Fig. 1.3). Our temporal estimation for the demographic expansion in the Mediterranean (93.8 – 205.2 kya) is coherent with the only

available fossil record (Stefanini, 1911). This is considerably younger than the times for expansion events found in other Mediterranean echinoderms using the same estimation method, which vary from 300 to 600 kya (Calderón et al., 2008; Borrero-Pérez et al., 2011) and fits with the possibility that the colonization of the Mediterranean by *A. lixula* took place as recently as during the last interglacial period (MIS 5e). This period was also the longest of all interglacial warm periods of the Pleistocene. The minimum winter surface temperature of the Mediterranean Sea stayed warmer than 19 ºC for several thousands of years (Bardají et al., 2009). This probably enabled tropical Atlantic populations of *A. lixula* to cross the Strait of Gibraltar and colonize the Mediterranean.

In the case of Eastern Atlantic populations, the exponential demographic expansion is even more apparent, since the mismatch distribution follows a sharp unimodal curve which fits to a sudden expansion model with a very high value for θ_1. This expansion probably occurred more recently than in the Mediterranean (31.3 – 68.4 kya). This estimation falls within the Late Pleistocene, an epoch generally dominated by the last glaciation (Würm), during which the mean sea level dropped down to 80 m below the present level (Guilcher, 1969; Waelbroeck et al., 2002). Changes in ocean circulation related to this sea level drop can be related to the population expansion of *A. lixula* in the Eastern Atlantic. Contrary to what happens in the Mediterranean, the fossils available show that the species was present in Macaronesia before this expansion (Madeira et al., 2011), so the demographic history of the Atlantic

populations of *A. lixula* seems to be more complex than that of the Mediterranean populations. To complete the picture of the colonization of Atlantic archipelagos, data from continental African shores would be highly valuable.

An invasive species can be defined as a "species that threatens the diversity or abundance of native species, the ecological stability of infested ecosystems, economic activities (e.g., agricultural, aquacultural, commercial, or recreational) dependent on these ecosystems and/or human health" (Occhipinti-Ambrogi and Galil, 2004). Although the term is generally applied to species introduced as a result of human activities, it should not be necessarily so. Moreover, ecosystem engineer species such as *Arbacia lixula*, that have shaped contemporary communities as the result of a colonization event that took place many years ago, can be falsely viewed as native (Haydar, 2012). According to our molecular data, *A. lixula* has indeed colonized the Mediterranean recently and complies with the terms of the former definition, even if it is usually viewed as native because its colonization took place following natural climatic changes, without human intervention.

Whether considered as an "old natural invader" or as native, the present trend of global warming can potentially boost the negative impact of *A. lixula* in Mediterranean ecosystems, thus possibly turning a "natural" colonization into an ecological problem related (at least partially) to human intervention. The ongoing warming (Burrows et al., 2011) may facilitate population blooms of *A. lixula* in Northern Mediterranean, by releasing the constraint to larval development due to low water temperature. Warnings have

been issued about its potential population increase and the generation of barren grounds in sublittoral habitats (Gianguzza et al., 2011; Privitera et al., 2011).

Thus, genetic data are in agreement with the consideration of *Arbacia lixula* as a thermophilous species that has recently colonised the Mediterranean and whose densities may increase in the foreseeable future. Monitoring of populations seems highly recommendable as a management tool in the near future for protecting the threatened Mediterranean shallow water ecosystems.

SUPPORTING INFORMATION

Table S1. Haplotype frequencies of *Arbacia lixula* COI for all sampled localities, is included in **Appendix I**.

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CHAPTER 2 – A WOLF IN SHEEP'S CLOTHING

Arbacia lixula and *Balanus perforatus* at Tossa de Mar. Photo: **O. S. Wangensteen**

Chapter 2. A wolf in sheep's clothing: carnivory in dominant sea urchins in the Mediterranean

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ABSTRACT

Arbacia lixula and *Paracentrotus lividus* are the dominant sea urchins in the Mediterranean sublittoral, where they are key structuring species due to their grazing activity. It has been commonly accepted that competition between both species is minimized by specializing in different algal foods: *A. lixula* is considered to feed mainly on encrusting coralline algae, while *P. lividus* prefers fleshy macroalgae. We used stable isotope analysis to test if these species occupy different trophic positions at three western Mediterranean and one Macaronesian locations. Our results unambiguously show that *A. lixula* always occupies a higher trophic level than *P. lividus*, with a $\delta^{15}N$ comparable in some locations to strict carnivores such as *Actinia schmidti* or *Marthasterias glacialis*. A temporal monitoring at one locality showed that this signature of a higher trophic level is consistent throughout the year. These results are incompatible with the current belief of an herbivorous diet for *A. lixula* and suggest that it must be considered an omnivore tending to carnivory in Mediterranean ecosystems, feeding at least partially on sessile animals such as Cirripedia, Hydrozoa or Bryozoa. A parallel analysis of gut contents showed a predominance of vegetal items in both species, although *A. lixula* consistently had a higher abundance of animal components than *P. lividus*. Our results challenge the validity of using gut content observations alone for characterizing the trophic behaviour of omnivorous marine invertebrates that feed on a variety of food sources with different digestibility.

KEY-WORDS

Arbacia lixula, *Paracentrotus lividus*, trophic relationships, benthic community, stable isotope analysis.

RESUMEN

Arbacia lixula y *Paracentrotus lividus* son los erizos de mar dominantes en la comunidad sublitoral mediterránea, en la cual son especies estructurales clave debido a su actividad ramoneadora. Tradicionalmente, se ha pensado que la competencia entre ambas especies es mínima, debido a su especialización trófica en diferentes tipos de algas: se considera que *A. lixula* se alimenta principalmente de algas coralináceas incrustantes, mientras que *P. lividus* prefiere macroalgas blandas. En el presente trabajo, hemos utilizado el análisis de isótopos estables para probar si estas especies ocupan diferentes posiciones tróficas en tres localidades del Mediterráneo Occidental y una de la Macaronesia. Nuestros resultados muestran claramente que *A. lixula* ocupa siempre un nivel trófico superior al de *P. lividus*, con un $\delta^{15}N$ comparable en algunas localidades al de carnívoros estrictos como *Actinia schmidti* o *Marthasterias glacialis*. Un seguimiento temporal en una de las localidades mostró que este resultado de un nivel trófico superior es constante a lo largo de todo el año. Estos resultados son incompatibles con la creencia actual de una dieta herbívora para *A. lixula* y sugieren que se debe considerar como un omnívoro que tiende a la carnivoría en los ecosistemas mediterráneos, alimentándose, al menos parcialmente, de animales sésiles, tales como Cirripedia, Hydrozoa o Bryozoa. Un análisis paralelo del contenido digestivo mostró un

predominio de elementos vegetales en ambas especies, aunque *A. lixula* mostraba consistentemente una mayor abundancia de componentes animales que *P. lividus*. Nuestros resultados cuestionan la validez del uso de la observación de contenidos digestivos como técnica única para caracterizar el comportamiento trófico de los invertebrados marinos omnívoros que utilizan distintas fuentes de alimentos con diferente digestibilidad.

PALABRAS CLAVE

Arbacia lixula, *Paracentrotus lividus*, relaciones tróficas, comunidad bentónica, análisis de isótopos estables

INTRODUCTION

The edible common sea urchin *Paracentrotus lividus* (Lamarck, 1816) and the black sea urchin *Arbacia lixula* (Linnaeus, 1758) are the two dominant echinoid species in shallow rocky bottoms in the Mediterranean, where they coexist (Palacín et al., 1998b; Benedetti-Cecchi et al., 1998). Their grazing activity is commonly considered to greatly influence benthic communities (Palacín et al., 1998a; Sala et al., 1998a; Bulleri et al., 1999). Their coexistence has raised questions regarding how these two abundant species interact and, specifically, whether and how they partition resources (Bulleri et al., 1999; Chiantore et al., 2008; Privitera et al., 2008). The currently prevalent view is that they are competitors for algal foods, although such putative competition seems alleviated by a selective preference of *P. lividus* for erect seaweeds, while *A. lixula* tends to feed more on encrusting coralline algae (Kempf, 1962; Régis, 1978; Verlaque and Nédélec, 1983; Frantzis et al., 1988; Bulleri et al., 1999; Boudouresque and Verlaque, 2001; Privitera et al., 2008).

This herbivorous behaviour described in *A. lixula* is, however, in sharp contrast with other species in the genus *Arbacia,* where omnivorous or unambiguously carnivorous diets have been reported. North American *A. punctulata* feeds on sessile animals, sand dollars and other *Arbacia* individuals, besides some algae (Harvey, 1956; Karlson, 1978; Cobb and Lawrence, 2005). The diet of South Atlantic *A. dufresnii* is mainly carnivorous (Penchaszadeh, 1979; Penchaszadeh and Lawrence, 1999). The Pacific *A. spatuligera* showed preference for animal food over common

species of algae from its habitat (Silva et al., 2004). Moreover, some observations indicate omnivorous or carnivorous behaviour of *A. lixula* outside the Mediterranean (Marques, 1984; Oliveira, 1991; Tavares and Borzone, 2005).

The crucial importance of sea urchins in shaping benthic ecosystems (Lawrence, 1975) has been demonstrated by many ecological experiments along the Mediterranean coasts (e.g.: Benedetti-Cecchi and Cinelli, 1995; Sala and Zabala, 1996; Benedetti-Cecchi et al., 1998; Palacín et al., 1998a; Bulleri et al., 1999; Guidetti et al., 2004; Bonaviri et al., 2011). The underlying premise in these experiments is that sea urchins are predominantly herbivorous and that their effects are mainly due to their grazing on benthic algae. In particular, population outbreaks of both *A. lixula* and *P. lividus* are able to create barrens in rocky substrates (Verlaque, 1987; Hereu, 2004), affecting both productivity and diversity of benthic assemblages (Bulleri et al., 2002; Privitera et al., 2008). The feeding behaviour and the herbivorous nature of *P. lividus* have been repeatedly assessed; however, much less information is available about the ecological role played by *A. lixula* in Mediterranean ecosystems. In fact, Privitera et al. (2008) demonstrated that both species occupy different trophic niches in resource-limited (barren) areas, again in the sense that *A. lixula* fed mainly on encrusting corallines while *P. lividus* fed on non-encrusting macrophytes. A knowledge gap about the effective diet of *A. lixula,* essential for designing and interpreting ecological studies, still persists. Filling this gap seems necessary not only for

basic research, but also for management purposes (e.g. marine reserves or local fisheries).

We used a combination of stable isotope analysis and gut content examination for assessing the diet and establishing the trophic position of *A. lixula* and *P. lividus* coexisting in western Mediterranean rocky bottoms.

MATERIALS AND METHODS

Study sites and sampling procedures

Gut contents and isotopic signatures of both sea urchin species were explored both temporally, performing a year-round follow-up at a single site, and spatially, sampling at two additional western Mediterranean sites at a single time point. We sampled also a non-Mediterranean site for reference information. This design aimed at establishing the robustness of the patterns found.

The temporal sampling was performed at Tossa de Mar (NE Spain, 41º 43.2' N, 2º 56.4' E, Fig. 2.1) from December 2008 to December 2009. This location is fully described elsewhere (Ballesteros, 1988, 1989, 1992, 1993) and is characterized by gently sloping rocks extending from the surface to 12 m depth, which show a rich algal cover, being almost devoid of barren zones. We sampled between 2 and 6 m depth, where the dominant communities are the *Corallina elongata* community (Ballesteros, 1988) at zones with high hydrodynamism and the *Stypocaulon scoparium* community (Ballesteros, 1993) at zones with a moderate to low hydrodynamism. Sea urchin densities during the sampling

period were 0.6 ± 0.8 and 5.7 ± 4.7 adult individuals (±SD) m^{-2}, for *A. lixula* and *P. lividus* respectively, measured at a depth of 3 m following the transect method as in Turon et al. (1995).

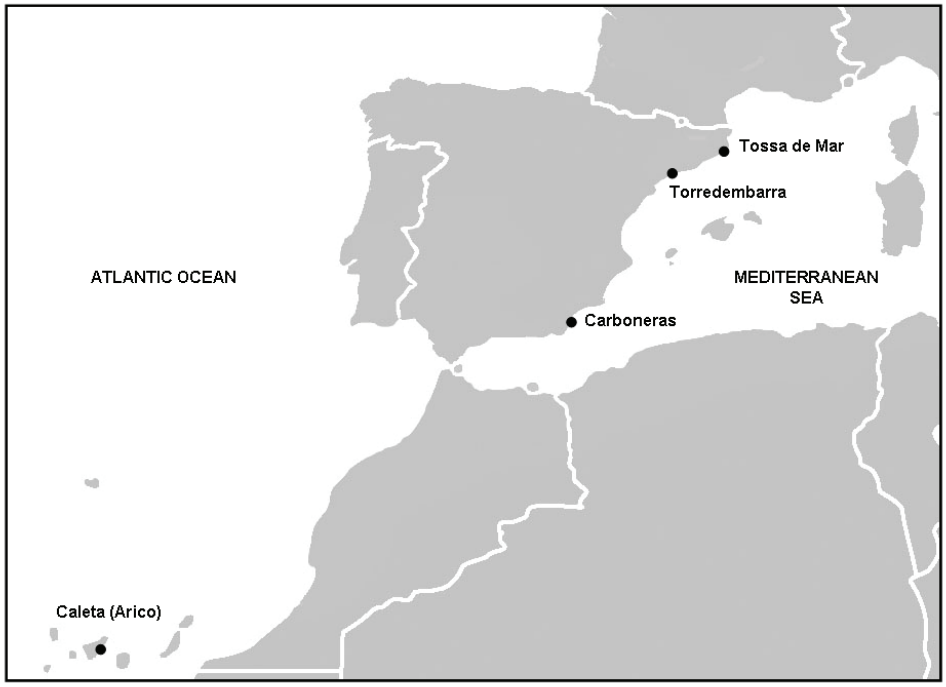

Fig. 2.1. Sampling locations

Ten *A. lixula* and ten *P. lividus* individuals were collected bimonthly by scuba diving. Only adults with test diameter >35 mm in *A. lixula* and >40 mm in *P. lividus* were sampled. The bimonthly sampling periodicity seems adequate to detect possible diet shifts (Tieszen et al., 1983; Hobson and Clark, 1992). Samples of the dominant taxa from the three macroalgal divisions (*Stypocaulon scoparium*, *Dictyota dichotoma* and *Padina pavonica:* Phaeophyta; *Codium vermilara* and *Flabellia petiolata*: Chorophyta and *Corallina elongata, Sphaerococcus coronopifolius, Peyssonnelia*

sp. and *Lithophyllum incrustans*: Rhodophyta) were collected at the same times. In addition, other invertebrates were also sampled throughout the year, including herbivores (*Patella* sp., *Amphitoe* sp.), detritivores (*Ophiothrix fragilis, Echinaster sepositus*), suspension feeders (*Balanus* spp.) and carnivores (*Actinia schmidti, Marthasterias glacialis, Ophioderma longicauda*), in order to characterize the different levels of the local trophic web. All samples were frozen (-20 ºC) shortly after collection for later analysis.

Additional sampling was carried out at two different locations (distant ca. 200 and 900 km from the previous one) in December 2009, in order to examine the consistency of the results. Although densities were not quantified, both sea urchin species were present at these localities (again with dominance of *P. lividus*) with largely overlapping depth distributions. These sampling points were Torredembarra, (NE Spain, 41º 7.9' N, 1º 23.7' E) and Carboneras (SE Spain, 36º 59.6' N, 1º 53.4' W) (Fig. 1). The location at Torredembarra is characterized by a shallow rocky habitat (0 - 3 m), surrounded by a sandy bottom. The macroalgal assemblages are poorly developed, and the main primary producer is *Jania rubens,* with scarce presence of other algae such as *Corallina elongata* or *Dictyota dichotoma*. The Carboneras site is a shallow rocky habitat (0 - 4 m) with a denser algal cover, where the dominant producers were *Jania rubens*, *Stypocaulon scoparium* and *Peyssonnelia* sp., with a well-developed *Posidonia oceanica* meadow located nearby. At these two sites, samples were obtained only of the two echinoids and of representative algal species, following the same procedures

as above. Thus, three communities with quite different characteristics were sampled in this study, accounting for some of the diversity of Mediterranean shallow habitats were *A. lixula* and *P. lividus* can coexist.

Finally, samples of sea urchins (of the same sizes detailed above) for stable isotope analysis were collected in November 2009 at one Atlantic site: Caleta (Arico, SE Tenerife, Canary Islands, 28º 6.1' N, 16º 28.7' W, Fig. 1), between 0 and 3 m depth. In this location rock boulders dominate at shallow depths, with a poorly developed algal community including sparse patches of *Caulerpa webbiana* and *Lobophora variegata.* While *A. lixula* and *P. lividus* are known to broadly share spatial niches at the Canary Islands (Tuya et al., 2007), in this locality, however, the former was only found in vertical walls, while the second was located under the stones at the bottom.

Stable isotope analyses

Muscles of the Aristotle's lantern of all collected sea urchins were used to perform isotopic analyses, and some of the same individuals were used for gut analyses (see below). Algae were sampled by slicing several pieces of different parts of the thalli after carefully scraping epibionts off their surface. For faunal specimens, we sliced a small portion of a specified part of the body: the foot for *Patella*, an arm for Ophiuroidea and Asteroidea and the body column for *Actinia*, while the whole body of amphipods and cirripeds (excluding the shell) was used.

Before isotopic analyses, samples were rinsed in distilled water, freeze-dried and ground to a fine powder. Isotopically lighter lipids may influence carbon isotope ratios in animal tissues (Attwood and Peterson, 1989; Hobson and Welch, 1992), so five samples of each species were reanalysed after lipid removal by chloroform-methanol 2:1 extraction (Folch et al., 1957). (Passing and Bablok, 1983) regression did not show any significant differences in the $\delta^{13}C$ for any species (data not shown), probably due to low lipid content in the sampled tissues. Thus, values of untreated samples were used thereinafter. Carbonate rich samples (Corallinaceae, *Padina pavonica,* Ophiuroidea, Asteroidea) were rinsed several times with HCl 0.1 M to remove inorganic carbonates (Tomas et al., 2006). As HCl treatment has been reported to alter the $\delta^{15}N$ values (Bunn et al., 1995), samples including calcareous elements were split into two subsamples, one analysed after acid treatment for $\delta^{13}C$ and the other, untreated, for $\delta^{15}N$.

Aliquots of 0.3 or 1 mg of dried powder from faunal or algal samples, respectively, were placed into tin capsules and crimped for combustion. Samples were oxidised in a Flash EA1112 furnace coupled to a Delta C stable isotope mass spectrometer through a Conflo III interface (Thermo-Finnigan). Isotope ratios are expressed as δ values in parts per thousand (‰) according to the equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X (‰) is ^{13}C or ^{15}N, and R is the ratio of corresponding element ($^{13}C/^{12}C$ or $^{15}N/^{14}N$), in sample or standard. The standard values were Pee Dee Belemnite for ^{13}C and atmospheric nitrogen for ^{15}N. IAEA standards were inserted every 12 samples for calibration. Replicate assays of standards

indicated measurement errors of ± 0.1 ‰ and ± 0.2 ‰ for carbon and nitrogen, respectively.

Trophic levels were calculated according to the equation of Hobson and Welch (1992): $TL = 1 + (N_m - N_b) / TE$. Where TL is the trophic level of the species, N_m is the mean $\delta^{15}N$ value of the species, N_b is the average basis $\delta^{15}N$ value of producers (baseline) and TE is the trophic enrichment factor in the ecosystem. A constant TE factor of 3 ‰, commonly accepted for aquatic benthic ecosystems involving invertebrates (Vander Zanden and Rasmussen, 2001; Jaschinski et al., 2008; Wan et al., 2010), was used. The baseline for $\delta^{15}N$ was estimated averaging the values obtained for the different algal species analysed, except in the Atlantic site, where we did not collect algae. In this case, we assigned *P. lividus* a value of TL = 2 (strict herbivore) and used it as a baseline for calculating the TL of *A. lixula*.

Gut content analyses

The gut contents of sea urchins of both species collected at Tossa de Mar in June and December, or at the other two Mediterranean locations in December, were analyzed. Sea urchins (from 5 to 10 individuals per species and month or locality) were dissected and the total gut contents of each specimen were examined under a binocular microscope after disaggregation of the pellets. Some small calcareous remnants were collected and examined under a scanning electron microscope. Algal fragments were identified to genus level, while faunal items were classified into the following taxonomic groups: Foraminifera, Porifera,

Hydrozoa, Polychaeta, Gastropoda, Bivalvia, Bryozoa, Cirripedia, Ostracoda, Copepoda, Amphipoda/Isopoda, Decapoda and Other. Echinoid fragments, which were present in the gut of some specimens, were not included in the analysis, since we cannot assure that they were not an artefact resulting from sample manipulation.

The frequency of occurrence of each food item in a species (FO_i) was calculated as the fraction of individuals having ingested this item (Pillay, 1952; Hyslop, 1980). The volumetric occupation of ingested items was assessed by quantifying 25 squares of a Petri dish with a 5 mm-grid. The surface occupied by the items present in each square was semiquantitatively estimated using a scale from 1 to 5, and the occupation indices of all items were calculated for every sea urchin individual, dividing the sum of the semiquantitative scores assigned to a given food item by the total sum of the scores for all measured squares. The volumetric index of each food item in a species (V_i) was then obtained as the mean value of all individuals. A feeding index (FI_i) reflecting the relative importance of each food item in the diet of each species at a given location, was calculated following Lauzane (1975), as $FI_i = FO_i \times V_i$ and then standardized as a percentage of the sum of the feeding indices for all items.

The relative contribution of animal and vegetal matter in the gut contents was quantified by addition of the standardized feeding indices of all items of either animal or vegetal origin (cumulative feeding indices). These indices summarize the carnivorous or herbivorous character of the diet, as inferred from gut contents.

Statistical analyses

Two-way ANOVA with species as fixed factor and month as random factor was performed to assess temporal variation of isotopic signatures of both sea urchin species at Tossa de Mar throughout the year. Two-way ANOVA with species as fixed factor and locality as random factor was used to compare the signatures and the calculated trophic levels of both sea urchin species at the sampled localities. We also used two-way ANOVA with species (fixed) and locality (random) as factors to formally analyse differences in the cumulative animal feeding indices. As in mixed models the expected mean square for the fixed factor (species in our case) includes the variance component for the interaction term, the fixed factor tests for the effect of species over and above the variation due to the interaction and the residual. It is therefore interpretable even in the presence of significant interaction (Quinn and Keough, 2002). Notwithstanding, when interaction was significant we performed separate *t*-tests with Bonferroni correction (unbalanced data prevented us from using other multiple comparison tests) at each locality to check that the effect was coherent across sites.

The assumptions of normality and homoscedasticity of the variables were checked with the Kolmogorov-Smirnov and Cochran tests, respectively. In some cases the data did not comply with these assumptions, and rank transformed data were used instead (detailed in Results). In two instances this transformation did not solve the lack of homoscedasticity (detailed in Results), but we performed the analysis anyway as the rank transformation is robust to deviations

from assumptions of parametric procedures (Conover and Iman, 1981; Potvin and Roff, 1993). All analyses were performed with STATISTICA 6.1 software.

RESULTS

Stable isotope analyses.

At Tossa de Mar, the annual average of $\delta^{15}N$ values found for *A. lixula* (8.2 ‰) was comparable to those of typical carnivores such as *Actinia schmidti* or *Marthasterias glacialis* (Table 2.1, Fig. 2.2). In contrast, herbivorous grazers and detritivores had lower $\delta^{15}N$ values comprised between 4.6 ‰ for the amphipod *Amphitoe* sp. and 5.3 ‰ for *Echinaster sepositus*, while *P. lividus* showed a slightly higher value of 5.9 ‰, possibly indicating a higher intake of animal items than its more strictly herbivorous counterparts. Seaweeds, as expected, showed lower $\delta^{15}N$ values, ranging from 1.9 ‰ for *Flabellia petiolata* to 4.2 ‰ for *Sphaerococcus coronopifolius*, while the most abundant species were within the range of 3 to 3.5 ‰. The mean value for all algae, used as the baseline for calculating consumers' trophic levels at Tossa de Mar, was 3.13 ‰.

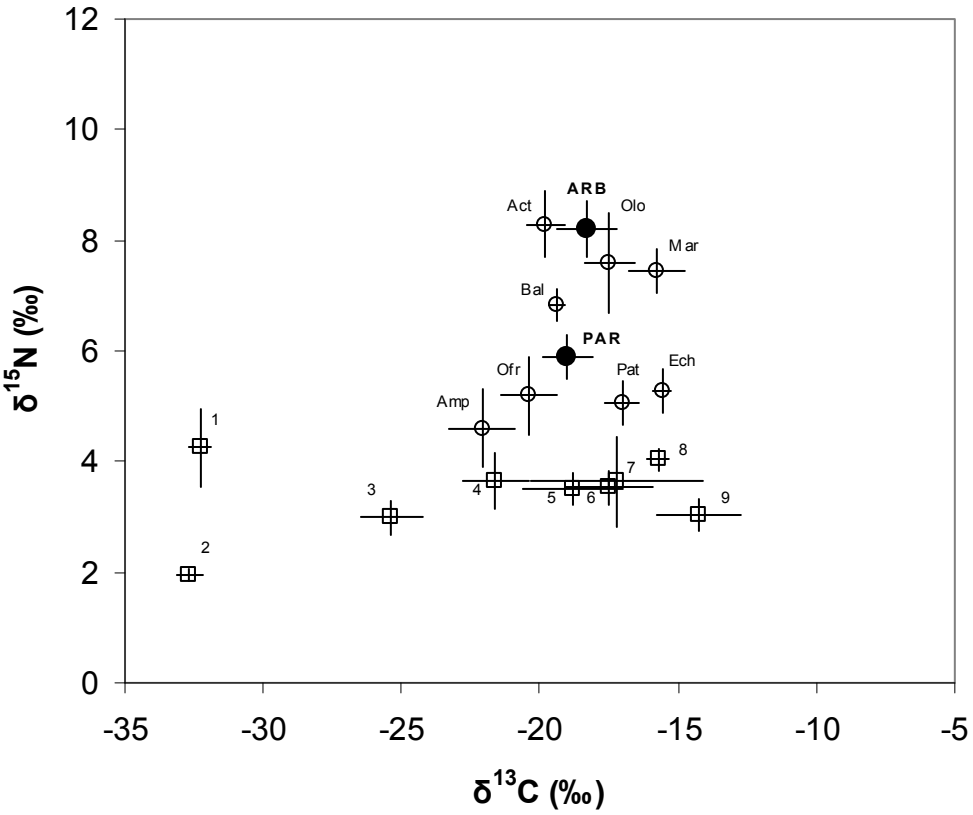

Fig. 2.2. Plot of $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures (mean ± SD) of species examined during the bimonthly sampling (Tossa de Mar). Producers are represented by open squares, whereas consumers are represented by open circles. Both sea urchin species are represented by solid circles

Metazoa: Act: *Actinia schmidti*, ARB: *Arbacia lixula*, Olo: *Ophioderma longicauda*, Mar: *Marthasterias glacialis*, Bal: *Balanus* spp., PAR: *Paracentrotus lividus*, Ech: *Echinaster sepositus*, Ofr: *Ophiothrix fragilis*, Pat: *Patella* sp., Amp: *Amphitoe* sp.

Algae: 1: *Sphaerococcus coronopifolius*, 2: *Flabellia petiolata,* 3: *Stypocaulon scoparius*, 4: *Corallina elongata*, 5: *Dictyota dichotoma*, 6: *Codium vermilara*, 7: *Peyssonnelia* spp. 8: *Lithophyllum incrustans*, 9: *Padina pavonica*

Table 2.1. Mean isotopic signatures (SD in parentheses) and derived trophic levels (TL=1 for primary producers) of animal species at the site where sampling was performed bimonthly (Tossa de Mar). n: number of individuals analysed

	n	***Trophic Level***	***$\delta^{15}N$ ‰***	***$\delta^{13}C$ ‰***
Actinia schmidti	5	2.7	8.3 (0.6)	-19.8 (0.7)
Arbacia lixula	**72**	**2.7**	**8.2 (0.5)**	**-18.3 (1.1)**
Ophioderma longicauda	5	2.5	7.6 (0.9)	-17.5 (0.9)
Marthasterias glacialis	5	2.4	7.5 (0.4)	-15.8 (1.0)
Balanus spp.	5	2.2	6.8 (0.3)	-19.4 (0.3)
Paracentrotus lividus	**71**	**1.9**	**5.9 (0.4)**	**-19.0 (0.9)**
Echinaster sepositus	5	1.7	5.3 (0.4)	-15.6 (0.3)
Ophiothrix fragilis	4	1.7	5.2 (0.7)	-20.4 (1.0)
Patella sp.	5	1.6	5.1 (0.4)	-17.0 (0.6)
Amphitoe sp.	4	1.5	4.6 (0.7)	-22.1 (1.2)

When analysed on a temporal basis (Fig. 2.3), $\delta^{15}N$ values in *A. lixula* were significantly higher than in *P. lividus* (species factor, $p < 0.001$, Table 2.2), while time and the interaction were not significant (Table 2.2), indicating that the difference in trophic levels is not subject to temporal variation. The mean difference was 2.3 ‰. Likewise, $\delta^{13}C$ values showed a high degree of individual variability (Fig. 2.3), but overall they were also significantly higher

(by ca. 0.7 ‰) for *A. lixula* than for *P. lividus* (species factor, $p < 0.001$, Table 2.2, Fig. 2.3), suggesting again a higher trophic level for *A. lixula* (the trophic enrichment factor for carbon in marine coastal trophic webs is ca. 0.8 ‰ according to France and Peters, 1997). No clear temporal trend was apparent for $\delta^{13}C$ values (time and interaction not significant, Table 2.2).

Table 2.2. Summary of factorial 2-way ANOVA for assessing significant differences in the isotopic signatures at Tossa de Mar between species (*A. lixula* and *P. lividus*, fixed factor) and sampling times (random factor)

Variable	***Effect***	***Df***	***MS***	**F**	**p-*level***
$\delta^{15}N$	Species (S)	1	187.663	493.66	**<0.001**
	Time (T)	6	0.632	1.66	0.28
	S x T	6	0.381	2.00	0.07
	Error	129	0.190		
$\delta^{13}C$ [a]	Species (S)	1	30481.07	15.82	**<0.001**
	Time (T)	6	6005.13	3.11	0.10
	S x T	6	1929.10	1.50	0.18
	Error	129	1282.21		

[a] Rank-transformed. No homogeneity of variance achieved.

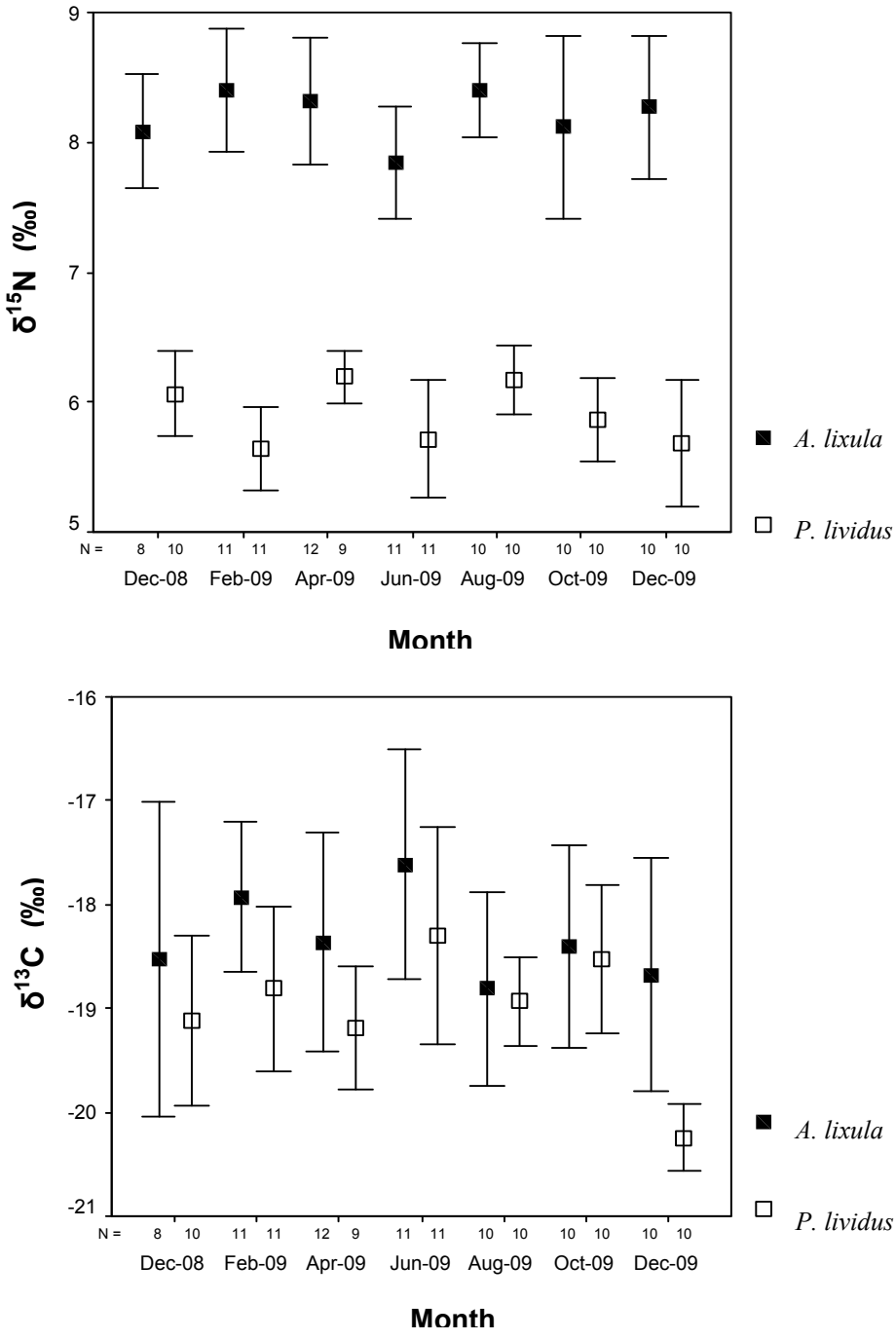

Fig. 2.3. Isotopic signatures of *A. lixula* and *P. lividus* at Tossa de Mar as a function of time. Mean values ± SD are displayed for every sampled month. The number of individuals analysed for each observation was from 8 to 12

In the two additional Mediterranean locations, as well as in the Atlantic one, $\delta^{15}N$ values obtained from *A. lixula*'s muscle exceeded those from *P. lividus*, as did the estimated trophic levels (Table 2.3). This suggests that the tendency to a more omnivorous/carnivorous diet of *A. lixula* is probably widespread through all its distribution range. A two-way ANOVA of $\delta^{15}N$ values revealed a significant effect of species (fixed) and locality (random), as well as a significant interaction term (Table 2.4), suggesting different adaptations of sea urchin species in different conditions (see below). Separate analyses (*t*-tests with Bonferroni correction) at fixed levels of the locality factor revealed that the differences in $\delta^{15}N$ values between sea urchin species were significant (all $p < 0.01$) at all sites. Likewise, the analysis of trophic level between species and localities revealed a significant effect of species and a significant interaction between species and locality (Table 2.4); the inter-specific differences being again significant in all localities (*t*-tests with Bonferroni correction, all $p < 0.01$). The significant interaction term is probably due to the plasticity that can be observed in the derived trophic levels of both species among the different localities and ecosystems. Thus, where algal cover was dense, as happened in Tossa de Mar and Carboneras, *P. lividus* showed a trophic level of around 2, compatible with a mainly herbivorous diet, whereas *A. lixula* showed trophic levels of around 2.7, corresponding to a predominantly carnivorous omnivore. On the contrary, where algal resources were scarce (as in Torredembarra) both sea urchins tended to increase their animal intake, rising their $\delta^{15}N$ values and trophic

levels. Our results showed a trophic level of 2.7 for *P. lividus* at Torredembarra, whereas *A. lixula* had a level of 3.0 (which would correspond to a strict carnivore) in this location. In Tenerife, as algal samples were not available for isotopic analysis, a baseline value for producers cannot be used, but the difference between $\delta^{15}N$ values of both sea urchins was the biggest of all locations sampled, and corresponded exactly to one trophic level.

Table 2.3. Calculated trophic levels and ^{15}N signatures for *A. lixula*, *P. lividus* and the mean value for seaweeds (baseline) at the four sampled locations (SD in parentheses). ^{13}C signatures are also shown for both sea urchin species. N/A: Data not available

	n	***Trophic level***	***$\delta^{15}N$ ‰***	***$\delta^{13}C$ ‰***
Tossa				
A. lixula	72	2.7 (0.17)	8.2 (0.5)	-18.3 (1.1)
P. lividus	71	1.9 (0.13)	5.9 (0.4)	-19.0 (0.9)
Seaweeds average	37	1.0	3.1 (0.7)	
Torredembarra				
A. lixula	10	3.0 (0.17)	10.6 (0.5)	-17.2 (0.6)
P. lividus	9	2.7 (0.12)	9.6 (0.4)	-17.9 (0.5)
Seaweeds average	12	1.0	4.6 (0.7)	
Carboneras				
A. lixula	9	2.7 (0.16)	10.4 (0.5)	-11.8 (0.6)
P. lividus	10	2.1 (0.19)	8.5 (0.6)	-16.7 (0.7)
Seaweeds average	18	1.0	5.3 (0.5)	
Tenerife				
A. lixula	10	3.0 (0.12)	10.7 (0.4)	-11.2 (0.7)
P. lividus	7	2.0 (0.14)	7.5 (0.4)	-17.8 (0.4)
Seaweeds average		N/A	N/A	

Table 2.4. Summary of factorial 2-way ANOVA for assessing significant differences in the isotopic signatures and calculated trophic levels between species (*A. lixula* and *P. lividus*, fixed factor) at the four sampled locations (random factor)

Variable	*Effect*	*Df*	*MS*	F	p-*level*
$\delta^{15}N$	Species (S)	1	98.355	31.27	**0.01**
	Location	3	87.491	22.04	**0.01**
	(L)	3	3.970	18.42	**<0.001**
	S x L	190	0.216		
	Error				
$\delta^{13}C$ [a]	Species (S)	1	35884.07	20.27	**0.007**
	Location	3	84754.14	46.79	**0.005**
	(L)	3	1811.29	1.11	0.34
	S x L	190	1624.53		
	Error				
Trophic Level	Species (S)	1	10.928	31.268	**0.01**
	Location	3	1.667	3.779	0.15
	(L)	3	0.441	18.418	**<0.001**
	S x L	190	0.024		
	Error				

[a] Rank-transformed. No homogeneity of variance achieved.

The $\delta^{13}C$ signatures at the additional localities revealed, as in Tossa de Mar, a higher enrichment in *A. lixula* (Table 2.3). For this variable, no significant interaction between species and locality was found, while the main factors were highly significant (Table 2.4), highlighting the higher $\delta^{13}C$ value in *A. lixula* as well as a noticeable spatial heterogeneity in isotopic signature. The increase in $\delta^{13}C$ in *A. lixula* relative to *P. lividus* in Torredembarra was similar to that in Tossa, but it was much higher at the other two localities (Carboneras and Tenerife, Table 2.3), suggesting a different carbon source for both sea urchins in these localities.

Table 2.5. Summary of standardized feeding indices (only the 12 highest values) for major food items (SD in parentheses) as derived from gut content analysis of *A. lixula* and *P. lividus* at three Mediterranean locations. Given the prominent seasonal changes in the algal assemblages, feeding indices have been calculated separately for June and December at the site where sampling was performed over time (Tossa de Mar). Animal items are shown in bold

Tossa de Mar - June

A. lixula *n = 6*		***P. lividus*** *n = 6*	
Cladophora	19.4 (9.4)	*Dictyota*	40.0 (21.6)
Polysiphonia	19.1 (9.4)	*Dasycladus*	22.3 (10.0)
Lithophyllum	17.3 (8.3)	*Stypocaulon*	11.9 (9.8)
Bryozoa	8.1 (12.7)	*Polysiphonia*	7.1 (6.1)
Hydrozoa	7.6 (4.9)	*Ceramium*	5.3 (4.7)
Cirripedia	5.6 (6.2)	**Bryozoa**	3.0 (3.1)
Polychaeta	5.2 (2.4)	*Corallina*	3.0 (3.6)
Stypocaulon	4.5 (4.5)	*Cladophora*	1.7 (2.5)
Foraminifera	3.6 (2.6)	*Jania*	1.4 (1.9)
Dictyota	2.5 (3.3)	*Colpomenia*	1.3 (1.8)
Ostracoda	2.3 (2.7)	Other seaweed	1.2 (0.9)
Porifera	1.1 (1.1)	*Sphaerococcus*	0.8 (1.4)

Tossa de Mar - December

A. lixula *n = 9*		***P. lividus*** *n = 5*	
Lithophyllum	19.6 (13.6)	*Corallina*	52.2 (7.1)
Polysiphonia	17.1 (10.6)	*Stypocaulon*	21.2 (5.3)
Hydrozoa	14.9 (10..9)	*Peyssonnelia*	9.6 (6.1)
Cirripedia	11.6 (6.1)	*Jania*	7.3 (5.4)
Cladophora	9.3 (7.9)	*Cladophora*	3.0 (2.2)
Polychaeta	8.3 (6.8)	*Polysiphonia*	1.7 (2.2)
Foraminifera	3.7 (1.4)	*Cystoseira*	1.3 (1.6)
Jania	3.2 (2.4)	**Porifera**	1.0 (1.0)
Corallina	2.8 (3.6)	**Polychaeta**	0.8 (0.8)
Porifera	2.0 (2.5)	*Lithophyllum*	0.8 (0.9)
Bryozoa	2.0 (2.0)	**Hydrozoa**	0.3 (0.3)
Stypocaulon	1.6 (3.8)	*Halimeda*	0.2 (0.4)

Torredembarra - December

A. lixula *n = 5*		***P. lividus*** *n = 5*	
Cirripedia	55.5 (12.8)	*Jania*	64.9 (5.5)
Hydrozoa	23.3 (6.9)	*Corallina*	8.8 (3.0)
Polysiphonia	8.9 (4.7)	*Posidonia*	8.4 (3.9)
Porifera	8.5 (4.7)	**Bryozoa**	5.7 (4.3)
Cladophora	1.3 (1.9)	*Polysiphonia*	3.0 (3.0)
Bivalvia	0.8 (1.1)	*Codium.*	2.1 (1.4)
Ceramium	0.7 (0.6)	*Stypocaulon*	1.9 (1.5)
Stypocaulon	0.3 (0.4)	*Cladophora*	1.6 (1.2)
Gastropoda	0.2 (0.4)	**Hydrozoa**	1.6 (1.3)
Polychaeta	0.2 (0.4)	Other seaweed	0.6 (1.1)
Bryozoa	0.2 (0.3)	*Peyssonnelia*	0.6 (0.6)
Other metazoa	0.1 (0.2)	*Padina*	0.2 (0.3)

Carboneras - December

A. lixula *n = 7*		***P. lividus*** *n = 10*	
Jania	34.1 (26.3)	*Jania*	59.9 (10.6)
Lithophyllum	21.2 (23.6)	*Posidonia*	11.6 (5.8)
Cladophora	17.2 (14.0)	*Peyssonnelia*	11.0 (11.0)
Porifera	8.3 (8.8)	*Cladophora*	5.0 (4.3)
Stypocaulon	5.8 (7.0)	*Stypocaulon*	4.1 (6.0)
Ceramium	4.0 (5.9)	*Flabellia*	3.3 (2.2)
Polysiphonia	2.2 (3.4)	Other seaweed	2.6 (5.7)
Peyssonnelia	1.9 (3.2)	*Lithophyllum*	1.6 (2.4)
Other seaweed	1.6 (3.2)	*Padina*	0.7 (0.8)
Foraminifera	1.4 (1.9)	*Corallina*	0.1 (0.2)
Corallina	0.9 (2.0)	**Polychaeta**	0.1 (0.1)
Hydrozoa	0.5 (1.1)	**Porifera**	0.0 (0.1)

Gut content analyses.

Gut content analyses in Tossa de Mar (Table 2.5) revealed a higher abundance of animal items in *A. lixula* than in *P. lividus*. In addition, the ingested material of *P. lividus* showed remarkable temporal differences. Thus, *Dictyota* and *Dasycladus*, the most frequent algal items found in June, did not appear in the gut contents of samples collected in December, when *Corallina* abundance increased. On the contrary, the gut contents of *A. lixula* showed very scarce seasonal changes, being dominated by small filamentous algae such as *Cladophora* and *Polysiphonia*, and crushed fragments of encrusting corallines (*Lithophyllum*). Sessile animals such as hydrozoans, cirripeds and polychaetes were also commonly found throughout the year. These six items, with the addition of bryozoans in June, constituted the main components of *A. lixula* gut contents, with little variation between seasons.

At the two other Mediterranean localities analysed, the results of the gut content analysis confirmed the higher prevalence of animal items in the diet of *A. lixula* relative to *P. lividus*, although with strong variability, probably associated to changes in benthic algal cover. Thus, at the Torredembarra site, where algae were less abundant, some animal items appeared frequently in *P. lividus* guts, such as the bryozoan *Schizoporella errata*, which was common in this habitat. Conversely, in Carboneras, a location with a well developed algal cover, the relative amount of animal remnants in the gut of both sea urchins was the least of all localities sampled. Remarkably, cirripeds were absent of this location, and *Jania* appeared as the main food source for both sea urchin species.

The cumulative feeding indices in the three localities showed that the diet of *A. lixula* has a significantly higher animal component than that of *P. lividus* (Fig. 2.4, Table 2.6). The locality factor was also highly significant, reflecting the marked spatial heterogeneity, but no significant interaction was detected. Whereas for *P. lividus* gut contents were always dominated by the algal fraction, that of *A. lixula* displayed a much higher variability in the relative contribution of animal and vegetal matter among the different localities, ranging from a predominantly animal component in Torredembarra to a dominance of vegetal diet in Carboneras (Fig. 2.4).

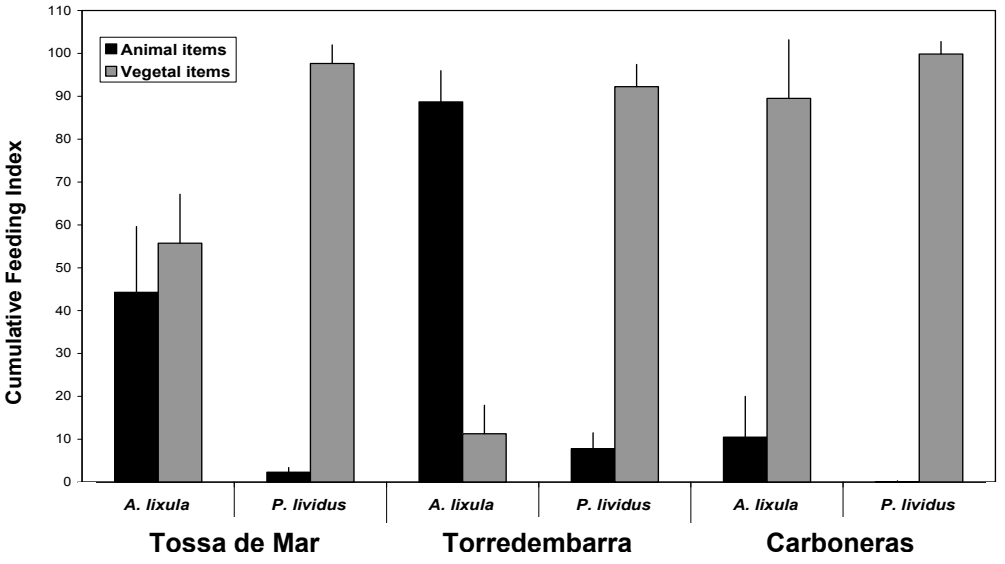

Fig. 2.4. Cumulative feeding indices (mean + SD) for animal and vegetal food items, derived from gut content analyses of *A. lixula* and *P. lividus* collected in December at three Mediterranean locations

Table 2.6. Summary of factorial 2-way ANOVA to assess significant differences in the cumulative animal feeding index (sum of standardized feeding indices of animal items), between both sea urchin species (fixed factor) and location (random factor).

