Time and space: genetic structure of the cohorts of the common sea urchin *Paracentrotus lividus* in Western Mediterranean

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Summary

Spatio-temporal variability in settlement and recruitment, high mortality during the first life-history stages, and selection may determine the genetic structure of cohorts of long-lived marine invertebrates at small scales. We conducted a spatial and temporal analysis of the common Mediterranean sea urchin *Paracentrotus lividus* to determine the genetic structure of cohorts at different scales. In Tossa de Mar (NW Mediterranean), recruitment was followed over 5 consecutive springs (2006-2010). In spring 2008, recruits and two-year old individuals were collected at 6 locations along East and South Iberian coasts separated from 200 to over 1100 km. All cohorts presented a high genetic diversity based on a fragment of mtCOI. Our results showed a marked genetic homogeneity in the temporal monitoring, and a low degree of spatial structure in 2006. In 2008, coupled with an abnormality in the usual circulation patterns in the area, the genetic structure of the southern populations studied changed markedly, with arrival of many private haplotypes. This fact highlights the importance of point events in renewing the genetic make-up of populations, which can only be detected through analysis of the cohort structure coupling temporal and spatial perspectives.

INTRODUCTION

The last few years have seen an increase in the number of studies aimed at discerning temporal genetic structure in marine invertebrates with large dispersal abilities. Long-lived larval phases theoretically ensure connectivity over large distances in species with benthic adult phases, determining the genetic composition of populations (Hedgecock 1986; Watts et al. 1990; Sponaugle et al. 2002; Cowen et al. 2006). However, the composition and survival of these dispersing stages depend on many factors, such as reproductive success of adult populations, sperm densities, settlement and recruitment variability, food availability, environmental conditions, currents, etc. These factors may change between reproductive events, considerably altering the genetic compositions of cohorts within a site (Planes and Lenfant 2002; Hogan et al. 2010). As a consequence, genetic exchange between populations may be variable over small temporal and spatial scales, varying from one site and from one generation to the next (Grosberg and Levitan 1992; Cowen et al. 2000; Hellberg et al. 2002; Hogan et al. 2010). A comprehensive study of dispersal patterns thus requires the analysis of multiple cohorts in time and in space (Selkoe et al. 2006), a crucial information that is overlooked when populations are sampled and analyzed without considering the underlying cohort structure.

Marine broadcast spawners with external fertilization have potentially a high fecundity, which can be offset by large variance in reproductive success (Cushing 1990; Levitan 2005). This random variance may affect the effective population size (N_e), leading to a reduction in genetic diversity of cohorts of recruits relative to adult populations (Hedgecock 1994). Several factors can determine this variance between reproductive events. Selection at pre-zygotic stages can play a crucial role in fertilization success (Metz and Palumbi 1996; Palumbi 1999; Zigler et al. 2005). The effect of selection may vary from one generation to the next depending on factors such as sperm density (i.e., Levitan 2002, 2004). During the dispersal phase, larvae may gather in non-homogenous pools resulting in spatial genetic patchiness (Johnson et al. 1993; Li and Hedgecock 1998). In addition, the crucial settlement and recruitment steps depend not only on predictable factors, such as substrate type, but also on other variable aspects such as adult density or food availability (Harrold et al. 1991; Tomas et al. 2004). Besides, many marine invertebrates present high mortalities over the first years due, among others, to physical and biological disturbances, predation, competition or selective pressures (i.e., Gosselin and Qian 1996; Hunt and Scheibling 1997). If some or all of these processes vary in space and time, fine-scale patchy genetic structure can build up even in the absence of restricted dispersal (Waples 1998; Banks et al. 2007; Hedgecock et al. 2007; Botsford et al. 2009). In the long term this genetic signal will be blurred, since adult populations usually comprise a mixture of several larval pools issued from different reproductive and dispersal events.

Sea urchins present a benthic adult phase while dispersal is generally achieved through planktonic larvae that can remain in the water column from several days to months, potentially ensuring gene flow between distant populations. Large variations in settlement and recruitment in sea urchins have been observed from one year to the next for reasons not fully understood (Ebert 1983; Hereu et al. 2004). In effect, many recent studies have shown a marked short-scale temporal genetic structure in some species of sea urchins (Edmands et al. 1996; Moberg and Burton 2000), but not in others (Flowers et al. 2002). The present paper addresses the study of cohort genetic structure, in space and time, in the common or purple Mediterranean sea urchin *Paracentrotus lividus* (Lamarck). This species presents an Atlanto-Mediterranean distribution, including the islands of Macaronesia (Boudouresque and Verlaque 2001). The common sea urchin is

the most abundant echinoid in the shallow Mediterranean sublittoral, and it is relevant both ecologically, as it is a keystone herbivorous (Boudouresque and Verlaque 2001) and commercially, as it is harvested for human consumption (Guidetti et al. 2004).

Paracentotus lividus is an external fertilizer that produces planktotrophic larvae with a life span of 20-40 days (Pedrotti 1993) that ensures dispersal over thousands of kilometers. However, changes in gene frequencies have been detected associated to a hydrological front close to the Gibraltar boundary (Duran et al. 2004; Calderón et al. 2008). Besides, recent studies suggest also a certain degree of genetic structure within the Mediterranean basin (Maltagliati et al. 2010). The long dispersing stage should theoretically enable the larval pool to homogenize during its pre-competent phase. Nevertheless, multiple factors affect the spatial, bathymetric and temporal structure of the larval pool, resulting in variable settlement and recruitment at small scales (Hereu et al. 2004; Tomas et al. 2004). In previous studies, neutral (microsatellite) markers have revealed a shallow differentiation between cohorts in *P. lividus* at a single locality, Tossa de Mar, in North-Western Mediterranean (Calderón et al. 2009). In another study at the same locality with a different temporal scale, no population structure was revealed through the analysis of a fragment of mtCOI of cohorts of different ages, including recruits (Calderón and Turon 2010). However, selection acting upon the gamete recognition protein bindin may have an important effect upon pre-zygotic stages, potentially determining a small-scale temporal structure of cohorts (Calderón and Turon 2010). These previous results suggest that there is a large effective number of progenitors (and thus a poor influence of sweepstake events) although pre-zygotic events may be important (gamete recognition mechanisms).

