Insights to the genetic structure of *Calanus helgolandicus* (Calanoida: Copepoda) from deep-sea specimens in the Balearic Sea

Diego F. Figueroa, Joan E. Cartes, Nicole J. Figueroa

**Abstract.**—*Calanus helgolandicus* is widely distributed across the northeast Atlantic and Mediterranean, and also found in the Black Sea where it is referred to as *Calanus euxinus*. Previous genetic studies do not include deep-water specimens despite high abundances at bathypelagic and mesopelagic depths. Our objective is to compare the genetic structure of *C. helgolandicus* from the deep Balearic Sea to that of coastal populations in the Northeastern Atlantic Ocean, the Adriatic Sea, and the Black Sea defined from previous research. We use a portion of the mitochondrial gene cytochrome oxidase I from 41 individuals of *C. helgolandicus* collected at 2170 m depth in the Balearic Sea to estimate genetic differentiation between geographic regions and elucidate phylogeographic patterns. Results show that populations do not follow an isolation by distance model. Instead, the lowest genetic differentiation is between two distant basins, the deep Balearic Sea and the Black Sea. The results can be explained by the presence of two types of *C. helgolandicus*, a coastal, shallow water, type and an oceanic, deep water, type that diapauses at great depths. Genetic differentiation between coastal populations is maintained by oceanographic barriers, while differentiation in oceanic populations is lower due to dispersal by deep ocean currents.

**Key Words:** Mediterranean, haplotype, genetic structure, phylogeography, Crustacea

**Introduction**

The geographical range occupied by *Calanus helgolandicus* is related with its depth distribution, its abundance/dominance in the environment, and with trophic dynamics and reproductive strategies (e.g., Bonnet *et al.*, 2005). *Calanus helgolandicus* is found from the Western to Eastern North Atlantic (including the North Sea) and throughout the Mediterranean Sea (e.g. Bonnet *et al.*, 2005). Within the Black sea, the species *Calanus euxinus* is considered to be the sister species of *C. helgolandicus* (Fleminger and Hulsemann, 1987). In fact, this species was considered a variety of *C. helgolandicus*, but its elevation to a separate species stems from various morphometric studies that have shown clear morphological differences between *C. euxinus* from the Black Sea and *C. helgolandicus* from the Atlantic and Mediterranean (e.g., review by Fleminger and Hulsemann, 1987). Nevertheless, phylogeographic research continues to treat *C. helgolandicus* and *C. euxinus* as members of a single species exhibiting gene flow between populations (Papadopoulos *et al.*, 2005; Unal *et al.*, 2006; Yebra *et al.*, 2011). Papadopoulos *et al.* (2015) analyzed specimens from the North Sea, the Adriatic Sea, and the Black Sea using the mitochondrial gene cytochrome oxidase I (COI). Their results show significant genetic differentiation between the three basins, but the observed differences are rather small, ranging from 0.22% to 0.57%, while differences between other *Calanus* species range from 7% to 25% (Bucklin *et al.*, 1999; Hill *et al.*, 2001).
Papadopoulos et al. (2005) also shows that haplotypes are shared between the three basins. One of their main conclusions is that the level of genetic divergence of *C. euxinus* from *C. helgolandicus* is not greater than the divergence of geographically separated populations of *C. helgolandicus* such as those from the Atlantic and those from the Adriatic Sea. A similar study was performed by Unal et al. (2006), also targeting COI and analyzing samples from the English Channel, the Adriatic Sea, and the Black Sea. Their results agree with those of Papadopoulos et al. (2005), demonstrating significant, yet very small genetic differences between the populations of the three basins. They conclude that the status of *C. euxinus* as a separate species is questionable and that *C. helgolandicus* and *C. euxinus* may be an example of incipient speciation. Another phylogeographic study of these two species was carried out by Yebra et al. (2011) using the mitochondrial 16s ribosomal RNA. Their study includes samples from the Northeast Atlantic, the North Sea (including various fjords), the Tyrrenian Sea, the Adriatic Sea, the Aegean Sea, and the Black Sea. Their results are in agreement with those of Papadopoulos et al. (2005) and Unal et al. (2006), demonstrating statistically significant genetic structuring albeit very small genetic differentiation.