Variable	*Effect*	*Df*	*MS*	F	p-*level*
Cumulative animal feeding index [a]	Species (S)	1	2799.113	450.15	**0.002**
	Location (L)	2	917.827	149.62	**0.007**
	S x L	2	6.135	0.60	0.55
	Error	35	10.220		

[a] Rank-transformed.

DISCUSSION

Our results show that *A. lixula* occupies a higher trophic level than *P. lividus*, as shown by its $\delta^{15}N$, consistently higher across the sampled localities in the former than in the latter. Their estimated trophic levels indicate that *A. lixula* is an omnivore tending to carnivory, while *P. lividus* is a predominantly herbivore that can turn into an omnivore in some instances. The (at least partial) carnivory in *A. lixula* is further supported by the analyses of gut contents, which reveal a consistently higher proportion of animal food items ingested in *A. lixula* as compared to *P. lividus*. However, gut content analysis alone do not reveal the full extent of the trophic gap between the two species, since vegetal components are the dominant ones in most situations analysed (except for *A. lixula* in Torredembarra). Finally, the results for $\delta^{13}C$ are coherent with those of $\delta^{15}N$, indicating an overall enrichment of the signature of *A. lixula* with respect to *P. lividus*. The results for carbon,

however, should be taken with caution as this isotope is best suited to detect differences in sources of food rather than trophic levels (Cardona et al., 2007). This implies that the role of *A. lixula* in the shallow subtidal in the Mediterranean should be, at least in part, re-evaluated. Specifically, the notion of a putative strong competition for food should be carefully re-examined.

The suitability of stable isotope analysis, and specifically $\delta^{15}N$, for identifying trophic levels in marine ecosystems has been clearly established (e.g.: Cherel et al., 2008). Much closer to the scope of the present study, this tool has revealed differences in the trophic levels of sympatric sea urchins (Vanderklift et al., 2006). These authors found that two littoral Australian echinoids previously thought to be herbivorous (*Phyllacanthus irregularis* and *Centrostephanus tenuispinus*) had actually an omnivorous behaviour tending to carnivory. The differences in $\delta^{15}N$ between *A. lixula* and *P. lividus* that we report here, based on a wide temporal and geographical scale, are comparable to those found between both Australian purportedly herbivorous species and *Heliocidaris erythrogramma*, which proved to be a true strict herbivore.

In previous studies, animal items had been reported in the gut contents of both Mediterranean sea urchins (Maggiore et al., 1987; Chiantore et al., 2008; Privitera et al., 2008), but were mostly disregarded as anecdotal or accidental captures, which may be true for *P. lividus* but certainly not for *A. lixula*. The long-held misconception about the herbivory of *A. lixula* may stem from several causes, but mainly from the fact that most primary information on this issue came from studies of gut contents, which

target ingested, rather than assimilated, food. While it is true that the ecological impact of an organism (in this case, *A. lixula*) feeding activity may depend mostly on what is ingested, rather than on what is assimilated, gut content analysis can introduce some biases on our perception of an animal diet if used alone. Gut content analyses cannot be dismissed, though, as they provide the only direct taxonomical information about what the sea urchins ingest and, in combination with stable isotope analyses, can shed light on important aspects of their feeding strategy.

In addition, if diverse kinds of foodstuff have differential digestibility, results can be biased towards less digestible material. It is remarkable in this sense that most faunal items found in the gut of *P. lividus* are nearly intact and easily identifiable, probably reflecting the little ability of this species for assimilating animal material. The opposite is true for *A. lixula*, which seems to perform complete digestions of animal tissues. Conversely, undigested filamentous algae, even the most delicate ones, are regularly found intact in the guts of *A. lixula*. In a study on *A. lixula* from Brazil, Oliveira (1991) found that 50% of the algae present in its faecal pellets survived digestion and were able to grow when cultured, in contrast to algae egested by herbivorous sea urchins such as *Lytechinus variegatus* or *Echinometra lucunter*.

Another cause that can contribute to the misconception about *A. lixula* herbivory is the fact that the gut contents of *A. lixula* that we examined consisted largely of small crushed pieces of pinkish-greyish carbonates, which can be easily interpreted as fragments of calcareous algae. However, using scanning electron microscopy, we

have unambiguously identified many of these pieces as fragments of shells of the common western Mediterranean barnacle *Balanus perforatus*. Thus, we must consider the possibility that cirriped shell remnants may have been mistaken for encrusting corallines in some studies which were carried out under the undisputed paradigm of an herbivorous *A. lixula*.

The finding that *A. lixula* is an omnivore tending to carnivory may shed light on unexpected results of some ecological experiments. For example, the removal of *P. lividus* had no effect and did not trigger an increase of the population of *A. lixula* (Gianguzza et al., 2006), as would be expected if inter-specific competition occurred between both species. Artificially reducing or increasing the density of *A. lixula* in selected patches had no effect on the percent cover of encrusting corallines (Benedetti-Cecchi et al., 1998; Bulleri et al., 1999), but the removal of *A. lixula* produced an increase in the density of *B. perforatus* and a decrease in the density of limpets (Bulleri et al., 1999), opposite to what would be expected if *A. lixula* was an herbivorous consumer of filamentous algae and trophic competitor of *Patella* sp.

Finally, our results add some information about the putative competition between the two main Mediterranean sea urchins. Densities of *P. lividus* in the NW Mediterranean are usually higher (an order of magnitude on average, Palacín et al., 1998b) than those of *A. lixula*. This fact challenges the idea that both species engage in strong competitive interactions or, at least, it suggests that *P. lividus* is able to outcompete *A. lixula*, whose shift to a different diet may help to avoid exclusion. However, both species can locally coexist

at high densities (Guidetti et al., 2004; Tuya et al., 2007), and *A. lixula* can be the dominant sea urchin in some communities (Benedetti-Cecchi et al., 1998). Furthermore, both species segregate spatially in some cases (Kempf, 1962; Chelazzi et al., 1997; Bulleri et al., 1999), as happens in our Atlantic location, where *A. lixula* is restricted to vertical walls. Thus, interference competition between these species is likely to happen in many places. Agonistic interactions (Shulman, 1990) have never been observed between them, so exploitative competition seems more likely, and feeding flexibility can be an important mechanism to alleviate its effects. On the other hand, it has to be emphasized that factors other than direct trophic competition, such as resistance to hydrodynamism (Tuya et al., 2007), resistance to predation (Guidetti, 2006) or presence of predators which could modulate sea urchin behaviour (Freeman, 2006) could also be involved in shaping the distribution and abundance of these two sea urchin species.

Few studies have addressed the possibly different foraging behaviour of these sea urchins species. Apparently, *A. lixula* shows a higher mobility than *P. lividus* in barren zones (Bonaviri et al., 2011), so that a wider area can be impacted by its grazing activity. The strong Aristotle's lantern that allows *A. lixula* to scrape the substrate for searching its prey, and the fact that this species tends to be more abundant than *P. lividus* in barren zones which offer relatively few algal food in comparison to animal prey (Guidetti and Dulcić, 2007), could be better explained in the light of its tendency to carnivory.

Studies comparing feeding strategies between these species in communities with dominance of *A. lixula* are necessary to ascertain the trophic position of both species under competitive pressures different from those found in the present study. Interestingly, at high densities, *A. lixula* may be expected to limit the abundance of its prey populations, and so it can compete with carnivores such as asteroids. Thus, the omnivory and feeding plasticity of *A. lixula* adds complexity to models of community structure, including possible trophic loops and increased connectivity between species (Camus et al., 2008).

In conclusion, the finding that *A. lixula* is an omnivore tending to carnivory has important implications for the dynamics of shallow water communities in the Mediterranean, as it suggests not only a reduced competition for food with the coexisting echinoid *P. lividus*, but also opens new views to understand biotic interactions in these communities. Given the important functional role of these echinoid species in shaping sublittoral assemblages, and the fact that one of them (*P. lividus*) sustains heavy fisheries in some areas, the results presented here should be taken into consideration both in basic studies of ecosystem functioning and in applied issues of environmental and fisheries management.

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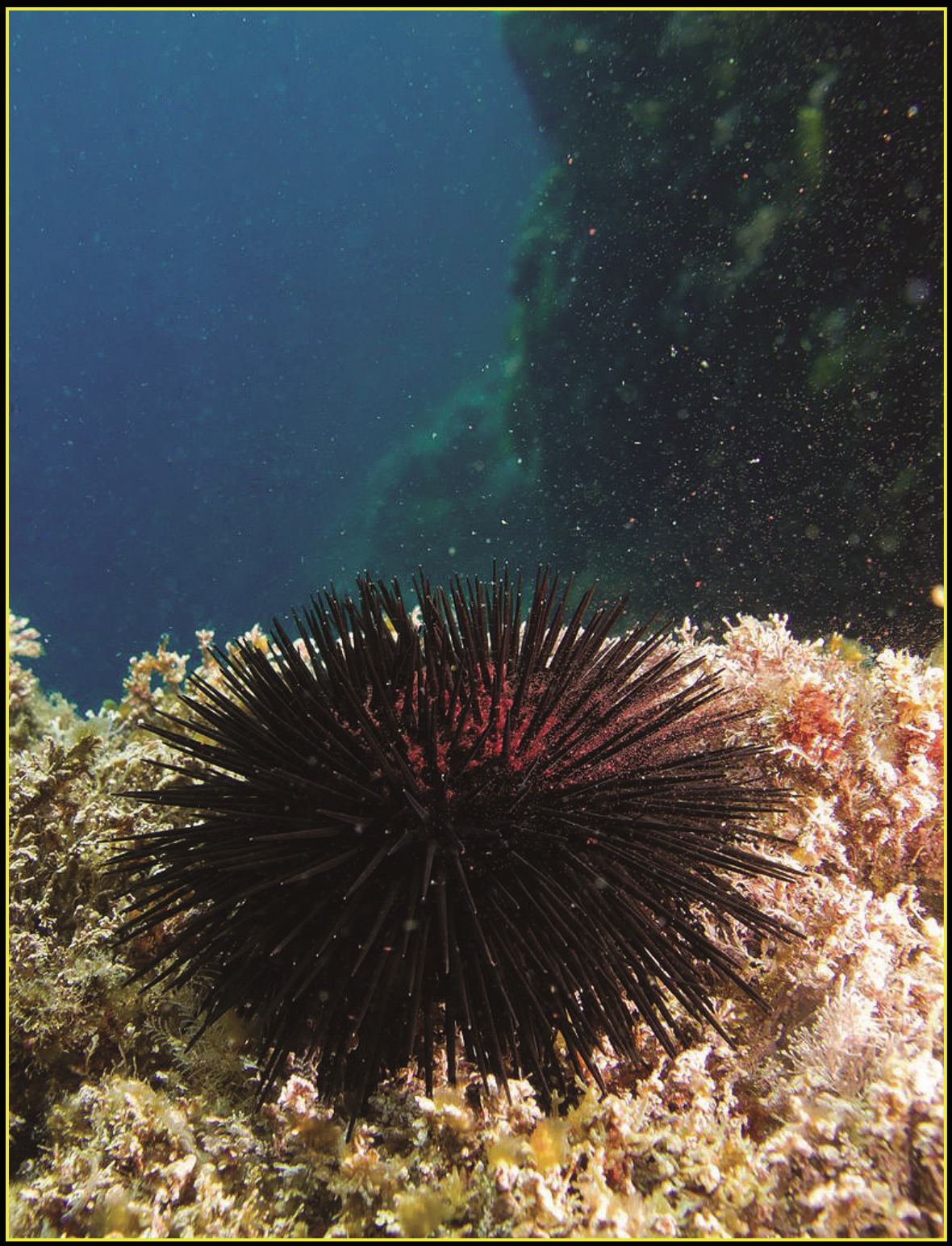
CHAPTER 3 - SPINY AFFAIRS HEATING UP



Arbacia lixula female spawning. Photo: **C. Palacín**

Chapter 3. Spiny affairs heating up: photoperiod, temperature and inter-annual variability in the reproductive cycle of the sea urchin *Arbacia lixula*

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ABSTRACT

We studied the reproductive cycle of the thermophilous sea urchin *Arbacia lixula* in the northwestern Mediterranean over four years using a gonadosomatic index and gonad histology. Our results show that the gonadosomatic index of *A. lixula* follows a seasonal cycle which peaks in May-July and attains its lowest values in October-November every year. The time course of the gonadosomatic index matched closely the photoperiod cycle. We also found a remarkable inter-annual variability in the maximum value of gonadosomatic index, which correlated with mean water temperature during the gonad growth period (winter and spring). Gonad histology was also in agreement with a single gametogenic cycle per year in this species. We explored the application of circular statistics to present and analyse gonadal development data, which allowed us to adequately handle the high intra-individual variability detected, with several developmental stages commonly found within the same gonad. The picture that emerged is one of a gametogenic timing driven by photoperiod, while the amount of reproductive output is determined by temperature. This is coherent with the thermophilous nature of the species and lends support to recent warnings about an increase in the abundance of this species in the Mediterranean as a result of global warming, with associated increased impact potential in sublittoral communities.

KEY WORDS

reproductive cycle regulation, Echinoidea, Mediterranean, gonad histology, polar plots, circular statistics

RESUMEN

Se ha estudiado el ciclo reproductivo del erizo termófilo *Arbacia lixula* en el Mediterráneo Noroccidental a lo largo de cuatro años, analizando la variación temporal de un índice gonadosomático y la histología gonadal. Los resultados muestran que el índice gonadosomático de *A. lixula* sigue un ciclo estacional que alcanza su máximo entre mayo y julio y sus valores más bajos en octubre y noviembre de cada año. La evolución temporal del índice gonadosomático se correlaciona estrechamente con el fotoperíodo. También se ha encontrado una elevada variabilidad interanual en el valor máximo del índice gonadosomático, que se correlaciona con la temperatura media del agua durante el período de crecimiento de las gónadas (invierno y primavera). Los resultados de los análisis histológicos también apoyan la existencia de un único ciclo gametogénico anual en esta especie. Se ha experimentado la utilización de la estadística circular para presentar y analizar datos sobre desarrollo gonadal, esto ha permitido manejar adecuadamente la alta variabilidad intraindividual detectada, debida a la coexistencia simultánea de varias etapas de desarrollo dentro de la misma gónada. El resultado global es congruente con la idea de que el fotoperiodo regula la componente temporal del ciclo gametogénico, mientras que la temperatura determina la cantidad de gametos producida. Estos datos son coherentes con el carácter termófilo de la especie y reafirman las advertencias sobre un posible incremento en la abundancia de esta especie en el Mediterráneo a consecuencia del calentamiento global, que iría asociado a un mayor potencial de impacto sobre las comunidades sublitorales.

PALABRAS CLAVE

regulación del ciclo reproductivo, Echinoidea, Mediterráneo, histología gonadal, coordenadas polares, estadística circular

INTRODUCTION

The black sea urchin *Arbacia lixula* (Linnaeus, 1758) is one of the most abundant sea urchins in the Mediterranean (Benedetti-Cecchi et al., 1998; Palacín et al., 1998b; Sala et al., 1998b). Despite its increasingly recognized ecological importance (Bulleri et al., 1999; Guidetti et al., 2003; Guidetti and Dulcić, 2007; Bonaviri et al., 2011; Gianguzza et al., 2011; Privitera et al., 2011), it has been traditionally less studied than the Atlanto-Mediterranean edible sea urchin *Paracentrotus lividus*. The reproductive cycle of *P. lividus* is now well understood (Fénaux, 1968; Lozano et al., 1995; Fernandez and Boudouresque, 1997; Sánchez-España et al., 2004; Barbaglio et al., 2007; Gianguzza et al., 2013a), but little information exists on the reproductive cycle of *A. lixula* in the Mediterranean (Fénaux, 1968; Régis, 1979), though Tavares (2004) studied its reproductive biology in Brazil.

Despite being commonly considered as a typical Mediterranean species, *Arbacia lixula* is actually a thermophilous species of tropical affinities (Stefanini, 1911; Mortensen, 1935; Tortonese, 1965) which probably spread through the Mediterranean in the Upper Pleistocene (Wangensteen et al., 2012). It is presently distributed along shores of the tropical Atlantic, including Brazil, the African coast, Macaronesian archipelagos and the Mediterranean, where it may reach high population densities (Palacín et al., 1998b; Bulleri et al., 1999; Guidetti et al., 2003). *A. lixula* is an omnivore tending to carnivory (Wangensteen et al., 2011) and has a high potential to impact shallow rocky areas. Its role in originating and maintaining barren zones is being

increasingly recognized (Guidetti et al., 2003; Bonaviri et al., 2011; Privitera et al., 2011). This species has experienced population increases in the past (Petit et al., 1950; Francour et al., 1994; Harmelin et al., 1995) and warnings have been issued about its potential future impact in the Mediterranean, considering the ongoing global warming trend (Gianguzza et al., 2011; Privitera et al., 2011).

Most echinoderm species show remarkable natural fluctuations (Uthicke et al., 2009), which may be related with the regulation of their reproductive processes by external factors. Thus, the study of the natural inter-annual variability of their reproductive cycles and the assessment of the possible physical or biological factors that regulate these cycles are invaluable tools to predict future trends in the context of the ongoing climate change. With this goal, we monitored a population of *A. lixula* in natural conditions in the northwestern Mediterranean during four years, in order to characterize the reproductive cycle of this species, to determine its inter-annual variability, and to assess the effects of temperature on the reproductive potential of this ecologically relevant echinoid.

MATERIALS AND METHODS

Sampling

Samples of *Arbacia lixula* were collected monthly by SCUBA diving at depths between 3 and 10 m at the littoral of Tossa de Mar (NE Spain, 41º 43.2' N, 2º 56.4' E) from November 2008 to September 2012. Specimens were fixed in 4% formaldehyde. Only

adult-sized sea urchins (test diameter size range 35.0 – 58.6 mm, mean 44.56 mm) were used for the study. Ten individuals per month were collected until August 2010, and twenty individuals per month were collected thereafter. December 2009 and October 2010 could not be sampled due to adverse meteorological conditions.

Gonadosomatic index analysis

The gonadosomatic index (GSI) was calculated as the ratio between the wet weight of the gonads and the total wet body weight, excluding coelomic fluid and digestive contents (Grant and Tyler, 1983; Pearse and Cameron, 1991), measured on a precision scale (0.001 g). A total of 695 individuals from 45 monthly samples were measured.

Histological analysis

Histological sections (14 μm thick) of one paraffin-embedded gonad per individual collected between October 2009 and September 2012 were obtained in a Microm HM325 microtome and stained with hematoxylin-eosin. A total of 596 individuals (295 males and 301 females) from 34 monthly samples were analysed. Sex was determined and gonadal acini were classified into one of five developmental stages (spent – recovery – growing – premature – mature) adapted from the staging method used by Yoshida (1952).

Due to high intra-individual heterogeneity, individual maturation states could not be adequately described by a single categorical stage. Instead, we used a circular coordinate system, in which evenly separated angles were assigned to each absolute

developmental stage (Spirlet et al., 1998). The maturation index (MI) was calculated for each individual as the vectorial mean of 10 examined acini per individual. Monthly averages of this MI were also calculated as vectorial means of individual MIs and represented in a polar-circular diagram. In this representation, the angular variable θ represents a continuous MI. The relative position of monthly average maturation vectors allows for the quick graphical comparison of the maturation progress between different reproductive cycles. In case that one yearly cycle is advanced relative to other, the average maturation vectors representing the corresponding months will have a greater value for the angular MI. The scalar value r (module) of a monthly average vector is a measure of the uniformity in the individual angular maturation indices. Thus, a value of r = 1 indicates that all the individual vectors are equal. The higher dispersion of individual maturation vectors, the shorter will the module of the resultant vector be.

The correlation between individual angular maturation indices and scalar gonadosomatic indices (circular-linear association) can also be graphically represented in a circular diagram. In this case, the value of the scalar variable represents the scalar gonadosomatic index. Any association between the scalar index and the angular index will be visualized as the accumulation of points with higher values of the scalar index in a given direction of the diagram, whereas the non-existence of correlation would generate a symmetrical distribution of points around the circular plane.

Temperature and photoperiod

Daily and monthly mean values for sea surface temperature (SST) were obtained from the nearby L'Estartit Meteorological Station (http://www.meteoestartit.cat). Measures of HOBO underwater temperature data loggers placed *in situ* at the sampling location during part of the study showed negligible differences with temperature recordings at L'Estartit (data not shown). Photoperiod data were obtained from the US Naval Observatory (http://aa.usno.navy.mil).

Statistical methods

Differences in GSI between sexes were assessed for every month using Mann-Whitney *U* tests and their significance was corrected using the Benjamini and Yekutieli (2001) FDR correction procedure. As no significant differences were found, both sexes were pooled for further analyses. Kruskal-Wallis non parametric ANOVAs, followed by Dunn's post hoc tests, were used to check for differences in GSI among months within each gonadal cycle and also to check for inter-annual differences among annual maximum values of GSI. Pearson correlation coefficients of monthly mean GSI with photoperiod were calculated separately for each gonadal cycle. The effect of SST on annual maximum values of GSI was assessed by calculating Pearson correlation coefficient of this annual maximum GSI with mean SST during the gonad maturation period (averaging daily temperatures during the six months previous to the GSI peak).

Small sample sizes ($n < 25$) prevented us from using circular

statistical tests such as Watson's U^2 (Fisher, 1993; Zar, 1996) for testing differences in MI. Instead, we used a balanced bootstrap procedure (Booth et al., 1993), where bias-corrected and accelerated confidence intervals (BCa; Efron, 1987) for the monthly mean circular MI were calculated using a modified procedure from the R package BOOT (Canty and Ripley, 2009), with 5,000 replicates. Differences of MI between sexes were then assessed by comparing these confidence intervals. As no differences between sexes were found, both sexes were pooled and bootstrap confidence intervals were recalculated for comparisons between months.

Circular-linear association between MI and GSI was assessed, for every cycle, calculating Mardia's (1976) circular-linear association coefficients, $R^2_{x\theta}$ and their significance was tested using a randomisation (permutation) procedure (Fisher, 1993) with 10^6 replicates.

All statistical analyses were performed with RStudio (Rstudio Inc., Boston, MA, USA) or Sigmastat 3.1 (Systat Software Inc., Point Richmond, CA, USA). Graphical functions included in R packages CIRCULAR (Lund and Agostinelli, 2010) and PLOTRIX (Lemon, 2006) were used for graphical representations.

RESULTS

Gonadosomatic index

Fig. 3.1 shows the periodic behaviour of the gonadosomatic index (GSI) of *Arbacia lixula* throughout four complete annual cycles. Males and females were pooled together, since no significant differences were found in any sampled month (Mann-Whitney *U* test, all $P > 0.05$). An annual peak is apparent whereby the maximum value is attained every year during May-June-July. The minimal values occur every year in October. So, each reproductive cycle can be considered to span from October to September of the following year. Kruskal-Wallis tests found significant differences among months within every cycle (Table 3.1).

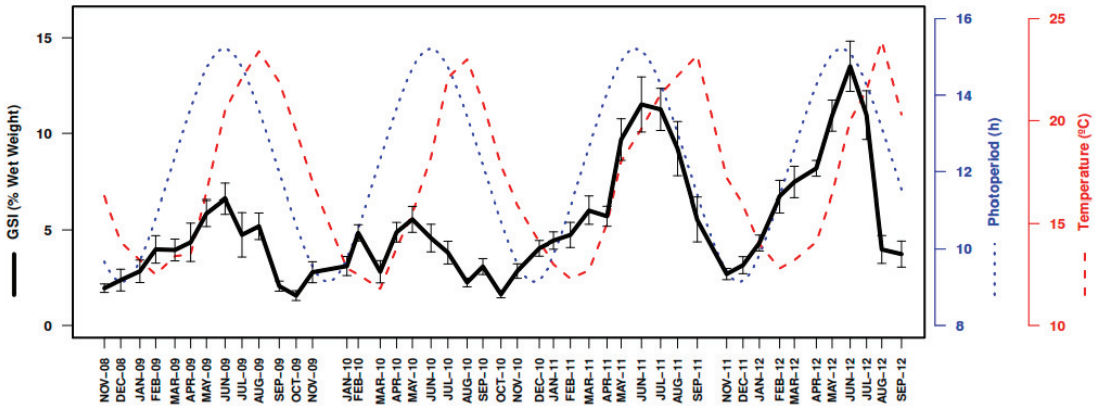


Fig. 3.1. Gonadosomatic index, GSI (% wet weight; means ± SE) of *Arbacia lixula* (pooled males and females) collected between November 2008 and September 2012 at Tossa de Mar (Spain, NW Mediterranean). Sea surface temperature data obtained from the Meteorological Station at L'Estartit. Photoperiod data obtained from the US Navy Observatory

Table 3.1. Kruskal-Wallis non parametric ANOVAs testing for differences among months in gonadosomatic indices of *Arbacia lixula* at Tossa de Mar (Spain) during four consecutive reproductive cycles. N: number of individuals used in each analysis

Cycle	***N***	**H**	***d.f.***	**P-*value***
Nov 2008 – Sep 2009	110	40.65	10	1.3×10^{-5}
Oct 2009 – Sep 2010	121	50.08	10	2.6×10^{-7}
Oct 2010 – Sep 2011	246	106.68	11	2.2×10^{-16}
Nov 2011 – Sep 2012	218	121.16	10	2.2×10^{-16}

An anomaly can be observed as a marked decrease of GSI during March of 2010, which could be explained by extreme low temperatures (see below). A remarkable correlation between monthly mean GSI and photoperiod was detected during three out of the four analysed cycles, with a somewhat less clear-cut relationship during the 2010 cycle (Table 3.2). Thus, the gonad build-up approximately starts with the winter solstice and the GSI peak occurs simultaneously with the summer solstice, suggesting that photoperiod may be the main factor regulating the timing of the gonadal cycle in *A. lixula*. Temperature, on the other hand, had cycles lagged by several months with respect to GSI cycles.

Table 3.2. Pearson correlation coefficients of monthly mean gonadosomatic index of *Arbacia lixula* with photoperiod during each reproductive cycle. Significant correlations were found in all cases, excluding the coldest cycle (2009-10), probably due to an anomalous gonadal cycle caused by cold temperatures

Cycle	*Pearson* r	P-*value*
2008-2009	0.92	0.0001
2009-2010	0.48	0.12
2010-2011	0.84	0.0007
2011-2012	0.95	0.00003

The magnitude of the annual maximum GSI showed a remarkable inter-annual variability, being significantly higher during the last two cycles than during the first two (Kruskal-Wallis followed by Dunn's test, $H_3 = 18.13$, $P = 0.0004$). If we average the sea surface temperature (SST) during the gonad growth period (the six months previous to the peak, i.e. from December to May), the annual maximum value for GSI shows a strong correlation ($r = 0.964$, $P = 0.04$) with this mean SST (Fig. 3.2). An increment of 1ºC in mean SST originated a 2.5-fold increase in the maximum GSI. The first two cycles were characterized by low mean SST values during the growth period and corresponded to GSI cycles showing a less defined peak (Fig. 3.1), whereas the last two cycles were warmer years characterized by high mean SST values and a well-defined GSI peak. This SST trend observed locally was similar to the more general temperature recorded for these years in the Western Mediterranean. Thus, the gonad growth of *A. lixula* can be considerably impaired during cold years with low winter and spring temperatures.

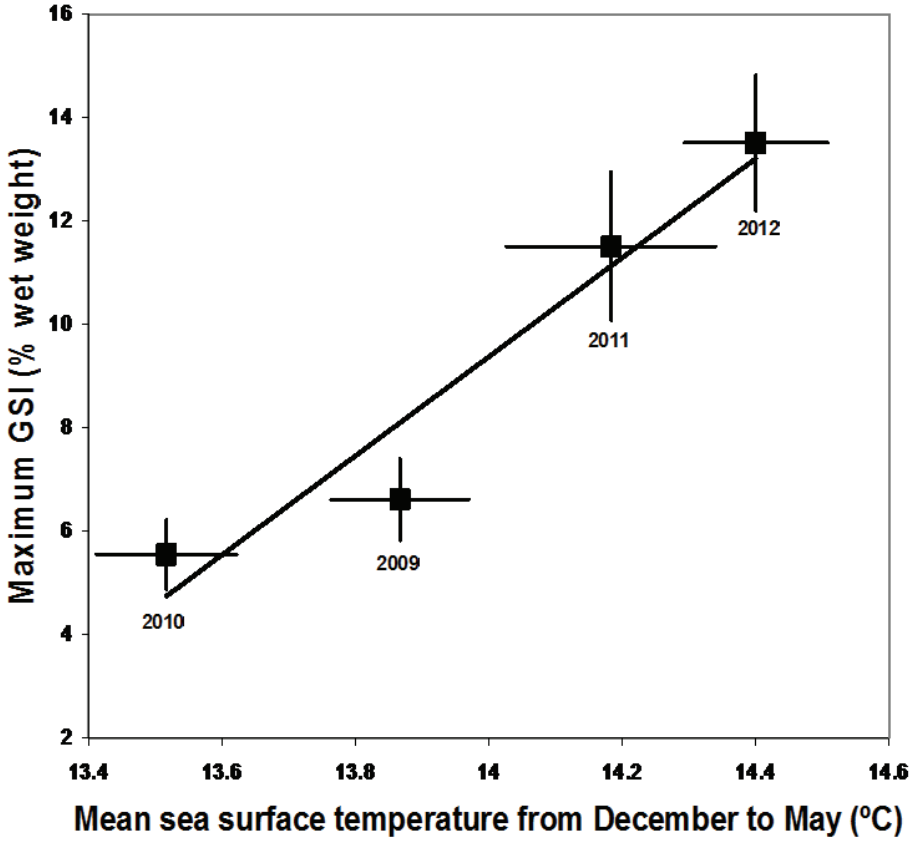


Fig. 3.2. Effect of sea surface temperature during the gonad growth period (averaged from December to May) on the maximum gonadosomatic index achieved by the thermophilous sea urchin *Arbacia lixula* at Tossa de Mar (Spain) over a four-year period. Error bars indicate standard errors

Histology of the gonads

From the examination of histological sections of *Arbacia lixula* gonads, we could differentiate five gonadal maturation stages, namely: spent, recovery, growing, premature and mature, both in males and females (Fig. 3.3).

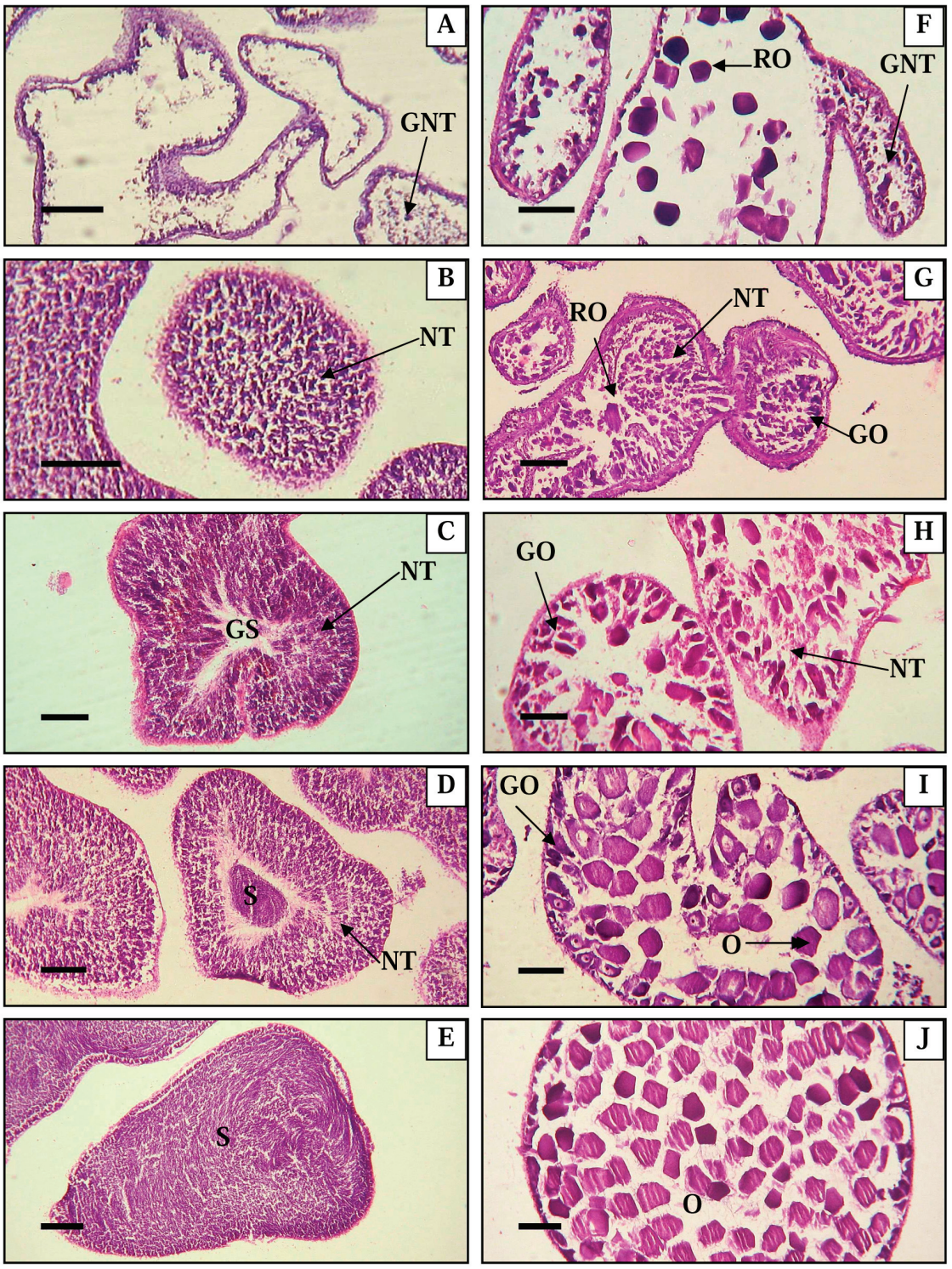


Fig. 3.3. Histological sections of *Arbacia lixula* male (A-E) and female (F-J) gonads illustrating the five maturation stages. A,F: Spent; B,G: Recovery; C,H: Growing; D,I: Premature and E,J: Mature. GNT: growing nutritive tissue; NT: nutritive tissue; GS: growing spermatozoa; S: spermatozoa; RO: relict ova; GO: growing ova; O: ova. Scale bar: 100 µm.

In both sexes, the gametogenic process begins with a spent gonad (Fig. 3.3A and 3.3F), in which the acini are enclosed by a thin wall and are mostly void of cellular material. Relict spermatozoa or ova from the previous spawning event may be observed. Some growing nutritive tissue may be present, but never occupying a significant portion of the acinus. In the recovery stage (Fig. 3.3B and 3.3G), a dense meshwork of nutritive phagocytes occupies most of the acinal space. Primary spermatogonia and oogonia may occur near the acinal walls. Relict spermatozoa or ova may be present in different degrees of lysis. In the growing stage (Fig. 3.3C), nutritive tissue still occupies a considerable portion of the acinus. An empty space opens in the central area of the male acini, where eosinophilic tails of developing spermatozoa can be observed (Fig. 3.3C). In the growing ovary (Fig. 3.3H), most developed oocytes are displaced towards the centre, while elongated, smaller ones are located near the acinal wall. In the premature testes (Fig. 3.3D), a mass of basophilic mature spermatozoa accumulates in the centre of the acinus, while a thick layer of nutritive tissue can still be observed in its periphery. In the premature ovary (Fig. 3.3I) oocytes at all stages of development occupy most of the space. Nucleoli and some remnant nutritive tissue are typically observed. The mature gonads (Fig. 3.3E and 3.3J) are densely packed with mature spermatozoa or ova and nutritive tissue is absent.

Arbacia lixula shows a striking intra-individual heterogeneity in gonad maturation, so that in most individuals, acini in different maturation stages can be found within one single gonad.

This prevented us from assigning a categorical state of maturarion to any individual. Thus, we used a continuous circular maturation index (MI) to correctly describe the gonad maturation state. Also, a high degree of inter-individual variability can be found, so that in any given month, individuals belonging to different maturation stages can coexist (Fig. 3.4).

Fig. 3.4 shows the temporal variation of the MI throughout 36 consecutive months (three complete reproductive cycles), corresponding to the last three cycles represented in the GSI graph (Fig. 3.1). The monthly mean vectors, as well as the individual MI for both males and females, are shown. Non-overlapping bootstrap confidence intervals for males and females were only found in one month (October 2009) out of 34 months compared, which is likely an artefact due to small sample size. Thus, both sexes were pooled for all the following analyses.

Although the inter-individual variability is consistently high, a remarkable match between mean MI vectors of corresponding months can be appreciated in Fig. 3.4 during the last two cycles (October 2010 – September 2011 and October 2011 – September 2012). These correspond to the warmest years, when the GSI curve featured well-defined peaks. The majority of individuals were mature during May-June-July. The only noticeable difference between these two cycles is in the spawning event, which took place one month earlier in 2012 (August), as compared with 2011 (September).

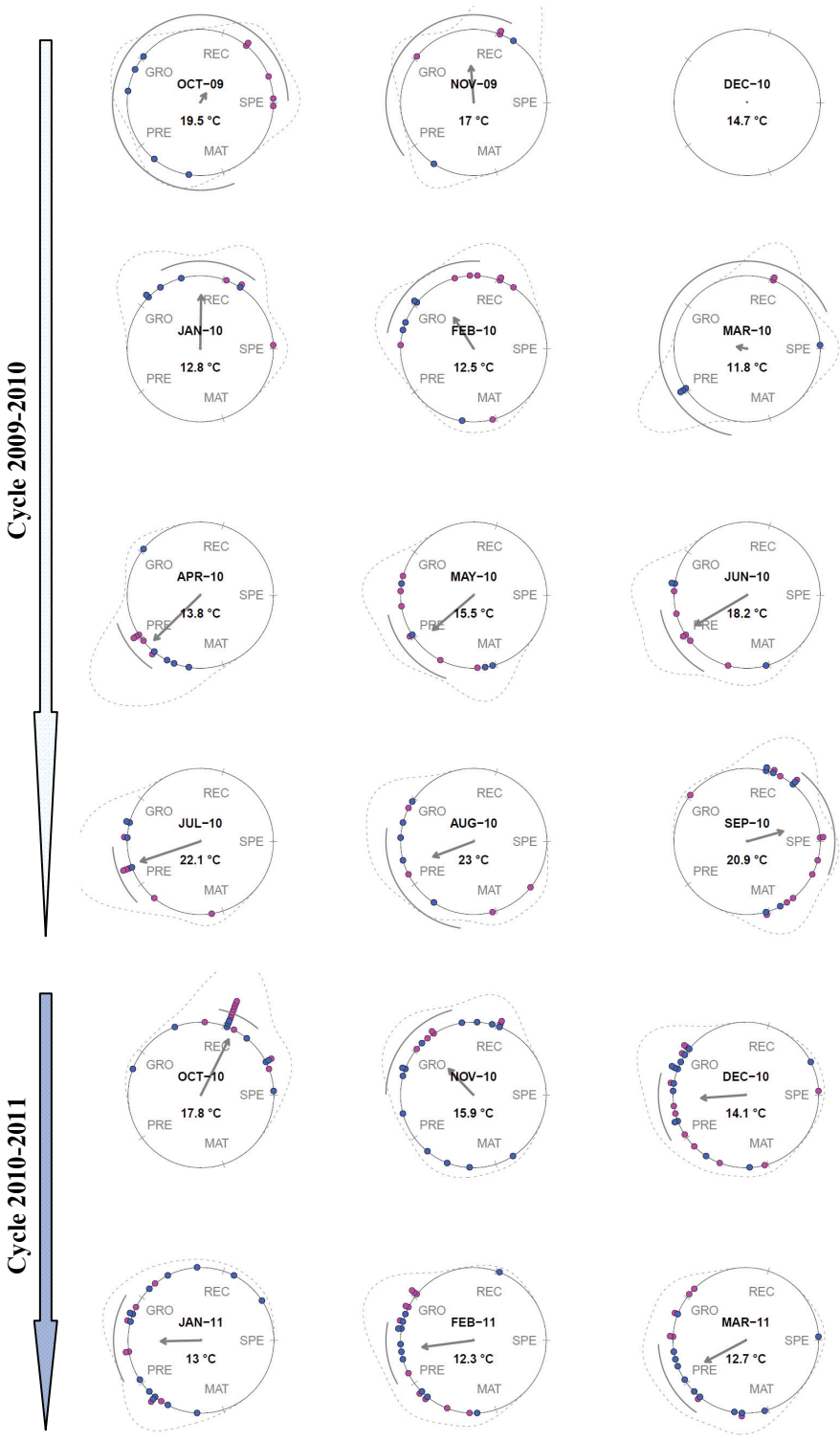
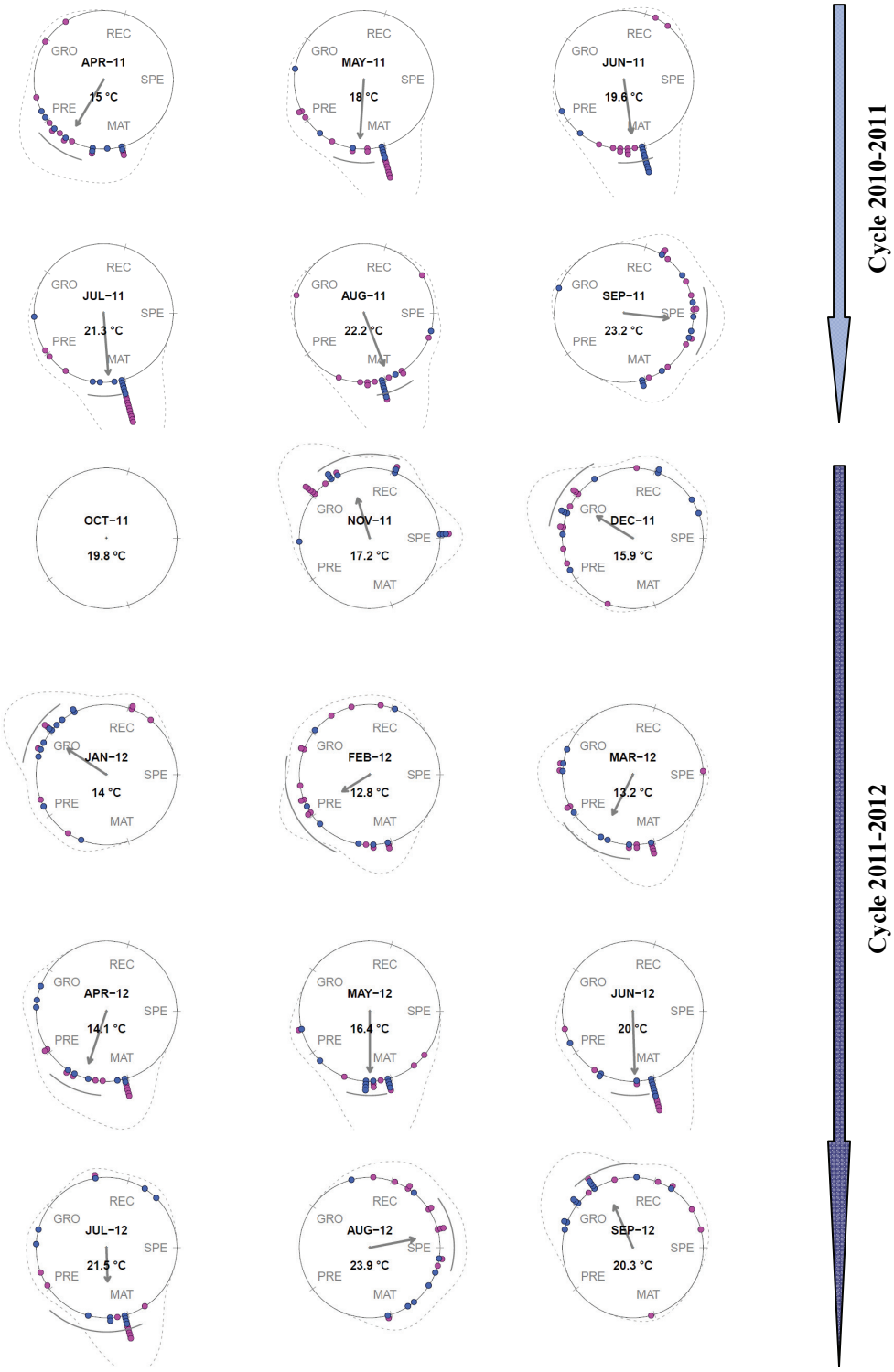


Fig. 3.4. Monthly circular maturation vector plots for *Arbacia lixula* at Tossa de Mar (Spain) throughout three consecutive reproductive cycles. Blue and pink bullets correspond to male and female individuals, respectively. Density estimates of the distributions are shown as dashed curves and 95% confidence interval for...



the mean direction (bootstrap) are shown as grey arcs. Average sea surface temperatures are shown. Maturation stages are indicated: SPE: spent, REC: recovery, GRO: growing, PRE: premature and MAT: mature. December 2009 and October 2010 could not be sampled due to adverse meteorological conditions.

In contrast, the first cycle in our histological data (October 2009 - September 2010), which is also the coldest one of our series, showed a very different behaviour from the two warmer cycles. The maturation vector is consistently retarded compared with the following cycles, not only during the coldest months, but also during the summer. Few mature individuals could be found, so that the mean MI vector did not attain the mature stage anytime during this cycle, halting instead at the premature stage.

Histologically mature *Arbacia lixula* individuals showed consistently high GSI values, and the individual GSI is significantly associated with the MI (Fig. 3.5 and Table 3.3). Nevertheless, Mardia's circular-linear association coefficient, $R^2_{x\theta}$, showed higher values and signification during the two warmest cycles, suggesting that, despite the significant association found, the GSI is less linked to the maturation stage during cold years, probably due to incomplete gonad maturation.

Table 3.3. Mardia's circular-linear association coefficients, $R^2_{x\theta}$, for testing the association between individual maturation indices (angular variable) and gonadosomatic indices (scalar variable) for *Arbacia lixula* throughout three consecutive reproductive cycles.