Temporal genetic processes can be examined by sequential sampling through time or by evaluating genetic data with respect to the age structure of the population sampled at a single point in time. Calderón and Turon (2010) took the latter approach to investigate the genetic variation of *Paracentrotus lividus* present in Tossa de Mar. In this approach, cohorts of different ages were analyzed, including recruits of the year (in 2006 and 2007), as well as 7 cohorts of different ages forming the current adult population (i.e., as identified by the band pattern, see Calderón and Turon 2010 for details). Previous data, therefore, had the caveats of having been obtained from a single locality and using mostly cohorts sampled when they were already several years old, missing all processes

that occurred before the year of sampling. In the present work we wanted to use the alternative, sequential approach to assess cohort variability in this species by sampling the recruits arrived over 5 consecutive years (2006-2010) at this same locality. At the same time, and in order to include a spatial perspective, we conducted a spatial survey at 6 different locations along South and East Iberian coast, sampling individuals recruited in spring 2006 and in spring 2008.

MATERIAL AND METHODS

Sampling design

Sampling was designed to capture the structure of cohorts at different temporal and spatial scales. *Paracentrotus lividus* presents two recruitment episodes in North-Western Mediterranean, a major event during spring and a minor event during the autumn (Lozano et al. 1995; Tomas et al. 2004; Ourens et al. 2011). Sampling was therefore conducted in June, in order to collect the newly arrived recruits of the main annual cohort. Samples were obtained by scraping off algae from 20 x 20 cm squares (at least 5 scrapings per sampling). In all localities samples were obtained in shallow (3-6 m deep) sublittoral communities on rocky shores, with well-developed algal assemblages. They were then preserved in 96% ethanol at -20ºC before being carefully sorted under the stereomicroscope to look for recruits (ca. 1 mm in diameter).

First, in order to assess the temporal genetic variability of recruitment in Tossa the Mar (41°43.1′N, 2°56.2′E; Fig. 1), where previous studies on this species were undertaken, recruits were sampled at exactly the same rocky wall over 5 consecutive springs [2006-2010; data from 2006 and 2007 were obtained from Calderón and Turon (2010)]. Second, in June 2008 sampling was also conducted at 5 additional locations along South and East Iberian coasts (separated between 200 and >1100 km; Fig. 1), from similar habitats to those sampled in Tossa de Mar. In this year, and in order to couple a spatial and a temporal survey, we collected both recruits, following the methodology described above, and two-year old individuals (recruited in spring 2006 and collected in June 2008) from the underside of boulders. Available data suggest that individuals between 20 and 30 mm in diameter most likely belong to this age category (Calderón and Turon 2010). We therefore targeted sea urchins within this size range, and their age was subsequently confirmed by analyzing the band pattern in interambulacral plates revealed after immersion in xylene under the stereomicroscope (following the method detailed in

Calderón and Turon 2010). Sea urchins belonging to the cohort recruited in spring 2006 were retained for genetic analyses. Previous studies have evidenced that the analysis of band patterns observed in the inside of tests, corresponding to dense deposits of $CaCO_3$ incorporated during growing periods, is an accurate method to estimate age in this species (Turon et al. 1995; Calderón et al. 2009; Calderón and Turon 2010).

Genomic DNA was directly extracted from whole recruits using DNeasy Tissue Kit (Qiagen, Valencia, CA) for samples collected between 2006 and 2008 and REDExtract-N-Amp^{TM} Tissue PCR (Sigma-Aldrich, Madrid, Spain) for samples collected in 2009 and 2010. For two-year old individuals, gonads (or Aristote´s lantern when gonads were absent) were obtained and used for genomic DNA extraction with REALPURE extraction kit (Durviz) following manufacturer's instructions. A fragment of the mitochondrial Cytochrome oxidase I (hereafter COI) was amplified with a specific primer *P. liv*-F (5'-TCC CAC TAA TGA TTG GAG CA-3'), designed with the program Primer3 v. 0.4.0 (Rozen and Skaletsky 2000), and the universal primer eCOI-R (Arndt et al. 1996) using conditions adapted from Duran et al. (2004). PCR products were vacuum-cleaned (Millipore) and sequenced using ABI PRISM Big Dye v. 3.1 (Applied Biosystems). All sequences were edited and aligned using BioEdit v. 7.0.5.3 (Hall 1999) and deposited in GenBank (accession n. XXXX-XXXX, submitted)

Sequence analyses

Analyses involved temporal comparisons of the cohorts recruited along 5 consecutive springs (2006-2010) in Tossa de Mar as well as spatial comparisons of the 2006 cohort (collected in 2008) and the 2008 cohort between localities. Furthermore, the cohorts from 2006 and from 2008 were compared within locations.

Haplotype and nucleotide diversities were calculated with DnaSP v.5.10.00 (Librado and Rozas 2009). The significance of the differences observed in haplotype diversities between cohorts was determined using an *ad hoc* randomization routine written in Turbo Pascal. Given the large variation in sample size (from 11 to 29 individuals, see Table 1), size-corrected haplotypic richness was calculated using the program Contrib 1.02 (Petit et al. 1998). Arlequin v.3.11 (Excoffier et al. 2005) was used to calculate genetic divergence (F_{ST}) between cohorts based on haplotype frequencies. The significance of comparisons was assessed by performing 10 000 permutations, and *P*-

values were corrected for multiple comparisons based on the false discovery rate (FDR) control following Benjamini and Yekutieli's (2001) method (B-Y), according to which the critical value is determined by:

$$\alpha / \sum_{i=1}^{k} (1/i)$$

where k is the number of hypothesis tests performed and α is the experiment-wise error rate sought (Narum 2006) which we set at 0.05.

Recent studies have questioned the suitability of the commonly used estimator F_{ST} to assess population differentiation, as it is highly dependent on the variability of the marker used (Hedrick 2005; Jost 2008). Therefore, we also computed the new index D_{est_Chao} (Chao et al. 2008; Jost 2008), hereafter D, using the program SPADE (Chao et al. 2008; Chao and Shen 2010). This program further calculated a confidence interval around the obtained value by 1 000 bootstrap replicates. We set this confidence interval, using the normal approximation, at the appropriate P-value following the FDR correction as explained above. If 0 is included within this confidence interval, no evidence for significant differentiation is demonstrated. Further, a MDS analysis was used to graphically represent the differentiation among cohorts based on D estimates.