These various phylogeographic studies (Papadopoulos et al., 2005; Unal et al., 2006; Yebra et al., 2011) lack specimens from the westernmost region of the Mediterranean and from deep oceanic waters. In the northwest Mediterranean, the Balearic sea forms a semi enclosed basin (Galarza et al., 2009). Within this basin is the Balearic Front, characterized by a significant density difference in the upper 200 m (Galarza et al., 2009). This front is formed from the inflow of the North Atlantic Central Water into the Mediterranean, where it is modified into a water mass of higher salinity known as the Modified Atlantic Water; when this water mass reaches the north western Mediterranean, it is deflected east by cyclonic circulation around the Balearic islands, resulting in the Balearic Front (Pascual et al., 2017). Galarza et al. (2009) show how the Balearic Front results in significant genetic differentiation in five species of fish. Similarly, Pascual et al. (2017) through a review of literature, demonstrate that the Balearic Front is a significant barrier to dispersal in marine species of varying life strategies, with significant genetic distances and reduction in gene flow between localities separated by this front. Given the strength of this barrier in reducing gene flow in many marine species (Galarza et al., 2009; Pascual et al., 2017), it is likely that *C. helgolandicus* from the Balearic sea also form a genetically unique population within the Mediterranean.

In addition to the potential separation of *C. helgolandicus* within the Balearic Sea by the Balearic Front, there may exist a vertical separation between *C. helgolandicus* in surface waters and those in the deep-sea. Early studies of *C. helgolandicus* in the Northern Atlantic, show evidence of two vertically separated populations in the Clyde Sea (Nicholls, 1933; Marshall et al., 1934) and the southern Norwegian Sea (Ostvedt, 1955; Krause, 1978). This observation is further tested by Hirche (1983) who performed a series of experiments to determine the physiological responses of *C. helgolandicus* to environmental stressors from individuals collected at the surface and from individuals collected in deep waters from two fjords, one in Sweden and the other in Norway. One of his key findings is that specimens collected at the surface were physiologically different than those collected in deep waters. Only the deep-water specimens showed overwintering features (Hirche, 1983). He concludes that there is likely a surface-living and a deep-living population of *C. helgolandicus* as suggested by earlier studies (Nicholls, 1933; Marshall et al., 1934; Ostvedt, 1955; Krause, 1978). The presence of distinct populations of *C. helgolandicus*...
that are separated by their vertical distribution has not been revisited since Hirche (1983).

The purpose of our study is to determine whether *C. helgolandicus* from deep waters of the western Mediterranean represent a separate population from those recovered in surface waters by earlier studies. We propose that the oceanographic barrier presented by the Balearic front combined with the vertical segregation observed in this species by earlier studies should result in high genetic differentiation. To test this hypothesis we collected specimens of *C. helgolandicus* within the Balearic Sea near the seafloor at a depth below 2,000 m. We target the mitochondrial gene COI and integrate our results with the genetic structure research by Papadopoulos *et al.* (2005) and Unal *et al.* (2006) who analyzed *C. helgolandicus* collected in the epipelagic zone from various sites within the Northeastern Atlantic Ocean and the Mediterranean (excluding the Balearic Sea).

### Materials and Methods

1. **Sample collection**

   Specimens were collected in the Balearic Sea (Fig. 1) on 04/05/2012 using a WP2 type net (PreTREND cruise, WP2–3) equipped with a net depressor. The net was deployed closed and opened near the bottom over soundings of 2,170 m, trawling the net at *ca.* 3–170 m above the bottom (2,000 m from surface). The net was equipped with 500 µm mesh and trawled at *ca.* 1.5–2 knots. The duration of tow was 10 min. Standard 2030 flowmeters (General Oceanics) were attached to the mouth of net to measure the amount of water filtered. Mechanical messengers were used to close the net upon retrieval. This method of collection prevents contamination of the sample from specimens occupying other depths in the water column. The deployment and location of the net in the water column and near the bottom was visualized using a Simrad Single-beam biological echo sounder EK500 and SCANMAR sensors (Cartes *et al.*, 2013). Specimens were preserved using 95% ethanol. All individuals of *C. helgolandicus* were identified (based on taxonomy described in Fleminger and Hulsemann, 1987 and Boxshall and Halsey, 2004) sorted, and preserved separately from the rest of the zooplankton for molecular analyses. Detailed morphological analyses were not possible because all specimens consisted of immature, CV copepodites. Additionally, the specimens were not intact with most swimming legs broken and incomplete. Nevertheless, positive identification of *C. helgolandicus* could be inferred due to its overwhelming abundance and unique body form within the sampled zooplankton community.