Cycle	***Males***	***Females***	***All***
2009-2010	$R^2_{x\theta} = 0.1832$ $P = 0.0096$ n = 49	$R^2_{x\theta} = 0.1696$ $P = 0.0046$ n = 59	$R^2_{x\theta} = 0.1791$ $P = 2.4 \times 10^{-5}$ n = 108
2010-2011	$R^2_{x\theta} = 0.3732$ $P < 10^{-6}$ n = 126	$R^2_{x\theta} = 0.4713$ $P < 10^{-6}$ n = 118	$R^2_{x\theta} = 0.4062$ $P < 10^{-6}$ n = 244
2011-2012	$R^2_{x\theta} = 0.4592$ $P < 10^{-6}$ n = 104	$R^2_{x\theta} = 0.5413$ $P < 10^{-6}$ n = 112	$R^2_{x\theta} = 0.4964$ $P < 10^{-6}$ n = 216

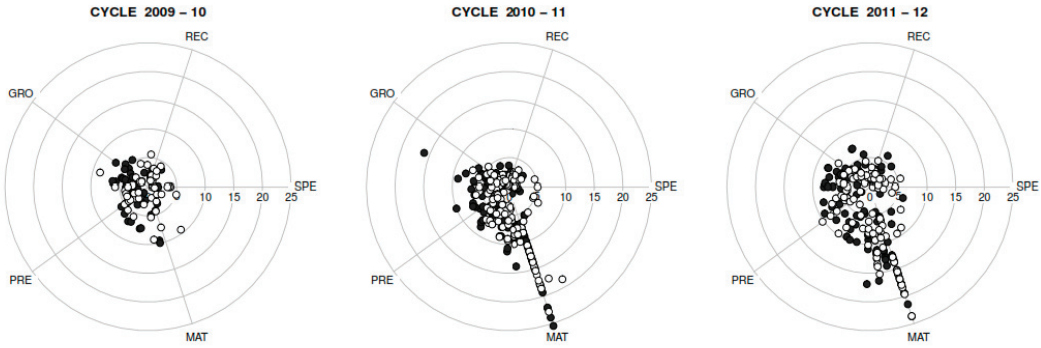


Fig. 3.5. Polar plots showing the association between individual maturation indices (angular axis) and gonadosomatic indices (radial axis) during three consecutive annual cycles for *Arbacia lixula* at Tossa de Mar (Spain). Solid and open bullets correspond to male and female individuals, respectively. Maturation stages are indicated: SPE: spent, REC: recovery, GRO: growing, PRE: premature and MAT: mature

DISCUSSION

The gonadosomatic index of *Arbacia lixula* in northwestern Mediterranean follows a seasonal cycle which peaks in May – July, coinciding with the summer solstice, and attains its lowest values in October-November every year. A single annual spawning event can be inferred from the GSI trend, which would take place during the summer and early autumn. The results from gonad histology are also in agreement with a single gametogenic cycle per year in this species.

As temperature and photoperiod co-vary with a lag of a few months, it could be difficult to disentangle the effect of both variables. However, in *Arbacia lixula* photoperiod matches quite

closely the time course of the GSI, especially in 2011 and 2012, while the temperature cycle is displaced by ca. 2-3 months (Fig. 3.1). This suggests that photoperiod rather than temperature drives the timing of gametogenesis. However, sea surface temperature (SST) seems to have also a critical effect in the reproductive cycle of *A. lixula*. The first two cycles of our series (November 2008 to September 2010) were characterized by low maximum values of the GSI and corresponded to years when SST stayed considerably cold during winter and spring months. Conversely, the last two cycles (October 2010 to September 2012) showed high values for the annual maximum GSI and corresponded to years when SST during winter and spring months was exceptionally warm, compared with the climatic SST averaged over a 30-year period (1973-2002) (www.meteoestartit.cat). There was a high correlation of annual maximal GSI with mean SST during winter and spring months (Fig. 3.2). This suggests that the temperature prevailing during these months (December to May), which corresponded to the period of gonadal recovery and growth after spawning (Fig. 3.4), may be the main factor determining the magnitude of the annual maximum for GSI. Temperature, therefore, can be directly related with the annual variability in the reproductive output of this species.

Our data show that the reproductive behaviour of *Arbacia lixula* can be considerably affected by atypical cold episodes. During the winter of 2009-2010, the North Atlantic Oscillation recorded its lowest values since at least 1950 (Cohen et al., 2010)which caused extremely low temperatures across the Northern Hemisphere. Indeed, the mean SST for March of 2010 (11.8ºC) is

the lowest of all temperatures recorded during our series, and lower than the historical mean over 30 years for this month (12.6ºC). This was reflected not only by a decline in the GSI of that month (Fig. 3.1) but also by a delay in the MI values during the following months of that reproductive cycle (Fig. 3.4). Conversely, the last two cycles correspond to warm years during which steady gonadal growth and maturation were observed, with predominance of mature individuals from May to July, and spawning events in August-September. The earlier spawning of August 2012 (compared with September 2011) could be related to the higher mean temperature during that month, compared with the same month of the previous year.

The inter-annual differences in the GSI-MI association plots (Fig. 3.5) also suggest a critical dependence of the reproductive cycle with temperature. The GSI was tightly correlated with maturation state during the two warmer cycles, attaining higher values for mature individuals, whereas this relationship was considerably weaker (albeit significant) during the coldest 2009-2010 cycle (Table 3.3), when all individuals had GSI values less than 10%.

An interesting question is whether GSI cycles with a sharp peak (such as those observed in the last two cycles of our study) correspond to the normal condition for *Arbacia lixula* reproduction in the northwestern Mediterranean or whether a gonadal cycle with a less-defined peak and low GSI values is the usual reproductive behaviour of the species in this area. It seems likely that a cycle with a sharp GSI peak is the normal condition of *A. lixula* in the

tropical Atlantic, where it originated. But our study area in the northwestern Mediterranean usually attains the coldest SST values of the whole Mediterranean basin. The climatic value for the SST, averaged from 1973 to 2002, for the months of December to May is 13.43ºC. If we compare this historical value with the ones that occurred in the last four years (Fig. 3.2), we can conclude that the average reproductive behaviour of the species during that three-decade period must have been more similar to the two first cycles of our study, and that the last two warmest years displaying sharp GSI peaks have to be considered as the abnormal situation. However, considering the current warming trend in the Mediterranean, these "abnormally high temperatures" could indeed become the rule in the near future, thus boosting the potential fecundity of this thermophilous species.

The reproduction of *Arbacia lixula* in the NW Mediterranean was first studied by Fénaux (1968). In this seminal work, she found that the GSI of *A. lixula* peaked regularly during May-June-July, with a spawning period extending from June to November, according to the presence of larvae in the plankton. She reported a delay between gonad maturation (which was achieved in March-April) and the beginning of spawning, concluding that the gametes would not be released until water temperature was over 20ºC. Thus, she argued that temperature was the main trigger of spawning in *A. lixula*. Our results for the GSI broadly agree with those of Fénaux, but they suggest that photoperiod is the main factor determining the timing of the gonad maturation process, which can nevertheless be considerably affected by temperature

during the growing period. Our histological results showed that low temperatures during winter and spring may impair the gonad maturation process throughout the reproductive cycle, which could probably prevent Mediterranean populations of *A. lixula* from successfully reproducing during cold years, even though the temperature during summer months reaches well over 20ºC.

Most works studying the gonad histology of echinoids assign a single, categorical maturation stage to any individual. This is useful only if the gonad maturation is a uniform process, producing individuals with homogenously matured gonads. However, the intra-individual variability in the maturation state of *A. lixula* gonads is strikingly high. Most individuals show gonadal acini in different maturation stages. For this reason, an integer scalar maturation index would not accurately describe most individuals. Alternatively, each individual is better characterized by a continuous maturation index, obtained from averaging the maturation state of several acini. To avoid problems arising from averaging mature and spent acini or individuals, this continuous index must not be a scalar number, but should be represented instead by a vector in a circular (polar) coordinate system (Spirlet et al., 1998). The use of a circular maturation index is a very powerful tool to analyse the gametogenic cycles of marine invertebrates, which can avoid the inherent problems of using scalar maturation indices to characterize a naturally cyclical process. The comparison among maturation states is straightforward in the circular monthly maturation vectors plots (Fig. 3.4). The polar coordinate system also allows to accurately compare between sexes or among months, and

it is particularly useful for quickly comparing different years. The method also allows to perform *ad hoc* statistical analyses designed for this kind of data. We advocate the use of polar methods for the study of reproduction of iteroparous species, whenever cyclical stages can be defined.

Arbacia lixula also shows remarkable inter-individual variability in its gonadal maturation state. The gonad maturation is highly asynchronous, so that individuals in different maturation states can be found at any given time, throughout most of the year. This behaviour is uncommon among temperate sea urchins, whose reproductive cycles are normally coupled with seasonal processes and thus show highly synchronous gonad maturation. This fact prompted us to increase sample size once we had the results of the first years of study. This asynchronicity and the presence of some mature individuals throughout most of the year could be a conserved trait from the tropical past of the genus *Arbacia*.

The main exogenous factors commonly reported to control the reproductive cycle of echinoids are temperature, photoperiod and food availability. Many works relate reproduction with temperature (Byrne, 1990; Zamora and Stotz, 1992; King et al., 1994; Lozano et al., 1995; López et al., 1998; Ling et al., 2008; Pecorino et al., 2013). It has been also demonstrated that GSI is correlated with food availability in many herbivorous sea urchin species (Fuji, 1960; Ebert, 1968; Pearse, 1981; Fernandez and Boudouresque, 1997; Guillou and Lumingas, 1998; Hernández et al., 2006; Martínez-pita et al., 2008). Thus, gonad index changes may depend on the amount of reserves accumulated rather than the

maturation stage, and therefore the GSI alone would not be a good parameter to assess the gametogenic state of these species. However, *A. lixula* is an omnivore tending to carnivory (Wangensteen et al., 2011) and thus food availability is unlikely to limit gonad growth in the study area at Tossa de Mar, which exhibits well developed sublittoral communities with high productivity throughout the year (Ballesteros, 1988, 1993). Photoperiod is known to control gonadal growth cycles in a variety of sea urchin species (Pearse et al., 1986; Bay-Schmith and Pearse, 1987; McClintock and Watts, 1990; Walker and Lesser, 1998; Kelly, 2001; Shpigel et al., 2004), including the congeneric *Arbacia dufresnii* (Brogger et al., 2010). Our results show that the GSI of *A. lixula* had a remarkable intra-cycle correlation with photoperiod, but the height of the maximum GSI peak was correlated with the mean temperature during the growing period. Thus, the photoperiod predicts "when" and the temperature predicts "how much" the gonads will grow. Our results with *A. lixula* agree with those of Spirlet et al. (2000) in *Paracentrotus lividus*, who suggested that temperature acted as an enhancer of the gametogenic process, but probably not as a trigger signal for the spawning in this species. In their study with *Strongylocentrotus droebachiensis*, Dumont et al. (2006) suggested that, once gametogenesis is initiated by photoperiod triggering, spawning cannot be halted by artificially altering the photoperiod. However, our results show that, in the case of *A. lixula*, the maturation process can be considerably disturbed by anomalous temperatures, which would then be a main modulator for the gametogenic process.

In accordance with its tropical origin, *A. lixula* shows a remarkable increase in its maximum gonadosomatic index with temperature. This may probably boost its reproductive output in the Mediterranean during warm years. This is in agreement with warning reports of increased abundance of *A lixula* in the Mediterranean attributable to increases in temperature (Francour et al., 1994; Harmelin et al., 1995). Given the ongoing global warming trend, *A. lixula* can potentially boost its negative impact (Privitera et al., 2011; Gianguzza et al., 2011) and become a serious threat for Mediterranean shallow rocky ecosystems in the near future. Other thermophilous sea urchins have been proven to be able to cause catastrophic shifts in newly colonized ecosystems as a consequence of climate change (Ling et al., 2009). A preventive monitoring of population densities of *A. lixula* would be desirable in potentially affected shallow water areas.

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CHAPTER 4 - SOME LIKE IT HOT

Arbacia lixula late 4-armed pluteus larva. Photo: **O. S. Wangensteen**

Chapter 4. Some like it hot: temperature and acidification modulate larval development and settlement of the sea urchin *Arbacia lixula*

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ABSTRACT

We studied the effects of temperature and pH on larval development, settlement and juvenile survival of a Mediterranean population of the sea urchin *Arbacia lixula*. Three temperatures (16, 17.5 and 19 ºC) were tested at present pH conditions (pH_T 8.1). At 19 ºC, two pH levels were compared to reflect present (pH_T 8.1) and near-future conditions (pH_T 7.7, expected by 2100). Larvae were reared for 52-days to achieve the full larval development and complete the metamorphosis to the settler stage. We analysed larval survival, growth, morphology and settlement success. We also tested the carry-over effect of acidification on juvenile survival after 3 days. Our results showed that larval survival and size significantly increased with temperature. Acidification resulted in higher survival rates and developmental delay. Larval morphology was significantly altered by low temperatures, which led to narrower larvae with relatively shorter skeletal rods, but larval morphology was only marginally affected by acidification. No carry-over effects between larvae and juveniles were detected in early settler survival, though settlers from larvae reared at lower pH were significantly smaller than their counterparts developed at current pH. These results suggest an overall positive effect of environmental parameters related to global change on the reproduction of *Arbacia lixula*, and reinforce the concerns about the increasing negative impact on shallow Mediterranean ecosystems of this post-glacial colonizer.

KEY WORDS

Echinoidea, pluteus, ocean warming, ocean acidification, Mediterranean, developmental delay, thermophilous sea urchins

RESUMEN

Se estudiaron los efectos de la temperatura y el pH en el desarrollo larvario, asentamiento y supervivencia de los juveniles de una población mediterránea del erizo de mar *Arbacia lixula*. Para ello, se compararon tres temperaturas (16, 17.5 y 19 ºC) y dos niveles de pH que reflejan las condiciones actuales (pH_T 8.1) y las del futuro próximo (pH_T 7.7, previsto para 2100). Las larvas se cultivaron durante 52 días para lograr el desarrollo larvario completo y completar la metamorfosis hasta el estado de asentados. Se analizaron la supervivencia larvaria, el crecimiento, la morfología y la tasa de asentamiento. También se estudió el posible efecto remanente de la acidificación durante el período larvario en la supervivencia a 3 días de los juveniles. Nuestros resultados mostraron que la tasa de supervivencia y el tamaño de las larvas se incrementaron significativamente al aumentar la temperatura. La acidificación se tradujo en mayores tasas de supervivencia y un ligero retraso en el desarrollo. Las temperaturas bajas produjeron alteraciones significativas en la morfología larvaria, dando lugar a larvas más pequeñas, más estrechas y con las varillas esqueléticas relativamente más cortas. Por el contrario, la acidificación prácticamente no produjo diferencias en las variables morfológicas. No se detectaron efectos remanentes de la exposición larvaria a

condiciones ácidas en la supervivencia a corto plazo de los asentados, aunque los asentados procedentes de larvas cultivadas a menor pH fueron significativamente más pequeños que sus equivalentes cultivados al pH actual. Estos resultados sugieren un efecto positivo de los parámetros ambientales relacionados con el cambio global en la reproducción de *Arbacia lixula*, y refuerzan la creciente preocupación por el posible impacto negativo de este colonizador post-glacial en los ecosistemas mediterráneos someros.

PALABRAS CLAVE

Echinoidea, pluteus, calentamiento oceánico, acidificación del océano, Mediterráneo, retraso en el desarrollo, erizos termófilos

INTRODUCTION

Global changes due to increased atmospheric CO_2 emissions are altering ocean ecosystems, though there is considerable uncertainty about the spatial and temporal details (Hoegh-Guldberg and Bruno, 2010). Major physicochemical changes in marine ecosystems come in two different ways: ocean warming and ocean acidification. In the Mediterranean Sea, long-term datasets have revealed temperature increases of 0.8–1.4 ºC over the last 30 years (Lejeusne et al., 2010 and references therein) and a further 2 ºC increase is expected by 2100 (Intergovernmental Panel on Climate Change, 2007; Meehl et al., 2007). On the other hand, the average pH of surface seawater has declined worldwide by approximately 0.1 units since the industrial revolution and future reductions are expected to be around 0.3–0.5 units by 2100 (Caldeira and Wickett, 2003, 2005; Royal Society, 2005).

Much research effort has been devoted to elucidate the effects of ocean acidification on the development of echinoderms (see, e.g., reviews by Kurihara, 2008, Dupont et al., 2010c; Dupont and Thorndyke, 2013). Some species show a clear impairment when their larvae are grown at lowered pH conditions, either as increased mortality (e.g. *Ophiothrix fragilis* Dupont et al., 2008), as delayed development (e.g. *Lytechinus pictus* O'Donnell et al., 2010, *Strongylocentrotus purpuratus* Stumpp et al., 2011) or as developmental malformations (e.g. *Sterechinus neumayeri* Byrne et al., 2013). But in many other species the effects are neutral or undetectable (e.g. *Arbacia punctulata* Carr et al., 2006, *Heliocidaris erythrogramma* Byrne et al., 2009, *Paracentrotus lividus* Martin et

al., 2011, *Arbacia dufresnei* Catarino et al., 2011, *Strongylocentrotus droebachiensis* Dupont et al., 2012), and a few species may even show enhanced development when grown at moderate levels of acidification (e.g. *Crossaster papposus*, Dupont et al., 2010b). Thus, with some exceptions, echinoderm larvae have shown to be robust to mild acidification (Dupont et al., 2010c).

Only a few previous works have studied the combined effects of increased temperature and ocean acidification on echinoderm larvae (Sheppard Brennand et al., 2010; Ericson et al., 2011; Foo et al., 2012; Nguyen et al., 2012; Gianguzza et al., 2013b; Padilla-Gamiño et al., 2013) and all of them were limited to the first stages of early endotrophic development (2 to 3 days exposure). From this limited dataset, it appears that interaction between temperature and ocean acidification is complex, from temperature being the main driver of change to temperature amplifying or diminishing the negative effects of ocean acidification. Gianguzza et al. (2013b) showed that temperature and pH had no significant effect on fertilization and larval survival (up to 2 days) of *Arbacia lixula* for temperatures <27 °C. However, both temperature and pH had effects on the developmental dynamics. Temperature appeared to modulate the impact of decreasing pH on the % of larvae reaching the pluteus stage, so a positive effect of low pH (faster growth as compared to pH 8.2) was found at 20°C, a neutral effect at 24°C and a negative effect (slower growth) at 26°C.

The black sea urchin *Arbacia lixula* (Linnaeus, 1758) is currently one of the most abundant sea urchins in the Mediterranean

(Palacín et al., 1998b; Benedetti-Cecchi et al., 1998; Hereu et al., 2012) and tropical Eastern Atlantic (Hernández et al., 2013). It is recognized as a thermophilous species of tropical affinities (Stefanini, 1911; Mortensen, 1935; Tortonese, 1965) which probably spread through the Mediterranean in the Upper Pleistocene (Wangensteen et al., 2012) where it may encounter suboptimal temperature conditions. Thus, it is a candidate species to be favoured by increased temperatures due to global change. *A. lixula* is an omnivore tending to carnivory (Wangensteen et al., 2011) which has a high potential to impact shallow rocky areas by originating or maintaining barren zones (Guidetti et al., 2003; Bonaviri et al., 2011). Despite its increasingly recognized ecological importance (e.g.: Bulleri et al., 1999; Guidetti et al., 2003; Bonaviri et al., 2011; Privitera et al., 2011; Gianguzza et al., 2011), it has been traditionally understudied compared with the sympatric edible sea urchin *Paracentrotus lividus* and its actual potential to modify shallow rocky ecosystems may be currently underestimated.

Arbacia lixula has undergone population increases in the past (Petit et al., 1950; Boudouresque et al., 1989; Francour et al., 1994; Harmelin et al., 1995). Its reproductive potential in the Mediterranean may be boosted by increasing temperature (Gianguzza et al., 2011, Chapter 3) and some results suggest that their larval survival may also increase with temperature (Privitera et al., 2011), supporting the view that their populations in the Mediterranean could be presently constrained by larval mortality due to low temperatures or to phytoplankton shortage and may then

benefit from ocean warming.

In this work, we studied the effect of temperature and acidification on the development (survival, growth, morphology and settlement success) of larvae from a northwestern Mediterranean population of *Arbacia lixula*. We also studied the carry-over effect of acidification on the 3-day survival of the settlers.

MATERIALS AND METHODS

Adult sea urchins collection

Adult *Arbacia lixula* individuals were collected by SCUBA diving at Tossa de Mar (NE Spain, 41º43'16" N, 2º56'24" E) in September 2012, kept in a 10 L plastic tank with seawater aerated by oxygen tablets and transported by airplane within 24 h to the Sven Lovén Centre for Marine Sciences - Kristineberg (Sweden). Induced spawning and *in vitro* fecundation were carried out shortly upon arrival.

In vitro fecundation and larval cultures

All filtered seawater (FSW) used in the experiments was supplemented with sea salts to achieve a salinity of 38 (comparable to Mediterranean water). Spawning was induced by intracoelomic injection of 1 mL of 0.5 M KCl in FSW. Seven females and one male were used for the fecundation. Eggs were collected in FSW, and sperm was collected dry and kept on ice until use. The number of eggs was estimated as the average of five counts of 50 µL of a 1

L egg dilution. Sperm stock solution in FSW was added to a final concentration of ~ 1,000 sperm mL^{-1}, allowing a fertilization success >80%. After fertilization, embryos were rinsed with FSW, after 2 hours they were aliquoted and inoculated in 5-L bottles filled with FSW at a density of 6000 embryos L^{-1} and the desired temperature and pH. Bottles were maintained in chambers with controlled temperature and continuously aerated to maintain oxygen concentrations close to air saturation by the slow convective current of a stream of single bubbles (~ 60 bubbles min^{-1}).

In the northwestern Mediterranean, the planktotrophic *A. lixula* larvae may be found in the water column between June and November and can be exposed to a wide range of temperatures (15 to 24°C; Fénaux, 1968; Pedrotti, 1993). Nevertheless, Pedrotti's (1993) results suggest that the highest planktonic concentrations occur in October-November, when the temperature ranges from 16 to 19 ºC. We compared four different scenarios: (i) Treatment I (16 ºC, pH_T 8.1), corresponding to the lower range of the present temperature variability; (ii) Treatment II (17.5 ºC, pH_T 8.1), an intermediate temperature; (iii) Treatment III (19 ºC, pH_T 8.1), corresponding to the higher range of temperature presently experienced by the autumnal larvae; (iv) Treatment IV (19 ºC, pH_T 7.7), corresponding to near-future ocean acidification scenario. Two replicates were used per treatment.

After three days, larvae were fed daily with the cryptophyte algae *Rhodomonas* sp., which were raised in B1 medium (Guillard and Ryther, 1962) at 20 ºC under a 12:12 h light:dark cycle. Algal strains were provided by the Marine Algal Culture Centre at

Gothenburg University (GUMACC). The carbon content of the algae was estimated based on volume measurements as equivalent spherical diameter with an electronic particle analyzer (Elzone 5380, Micrometrics, Aachen, Germany) and equations provided by Mullin et al. (1966). Algae concentration and size were checked daily using the same analyzer and then adjusted in the experimental bottles to a concentration of 150 μg C L^{-1}. The FSW of all cultures was changed twice a week, coinciding with chemistry measurements (see below). Larval densities were monitored daily for the first 15-day post-fertilization, and every second day thereafter until day 36. Every sampling day, four subsamples of 10 mL of each replicate were counted. Density at time t (N_t, number of larvae L^{-1}) was estimated as the mean of this four measures. Daily survival (SUR) was calculated as: $SUR = (N_t/N_0)*100$. Cultures were run until day 52 in order to get settlers to be used in the following experiment, except Treatment II, which was discontinued at day 26 due to logistical issues.

Larval morphology measures

For each treatment, 10 larvae, fixed in buffered 4% paraformaldehyde in FSW, were photographed every two days (2 to 8 days post-fertilization) or every three days (11 to 20 days post-fertilization) using a digital camera mounted on a dissecting microscope with polarized light to visualize the skeleton. Six morphometric lengths: body length (BL), body width (BW), body rod lengths (right BRR and left BRL) and post-oral rod lengths (right POR and left POL) were measured for each larva (Fig. 4.1)

using ImageJ 1.46r image analyzing software (Schneider et al., 2012). An asymmetry index (ASY) was calculated as the ratio between the shortest and the longest maximum total length (MTL=BR+PO at each side of the body).

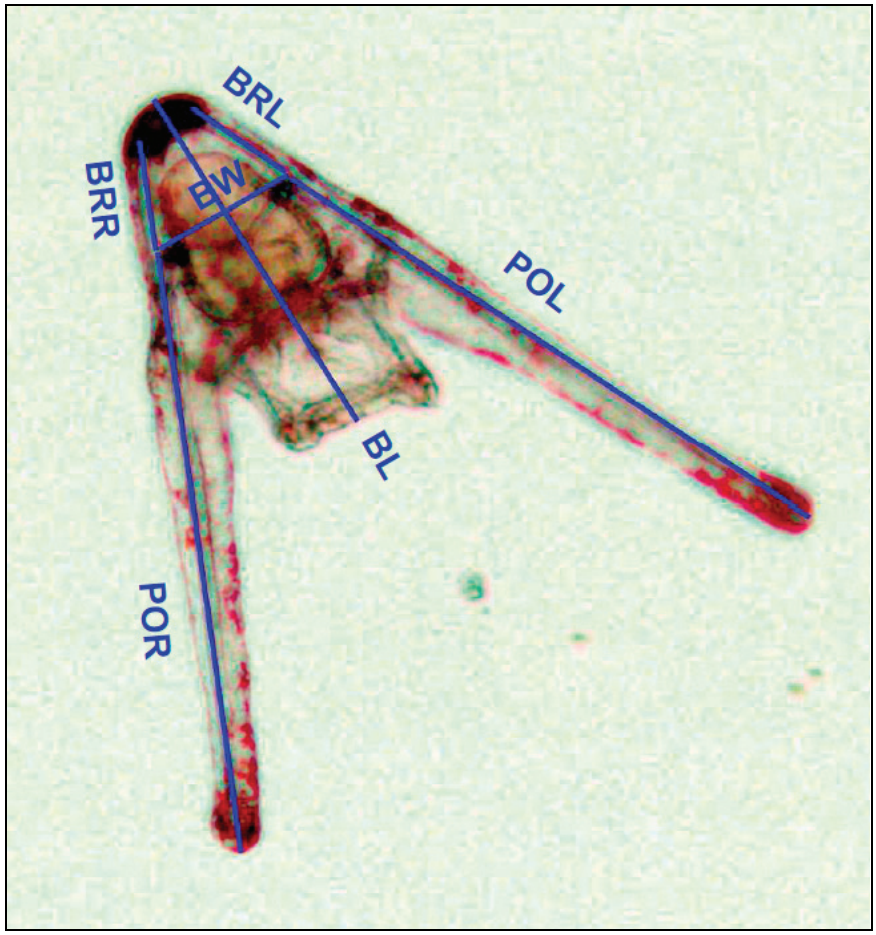

Fig. 4.1. Measured distances for the morphological study of *Arbacia lixula* pluteus larvae. BL: body length. BW: Body width, BRL & BRR: Body rods lengths (left and right); POL & POR: Post-oral rods lengths (left and right)

Experiments with settled post-larvae

After 40-42 days of culture, settlers appeared spontaneously in the experimental bottles kept at 19 ºC, both at pH_T 8.1 and pH_T 7.7. Living settlers were then recovered and the test diameter of 30 individuals from each treatment was measured. A survival experiment was performed in order to test the effect of pH on the survival of the settlers. For this experiment, we used a crossed design (pH during larval growth x pH during settler growth) with settlers grown at pH_T 8.1 or 7.7, transferred to plastic plates with 3-mL wells and kept in filtered seawater at 19 ºC and pH_T 8.1 or 7.7. We used three replicates for each treatment, with 18 settlers (6 wells; 3 individuals per well) per replicate (a total of 54 settlers per treatment). After three days, we counted the settlers which remained alive and calculated the survival rate as the % of surviving juveniles.

Seawater chemistry

Temperature was monitored daily. Total alkalinity (A_T) and pH_T were measured twice a week. A_T was determined on filtered samples with a titration system (TitroLine alpha plus, SI Analytics). pH_T (henceforth "pH") was measured with a Metrohm 827 pH-electrode adjusted for pH measurements at the total scale using Tris/HCl and 2-aminopyridine/HCl buffer solutions (provided by Unité d'Océanographie Chimique, Université de Liège, Belgium). Total carbon (C_T) and the carbonate system speciation (p_{CO2}, Ω_{Ca} and Ω_{Ar}) were calculated from temperature, pH and A_T using CO2CALC (Robbins et al., 2010), an application based on CO2SYS

(Lewis and Wallace, 1998), using the dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987). pH was maintained in each experimental bottle using a computerized feedback system (AquaMedic) that regulated pH by addition of pure gaseous CO_2 directly into the seawater (±0.02 pH units).

Statistical analyses

One-way ANOVA followed by SNK post hoc test was used to confirm that differences between measured temperatures and pH were as expected between the four treatments.

The effects of temperature and pH on larval size (BL) and on survival rate (SUR) at a given time of culture (TOC) were tested using separate ANCOVAs for each factor, to avoid problems arising from our not fully crossed experimental design; TOC (Ln-transformed) was the covariate. The following lineal model was used for each variable Y, where Y represents the dependent variable (BL or SUR) and X represents the factor (either temperature or pH): $Y = \mu + \beta_1 \text{Ln(TOC)} + \beta_2 X + \beta_3 \text{Ln(TOC)} \times X + \beta_4 \text{Replicate(X)}$. X was considered as a fixed factor and the replicate (bottle) was nested within it. Similar linear models were used to assess the effects of the two physicochemical factors in the relations between SUR and BL as a covariate (also Ln-transformed).

The effects of temperature and pH in the morphological variables of the larvae were also tested separately using BL as a covariate. Linear regressions (not shown) were used for each experimental treatment to check the linearity of the relationships between morphological variables and BL. The following lineal

model was used for each variable Y, where Y represents a morphological variable and X represents either temperature or pH: $Y = \mu + \beta_1 BL + \beta_2 X + \beta_3 BL \times X + \beta_4 Replicate(X)$.

The survival curves for the larvae were considered to be derived from a hazard function following a 2-parameter Weibull distribution (Cox and Oakes, 1984). Thus, the ratio of surviving larvae (SUR) at a given TOC, is given by $SUR = \exp(-\lambda \cdot TOC^{\beta})$, where λ is the scale parameter and β is the shape parameter. We calculated both parameters separately for every replicate using non-linear least-squares regressions (Bates and Watts, 1988), and pooled the replicates for each treatment, after verifying the absence of significant differences.

Differences in the diameter of settlers derived from larvae reared under normal or acid pH were tested using a t-test and differences in settler survival were tested using one-way ANOVA. Homogeneity of variances and normality of residuals were tested in all models using the Bartlett and Shapiro-Wilk tests respectively. All statistical analyses were performed in R using the RStudio interface (RStudio Inc., Boston, MA, USA).

RESULTS

Physico-chemical variables

The experimental means and standard deviations of the measured physico-chemical parameters for the four treatments are summarized in Table 4.1. As expected, ANOVA followed by SNK post hoc test found significant differences for temperatures between treatments I, II and III (all $P < 0.001$) but not between treatments III and IV ($P = 0.67$). Concerning pH, ANOVA followed by SNK found no differences between treatments I, II and III (all $P > 0.33$), whereas treatment IV was significantly different from the former three treatments (all $P < 0.001$).

Table 4.1. Physico-chemical variables measured in the four experimental treatments (mean ± SD). Partial pressure of carbon dioxide (p_{CO2}), total dissolved inorganic carbon (C_T) and calcium carbonate saturation state for calcite and aragonite (Ω_{Ca}, Ω_{Ar}) were calculated from temperature, pH_T and total alkalinity (A_T)

Treatment	*T (ºC)*	*pH_T*	*A_T (µmol/kg)*	*p_{CO2} (µatm)*	*C_T (µmol/kg)*	*Ω_{Ca}*	*Ω_{Ar}*
I. 16 ºC pH 8.1	16.3±0.4	8.09±0.05	2638±39	547±79	2403±56	4.13±0.38	2.67±0.24
II. 17.5 ºC pH 8.1	17.5±0.3	8.08±0.03	2637±78	548±53	2384±77	4.45±0.29	2.88±0.19
III. 19 ºC pH 8.1	18.8±0.3	8.09±0.04	2630±44	548±60	2379±53	4.42±0.27	2.87±0.17
IV. 19 ºC pH 7.7	18.8±0.3	7.69±0.04	2658±61	1575±153	2590±56	1.95±0.15	1.27±0.10

Larval growth and survival

The variation over time of larval size at different temperatures and pH is displayed in Fig. 4.2 and the ANCOVAs are listed in Table 4.2. No significant differences between replicates were found for any variable throughout all analyses, so replicates have been pooled for clarity in the graphical representations. The larval size, measured as body length (BL) grew significantly faster with increasing temperatures (treatments I, II and III, Table 4.2a). The effect of a pH decrease from 8.1 to 7.7 at 19 ºC produced no appreciable difference in BL during the first eight days of culture, but originated significantly smaller larvae from then on (treatments III and IV, Table 4.2b).

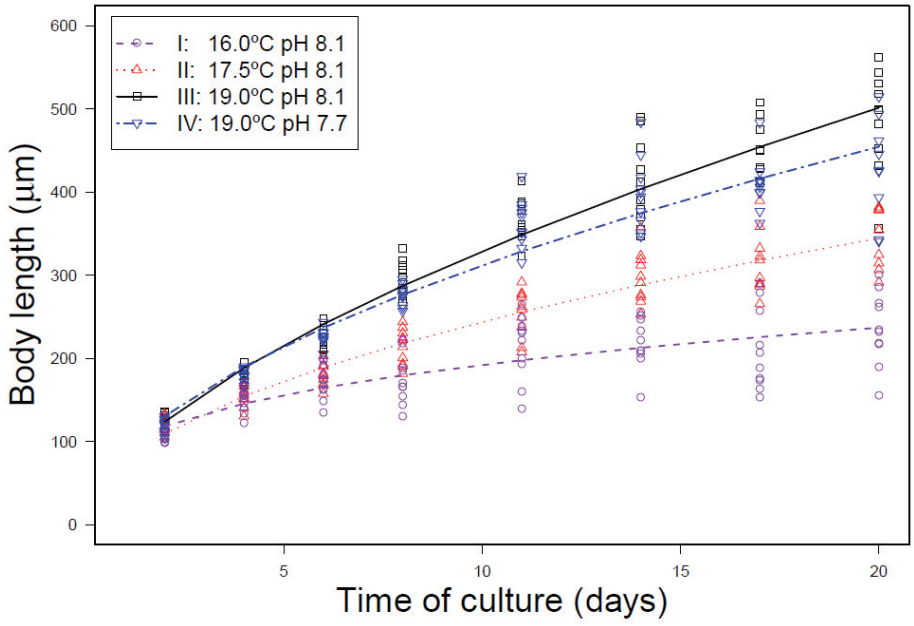

Fig. 4.2. Effect of temperature and pH on individual growth (body length) of *Arbacia lixula* larvae. Since no differences were found between replicate cultures, replicates have been pooled for clarity. Trends are indicated by curves adjusted to potential equations $BL = a(TOC)^b$

Table 4.2. Analysis of covariance testing the effects of temperature (a) and pH (b) on *Arbacia lixula* larval growth. BL: body length, TOC: time of culture, T: temperature.

a. BL ~ Ln(TOC) + T + Ln(TOC) x T + Replicate(T)			
Source	**d.f.**	***F***	***P***
Ln(TOC)	1	1219.23	< 0.0001
T	2	299.62	< 0.0001
Ln(TOC) x T	2	115.90	< 0.0001
Replicate(T)	3	0.06	0.98
Residuals	230		
b. BL ~ Ln(TOC) + pH + Ln(TOC) x pH + Replicate(pH)			
Source	**d.f.**	***F***	***P***
Ln(TOC)	1	1475.94	< 0.0001
pH	1	9.17	0.0031
Ln(TOC) x pH	1	6.68	0.011
Replicate(pH)	2	2.00	0.14
Residuals	153		

The survival curves are shown in Fig. 4.3 for the four treatments tested. The results of the ANCOVAs are listed in Table 4.3. Temperature increase from 16 to 19 ºC had a positive significant effect on larval survival (Table 4.3a). The effect of pH on survival was more complex, as reflected by the significant Ln(TOC) x pH interaction of the ANCOVA (Table 4.3b). The survival was similar at pH 8.1 and 7.7 during the first 14 days, but it was significantly higher from then on at the lower pH. The significant ANCOVAs of survival rate (SUR) with BL as covariate suggest that the differences in survival can be ascribed to the effects of temperature (Table 4.3c) and pH (Table 4.3d), and are not attributable to a hidden effect of body length due to developmental delay (in which case survival at given BL values would be the same

irrespective of treatment). The significant Ln(BL) x pH interaction (Table 4.3d) reflects the fact that at smaller sizes the survival rate was higher at pH 8.1, but at bigger sizes the survival rate was higher at pH 7.7.

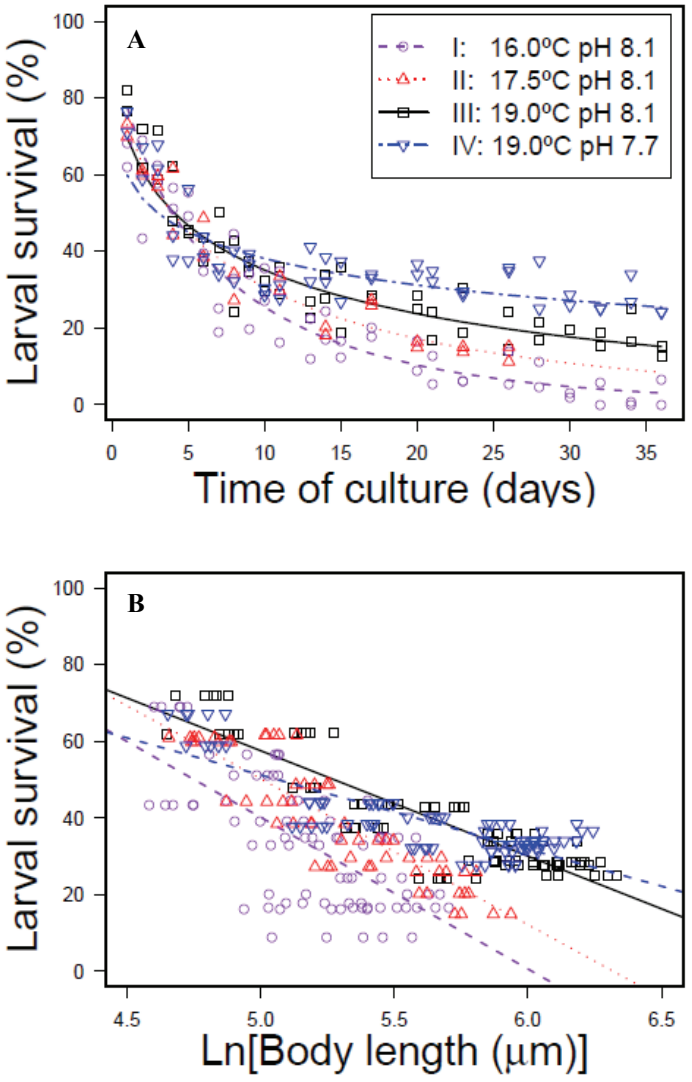

Fig. 4.3. Survival curves for *Arbacia lixula* larvae cultured at different temperatures and pH in function of time of culture (A) or body length (B). The interpolation curves in A were calculated assuming hazard functions following a Weibull distribution. Replicates have been pooled for clarity

Table 4.3. Analysis of covariance for *Arbacia lixula* larvae survival data. SUR: Survival rate, TOC: Time of culture, T: Temperature, BL: Body length

a. SUR ~ Ln(TOC) + T + Ln(TOC) x T + Replicate(T)			
Source	**d.f.**	***F***	***P***
Ln(TOC)	1	849.33	< 0.0001
T	2	20.71	< 0.0001
Ln(TOC) x T	2	1.11	0.33
Replicate(T)	3	0.93	0.43
Residuals	102		
b. SUR ~ Ln(TOC) + pH + Ln(TOC) x pH + Replicate(pH)			
Source	**d.f.**	***F***	***P***
Ln(TOC)	1	420.62	< 0.0001
pH	1	4.69	0.033
Ln(TOC) x pH	1	15.98	0.00014
Replicate(pH)	2	1.16	0.32
Residuals	82		
c. SUR ~ Ln(BL) + T + Ln(BL) x T + Replicate(T)			
Source	**d.f.**	***F***	***P***
Ln(BL)	1	283.90	< 0.0001
T	2	192.07	< 0.0001
Ln(BL) x T	2	13.70	0.0003
Replicate(T)	3	0.52	0.47
Residuals	230		
d. SUR ~ Ln(BL) + pH + Ln(BL) x pH + Replicate(pH)			
Source	**d.f.**	***F***	***P***
Ln(BL)	1	490.20	< 0.0001
pH	1	1.37	0.24
Ln(BL) x pH	1	14.45	0.0002
Replicate(pH)	2	2.17	0.14
Residuals	153		

The calculated values for the parameters of the hazard functions for the four different treatments are listed in Table 4.4. The values of the shape parameter β were < 1 in all cases, showing that the survival curves departed from the exponential function. That is, the hazard rates were not constant and were higher during

the first days of development. The hazard rate variation was most apparent in the pH 7.7 treatment ($\beta = 0.338 \pm 0.035$).

Larval morphology

The variation of larval morphology (allometry) using body length as covariate at different temperatures and pH is summarized in Fig. 4 and the results of the ANCOVAs for the studied variables are listed in Table 4.5.

Table 4.4. Calculated values for the parameters of the hazard functions (Weibull distributions) describing the survival of *Arbacia lixula* larvae raised at different temperature and pH. SSR: sum of squared residuals of the nonlinear regression. The survival function against time of culture can be modelled by $SUR = \exp(-\lambda \cdot TOC^{\beta})$

Treatment	λ ($day^{-\beta}$)	β	*SSR*	R^2
I: 16.0ºC pH 8.1	0.304 ± 0.034	0.642 ± 0.050	0.223	0.87
II: 17.5ºC pH 8.1	0.313 ± 0.025	0.572 ± 0.035	0.050	0.95
III: 19.0ºC pH 8.1	0.301 ± 0.026	0.531 ± 0.035	0.149	0.89
IV: 19.0ºC pH 7.7	0.434 ±0.039	0.338 ±0.035	0.200	0.72

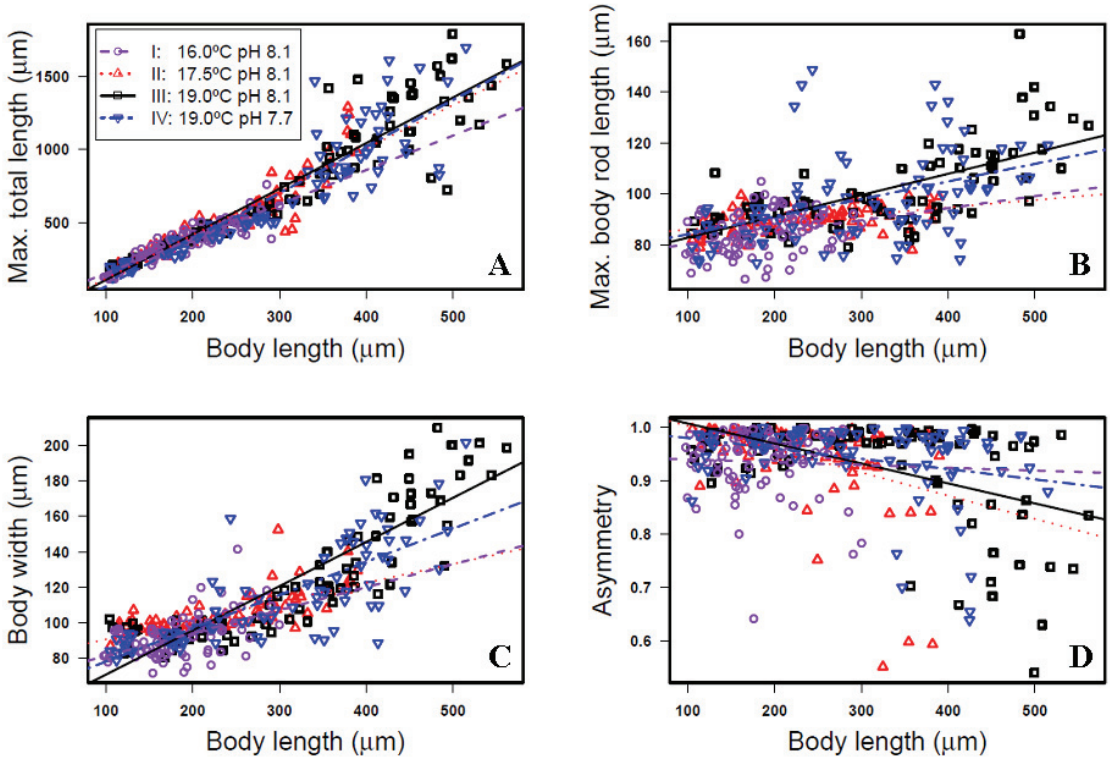

Fig. 4.4. Morphological variables of *Arbacia lixula* larvae grown at different conditions of temperature and pH. Maximum total length (A), maximum body rod length (B), body width (C) and asymmetry index (D) plotted against body length. Since no differences were found between replicate cultures, replicates have been pooled for clarity

Changes in temperature affected significantly all the morphological variables studied (Tables 4.5a, 4.5c, 4.5e and 4.5g). Maximum total length (Fig. 4.4A) varied similarly with body length for treatments II, III and IV, but a significant BL x T interaction indicates that, in treatment I (16 ºC), larvae tended to have significantly smaller post-oral rods when reaching BL > 250 µm.

The variation of body rod length (Fig. 4.4B) and of body width (Fig. 4.4C) with body size was similar at 16 and 17.5 ºC, but was significantly different at 19 ºC, implying that larvae grown at the higher temperature were relatively wider and with longer body rods than those grown at colder temperatures, for similar values of BL. All BL x T interaction terms were significant for these variables, thus the observed effects of temperature on larval morphology were complex and changing over the size range.

Conversely, the effects of pH were nonsignificant for almost all morphological variables (Tables 4.5b, 4.5d and 4.5h), and thus larvae grown at 19 ºC had the same overall morphology independently of pH, except for a significant BL x pH interaction effect on body width (Table 4.5f). Larvae grown at pH 8.1 and 19 ºC tend to grow wider than those reared at pH 7.7 and the same temperature, when BL > 400 μm.

The asymmetry index showed a high degree of dispersion for BL > 150 μm (Fig. 4.4D) and these results (a slightly significant effect of temperature, Table 5g) must then be taken with caution.

Fig. 4.5 graphically compares the size and morphology of average larvae reared using the four different treatments at two different times. Overall, we found developmental delay in all treatments when compared to pH 8.1 and 19 ºC. The growth rate and morphology of the larvae was remarkably affected by changes in temperature, but the effects of pH change were subtler and almost all the morphological differences between treatments III and IV may be attributable to the delay in the development.