Finally, a Mantel test was performed to determine whether a correlation exists between genetic differentiation of a single cohort at different locations (using both F_{ST} and D) and geographic distances between locations. Distances between sampling sites were calculated using Google Earth (http://earth.google.com/) by measuring the shortest path by sea between localities.

RESULTS

A total of 290 individuals (including 199 recruits and 91 two-year old individuals) were analyzed (Table 1). A fragment of COI 541-bp long was sequenced, showing 79 variable positions of which 64 corresponded to changes in third positions. Most sequence changes were neutral, with only 11 amino acid changes determined by all the substitutions found. Thirty-nine of the variable positions presented substitutions only once in our data set. Global haplotype diversity was high (H=0.894±0.016) and nucleotide diversity was low (π=0.00638±0.00029). We identified a total of 108

haplotypes of which 77 (71%) were singletons. One single haplotype was present in 89 individuals (ca. 31%) individuals.

We analyzed a total of 117 recruits arrived at Tossa de Mar over 5 consecutive springs (2006, 2007, 2008, 2009 and 2010). The fragment of COI sequenced corresponded to 38 haplotypes, globally revealing high haplotype diversity (H=0.821±0.033) and low nucleotide diversity (π=0.0049±0.00043), as was also observed for each cohort independently (Fig. 2). No significant differentiation was observed in haplotype diversity between cohorts (randomization test, all pairwise comparisons P>0.125). Haplotypic richness did not show large variations between cohorts of recruits arrived at Tossa de Mar (Table 1). Except for Calafat, all populations presented higher haplotypic richness in the cohorts recruited in 2008 than in two-year old cohorts (recruited in 2006; Table 1).

Concerning the spatial study along the Iberian coast, from 38 to 58% of the sea urchins collected in 2008 actually corresponded to two-year old individuals recruited in spring 2006 according to the band pattern observed in the inside of the tests. Two-year old individuals were not found in Tarifa. A significant change in haplotype diversity between cohorts was observed only in one of the 5 localities where both cohorts were available (randomization test, Jávea, P=0.038), where the two-year old individuals had a significantly higher diversity than the recruits of 2006 (Fig. 3), as reflected also by a higher haplotypic richness of the former in spite of a lower sample size (Table 1).

Temporal cohort differentiation was studied at Tossa de Mar. No significant differentiation in haplotype frequencies was observed in any pairwise comparison of cohorts recruited between 2006 and 2010 based on F_{ST} (mean value=-0.0119) and the same results were obtained when differentiation was computed based on Jost's D (mean value=0.0124; Table 2).

We also computed genetic differentiation between cohorts coupling the temporal and spatial perspective. The comparison of haplotype frequencies between the two cohorts (2006 sampled in 2008 and 2008, Table 3) recruited at the same location in the 5 localities for which we have data of both years yielded significantly different results based on F_{ST} in Jávea and Carboneras, with Cádiz being marginally significant

(*P*=0.051, Table 3), or in Jávea, Carboneras and Cádiz according to *D*. It is noteworthy that no haplotype was shared between the Carboneras samples of 2006 and 2008 (thus *D*=1). No significant differentiation was observed in Tossa de Mar or Calafat based on either estimator (Table 3).

Concerning spatial differentiation, no significant differentiation was observed in comparisons between two-year old individuals recruited in 2006 from populations separated up to 1100 km (Table 4), and results were coherent for both F_{ST} and *D* estimators. Interestingly, however, a large number of significant differentiations were observed when recruits arrived and collected in 2008 at the same geographic locations were compared to each other (Table 5). Average differentiation between two-year old cohorts (F_{ST}=0.0136; *D*=0.111) was markedly smaller than that between cohorts of recruits (F_{ST}=0.0467; *D*=0.801). Finally, a significant differentiation based on haplotype frequencies [F_{ST}=0.0123, *P*<0.01; *D*=0.270 (0.067, 0.506)] was observed when comparing all recruits arrived in 2008 as a single cohort with all two-year old populations pooled together.

A graphical representation of the genetic structure can be obtained by combining the *D* values estimated between all cohorts and localities in a MDS plot (Fig. 4). This figure further evidences the higher differentiation found in the recruits of 2008. Samples of 2008 from Jávea and Carboneras (on the first axis) and from Tenerife and Cádiz (on the second axis) are set apart from a tight group containing all samples from Tossa de Mar and all samples from the two-year old individuals (plus the 2008 recruits from Calafat).

Finally, a Mantel test did not reveal a significant correlation between genetic differentiation and geographic distance between cohorts for the global dataset (F_{ST}: *r*=0.177, *P*=0.092; *D*: 0.221, *P*=0.064). Nonetheless, when cohorts were analyzed separately, this same result was obtained for the cohort recruited in 2006 (F_{ST}: *r*=-0.349, *P*=0.886; *D*: *r*=-0.406, *P*=0.880), but a significant correlation was observed for the cohort recruited in 2008 (F_{ST}: *r*=0.602, *P*=0.034; *D*: *r*=0.488, *P*=0.043).