2. **Molecular methods**

   Random individuals of *C. helgolandicus* preserved in ethanol were rehydrated in molecular-grade water for thirty minutes. Genomic DNA extraction was performed by placing individuals in a 2 mL tube with 100 µL of Bio-Rad’s Instagene Matrix. The specimens were
then placed in a thermomixer and incubated at 56°C overnight. After incubation, samples were heated to 100°C for 8 minutes. Samples were then centrifuged for 1 minute at 10,000 g’s. The supernatant containing the DNA was removed with a pipette and placed in a new 2 mL tube and stored in a −20°C freezer until further use.

Quantification of extracted DNA was performed using Thermofisher Scientific’s Qubit fluorometer set to OD260. Polymerase Chain Reaction (PCR) was used to amplify 515 base pairs of the first half of the mitochondrial gene cytochrome oxidase I (COI) using primers developed by Papadopoulos et al. (2005): Chelg-COI-F (5’-GGGAAACGAGGAGAGATA-3’) and ChelgCOI-R (5’-CGGGACTCAGTATAAT TATTTCTA-3’). The PCR reaction was carried out in a 25 µL volume: 7.55 µL PCR water, Invitrogen’s 10X PCR Buffer (2.0 µL), 1.25 µL Invitrogen’s 50 mM MgCl2, 2.0 µL of 10 mM dNTP, 1.0 µL of 10 mM forward primer (ChelgCOI-F), 1.0 µL of 10 mM reverse primer (ChelgCOI-R), 0.2 µL Thermo Fisher’s Invitrogen Platinum TAQ DNA Polymerase, and 10.0 µL DNA. The following thermocycler conditions were employed: 94°C for 2 mins, followed by 40 cycles of 94°C for 1 min, 46°C for 1 min, 72°C for 1.5 mins, followed by 72°C for 1 additional min, and then cooled at 4°C ∞.

PCR products were visualized by agarose-gel electrophoresis and those with a single band of ~500 bp were purified using Sigma Aldrich’s GenElute PCR clean-up kit. Purified PCR products were sent to Eurofins MWG Operon LLC for standard Sanger sequencing of both forward and reverse strands.

3. Population genetic analyses

For each specimen, the sequences for the forward and reverse strands were assembled with the software CLC Workbench 7.9.1 (CLC Bio, Aarhus, Denmark) using default settings. Chromatograms were visually inspected for conflicts between the two strands and conflicts were resolved manually. Base quality scores were visually examined for quality control and a consensus sequence was generated from the alignment. Consensus sequences were checked for the presence of indels and in-frame stop codons which are characteristic of nuclear mitochondrial pseudogenes (Song et al., 2008). All available sequences of C. helgolandicus and C. euxinus were downloaded from GenBank (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/genbank/) and added to our analyses. A total of 219 sequences were analyzed, 41 from this study, 72 (GenBank PopSet: 62868693) from Papadopoulos et al. (2005), and 99 (GenBank PopSet: 443907159) from Unal et al. (2006). All sequences were aligned using MUSCLE v3.8 (Edgar, 2004) with default parameters and visually inspected for consistency. Since the sequences used came from various studies, their length varies, therefore the alignment was trimmed to 416 base pairs; ensuring that all sequences are of the same length, which is essential for accurate definition of haplotypes. This alignment was imported to the software DnaSP v5 (Librado and Rozas, 2009) which was used to generate a haplotype list. This resulted in 57 haplotypes that were used to generate a median-joining network with PopArt v 1.7.2 (Leigh and Bryant, 2015), using an epsilon of zero.