Table 4.5. Analysis of covariance for *Arbacia lixula* larval morphology against body length and temperature or pH. BL: body length, T: temperature, MTL: maximum total length, MBR: maximum body rod length, BW: body width, ASY: asymmetry index

a. MTL ~ BL+ T + BL x T + Replicate(T)				**b. MTL ~ BL+ pH + BL x pH + Replicate(pH)**			
Source	**d.f.**	***F***	***P***	**Source**	**d.f.**	***F***	***P***
BL	1	1951.94	< 0.0001	BL	1	779.96	< 0.0001
T	2	1.34	0.26	pH	1	1.19	0.28
BL x T	2	3.90	0.03	BL x pH	1	0.12	0.73
Replicate(T)	3	2.24	0.09	Replicate(pH)	2	1.29	0.28
Residuals	230			Residuals	153		
c. MBR ~ BL+ T + BL x T + Replicate(T)				**d. MBR ~ BL+ pH + BL x pH + Replicate(pH)**			
Source	**d.f.**	***F***	***P***	**Source**	**d.f.**	***F***	***P***
BL	1	251.42	< 0.0001	BL	1	70.80	< 0.0001
T	2	13.16	< 0.0001	pH	1	0.54	0.46
BL x T	2	7.79	0.0005	BL x pH	1	0.68	0.41
Replicate(T)	3	0.80	0.50	Replicate(pH)	2	0.62	0.53
Residuals	230			Residuals	153		
e. BW ~ BL+ T + BL x T + Replicate(T)				**f. BW ~ BL+ pH + BL x pH + Replicate(pH)**			
Source	**d.f.**	***F***	***P***	**Source**	**d.f.**	***F***	***P***
BL	1	801.67	< 0.0001	BL	1	390.67	< 0.0001
T	2	3.35	0.037	pH	1	3.83	0.052
BL x T	2	27.42	< 0.0001	BL x pH	1	7.44	0.007
Replicate(T)	3	0.36	0.78	Replicate(pH)	2	0.39	0.68
Residuals	230			Residuals	153		
g. ASY ~ BL+ T+ BL x T + Replicate(T)				**h. ASY ~ BL+ pH + BL x pH + Replicate(pH)**			
Source	**d.f.**	***F***	***P***	**Source**	**d.f.**	***F***	***P***
BL	1	37.65	< 0.0001	BL	1	32.01	< 0.0001
T	2	3.81	0.02	pH	1	0.60	0.44
BL x T	2	1.86	0.16	BL x pH	1	2.65	0.11
Replicate(T)	3	0.94	0.42	Replicate(pH)	2	1.26	0.29
Residuals	230			Residuals	153		

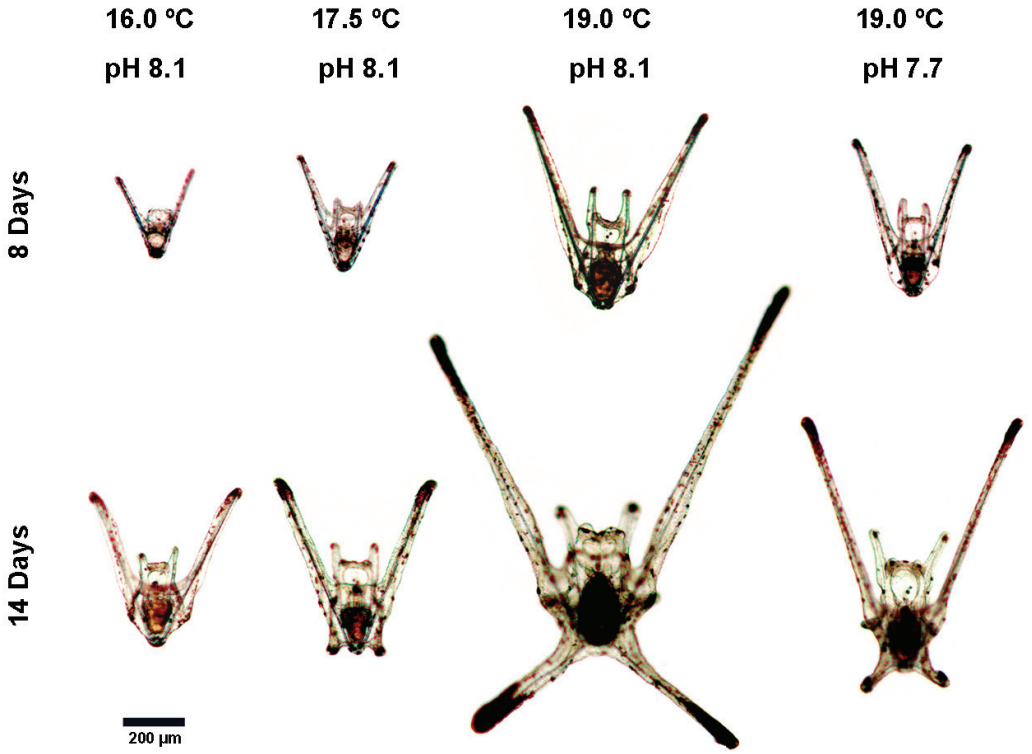

Fig. 4.5. Typical morphology and size of *Arbacia lixula* larvae grown under different conditions, after eight (upper row) or fourteen (lower row) days of culture. The four treatments tested are shown

Settlers count, size and survival

The first settlers appeared at day 40-42 in the cultures at 19 ºC, both at pH 8.1 and 7.7, whereas only a few settlers appeared at day 48-50 in the cultures at 16 ºC. These cultures were stopped at day 52 and all the living settlers were counted. Overall, we obtained 480 ± 341 (mean ± SE) settlers in the cultures at 19 ºC and pH 8.1, 149 ± 117 settlers in the cultures at 19 ºC and pH 7.7 and only 12 ± 12 settlers in the cultures at 16 ºC. The settlers reared at 19 ºC and pH 8.1 had diameters of 489 ± 5 µm (mean ± SE) and were

significantly bigger (t_{58} = 6.62; p < 0.0001) than those reared at pH 7.7 (diameter = 433 ± 7 µm; Fig.4.6).

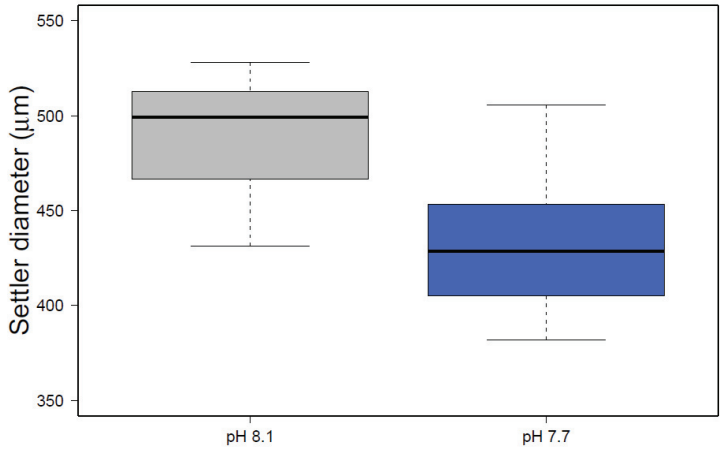

Fig. 4.6. Diameter of early settlers (n=30) reared from *Arbacia lixula* larvae grown at pH 8.1 or pH 7.7

The survival experiment was carried out using only settlers grown at 19 ºC, in pH 8.1 ot 7.7 (treatments III and IV), which were transferred to FSW at 19 ºC and pH 8.1 or 7.7 (all combinations) and cultured for three days. The survival rate did not differ between the four treatments (ANOVA F=2.43, P = 0.14; Fig. 4.7).

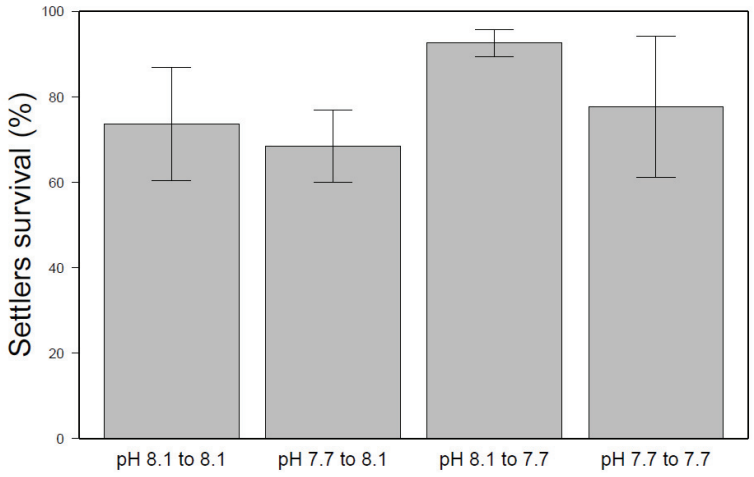

Fig 4.7. Effect of water acidification on the survival of *Arbacia lixula* settlers reared from larvae grown at pH 8.1 or 7.7 and then transferred to either pH 8.1 or 7.7 after settlement. No significant differences between treatments were found.

DISCUSSION

The main conclusion arising from our results is that temperature is a key factor affecting the developmental timing and survival rate of *Arbacia lixula* larvae (a temperature increases from 16 to 17.5 to 19 ºC improved their survival and accelerated their growth), whereas a moderate drop in pH (such as that predicted for 2100) affected the development only to a lesser degree.

Nevertheless, our results show that *A. lixula* larvae can be cultured and complete their development at temperatures between 16 and 19 ºC, though the survival curve showed quite elevated mortality rates, especially during the first days of culture. The advantage of using Weibull distributions to describe the survival curve is their flexibility for modelling both increasing and decreasing hazard functions, depending on the value of the shape

parameter β. All values obtained for β in our study were smaller than 1 (Table 4.3), implying that the hazard functions decreased over time; that is, in the conditions of our experiments, the larval mortality was higher during the first days of the development and it diminished over time. Also, the parameter β showed a clear trend to decrease with warming (Table 4.3), which suggests that the mortality remained more constant over time at low temperatures.

Gianguzza et al. (2013b) reported that mild acidification could have a positive effect in the early developmental dynamics (two days) of *A. lixula* larvae raised at 20 ºC. Our results did not detect any positive effect of lowered pH on the growth rate of the early larvae, but showed that a decrease of pH from 8.1 to 7.7 led to an enhancement of survival rate of the larvae in the long-term. Actually, the difference with the survival at natural pH increased over time, as reflected by the low value of parameter β, the shape of the survival curve (Fig. 4.3A) and the significant Ln(TOC) x pH interaction term in the ANCOVA (Table 4.3b). However, this increase in the survival rate by lowered pH is accompanied by a significant decrease in body length (Fig. 4.2) and body width (Fig. 4.4C).

The overall shape of *A. lixula* larvae was remarkably affected by changes in temperature (Fig. 4.5). Lower temperatures produced smaller larvae (Fig. 4.2) with relatively shorter post-oral and body rods (Fig 4.4A, 4.4B) and narrower bodies (Fig. 4.4C). These morphological changes associated with temperature cannot be attributed to a hidden effect due to developmental delay (Table 4.5). Conversely, pH affected larval morphology to a lesser degree

(Table 4.5), and only the body width showed some dependence of pH (Table 4.5f).

Our results also demonstrate that, despite the significant differences in body size, the survival of early settlers of *Arbacia lixula* is resilient against changes induced by slight acidification, either if exposed to it as larvae, as settlers, or both. No significant difference in the settler survival after 3 days was found between treatments. Only one previous work (Dupont et al. 2012) studied the possible carry-over effect of ocean acidification from sea urchin (*Strongylocentrotus droebachiensis*) larvae to settlers. Their results with this cold water species are not in good agreement with our results with *A. lixula*. They found that the combined exposition to pH 7.7 during larval development, continued as settlers, led to a higher mortality than that observed in individuals exposed to pH 8.1 as larvae, as settlers, or as both. These experiments were run for 3 months and the settlers were fed, which could explain the differences with our results. The difficulty to find a suitable food source for *Arbacia lixula* settlers prevented us from running a longer survival experiment. Further research is needed to produce robust evidence, as settlers are probably one of the most sensitive life-history stages to ocean acidification (Dupont and Thorndyke, 2013).

George et al. (1990) cultured Mediterranean *A. lixula* larvae at 22 ºC and achieved metamorphosis at 26-30 days after fertilization. Their results also suggest the existence of natural variability in developmental growth rates, depending on the initial quality of the eggs (egg size and protein and lipids content). In our

experiments, the first settlers appeared at days 40-42 at 19 ºC and at days 48-50 at 16 ºC. Thus, temperature may be a main factor affecting the developmental time of *A. lixula* in natural environments.

Another recent work studied the culture and settlement of *A. lixula*. Privitera et al. (2011) reported that larvae from Genoa populations cultured at 18 ºC suffered 100% mortality at 7 days, while the same larvae reared at 22 ºC survived and reached the competent stage at approximately 20 days. Our results show that *A. lixula* larvae from northwestern Mediterranean are indeed able to develop at lower temperatures, down to 16 ºC, and even achieve metamorphosis and reach the settler stage, albeit with reduced survival and slower growth. This discrepancy in the results may arise from differences in the culture methods (container volume, algal species, feeding dose and timing, sterilization of FSW by autoclaving or the use of agitation by swinging paddles), since it is hardly attributable to genetic differences between Ligurian and Catalan populations (Wangensteen et al. 2012).

On the other hand, Gianguzza et al. (2013b) recently studied the development of *A. lixula* during the early endotrophic stages (up to 2 days) using temperatures from 20 to 27 ºC at two different pH values. They reported an interesting interaction between pH and temperature. Thus, slightly acidic pH accelerated growth at 20 ºC, while it has a neutral effect at 24 ºC and a negative effect at 26 ºC. Our results showing enhanced survival rates using pH 7.7 at 19 ºC are in accordance with a positive effect of slight acidification for *A. lixula* at temperatures around 20 ºC, but we found a detectable

enhanced survival rate only after approximately 14 days of culture and this change was concurrent with developmental delay.

Delay in the development is the most documented effect of ocean acidification on echinoderm larvae, with 16 out of 19 tested species showing some degree of retarded development (Dupont and Thorndyke, 2013). More sophisticated experiments have to be conducted in order to test the outcomes of this delay in natural ecosystems. It can be argued that larvae suffering arrested growth would have to develop for longer time and thus be more vulnerable to predation, drastically affecting their fitness (Dupont et al., 2010a), but more work is needed to test this hypothesis. Interestingly, in our case this delayed development did not translate into longer larval periods, as settlers appeared at about the same days in cultures kept at natural and slightly acidic conditions, though the latter had lower settlement success and smaller size after metamorphosis (Fig. 4.6).

In the present work we report data of experiments spanning the whole larval development and the early post-settlement period on the thermophilous species *Arbacia lixula*. Further laboratory experiments, using a wider range of pH and temperature conditions and longer follow-up of settlers, supported by thorough field monitoring of larval and adult densities throughout several years should be carried out in order to acquire a full view of the possible impact of ocean acidification and global warming on the ecology of this significant species. A plethora of physical and biological factors other than temperature or acidification may modulate larval development and survival of sea urchins in natural environments,

and many of them are subject to unpredictable changes in the near future. Some recent works have also proved that sea urchins feature high levels of genetic and larval phenotypic variability and thus show a high potential for adaptation to changing environmental conditions (Sunday et al., 2011; Pespeni et al., 2012).

Although the conditions of any experimental setup may be too simplistic to accurately predict the behavior of complex systems, our results so far suggest that warming will contribute to enhance the reproductive success of *A. lixula* and that a mild acidification, coherent with the foreseeable situation in the near future, would reduce larval growth rates but improve larval survival. Overall, then, the impact of *A. lixula* on Mediterranean communities may be expected to increase in the forthcoming decades.

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GENERAL DISCUSSION

Arbacia lixula settlers from laboratory bottles. Photo: **O. S. Wangensteen**

GENERAL DISCUSSION

The research work presented in this dissertation is intended to contribute to a better understanding of the biology, ecology and past history of *Arbacia lixula*, an ecologically significant species in coastal ecosystems from the Mediterranean Sea which remained relatively understudied. New findings have been discovered which challenge some previously established views. In the following subsections we will try to summarize the main conclusions of the different studies presented and discuss their possible implications.

Phylogeographical history of *Arbacia lixula*

Our phylogeographical study (Chapter 1) concluded that, despite being widely considered a typical representative of the Mediterranean fauna (Riedl, 1983), *Arbacia lixula* colonized the Mediterranean Sea in relatively recent times. *A. lixula* did not have any exclusively Mediterranean mitochondrial lineage (Fig. 1.2), as is the case for other Atlanto-Mediterranean echinoderms of similar larval dispersion capacity such as *Marthasterias glacialis* (Pérez-Portela et al., 2010), *Holothuria mammata* (Borrero-Pérez et al., 2011) or *Paracentrotus lividus* (Calderón et al., 2008; Maltagliati et al., 2010). Moreover, the population pairwise differentiation analysis of mitochondrial COI haplotype frequencies (Table 1.3) showed that *A. lixula* populations were not genetically differentiated throughout the whole Mediterranean Sea. The analysis of the mismatch distribution for the Mediterranean populations found a

clear signature of a demographical expansion which could be dated to 94 – 205 kya (Fig. 1.6). This estimation for the expansion time is compatible with the view that *A. lixula* entered the Mediterranean assisted by the warm temperatures which occurred during the last interglacial period (Riss-Würm, Eemian or MIS 5e, ca. 125 kya). This period was also the longest of all interglacial warm periods of the Pleistocene. The minimum winter surface temperature of the Mediterranean Sea stayed warmer than 19 ºC for several thousands of years (Bardají et al., 2009), which probably enabled subtropical Atlantic populations of *A. lixula* to cross the Strait of Gibraltar and colonize the Mediterranean.

Considering its current abundance and distribution along areas that have been extensively sampled by palaeontologists, the almost complete absence of *A. lixula* from the Mediterranean fossil record is revealing. The only fossil individual ever found in the Mediterranean (Stefanini, 1911) was retrieved from very young deposits (*panchina* of Livorno). *A. lixula* is even absent from assemblages of the so-called "Senegalese fauna" which was present in the Mediterranean along several interglacial periods of the Pleistocene. This fauna has been thoroughly studied and is well documented (Issel, 1914; Bonifay and Mars, 1959; Hearty et al., 1986; Lario et al., 1993; Zazo, 1999; Belluomini et al., 2002; Hearty and Dai Pra, 2003; Zazo et al., 2003; Thiel et al., 2010), but *A. lixula* has never been reported from these assemblages, suggesting again that its presence in the Mediterranean is very recent indeed.

These results imply that *Arbacia lixula* has been able to colonize and become dominant in the whole Mediterranean basin in

approximately one hundred thousand years and emphasizes its high colonizing potential, comparable to other invasive species which currently pose serious threats in many marine ecosystems around the World (Bax et al., 2003; Molnar et al., 2008). Haydar (2012) pointed out that ecosystem engineer species that have shaped contemporary communities as the result of very successful colonization events that took place many years ago may be falsely viewed as native. This could explain why *A. lixula* is widely considered a Mediterranean native species, which could lead to underestimate its potential to negatively impact Mediterranean ecosystems.

The implication of *Arbacia lixula* being a recent colonizer is that its populations in the Mediterranean may still be subject to adaptation pressures from potentially suboptimal conditions. *A. lixula* is a thermophilous animal of tropical origin, and it lives at higher temperatures in its original area in Tropical East Atlantic. We did not find strong genetic differences between *A. lixula* populations from the Mediterranean and East Atlantic, at least in the mitochondrial genome. Probably this is due to the fact that these populations have not had enough time to diverge. New genetic variants take more time to become frequent in species with large population sizes, as is the case for *A. lixula*. Thus, the genome of *A. lixula* is still probably better adapted to situations of warmer temperature. The implication that the foreseeable ocean warming due to global change will promote future dynamics of Mediterranean *Arbacia lixula* populations is straightforward.

Trophic ecology of *Arbacia lixula*

The trophic position traditionally assigned to *Arbacia lixula* as an encrusting coralline algae grazer must be reconsidered. The results from our stable isotopes analysis (SIA) study (Chapter 2) clearly showed that *Arbacia lixula* consistently occupies a higher trophic level than *Paracentrotus lividus*. The $\delta^{15}N$ signature in the former was consistently higher than in the latter (Fig. 2.2 and 2.3), with differences in the estimated trophic level ranging from 0.3 to 1 (Table 2.3). Thus, *A. lixula* must be considered an omnivore with a strong tendency to carnivory, while *P. lividus* is a predominantly herbivore that may turn into an omnivore in some instances. Furthermore, the results for $\delta^{13}C$ of *A. lixula* were not compatible with a diet having a high component of encrusting coralline algae (Fig. 2.2). Thus, the traditional views that deemed *A. lixula* a specialized grazer of such algae (Kempf, 1962; Régis, 1978; Verlaque and Nédelec, 1983; Frantzis et al., 1988; Bulleri et al., 1999; Boudouresque and Verlaque, 2001; Privitera et al., 2008) can not be considered valid anymore. These previous results were based on analyses of gut contents rather than on analyses of assimilated food. This new view implies that the role of *A. lixula* in the shallow subtidal community in the Mediterranean should be, at least in part, re-evaluated. Specifically, the potential impact of *A. lixula* on benthic invertebrate communities and the notion of a putative strong competition for food with *P. lividus* and other herbivores should be carefully re-examined.

The analyses of gut contents, which revealed a consistently higher proportion of animal food items ingested in *Arbacia lixula* as compared to *Paracentrotus lividus* (Fig. 2.4 and Table 2.5) supported the results from the SIA. However, gut content analysis alone did not reveal the full extent of the trophic gap between the two species, since vegetal components were the dominant items inside the guts of both sea urchins in most situations analysed (Fig. 2.4). This can be a consequence of the different digestibility of food material of animal or vegetal origin in both species. After the publication of our trophic studies (Wangensteen et al., 2011), Trenzado et al. (2012) studied the enzymatic activity inside the guts of three Mediterranean species of sea urchins, and concluded that *A. lixula* had the highest total digestive ability (i.e. highest lipase, proteases and trypsin activities) enabling it to digest complex animal tissues, while *P. lividus* had a higher cellulolytic and amylase activities, consistent with its herbivorous diet.

A recent work by Agnetta et al. (2013) compared the trophic behaviour of *Arbacia lixula* and *Paracentrotus lividus* in two different habitats (barren and algal forest) at Ustica Island (northern Sicily) using SIA and gut contents. They completely confirmed our results, concluding that *A. lixula* was carnivorous and showed a higher trophic level than *P. lividus* in both studied habitats.

Other trophic studies carried out using SIA on sympatric echinoderms have resulted in similar findings. Thus, Vanderklift et al. (2006) studied three abundant echinoids along south-western Australia coast and found that two species previously thought to be herbivores (*Phyllacanthus irregularis* and *Centrostephanus*

tenuispinus) actually showed omnivorous behaviour tending to carnivory, while the third (*Heliocidaris erythrogramma*) was indeed an herbivore.

The lack of a strong trophic competition with *Paracentrotus lividus* has important implications for the ecology of *Arbacia lixula* in the Mediterranean ecosystems, where *P. lividus* is usually the dominant sea urchin. It implies that *A. lixula* populations are not limited by inter-specific trophic competition with *P. lividus*, since such competition, if any, must be of very low intensity. Also, the carnivorous nature of *A. lixula*, which shows a preference for sessile filter-feeders (such as cirripedes) and other fauna associated to encrusting algae, suggests that this sea urchin would rarely face situations of food shortage, even in barren zones mostly devoid of algae, since small sessile suspension-feeding animals are usually abundant in most coastal rocky habitats in the Mediterranean and Eastern Atlantic.

Reproductive biology of *Arbacia lixula* and effects of temperature

Despite its present abundance in the Mediterranean coastal ecosystems, the gonadal cycle of *Arbacia lixula* was poorly known, and few research efforts had been carried out, compared to those addressed to the sympatric *Paracentrotus lividus*. The seminal work by Fénaux (1968) was the only serious attempt to study the reproductive biology of the black sea urchin. She studied the gonadosomatic index through one and a half cycles at Villefranche

Sur Mer (Southern France) and found a clear gonadal peak in May-June-July. The results we present here (Chapter 3), including samples spanning four years, are overall in agreement with Fénaux's results, but suggest interesting relationships with other variables. We found a clear correlation between gonadal cycle and photoperiod (Fig. 3.1 and Table 3.2), suggesting that daylength is a main factor regulating gonadal development in *A. lixula*. Remarkable increments in gonadosomatic index were found during May-June-July, peaking around the summer solstice. Drops in the GSI, indicative of spawning events, were recorded in August-September-October.

Besides this general trend, the inter-annual variability found in the gonadal cycle of *A. lixula* was striking. The behaviour ranged from curves with very marked peaks, during which virtually all individuals were histologically mature, to nearly flat curves with faint, almost undistinguishable peaks when only a few individuals attained full histological maturity (Fig. 3.4). The long follow-up (four years) allowed us to assess the relationship between mean temperatures and the reproductive output of the species, measured as the maximum annual value of the gonadosomatic index. The observed inter-annual variability in this maximum value was closely correlated with the variation in mean water temperature during the gonad growth period (the six months previous to the peak, i.e. from December to May; Fig. 3.2). Temperature may affect gonadal development in natural environments through direct or indirect effects. Sea urchins use their gonads for nutrient storage and thus temperature may have a direct metabolic impact in gonad

size, since cold temperatures may represent a serious physiological challenge for species of tropical affinities. This effect may probably be observed in the short-term and is more evident in case of extreme temperature events (cold or hot thermal episodes), where individuals would need to mobilize the reserves stored in the gonads in order to obtain energy for somatic maintenance. Secondly, mean temperature may affect ecosystem productivity and thus decrease the availability of food, which could turn out in starvation periods and shortage of reserves. This effect probably requires longer times of sustained cold temperatures.

In summary, our results suggest that warmer temperatures prolonged over time during gonadal growth enhance gonadal development. Though we do not have a direct measure of fertility (such as number of eggs produced by female individuals), it is reasonable to expect a greater reproductive output of individuals having bigger gonadosomatic indices. The conclusion is that the reproductive output of the Mediterranean populations of *A. lixula* may be boosted in years with warm winter and spring temperatures. Since these warmer years are expected to increment their occurrence in the next decades, in view of the ongoing global change, a fecundity boost may be expected for *A. lixula* in the Mediterranean.

Effect of climate change on the larval biology of *Arbacia lixula*

In Chapter 4 we explored the effects of temperature and acidification on the larval development of *Arbacia lixula*. The main conclusion of these experiments is that the temperature is a key enhancer of larval development in this sea urchin, since increments of temperature from 16 to 17.5 to 19 ºC allowed for accelerated growth rates (Fig. 4.2) and higher survival (Fig. 4.3). The effects on the development of a slight degree of acidification (from pH 8.1 to 7.7, the expected value by 2100) were also tested at 19 ºC. The results showed that the survival rate of the larvae was enhanced, albeit with slower growth, and the settlers reared at pH 7.7 were significantly smaller than those reared at pH 8.1 (Fig. 4.7). However, acidification did not affect the early settlers survival rate (Fig. 4.7).

In northwestern Mediterranean, the planktotrophic *A. lixula* larvae have been found in the water column between June and November (Fénaux, 1968; Pedrotti, 1993) and thus may be exposed to a wide range of temperatures (15 to 24°C). Nevertheless, Pedrotti's (1993) results suggest that the highest planktonic concentrations occur in October-November, when the temperature currently ranges from 16 to 19 ºC. Our results show that the larval development takes more than 40 days at 19 ºC. Thus, the larvae settled in the middle November have been growing in the water column since, at least, early October. The water temperature gradually decreases during these months in northwestern Mediterranean. So, the autumnal larvae are undoubtedly exposed to

changing (decreasing) temperatures along their development in natural ecosystems. The settlement success rate was considerably enhanced by an increase from 16 to 19 ºC in our experimental bottles (from only 12 ± 12 settlers per bottle reared at 16 ºC to 480 ± 341 settlers per bottle at 19 ºC. Thus, a remarkable enhancement in the settlement success rate of *A. lixula* may be expected in natural ecosystems if the autumnal water temperature raises or if the winter decrease is delayed as a consequence of global warming.

Factors modulating the life cycle of *Arbacia lixula* and future trends

As most echinoderms, *Arbacia lixula* has a bentho-pelagic life cycle, with long-lived, planktotrophic larvae showing high dispersal capacity. In marine invertebrates with a bentho-pelagic life-history, a plethora of physico-chemical and biological factors may regulate or modulate the reproductive cycle and determine population dynamics (Fig. D.1). Most of these factors may be affected in the future (in many cases, in unpredictable ways) by the current global change.

The juvenile and adult populations of sea urchins may be challenged by physical events, such as extreme temperatures (Girard et al., 2012) and severe storms (Hereu et al., 2012; Pagès et al., 2013), or biological factors such as food shortages (Himmelman, 1986), predators (Cowen, 1983; Hereu et al., 2005; Clemente et al., 2009; Bonaviri et al., 2012), or mass mortalities due to parasites or other disease vectors (Lessios et al., 1984; Jones,

1985; Jones and Hagen, 1987; Lessios, 1988). Moreover, complex interactions between physico-chemical and biological factors, such as links between hydrodynamism and diseases producing mass mortality events, may exist (Scheibling and Hennigar, 1997; Scheibling and Lauzon-Guay, 2010; Girard et al., 2012).

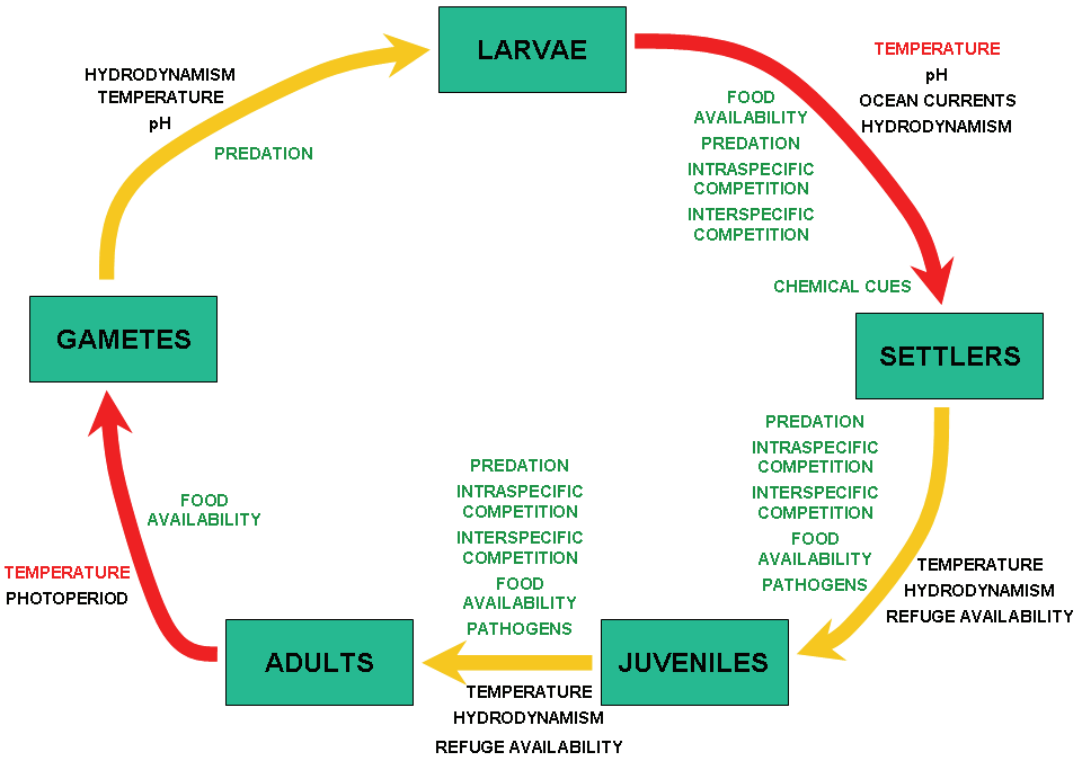

Fig. D.1. Conceptual model for the life-cycle of a broadcast spawner echinoid, indicating some of the physico-chemical (in black) and biological factors (in green) involved in its regulation. Production of gametes and larval development, processes probably limiting *Arbacia lixula* population in north-western Mediterranean, are highlighted by red arrows. Both processes could be significantly enhanced by ocean warming (illustrated by "TEMPERATURE" in red).

Larval growth and survival are modulated, in turn, by physico-chemical or biological factors. The egg quality, which itself depends on the condition of adult females, may influence these variables, not only during the initial stages of endotrophic development, but also along the whole developmental process (George et al., 1990). Other important factors affecting larval survival are temperature and phytoplankton availability, which may be the two crucial factors determining the developmental success. The timings for the spawning of different sea urchin species have been shown to be adapted to both optimal temperature range for the larvae (Fujisawa and Shigei, 1990) and phytoplankton blooms (Himmelman, 1975; Starr et al., 1990).

Of the five processes which shape the life-cycle of *A. lixula*, the maturation of the gametes is affected by low temperatures and may be currently suboptimal during average years in north-western Mediterranean (Chapter 3), whereas the larval development is also negatively affected by low temperatures (Chapter 4) or other factors. Thus, these two processes are likely limiting current population dynamics of *A. lixula* in north-western Mediterranean. Conversely, the fecundation of gametes and early development of the embryos have been studied by Gianguzza et al. (2013b), who proved these processes were robust against changes in temperature and pH.

Considering the scarcity of settlers and the abundance of adults of *A. lixula* in the Mediterranean, it seems that the transition from settlers to juveniles to adults is not a limiting process either. The natural settlement rate of *A. lixula* in the Mediterranean is

currently unknown. However, our own recent observations show that, during a two-year follow up carried out at Tossa de Mar (Costa Brava) using different types of artificial collectors to measure settlement rates, only four *A. lixula* individuals settled on the collectors (three in November and one in July), compared with several thousands of individuals of *P. lividus* settled during the same period (unpublished results). The rarity of *A. lixula* settlers is reflected by the few times that *Arbaciella elegans* (i.e., *Arbacia lixula* settlers and juveniles) has been reported in the literature. Actually, the discovery and consequent description of *Arbaciella elegans* as a different species is itself a reflection of how unused the European specialists were to this stage of the life cycle of *A. lixula*. Low settlement rates are in agreement with the skewed size distribution of adults (Kempf, 1962; Guidetti et al., 2003; Hereu et al., 2012). So, settlement events of *A. lixula* are probably sporadic in the north-western Mediterranean, where settlement failure is probably the rule, rather than the exception, in average years. Irregular settlement events have also been described in other sea urchin species, such as *Strongylocentrotus purpuratus* and *Mesocentrotus franciscanus* (Pearse and Hines, 1987). This strongly contrasts with the regular settlement rates observed for *A. lixula* in the Canary Islands, where hundreds of settled individuals are recorded in each collector every year from December to February (Hernández et al., *pers. comm.*). This discrepancy is probably due to the contrasting larval mortality rates between northwestern Mediterranean and the area of origin of this species in the Atlantic.

Arbacia lixula is a species of tropical origin which colonized the temperate Mediterranean Sea in relatively recent times (Chapter 1). Although the intensity of the gametogenesis seems modulated by temperature, the timing of its gonadal cycle is determined by photoperiod (Chapter 3), as well as in other congeneric species (Brogger et al., 2010). Thus, an adaptive potential for shifts in the gonadal cycle timing so to match the optimal conditions for the larvae in the Mediterranean seems unlikely for this species. The gonadosomatic index drops every year during August-September-October, indicating spawning events, so larvae are expected to be abundant during September-November. In August and September, the phytoplankton concentration is low in northwestern Mediterranean (Satta et al., 1996; Agustí and Duarte, 2000). So, in these months, the larval development may be impaired by food shortage. Thus, the fact that *A. lixula* spawns from August on, even if mature gametes are already present in May, likely reflects an adaptation for a delayed spawning period, in order to have developing larvae during the relatively colder months of autumn, when the phytoplankton concentration begins to rise again. Thus the reproductive strategy of *A. lixula* could be a compromise between taking advantage of the warm periods for gonad growth and gamete provisioning, but delaying release until periods when food is available for the larvae, even if temperature conditions are not optimal for them. The yearly fine-tuning of the spawning periods in echinoids may be triggered by chemical cues indicating phytoplankton build-up (Himmelman, 1975). From our results of Chapter 4, we can predict that a rise in

the water temperatures during the autumnal months would improve the survival and accelerate the growth of *A. lixula* larvae in the north-western Mediterranean.

Thus, the current ocean warming trend may considerably improve the two most important processes which are limiting the populations of this species (production of gametes and larval survival). It would probably lead to population boosts, which would translate into a higher impact of this omnivorous predator (Chapter 2) on shallow rocky ecosystems along the northern Mediterranean coasts, including the potential to originate sea urchin barrens of reduced diversity and productivity.

Monitoring of Mediterranean populations of *Arbacia lixula* is highly recommendable in the near future, in order to detect possible demographic expansions which could become a threat for endangered coastal ecosystems. This monitoring should be carried out at several levels: measures of adult density over time, measures of larval abundance in the plankton at the appropriate seasons and measures of settlement rates, using artificial collectors.

If such a potential threat becomes real, the possible range of recommendations to address for a proper management of the affected coastal areas is scarce. A mitigation of the global warming trend is highly improbable in the next decades, and the effectiveness of the management of *A. lixula* populations in this scenario using ecological tools (such as marine protected areas) is far from being guaranteed.

Considering the complexity of biological interactions in natural ecosystems and the multiple processes that may be up- or

down- regulated by global change, the more field data or experimental results we gather and the more we know about the behaviour of complex ecological processes, the more unpredictable implications of global change seem to be. In the words of J. B. S. Haldane (1927), *the world is not only queerer than we suppose, but queerer than we can suppose*. That is, though the general future trend of physical parameters is well known and may be calculated within certain confidence intervals, the details of biological interactions and the future behaviour of population dynamics of any particular species are mostly unpredictable.

And the Devil is in the details. Of course, there will be losers and there will be winners among marine species. In the next decades, lots of them will see their populations decline and many will become extinct, but others will be promoted by global change, and their range expansions or population boosts will become a serious threat for other species and for whole communities. It is important that we are aware.

CONCLUSIONS

Chapter 1. Natural or naturalized? phylogeography suggests that the abundant sea urchin *Arbacia lixula* is a recent colonizer of the Mediterranean

1. The mitochondrial marker COI from *Arbacia lixula* shows high haplotype diversity but relatively low nucleotide diversity.
2. The populations from Brazil are differentiated from those from the Eastern Atlantic, and probably derive from Eastern Atlantic ancestors.
3. The populations from the Mediterranean and from the Eastern Atlantic are differentiated only by their haplotype frequencies, but they belong to the same two haplogroups
4. The Mediterranean populations show striking similarity among sub-basins and have undergone a demographic expansion event which may be dated 94 – 205 kya.
5. All these evidences suggest that *Arbacia lixula*, despite its present abundance, colonized the Mediterranean in relatively recent times, during the last interglacial period.

Chapter 2. A wolf in sheep's clothing: carnivory in dominant sea urchins in the Mediterranean

6. *Arbacia lixula* is an omnivore tending to carnivory throughout its area of distribution. Thus, the traditional

belief that it was an herbivorous grazer of encrusting coralline algae cannot be considered valid anymore.

7. The analysis of gut contents alone can not be considered a reliable method to assess the trophic position of omnivorous marine invertebrates, and it should be complemented with stable isotopes analysis.
8. Given the differences in trophic positions between both species, a very low degree (in any) of trophic competition may be established between *Arbacia lixula* and *Paracentrotus lividus*.

Chapter 3. Spiny affairs heating up: photoperiod, temperature and inter-annual variability in the reproductive cycle of the thermophilous sea urchin *Arbacia lixula*

9. The timing of the gonadal cycle of *Arbacia lixula* in the Mediterranean is regulated by the photoperiod, but its reproductive output is highly correlated with the mean sea surface temperature during the gonadal growth period.
10. The production of gametes in Mediterranean populations of *Arbacia lixula* may be considerably affected by cold temperatures, whereas warmer temperatures produce a remarkable increase in its gonadosomatic index.
11. The use of polar coordinates and related circular statistics methods allows to adequately analyse reproductive histological data with high intra-gonadal and inter-individual variability.

Chapter 4. Some like it hot: temperature and acidification modulate larval development and settlement of the sea urchin *Arbacia lixula*

12. The larval development of *Arbacia lixula* may be enhanced by raising temperatures, which accelerate growth and increase larval survival rates.
13. A slight degree of acidification produces only small effects on the larval development of *Arbacia lixula*, delaying the development but enhancing survival rates
14. *Arbacia lixula* may complete its larval development at temperatures as low as 16 ºC, albeit with reduced survival rates, slower growth rates and altered larval morphology compared with the development at 19 ºC.
15. Mild acidification did not have a significant effect on the early survival of the settlers of *Arbacia lixula*, albeit settlers grown from larvae reared at pH 7.7 were significantly smaller than those grown from larvae reared at pH 8.1.

General conclusion

16. *Arbacia lixula* is a thermophilous species of tropical affinities which is probably facing suboptimal conditions in northern Mediterranean. Its populations may be promoted by global change, since the current warming trend would eventually enhance the processes which are

limiting its populations in this region. Thus, its negative impact on the Mediterranean coastal ecosystems may be increased in the future.

RESUMEN EN CASTELLANO

Arbacia lixula settler from Tossa de Mar. Oral side. Photo: **O. S. Wangensteen**

RESUMEN EN CASTELLANO

INTRODUCCIÓN

El erizo de mar negro *Arbacia lixula* (Linnaeus, 1758) es, en la actualidad, uno de los macroinvertebrados más conspicuos en los ecosistemas rocosos someros del Mediterráneo. Se trata de un erizo regular con una coloración epitelial uniformemente negra, caparazón relativamente achatado y espinas largas y duras (Fig. I.1). Se puede encontrar entre el nivel del mar y aproximadamente 50 m de profundidad, preferentemente en zonas rocosas semi-verticales y expuestas, aunque también puede estar presente en otros hábitats, como fondos rocosos planos e incluso en fondos arenosos con piedras.

Linneo describió esta especie en 1758, en la décima edición de su *Systema Naturae*, como *Echinus lixula,* un "erizo de mar hemisférico con diez ambulacros situados por parejas, áreas transversales puntuadas con tubérculos cortos y duros". Aunque esta descripción podría referirse posiblemente a cualquier especie de erizo regular, se acepta normalmente que corresponde a una especie del género *Arbacia*. El nombre del género *Arbacia* fue acuñado por John Edward Gray (1835), el cual desplazó *E. lixula* del género *Echinus*. La etimología de *Arbacia* es un poco desconcertante. Tanto Agassiz (1842-1846) como Mortensen (1935) lo consideraron un nombre "sin sentido", mientras que Harvey (1956) indicó que podía derivar de *Arbaces*, un personaje secundario del poema histórico *Sardanapalus* de Lord Byron, publicado en 1821, unos cuantos años

antes del trabajo de Gray. El nombre específico utilizado por Linneo, *lixula*, probablemente deriva del nombre de una galleta redondeada y aplanada, que los antiguos romanos hacían con harina y queso, tal como se define en el *Lexicon Philologicum* de Matthias Martinius (1623), derivada, a su vez, del verbo latino *lixare* (hervir en agua).

Los Arbacioida (Gregory, 1900) constituyen un orden englobado en el Superorden Echinacea Claus, 1876 (Kroh y Smith, 2010). Este superorden incluye los erizos regulares que tienen caparazón duro con diez placas peristómicas, espinas sólidas y presencia de branquias (Ruppert, Fox y Barnes, 2004). Los Arbacioida se distinguen por tener la llamada "placa arbacioide" (Fig. I.2; Duncan, 1855), que consiste en una placa ambulacral compuesta por tres elementos, de los cuales el central es siempre el mayor, y el inferior y el superior no llegan a la sutura perradial.

Los Arbacioida son el grupo hermano de los Camarodonta (que constituyen el orden más diversificado de equinoideos regulares, incluyendo a los Echinidae y otros erizos de importancia ecológica). El orden incluye una única familia actual, los Arbaciidae (Gray, 1855), cuyos representantes son en su mayoría especies omnívoras o carnívoras. El género *Arbacia* es de origen Neotropical, y cuatro especies fósiles han sido descritas del Mioceno Tardío de Norteamérica (Fig. I.3), aunque existen dudas razonables acerca de su sinonimia (Kier, 1972). Sus morfologías están estrechamente relacionadas con la de la especie *A. punctulata,* presente en la actualidad en la misma región.

Tras la reciente sinonimización de *Arbacia crassispina* con *Arbacia dufresnii* (Lessios et al., 2012), el género *Arbacia* incluye

cinco especies válidas actuales, cuatro de las cuales poseen una distribución exclusivamente Neotropical (Fig. I.4). Todas ellas habitan ecosistemas someros, hasta aproximadamente 50 m de profundidad.

La filogeografía intragenérica de *Arbacia* ha sido estudiada por Metz et al. (1998) y revisada por Lessios et al. (2012). Ambos estudios utilizaron el gen mitocondrial COI y secuencias nucleares de la proteína de reconocimiento de gametos bindina. La reconstrucción de la secuencia de los eventos de especiación sugiere que el género se originó en la costa pacífica de Sudamérica, desde donde se extendió hasta el Océano Atlántico a través del Estrecho Centroamericano, antes del alzamiento del Istmo de Panamá, que produjo la separación de la especie pacífica *A. stellata* del clado atlántico (*A. punctulata/A. lixula*). Así, el atlanto-mediterráneo *A. lixula* y el caribeño *A. punctulata* son especies hermanas, que divergieron pronto tras el cierre del Istmo de Panamá, hace alrededor de 2.6 Ma (Fig. I.5; Lessios et al., 2012).

Existe cierta controversia sobre la sinonimia de *Arbacia lixula* con *Arbaciella elegans* Mortensen, 1910. Esta especie fue descrita por Mortensen a partir de material hallado en la costa Oeste de África. *Arbaciella* presenta características similares a *Arbacia* en muchos aspectos, pero su caparazón es aplanado y mucho menor, y tiene un anillo de quince espinas planas con forma de remo alrededor del ámbito (Fig. I.6). Desde su descripción, *Arbaciella elegans* ha sido también citado en las Islas Canarias, Azores, Madeira y el Sur del Mar Mediterráneo. Algunos autores habían expresado serias dudas sobre la validez del género *Arbaciella*. Tommasi (1964)

sugirió que este nombre designaba, en realidad, a los juveniles de *Arbacia lixula*. Sin embargo, estas dudas parecieron despejarse cuando Régis (1982), comparó la microestructura de las púas de *Arbaciella elegans* con las de *Arbacia lixula* y concluyó que eran dos especies morfológicamente diferentes.

Recientes hallazgos han arrojado nuevas dudas sobre la invalidez del género *Arbaciella*. Probablemente, la prueba más concluyente procede de los estudios de asentamiento de Hernández et al. (2005) en las Islas Canarias. Tres especies de erizos dominan completamente las orillas rocosas someras de las Islas Canarias: *Diadema africanum, Paracentrotus lividus* y *Arbacia lixula*, mientras que *Arbaciella elegans* se considera poco abundante. En su estudio realizado con colectores artificiales, Hernández et al. (2005) encontraron abundantes asentados de *Diadema africanum*, *Paracentrotus lividus* y de un tercer erizo que era indistinguible de *Arbaciella elegans*. Sin embargo, a pesar de su dominancia en el archipiélago, no hallaron ni rastro de un cuarto erizo que pudiera corresponder al asentado de *Arbacia lixula*. Así, con toda probabilidad, los *Arbaciella elegans* eran, en realidad, los juveniles de *Arbacia lixula*. Recientemente, las sospechas fueron confirmadas por Kroh et al. (2011) al secuenciar el gen mitocondrial COI de cuatro especímenes de las Azores morfológicamente indistinguibles del holotipo de *Arbaciella*, confirmando su identidad con *Arbacia lixula*. Los mismos resultados moleculares fueron obtenidos por López et al. (2012), con muestras de *Arbaciella* de las Islas Canarias. La confusión probablemente se originó por el hecho de que en los juveniles de *Arbacia lixula*, a diferencia de otros erizos, los

gonoporos se desarrollan en una etapa muy temprana del crecimiento, cuando su diámetro es de apenas 6 mm (Kroh et al., 2011; Fig. I.2). Este detalle llevó a Mortensen a considerar erróneamente que los especímenes observados eran individuos sexualmente maduros correspondientes a una especie diferente.