DISCUSSION

285 The data presented in this study constitute an approach to the spatial and temporal
286 genetic structure of cohorts of the common Mediterranean sea urchin *Paracentrotus*
287 *lividus*. Our results showed that the partition of genetic variability between temporal and
288 spatial components is a dynamic feature, and that conclusions extracted either from a
289 single point in time (e.g., a cohort monitored at different localities) or a single point of
290 space (e.g., different cohorts sampled at a given locality) may be misleading. Although
291 our work is based on a single marker (mtDNA sequence data) and more variable genetic
292 markers (e.g., microsatellites) could reveal more subtle genetic patterns, it is clear that
293 important structuring in the genetic composition of the recruiting cohorts can be missed
294 unless sampling in space and time is made at scales wide enough to encompass
295 potentially infrequent episodes.
296
297 The use of F_{ST}-related statistics as a measure of population differentiation has been
298 recently challenged (Hedrick 2005; Jost 2008) because of its dependency on within-
299 population diversity, leading to counter-intuitive results when markers are highly
300 variable. Alternatives to these statistics have been proposed and discussed (Hedrick
301 2005; Jost 2008, 2009; Ryman and Leimar 2009). In the present study, we follow the
302 criterion in Meirmans and Hedrick (2011), who advocate a combined use of F_{ST} and
303 other estimators such as *D* to obtain the maximum possible information based on the
304 properties of the different statistics. F_{ST} and related measures may be more suited to
305 analysis of demographic parameters, such as migration rate, while *D* has the right
306 properties to measure differentiation based on allele frequencies (Jost 2009). In
307 addition, F_{ST} measures have been used for decades and reporting them is useful for
308 comparative purposes (Meirmans and Hedrick 2011). In the present study, both
309 statistics provided similar results for the 2006 cohort although, globally, *D* estimator
310 yielded more significant outcomes, especially for the 2008 cohort, and more accurately
311 detected differentiation between cohorts (i.e., two cohorts with no shared allele have a *D*
312 value of 1), which was the primary interest of this paper.
313
314 A summary of our results based on the *D* estimator of genetic differentiation is
315 presented in Fig. 5. An additional measure of the differentiation among adult
316 populations was obtained by reanalyzing data to obtain *D* values from Duran et al.
317 (2004) from 6 so-called adult populations within the same spatial area (localities 1, 2, 4,
318 5, 6, and 7 in Duran et al. 2004) using the same marker. Those populations were

sampled without regard to their age structure and thus represent a mixture of cohorts potentially averaging out yearly differences. Variability between those adult populations is ca. 20 times higher than that found between cohorts from Tossa de Mar (Fig. 5). Likewise, spatial differentiation within a single recruitment event (spring 2006 or spring 2008) was higher than values of inter-cohort comparisons in Tossa de Mar (Fig. 5). Taken together, these results would lead to the conclusion that the spatial component plays a stronger role than the temporal component in differentiation between cohorts within this species. However, the situation was drastically different between 2006 and 2008. *D* values between cohorts arrived at different localities during the recruitment event in 2008 were 8 times higher than in 2006, and differentiation in temporal comparisons between the two cohorts at a given locality was 40 times higher than the inter-cohort comparisons in Tossa de Mar (Fig. 5).

Had we only undertaken the temporal study in Tossa de Mar and the spatial analysis of the cohort recruited in 2006, we would have concluded that temporal variation within localities was negligible and that differentiation among localities for a single cohort, although higher, was not significant at this spatial scale. However, the analysis of the 2008 cohort clearly showed that marked spatial and temporal cohort differentiation can occur at some years at least. Our results were largely dependent on patterns occurred in the southern localities studied (Cádiz, Tarifa, Carboneras and, to a lesser extent, Jávea), while genetic composition of recruits of northern localities (Calafat and, in particular, Tossa de Mar), remained constant within the time-frame studied (see Fig. 3). A thorough analysis of the composition of the 2008 cohort evidenced the arrival of a large number of private haplotypes, mainly in Carboneras (12 out of 12 haplotypes) and Cádiz (9 out of 15). This suggests that individuals recruited in 2008 at these localities most likely belonged to different genetic pools, apparently with a marked spatial heterogeneity. This heterogeneity is probably behind the significant relationship between genetic differentiation and geographic distance (Mantel test) detected in 2008, but not in the cohort recruited in 2006, which was much more homogeneous.

The contrasting patterns of genetic structure in 2006 and 2008 raise questions about the reasons behind such variation and which the “anomalous” year is (if any can be described as such). To advance possible answers to these questions, we sought for hydrological features in the zone studied. The sampled shores are located in the Balearic

353 Sea and the Alborán Sea, two sub-basins of the Western Mediterranean whose
354 circulation patterns are well known (López-Jurado et al. 1996; Pinot et al. 1996;
355 Bouffard et al. 2010). In short, in the Iberian coast, a predominantly southward current
356 flows from the Gulf of Lions to the Alborán Sea, keeping Atlantic waters restricted to
357 the southern part of the basin. However, alterations of this pattern have been described.
358 For instance, an intense anticyclonic eddy was established in 1998 in the Balearic Sea,
359 which reversed the usual pattern of southward circulation along the littoral (Pascual et
360 al. 2002). This abnormality had important effects on larval distribution of several
361 species of fish (Olivar et al. 2003). Interestingly, in spring 2008, another powerful
362 anticyclonic gyre became established in the Balearic Sea (Bouffard et al. 2010),
363 allowing Atlantic waters to reach further North than usual along the Iberian
364 Mediterranean shores (Tintoré, pers. comm.). This abnormality in 2008 nicely fits our
365 results, as the genetic patterns in the southern populations were changed (particularly in
366 Carboneras), while no changes were observed in the north (Calafat and Tossa).
367 Although evidence is purely correlational, the fact that 2008 was an abnormal year as
368 regards patterns of circulation in the study area strongly points towards hydrological
369 features as the cause of the genetic structures found. These results highlight the potential
370 role of punctual, infrequent episodes of altered circulation in providing a genetic
371 renewal to established populations.
372
373 Of course, other factors can also determine changes in the genetic composition of
374 cohorts recruiting in a given area between consecutive reproductive events. Some
375 populations may experience systematically a higher degree of self-recruitment than
376 others due to micro or mesoscale topographic patterns. Reproductive periods in
377 *Paracentrotus lividus* may be slightly variable from year to year (e.g., Lozano et al.
378 1995) and from one area to another (Ourens et al. 2011). As a result, larval batches can
379 be subject to various prevailing oceanographic conditions at different points of space or
380 time. This may explain the marked variability in the strength of the yearly cohorts
381 which has been repeatedly noted in this species (López et al. 1998; Tomas et al. 2004).
382 Differences also exist between different habitats (Tomas et al. 2004) but were
383 minimized here by sampling the same type of habitat in all localities. In addition,
384 selection can be acting at different pre-zygotic (e.g., Calderón and Turon 2010) or post-
385 zygotic stages, and selection pressures themselves may vary spatially and temporally
386 (e.g., years of rich or poor planktonic food sources for the larvae). Finally, it must be

noted that we have analyzed only the main recruitment event occurring in June of every year, but *P. lividus* has a second, smaller, recruitment in autumn in the study area (López et al. 1998; Tomas et al. 2004). It would have been instructive to assess whether this second recruitment event showed patterns similar to the June episode. Overall, longer time series coupled with circulation data and models are necessary to reliably establish a causal link between the different potential factors and the realized recruitment of species with long-lived larvae in the South-East Iberian shores.