DnaSP v5 was also used to calculate population diversity indices, including number of segregating sites (S), haplotypes number (h), haplotype diversity (Hd), nucleotide diversity (π), average number of pairwise nucleotide differences (K), and mutation rate (θ). The same software was used to test neutrality of mutations by calculating Tajima’s D and Fu’s F indices. The significance of each index was determined by using coalescent simulations using segregating sites with 5,000 replicates.

Genetic structure was inferred using the software Arlequin v3.5.1.2 (Excoffier and Lischer, 2010). The 219 sequences were grouped by geographic regions corresponding to sampling
locations. Four regions were compared (Figure 2): North East Atlantic (58 individuals from previous studies), Balearic Sea (41 individuals from this study), Adriatic Sea (51 individuals from previous studies), and Black Sea (69 individuals from previous studies). Standard AMOVA was performed using conventional F-statistics with 1000 permutations. Pairwise population comparisons were performed to determine genetic differentiation by calculating Fst using 3,000 permutations to estimate statistical significance. Isolation by distance analyses were carried out using Rousset’s distance (F/(1-F)) with the software IBD (Bohonak 2002).

Results

*Calanus helgolandicus* from 2,000 m depth in the Balearic Sea are the dominant zooplankton taxa and make up the vast majority (92%) of the copepods. The abundance of *C. helgolandicus* in our samples is approximately 3,921 individuals/1000 m³. There are no mature individuals; all the specimens are CV copepodites. Furthermore, none of the specimens are intact, and therefore they are unsuitable for morphometric analyses. At total of 41 individuals generated high quality DNA and PCR product, resulting in 41 sequences of mtCOI. The single bands observed during gel electrophoresis visualization and the lack of indels or stop-codonts within the sequences suggests that nuclear mitochondrial pseudogenes were not amplified (Song et al., 2008). BLAST analyses against GenBank’s database results in positive matches for *C. helgolandicus* for all 41 sequences.

The 41 sequences of mtCOI from our study are analyzed with 178 additional sequences downloaded from GenBank (see methods). These 219 sequences reveal 57 haplotypes with 44 of them unique, represented by a single individual (Table 1, Fig. 2). Haplotypes from the Balearic Sea are deposited in GenBank with accession numbers MN503866-MN503880. The populations from the Northeast Atlantic and the Black Sea have the most haplotypes with 21 each; they are followed by the population in the Balearic Sea with 15 and finally the Adriatic Sea with only 14 (Table 1, Fig. 2). The Northeast Atlantic has the highest percentage of individuals with unique haplotypes (24%), followed by the Balearic Sea (22%), the Adriatic Sea (18%) and the Black Sea (17%, Table 1, Figs. 2 and 3). The haplotype network shows two major haplotypes (H1 and H2) with other haplotypes radiating from these two (Fig. 3). Haplotype H1 is found in all four regions while haplotype H2 is absent in the Adriatic Sea but present in the other three regions (Table 1, Fig. 2).
The majority of individuals from the Black Sea belong to haplotype H1 (52%, Table 1, Figs. 2 and 3). This is also the most prevalent haplotype in the Balearic Sea (32%); it is less frequent in the Adriatic Sea (24%) and barely present in the Northeast Atlantic (3%, Table 1, Figs. 2 and 3). The majority of individuals in the Northeast Atlantic belong to haplotype H2 (60%, Table 1, Figs. 2 and 3). This haplotype is the second most abundant haplotype in the Balearic Sea (20%); it is barely present in the Black Sea (1%) and absent in the Adriatic Sea (Table 1, Figs. 2 and 3). The most prevalent haplotype in the Adriatic Sea is haplotype H11 (33%); this haplotype is absent in all other regions (Table 1, Figs. 2 and 3).