Si se admite que los especímenes de *Arbaciella* son juveniles de *Arbacia lixula*, se puede inferir considerable información sobre el ciclo biológico de *A. lixula*. Así, Salas y Hergueta (1994) hallaron *Arbaciella elegans* (es decir, juveniles de *A. lixula*) durante el otoño e invierno en el Sur de España. Giacobbe y Rinelli (1992) los encontraron asociados a conchas de *Pinna* en el Estrecho de Messina, y Aliani y Meloni (1999) los recolectaron en diciembre en boyas situadas en el Canal de Córcega. Las recientes "nuevas citas" de *Arbaciella elegans* en el Norte del Mediterráneo (Solinas et al., 1990; Merella et al., 1994; Signorelli y Zamboni, 1998; Grubelic y Antolic, 2000) y en la Cuenca Oriental del Mediterráneo (Koukouras et al., 2007) pueden estar asociadas a un incremento en la frecuencia de eventos exitosos de asentamiento de *A. lixula*, aunque no puede descartarse que se deban a un aumento en el esfuerzo de muestreo, especialmente durante los meses invernales.

La filogeografía basada en marcadores mitocondriales ha sido extensamente usada para estudiar la variabilidad intra-específica de los equinodermos y otros invertebrados marinos (e.g.: Dawson, 2001; Kelly y Palumbi, 2010). El uso de las redes de haplotipos (Bandelt et al., 1999) permite la reconstrucción de la historia evolutiva de los diferentes linajes mitocondriales. El análisis de la distribución de sustituciones por parejas (mismatch distribution;

Rogers y Harpending, 1992) permite inferir la historia demográfica de poblaciones seleccionadas. En combinación con el uso de valores bien calibrados para las tasas de mutación, se puede realizar una estimación temporal que permite la datación de expansiones y otros eventos demográficos (Rogers, 1995).

En el contexto de los equinodermos atlanto-mediterráneos, la filogeografía mitocondrial ha sido recientemente empleada para estudiar la estructura poblacional y la historia demográfica de algunas de las especies más representativas en los ecosistemas costeros poco profundos, como *Paracentrotus lividus* (Calderón et al., 2008; Maltagliati et al. 2010), *Marthasterias glacialis* (Pérez-Portela et al., 2010) o *Holothuria mammata* (Borrero-Pérez et al., 2011). En la mayoría de los casos, estas especies han estado presentes en el Mediterráneo desde tiempos muy antiguos (la mayoría de ellos, probablemente, colonizaron el Mediterráneo rápidamente tras la inundación Zancleense que puso fin a la crisis salina del Mesiniense) y sus poblaciones presentan, en la actualidad, una marcada diferenciación genética a lo largo del Mediterráneo, existiendo diferencias inter-poblacionales en las secuencias de los haplotipos o en sus frecuencias. El origen de la mayor parte de esta estructura poblacional puede ser atribuido al reducido flujo génico entre sub-cuencas del Mediterráneo. La circulación de las aguas superficiales entre estas sub-cuencas está limitada debido a la existencia de accidentes geográficos y/o frentes oceánicos, que impiden la mezcla de masas de agua y reducen, así, el flujo génico entre los organismos que tienen larvas planctónicas. En muchas de estas especies de equinodermos atlanto-mediterráneos, si la especie

ha dispuesto de suficiente tiempo para divergir, han aparecido linajes exclusivamente mediterráneos. En la mayoría de los casos, estos linajes no pueden colonizar el Atlántico y permanecen endémicos del Mediterráneo, debido al intenso flujo de entrada de agua superficial atlántica a través del Estrecho de Gibraltar, que impide a las larvas planctónicas superficiales salir al Océano Atlántico.

Los erizos herbívoros son, a menudo, el factor que determina la abundancia y la distribución de las algas y fanerógamas marinas en los ecosistemas marinos costeros. La importancia crucial de los erizos de mar para el funcionamiento de los ecosistemas someros (Lawrence, 1975; Lawrence y Sanmarco, 1982; Sala et al., 1998a) ha sido puesta de manifiesto en numerosos experimentos ecológicos a lo largo de las costas del Mediterráneo (e.g. Benedetti-Cecchi y Cinelli, 1995; Sala y Zabala, 1996; Benedetti-Cecchi et al., 1998; Palacín et al., 1998a; Bulleri et al., 1999; Guidetti et al., 2004; Bonaviri et al., 2011). El papel ecológico de *Arbacia lixula* no puede ser comprendido en su totalidad si no es en el contexto de sus interacciones con el erizo de mar común europeo, *Paracentrotus lividus*. Ambos erizos comparten sus hábitats y distribuciones de una forma tan entrelazada que los pescadores mediterráneos tradicionalmente han tenido la creencia de que ambos erizos eran el macho y la hembra de una única especie (Tortonese, 1965; Corcoll, 2012). Los nombres comunes en italiano para *A. lixula* y *P. lividus* (*riccio maschio* y *riccio femina*, respectivamente) reflejan esta coexistencia. Las mayores densidades alcanzadas normalmente por *P. lividus* en comparación con las de *A. lixula* han llevado frecuentemente a los ecólogos marinos a centrar sus investigaciones

en el primero de ellos. Así, *P. lividus* ha sido considerado una especie estructural primaria en los ecosistemas mediterráneos, no sólo en las zonas de blanquizal con densidades altas o muy altas de erizos, sino también en comunidades con escasa densidad de erizos (Palacín et al., 1998a), más representativas de gran parte del área de distribución geográfica de la especie (Boudouresque y Verlaque, 2001). Por el contrario, a *A. lixula* se le ha atribuido tradicionalmente un papel ecológico secundario. Sin embargo, este punto de vista está cambiando en los últimos años, y la importancia ecológica de *A. lixula* está siendo cada vez más reconocida (Bulleri et al., 1999; Guidetti et al., 2003; Guidetti y Dulcic, 2007; Bonaviri et al., 2011; Gianguzza et al., 2011; Privitera et al., 2011).

Muchos de los trabajos clásicos sobre erizos hechos en el litoral Mediterráneo han abordado la posible competencia entre las especies simpátricas *Paracentrotus lividus* y *Arbacia lixula*. Los estudios tróficos basados en la observación de contenidos digestivos llevaron a la conclusión de que *A. lixula* se alimentaba principalmente de algas coralináceas incrustantes, mientras *P. lividus* tendía a alimentarse de macroalgas blandas erectas y de hojas y epifitos de *Posidonia oceanica* (Kempf, 1962; Régis, 1978; Verlaque y Nédelec, 1983; Frantzis et al., 1988; Bulleri et al., 1999; Boudouresque y Verlaque, 2001). Así, la visión tradicional es que no se establecía una fuerte competencia por los recursos tróficos entre ambos equinoideos. De hecho, Privitera et al. (2008) demostraron que las dos especies ocupaban diferentes nichos tróficos en áreas de blanquizal con recursos limitados, de nuevo en el sentido de que *A.*

lixula se alimentaba principalmente de coralináceas incrustantes y *P. lividus* de macrófitos no incrustantes.

A pesar de la consideración de *Arbacia lixula* como una especie ramoneadora de algas coralináceas incrustantes, otras especies del género *Arbacia* muestran un comportamiento omnívoro o decididamente carnívoro. Así, la especie norteamericana *A. punctulata* se alimenta de invertebrados sésiles, erizos irregulares y otros individuos de *Arbacia*, así como de algunas algas (Harvey, 1956; Karlson, 1978; Cobb y Lawrence, 2005). La dieta de la especie austral *A. dufresnii* es principalmente carnívora (Penchaszadeh, 1979; Vasquez et al., 1984; Penchaszadeh y Lawrence, 1999; Zaixso, 2004). La especie del Pacífico *A. spatuligera* muestra preferencia por el alimento de origen animal, antes que por algunas especies de algas comunes en su hábitat (Silva et al., 2004). Así mismo, algunas observaciones indican también un comportamiento omnívoro o carnívoro de *A. lixula* fuera del Mediterráneo (Marques, 1984; Oliveira, 1991; Tavares y Borzone, 2005).

Los métodos tradicionales basados en la observación de contenidos digestivos están sujetos al posible sesgo derivado de las diferencias en la digestibilidad del material ingerido. El método que actualmente se considera más adecuado para estudiar las relaciones tróficas en los ecosistemas naturales es el análisis de isótopos estables (Peterson y Fry, 1987; Owens, 1988). Esta técnica analiza y compara muestras de tejido de los consumidores y de sus posibles presas, obteniendo así información del material asimilado en lugar del ingerido. Ha sido aplicada con éxito en los ecosistemas marinos

(e.g.: Hobson y Welch 1992; Cardona et al., 2007; Jaschinski et al., 2008) y ha demostrado ser muy útil para estudiar las preferencias alimentarias y los niveles tróficos de equinoideos de aguas someras en Australia Occidental (Vanderklift et al., 2006). En el Mediterráneo, se ha utilizado para estudiar la dieta de *Paracentrotus lividus* en praderas de *Posidonia* (Tomas et al., 2006), pero nunca antes del presente trabajo se ha utilizado para comparar los niveles tróficos de *A. lixula* y *P. lividus*.

En las áreas de distribución geográfica de *Arbacia lixula* y *Paracentrotus lividus*, los *blooms* poblacionales de ambas especies son capaces de crear zonas de blanquizal en los sustratos rocosos (Kempf, 1962; Verlaque, 1987; Hereu, 2004; Guidetti y Dulcic, 2007; Bulleri, 2013), afectando tanto a la productividad como a la diversidad de las comunidades bentónicas (Bulleri et al., 2002; Sala, 2004; Privitera et al., 2008). Las zonas de blanquizal no son tan frecuentes en el Mediterráneo Noroccidental como en otras regiones templadas o tropicales (e.g.: los extensos blanquizales producidos por *Diadema africanum* en Madeira y en las Islas Canarias; Tuya et al., 2004; Hernández et al., 2008). No obstante, su importancia va en aumento en el Sur del Mediterráneo (Guidetti et al., 2003; Gianguzza et al., 2006; Privitera et al., 2008). Algunos de los primeros experimentos que trataban de estudiar las relaciones entre blanquizales y erizos de mar en el Mediterráneo no distinguían entre *P. lividus* y *A. lixula* (e.g. Bulleri et al., 2002) y, por tanto, asignaban el papel principal como creador de blanquizales a *P. lividus*, debido a sus mayores densidades. Sin embargo, en algunos estudios se han encontrado asociaciones significativas entre la densidad de *A. lixula*

y la extensión ocupada por el blanquizal (Micheli et al., 2005; Guidetti y Dulcic, 2007), así que el papel de *A. lixula* en la creación o mantenimiento de los blanquizales está siendo cada vez más reconocido (Gianguzza et al., 2011; Privitera et al., 2011).

Existen crecientes evidencias de que las poblaciones de *Arbacia lixula* en el Mediterráneo noroccidental han sufrido incrementos demográficos durante las últimas décadas. Marion (1883) y Koehler (1883) lo habían descrito como muy raro en las costas de Provenza. Sin embargo, Petit et al. (1950) afirmaron que se había convertido en abundante en la misma región en un período de unos 30 años. Boudouresque et al. (1989) registraron que sus densidades se habían cuadruplicado en Córcega entre 1980 y 1988. Francour et al. (1994) informaron de incrementos de hasta 12 veces en la Reserva de Scandola (Córcega) entre 1983 y 1992, y especularon que un aumento a largo plazo en la temperatura del agua podría haber sido la causa de esta proliferación. En el mismo período (1982 a 1995), aumentos de hasta 5 veces en las densidades de *A. lixula* fueron descritos en la Reserva Marina de Port-Cros (Francia) (Harmelin et al., 1995). Por el contrario, en un estudio comparativo de las densidades de erizos entre 1992 y 2007 a lo largo de la costa catalana, Corbacho et al. (*comunic. pers.*) no hallaron diferencias significativas en las abundancias de *A. lixula*. Tampoco Hereu et al. (2012) encontraron aumentos significativos en las densidades de *A. lixula* en la Reserva Marina de las Islas Medas, en el seguimiento a más largo plazo publicado hasta el momento (entre 1995 y 2010). Desafortunadamente, existen muy pocos estudios que comparen las abundancias de erizos de mar a largo plazo.

El primer trabajo sobre el ciclo reproductor de *Arbacia lixula* en el Mediterráneo Noroccidental fue publicado por Fénaux (1968). Estudió el ciclo gonadosomático y la aparición de larvas en el plancton a lo largo de tres años en Villefranche-Sur-Mer (Riviera Francesa) y concluyó que el índice gonadadosomático presentaba un máximo anual entre mayo y junio. Este período era coherente con la aparición de larvas pluteus en el plancton entre julio y noviembre, es decir, durante los meses más cálidos del año. Fénaux argumentó que este comportamiento se ajustaba a la naturaleza termófila de *A. lixula*.

Pedrotti (1993) también estudió los patrones de distribución espacial y temporal de las larvas de varias especies de equinodermos, incluyendo *Arbacia lixula*, en el Mar Ligur, cerca de Villefranche-Sur-Mer. Encontró abundantes larvas de *A. lixula* entre octubre y noviembre, y un pico secundario en junio-julio (Fig. I.7). Este resultado coincide con el de Salas y Hergueta (1994) sobre el período de aparición de *Arbaciella elegans* (presumiblemente, asentados de *A. lixula*) en las costas del sur de España. La ausencia de larvas durante los meses intermedios (entre agosto y septiembre) en los resultados de Pedrotti (1993) es notable, ya que coincide con los meses más cálidos y con el período de emisión de gametos predicho por la caída en el índice gonadosomático, según los resultados de Fénaux (1968).

A pesar de la elevada densidad de individuos adultos, los juveniles de *Arbacia lixula* resultan muy poco comunes en la costa catalana. Tras estudiar la distribución de tamaños de *A. lixula* en la Reserva Marina de las Islas Medas, Sala et al. (1998b) y Hereu et al.

(2012) comentaron la abundancia de individuos de las clases de 4 - 5 cm de diámetro, así como la escasez de individuos menores de 30 mm. Distribuciones de tamaño similares fueron registradas en Marsella por Kempf (1962) y en las costas de la Apulia (SE de Italia) por Guidetti et al. (2003). Estos resultados sugieren que, a diferencia del patrón anual regularmente observado para el asentamiento de *Paracentrotus lividus* (Lozano et al., 1995; López et al., 1998; Tomas et al., 2004), la frecuencia de los procesos de reclutamiento de *A. lixula* parece ser altamente irregular en el Mediterráneo Noroccidental. Es probable que los procesos de asentamiento estén modulados por diversos factores físicos y/o biológicos aperiódicos, aún no determinados. Cabe destacar que Harvey (1956) encontró resultados similares, en lo que respecta a la ausencia de ejemplares de pequeño tamaño, en su trabajo sobre *Arbacia punctulata* en Massachusetts. Los juveniles de esta especie americana eran muy escasos en los alrededores de Woods Hole. De hecho, la mayoría de las comunidades estaban formadas, casi exclusivamente, por grandes individuos adultos.

Aunque existe una considerable incertidumbre acerca de los detalles espaciales y temporales, es evidente que el cambio climático global está alterando profundamente los ecosistemas marinos (Hoegh-Guldberg y Bruno 2010). Estos cambios se están produciendo de dos modos fundamentales. Por un lado, el calentamiento del agua marina es un fenómeno cada vez más documentado, que afecta a todos los océanos del Mundo. Por otro lado, el aumento de las emisiones de CO_2 a la atmósfera está produciendo cambios en la química marina del carbono,

disminuyendo el pH del agua de mar, un fenómeno que se conoce como acidificación del océano. El pH medio del agua marina superficial ha disminuido aproximadamente en 0.1 unidades desde el comienzo de la revolución industrial, y se espera que se reduzca en 0.3-0.5 unidades para 2100, y en 0.7-0.8 unidades para 2300 (Caldeira y Wickett, 2003, 2005; IPCC, 2007). El resultado es una disminución de la concentración de ión carbonato ($CO_3^{=}$) que produce una reducción en el estado de saturación de las especies minerales del carbonato de calcio (Ω_{CaCO3}) (Feely et al., 2004; Orr et al., 2005). En teoría, esto debería tener un efecto negativo en los organismos calcificadores (aquellos que producen esqueletos de carbonato de calcio), como las larvas de equinodermos. Sin embargo, los resultados experimentales recientes demuestran que las larvas de equinodermos son más robustas ante la acidificación del océano de lo que se pensaba inicialmente (Dupont et al. 2010c; Dupont y Thorndyke, 2013). La acidificación, normalmente, produce sólo efectos subletales en la mayoría de las especies de equinodermos, que pueden ser explicados por cambios en el balance energético metabólico (Dupont y Thorndyke, 2013).

A pesar del gran esfuerzo dedicado al estudio de los efectos de la acidificación en el desarrollo larvario de los equinodermos, los resultados han sido, en cierto modo, ambiguos. Algunas especies muestran evidentes alteraciones del desarrollo cuando se exponen a valores de pH disminuidos (*Ophiothrix fragilis*, Dupont et al., 2008). En otras especies, sin embargo, los efectos son neutros o indetectables e, incluso, en algunas especies el desarrollo puede ser favorecido por niveles moderados de acidificación (*Crossaster*

papposus, Dupont et al., 2010b). Además, los efectos del cambio global en las poblaciones futuras de organismos calcificadores termófilos son inciertos. Por un lado, su desarrollo larvario se puede ver favorecido por el aumento de temperatura, mientras que podría verse perjudicado por el descenso de pH.

El desarrollo larvario es uno de los estadios más vulnerables del ciclo de vida de los equinoideos, que normalmente tienen larvas pelágicas, siendo las tasas de mortalidad generalmente muy elevadas durante este período (Rumrill, 1990; López et al., 1998). Así, muchas poblaciones de equinoideos pueden estar limitadas, al menos parcialmente, por la mortalidad larvaria. Si los principales factores limitantes de la supervivencia larvaria se relajan, entonces pueden ocurrir explosiones demográficas de erizos adultos, que tienen el potencial para impactar de forma drástica en las comunidades bentónicas. El cambio global está alterando la práctica totalidad de los procesos físicos y biológicos en los ecosistemas marinos, afectando tanto a las comunidades planctónicas como a las bentónicas. Existe una multitud de interacciones complejas implicadas en la regulación del ciclo de vida de los erizos de mar, que determina la densidad poblacional de los adultos. Virtualmente todos los factores físicos y biológicos moduladores de estas interacciones pueden verse alterados por el cambio global. Así, el comportamiento futuro de la dinámica poblacional de los erizos de mar es prácticamente impredecible. Sin embargo, los efectos en el desarrollo larvario de determinadas variables críticas, tales como la temperatura o el pH, pueden estudiarse mediante experimentación en condiciones controladas, y así pueden llegar a inferirse, hasta un

cierto grado, predicciones acerca de la posible tendencia futura de la dinámica poblacional de algunas especies de importancia ecológica.

OBJETIVOS

En este trabajo se aborda el estudio de los factores que afectan a la filogeografía, biología y ecología trófica del erizo de mar negro *Arbacia lixula* en los ecosistemas mediterráneos, con el objeto de contribuir a la comprensión de su papel ecológico y estimar su posible impacto futuro en las comunidades bentónicas.

Para ello, nos propusimos desarrollar los siguientes objetivos específicos:

1. Estudiar la genética de poblaciones y la filogeografía de *A. lixula* a lo largo de su rango geográfico de distribución, empleando marcadores moleculares mitocondriales.

2. Estudiar las relaciones tróficas entre las dos especies simpátricas *A. lixula* y *Paracentrotus lividus* y sus posibles fuentes de alimento, empleando para ello análisis de isótopos estables y análisis de los contenidos digestivos.

3. Estudiar el ciclo reproductor de *A. lixula* en el Mediterráneo y los factores que lo regulan.

4. Estudiar el desarrollo larvario de *A. lixula* en distintas condiciones de temperatura y acidificación, con el objeto de

estudiar su vulnerabilidad a nivel de organismo en el contexto del cambio climático global.

RESULTADOS Y DISCUSIÓN

Con este trabajo se pretende contribuir a una mejor comprensión de la biología, ecología e historia evolutiva del erizo de mar *Arbacia lixula*, una especie de elevada importancia ecológica en los ecosistemas costeros del Mar Mediterráneo que permanecía relativamente poco estudiada. Se han encontrado nuevos resultados experimentales que desafían algunos de los conceptos tradicionalmente aceptados sobre esta especie. En las secciones siguientes se intentará resumir las principales conclusiones de los diferentes estudios presentados, así como discutir sus posibles implicaciones.

Filogeografía de *Arbacia lixula*

A partir de nuestro estudio filogeográfico (Capítulo 1) se concluye que, a pesar de ser considerado un representante típico de la fauna mediterránea (Riedl, 1983), *Arbacia lixula* colonizó el Mar Mediterráneo en épocas relativamente recientes. *A. lixula* no presenta un linaje mitocondrial exclusivamente mediterráneo (Fig. 1.2), a diferencia de otros equinodermos atlanto-mediterráneos con larvas de similar capacidad dispersiva, como *Marthasterias glacialis* (Pérez-Portela et al., 2010), *Holothuria mammata* (Borrero-Pérez et al., 2011) o *Paracentrotus lividus* (Calderón et al., 2008; Maltagliati

et al., 2010). Así mismo, el análisis de la diferenciación entre pares de poblaciones de las frecuencias haplotípicas del marcador mitocondrial COI (Tabla 1.3) no encontró ninguna diferencia entre las poblaciones de esta especie a lo largo de toda la Cuenca Mediterránea. En el análisis de la distribución de sustituciones por parejas (*mismatch distribution*; Rogers y Harpending, 1992) para las poblaciones del Mediterráneo se encontró una señal clara de expansión demográfica (Fig. 1.6), cuya datación puede estimarse entre 94.000 y 205.000 años. Esta estimación para el tiempo transcurrido desde la expansión es compatible con la idea de que *A. lixula* colonizó el Mediterráneo aprovechando las temperaturas cálidas que ocurrieron durante el último período interglaciar (Riss-Würm, Eemiense o MIS 5e, hace alrededor de 125.000 años). Este período fue también el más prolongado de todos los períodos interglaciares del Pleistoceno. La temperatura mínima invernal del agua de mar permaneció en valores superiores a 19 ºC durante varios miles de años (Bardají et al., 2009) lo que, probablemente, permitió a las poblaciones de *A. lixula* del Atlántico Tropical cruzar el estrecho de Gibraltar y colonizar el Mediterráneo.

Considerando su abundancia actual y su distribución en zonas geográficas que han sido extensivamente muestreadas por los paleontólogos, la casi total ausencia de *A. lixula* del registro fósil del Mediterráneo resulta muy significativa. El único individuo fósil encontrado en el Mediterráneo (Stefanini, 1911) procede de depósitos muy recientes (*panchina* de Livorno). Además, *A. lixula* está ausente de los yacimientos de la llamada "Fauna Senegalesa" que estuvo presente en el Mediterráneo a lo largo de varios períodos

interglaciares del Pleistoceno. Está fauna ha sido profusamente estudiada y está bien documentada (Issel, 1914; Bonifay y Mars, 1959; Hearty et al., 1986; Lario et al., 1993; Zazo, 1999; Bellouomini et al., 2002; Hearty y Dai Pra, 2003; Zazo et al., 2003; Thiel et al., 2010), pero *A. lixula* nunca ha sido encontrado en estos yacimientos, sugiriendo, de nuevo, que su presencia en el Mediterráneo es verdaderamente muy reciente.

Estos resultados implican que *Arbacia lixula* ha sido capaz de colonizar, de forma exitosa, toda la Cuenca Mediterránea en alrededor de 100.000 años, lo que enfatiza su potencial colonizador, comparable al de otras especies invasivas que suponen en la actualidad serias amenazas para los ecosistemas marinos de todo el mundo (Bax et al., 2003; Molnar et al., 2008). Haydar (2012) señaló que las especies que son ingenieras de ecosistemas, capaces de alterar las comunidades actuales como resultado de eventos colonizadores de elevado éxito, pueden llegar a ser falsamente reconocidas como especies nativas de estos ecosistemas. Esto podría explicar por qué *A. lixula*, considerado habitualmente una especie nativa del Mediterráneo, puede estar infravalorado en cuanto a su potencial para alterar negativamente los ecosistemas mediterráneos.

El hecho de que *Arbacia lixula* sea un colonizador reciente implica que, probablemente, sus poblaciones en el Mediterráneo se encuentran en la actualidad sometidas a condiciones subóptimas. *A. lixula* es un animal termófilo de origen tropical, y, actualmente, vive a mayores temperaturas en su área de distribución original en el Atlántico Tropical. En el presente trabajo, no se han hallado diferencias importantes a nivel genético entre las poblaciones del

Mediterráneo y las del Atlántico Este, al menos en cuanto al genoma mitocondrial. Probablemente esto se deba al hecho de que las poblaciones no han tenido tiempo suficiente para divergir. Las nuevas variantes genéticas aparecidas en especies con tamaños poblacionales muy grandes, como es el caso de *A. lixula*, tardan más tiempo en convertirse en frecuentes. Así, probablemente, el genoma de *A. lixula* todavía está mejor adaptado a temperaturas más cálidas. Las posibles consecuencias para la dinámica poblacional futura de las poblaciones mediterráneas de *A. lixula* causadas por el previsible calentamiento del agua del Mediterráneo, son evidentes.

Ecología trófica de *Arbacia lixula*

La posición trófica que, tradicionalmente, se ha asignado a *Arbacia lixula* como un ramoneador de algas coralináceas incrustantes debe ser reconsiderada. Los resultados de nuestro estudio de isótopos estables (SIA) (Capítulo 2) mostraron que *A. lixula* ocupa un nivel trófico claramente superior al de *Paracentrotus lividus*. La signatura de $\delta^{15}N$ del primero fue siempre significativamente más alta que la del segundo (Figs. 2.2 y 2.3), con diferencias en el nivel trófico calculado entre ambas especies que oscilaban entre 0.3 y 1 (Tabla 2.3). Por tanto, *A. lixula* debe ser considerado un omnívoro con una fuerte tendencia a la carnivoría, mientras que *P. lividus* es predominantemente un herbívoro, que en algunos casos puede tender a la omnivoría. Además, los resultados para la signatura de $\delta^{13}C$ de *A. lixula* no son compatibles con el punto de vista de que su dieta esté formada en gran parte por

coralináceas incrustantes (Fig. 2.2). La visión tradicional de *A. lixula* como un ramoneador especializado de dichas algas (Kempf, 1962; Régis, 1978; Verlaque y Nédelec, 1983; Frantzis et al., 1988; Bulleri et al., 1999; Boudouresque y Verlaque, 2001; Privitera et al., 2008) no puede ser considerada ya válida. Esto implica que el papel asignado a *A. lixula* en los ecosistemas someros del Mediterráneo debe ser, al menos en parte, reevaluado. Específicamente, el impacto potencial de *A. lixula* en las comunidades de invertebrados bentónicos y la existencia de un elevado grado de competencia trófica con *P. lividus* y otros herbívoros, debe ser reexaminado cuidadosamente.

Los análisis de los contenidos digestivos, que siempre mostraron una proporción consistentemente mayor de alimentos ingeridos de origen animal en *Arbacia lixula* que en *Paracentrotus lividus* (Fig. 2.4 y Tabla 2.5), apoyaron los resultados del SIA. Sin embargo, la observación de los contenidos digestivos no revela en toda su extensión la diferencia de nivel trófico existente entre ambas especies, ya que los componentes de origen vegetal fueron mayoritarios en el interior del tracto digestivo de ambos erizos en la mayoría de las situaciones analizadas (Fig. 2.4). Esto puede ser una consecuencia de la diferente digestibilidad del material alimenticio de origen animal o vegetal entre ambas especies. Tras la publicación del trabajo que se presenta en esta tesis doctoral (Wangensteen et al., 2011), Trenzado et al. (2012) estudiaron la actividad enzimática en muestras del sistema digestivo de tres especies de erizos del Mediterráneo, y concluyeron que *A. lixula* mostraba la mayor capacidad digestiva total (es decir, la mayor actividad de lipasa,

proteasas y tripsina), lo que le permitiría digerir tejidos animales complejos, mientras que *P. lividus* mostró una mayor actividad celulolítica y de amilasa, congruente con su dieta herbívora.

Un reciente trabajo de Agnetta et al. (2013) ha comparado el comportamiento trófico de *Arbacia lixula* y *Paracentrotus lividus* en dos hábitats diferentes (blanquizal y bosque de algas) en la isla de Ustica (al Norte de Sicilia) utilizando SIA y contenidos digestivos. Sus resultados confirman completamente los nuestros, concluyéndose que *A. lixula* es un carnívoro con un nivel trófico más alto que *P. lividus* en los dos tipos de hábitats estudiados.

La limitada competencia por los recursos tróficos con *Paracentrotus lividus* tiene importantes implicaciones para la ecología de *Arbacia lixula* en los ecosistemas mediterráneos, en los cuales *P. lividus* es normalmente el erizo dominante. Las poblaciones de *A. lixula* difícilmente pueden estar limitadas por la competencia trófica inter-específica con *P. lividus*, ya que dicha competencia, si existe, debe ser de muy baja intensidad. Así mismo, la naturaleza carnívora de *A. lixula*, que parece mostrar preferencia por los invertebrados sésiles (frecuentemente cirrípedos o formas crípticas asociadas a algas coralináceas), sugiere que este erizo raramente se debe enfrentar a situaciones de escasez de alimento, incluso en las zonas de blanquizales con escasa cobertura algal, ya que los pequeños animales sésiles suspensívoros son normalmente muy abundantes en la mayoría de los hábitats costeros rocosos del Mediterráneo y del Atlántico Este.

Biología reproductiva de *Arbacia lixula* y efecto de la temperatura

A pesar de su abundancia actual en los ecosistemas costeros del Mediterráneo, la información sobre el ciclo gonadal de *Arbacia lixula* era escasa, y muy pocos trabajos habían abordado este tema, en comparación con los que habían estudiado el ciclo de *Paracentrotus lividus*. El trabajo pionero de Fénaux (1968) constituía el único intento serio de estudiar el ciclo reproductivo de esta especie de erizo. En dicho estudio, se midió el índice gonadosomático a lo largo de 18 meses en Villefranche Sur Mer (Sur de Francia) y se encontró un claro máximo en los meses de mayo-junio-julio. Los resultados que se presentan en esta tesis (Capítulo 3), y que incluyen muestras recogidas a lo largo de cuatro años, están de acuerdo con estos resultados iniciales de Fénaux. Se observa una clara correlación entre el ciclo gonadal y el fotoperiodo (Fig. 3.1 y Tabla 3.2), que sugiere que la duración del día actúa de regulador principal del desarrollo gonadal de *A. lixula*. El índice gonadosomático aumenta considerablemente durante mayo-junio-julio, coincidiendo con el solsticio de verano. Disminuciones del índice gonadosomático, indicativas de eventos de emisión de gametos, se registraron en agosto-septiembre-octubre.

Aparte de esta tendencia general, se encontró una notable variabilidad interanual en el comportamiento de las curvas de índice gonadosomático en *A. lixula*. El comportamiento varió entre un ciclo y otro, desde curvas con máximos muy marcados, en los cuales la práctica totalidad de los individuos se encontraban histológicamente

maduros, hasta curvas prácticamente planas, con máximos casi imperceptibles, en los cuales pocos individuos alcanzaron la madurez histológica completa (Fig. 3.4). El seguimiento a largo plazo (cuatro años) permitió estudiar la relación entre las temperaturas medias y la producción de gametos de la especie, estimada como el valor máximo anual del índice gonadosomático. La variabilidad interanual observada en este valor máximo anual se correlacionó estrechamente con la variación en la temperatura media del agua durante los meses de crecimiento gonadal (los seis meses previos al máximo, es decir, de diciembre a mayo; Fig. 3.2). La temperatura puede afectar al desarrollo gonadal de los invertebrados en los ecosistemas naturales mediante efectos directos o indirectos. Los erizos de mar utilizan sus gónadas también para el almacenamiento de nutrientes y, por tanto, la temperatura puede tener un impacto metabólico directo en el tamaño de las gónadas. Las temperaturas frías pueden representar un importante desafío para la fisiología de las especies de afinidades tropicales. Este efecto puede probablemente ser observado a corto plazo, y se puede dar en el caso de eventos de temperaturas extremas (por frío o por calor), durante los cuales los individuos pueden necesitar movilizar las reservas acumuladas en las gónadas para obtener la energía necesaria para hacer frente a los gastos de mantenimiento somático durante el período de estrés. En segundo lugar, la temperatura media puede afectar a la productividad del ecosistema, disminuyendo la disponibilidad de alimento, lo cuál podría traducirse en períodos de ayuno y disminución de las reservas acumuladas. Este efecto

probablemente requiere de bajas temperaturas mantenidas durante mayores intervalos de tiempo.

En resumen, los resultados del presente trabajo sugieren que las temperaturas cálidas sostenidas durante cierto tiempo durante la época de maduración gonadal, mejoran el desarrollo gonadal de *A. lixula*. Aunque no se disponga de una medida directa de la fecundidad (como sería, por ejemplo el número de huevos producidos por cada hembra), cabe esperar una mayor producción de gametos al aumentar el índice gonadosomático. La conclusión es que la eficacia reproductiva de las poblaciones mediterráneas de *A. lixula* podría aumentar durante los años con inviernos y primaveras relativamente cálidos. Como, probablemente, la frecuencia de estos años cálidos aumente en las próximas décadas por el efecto del cambio global, cabe esperar que la fecundidad de *A. lixula* en el mediterráneo se incremente en el futuro próximo.

Efecto del cambio global sobre la biología larvaria de *Arbacia lixula*

En el Capítulo 4 estudiamos los efectos de la temperatura y de la acidificación en el desarrollo larvario de *Arbacia lixula*. La conclusión principal del estudio es que el aumento de temperatura facilita el desarrollo larvario de esta especie, ya que los incrementos de temperatura de unos pocos grados centígrados (desde 16 a 17.5 y 19 ºC) aceleraron significativamente su crecimiento (Fig. 4.2) y aumentaron sus tasas de supervivencia (Fig. 4.3). En lo que respecta a la acidifación, se estudió el efecto sobre el desarrollo de un descenso del pH (desde 8.1, valor actual, hasta 7.7, valor predicho

para 2100). Los resultados mostraron un aumento en la tasa de supervivencia de las larvas, al disminuir el pH aunque también se produjo un ligero retraso en el crecimiento. Los asentados procedentes de larvas cultivadas a pH 7.7 fueron, así mismo, de un tamaño significativamente menor que los procedentes de larvas cultivadas a pH 8.1 (Fig. 4.6), aunque la acidificación no afectó a su supervivencia a corto plazo (Fig. 4.7).

En el Mediterráneo Noroccidental las larvas planctotróficas de *A. lixula* se han localizado en la columna de agua entre los meses de junio y noviembre (Fénaux, 1968; Pedrotti, 1993). Por esta razón, pueden estar expuestas a un amplio rango de temperaturas (desde 15 hasta 24 °C). Sin embargo, los resultados de Pedrotti (1993) sugieren que las mayores concentraciones de larvas en el plancton ocurren entre octubre y noviembre, cuando las temperaturas varían entre 16 y 19 ºC. Nuestros resultados muestran que el desarrollo larvario dura más de 40 días a 19 ºC, lo que sería congruente con que las larvas asentadas a mediados de noviembre estuvieran presentes en el plancton desde comienzos de octubre. La temperatura del agua disminuye gradualmente durante dichos meses en el Mediterráneo Noroccidental. Así, las larvas otoñales están sin duda expuestas a temperaturas variables (decrecientes) a lo largo de su desarrollo en condiciones naturales. La tasa de asentamiento se incrementó considerablemente en nuestros recipientes experimentales al pasar de 16 a 19 ºC (desde tan sólo 12 ± 12 asentados por recipiente obtenidos a 16 ºC hasta 480 ± 341 asentados por recipiente a 19 ºC). Así, si la temperatura del agua durante el otoño aumentara en la

naturaleza un par de grados centígrados, cabría esperar un posible aumento en la tasa de asentamiento de *A. lixula*.

Factores moduladores del ciclo vital de *Arbacia lixula* y tendencias futuras

Como la mayoría de los equinodermos, *Arbacia lixula* posee un ciclo vital bento-pelágico, con larvas planctotróficas de vida larga y elevada capacidad dispersiva (Fig. D.1). En los invertebrados marinos con ciclos de vida bento-pelágicos, puede existir una multitud de factores físico-químicos y biológicos que regulen el ciclo reproductor y determinen la dinámica poblacional.

Las poblaciones de juveniles o adultos de erizos de mar pueden verse sometidas a diversos desafíos de carácter físico, como temperaturas extremas (Girard et al., 2012) o tormentas intensas (Hereu et al., 2012; Pagès et al., 2013), o a factores biológicos, como la escasez de alimento (Himmelman, 1986), los depredadores (Cowen, 1983; Hereu et al., 2005; Clemente et al., 2009; Bonaviri et al., 2012) o eventos de mortalidad masiva producidos por parásitos u otros vectores de enfermedades (Lessios et al., 1984; Jones, 1985; Jones y Hagen, 1987; Lessios 1988). Además, pueden darse complejas interacciones entre los factores físico-químicos y los biológicos, como, por ejemplo, los vínculos hallados entre el hidrodinamismo y las enfermedades causantes de mortalidad masiva (Scheibling y Hennigar, 1997; Scheibling y Lauzon-Guay, 2010; Girard et al., 2012).

A su vez, el crecimiento y la supervivencia larvarios pueden estar modulados por otros factores físicos o biológicos. La calidad del huevo, que a su vez depende del estado físico de las hembras adultas, puede influir en esas variables, no sólo durante las etapas iniciales de desarrollo endotrófico, sino a lo largo de todo el proceso de desarrollo (George et al., 1990). Otros importantes factores son la temperatura y la disponibilidad de fitoplancton, que podrían ser los dos factores principales que afecten al éxito del desarrollo larvario. Se ha demostrado que las épocas de desove de diferentes especies de erizo pueden estar adaptadas tanto al rango de temperatura óptima para el desarrollo larvario (Fujisawa y Shigei, 1990) como a la dinámica de las proliferaciones (blooms) de fitoplancton (Himmelman, 1975; Starr et al., 1990).

La tasa de asentamiento natural de *Arbacia lixula* en el Noroeste del Mediterráneo es desconocida y sus eventos de asentamiento son probablemente esporádicos. Nuestras propias observaciones recientes muestran que, en un estudio para determinar la tasa de asentamiento en Tossa de Mar (Costa Brava) usando diferentes tipos de colectores artificiales a lo largo de dos años, únicamente cuatro individuos de *A. lixula* se asentaron en los colectores utilizados (un individuo en julio y tres en noviembre), en comparación con varios miles de *Paracentrotus lividus* asentados durante el mismo período (*resultados sin publicar*). La rareza del asentamiento de *A. lixula* en el Norte del Mediterráneo se refleja en las escasas ocasiones en que los hallazgos de *Arbaciella elegans* (es decir, de asentados y juveniles de *A. lixula*) han sido citados en la bibliografía. De hecho, la propia descripción como especie distinta

de *Arbaciella elegans* pone de manifiesto la falta de familiaridad de los especialistas europeos con este estadio del ciclo vital de *A. lixula*. Esto contrasta completamente con la regularidad y abundancia de los procesos de asentamiento de *A. lixula* en las Islas Canarias, donde se registran centenares de individuos por colector que se asientan anualmente durante los meses de Diciembre a Febrero (Hernández et al., *comunicación personal*). Muy probablemente, esta discrepancia aparece como consecuencia de las diferentes tasas de mortalidad larvaria entre el Mediterráneo Noroccidental y el área de origen de la especie en el Atlántico Tropical.

Arbacia lixula es una especie de origen tropical que ha colonizado recientemente el Mediterráneo (Capítulo 1). Su ciclo gonadal está regulado por el fotoperiodo (Capítulo 3), igual que ocurre en otras especies del género (Brogger et al., 2010). Así, un cambio adaptativo que consiga desplazar la época de desove para que coincida con las condiciones óptimas para el desarrollo de las larvas en unas condiciones geográficas distintas a las de su área de origen no es muy probable que ocurra. Los descensos del índice gonadosomático, indicativos de eventos de liberación de gametos, ocurren cada año durante agosto-septiembre-octubre, así que cabría esperar que las larvas fueran abundantes durante estos meses. En agosto y septiembre, sin embargo, la concentración de fitoplancton es baja en el Mediterráneo Noroccidental (Satta et al., 1996; Agustí y Duarte, 2000); así que, durante estos meses, es posible que el desarrollo esté limitado por la falta de alimento. El hecho de que *A. lixula* emita sus gametos a partir de agosto, aunque estén maduros ya desde mayo, refleja su estrategia de retardar la emisión de gametos

para que el desarrollo larvario ocurra durante los meses más fríos de octubre y noviembre, cuando la concentración de fitoplancton vuelve a subir. La estrategia reproductiva de *A. lixula* podría ser, por tanto, un compromiso entre aprovechar la estación cálida para el crecimiento gonadal y el almacenamiento de gametos y retrasar la liberación de éstos hasta periodos en que haya disponibilidad de alimento para las larvas, aunque ello implique que las condiciones de temperatura no sean las óptimas para el desarrollo larvario. El ajuste fino del periodo de liberación de gametos en los equinoideos podría estar regulado por señales químicas procedentes del crecimiento del fitoplancton (Himmelman, 1975). A partir de nuestros resultados del Capítulo 4, podemos predecir que un aumento en la temperatura del agua durante los meses de otoño, produciría un aumento de la supervivencia y aceleraría el crecimiento de las larvas de *A. lixula* en el Mediterráneo Noroccidental.

Así, la actual tendencia al calentamiento de las aguas del Mediterráneo podría mejorar precisamente los dos procesos que probablemente están limitando la población de esta especie (la producción de gametos y la supervivencia larvaria). Probablemente, esto se traduzca en futuros incrementos poblacionales, con el consiguiente aumento del impacto de esta especie omnívora (Capítulo 2) en los ecosistemas rocosos someros del Mediterráneo Noroccidental, incrementándose la posibilidad de que aparezcan zonas de blanquizal, de diversidad y productividad disminuidas.

Sería recomendable un seguimiento de las poblaciones mediterráneas de *Arbacia lixula* en el futuro próximo, con el fin de detectar posibles expansiones demográficas que pudieran convertirse

en una amenaza para los ecosistemas costeros. Este seguimiento se podría llevar a cabo a varios niveles: mediciones de la densidad de adultos a lo largo del tiempo, medidas de la concentración de larvas en diferentes épocas del año, o medidas de la tasa de asentamiento, empleando colectores artificiales.

Si tal amenaza potencial se transforma en real, el posible rango de recomendaciones que podrían proponerse para una adecuada gestión de los ecosistemas afectados es escaso. Una mejora de la tendencia al calentamiento global es muy improbable que ocurra en las próximas décadas y la posible eficacia del uso de herramientas ecológicas (e.g., la creación de reservas marinas) para controlar las poblaciones de esta especie no está, en absoluto, garantizada.

Si consideramos la complejidad de las interacciones biológicas en los ecosistemas naturales y los múltiples procesos cuya intensidad puede verse incrementada o reducida por el cambio global, cuantos más datos de campo y resultados experimentales recopilamos, más impredecibles parecen ser las consecuencias del cambio global. En palabras de J. B. S. Haldane (1927), *el mundo no es sólo más extraño de lo que suponemos, sino más extraño de lo que somos capaces de suponer*. Es decir, la tendencia futura general de los parámetros físicos es bien conocida y puede predecirse, dentro de unos ciertos intervalos de confianza, pero los detalles de las interacciones biológicas y el comportamiento futuro de la dinámica poblacional de cualquier especie en concreto son básicamente impredecibles.

Y el diablo está en los detalles. Por supuesto, habrá ganadores y perdedores entre las especies marinas. En las próximas décadas, muchas de ellas verán sus poblaciones decrecer y unas cuántas se extinguirán, pero otras serán favorecidas por el cambio global y las expansiones de sus áreas de distribución o sus explosiones demográficas las transformarán en una seria amenaza para otras especies y para comunidades enteras. Es importante que estemos prevenidos.

Conclusiones

Del Capítulo 1:

1. El marcador mitocondrial COI de *Arbacia lixula* muestra una elevada diversidad haplotípica y una diversidad nucleotídica relativamente baja.
2. Las poblaciones de *A. lixula* de Brasil están genéticamente diferenciadas de las del Atlántico Oriental, y probablemente derivan de antecesores del Atlántico Oriental.
3. Las poblaciones de *A. lixula* del Mediterráneo y del Atlántico Oriental se diferencian en sus frecuencias haplotípicas, pero pertenecen a los mismos dos haplogrupos. A diferencia de lo que ocurre en otros equinodermos, no existe un linaje de *A. lixula* endémico del Mediterráneo.

4. Las poblaciones de *A. lixula* del Mediterráneo no presentan diferencias genéticas entre sub-cuencas y han sufrido una expansion demográfica que se puede datar entre 94,000 y 205,000 años.
5. Todas estas evidencias sugieren que, a pesar de su actual abundancia, *A. lixula* colonizó el Mediterráneo en tiempos relativamente recientes, probablemente durante el ultimo período interglaciar.

Del Capítulo 2:

6. *Arbacia lixula* es un omnívoro con tendencia a la carnivoría en toda su área de distribución. Así, la creencia tradicional de que es una especie herbívora que se alimenta de algas coralináceas incrustantes no puede ser considerada ya válida.
7. El análisis de contenidos digestivos, por sí solo, no puede considerarse un método fiable para estudiar las relaciones tróficas de invertebrados marinos omnívoros y debería complementarse siempre con el análisis de isótopos estables.
8. Dadas las diferencias de posición trófica existentes entre *Arbacia lixula* y *Paracentrotus lívidus*, la competencia trófica entre ellos, si es que existe, ha de ser de muy baja intensidad.

Del Capítulo 3:

9. El fotoperiodo es el factor que regula el curso temporal del ciclo gonadal de *Arbacia lixula* en el Mediterráneo, pero su producción de gametos se correlaciona estrechamente con la temperatura superficial media del agua durante el período de crecimiento gonadal.
10. La producción de gametos en las poblaciones mediterráneas de *Arbacia lixula* puede verse muy afectada por las bajas temperaturas, mientras que las temperaturas más cálidas producen un notable incremento de su índice gonadosomático.
11. El uso de coordenadas polares y los métodos relacionados de estadística circular permite el análisis adecuado de datos de histología reproductiva en especies con elevada variabilidad intra-gonadal e inter-individual.

Del Capítulo 4:

12. El desarrollo larvario de *Arbacia lixula* se ve facilitado por un aumento de temperatura, que produce una aceleración en el crecimiento y un aumento en la tasa de supervivencia.
13. Un ligero grado de acidificación (pH 7.7) produce sólo un efecto menor en el desarrollo larvario de *Arbacia lixula*, retardando el crecimiento, pero aumentando las tasas de supervivencia.

14. *Arbacia lixula* puede completar su desarrollo a temperatura tan baja como 16 ºC, aunque con tasas de supervivencia reducidas, crecimiento más lento y morfología larvaria alterada, respecto al desarrollo a 19 ºC.
15. Un ligero grado de acidificación no produce efectos en la supervivencia de los asentados a corto plazo, aunque los asentados procedentes de larvas crecidas a pH 7.7 fueron significativamente de menor tamaño que los procedentes de larvas crecidas a pH 8.1,

Conclusión general:

16. *Arbacia lixula* es una especie termófila de afinidades tropicales que, probablemente, se encuentra sometida a condiciones por debajo de las óptimas en el Mediterráneo Septentrional. Sus poblaciones podrían verse beneficiadas por el cambio global, ya que la tendencia al calentamiento puede mejorar la eficacia de los procesos que actualmente están limitando sus poblaciones en esta región. Por consiguiente, cabe esperar un futuro aumento de su impacto negativo en los ecosistemas costeros mediterráneos.