In Tossa de Mar, combining the present work with data from Calderón and Turon (2010) we could monitor the cohort of 2006 at the year of recruitment, and after one (2007) and two (2008) years. In this cohort, no significant change occurred in genetic diversity over time (mean±SD: 0.847±0.047, 0.819±0.082, 0.825±0.059, in 2006, 2007 and 2008, respectively, all randomization tests $P>0.5$). Likewise, no differences in allele frequencies were found (overall $F_{ST}=0.028$, overall $D=0.117$, not different from 0). This result suggests that genetic composition of cohorts does not experience important changes as the cohort grows older. Large mortalities occur during the first stages of benthic life in most marine invertebrates in general (Gosselin and Qian 1996; Hunt and Scheibling 1997), and *Paracentrotus lividus* in particular (Turon et al. 1995; López et al. 1998). A reduction of genetic diversity over these crucial first stages of benthic existence would therefore be expectable (e.g., Pini et al. 2011). However, our results do not support this idea, possibly due to the huge numbers of recruits that can settle in a given locality (López et al. 1998).

Previous studies have shown contradictory results about the temporal structure in sea urchins (Palumbi and Wilson 1990; Edmands et al. 1996; Moberg and Burton 2000; but see Flowers et al. 2002). Sweepstake events, whereby only a random subset of adult individuals contributes to each reproductive event (Hedgecock 1994), can explain stochastic changes in recruits' composition. In our case, however, sweepstake effects were not detected for *Paracentrotus lividus* using nuclear and mitochondrial markers (Calderón et al. 2009; Calderón and Turon 2010). It might be noted, however, that those studies were performed in Tossa de Mar, where a marked constancy in the genetic make-up of the cohorts in the 5 years monitored has been found in the present study. This pattern may be far from general, as shown in other localities of the present study. Thus, a stochastic component of variability related to arrival of different pools of larvae

may be determinant to obtain genetically diverse populations, at least in some areas and over long temporal frames. Care must be taken when interpreting results of connectivity derived from single time-point genetic analyses between populations of this commercially interesting species for which massive collection is leading to population depletion in many areas (Guidetti et al. 2004).

In conclusion, our study showed a hidden source of genetic variability that could only be unveiled by tracing the genetic structure of cohorts over a spatial and temporal scenario. This is likely applicable to other marine species. The widely used approach of studying populations by combining different cohorts neglects this important source of variability. Relatively high genetic homogeneity among cohorts recruiting over the years at a given locality may be common. However, episodes of genetic "renewal" do occur, which contribute to the diversity and variability of the populations by seeding them with larvae coming from different sources. This temporal variability may be linked to changes in current patterns, to differences in reproduction timing and success, or to a combination of these and other factors. Analyses focusing on cohorts of settling recruits can yield important spatial and mechanistic insights into patterns of larval dispersal (Selkoe et al. 2006). Further research should try to couple genetic variability of cohorts at larger spatio-temporal scales with biological and oceanographic features. *Paracentrotus lividus* is an ideal case study for the analysis of the implications of dispersal and genetic diversity of cohorts, for its long-lived larval strategy, for its ecological importance as a keystone herbivore and because it is an economic resource fished in much of its distribution range.

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598

Figure legends

Fig. 1 Sampling locations for *Paracentrotus lividus* along East and South Iberian coast. Tossa de Mar was sampled over 5 consecutive springs (June 2006-2010). The 5 additional locations were only sampled in June 2008, both for recruits and two-year old individuals

Fig. 2 Haplotype diversity of 5 cohorts of recruits arrived at Tossa de Mar in 5 consecutive years (spring 2006-spring 2010). Vertical bars represent standard deviations

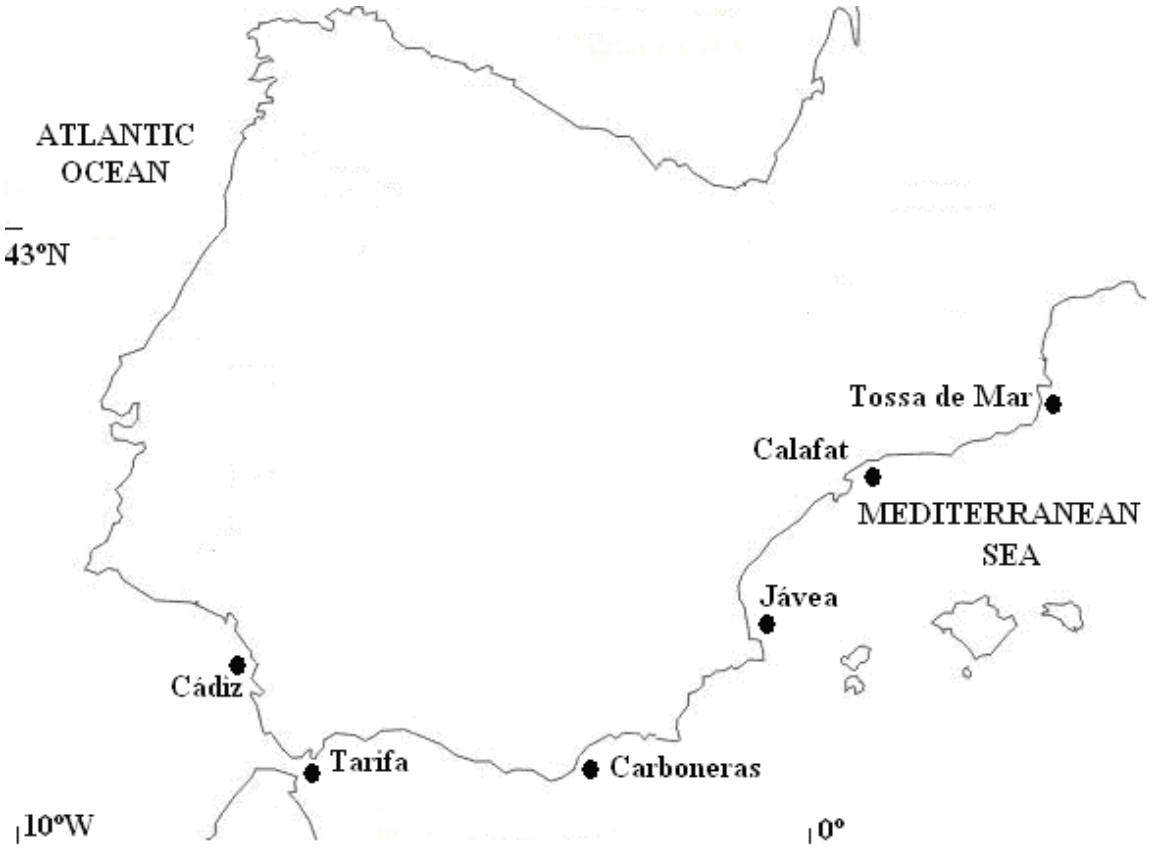
Fig. 3 Values of haplotype diversity between two different cohorts (2006, two-years old, in black) and 2008 (recruits of the year, in grey) at the 6 localities studied. Bars represent standard deviations. Asterisk mark significant differences in haplotype diversity assessed by a randomization test