Table 1. These counts show for each region the total number of individual sequences used in the analyses, the total number of haplotypes, the frequency for each haplotype and the number of unique haplotypes (those represented by only one individual).

| Sequences | Haplotypes | H1 | H2 | H11 | H10 | H7 | H40 | H45 | H3 | H37 | H28 | H53 | H27 | H42 | Unique |
|-----------|------------|----|----|-----|-----|----|-----|-----|----|-----|-----|-----|-----|-----|---------|
| NE Atlantic | 58 | 21 | 2 | 35 | 0 | 1 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 14 |
| Adriatic Sea | 51 | 14 | 12 | 0 | 17 | 0 | 9 | 0 | 3 | 0 | 0 | 0 | 0 | 1 | 0 | 9 |
| Black Sea | 69 | 21 | 36 | 1 | 0 | 10 | 1 | 1 | 2 | 2 | 0 | 3 | 0 | 1 | 0 | 12 |
| Balearic Sea | 41 | 15 | 13 | 8 | 0 | 0 | 0 | 5 | 0 | 2 | 2 | 0 | 2 | 0 | 0 | 9 |
| Total | 219 | 57 | 63 | 44 | 17 | 11 | 11 | 7 | 5 | 4 | 4 | 3 | 2 | 2 | 2 | 44 |

Fig. 3. Median Joining network of C. helgolandicus and C. euxinus based on mtCOI. Color corresponds to the four geographic regions: Northeast Atlantic-blue, Adriatic Sea-green, Black Sea-orange, and Balearic Sea-red. Black nodes represent an inferred ancestral haplotype. The size of the nodes is proportional to the number of individuals with that haplotype and tick marks represent number of single mutations between connecting haplotypes.
The highest haplotype diversity is in the Balearic Sea (Hd = 0.855) followed by the Adriatic Sea (Hd = 0.811), the Black Sea (Hd = 0.71) and finally the Northeast Atlantic (Hd = 0.638). Tajima’s D is negative and statistically significant (p < 0.05) for each region, ranging from −1.990 to −2.255, with a value of −2.432 for the entire population (Table 2). Fu’s F is also negative and statistically significant (p < 0.05) for each region, except for the Balearic Sea (p = 0.210). The values of Fu’s F range from −2.637 to −19.385 for each region, with a value of −33.441 for the entire population (Table 2). Ramos-Onsins and Rozas’s R2 is statistically significant (p < 0.05) and greater than 0 for each region, ranging from 0.028 to 0.060, with a value of 0.019 for the entire population (Table 2). The AMOVA results show that 17.8% of the variation is among populations and 82.2% of the variation is within populations (Table 3). The Fixation Index (Fst) for the entire population is 0.178. The results of the AMOVA are significant p < 0.001 based on 1023 permutations (Table 3).

Pairwise Fst values range from 0.06 to 0.27 and all are statistically significant (p < 0.05 and Figure 4). The highest genetic differentiation is between populations of the Northeast Atlantic and Black Sea (Fst = 0.27) and the Northeast Atlantic and Adriatic Sea (Fst = 0.24). Genetic differentiation is lower between populations of the Northeast Atlantic and Balearic Sea (Fst = 0.16), Adriatic Sea and Black Sea (Fst = 0.13), and Adriatic Sea and Balearic Sea (0.10). The Balearic Sea and Black Sea are the least genetically differentiated populations (Fst = 0.06). The results of the isolation by distance test using Rousset’s distance did not show a clear pattern and were not significant (p > 0.05) accord-

### Table 2. Haplotype statistics and diversity indices: n = number of sequences, h = number of haplotypes, Hd = haplotype diversity, S = number of segregating sites, θ = population mutation rate, π = nucleotide diversity, k = number of nucleotide differences between any two sequences. Statistical significance of Tajima’s D, Ramos-Onsins and Rozas’s R2, and Fu’s F neutrality tests calculated from coalescent simulations.