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Tossa de Mar beach. Photo: **X. Turon**

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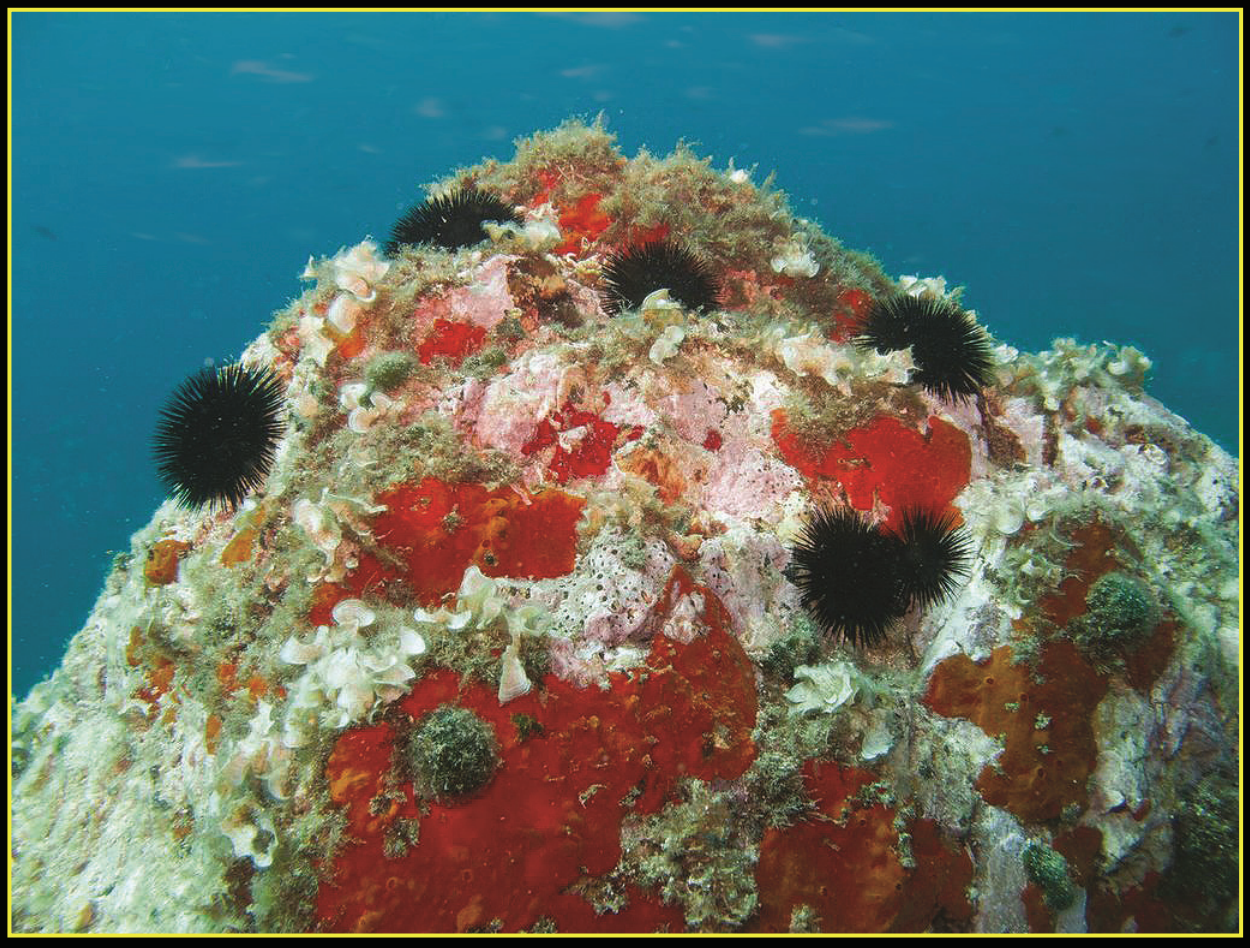
APPENDICES



A group of *Arbacia lixula* at Tossa de Mar. Photo: **C. Palacín**

APPENDIX I

Table S1 Haplotype frequencies of *Arbacia lixula* COI for all sampled (24) localities. Haplotypes shared by two or more localities are represented in bold, while numbers not in bold correspond to private haplotypes. Background colours correspond to the three different haplogroups (A, B or C).

Haplotype	ITA	CFR	BOA	TEN	GIG	FAI	PIC	TOR	HER	CAR	PAL	VIL
A1	**0**	**0**	**0.074**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A2	**0**	**0**	**0.259**	**0.250**	**0.375**	**0.250**	**0.167**	**0.148**	**0.077**	**0.231**	**0.179**	**0.133**
A3	0	0	0.074	0	0	0	0	0	0	0	0	0
A4	**0**	**0**	**0.111**	**0.042**	**0**	**0.083**	**0**	**0.037**	**0**	**0.038**	**0.036**	**0.033**
A5	0	0	0.037	0	0	0	0	0	0	0	0	0
A6	0	0	0.037	0	0	0	0	0	0	0	0	0
A7	0	0	0.074	0	0	0	0	0	0	0	0	0
A8	0	0	0.037	0	0	0	0	0	0	0	0	0
A9	0	0	0.037	0	0	0	0	0	0	0	0	0
A10	0	0	0.074	0	0	0	0	0	0	0	0	0
A11	0	0	0.037	0	0	0	0	0	0	0	0	0
A12	0	0	0.037	0	0	0	0	0	0	0	0	0
A13	**0**	**0**	**0.037**	**0**	**0**	**0**	**0**	**0**	**0**	**0.038**	**0**	**0**
A14	0	0	0	0.042	0	0	0	0	0	0	0	0
A15	**0**	**0**	**0**	**0.042**	**0**	**0**	**0**	**0.037**	**0.038**	**0**	**0**	**0**
A16	0	0	0	0.042	0	0	0	0	0	0	0	0
A17	**0**	**0**	**0**	**0.083**	**0**	**0.042**	**0.167**	**0**	**0.192**	**0.038**	**0.143**	**0.067**
A18	0	0	0	0.042	0	0	0	0	0	0	0	0
A19	0	0	0	0.042	0	0	0	0	0	0	0	0
A20	**0**	**0**	**0**	**0.042**	**0.042**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A21	**0**	**0**	**0**	**0.042**	**0.125**	**0.125**	**0.125**	**0**	**0**	**0**	**0**	**0**
A22	0	0	0	0.042	0	0	0	0	0	0	0	0
A23	0	0	0	0.042	0	0	0	0	0	0	0	0
A24	0	0	0	0.042	0	0	0	0	0	0	0	0
A25	0	0	0	0.042	0	0	0	0	0	0	0	0
A26	**0**	**0**	**0**	**0**	**0.042**	**0**	**0**	**0**	**0.038**	**0**	**0**	**0.033**
A27	**0**	**0**	**0**	**0**	**0.042**	**0.042**	**0**	**0.037**	**0.077**	**0.077**	**0.071**	**0.100**
A28	0	0	0	0	0.083	0	0	0	0	0	0	0
A29	0	0	0	0	0.042	0	0	0	0	0	0	0
A30	0	0	0	0	0.042	0	0	0	0	0	0	0
A31	0	0	0	0	0	0.042	0	0	0	0	0	0
A32	**0**	**0**	**0**	**0**	**0**	**0.042**	**0.042**	**0**	**0**	**0**	**0**	**0**
A33	**0**	**0**	**0**	**0**	**0**	**0.042**	**0**	**0**	**0**	**0.038**	**0**	**0**
A34	0	0	0	0	0	0.083	0	0	0	0	0	0
A35	0	0	0	0	0	0.042	0	0	0	0	0	0
A36	0	0	0	0	0	0.042	0	0	0	0	0	0
A37	**0**	**0**	**0**	**0**	**0**	**0.042**	**0.042**	**0**	**0**	**0**	**0**	**0**
A38	0	0	0	0	0	0.042	0	0	0	0	0	0
A39	0	0	0	0	0	0.042	0	0	0	0	0	0
A40	0	0	0	0	0	0	0.042	0	0	0	0	0
A41	**0**	**0**	**0**	**0**	**0**	**0**	**0.042**	**0.037**	**0**	**0**	**0**	**0.033**
A42	**0**	**0**	**0**	**0**	**0**	**0**	**0.042**	**0**	**0**	**0**	**0**	**0**
A43	0	0	0	0	0	0	0.042	0	0	0	0	0
A44	0	0	0	0	0	0	0.042	0	0	0	0	0
A45	0	0	0	0	0	0	0.042	0	0	0	0	0
A46	0	0	0	0	0	0	0	0.037	0	0	0	0
A47	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.037**	**0**	**0.038**	**0.036**	**0.033**
A48	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.037**	**0**	**0.038**	**0**	**0**
A49	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.038**	**0**	**0**	**0**
A50	0	0	0	0	0	0	0	0	0.038	0	0	0
A51	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.038**	**0**	**0**	**0**

Haplotype	ITA	CFR	BOA	TEN	GIG	FAI	PIC	TOR	HER	CAR	PAL	VIL
A52	0	0	0	0	0	0	0	0	0.038	0	0	0
A53	0	0	0	0	0	0	0	0	0.038	0	0	0
A54	0	0	0	0	0	0	0	0	0.038	0	0	0
A55	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.038**	**0**	**0**	**0**
A56	0	0	0	0	0	0	0	0	0.038	0	0	0
A57	0	0	0	0	0	0	0	0	0.038	0	0	0
A58	0	0	0	0	0	0	0	0	0	0.038	0	0
A59	0	0	0	0	0	0	0	0	0	0.038	0	0
A60	0	0	0	0	0	0	0	0	0	0.038	0	0
A61	0	0	0	0	0	0	0	0	0	0.038	0	0
A62	0	0	0	0	0	0	0	0	0	0	0.036	0
A63	0	0	0	0	0	0	0	0	0	0	0.036	0
A64	0	0	0	0	0	0	0	0	0	0	0.036	0
A65	0	0	0	0	0	0	0	0	0	0	0.036	0
A66	0	0	0	0	0	0	0	0	0	0	0	0.033
A67	0	0	0	0	0	0	0	0	0	0	0	0.033
A68	0	0	0	0	0	0	0	0	0	0	0	0.033
A69	0	0	0	0	0	0	0	0	0	0	0	0.033
A70	0	0	0	0	0	0	0	0	0	0	0	0.033
A71	0	0	0	0	0	0	0	0	0	0	0	0
A72	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A73	0	0	0	0	0	0	0	0	0	0	0	0
A74	0	0	0	0	0	0	0	0	0	0	0	0
A75	0	0	0	0	0	0	0	0	0	0	0	0
A76	0	0	0	0	0	0	0	0	0	0	0	0
A77	0	0	0	0	0	0	0	0	0	0	0	0
A78	0	0	0	0	0	0	0	0	0	0	0	0
A79	0	0	0	0	0	0	0	0	0	0	0	0
A80	0	0	0	0	0	0	0	0	0	0	0	0
A81	0	0	0	0	0	0	0	0	0	0	0	0
A82	0	0	0	0	0	0	0	0	0	0	0	0
A83	0	0	0	0	0	0	0	0	0	0	0	0
A84	0	0	0	0	0	0	0	0	0	0	0	0
A85	0	0	0	0	0	0	0	0	0	0	0	0
A86	0	0	0	0	0	0	0	0	0	0	0	0
A87	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A88	0	0	0	0	0	0	0	0	0	0	0	0
A89	0	0	0	0	0	0	0	0	0	0	0	0
A90	0	0	0	0	0	0	0	0	0	0	0	0
A91	0	0	0	0	0	0	0	0	0	0	0	0
A92	0	0	0	0	0	0	0	0	0	0	0	0
A93	0	0	0	0	0	0	0	0	0	0	0	0
A94	0	0	0	0	0	0	0	0	0	0	0	0
A95	0	0	0	0	0	0	0	0	0	0	0	0
A96	0	0	0	0	0	0	0	0	0	0	0	0
A97	0	0	0	0	0	0	0	0	0	0	0	0
A98	0	0	0	0	0	0	0	0	0	0	0	0
A99	0	0	0	0	0	0	0	0	0	0	0	0
A100	0	0	0	0	0	0	0	0	0	0	0	0
A101	0	0	0	0	0	0	0	0	0	0	0	0
A102	0	0	0	0	0	0	0	0	0	0	0	0
A103	0	0	0	0	0	0	0	0	0	0	0	0
A104	0	0	0	0	0	0	0	0	0	0	0	0
A105	0	0	0	0	0	0	0	0	0	0	0	0
A106	0	0	0	0	0	0	0	0	0	0	0	0
A107	0	0	0	0	0	0	0	0	0	0	0	0
B1	**0**	**0**	**0.037**	**0.042**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B2	0	0	0.037	0	0	0	0	0	0	0	0	0
B3	0	0	0	0.042	0	0	0	0	0	0	0	0
B4	**0**	**0**	**0**	**0.042**	**0.042**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B5	0	0	0	0.042	0	0	0	0	0	0	0	0
B6	**0**	**0**	**0**	**0**	**0.083**	**0**	**0.083**	**0.407**	**0.231**	**0.231**	**0.321**	**0.300**
B7	0	0	0	0	0.042	0	0	0	0	0	0	0

Haplotype	ITA	CFR	BOA	TEN	GIG	FAI	PIC	TOR	HER	CAR	PAL	VIL
B8	0	0	0	0	0.042	0	0	0	0	0	0	0
B9	0	0	0	0	0	0.042	0	0	0	0	0	0
B10	**0**	**0**	**0**	**0**	**0**	**0**	**0.042**	**0**	**0**	**0**	**0**	**0**
B11	0	0	0	0	0	0	0.083	0	0	0	0	0
B12	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.037**	**0**	**0**	**0.036**	**0**
B13	0	0	0	0	0	0	0	0.037	0	0	0	0
B14	0	0	0	0	0	0	0	0.037	0	0	0	0
B15	0	0	0	0	0	0	0	0.037	0	0	0	0
B16	0	0	0	0	0	0	0	0.037	0	0	0	0
B17	0	0	0	0	0	0	0	0	0	0.038	0	0
B18	0	0	0	0	0	0	0	0	0	0.038	0	0
B19	0	0	0	0	0	0	0	0	0	0	0.036	0
B20	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.033**
B21	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.033**
B22	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.033**
B23	0	0	0	0	0	0	0	0	0	0	0	0
B24	0	0	0	0	0	0	0	0	0	0	0	0
B25	0	0	0	0	0	0	0	0	0	0	0	0
B26	0	0	0	0	0	0	0	0	0	0	0	0
B27	0	0	0	0	0	0	0	0	0	0	0	0
B28	0	0	0	0	0	0	0	0	0	0	0	0
B29	0	0	0	0	0	0	0	0	0	0	0	0
B30	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B31	0	0	0	0	0	0	0	0	0	0	0	0
B32	0	0	0	0	0	0	0	0	0	0	0	0
B33	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B34	0	0	0	0	0	0	0	0	0	0	0	0
B35	0	0	0	0	0	0	0	0	0	0	0	0
B36	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B37	0	0	0	0	0	0	0	0	0	0	0	0
B38	0	0	0	0	0	0	0	0	0	0	0	0
B39	0	0	0	0	0	0	0	0	0	0	0	0
B40	0	0	0	0	0	0	0	0	0	0	0	0
B41	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B42	0	0	0	0	0	0	0	0	0	0	0	0
B43	0	0	0	0	0	0	0	0	0	0	0	0
B44	0	0.067	0	0	0	0	0	0	0	0	0	0
B45	0	0.067	0	0	0	0	0	0	0	0	0	0
B46	0	0.067	0	0	0	0	0	0	0	0	0	0
C1	**0.750**	**0.467**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
C2	0.050	0	0	0	0	0	0	0	0	0	0	0
C3	0.150	0	0	0	0	0	0	0	0	0	0	0
C4	0.050	0	0	0	0	0	0	0	0	0	0	0
C5	0	0.067	0	0	0	0	0	0	0	0	0	0
C6	0	0.133	0	0	0	0	0	0	0	0	0	0
C7	0	0.067	0	0	0	0	0	0	0	0	0	0
C8	0	0.067	0	0	0	0	0	0	0	0	0	0

Table S1 (continued)

Haplotype	BEN	XAB	CLM	TOS	COL	CAB	FOR	SCA	POP	CRE	RHO	KOS
A1	**0**	**0**	**0**	**0.034**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A2	**0.207**	**0.111**	**0.280**	**0.034**	**0.080**	**0.250**	**0.111**	**0.143**	**0.148**	**0.207**	**0.111**	**0.296**
A3	0	0	0	0	0	0	0	0	0	0	0	0
A4	**0**	**0**	**0.040**	**0**	**0.040**	**0**	**0.037**	**0**	**0**	**0.138**	**0**	**0**
A5	0	0	0	0	0	0	0	0	0	0	0	0
A6	0	0	0	0	0	0	0	0	0	0	0	0
A7	0	0	0	0	0	0	0	0	0	0	0	0
A8	0	0	0	0	0	0	0	0	0	0	0	0
A9	0	0	0	0	0	0	0	0	0	0	0	0
A10	0	0	0	0	0	0	0	0	0	0	0	0
A11	0	0	0	0	0	0	0	0	0	0	0	0
A12	0	0	0	0	0	0	0	0	0	0	0	0
A13	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A14	0	0	0	0	0	0	0	0	0	0	0	0
A15	**0**	**0.037**	**0**	**0**	**0.040**	**0**	**0**	**0**	**0.074**	**0**	**0**	**0**
A16	0	0	0	0	0	0	0	0	0	0	0	0
A17	**0.103**	**0.111**	**0.120**	**0.241**	**0.320**	**0.063**	**0.222**	**0.238**	**0.222**	**0.069**	**0.185**	**0.148**
A18	0	0	0	0	0	0	0	0	0	0	0	0
A19	0	0	0	0	0	0	0	0	0	0	0	0
A20	**0**	**0.037**	**0**	**0.034**	**0**	**0**	**0**	**0**	**0.074**	**0**	**0**	**0**
A21	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A22	0	0	0	0	0	0	0	0	0	0	0	0
A23	0	0	0	0	0	0	0	0	0	0	0	0
A24	0	0	0	0	0	0	0	0	0	0	0	0
A25	0	0	0	0	0	0	0	0	0	0	0	0
A26	**0.069**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A27	**0.034**	**0.074**	**0**	**0.034**	**0.040**	**0**	**0.037**	**0.048**	**0.037**	**0.069**	**0.037**	**0**
A28	0	0	0	0	0	0	0	0	0	0	0	0
A29	0	0	0	0	0	0	0	0	0	0	0	0
A30	0	0	0	0	0	0	0	0	0	0	0	0
A31	0	0	0	0	0	0	0	0	0	0	0	0
A32	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A33	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A34	0	0	0	0	0	0	0	0	0	0	0	0
A35	0	0	0	0	0	0	0	0	0	0	0	0
A36	0	0	0	0	0	0	0	0	0	0	0	0
A37	**0.034**	**0**	**0**	**0.069**	**0**	**0**	**0.037**	**0**	**0**	**0.034**	**0.037**	**0.037**
A38	0	0	0	0	0	0	0	0	0	0	0	0
A39	0	0	0	0	0	0	0	0	0	0	0	0
A40	0	0	0	0	0	0	0	0	0	0	0	0
A41	**0**	**0.074**	**0**	**0**	**0.040**	**0**	**0.037**	**0**	**0**	**0.069**	**0**	**0**
A42	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.037**	**0**
A43	0	0	0	0	0	0	0	0	0	0	0	0
A44	0	0	0	0	0	0	0	0	0	0	0	0
A45	0	0	0	0	0	0	0	0	0	0	0	0
A46	0	0	0	0	0	0	0	0	0	0	0	0
A47	**0**	**0**	**0**	**0.034**	**0**	**0**	**0.037**	**0**	**0.037**	**0**	**0**	**0**
A48	**0.034**	**0**	**0**	**0**	**0.040**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
A49	**0**	**0**	**0**	**0**	**0**	**0**	**0.037**	**0**	**0**	**0**	**0**	**0**
A50	0	0	0	0	0	0	0	0	0	0	0	0
A51	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.048**	**0**	**0**	**0**	**0**
A52	0	0	0	0	0	0	0	0	0	0	0	0
A53	0	0	0	0	0	0	0	0	0	0	0	0
A54	0	0	0	0	0	0	0	0	0	0	0	0
A55	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.037**	**0**	**0**	**0**
A56	0	0	0	0	0	0	0	0	0	0	0	0
A57	0	0	0	0	0	0	0	0	0	0	0	0
A58	0	0	0	0	0	0	0	0	0	0	0	0
A59	0	0	0	0	0	0	0	0	0	0	0	0
A60	0	0	0	0	0	0	0	0	0	0	0	0
A61	0	0	0	0	0	0	0	0	0	0	0	0

Haplotype	BEN	XAB	CLM	TOS	COL	CAB	FOR	SCA	POP	CRE	RHO	KOS
A62	0	0	0	0	0	0	0	0	0	0	0	0
A63	0	0	0	0	0	0	0	0	0	0	0	0
A64	0	0	0	0	0	0	0	0	0	0	0	0
A65	0	0	0	0	0	0	0	0	0	0	0	0
A66	0	0	0	0	0	0	0	0	0	0	0	0
A67	0	0	0	0	0	0	0	0	0	0	0	0
A68	0	0	0	0	0	0	0	0	0	0	0	0
A69	0	0	0	0	0	0	0	0	0	0	0	0
A70	0	0	0	0	0	0	0	0	0	0	0	0
A71	0.034	0	0	0	0	0	0	0	0	0	0	0
A72	**0**	**0.037**	**0**	**0**	**0**	**0.063**	**0**	**0.048**	**0**	**0.034**	**0**	**0.037**
A73	0	0.037	0	0	0	0	0	0	0	0	0	0
A74	0	0	0.040	0	0	0	0	0	0	0	0	0
A75	0	0	0.040	0	0	0	0	0	0	0	0	0
A76	0	0	0.040	0	0	0	0	0	0	0	0	0
A77	0	0	0.040	0	0	0	0	0	0	0	0	0
A78	0	0	0.040	0	0	0	0	0	0	0	0	0
A79	0	0	0	0.034	0	0	0	0	0	0	0	0
A80	0	0	0	0.034	0	0	0	0	0	0	0	0
A81	0	0	0	0.034	0	0	0	0	0	0	0	0
A82	0	0	0	0	0.040	0	0	0	0	0	0	0
A83	0	0	0	0	0.040	0	0	0	0	0	0	0
A84	0	0	0	0	0.040	0	0	0	0	0	0	0
A85	0	0	0	0	0	0.063	0	0	0	0	0	0
A86	0	0	0	0	0	0.063	0	0	0	0	0	0
A87	**0**	**0**	**0**	**0**	**0**	**0.063**	**0**	**0**	**0**	**0.034**	**0**	**0**
A88	0	0	0	0	0	0.063	0	0	0	0	0	0
A89	0	0	0	0	0	0	0.037	0	0	0	0	0
A90	0	0	0	0	0	0	0.037	0	0	0	0	0
A91	0	0	0	0	0	0	0.037	0	0	0	0	0
A92	0	0	0	0	0	0	0	0.048	0	0	0	0
A93	0	0	0	0	0	0	0	0	0.037	0	0	0
A94	0	0	0	0	0	0	0	0	0	0.034	0	0
A95	0	0	0	0	0	0	0	0	0	0.034	0	0
A96	0	0	0	0	0	0	0	0	0	0.034	0	0
A97	0	0	0	0	0	0	0	0	0	0	0.037	0
A98	0	0	0	0	0	0	0	0	0	0	0.037	0
A99	0	0	0	0	0	0	0	0	0	0	0.037	0
A100	0	0	0	0	0	0	0	0	0	0	0.037	0
A101	0	0	0	0	0	0	0	0	0	0	0.037	0
A102	0	0	0	0	0	0	0	0	0	0	0.037	0
A103	0	0	0	0	0	0	0	0	0	0	0	0.037
A104	0	0	0	0	0	0	0	0	0	0	0	0.037
A105	0	0	0	0	0	0	0	0	0	0	0	0.037
A106	0	0	0	0	0	0	0	0	0	0	0	0.037
A107	0	0	0	0	0	0	0	0	0	0	0	0.037
B1	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B2	0	0	0	0	0	0	0	0	0	0	0	0
B3	0	0	0	0	0	0	0	0	0	0	0	0
B4	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B5	0	0	0	0	0	0	0	0	0	0	0	0
B6	**0.345**	**0.259**	**0.200**	**0.276**	**0.160**	**0.375**	**0.259**	**0.238**	**0.222**	**0.172**	**0.296**	**0.185**
B7	0	0	0	0	0	0	0	0	0	0	0	0
B8	0	0	0	0	0	0	0	0	0	0	0	0
B9	0	0	0	0	0	0	0	0	0	0	0	0
B10	**0**	**0**	**0**	**0.034**	**0.040**	**0**	**0**	**0**	**0.074**	**0.034**	**0.037**	**0.037**
B11	0	0	0	0	0	0	0	0	0	0	0	0
B12	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B13	0	0	0	0	0	0	0	0	0	0	0	0
B14	0	0	0	0	0	0	0	0	0	0	0	0
B15	0	0	0	0	0	0	0	0	0	0	0	0

Haplotype	BEN	XAB	CLM	TOS	COL	CAB	FOR	SCA	POP	CRE	RHO	KOS
B16	0	0	0	0	0	0	0	0	0	0	0	0
B17	0	0	0	0	0	0	0	0	0	0	0	0
B18	0	0	0	0	0	0	0	0	0	0	0	0
B19	0	0	0	0	0	0	0	0	0	0	0	0
B20	**0**	**0**	**0**	**0**	**0.040**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B21	**0.034**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B22	**0**	**0.037**	**0.040**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
B23	0.034	0	0	0	0	0	0	0	0	0	0	0
B24	0.034	0	0	0	0	0	0	0	0	0	0	0
B25	0.034	0	0	0	0	0	0	0	0	0	0	0
B26	0	0.037	0	0	0	0	0	0	0	0	0	0
B27	0	0.037	0	0	0	0	0	0	0	0	0	0
B28	0	0.037	0	0	0	0	0	0	0	0	0	0
B29	0	0.037	0	0	0	0	0	0	0	0	0	0
B30	**0**	**0.037**	**0**	**0**	**0**	**0**	**0**	**0.048**	**0**	**0**	**0**	**0**
B31	0	0	0.040	0	0	0	0	0	0	0	0	0
B32	0	0	0.040	0	0	0	0	0	0	0	0	0
B33	**0**	**0**	**0.040**	**0**	**0**	**0**	**0.037**	**0**	**0**	**0**	**0**	**0**
B34	0	0	0	0.034	0	0	0	0	0	0	0	0
B35	0	0	0	0.034	0	0	0	0	0	0	0	0
B36	**0**	**0**	**0**	**0.034**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.037**
B37	0	0	0	0	0.040	0	0	0	0	0	0	0
B38	0	0	0	0	0	0	0.037	0	0	0	0	0
B39	0	0	0	0	0	0	0	0.095	0	0	0	0
B40	0	0	0	0	0	0	0	0.048	0	0	0	0
B41	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.037**	**0**	**0**	**0.037**
B42	0	0	0	0	0	0	0	0	0	0.034	0	0
B43	0	0	0	0	0	0	0	0	0	0	0.037	0
B44	0	0	0	0	0	0	0	0	0	0	0	0
B45	0	0	0	0	0	0	0	0	0	0	0	0
B46	0	0	0	0	0	0	0	0	0	0	0	0
C1	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
C2	0	0	0	0	0	0	0	0	0	0	0	0
C3	0	0	0	0	0	0	0	0	0	0	0	0
C4	0	0	0	0	0	0	0	0	0	0	0	0
C5	0	0	0	0	0	0	0	0	0	0	0	0
C6	0	0	0	0	0	0	0	0	0	0	0	0
C7	0	0	0	0	0	0	0	0	0	0	0	0
C8	0	0	0	0	0	0	0	0	0	0	0	0

APPENDIX II

Published papers. Copies of the two chapters of this thesis already published are included below in their original format.

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PLOS ONE

Natural or Naturalized? Phylogeography Suggests That the Abundant Sea Urchin *Arbacia lixula* Is a Recent Colonizer of the Mediterranean

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Abstract

We present the global phylogeography of the black sea urchin *Arbacia lixula*, an amphi-Atlantic echinoid with potential to strongly impact shallow rocky ecosystems. Sequences of the mitochondrial cytochrome *c* oxidase gene of 604 specimens from 24 localities were obtained, covering most of the distribution area of the species, including the Mediterranean and both shores of the Atlantic. Genetic diversity measures, phylogeographic patterns, demographic parameters and population differentiation were analysed. We found high haplotype diversity but relatively low nucleotide diversity, with 176 haplotypes grouped within three haplogroups: one is shared between Eastern Atlantic (including Mediterranean) and Brazilian populations, the second is found in Eastern Atlantic and the Mediterranean and the third is exclusively from Brazil. Significant genetic differentiation was found between Brazilian, Eastern Atlantic and Mediterranean regions, but no differentiation was found among Mediterranean sub-basins or among Eastern Atlantic sub-regions. The star-shaped topology of the haplotype network and the unimodal mismatch distributions of Mediterranean and Eastern Atlantic samples suggest that these populations have suffered very recent demographic expansions. These expansions could be dated 94–205 kya in the Mediterranean, and 31–67 kya in the Eastern Atlantic. In contrast, Brazilian populations did not show any signature of population expansion. Our results indicate that all populations of *A. lixula* constitute a single species. The Brazilian populations probably diverged from an Eastern Atlantic stock. The present-day genetic structure of the species in Eastern Atlantic and the Mediterranean is shaped by very recent demographic processes. Our results support the view (backed by the lack of fossil record) that *A. lixula* is a recent thermophilous colonizer which spread throughout the Mediterranean during a warm period of the Pleistocene, probably during the last interglacial. Implications for the possible future impact of *A. lixula* on shallow Mediterranean ecosystems in the context of global warming trends must be considered.

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Introduction

The European black sea urchin *Arbacia lixula* (Linnaeus, 1758) is currently one of the most abundant echinoids in shallow rocky habitats of the Mediterranean [1], where it has the potential to greatly influence benthic communities with their grazing activity [2–4]. *A. lixula* has a considerable trophic plasticity, ranging from omnivory to strict carnivory [5] and its scraping predatory behaviour can bulldoze the substrate bare of erect and encrusting algae and sessile animals. *A. lixula* broadly overlaps its habitat with the common edible sea urchin *Paracentrotus lividus* (Lamarck, 1816). Both species are traditionally thought to have the ability to trigger the development of subtidal barren zones of reduced benthic productivity and diversity [6–9]. However, new and increasing evidence suggests that *A. lixula* could actually be playing the principal role in producing and maintaining these barrens [10] and that this trend could be worsening in the near future due to foreseeable climatic changes [11].

Arbacia lixula is commonly regarded as a typical native species in the Mediterranean fauna [12], since it is currently found in shallow rocky shores all along the Mediterranean, often at high densities, and has been so since historical times. However, its tropical affinities have been suggested for a long time. Based on the lack of Mediterranean fossil record, Stefanini [13] and Mortensen [14] stated that *A. lixula* (reported as *A. pustulosa*), probably originated at the Tropical Atlantic region, from where it spread into the Mediterranean. Kempf [15], Tortonese [16] and Fenaux [17] also considered that *A. lixula* was a thermophilous species.

In NW Mediterranean, increasing abundances over time have been reported for this species. In 1950, Petit *et al.* reported that *Arbacia lixula* had become abundant in Marseilles during the previous 30 years [18], despite Marion had described it as rare in the same area in 1883 [19]. More recently, Francour *et al.* reported a 12–fold increase in the abundance of *A. lixula* in Corsica over a period of nine years (1983–1992) and speculated that a long term rise in the water temperature could have been the cause for this

proliferation [20]. In the same period (1982 to 1995), a 5-fold increase in *A. lixula* densities was reported at the Port-Cros Marine Reserve (France) [21]. On the other hand, in a recent 5-year follow-up (2003–2008) at Ustica Island (Southern Thyrrenian Basin), a positive correlation was found between the gonado-somatic index of adult *A. lixula* and summer surface water temperature, suggesting increased reproductive potential with temperature [10].

Arbacia is an ancient genus with a fossil record that dates back to the Paleocene [22] whose distribution is mainly Neotropical. Unlike other sea urchin genera, *Arbacia* has a history of latitudinal shifts [23], and the five extant species inhabit mainly temperate and tropical shallow waters [24], being mostly allopatric. Only one species, *A. dufresnii*, is able to live in cold Subantarctic waters. *A. lixula* is the only species in the genus that lives in the Old World. Its present distribution includes Brazil, the African Atlantic coast from Morocco to Angola, the East Atlantic archipelagos of Cape Verde, Canaries, Madeira and Azores, and the whole Mediterranean basin, excluding the Black Sea. It has never been reported from the Atlantic European coast north of Gibraltar (J. Cristobo, X. Troncoso, N. V. Rodrigues; pers. comms.), probably due to the low sea surface temperature originated by the southward Portugal Current [25].

Recently, Lessios *et al.* [26] presented an exhaustive phylogenetic study of genus *Arbacia*, using sequences of the mitochondrial COI (cytochrome *c* oxidase I) and the nuclear gamete recognition protein bindin, which has clarified many interesting questions on inter-specific relationships within this remarkable genus. Notably, the sequence of speciation events was consistently reconstructed and their divergence times were reliably estimated. Thus, the splitting between *A. lixula* and its sister species, the NW Atlantic *A. punctulata*, was estimated to have taken place some 2.2–3.0 Mya (millions years ago) based on COI sequences, or 1.9–3.3 Mya based on bindin sequences. The phylogeny of bindin sequences also allowed these authors to infer that Brazil populations separated from the rest of *A. lixula* some 1.8–3.4 Mya; i.e. very early in the evolution of this species (however, only 5 individuals from Brazil were used in the analysis, and no estimation could be inferred for the same event from mitochondrial sequences, due to the unresolved position of the Brazilian clade within other *A. lixula* haplotypes).

Yet, many questions remain open about the intra-specific relationships of *Arbacia lixula*. Considering its unusually wide present distribution area, which ranges from equatorial waters to temperate Mediterranean, the great colonizing potential shown by this species, including the ability to cross trans-oceanic barriers to gene flow [26], and the massive potential impact of its behaviour on coastal ecosystems, further research on its phylogeography and population genetics is necessary in order to elucidate the history and ongoing processes that shape the distribution of the species. In this work, we present a phylogeographic study using the mitochondrial marker COI, based on a representative sample of individuals covering most of the distribution area of *Arbacia lixula*. Our goals were to answer relevant questions concerning the history and present-day distribution of the species: What are the relationships between the main geographic areas where the species is found? Do the main geographic barriers to gene flow, that are known to regulate the genetic structure of many other marine organisms, affect the present-day genetic structure of this species? Can recent geographic and/or population expansion events be traced and reconstructed by analysing the signature left in sequence data of this species?

Methods

Ethics Statement

Field sampling required for this work involved only invertebrate species which are neither endangered nor protected. All necessary permits for sampling at localities placed inside protected areas (Cabrera National Park, Columbretes Islands Marine Reserve & Scandola Nature Reserve) were previously obtained from the competent authorities. Non-destructive sampling techniques (external soft tissue biopsy) were used in these localities in order to minimize impact on the ecosystems.

Sampling

Between April 2009 and July 2011, we obtained samples from 24 localities belonging to three predefined regions: West Atlantic, East Atlantic and Mediterranean (see Fig. 1 and Table 1). For more detailed analyses, we further subdivided the East Atlantic region in two sub-regions (Cape Verde and Macaronesia), while the Mediterranean was divided in three sub-basins (Alboran Sea, West Mediterranean and East Mediterranean). The sampled localities were: two from Brazil, one from Cape Verde, four from Macaronesian archipelagos, two from the Alboran Sea, twelve from West Mediterranean and three from East Mediterranean. 15 to 30 adult *Arbacia lixula* individuals (average: 25.2) per location were sampled. In all cases, tissue samples were stored in absolute ethanol at -20°C until processed.

DNA Amplification and Sequencing

Total DNA was extracted using REDExtract-N-Amp Tissue kit (Sigma–Aldrich, www.sigma.com) from either one tube foot or a tiny portion (5–10 mg) of gonad. A fragment of the COI gene was amplified and sequenced using specific primers designed using the complete genome sequence of *A. lixula* mitochondrion [27] with PRIMER 3.0 [28], as follows: COIARB-F: 5′-TTC TCT GCT TCA AGA TGA C-3′, COIARB-R: 5′-CTA TAA TCA TAG TCG CTG CT-3′, COIAL-R: 5′-GCT CGG GTA TCT AGG TCC AT-3′. Most individuals were amplified using the COIARB-F/COIARB-R pair, but some individuals belonging to Atlantic populations had to be amplified using COIARB-F/COIAL-R instead. PCR amplification reactions were performed in a 20 µl total-reaction volume with 10 µl of REDExtract-N-Amp PCR reaction mix (Sigma–Aldrich), 0.8 µl of each primer (10 µM), 6.4 µl of ultrapure water (Sigma–Aldrich) and 2 µl of template DNA. A single denaturing step at 94°C for 5 min was followed by 40 cycles (denaturation at 94°C for 40 s, annealing at 43°C for 45 s and extension at 72°C for 45 s) and a final extension at 72°C for 5 min in a S1000 dual thermal cycler (BioRad, www.bio-rad.com). The PCR products were purified and both strands sequenced in Macrogen (www.macrogen.com) using the same primers for the sequencing reaction.

Genetic Diversity Analyses

All the sequences were edited in BIOEDIT [29] and aligned using CLUSTALW as implemented in MEGA 5 [30]. The single nucleotide mutations found were double-checked by contrasting the agreement and quality of forward and reverse sequencing chromatograms. The Nei & Gojobori procedure with the Jukes & Cantor correction [31–32] implemented in MEGA 5 was used for detecting positive natural selection. Sequences of the haplotypes found have been deposited in GenBank (accession numbers from JQ745096 to JQ745256).

Number of haplotypes (N_h), haplotype diversity (H_d) and nucleotide diversity (π) were computed with DNASP v. 5.10 [33]. Haplotype richness was calculated with CONTRIB v. 1.02 [34] using

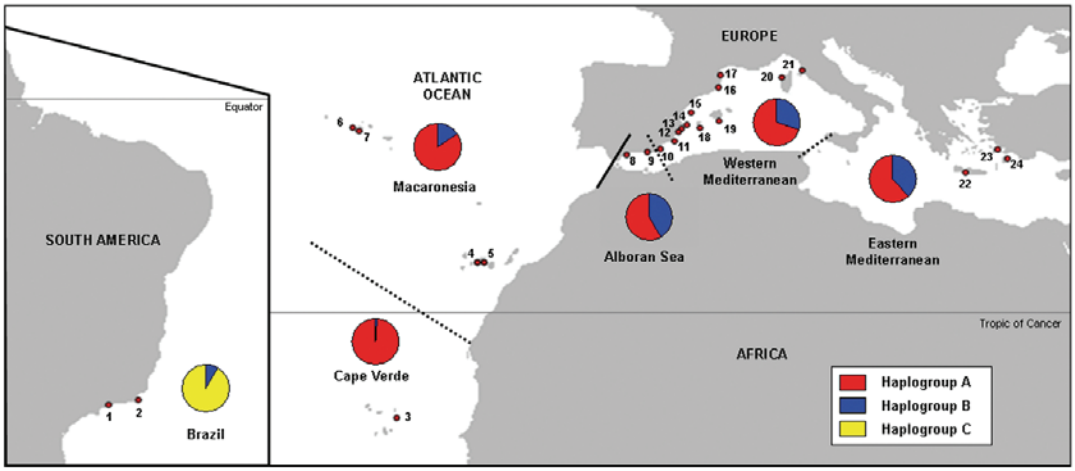

Figure 1. Sampling localities for *Arbacia lixula* populations. See Table 1 for locality names and coordinates. Borders between regions are indicated by solid bold lines and borders between sub-regions are represented by dotted lines. Pie charts of haplogroup frequencies are shown for the six sub-regions in which the three studied regions have been subdivided.
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a rarefaction size equal to the smallest sample size (n = 15) and Student's *t*-test was used for comparing its values between regions having more than two sampled locations (i.e., Eastern Atlantic and Mediterranean).

We used BAPS v. 5.2 (Bayesian Analysis of Population Structure) [35–36] for clustering the sampled haplotypes into monophyletic clusters of haplotypes (haplogroups). We ran five replicates for every value of the maximum number of clusters (k) up to k = 10. Haplotypes were assigned to one of the clusters by admixture analysis, performing 50 simulations from posterior haplotype frequencies. The assigned haplotype names reflect the haplogroup they belong.

Phylogeography and Phylogeny

Relationships and geographical distribution of the haplotypes were analysed in a haplotype network constructed with NETWORK v. 4.6.0.0 (http://www.fluxus-engineering.com/sharenet.htm), which implements the median-joining method, in the absence of recombination [37]. The network was optimized using maximum parsimony criterion and the obtained loops were solved using criteria derived from coalescent theory [38–39]. In order to determine the putative ancestral haplotypes, the outgroup weights based on haplotype frequency and connectivity [40] were calculated for each haplotype using the TCS v. 1.21 program [41].

For phylogenetic analysis of the haplotypes obtained, we included a sequence of *Strongylocentrotus purpuratus* from GenBank (Acc. number NC_001453 [42]). Though the use of an outgroup sequence for rooting intraspecific genealogies has been shown to have little resolution [43], we nevertheless used it since the resulting tree is coherent with the outgroup weights calculated using TCS. We used JMODELTEST v. 0.1.1 [44], based on a hierarchical series of likelihood ratio tests [45] and the Bayesian Information Criterion (BIC), to assess the most appropriate nucleotide substitution model for our data. This condition was satisfied by the Tamura & Nei model [46] with a gamma correction ($\alpha = 0.240$) (TrN + G). This evolution model was fed into MRBAYES software v. 3.1.2 [47] and the haplotype tree was estimated under the BIC after 1 million generations of 8 MCMC chains with a sample frequency of 100 (10,000 final trees). After verifying that stationarity had been reached, the first 2,000 trees were discarded, an independent majority-rule consensus tree was generated from the remaining (8,000 trees), and it was drawn using MESQUITE v. 2.75 [48].

Population Structure Analyses

Pairwise genetic distances between populations (F_{st}) were calculated with ARLEQUIN v. 3.1 [49] considering the genetic distance between haplotypes, and their significances were tested by performing 40,000 permutations. The level of significance for these multiple tests was corrected by applying the B–Y false discovery rate (FRD) procedure [50–51]. Kruskal's non-metric multidimensional scaling (MDS [52]) of F_{st} values was performed with RSTUDIO [53] to graphically visualise these results. In order to have a different differentiation measure based only on haplotype frequencies, Jost's *D* [54] was calculated using SPADE [55]. Negative values for *D* were corrected to zero. We calculated a confidence interval around the obtained values by 1,000 bootstrap replicates. We set this confidence interval, using the normal approximation, at the appropriate *P*-value following the B-Y correction as explained above. Significant differentiation was inferred when this confidence interval excluded zero.

Analyses of molecular variance (AMOVA) were performed to assess population structure, using conventional *F*-statistics (i.e. only with haplotype frequencies), and their significances were tested running 90,000 permutations in ARLEQUIN [56]. AMOVAs were performed using different population sets in order to test the significance of population structure among regions, or among sub-basins within regions. These AMOVAs were repeated also considering genetic distances between haplotypes, in order to check the robustness of the results.

The effect of isolation by geographical distance was assessed, for the whole dataset or separately for different populations sets, by the correlation of linearized genetic distances ($F_{st}/1–F_{st}$) [57] with geographical distances between localities. Though ideally the

Table 1. *Arbacia lixula.* Sampling localities.

Label	Locality	Code	Region	Sub-region	Latitude/Longitude
1	Itaipu	ITA	W. Atlantic	Brazil	−22.974910/−43.050456
2	Cabo Frio	CFR	W. Atlantic	Brazil	−22.890409/−41.998186
3	Boavista	BOA	E. Atlantic	Cape Verde	16.136858/−22.941055
4	Los Gigantes	GIG	E. Atlantic	Macaronesia	28.200925/−16.8294084
5	Tenerife (East)	TEN	E. Atlantic	Macaronesia	28.100823/−16.478088
6	Faial	FAI	E. Atlantic	Macaronesia	38.522720/−28.620937
7	Pico	PIC	E. Atlantic	Macaronesia	38.423336/−28.415823
8	Torremuelle	TOR	Mediterranean	Alboran Sea	36.577369/−4.565396
9	La Herradura	HER	Mediterranean	Alboran Sea	36.721044/−3.728487
10	Carboneras	CAR	Mediterranean	W. Medit.	36.993869/−1.890274
11	Palos	PAL	Mediterranean	W. Medit.	37.634580/−0.693749
12	Villajoyosa	VIL	Mediterranean	W. Medit.	38.509007/−0.212885
13	Benidorm	BEN	Mediterranean	W. Medit.	38.502530/−0.128329
14	Xabia	XAB	Mediterranean	W. Medit.	38.752880/0.224511
15	Columbretes	CLM	Mediterranean	W. Medit.	39.898115/0.685179
16	Tossa	TOS	Mediterranean	W. Medit.	41.722109/2.939914
17	Colera	COL	Mediterranean	W. Medit.	42.391077/3.155390
18	Formentera	FOR	Mediterranean	W. Medit.	38.693415/1.376867
19	Cabrera	CAB	Mediterranean	W. Medit.	39.155689/2.944236
20	Scandola	SCA	Mediterranean	W. Medit.	42.361842/8.549023
21	Populonia	POP	Mediterranean	W. Medit.	42.993752/10.498702
22	Crete	CRE	Mediterranean	E. Medit.	35.171626/24.400875
23	Kos	KOS	Mediterranean	E. Medit.	36.888477/27.308822
24	Rhodes	ROD	Mediterranean	E. Medit.	36.319364/28.207868

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oceanic current patterns should be included in the geographical distances calculation, currently we do not know of any reliable method for accurately quantifying this, so we used the shortest distance by sea on GOOGLE EARTH 6 (http://www.google.com/earth). The significance of the correlation was tested by the Mantel test procedure [58], implemented in ARLEQUIN, with 20,000 permutations for each analysis.

Demographic History Inference

Demographic history was inferred for the three studied regions and for each sub-basin by analysing the mismatch distributions. Populations that have recently experienced a sudden demographic growth show unimodal distributions, whereas those at demographic equilibrium show multimodal distributions [59]. The expected mismatch distributions under a sudden expansion model were computed in ARLEQUIN using Monte Carlo simulations with 10,000 random samples. The sum of squared deviations (SSD) between observed and expected distributions was used as a measure of fit, and the probability of obtaining a simulated SSD greater than or equal to the expected was computed by randomisation. If this probability was >0.05, the expansion model was accepted, and its parameters θ_0, θ_1 and τ were calculated. For those populations showing large values for the final effective population size θ_1, this method does not usually converge and flawed results could be obtained. In this case, we kept the value of τ calculated by this method, which is consistently robust [60], and used DNASP to calculate the value of θ_0 which minimized the SSD, letting θ_1 have an arbitrary large value of 1000 [61]. In the case that the mismatch distribution was not unimodal, the data were fitted to a constant population size model [62–63] for graphical representation.

To estimate the approximate time of a demographic expansion (t) from coalescence methods, the relationship $\tau = 2\ \mu kt$ was used [59] where τ is the mode of the mismatch distribution, μ is the mutation rate per nucleotide and k is the number of nucleotides of the analysed fragment. A range of mutation rates from 1.6% to 3.5% per million years was used for the COI gene, as calculated previously for echinoids [64–65].