Fig. 4 MDS plot obtained from the dissimilarity matrix of *D* values among all cohorts and localities. The inset shows an enlarged view of the cluster of points indicated. The stress of the final configuration was 0.038

Fig. 5 Representation of *D* values of dissimilarity obtained among adult populations (data from Duran et al. 2004) and from the different sets of cohort comparisons performed in the present study. Bars represent standard errors

Figure

Fig. 1


ATLANTIC
OCEAN
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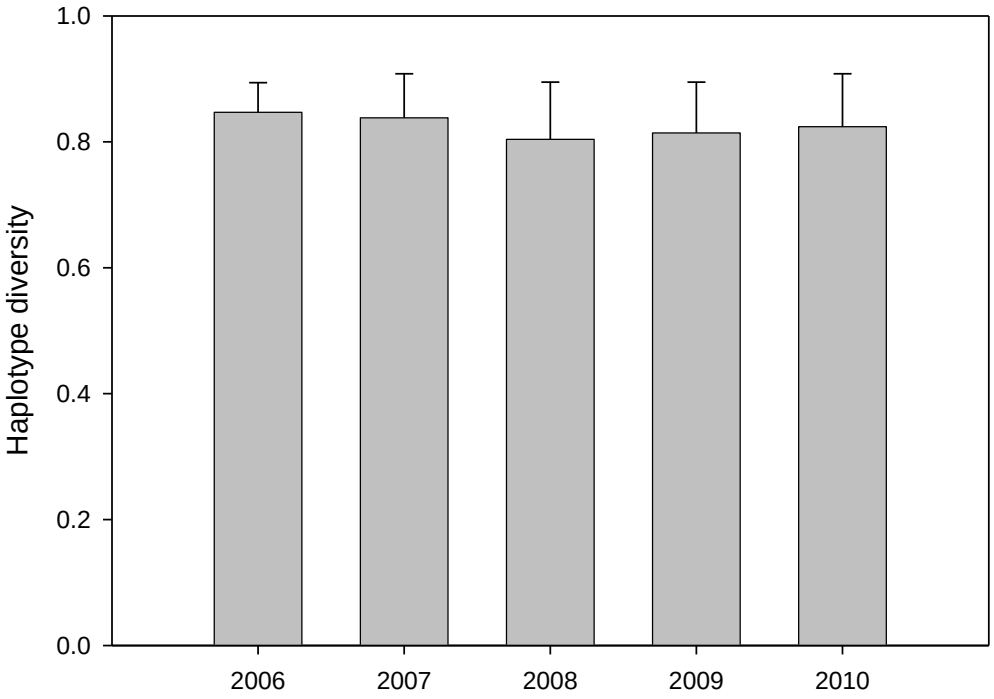
Tossa de Mar
Calafat
MEDITERRANEAN
SEA
Jávea
Cádiz
Tarifa
Carboneras
10°W
0°

Fig. 2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Haplotype diversity
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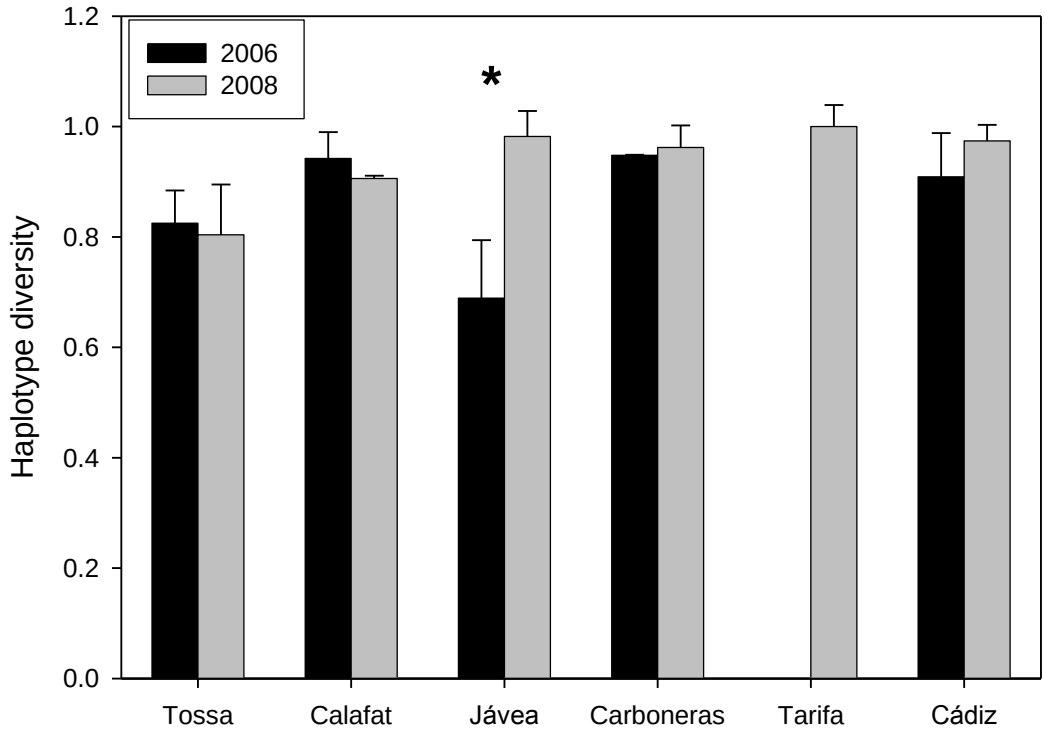
Fig. 3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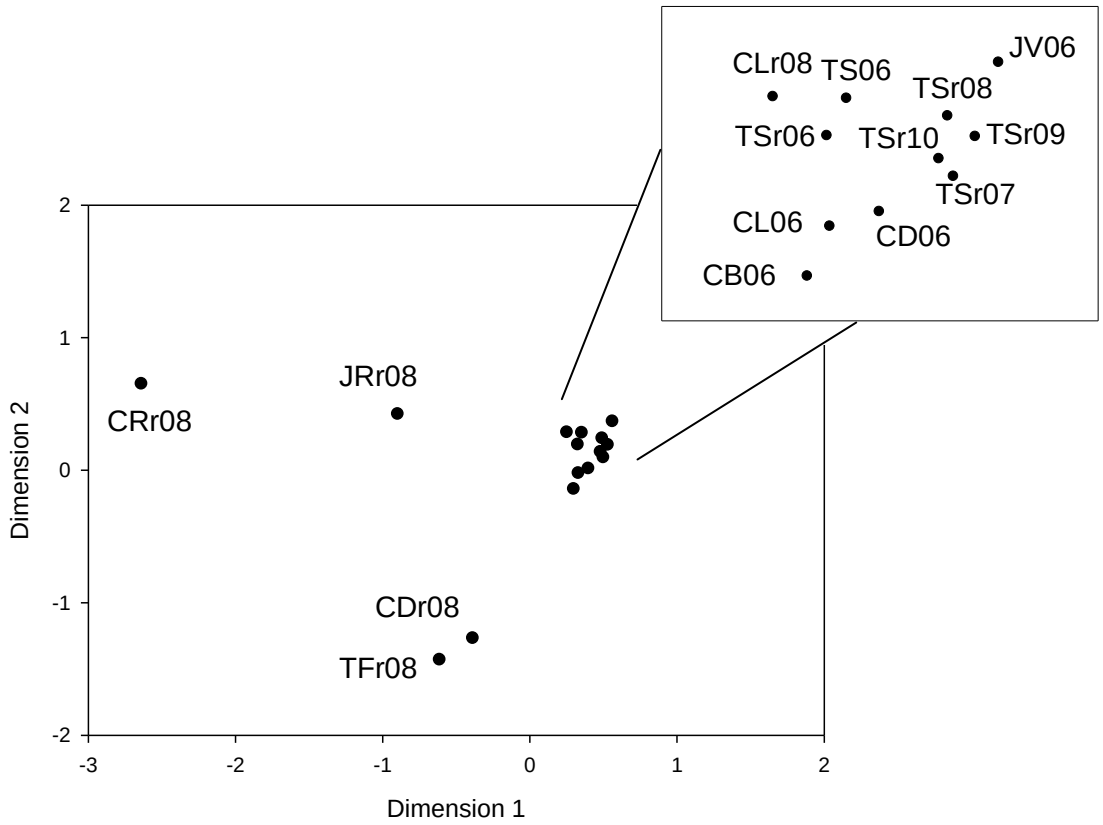
Cádiz

Fig. 4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TS06
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TSr09
TSr07
CL06
CD06
CB06
JRr08
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TFr08
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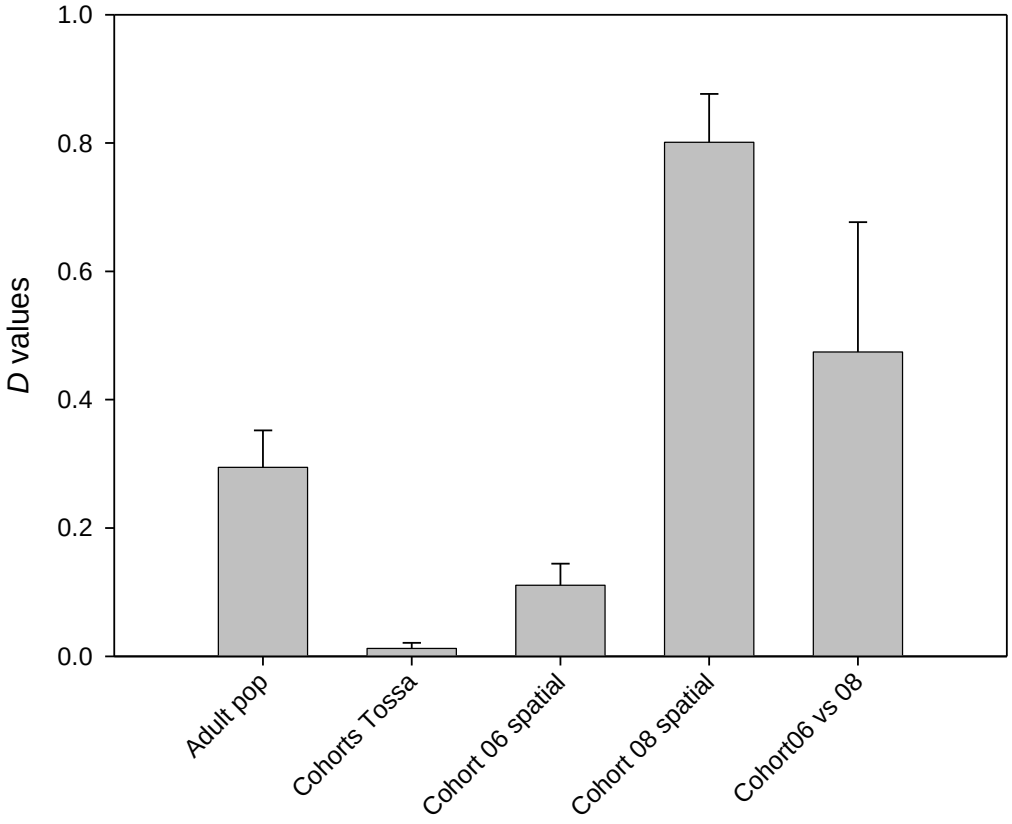
Fig. 5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Cohort 08 spatial
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Table