In order to add more statistical support for population expansions, Tajima's D test of neutrality [66], Fu's F_s [67], and Ramos-Onsins & Rozas' R_2 [68] indices of population expansion were calculated using DNASP. The confidence limits of Tajima's D were obtained assuming that it follows the beta distribution [66], while statistical tests and confidence intervals for F_s and R_2 were based on a coalescent simulation algorithm implemented in DNASP, with 20,000 simulations. Harpending's raggedness index r [69] was calculated using ARLEQUIN and its significance was tested using parametric bootstrapping (10,000 replicates). These indices were calculated for the three regions and the six predefined subregions.

Results

Genetic Diversity

We sequenced 635 bp of the mitochondrial gene COI from 604 *Arbacia lixula* individuals from 24 localities (Fig. 1 and Table 1). We

Table 2. *Arbacia lixula*. Estimates of genetic diversity for all locations and regions sampled.

Locality or region	*N*	N_h (N_{priv})	r_{hap}	$H \pm SD$	$\pi \pm SD$
Itaipu	20	4 (3)	3.491	0.432±0.126	0.00074±0.00024
Cabo Frio	15	8 (7)	8.000	0.790±0.105	0.00594±0.00156
Total W. Atlantic	**35**	**11 (11)**	**5.935**	**0.605±0.096**	**0.00317±0.00098**
Boavista	27	15 (10)	10.172	0.920±0.038	0.00358±0.00067
Los Gigantes	24	12 (5)	8.698	0.851±0.064	0.00389±0.00092
Tenerife (East)	24	18 (10)	11.869	0.942±0.040	0.00577±0.00089
Faial	24	15 (7)	10.572	0.928±0.039	0.00444±0.00095
Pico	24	14 (5)	10.299	0.938±0.028	0.00528±0.00069
Total E. Atlantic	**123**	**56 (41)**	**10.924**	**0.921±0.019**	**0.00461±0.00040**
Torremuelle	27	14 (5)	8.638	0.826±0.069	0.00480±0.00065
La Herradura	26	15 (6)	9.999	0.917±0.037	0.00517±0.00040
Carboneras	26	15 (6)	9.750	0.905±0.041	0.00451±0.00051
Palos	28	12 (5)	8.031	0.860±0.047	0.00530±0.00062
Villajoyosa	30	16 (5)	9.596	0.894±0.044	0.00542±0.00058
Benidorm	29	12 (4)	7.808	0.842±0.051	0.00410±0.00033
Xabia	27	15 (5)	10.028	0.917±0.038	0.00544±0.00051
Columbretes	25	13 (7)	8.943	0.887±0.045	0.00549±0.00068
Tossa	29	15 (5)	8.980	0.877±0.044	0.00588±0.00068
Colera	25	14 (4)	9.433	0.883±0.052	0.00534±0.00069
Formentera	27	14 (4)	9.032	0.889±0.041	0.00511±0.00041
Cabrera	16	8 (3)	7.625	0.825±0.076	0.00493±0.00067
Scandola	21	10 (3)	8.199	0.886±0.043	0.00589±0.00069
Populonia	27	11 (1)	8.179	0.889±0.035	0.00529±0.00057
Crete	29	14 (4)	9.400	0.916±0.029	0.00492±0.00068
Kos	27	13 (5)	8.517	0.875±0.044	0.00503±0.00063
Rhodes	27	14 (7)	9.026	0.883±0.045	0.00550±0.00053
Total Mediterranean	**446**	**109 (94)**	**8.930**	**0.881±0.010**	**0.00519±0.00014**
TOTAL	**604**	**161**	**9.954**	**0.912±0.007**	**0.00658±0.00026**

N: sample size, N_h: number of haplotypes, N_{priv}: number of private haplotypes, r_{hap}: haplotype richness after rarefaction to a sample size of 15, *H*: haplotype diversity, π: nucleotide diversity, *SD*: standard deviation.

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found 135 polymorphic sites (21%), with a total of 144 mutations. All differences between haplotypes were substitutions, 42 of which were non-synonymous. The Nei-Gojobori Z-test did not detect any significant positive selection ($P>0.95$). A total of 161 haplotypes were obtained from all the sequences (Table S1). Of them, 126 (78.3%) were private haplotypes (found in only one locality) and 117 (72.7%) were represented by only one sampled individual. The number of haplotypes per locality ranged between 4 and 18. Haplotype diversity (H_d) and nucleotide diversity (π) calculated for the whole geographical range were 0.912 (±0.007 SD) and 0.00658 (±0.00026 SD), respectively (Table 2). All diversity measures were remarkably uniform among localities within each East Atlantic or Mediterranean regions, but were quite different in the case of the two sampled localities in Brazil, having the smallest values in Itaipu (the westernmost and southernmost locality in our study). The haplotype richness in the Eastern Atlantic samples was higher than in the Mediterranean ($t=3.336$, 20 d.f.; $P=0.0033$), indicating that the Eastern Atlantic populations are more genetically diverse than their Mediterranean counterparts. The small number of samples available from Brazil prevented us from performing any diversity comparison of this area with other regions.

The analysis of haplotype relationships using BAPS clustered the sampled haplotypes into three haplogroups (henceforth named A, B & C). Haplogroup A is the most abundant in all Eastern Atlantic and Mediterranean populations, but it is absent from Brazil, haplogroup B can be found in all three regions and haplogroup C is exclusive from Brazilian populations (Fig. 1).

Haplotype Network and Phylogenetic Inference

The haplotype network (Fig. 2) showed a strikingly star-shaped topology with a high ratio of singletons (81.4% of all haplotypes), which is typical of populations that have suffered a recent demographic expansion. The three most abundant haplotypes (A2, A17, B6) occupy central positions. All initial loops obtained by the MP criterion could be resolved using coalescent theory, except one, comprising 2 of the most frequent haplotypes (A2, A17), plus haplotypes, A4 & A20, which is therefore left unresolved in the figure. The outgroup weights calculated by the TCS program identified A2 as the ancestral haplotype (Table S1). This is the second most frequent haplotype and the only which is

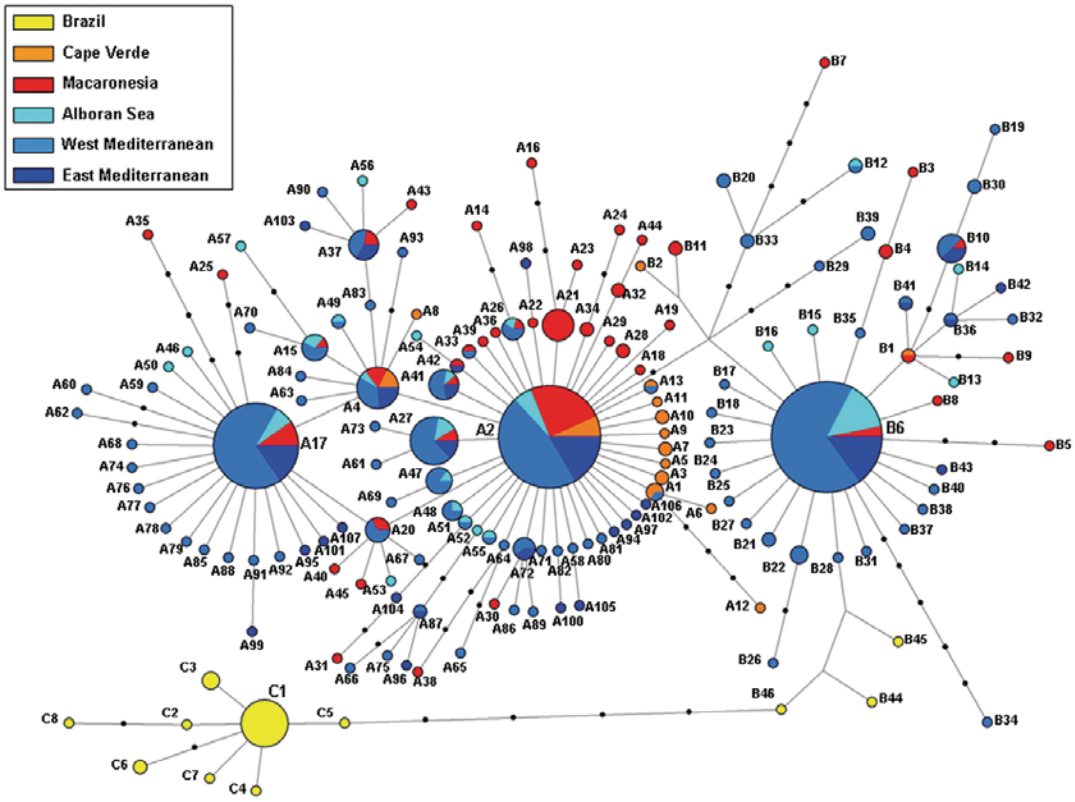

Figure 2. Median-joining haplotype network for *Arbacia lixula* COI. Haplotype numbers are preceded by a letter indicating the haplogroup they belong, A, B or C. Each haplotype is depicted by a circle coloured after the sub-region where it has been sampled. Areas are proportional to haplotype frequency. Each line represents a single nucleotide substitution step and additional mutations are represented by black bullets. The four haplotypes occupying central positions in each haplogroup, A2, A17, B6 and C1 are labelled in bigger font size.
doi:10.1371/journal.pone.0045067.g002

present in all localities except in the Brazilian ones. Haplotypes of groups A & B, widely shared among Eastern Atlantic and Mediterranean populations, appear close together in the network. Conversely, the Brazilian private haplogroup C is separated by six mutation steps from haplogroup B. The three haplotypes belonging to group B that are present in Brazilian populations are the most closely related to haplogroup C.

The consensus phylogenetic tree obtained by Bayesian Inference (Fig. 3) is coherent with the topology of the haplotype network. Haplotypes belonging to haplogroup A were collapsed at the base of the phylogram, indicating that this group is paraphyletic and ancestral, in accordance with the results of the outgroup weights analysis. Haplotypes of group B form a homogenous clade from which haplogroup C derives. The collapsed comb-like shape of haplogroups A and B suggests a recent demographic expansion. Interestingly, Brazilian haplotypes B44, B45 & B46 formed a monophyletic clade with haplogroup C, supported by a PP value of 0.81. This is consistent with previous results by Lessios *et al.* [26] which found that the samples from Brazil included in their analysis formed a clade nested within Eastern Atlantic (and Mediterranean) sequences.

Population Structure

The analyses of population pairwise genetic differentiation (F_{st} and Jost's D, Table 3) reflected a lack of population structure within both Eastern Atlantic and Mediterranean regions, but a clear differentiation between them and a complete differentiation (no alleles shared) of both regions from the Brazilian samples. Results from F_{st} and D were largely consistent. No significant differences could be found between any pair of localities from Cape Verde and Macaronesia, suggesting a high level of genetic flow among these Eastern Atlantic sub-regions. Likewise, no significant differences were found between any pair of Mediterranean localities (out of 136 possible pairs), with the exception of Torremuelle (the westernmost Mediterranean locality) where F_{st} analysis showed significant differences with two other Mediterranean localities, though these differences were not significant when D measures were analysed. Between Eastern Atlantic and Mediterranean, however, 38 (D) and 31 (F_{st}) comparisons (out of 85) were significant. Remarkably, the localities of Carboneras (Western Mediterranean), Crete and Kos (Eastern Mediterranean) did not show any significant difference to any other Eastern Atlantic or Mediterranean population, despite the large geographical distances involved in the case of the two latter localities.

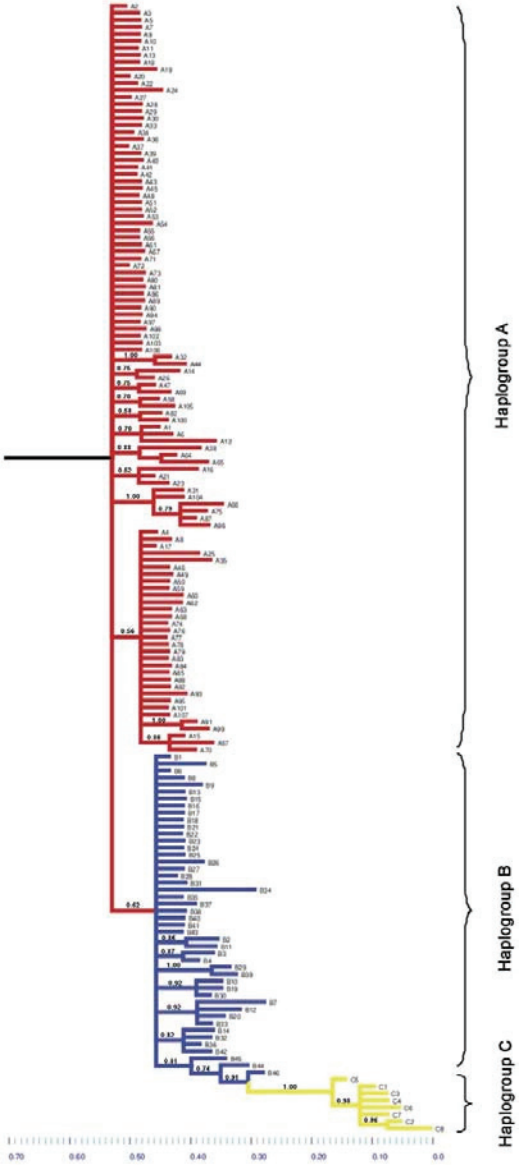

Figure 3. Bayesian inference consensus tree for haplotypes of *Arbacia lixula* COI. The tree is rooted using *Strongylocentrotus purpuratus* as outgroup (not shown); values for posterior probabilities >0.5, supporting non-collapsed clades, are indicated.
doi:10.1371/journal.pone.0045067.g003

The MDS analysis (Fig. 4) graphically expresses the relationships among populations obtained from F_{st} measures. Brazilian localities are widely separated in the first dimension from Eastern Atlantic and Mediterranean populations, whereas the Mediterranean and Eastern Atlantic populations were separated along the second axis. The lack of structure between sub-regions within the Eastern Atlantic and the Mediterranean is also apparent in the graphical arrangement. The same analysis using D measures (not shown) reflected the same overall structure.

Consistent with the pairwise differentiation analysis, the AMOVA found significant differences between the three regions (Table 4), which remained significant when only Eastern Atlantic *vs.* Mediterranean regions were compared (Table 5). Conversely, and again in agreement with the pairwise differentiation analyses, no significant differences within regions between Eastern Atlantic sub-regions (Table 6) or among the three Mediterranean sub-basins (Table 7) were detected by AMOVA. The same results were obtained when these AMOVAs were repeated considering genetic distances between haplotypes (data not shown).

The Mantel test showed significant isolation by distance when the whole dataset was analyzed (Fig. 5A). This result remained significant when populations from Brazil were excluded (Fig. 5B). Contrarily, no significant correlation between genetic differentiation and geographical distance was found when populations within just one region, either East Atlantic or Mediterranean, were analyzed (Fig. 5C & 5D).

Historical Demography

The mismatch distribution of *Arbacia lixula* populations from the Brazilian region (Fig. 6A) did not fit the sudden expansion model (Table 8). Conversely, the mismatch distribution for the Eastern Atlantic region (Fig. 6B) was remarkably unimodal. This indicates that a recent demographic expansion has occurred in this population. Similar results were obtained when only the Macaronesian sub-region was analyzed (Table 8). However, the distribution for the Cape Verde sub-basin did not fit the sudden expansion model, as reflected by a high SSD (Table 8). Nevertheless, this result may be an artefact due to small sample size (n = 27). The demographic expansion in the Eastern Atlantic populations could be dated, from the value of τ and the known mutation rate for the COI of Echinoidea, between 30.6–66.9 kya (thousand years ago), which is a surprisingly recent time.

The mismatch distribution obtained for the Mediterranean region (Fig. 6C) was also typically unimodal. The parameters of the theoretical curves calculated individually for each Mediterranean sub-basin had all similar values, comparable to those of the whole Mediterranean region (Table 8), reinforcing the idea that all the Mediterranean *Arbacia lixula* populations belong to the same genetic pool. The demographic expansion in the Mediterranean could be dated between 93.8–205.2 kya. This estimation is a little older than that obtained for the Eastern Atlantic expansion, but is still a recent time.

The neutrality and population expansion tests calculated for the different regions and sub-basins (Table 9) were largely coherent with the results inferred from the mismatch distributions. Tajima's D detected significant differences from neutrality in all cases, except for Brazil and the Eastern Mediterranean sub-basin. Fu's F_s test for demographic expansion was significant in all cases (though just marginally so in the case of Brazil). Ramos-Onsins & Rozas' R_2 was significant for all cases except the Eastern Mediterranean sub-basin, and the raggedness value r was consistent with unimodal distributions, except for Brazilian and Cape Verdean populations.

Discussion

COI and other mitochondrial markers have proven to be the most useful tool for tracing both intraspecific and intrageneric genealogies of many echinoid species [26,64–65,70–73] and usually yield easily interpretable results which are consistent with

Table 3. Genetic differentiation between *Arbacia lixula* populations, F_{st} (below the diagonal) and Jost's *D* (above the diagonal).

	Brazil		**East Atlantic**					**Mediterranean**																
	ITA	**CFR**	**BOA**	**GIG**	**TEN**	**FAI**	**PIC**	**TOR**	**HER**	**CAR**	**PAL**	**VIL**	**BEN**	**XAB**	**CLM**	**TOS**	**COL**	**FOR**	**CAB**	**SCA**	**POP**	**CRE**	**KOS**	**ROD**
ITA		0.115	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***
CFR	0.132*		**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***	**1***
BOA	**0.876***	**0.740***		0.170	0.012	0.064	0.413	**0.671***	0.763*	0.275	**0.553***	**0.598***	**0.557***	**0.656***	0.225	**0.889***	**0.751***	**0.664***	0.510	**0.632***	**0.608***	0.181	0.270	**0.715***
GIG	**0.865***	**0.712***	0.030		0.039	0.024	0.215	0.445	0.555*	0.129	0.343	0.380	0.290	0.427	0.093	0.720*	**0.669***	0.512*	0.252	0.443	0.407	0.203	0.096	0.500*
TEN	**0.825***	**0.676***	0.015	0.002		0	0.037	**0.662***	0.499*	0.216	0.431	0.524*	**0.454***	0.453*	0.081	0.677*	0.448	0.453*	0.444	0.382	0.295	0.142	0.087	0.522*
FAI	**0.861***	**0.719***	0.007	0.022	0.000		0.038	**0.670***	0.623*	0.226	**0.482***	**0.531***	**0.500***	**0.559***	0.185	**0.772***	**0.608***	**0.544***	0.496	**0.510***	**0.495***	0.130	0.197	**0.605***
PIC	**0.832***	**0.679***	0.035	0.010	−0.006	0.013		**0.501***	0.150	0.214	0.226	0.309	0.272	0.178	0.081	0.230	0.097	0.102	0.326	0.092	0.067	0.124	0.040	0.146
TOR	**0.830***	**0.674***	**0.228***	0.136*	**0.143***	**0.222***	**0.135***		0.164	0.015	0	0	0	0.015	0.150	0.205	0.430	0.121	0	0.187	0.187	0.153	0.216	0.064
HER	**0.833***	**0.687***	0.053	0.043	0.002	0.043	0.001	0.113*		0.084	0	0	0.018	0	0.103	0	0	0	0.122	0	0	0.136	0.121	0
CAR	**0.846***	**0.697***	0.063	0.015	0.019	0.056	0.017	0.053	0.002		0	0	0	0	0	0.241	0.322	0.059	0	0.071	0.073	0	0	0.045
PAL	**0.820***	**0.670***	**0.101***	0.047	0.039	**0.092***	0.028	0.024	0.003	−0.014		0	0	0	0	0.008	0.118	0	0	0	0	0.020	0.013	0
VIL	**0.810***	**0.662***	**0.100***	0.038	0.043	**0.095***	0.036	0.015	0.013	−0.017	−0.020		0	0	0.037	0.074	0.227	0	0	0.019	0.042	0	0.110	0
BEN	**0.849***	**0.701***	**0.177***	0.090	**0.097***	**0.172***	0.083	−0.010	0.061	0.011	−0.006	−0.012		0	0	0.083	0.231	0	0	0.010	0.039	0.082	0.015	0
XAB	**0.814***	**0.659***	**0.148***	0.072	0.075	**0.142***	0.061	−0.006	0.039	0.002	−0.012	−0.020	−0.020		0.030	0	0.089	0	0	0	0	0	0.074	0
CLM	**0.822***	**0.667***	0.093*	0.057	0.027	0.084*	0.019	0.051	−0.012	−0.002	−0.019	−0.011	0.015	0.003		0.224	0.201	0.022	0	0.011	0.022	0	0	0.045
TOS	**0.805***	**0.659***	**0.123***	0.077	0.051	**0.115***	0.035	0.028	0.008	0.005	−0.018	−0.007	−0.000	−0.007	−0.021		0	0	0.174	0	0	0.269	0.194	0
COL	**0.831***	**0.682***	**0.092***	**0.084***	0.025	**0.077***	0.020	0.113*	−0.020	0.024	0.005	0.025	0.073	0.046	−0.017	0.001		0	0.338	0	0	0.236	0.175	0.009
FOR	**0.829***	**0.681***	**0.085***	0.053	0.020	**0.074***	0.013	0.065	−0.018	−0.009	−0.019	−0.006	0.024	0.010	−0.026	−0.017	−0.020		0.062	0	0	0.054	0.047	0
CAB	**0.858***	**0.676***	0.099*	0.037	0.026	0.088*	0.020	0.022	−0.001	−0.023	−0.028	−0.029	−0.015	−0.022	−0.029	−0.022	0.010	−0.023		0.051	0.093	0.069	0.001	0.003
SCA	**0.819***	**0.651***	**0.122***	0.069	0.044	**0.111***	0.026	0.037	0.002	0.002	−0.018	−0.007	0.006	−0.014	−0.022	−0.020	0.000	−0.017	−0.025		0	0.101	0	0
POP	**0.826***	**0.680***	**0.096***	0.067	0.027	**0.087***	0.018	0.063	−0.014	0.001	−0.014	0.000	0.028	0.011	−0.023	−0.021	−0.019	−0.020	−0.015	−0.017		0.105	0	0
CRE	**0.833***	**0.693***	0.037	0.016	0.003	0.030	0.004	0.088	−0.008	−0.011	0.001	0.002	0.047	0.027	−0.003	0.016	0.007	−0.005	−0.014	0.014	−0.001		0	0.105
KOS	**0.833***	**0.686***	0.064	0.025	0.013	0.058	0.005	0.053	−0.004	−0.016	−0.013	−0.008	0.013	0.007	−0.016	−0.011	0.008	−0.014	−0.029	−0.005	−0.016	−0.019		0.047
ROD	**0.817***	**0.666***	**0.100***	0.055	0.034	**0.091***	0.014	0.037	−0.004	−0.008	−0.021	−0.011	0.003	−0.009	−0.023	−0.024	−0.004	−0.023	−0.026	−0.024	−0.023	−0.000	−0.020	

Consistently significant differences obtained by both methods after false discovery rate correction are represented in bold.
Significant *P* values for F_{st} obtained from randomization. *: significant after false discovery rate correction ($P<0.0085$).
Significant *P* values for *D* indicate that confidence interval obtained by bootstrapping excludes 0.
*: significant after false discovery rate correction ($P<0.0085$).
doi:10.1371/journal.pone.0045067.t003

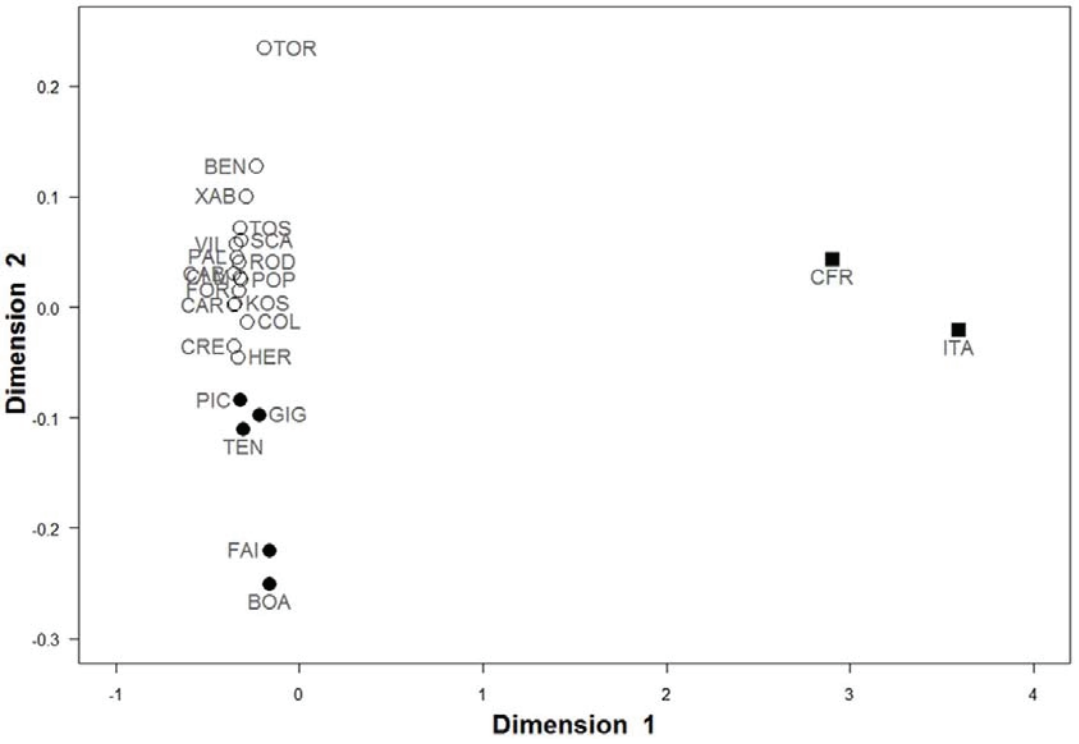

Figure 4. Multidimensional scaling (MDS) for F_{st} differentiation of *Arbacia lixula* COI haplotypes. Filled squares (■) represent Brazilian populations, whereas filled circles (●) represent Eastern Atlantic populations and open circles (○) correspond to Mediterranean populations.
doi:10.1371/journal.pone.0045067.g004

those of other nuclear markers. Nevertheless, our analyses are based on a single mitochondrial marker (COI). Thus, these results must be taken with caution, and further analyses using nuclear markers would be desirable. On the other hand, previous works in Echinoidea have shown that other nuclear markers were mainly used only to confirm the evolutionary history depicted by mtDNA [26,72] or else displayed too much diversity to produce interpretable results [73].

The *Arbacia lixula* populations sampled showed high values of haplotype diversity and haplotype richness, but relatively low values of nucleotide diversity. The lowest diversity was found in Brazilian populations and, specifically, in the westernmost locality (Itaipu), which is close to the distribution limit of the species and separated from the other Brazilian locality by the Cabo Frio upwelling. In contrast, the highest diversity was found in the East Atlantic, as expected if this region is the geographical origin of the species [16,26]. We detected three haplogroups in *A. lixula*. One of them (Group A) seems to be ancestral and is found only in Eastern Atlantic and Mediterranean populations, while another (Group B) is present at both sides of the Atlantic. The third one (Group C) is derived from Group B and found only in Brazil.

In a recent work, Lessios *et al.* [26] concluded that *Arbacia lixula* split from a common ancestor with *A. punctulata* ca. 2.6 Mya, and attributed this split to the mid-Atlantic barrier, separating the western *A. punctulata* from the eastern *A. lixula*, which would later have crossed back this barrier to establish itself, as an isolated

Table 4. Analysis of molecular variance (AMOVA) among regions using COI haplotype frequencies. Brazil vs. East Atlantic vs. Mediterranean.

Source of variation	df	Sum of squares	Variance components	Variation %	*P* value	Fixation index
Among groups	2	12.530	0.04690	9.69	<**0.0001*****	0.09692
Among populations within groups	21	9.728	0.00107	0.22	0.2583	0.00245
Within populations	580	252.833	0.43592	90.09	<**0.0001*****	0.09913
Total	603	275.091	0.48389			

doi:10.1371/journal.pone.0045067.t004

Table 5. Analysis of molecular variance (AMOVA) among regions using COI haplotype frequencies. East Atlantic vs. Mediterranean.

Source of variation	df	Sum of squares	Variance components	Variation %	*P* value	Fixation index
Between groups	1	4.104	0.01893	4.08	<**0.0001*****	0.04081
Among populations within groups	20	9.075	0.00035	0.08	0.3916	0.00080
Within populations	547	243.200	0.44461	95.84	**0.0002*****	0.04157
Total	568	256.380	0.46389			

doi:10.1371/journal.pone.0045067.t005

clade, in the coast of Brazil. A problem with this view is that the mid-Atlantic barrier was fully in place long before the estimated date of the split, so the separation of the two species could not be a vicariance event but a range expansion event (on the part of the lineage that would become *A. lixula*), and two crossings of the barrier are required to fully explain the present-day distribution of the species (though the second crossing could be facilitated by the South Equatorial Current system [74]). An alternative scenario would be that the two Atlantic species diverged in Western Atlantic, after the rise of the Panama isthmus isolated their ancestor from the eastern Pacific region (the possible origin of the genus *Arbacia* [26]), and that *A. lixula* crossed the Atlantic ridge only once to colonize the Eastern Atlantic. Our results favour the first (Lessios') view, as the haplotypes from Brazil formed a derived monophyletic group nested within the amphi-Atlantic Group B, rather than the opposite. This indicates a derived lineage in Western Atlantic, old enough to have had time to evolve forming the haplotype Group C. A more thorough sampling of the whole range of the Western Atlantic distribution and the inclusion of more data from Western Africa, are necessary before firm evidence can be obtained about the historical whereabouts of the main lineages of *A. lixula*.

Overall, the pattern of distribution of genetic variability (as shown in F_{st}, Jost's *D*, MDS and AMOVA analyses) showed three groups of populations that differed significantly from each other (Brazilian, Eastern Atlantic and Mediterranean), while little structure could be found within these groups. It is remarkable that the F_{st} measures based on sequence distance metrics and the differentiation measure *D* based on haplotype frequencies yielded essentially the same results. This is attributable to the prevalence of close haplotypes separated by small number of mutations (hence the low nucleotide diversity in general) that are widespread among populations. Thus, haplotype genetic differences had relatively little weight and most population structure derives from haplotype frequency differences.

Another striking pattern resulting from our molecular analyses is that recent demographic phenomena have shaped the present-day genetic structure of *Arbacia lixula* populations in the Eastern Atlantic and the Mediterranean. This does not seem to be the case of the Brazilian population but, given the small sample size, it is unclear if the resulting mismatch distribution (Fig. 6A) is either multimodal or L-shaped in this population. Multimodal curves are typical of populations at demographic equilibrium, but L-shaped distributions may result from very recent demographic bottlenecks [75]. More extensive sampling would be required to get the full picture of the demographic processes that have shaped the Brazilian populations of *A. lixula*.

The lack of an exclusively Mediterranean mitochondrial lineage of *Arbacia lixula* is remarkable. Other Atlanto-Mediterranean echinoderms such as *Marthasterias glacialis* [76], *Holothuria mammata* [77] or *Paracentrotus lividus* [73,78] do have lineages exclusive of the Mediterranean. These species have been probably present in the Mediterranean for several million years and their populations may have suffered several episodes of impaired gene flow during the Pleistocene glaciations. The genetic structure shown by *A. lixula* probably reflects a different demographic history from these other species.

Even if there is no phylogenetic break in the Mediterranean (as also found by Lessios *et al.* [26]) and alleles are widely shared at both sides of the Gibraltar boundary, this barrier seems nevertheless to restrict gene flow in *Arbacia lixula*, so as to establish significant differences in terms of haplotype frequencies between Mediterranean and Eastern Atlantic populations. The AMOVA (and pairwise comparisons) detected significant genetic differentiation between these groups of populations (Table 5), suggesting a reduced gene flow through the Strait of Gibraltar. Differently to what can be found in other marine organisms [79], the Strait itself, and not the Almeria-Oran Front (some 350 Km east of Gibraltar), is the place of the phylogeographic break, as the populations from the Alboran Sea are undistinguishable from other Mediterranean populations, but are significantly differentiated from most Atlantic populations (Fig. 4, Tables 3 and 7). Thus, *A. lixula* does not show any genetic differentiation among populations throughout the whole Mediterranean Sea. This could be due to recurrent gene flow, but oceanographic barriers such as the Almeria-Oran Front or the Siculo-Tunisian Strait [79] are strong enough to maintain genetic differentiation among different sub-basins in the case of other echinoderms of similar larval dispersive capacity [73,77–78].

Table 6. Analysis of molecular variance (AMOVA) among sub-regions within Eastern Atlantic region, using COI haplotype frequencies: Macaronesia vs. Cape Verde.

Source of variation	df	Sum of squares	Variance components	Variation %	*P* value	Fixation index
Among groups	1	0.681	0.00483	1.04	0.400	0.01042
Among populations within groups	3	1.427	0.00074	0.16	0.385	0.00161
Within populations	118	54.046	0.45802	98.80	0.179	0.01201
Total	122	56.154	0.46359			

doi:10.1371/journal.pone.0045067.t006

Table 7. Analysis of molecular variance (AMOVA) among sub-regions within the Mediterranean, using COI haplotype frequencies: Alboran vs. Western Mediterranean vs. Eastern Mediterranean.

Source of variation	df	Sum of squares	Variance components	Variation %	*P* value	Fixation index
Between groups	2	0.829	−0.00023	−0.05	0.495	−0.00052
Among populations within groups	14	6.138	−0.00009	−0.02	0.482	−0.00021
Within populations	429	189.154	0.44092	100.07	0.514	−0.00073
Total	445	196.121	0.44059			

doi:10.1371/journal.pone.0045067.t007

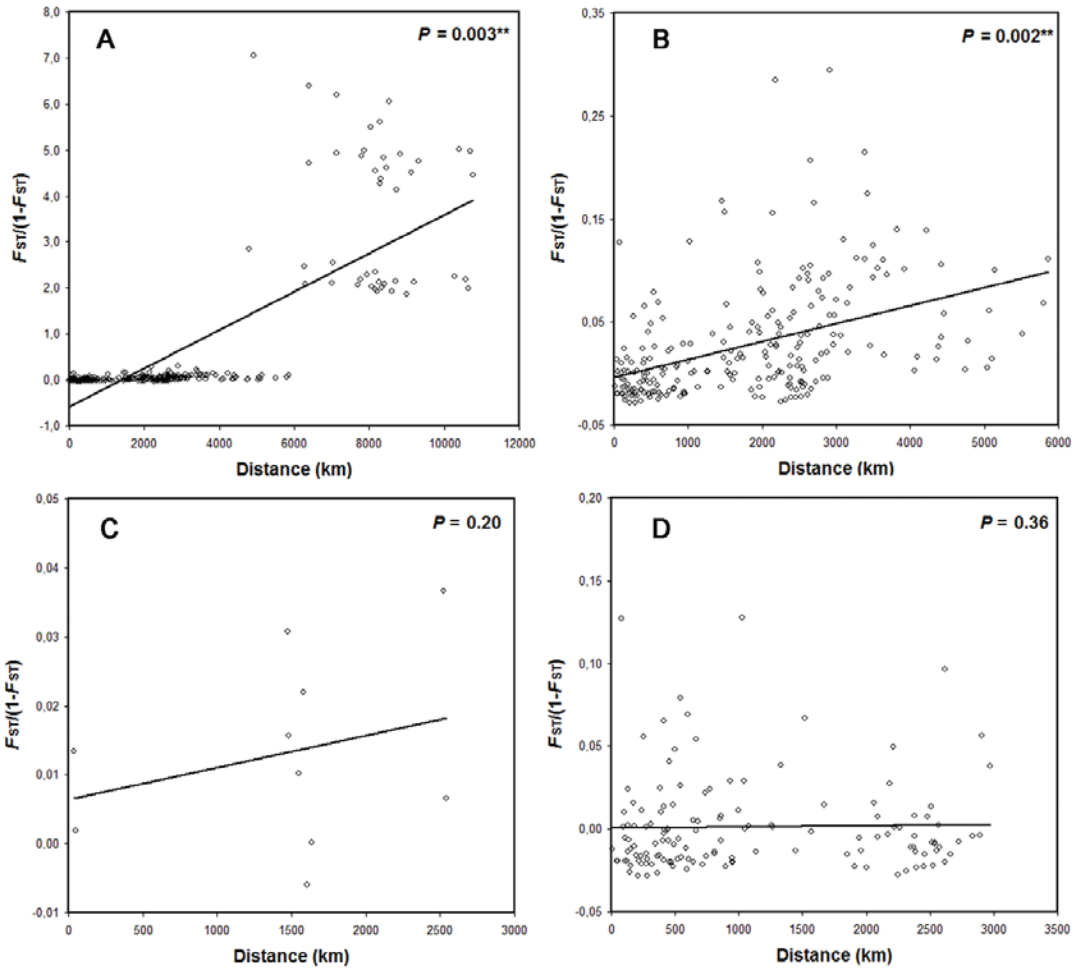

Figure 5. Relationships between genetic and geographic distances for different datasets of *Arbacia lixula* populations. Results of the Mantel test for isolation by distance are indicated.
doi:10.1371/journal.pone.0045067.g005

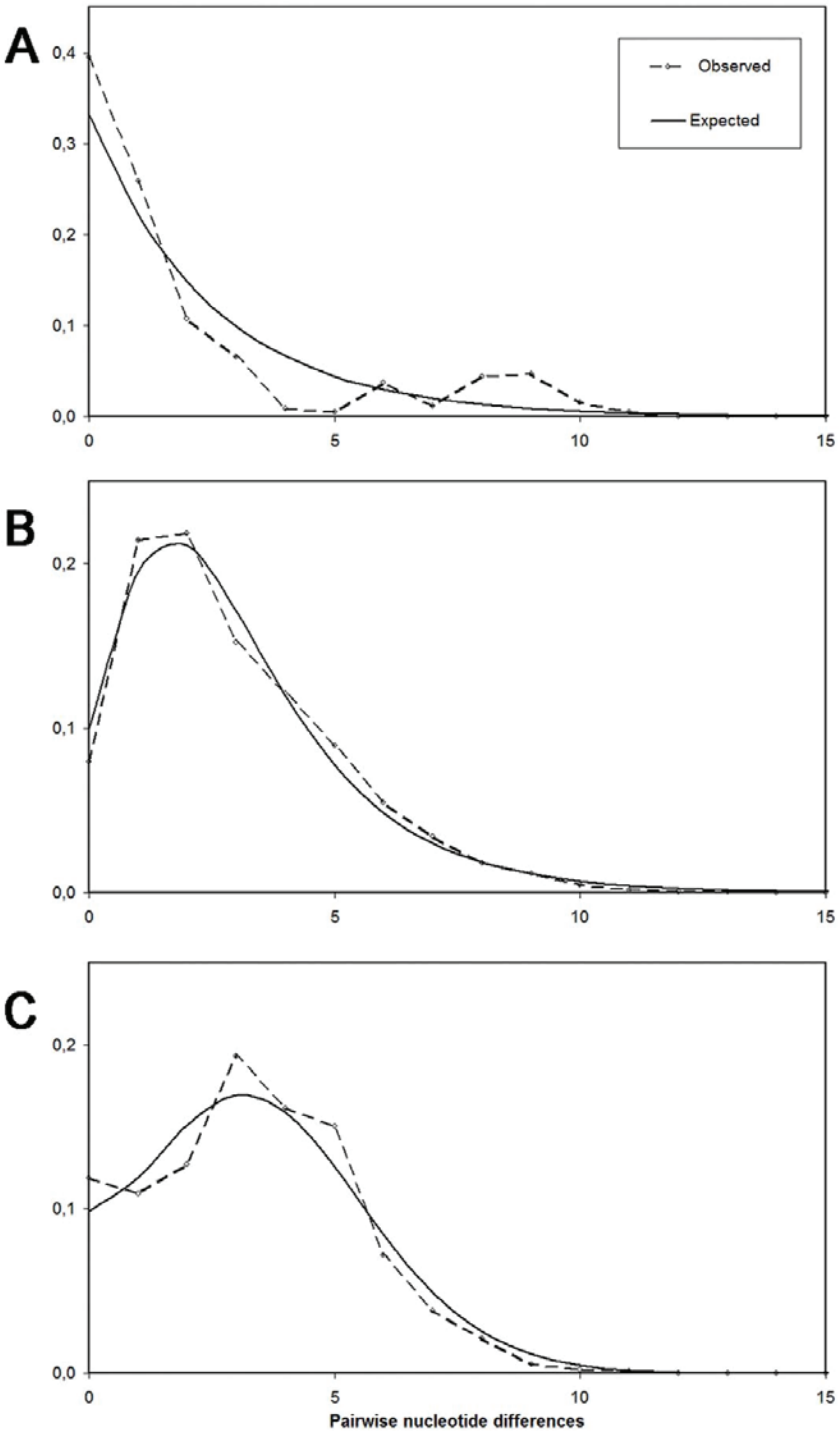
A
B
C
Observed
Expected
0,4
0,3
0,2
0,1
0,0
0
5
10
15
Pairwise nucleotide differences

Figure 6. Mismatch distributions of *Arbacia lixula* populations in the three studied regions. Observed data and theoretical expected distributions are represented by discontinuous and solid lines, respectively. For Brazil (A), the theoretical expected distribution shown is that of a population of constant size. In the case of the East Atlantic (B) and the Mediterranean (C), data were fitted to a sudden expansion model.
doi:10.1371/journal.pone.0045067.g006

We favour the alternative explanation (for the lack of genetic structure) that the colonization of the Mediterranean by *A. lixula* is so recent (see below) that populations in the different Mediterranean sub-basins have not had yet enough time to diverge from each other.

In the case of Macaronesian and Cape Verdean populations (Table 6), it seems likely that the present-day genetic similarity could be the result of a recent demographic expansion (see below), which could have swamped any trace of previous differentiated lineages potentially formed during periods of restricted gene flow among archipelagos.

Brazilian populations of *Arbacia lixula* are completely differentiated from Eastern Atlantic and Mediterranean populations (Tables 3 & 4). In addition, they showed the lowest genetic diversity and did not show any signature of demographic expansion. Nevertheless, our sample size is small, and Northern and Central Brazilian populations of *A. lixula* have never been sampled for phylogeographic studies. More extensive sampling along the Brazilian coast would be required for a full understanding of factors shaping the genetic structure of the West Atlantic populations of *A. lixula*.

The almost complete lack of fossil record for *Arbacia lixula* in the Mediterranean is most revealing. At present, the species is highly abundant and occurs in areas that have been thoroughly sampled by palaeontologists. Other Mediterranean echinoids currently co-occurring in the same habitats are commonly found in assemblages of the Pleistocene and have been abundantly reported in the paleontological literature [80–83]. In contrast, only one fossil individual of *A. lixula* from the Mediterranean has ever been reported in the literature [13]. It was found in very young deposits from Livorno (Italy) whose recency led Stefanini to speculate that *A. lixula* had an exotic origin and had entered the Mediterranean in recent times [13]. *A. lixula* is consistently absent from fossil assemblages of the so-called "Senegalese fauna" that characterize the warmer periods from the Tyrrhenian stage (ca. 260–11.4 kya), which have been extensively sampled and thoroughly described [84–89].

As for the Atlantic archipelagos, recent work on the fossil echinoid fauna of Azores Islands [90] has revealed the presence of *A. lixula*, providing several tens of pieces of individuals, including the oldest known record of this species. These deposits are currently dated to 130–120 kya [91], which corresponds to the last interglacial or Riss-Würm (also called MIS 5e, ca. 130–114 kya). These specimens add up to the only other Atlantic *A. lixula* fossil specimen known from the Pleistocene of Madeira [13] whose dating is more uncertain.

Thus, there is scarce paleontological evidence of the occurrence of *Arbacia lixula* in the Mediterranean, and somewhat more, but still scarce, evidence of the colonization of the Atlantic archipelagos of Azores and Madeira, which probably occurred during the last interglacial period of the Pleistocene (MIS 5e). These observations are in agreement with the genetic signatures we observed in the mismatch distributions, which clearly show that recent sudden expansions have occurred in the Mediterranean and Macaronesian populations (Fig. 6). This is also supported by the strikingly star-shaped topologies of the haplotype network (Fig. 2) and by the comb-like clades in the BI phylogenetic tree (Fig. 3). Our temporal estimation for the demographic expansion in the Mediterranean (93.8–205.2 kya) is coherent with the only available fossil record [13]. This is considerably younger than the times for expansion events found in other Mediterranean echinoderms using the same estimation method, which vary from 300 to 600 kya [73,77] and fits with the possibility that the colonization of the Mediterranean by *A. lixula* took place as recently as during the last interglacial period (MIS 5e). This period was also the longest of all interglacial warm periods of the Pleistocene. The minimum winter surface temperature of the Mediterranean Sea stayed warmer than 19°C for several thousands of years [89]. This probably enabled tropical Atlantic populations of *A. lixula* to cross the Strait of Gibraltar and colonize the Mediterranean.

Table 8. Mismatch distribution parameters for *Arbacia lixula* populations.

Region	*SSD*	τ	θ_0	θ_1	Estimated expansion time (kya)
Brazil	0.3525 **	N.A.	N.A.	N.A.	N.A.
Cape Verde	0.0265 *	N.A.	N.A.	N.A.	N.A.
Macaronesia	0.0004 ^{ns}	1.39	1.850	1000	31.3–68.4
Pooled East Atlantic	0.0014 ^{ns}	1.36	1.286	1000	30.6–66.9
Alboran Sea	0.0067 ^{ns}	4.24	0.000	12.54	95.4–208.7
West Mediterranean	0.0028 ^{ns}	4.20	0.000	9.75	94.5–206.7
East Mediterranean	0.0024 ^{ns}	3.90	0.001	10.86	87.7–191.9
Pooled Mediterranean	0.0026 ^{ns}	4.17	0.000	10.20	93.8–205.2
Whole Dataset	0.0030 ^{ns}	2.91	1.376	13.13	65.5–143.2

SSD values and their significances are presented along with sudden expansion model parameters and estimated time for the expansion (where applicable), for the studied regions and sub-regions and for the whole dataset.
*: Significant at $P<0.05$.
**: Significant at $P<0.01$.
^{ns}: Not significant.
N.A.: Not applicable (sudden expansion model rejected).
doi:10.1371/journal.pone.0045067.t008

Table 9. Neutrality and population expansion tests for *Arbacia lixula* in the studied regions or sub-regions and for the whole dataset.

Region	*N*	*D*	F_s	R_2	*r*
Brazil	35	−1.80405 ns	−3.712 *	0.0566 **	0.0503 *
Cape Verde	27	−2.08319 *	−9.809 ***	0.0527 ***	0.1254 *
Macaronesia	96	−2.40571 **	−45.988 ***	0.0234 ***	0.0167 ^{ns}
Pooled East Atlantic	123	−2.51677 ***	−70.825 ***	0.0185 ***	0.0265 ^{ns}
Alboran Sea	53	−1.83549 *	−15.648 ***	0.0421 **	0.0221 ^{ns}
West Mediterranean	310	−2.25417 **	−98.101 ***	0.0187 **	0.0137 ^{ns}
East Mediterranean	83	−1.49411 ns	−17.677 ***	0.0494 ns	0.0160 ^{ns}
Pooled Mediterranean	446	−2.28043 **	−155.806 ***	0.0162 ***	0.0137 ^{ns}
Whole Dataset	604	−2.32451 **	−256.026 ***	0.0150 **	0.0094 ^{ns}

Tajima's *D*, Fu's F_s statistic, Ramos-Onsins & Rozas' statistic (R_2), and raggedness index (*r*).
*: Significant at $P<0.05$;
**: Significant at $P<0.01$;
***: Significant at $P<0.001$;
^{ns}: Not significant.
doi:10.1371/journal.pone.0045067.t009

In the case of Eastern Atlantic populations, the exponential demographic expansion is even more apparent, since the mismatch distribution follows a sharp unimodal curve which fits to a sudden expansion model with a very high value for θ_1. This expansion probably occurred more recently than in the Mediterranean (31.3–68.4 kya). This estimation falls within the Late Pleistocene, an epoch generally dominated by the last glaciation (Würm), during which the mean sea level dropped down to 80 m below the present level [92–93]. Changes in ocean circulation related to this sea level drop can be related to the population expansion of *A. lixula* in the Eastern Atlantic. Contrary to what happens in the Mediterranean, the fossils available show that the species was present in Macaronesia before this expansion [90], so the demographic history of the Atlantic populations of *A. lixula* seems to be more complex than that of the Mediterranean populations. To complete the picture of the colonization of Atlantic archipelagos, data from continental African shores would be highly valuable.

An invasive species can be defined as a "species that threatens the diversity or abundance of native species, the ecological stability of infested ecosystems, economic activities (e.g., agricultural, aquacultural, commercial, or recreational) dependent on these ecosystems and/or human health" [94]. Although the term is generally applied to species introduced as a result of human activities, it should not be necessarily so. Moreover, ecosystem engineer species such as *Arbacia lixula*, that have shaped contemporary communities as the result of a colonization event that took place many years ago, can be falsely viewed as native [95]. According to our molecular data, *A. lixula* has indeed colonized the Mediterranean recently and complies with the terms of the former definition, even if it is usually viewed as native because its colonization took place following natural climatic changes, without human intervention.

Whether considered as an "old natural invader" or as native, the present trend of global warming can potentially boost the negative impact of *A. lixula* in Mediterranean ecosystems, thus possibly turning a "natural" colonization into an ecological problem related (at least partially) to human intervention. The ongoing warming [96] may facilitate population blooms of *A. lixula* in Northern Mediterranean, by releasing the constraint to larval development due to low water temperature. Warnings have been issued about its potential population increase and the generation of barren grounds in sublittoral habitats [10–11].

Thus, genetic data are in agreement with the consideration of *Arbacia lixula* as a thermophilous species that has recently colonised the Mediterranean and whose densities may increase in the foreseeable future. Monitoring of populations seems highly recommendable as a management tool in the near future for protecting the threatened Mediterranean shallow water ecosystems.

Supporting Information

Table S1 Haplotype frequencies of *Arbacia lixula* COI for all sampled localities. Haplotypes shared by two or more localities are represented in **bold**, while numbers not in bold correspond to private haplotypes. Background colours correspond to the three different haplogroups. Outgroups weights calculated by TCS are also displayed for each haplotype, and that with the highest outgroup weight (A2) is highlighted in green background.
(XLS)

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Author Contributions

Conceived and designed the experiments: XT CP. Performed the experiments: OSW RPP. Analyzed the data: OSW XT RPP. Contributed reagents/materials/analysis tools: OSW XT RPP CP. Wrote the paper: OSW XT RPP CP.

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A wolf in sheep's clothing: carnivory in dominant sea urchins in the Mediterranean

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ABSTRACT: *Arbacia lixula* and *Paracentrotus lividus* are the dominant sea urchins in the Mediterranean sublittoral, where they are key structuring species due to their grazing activity. It has been commonly accepted that competition between both species is minimized by specializing in different algal foods. *A. lixula* is considered to feed mainly on encrusting coralline algae, while *P. lividus* prefers fleshy macroalgae. We used stable isotope analysis to test if these species occupy different trophic positions at 3 locations in the western Mediterranean and one in Macaronesia. Our results show unambiguously that *A. lixula* always occupies a higher trophic level than *P. lividus*, with a $\delta^{15}N$ comparable in some locations to strict carnivores such as *Actinia schmidti* or *Marthasterias glacialis*. A temporal monitoring at one locality showed that this signature of a higher trophic level is consistent throughout the year. These results are incompatible with the current belief of an herbivorous diet for *A. lixula* and suggest that it must be considered an omnivore tending to carnivory in Mediterranean ecosystems, feeding at least partially on sessile animals such as Cirripedia, Hydrozoa or Bryozoa. A parallel analysis of gut contents showed a predominance of vegetal items in both species, although *A. lixula* consistently had a higher abundance of animal components than *P. lividus*. Our results challenge the validity of using gut content observations alone for characterizing the trophic behaviour of omnivorous marine invertebrates that feed on a variety of food sources with different digestibility.

KEY WORDS: *Arbacia lixula* · *Paracentrotus lividus* · Trophic relationships · Benthic community · Stable isotope analysis.

INTRODUCTION

The edible common sea urchin *Paracentrotus lividus* (Lamarck, 1816) and the black sea urchin *Arbacia lixula* (Linnaeus, 1758) are the 2 dominant echinoid species in shallow rocky bottoms in the Mediterranean, where they coexist (Palacín et al. 1998b, Benedetti-Cecchi et al. 1998). Their grazing activity is commonly considered to greatly influence benthic communities (Sala et al. 1998, Palacín et al. 1998a, Bulleri et al. 1999). Their coexistence has raised questions regarding how these 2 abundant species interact and, specifically, whether and how they partition resources (Bulleri et al. 1999, Chiantore et al. 2008, Privitera et al. 2008). The currently prevalent view is that they are competitors for algal foods, although this putative competition seems alleviated by a selective preference of *P. lividus* for erect seaweeds, while *A. lixula* tends to feed more on encrusting coralline algae (Kempf 1962, Régis 1978, Verlaque & Nédelec 1983, Frantzis et al. 1988, Bulleri et al. 1999, Boudouresque & Verlaque 2001, Privitera et al. 2008).

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This herbivorous behaviour described in *Arbacia lixula* is, however, in sharp contrast with other species in the genus *Arbacia,* where omnivorous or unambiguously carnivorous diets have been reported. North American *A. punctulata* feeds on sessile invertebrate species, sand dollars and other *Arbacia* individuals, as well as some algae (Harvey 1956, Karlson 1978, Cobb & Lawrence 2005). The diet of South Atlantic *A. dufresnei* is mainly carnivorous (Penchaszadeh 1979, Penchaszadeh & Lawrence 1999). The Pacific *A. spatuligera* showed preference for animal food over common species of algae from its habitat (Silva et al. 2004). Moreover, some observations indicate omnivorous or carnivorous behaviour of *A. lixula* outside the Mediterranean (Marques 1984, Oliveira 1991, Tavares & Borzone 2005).

The crucial importance of sea urchins in shaping benthic ecosystems (Lawrence 1975) has been demonstrated by many ecological experiments along the Mediterranean coasts (e.g. Benedetti-Cecchi & Cinelli 1995, Sala & Zabala 1996, Benedetti-Cecchi et al. 1998, Palacín et al. 1998a, Bulleri et al. 1999, Guidetti et al. 2004, Bonaviri et al. 2011). The underlying premise in these experiments is that sea urchins are predominantly herbivorous and that their effects are mainly due to their grazing on benthic algae. In particular, population outbreaks of both *Arbacia lixula* and *Paracentrotus lividus* are able to create barrens in rocky substrates (Verlaque 1987, Hereu 2004), affecting both productivity and diversity of benthic assemblages (Bulleri et al. 2002, Privitera et al. 2008). The feeding behaviour and the herbivorous nature of *P. lividus* have been repeatedly assessed; however, much less information is available about the ecological role played by *A. lixula* in Mediterranean ecosystems. In fact, Privitera et al. (2008) demonstrated that both species occupy different trophic niches in resource-limited (barren) areas, again in the sense that *A. lixula* feeds mainly on encrusting corallines while *P. lividus* feeds on non-encrusting macrophytes. A knowledge gap persists about the effective diet of *A. lixula*; however information on this is essential for designing and interpreting ecological studies. Filling this gap seems necessary not only for basic research, but also for management purposes (e.g. of marine reserves or local fisheries).

We used a combination of stable isotope analysis and gut content examination to assess the diet and establish the trophic position of *Arbacia lixula* and *Paracentrotus lividus* coexisting in western Mediterranean rocky bottoms.

MATERIALS AND METHODS

Study sites and sampling procedures

Gut contents and isotopic signatures of both sea urchin species were explored both temporally, performing a year-round follow-up at a single site, and spatially, sampling at 2 additional western Mediterranean sites at a single time point. We also sampled a non-Mediterranean site for reference information. This design aimed at establishing the robustness of the patterns found.

The temporal sampling was performed at Tossa de Mar (NE Spain, 41° 43.2′ N, 2° 56.4′ E, Fig. 1) from December 2008 to December 2009. This location is fully described elsewhere (Ballesteros 1988, 1989, 1992, 1993) and is characterized by gently sloping rocks extending from the surface to 12 m depth, with a rich algal cover and almost devoid of barren zones. We sampled between 2 and 6 m depth, where the dominant communities are the *Corallina elongata* community (Ballesteros 1988) in zones with high hydrodynamism and the *Stypocaulon scoparium* community (Ballesteros 1993) in zones with moderate to low hydrodynamism. Sea urchin densities during the sampling period (mean ± SD) were 0.6 ± 0.8 and 5.7 ± 4.7 adult ind. m^{-2}, for *Arbacia lixula* and *Paracentrotus lividus* respectively, measured at a depth of 3 m following the transect method as in Turon et al. (1995).

Ten *Arbacia lixula* and 10 *Paracentrotus lividus* individuals were collected every 2 mo by scuba diving. Only adults with test diameter >35 mm in *A. lixula* and >40 mm in *P. lividus* were sampled. The 2-monthly sampling periodicity seems adequate to

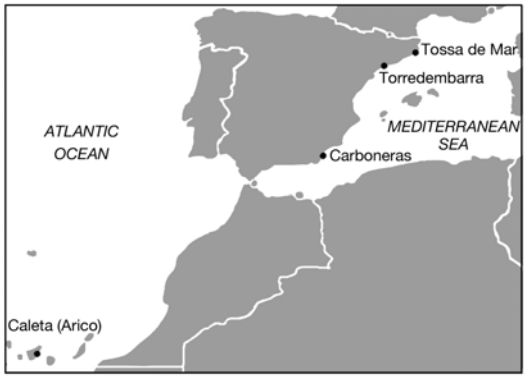

Fig. 1. Sampling locations for 2 Mediterranean sea urchin species, *Arbacia lixula* and *Paracentrotus lividus*

detect possible diet shifts (Tieszen et al. 1983, Hobson & Clark 1992). Samples of the dominant taxa from the 3 macroalgal divisions (Phaeophyta: *Stypocaulon scoparium*, *Dictyota dichotoma* and *Padina pavonica*; Chorophyta: *Codium vermilara* and *Flabellia petiolata*; Rhodophyta: *Corallina elongata, Sphaerococcus coronopifolius, Peyssonnelia* spp. and *Lithophyllum incrustans*) were collected at the same times. In addition, other invertebrates were also sampled throughout the year, including herbivores (*Patella* sp., *Amphitoe* sp.), detritivores (*Ophiothrix fragilis, Echinaster sepositus*), suspension feeders (*Balanus* spp.) and carnivores (*Actinia schmidti, Marthasterias glacialis, Ophioderma longicauda*), in order to characterize the different levels of the local trophic web. All samples were frozen (–20 °C) shortly after collection for later analysis.

For comparison, additional sampling was carried out at 2 other locations, ca. 200 and 900 km distant from Tossa de Mar, in December 2009. Although densities were not quantified, both sea urchin species were present at these localities (again with dominance of *Paracentrotus lividus*) with largely overlapping depth distributions. These sampling points were Torredembarra, (NE Spain, 41° 7.9′ N, 1° 23.7′ E) and Carboneras (SE Spain, 36° 59.6′ N, 1° 53.4′ W) (Fig. 1). The site at Torredembarra is characterized by a shallow rocky habitat (0 to 3 m depth), surrounded by a sandy bottom. The macroalgal assemblages are poorly developed, and the main primary producer is *Jania rubens,* with scarce presence of other algae such as *Corallina elongata* or *Dictyota dichotoma*. The Carboneras site is a shallow rocky habitat (0 to 4 m depth) with a denser algal cover, where the dominant producers are *Jania rubens*, *Stypocaulon scoparium* and *Peyssonnelia* spp., with a well-developed *Posidonia oceanica* meadow located nearby. At these 2 sites, samples were obtained only of the 2 echinoids and of representative algal species, following the same procedures as above. Thus, 3 communities with quite different characteristics were sampled in this study, representing some of the diversity of Mediterranean shallow habitats where *Arbacia lixula* and *P. lividus* can coexist.

Finally, samples of sea urchins (of the same sizes detailed above) for stable isotope analysis were collected at 1 Atlantic site in November 2009. This was Caleta, located near Arico, SE Tenerife, Canary Islands (28° 6.1′ N, 16° 28.7′ W, Fig. 1), where samples were collected between 0 and 3 m depth. In this location rock boulders dominate at shallow depths, with a poorly developed algal community including sparse patches of *Caulerpa webbiana* and *Lobophora variegata.* While *Arbacia lixula* and *Paracentrotus lividus* are known to broadly share spatial niches at the Canary Islands (Tuya et al. 2007), in this locality, the former was only found in vertical walls, while the latter was located under the stones at the bottom.

Stable isotope analyses

Muscles of the Aristotle's lantern of all collected sea urchins were used to perform isotopic analyses, and some of the same individuals were used for gut analyses (see next section). Algae were sampled by slicing several pieces of different parts of the thalli after carefully scraping epibionts off their surface. For faunal specimens, we sliced a small portion of a specified part of the body (the foot for *Patella* sp., an arm for Ophiuroidea and Asteroidea and the body column for *Actinia schmidti*), while the whole body of amphipods and cirripeds (excluding the shell) was used.

Before isotopic analyses, samples were rinsed in distilled water, freeze-dried and ground to a fine powder. Isotopically lighter lipids may influence carbon isotope ratios in animal tissues (Attwood & Peterson 1989, Hobson & Welch 1992), so 5 samples of each species were reanalyzed after lipid removal by chloroform-methanol 2:1 extraction (Folch et al. 1957). Passing & Bablok (1983) regression did not show any significant differences in the $\delta^{13}C$ for any species (data not shown), probably due to low lipid content in the sampled tissues. Thus, values of untreated samples were used thereafter. Carbonate rich samples (Corallinaceae, *Padina pavonica,* Ophiuroidea, Asteroidea) were rinsed several times with HCl 0.1 M to remove inorganic carbonates (Tomas et al. 2006). As HCl treatment has been reported to alter the $\delta^{15}N$ values (Bunn et al. 1995), samples including calcareous elements were split into 2 subsamples, one analyzed after acid treatment for $\delta^{13}C$ and the other, untreated, for $\delta^{15}N$.

Aliquots of 0.3 or 1 mg of dried powder from faunal or algal samples, respectively, were placed into tin capsules and crimped for combustion. Samples were oxidized in a Flash EA1112 furnace coupled to a Delta C stable isotope mass spectrometer through a Conflo III interface (Thermo-Finnigan). Isotope ratios are expressed as δ values in parts per thousand (‰) according to the equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X (‰) is ^{13}C or ^{15}N, and R is the ratio of corresponding element ($^{13}C/^{12}C$ or $^{15}N/^{14}N$), in sample or standard. The standard values were Pee Dee Belemnite for ^{13}C and atmospheric nitrogen for

^{15}N. IAEA standards were inserted every 12 samples for calibration. Replicate assays of standards indicated measurement errors of ±0.1‰ and ±0.2‰ for carbon and nitrogen, respectively.

Trophic levels were calculated according to the equation of Hobson & Welch (1992): $TL = 1 + (N_m - N_b) / TE$. Where TL is the trophic level of the species, N_m is the mean $\delta^{15}N$ value of the species, N_b is the average basis $\delta^{15}N$ value of producers (baseline) and TE is the trophic enrichment factor in the ecosystem. A constant TE factor of 3‰, commonly accepted for aquatic benthic ecosystems involving invertebrates (Vander Zanden & Rasmussen 2001, Jaschinski et al. 2008, Wan et al. 2010), was used. The baseline for $\delta^{15}N$ was estimated averaging the values obtained for the different algal species analyzed, except in the Atlantic site, where we did not collect algae. In this case, we assigned *Paracentrotus lividus* a value of TL = 2 (strict herbivore) and used it as a baseline for calculating the TL of *Arbacia lixula*.

Gut content analyses

The gut contents of sea urchins of both species collected at Tossa de Mar in June and December, and at the other 2 Mediterranean locations in December, were analyzed. Sea urchins (from 5 to 10 individuals per species and locality, and for two different months in the case of Tossa de Mar) were dissected and the total gut contents of each specimen were examined under a binocular microscope after disaggregation of the pellets. Some small calcareous remnants were collected and examined under a scanning electron microscope. Algal fragments were identified to genus level, while faunal items were classified into the following taxonomic groups: Foraminifera, Porifera, Hydrozoa, Polychaeta, Gastropoda, Bivalvia, Bryozoa, Cirripedia, Ostracoda, Copepoda, Amphipoda/Isopoda, Decapoda and 'other'. Echinoid fragments, which were present in the gut of some specimens, were not included in the analysis, since we cannot assure that they were not an artifact resulting from sample manipulation.

The frequency of occurrence of each food item in a species (FO_i) was calculated as the fraction of individuals having ingested this item (Pillay 1952, Hyslop 1980). The volumetric occupation of ingested items was assessed by quantifying 25 squares of a Petri dish with a 5 mm grid. The surface occupied by the items present in each square was semiquantitatively estimated using a scale from 1 to 5, and the occupation indices of all items were calculated for every sea urchin individual, dividing the sum of the semiquantitative scores assigned to a given food item by the total sum of the scores for all measured squares. The volumetric index of each food item in a species (V_i) was then obtained as the mean value of all individuals. A feeding index (FI_i) reflecting the relative importance of each food item in the diet of each species at a given location, was calculated following Lauzanne (1975), as $FI_i = FO_i \times V_i$ and then standardized as a percentage of the sum of the feeding indices for all items.

The relative contribution of animal and vegetal matter in the gut contents was quantified by addition of the standardized feeding indices of all items of either animal or vegetal origin (cumulative feeding indices). These indices summarize the carnivorous or herbivorous character of the diet, as inferred from gut contents.

Statistical analyses

Two-way ANOVA with species as fixed factor and month as random factor was performed to assess temporal variation of isotopic signatures of both sea urchin species at Tossa de Mar throughout the year. Two-way ANOVA with species as fixed factor and locality as random factor was used to compare the signatures and the calculated trophic levels of both sea urchin species at the sampled localities. We also used 2-way ANOVA with species (fixed) and locality (random) as factors to formally analyze differences in the cumulative animal feeding indices. In mixed models, the expected mean square for the fixed factor (species in our case) includes the variance component for the interaction term. Thus the fixed factor tests for the effect of species over and above the variation due to the interaction and the residual. It is therefore interpretable even in the presence of significant interaction (Quinn & Keough 2002). Notwithstanding, when interaction was significant, we performed separate *t*-tests with Bonferroni correction (unbalanced data prevented us from using other multiple comparison tests) at each locality to check that the effect was consistent across sites.

The assumptions of normality and homoscedasticity of the variables were checked with the Kolmogorov-Smirnov and Cochran tests, respectively. In some cases the data did not comply with these assumptions, and rank transformed data were used instead (detailed in Results). In 2 instances this transformation did not solve the lack of homoscedasticity (detailed in Results), but we performed the analysis

anyway as rank transformation is robust to deviations from assumptions of parametric procedures (Conover & Iman 1981, Potvin & Roff 1993). All analyses were performed with STATISTICA 6.1 software.

RESULTS

Stable isotope analyses

At Tossa de Mar, the annual average of $\delta^{15}N$ values found for *Arbacia lixula* (8.2‰) was comparable to those of typical carnivores such as *Actinia schmidti* or *Marthasterias glacialis* (Table 1, Fig. 2). In contrast, herbivorous grazers and detritivores had lower $\delta^{15}N$ values (between 4.6‰ for the amphipod *Amphitoe* sp. and 5.3‰ for *Echinaster sepositus*) while *Paracentrotus lividus* showed a slightly higher value of 5.9‰, possibly indicating a higher intake of animal items than its more strictly herbivorous counterparts. Seaweeds, as expected, showed lower $\delta^{15}N$ values, ranging from 1.9‰ for *Flabellia petiolata* to 4.2‰ for *Sphaerococcus coronopifolius*, while the most abundant species were within the range of 3 to 3.5‰. The mean value for all algae, used as the baseline for calculating consumers' trophic levels at Tossa de Mar, was 3.13‰.

When analyzed on a temporal basis (Fig. 3), $\delta^{15}N$ values in *Arbacia lixula* were significantly higher than in *Paracentrotus lividus* (species factor, $p < 0.001$, Table 2), while time and the interaction were not significant (Table 2), indicating that the difference in trophic levels is not subject to temporal variation. The mean difference was 2.3‰. Likewise, $\delta^{13}C$ values showed a high degree of individual variability (Fig. 3), but overall they were also significantly higher (by ca. 0.7‰) for *A. lixula* than for *P. lividus* (species factor, $p < 0.001$, Table 2, Fig. 3), again suggesting a higher trophic level for *A. lixula* (the trophic enrichment factor for carbon in marine coastal trophic webs is ca. 0.8‰ according to France & Peters 1997). No clear temporal trend was apparent for $\delta^{13}C$ values (time and interaction not significant, Table 2).

Table 1. Isotopic signatures (mean ± SD) and derived trophic levels (TL = 1 for primary producers) of invertebrate species in the Mediterranean sublittoral, based on results of 2-monthly sampling at Tossa de Mar, NE Spain. Sea urchin species are shown in **bold**

Species	n	Trophic level	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)
Actinia schmidti	5	2.7	8.3 ± 0.6	−19.8 ± 0.7
Arbacia lixula	**72**	**2.7**	**8.2 ± 0.5**	**−18.3 ± 1.1**
Ophioderma longicauda	5	2.5	7.6 ± 0.9	−17.5 ± 0.9
Marthasterias glacialis	5	2.4	7.5 ± 0.4	−15.8 ± 1.0
Balanus spp.	5	2.2	6.8 ± 0.3	−19.4 ± 0.3
Paracentrotus lividus	**71**	**1.9**	**5.9 ± 0.4**	**−19.0 ± 0.9**
Echinaster sepositus	5	1.7	5.3 ± 0.4	−15.6 ± 0.3
Ophiothrix fragilis	4	1.7	5.2 ± 0.7	−20.4 ± 1.0
Patella sp.	5	1.6	5.1 ± 0.4	−17.0 ± 0.6
Amphitoe sp.	4	1.5	4.6 ± 0.7	−22.1 ± 1.2

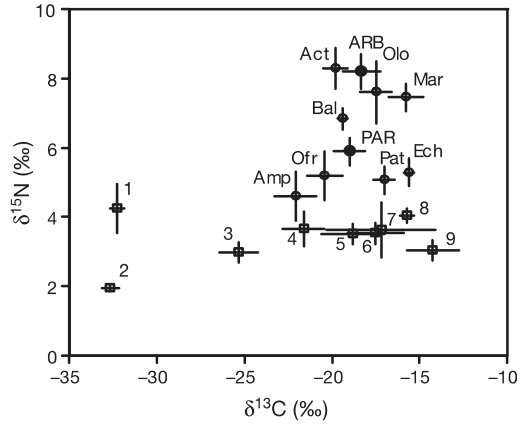

Fig. 2. Plot of $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures (mean ± SD) of sublittoral species examined during 2-monthly sampling at Tossa de Mar (NE Spain): (●) sea urchin species; (○) other consumers; (□) producers. Metazoa: Act = *Actinia schmidti*, ARB = *Arbacia lixula*, Olo = *Ophioderma longicauda*, Mar = *Marthasterias glacialis*, Bal = *Balanus* spp., PAR = *Paracentrotus lividus*, Ech = *Echinaster sepositus*, Ofr = *Ophiothrix fragilis*, Pat = *Patella* sp., Amp = *Amphitoe* sp. Algae: 1 = *Sphaerococcus coronopifolius*, 2 = *Flabellia petiolata*, 3 = *Stypocaulon scoparius*, 4 = *Corallina elongata*, 5 = *Dictyota dichotoma*, 6 = *Codium vermilara*, 7 = *Peyssonnelia* spp. 8 = *Lithophyllum incrustans*, 9 = *Padina pavonica*

In the 2 additional Mediterranean locations, as well as in the Atlantic one, $\delta^{15}N$ values obtained from *Arbacia lixula* exceeded those from *Paracentrotus lividus*, as did the estimated trophic levels (Table 3). This suggests that the tendency towards a more omnivorous or carnivorous diet of *A. lixula* is probably widespread throughout its distribution range. A 2-way ANOVA of $\delta^{15}N$ values revealed a significant effect of species (fixed) and locality (random), as well as a significant interaction term (Table 4), suggesting different adaptations of sea urchin species in different conditions (see below). Separate analyses (*t*-tests with Bonferroni correction) at fixed levels of the locality factor revealed that the differences in $\delta^{15}N$ values between sea urchin species were significant (all $p < 0.01$) at all sites. Likewise, the analysis of trophic level between species and localities revealed a significant effect of species and a significant interaction between species and locality (Table 4); the

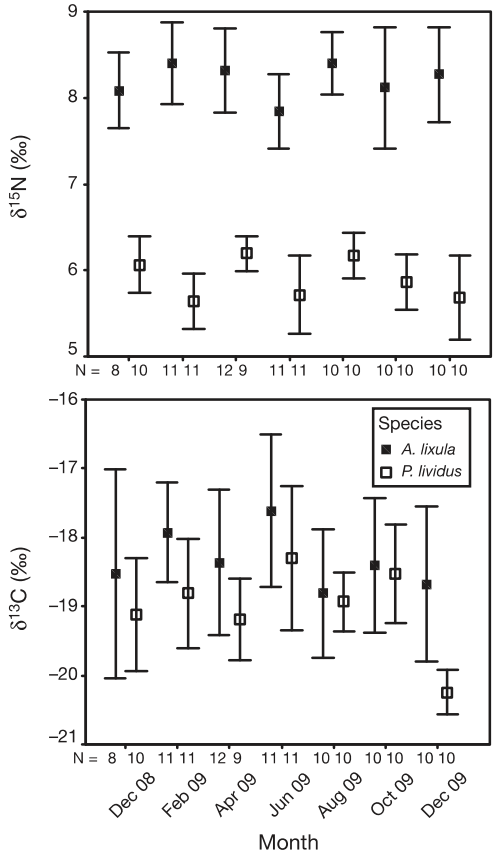

Fig. 3. *Arbacia lixula* and *Paracentrotus lividus*. Isotopic signatures of 2 sea urchin species at Tossa de Mar (NE Spain) as a function of time. Means ± SD are displayed for every sampled month. The number of individuals analyzed for each observation was from 8 to 12

Table 2. *Arbacia lixula* and *Paracentrotus lividus*. Factorial 2-way ANOVA to assess significant differences in isotopic signatures between species (fixed factor) and sampling times (random factor). Data for $\delta^{13}C$ was rank-transformed as variance was not homogeneous. Significant p-values in **bold**

Variable	Effect	df	MS	*F*	p
$\delta^{15}N$	Species (S)	1	187.663	493.66	**<0.001**
	Time (T)	6	0.632	1.66	0.28
	S × T	6	0.381	2.00	0.07
	Error	129	0.190		
$\delta^{13}C$	Species (S)	1	30481.07	15.82	**<0.001**
	Time (T)	6	6005.13	3.11	0.10
	S × T	6	1929.10	1.50	0.18
	Error	129	1282.21		

Table 3. *Arbacia lixula* and *Paracentrotus lividus*. Calculated trophic levels based on ^{15}N signatures for the 2 sea urchin species and values for seaweeds (baseline) from 4 sample locations, means ± SD. ^{13}C signatures are also shown for both sea urchin species. na: data not available

Species	n	Trophic level	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)
Tossa				
A. lixula	72	2.7 ± 0.17	8.2 ± 0.5	−18.3 ± 1.1
P. lividus	71	1.9 ± 0.13	5.9 ± 0.4	−19.0 ± 0.9
Seaweeds	37	1.0	3.1 ± 0.7	
Torredembarra				
A. lixula	10	3.0 ± 0.17	10.6 ± 0.5	−17.2 ± 0.6
P. lividus	9	2.7 ± 0.12	9.6 ± 0.4	−17.9 ± 0.5
Seaweeds	12	1.0	4.6 ± 0.7	
Carboneras				
A. lixula	9	2.7 ± 0.16	10.4 ± 0.5	−11.8 ± 0.6
P. lividus	10	2.1 ± 0.19	8.5 ± 0.6	−16.7 ± 0.7
Seaweeds	18	1.0	5.3 ± 0.5	
Tenerife				
A. lixula	10	3.0 ± 0.12	10.7 ± 0.4	−11.2 ± 0.7
P. lividus	7	2.0 ± 0.14	7.5 ± 0.4	−17.8 ± 0.4
Seaweeds		na	na	

Table 4. *Arbacia lixula* and *Paracentrotus lividus*. Factorial 2-way ANOVA to assess significant differences in the isotopic signatures and calculated trophic levels between species (fixed factor) at the 4 sampled locations (random factor). Data for $\delta^{13}C$ was rank-transformed as variance was not homogeneous. Significant p-values in **bold**

Variable	Effect	df	MS	*F*	p
$\delta^{15}N$	Species (S)	1	98.355	31.27	**0.01**
	Location (L)	3	87.491	22.04	**0.01**
	S × L	3	3.970	18.42	**<0.001**
	Error	190	0.216		
$\delta^{13}C$	Species (S)	1	35884.07	20.27	**0.007**
	Location (L)	3	84754.14	46.79	**0.005**
	S × L	3	1811.29	1.11	0.34
	Error	190	1624.53		
Trophic level	Species (S)	1	10.928	31.268	**0.01**
	Location (L)	3	1.667	3.779	0.15
	S × L	3	0.441	18.418	**<0.001**
	Error	190	0.024		

inter-specific differences were again significant in all localities (*t*-tests with Bonferroni correction, all $p < 0.01$). The significant interaction term is probably due to the plasticity that can be observed in the derived trophic levels of both species among the different localities and ecosystems. Thus, where algal cover was dense, as happened in Tossa de Mar and Carboneras, *P. lividus* showed a trophic level of around 2, compatible with a mainly herbivorous diet, whereas *A. lixula* showed trophic levels of around

2.7, corresponding to a predominantly carnivorous omnivore. In contrast, where algal resources were scarce (as in Torredembarra) both sea urchins tended to increase their animal intake, raising their $\delta^{15}N$ values and trophic levels. Our results showed a trophic level of 2.7 for *P. lividus* at Torredembarra, whereas *A. lixula* had a level of 3.0 (which would correspond to a strict carnivore) in this location. In Tenerife, as algal samples were not available for isotopic analysis, a baseline value for producers cannot be used, but the difference between $\delta^{15}N$ values of the 2 sea urchins was the biggest of all locations sampled, and corresponded to exactly one trophic level.

The $\delta^{13}C$ signatures at the additional localities revealed, as in Tossa de Mar, a higher enrichment in *Arbacia lixula* (Table 3). For this variable, no significant interaction between species and locality was found, while the single factors were highly significant (Table 4), highlighting the higher $\delta^{13}C$ value in *A. lixula* as well as a noticeable spatial heterogeneity in isotopic signature. The increase in $\delta^{13}C$ in *A. lixula* relative to *Paracentrotus lividus* in Torredembarra was similar to that in Tossa, but it was much higher at the other 2 localities (Carboneras and Tenerife, Table 3), suggesting a different carbon source for both sea urchins in these localities.

Gut content analyses

Gut content analyses in Tossa de Mar (Table 5) revealed a higher abundance of animal items in *Arbacia lixula* than in *Paracentrotus lividus*. In addition, the ingested material of *P. lividus* showed remarkable temporal differences. Thus, *Dictyota* and *Dasycladus*, the most frequent algal items found in June, did not appear in the gut contents of samples collected in December, when *Corallina* abundance increased. In contrast, the gut contents of *A. lixula* showed very little seasonal changes, being dominated by small filamentous algae such as *Cladophora* and *Polysiphonia*, and crushed fragments of encrust-

Table 5. *Arbacia lixula* and *Paracentrotus lividus*. Standardized feeding indices (only the 12 highest values shown, means ± SD) for major food items, as derived from gut content analysis of 2 sea urchin species at 3 Mediterranean locations. Given the prominent seasonal changes in the algal assemblages, feeding indices have been calculated separately for June and December at the site where sampling was performed over time (Tossa de Mar). Animal items are shown in **bold**

A. lixula		*P. lividus*	
Tossa de Mar			
June: n = 6		n = 6	
Cladophora	19.4 ± 9.4	*Dictyota*	40.0 ± 21.6
Polysiphonia	19.1 ± 9.4	*Dasycladus*	22.3 ± 10.0
Lithophyllum	17.3 ± 8.3	*Stypocaulon*	11.9 ± 9.8
Bryozoa	8.1 ± 12.7	*Polysiphonia*	7.1 ± 6.1
Hydrozoa	7.6 ± 4.9	*Ceramium*	5.3 ± 4.7
Cirripedia	5.6 ± 6.2	**Bryozoa**	3.0 ± 3.1
Polychaeta	5.2 ± 2.4	*Corallina*	3.0 ± 3.6
Stypocaulon	4.5 ± 4.5	*Cladophora*	1.7 ± 2.5
Foraminifera	3.6 ± 2.6	*Jania*	1.4 ± 1.9
Dictyota	2.5 ± 3.3	*Colpomenia*	1.3 ± 1.8
Ostracoda	2.3 ± 2.7	Other seaweed	1.2 ± 0.9
Porifera	1.1 ± 1.1	*Sphaerococcus*	0.8 ± 1.4
December: n = 9		n = 5	
Lithophyllum	19.6 ± 13.6	*Corallina*	52.2 ± 7.1
Polysiphonia	17.1 ± 10.6	*Stypocaulon*	21.2 ± 5.3
Hydrozoa	14.9 ± 10..9	*Peyssonnelia*	9.6 ± 6.1
Cirripedia	11.6 ± 6.1	*Jania*	7.3 ± 5.4
Cladophora	9.3 ± 7.9	*Cladophora*	3.0 ± 2.2
Polychaeta	8.3 ± 6.8	*Polysiphonia*	1.7 ± 2.2
Foraminifera	3.7 ± 1.4	*Cystoseira*	1.3 ± 1.6
Jania	3.2 ± 2.4	**Porifera**	1.0 ± 1.0
Corallina	2.8 ± 3.6	**Polychaeta**	0.8 ± 0.8
Porifera	2.0 ± 2.5	*Lithophyllum*	0.8 ± 0.9
Bryozoa	2.0 ± 2.0	**Hydrozoa**	0.3 ± 0.3
Stypocaulon	1.6 ± 3.8	*Halimeda*	0.2 ± 0.4

A. lixula		*P. lividus*	
Torredembarra			
December: n = 5		n = 5	
Cirripedia	55.5 ± 12.8	*Jania*	64.9 ± 5.5
Hydrozoa	23.3 ± 6.9	*Corallina*	8.8 ± 3.0
Polysiphonia	8.9 ± 4.7	*Posidonia*	8.4 ± 3.9
Porifera	8.5 ± 4.7	**Bryozoa**	5.7 ± 4.3
Cladophora	1.3 ± 1.9	*Polysiphonia*	3.0 ± 3.0
Bivalvia	0.8 ± 1.1	*Codium*	2.1 ± 1.4
Ceramium	0.7 ± 0.6	*Stypocaulon*	1.9 ± 1.5
Stypocaulon	0.3 ± 0.4	*Cladophora*	1.6 ± 1.2
Gastropoda	0.2 ± 0.4	**Hydrozoa**	1.6 ± 1.3
Polychaeta	0.2 ± 0.4	Other seaweed	0.6 ± 1.1
Bryozoa	0.2 ± 0.3	*Peyssonnelia*	0.6 ± 0.6
Other metazoa	0.1 ± 0.2	*Padina*	0.2 ± 0.3
Carboneras			
December: n = 7		n = 10	
Jania	34.1 ± 26.3	*Jania*	59.9 ± 10.6
Lithophyllum	21.2 ± 23.6	*Posidonia*	11.6 ± 5.8
Cladophora	17.2 ± 14.0	*Peyssonnelia*	11.0 ± 11.0
Porifera	8.3 ± 8.8	*Cladophora*	5.0 ± 4.3
Stypocaulon	5.8 ± 7.0	*Stypocaulon*	4.1 ± 6.0
Ceramium	4.0 ± 5.9	*Flabellia*	3.3 ± 2.2
Polysiphonia	2.2 ± 3.4	Other seaweed	2.6 ± 5.7
Peyssonnelia	1.9 ± 3.2	*Lithophyllum*	1.6 ± 2.4
Other seaweed	1.6 ± 3.2	*Padina*	0.7 ± 0.8
Foraminifera	1.4 ± 1.9	*Corallina*	0.1 ± 0.2
Corallina	0.9 ± 2.0	**Polychaeta**	0.1 ± 0.1
Hydrozoa	0.5 ± 1.1	**Porifera**	0.0 ± 0.1

ing corallines (*Lithophyllum* spp.). Sessile invertebrate species such as hydrozoans, cirripeds and polychaetes were also commonly found throughout the year. These 6 items, with the addition of bryozoans in June, constituted the main components of *A. lixula* gut contents, with little variation between seasons.

At the 2 other Mediterranean localities analyzed, the results of the gut content analysis confirmed the higher prevalence of animal items in the diet of *Arbacia lixula* relative to *Paracentrotus lividus*, although with strong variability, probably associated with changes in benthic algal cover. Thus, at Torredembarra , where algae were less abundant, some animal items appeared frequently in *P. lividus* guts, such as the bryozoan *Schizoporella errata*, which was common in this habitat. Conversely, in Carboneras, a location with a well developed algal cover, the relative amount of animal remnants in the gut of both sea urchins was the least of all localities sampled. Remarkably, cirripeds were absent from this location, and *Jania rubens* appeared as the main food source for both sea urchin species.

The cumulative feeding indices in the 3 localities showed that the diet of *Arbacia lixula* has a significantly higher animal component than that of *Paracentrotus lividus* (Fig. 4, Table 6). The locality factor was also highly significant, reflecting the marked spatial heterogeneity, but no significant interaction was detected. Whereas for *P. lividus* gut contents were always dominated by the algal fraction, that of *A. lixula* displayed a much higher variability in the relative contribution of animal and vegetal matter among the different localities, ranging from a predominantly animal component in Torredembarra to a dominance of vegetal diet in Carboneras (Fig. 4).

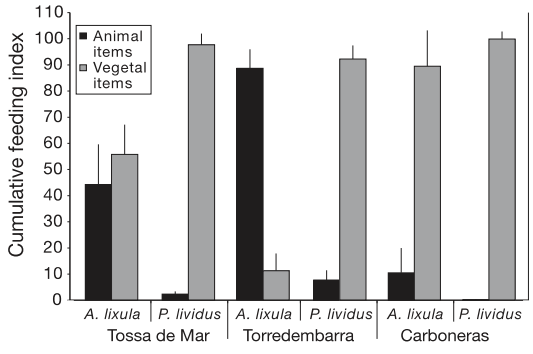

Fig. 4. *Arbacia lixula* and *Paracentrotus lividus*. Cumulative feeding indices (mean + SD) for animal and vegetal food items, derived from gut content analyses of 2 sea urchin species collected in December at 3 Mediterranean locations

Table 6. *Arbacia lixula* and *Paracentrotus lividus*. Factorial 2-way ANOVA to assess significant differences in the cumulative animal feeding index (calculated as the rank transformed sum of standardized feeding indices of animal items), between the 2 sea urchin species (fixed factor) and location (random factor). Significant p-values in **bold**

Variable	Effect	df	MS	*F*	p
Cumulative animal feeding index	Species (S)	1	2799.113	450.15	**0.002**
	Location (L)	2	917.827	149.62	**0.007**
	S × L	2	6.135	0.60	0.55
	Error	35	10.220		

DISCUSSION

Our results show that *Arbacia lixula* occupies a higher trophic level than *Paracentrotus lividus*, as shown by its consistently higher $\delta^{15}N$ across the sampled localities. The estimated trophic levels indicate that *A. lixula* is an omnivore tending to carnivory, while *P. lividus* is predominantly a herbivore that can become an omnivore in some instances. The (at least partial) carnivory in *A. lixula* is further supported by the analyses of gut contents, which reveal a consistently higher proportion of animal food items ingested in *A. lixula* as compared to *P. lividus*. However, gut content analyses alone do not reveal the full extent of the trophic gap between the 2 species, since vegetal components are dominant in most situations analyzed (except for *A. lixula* in Torredembarra). Finally, the results for $\delta^{13}C$ are consistent with those of $\delta^{15}N$, indicating an overall enrichment of the signature of *A. lixula* with respect to *P. lividus*. The results for carbon, however, should be taken with caution as this isotope is best suited to detect differences in sources of food rather than trophic levels (Cardona et al. 2007). This implies that the role of *A. lixula* in the shallow subtidal in the Mediterranean should be, at least in part, re-evaluated. Specifically, the putative strong competition for food should be carefully re-examined.

The suitability of stable isotope analysis, and specifically $\delta^{15}N$, for identifying trophic levels in marine ecosystems has been clearly established (e.g. Cherel et al. 2008). Much closer to the scope of the present study, this tool has revealed differences in the trophic levels of sympatric sea urchins (Vanderklift et al. 2006). These authors found that 2 littoral Australian echinoids previously thought to be herbivorous (*Phyllacanthus irregularis* and *Centrostephanus tenuispinus*) actually had an omnivorous behaviour tending towards carnivory. The differences in $\delta^{15}N$

between *Arbacia lixula* and *Paracentrotus lividus* that we report here, based on a wide temporal and geographical scale, are comparable to those found between both Australian purportedly herbivorous species and *Heliocidaris erythrogramma*, which proved to be a true strict herbivore.

In previous studies, animal items had been reported in the gut contents of both Mediterranean sea urchins (Maggiore et al. 1987, Privitera et al. 2008, Chiantore et al. 2008), but were mostly disregarded as anecdotal or accidental captures, which may be true for *Paracentrotus lividus* but is certainly not for *Arbacia lixula*. The long-held misconception about the herbivory of *A. lixula* may stem from several causes, but mainly from the fact that most primary information on this issue came from studies of gut contents, which target ingested, rather than assimilated, food. While it is true that the ecological impact of the feeding activity of an organism (in this case, *A. lixula*) may depend mostly on what is ingested, rather than on what is assimilated, gut content analysis can introduce some biases on our perception of an animal diet if used alone. Gut content analyses cannot be dismissed, though, as they provide the only direct taxonomical information about what the sea urchins ingest and, in combination with stable isotope analyses, can shed light on important aspects of their feeding strategy.

In addition, if diverse kinds of foodstuff have differential digestibility, results can be biased towards less digestible material. It is remarkable in this sense that most faunal items found in the gut of *Paracentrotus lividus* are nearly intact and easily identifiable, probably reflecting the reduced ability of this species to assimilate animal material. The opposite is true for *Arbacia lixula*, which seems to digest animal tissues completely. Conversely, undigested filamentous algae, even the most delicate ones, are regularly found intact in the guts of *A. lixula*. In a study on *A. lixula* from Brazil, Oliveira (1991) found that 50% of the algae present in its faecal pellets survived digestion and were able to grow when cultured, in contrast to algae egested by herbivorous sea urchins such as *Lytechinus variegatus* or *Echinometra lucunter*.

Another reason for the misconception about *Arbacia lixula* herbivory is suggested by the fact that the gut contents of *A. lixula* that we examined consisted largely of small crushed pieces of pinkish-grey carbonates, which can be easily interpreted as fragments of calcareous algae. However, using scanning electron microscopy, we have unambiguously identified many of these pieces as fragments of shells of the common western Mediterranean barnacle *Balanus perforatus*. Thus, we must consider the possibility that cirriped shell remnants may have been mistaken for encrusting corallines in some studies which were carried out under the undisputed paradigm of an herbivorous *A. lixula*.

The finding that *Arbacia lixula* is an omnivore tending to carnivory may shed light on unexpected results of some ecological experiments. For example, the removal of *Paracentrotus lividus* had no effect and did not trigger an increase of the population of *A. lixula* (Gianguzza et al. 2006), as would be expected if inter-specific competition occurred between both species. Artificially reducing or increasing the density of *A. lixula* in selected patches had no effect on the percent cover of encrusting corallines (Benedetti-Cecchi et al. 1998, Bulleri et al. 1999), but the removal of *A. lixula* produced an increase in the density of *Balanus perforatus* and a decrease in the density of limpets (Bulleri et al. 1999), opposite to what would be expected if *A. lixula* was an herbivorous consumer of filamentous algae and trophic competitor of *Patella* sp.

Finally, our results add some information about the putative competition between the 2 most abundant Mediterranean sea urchins. Densities of *Paracentrotus lividus* in the NW Mediterranean are on average 10× higher (Palacín et al. 1998b) than those of *Arbacia lixula*. This fact challenges the idea that the 2 species engage in strong competitive interactions. Alternatively, at least, it suggests that *P. lividus* is able to outcompete *A. lixula*, whose shift to a different diet may help to avoid exclusion. However, both species can locally coexist at high densities (Guidetti et al. 2004, Tuya et al. 2007), and *A. lixula* is the dominant sea urchin in some communities (Benedetti-Cecchi et al. 1998). Furthermore, the 2 species segregate spatially in some cases (Kempf 1962, Chelazzi et al. 1997, Bulleri et al. 1999), as happens in our Atlantic location, where *A. lixula* is restricted to vertical walls. Thus, interference competition between these species is likely to occur in many places. Agonistic interactions (as those reported by Shulman 1990) have never been observed between them, so exploitative competition seems more likely, and feeding flexibility can be an important mechanism to alleviate its effects. On the other hand, it has to be emphasized that factors other than direct trophic competition, such as resistance to hydrodynamism (Tuya et al. 2007), resistance to predation (Guidetti 2006) or presence of predators which could modulate sea urchin behaviour (Freeman 2006) could also be involved in shaping the distribution and abundance of these 2 sea urchin species.

Few studies have addressed the possibly different foraging behaviour of these sea urchins species. Apparently, *Arbacia lixula* shows a higher mobility than *Paracentrotus lividus* in barren zones (Bonaviri et al. 2011), so that a wider area can be impacted by its grazing activity. The strong Aristotle's lantern that allows *A. lixula* to scrape the substrate for searching its prey, and the fact that this species tends to be more abundant than *P. lividus* in barren zones which offer relatively few algal food resources in comparison to animal prey (Guidetti & Dulcic 2007), could be better explained in the light of its tendency to carnivory.

Studies comparing feeding strategies between these species in communities with dominance of *Arbacia lixula* are necessary to ascertain the trophic position of both species under competitive pressures different from those found in the present study. Interestingly, at high densities, *A. lixula* may be expected to limit the abundance of its prey populations, and so it can compete with carnivores such as asteroids. Thus, the omnivory and feeding plasticity of *A. lixula* adds complexity to models of community structure, including possible trophic loops and increased connectivity between species (Camus et al. 2008).

In conclusion, the finding that *Arbacia lixula* is an omnivore tending to carnivory has important implications for the dynamics of shallow water communities in the Mediterranean, as it suggests not only a reduced competition for food with the coexisting echinoid *Paracentrotus lividus*, but also opens up new perspectives on biotic interactions in these communities. Given the important functional role of these echinoid species in shaping sublittoral assemblages, and the fact that one of them (*P. lividus*) sustains heavy fisheries in some areas, the results presented here should be taken into consideration both in basic studies of ecosystem functioning and in applied issues of environmental and fisheries management.

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