Location	Year of recruitment	Year of collection	Code	Sample size	Number of haplotypes (private haplotypes)	Haplotypic richness
Tossa de Mar	2006	2006	TSr06	28	11 (4)	5.238
Tossa de Mar	2007	2007	TSr07	27	15 (5)	6.205
Tossa de Mar	2006 2008	2008 2008	TS06 TSr08	29 18	13 (7) 9 (6)	5.376 5.392
Tossa de Mar	2009	2009	TSr09	23	12 (5)	5.783
Tossa de Mar	2010	2010	TSr10	21	12 (3)	6.024
Calafat	2006 2008	2008 2008	CL06 CLr08	16 19	12 (4) 12 (7)	7.789 6.767
Jávea	2006 2008	2008 2008	JV06 JVr08	20 11	7 (3) 10 (5)	3.937 9.000
Carboneras	2006 2008	2008 2008	CB06 CBr08	22 15	16 (8) 12 (12)	8.006 8.267
Cádiz	2006 2008	2008 2008	CD06 CDr08	12 18	9 (3) 15 (9)	7.333 8.764
Tarifa	2008	2008	TFr08	11	11 (5)	10.000
Total number of recruits				191	82	
Total number of two-year old adults				99	41	
Total number of individuals				290	108	

Table 1. Locations of collection, year of recruitment, collection and sample sizes for the cohorts analyzed. Code lists short names given to each sample: two upper case letters designate locality, two numbers refer to year of recruitment, and lower case “r” means that the sample in question consisted of recruits of the year. Note that the 2006 cohort at Tossa de Mar was collected in 2006 (TSr06) and two years later (TS06). Number of haplotypes (haplotypes exclusive to one population in brackets) and haplotypic richness (corrected for sample size) are also presented.

Tossa de Mar	2006	2007	2008	2009	2010
2006	0	0.077 (0, 0.395)	0 (0, 0.310)	0.047 (0, 0.356)	0 (0, 0.299)
2007	0.0115 (0.1942)	0	0 (0, 0.292)	0 (0, 0.181)	0 (0, 0.171)
2008	-0.0099 (0.5604)	-0.0128 (0.7496)	0	0 (0, 0.245)	0 (0, 0.225)
2009	0.0058 (0.2871)	-0.0117 (0.7545)	-0.0213 (0.8749)	0	0 (0, 0.152)
2010	-0.0086 (0.5738)	-0.0196 (0.9453)	-0.0256 (0.9529)	-0.0275 (0.9999)	0

Table 2. Cohort differentiation between temporal samples from Tossa de Mar. Only recruits collected at the same year of arrival (spring 2006 to spring 2010) are analyzed. Lower diagonal are F_{ST} estimates (*P*-values in brackets). Upper diagonals represent *D* values (confidence intervals bounded between 0 and 1 in brackets). Following a FDR correction, *P*-values for significance (and confidence interval limits) were set at 0.017.

Locality	F_{ST} (*P*-value)	*D* ($IC_{95\%}$)
Tossa	-0.01258 (0.6498)	0 (0, 0.256)
Calafat	-0.0067 (0.5102)	0 (0, 0.419)
Jávea	0.1323 (**0.0063**)	0.713 **(0.233, 0.930)**
Carboneras	0.04523(**0.0039**)	1 **(1,1)**
Cádiz	0.03505 (0.0512)	0.659 **(0.241, 0.854)**

Table 3. F_{ST} and *D* estimates of genetic differentiation between the cohorts of 2006 (sampled in 2008) and 2008 at 5 localities; *P*-values for F_{ST} and confidence interval for *D* are presented in brackets. Significant values at *P*=0.05 are presented in bold.

	TS06	**CL06**	**JV06**	**CB06**	**CD06**
TS06	0	0.095 (0, 0.516)	0.097 (0, 0.367)	0.220 (0, 0.585)	0.079 (0, 0.526)
CL06	0.0096 (0.2499)	0	0.229 (0, 0.648)	0 (0, 0.351)	0 (0, 0.345)
JV06	0.0243 (0.1197)	0.0466 (0.0613)	0	0.302 (0, 0.656)	0.085 (0, 0.557)
CB06	0.0257 (0.0653)	-0.0109 (0.7795)	0.0583 (0.0233)	0	0 (0, 0.303)
CD06	0.0066 (0.2888)	-0.0214 (0.9192)	0.0164 (0.2289)	-0.0221 (0.9227)	

Table 4. Differentiation between samples from different Iberian localities for the cohort of 2006 sampled in 2008. Codes as in Table 1. Lower diagonal shows F_{ST} estimates (*P*-values in brackets). Upper diagonal represents *D* values (confidence intervals bounded between 0 and 1 in brackets). Following a FDR correction, *P*-values for significance (and confidence interval limits) were set at 0.017.

	TSr08	**CLr08**	**JVr08**	**CBr08**	**TFr08**	**CDr08**
TSr08	0	0.028 (0, 0.427)	**0.657** **(0.162, 0.884)**	**1 (1, 1)**	**1 (1, 1)**	**0.789** **(0.446, 0.948)**
CLr08	-0.0018 (0.4057)	0	**0. 566** **(0.057, 0.796)**	**1 (1, 1)**	**1 (1, 1)**	**0.776** **(0.463, 0.908)**
JVr08	0.0745 **(0.0146)**	0.0298 (0.1123)	0	**1 (1, 1)**	**1 (1, 1)**	**0.845** **(0.586, 0.901)**
CBr08	0.1188 **(0.0006)**	0.06644 **(0.0008)**	0.0284 (0.0572)	0	**1 (1, 1)**	**1 (1, 1)**
TFr08	0.1039 **(0.0038)**	0.0492 (0.0278)	0.0091 (0.4741)	0.0196 (0.1465)	0	0.357 (0, 0.537)
CDr08	0.0886 **(0.0009)**	0.0461 (0.0111)	0.0174 (0.1316)	0.0320 **(0.0072)**	-0.0016 (0.5355)	0

Table 5. Differentiation between samples from different Iberian localities for the cohort of 2008 sampled the same year. Codes as in Table 1. Lower diagonal represents F_{ST} estimates (*P*-values in brackets). Upper diagonal represents *D* values (confidence intervals bounded between 0 and 1 in brackets). Values in bold indicate significant comparisons. Following a FDR correction, *P*-values for significance (and confidence interval limits) were set at 0.015.