| Region         | n   | h   | Hd   | S    | θ    | π    | max. k | average k | Tajima’s D | Prob. (D) > 0 | R2 | Prob. R2 > 0 | Fu’s F | Prob. (F) > 0 |
|----------------|-----|-----|------|------|------|------|--------|-----------|------------|---------------|----|--------------|--------|---------------|
| NE Atlantic    | 58  | 21  | 0.638| 23   | 0.0119| 0.0037| 10     | 1.52      | −2.000     | 0.002         | 0.031| 0.000        | −19.385| 0.000         |
| Adriatic Sea   | 51  | 14  | 0.811| 14   | 0.0075| 0.0035| 5      | 1.46      | −1.610     | 0.030         | 0.050| 0.004        | −8.318 | 0.004         |
| Black Sea      | 69  | 21  | 0.710| 21   | 0.0105| 0.0028| 4      | 1.15      | −2.255     | 0.000         | 0.028| 0.000        | −22.232| 0.000         |
| Balearic Sea   | 41  | 15  | 0.855| 43   | 0.0242| 0.0105| 31     | 4.37      | −1.990     | 0.007         | 0.060| 0.015        | −2.637 | 0.210         |
| All            | 219 | 57  | 0.866| 69   | 0.0278| 0.0054| 31     | 2.24      | −2.432     | 0.000         | 0.019| 0.000        | −33.441| 0.000         |

### Table 3. AMOVA results using conventional F-statistic.

| Source of variation | d.f | Sum of squares | Variance components | Percentage of variation |
|---------------------|-----|---------------|---------------------|------------------------|
| Among populations   | 3   | 16.168        | 0.08180 Va           | 17.8                   |
| Within populations  | 249 | 94.05         | 0.37771 Vb           | 82.2                   |
| Total               | 252 | 110.217       | 0.45951             |                        |

Significance Tests
(1023 permutations)
P (rand. value > obs. value) 0.000
P (rand. value = obs. value) 0.000
P-value 0.000

and 3).
ing to the Mantel test (not shown).

Discussion

Our results show that \textit{C. helgolandicus} from deep waters of the Balearic Sea form a genetically distinct population from those in surface waters of the North East Atlantic (pairwise $F_{st}$ 0.16, $p < 0.05$) and from those in surface waters of the Adriatic Sea (pairwise $F_{st}$ 0.10, $p < 0.05$). But, when compared to the population of \textit{C. euxinus} in the Black Sea, the genetic differentiation is much smaller (pairwise $F_{st}$ 0.06, $p < 0.05$). Our hypothesis that \textit{C. helgolandicus} from the deep Balearic Sea should represent a genetically unique population due to both, the regional retention by the Balearic Front and the vertical separation in distribution from shallow water populations is only partly supported. If both of these processes are effective barriers to gene flow, then the expectation would be to have high genetic differentiation between the Balearic Sea and all other regions. Instead, the deep Balearic Sea population exhibits the greatest genetic differentiation with the neighboring populations in the North East Atlantic and Adriatic Sea, while showing a greater genetic similarity to the distant population of \textit{C. euxinus} in the Black Sea. These observations can be explained by the circulation of deep-water masses throughout the Mediterranean and by the ontogenetic distribution and presence of a coastal and an oceanic population of \textit{C. helgolandicus}.

Similar to previous research on the genetic structure of \textit{C. helgolandicus} (Papadopoulos et al., 2005; Unal et al., 2006; Yebra et al., 2011), our study demonstrates that there are small, yet statistically significant genetic differences across geographic regions. By adding specimens from the deep Balearic Sea and combining the data from Papadoupoulos et al. (2005) and Unal et al. (2006), we show that these genetic differences between \textit{C. helgolandicus} from the Atlantic, \textit{C. helgolandicus} from the Mediterranean and \textit{C. euxinus} from the Black Sea...
sea are even lower than those reported in the literature (Papadopoulos et al., 2005; Unal et al., 2006; Yebra et al., 2011). The population of \textit{C. helgolandicus} in the Northeast Atlantic is genetically more similar to that of the neighboring Balearic Sea ($F_{st}$ 0.16), followed by that of the Adriatic Sea ($F_{st}$ 0.24), and most different to the population of \textit{C. euxinus} in the Black Sea ($F_{st}$ 0.27). The same relative pattern but with much higher genetic differentiation was observed by Papadopoulos et al. (2005) and Unal et al. (2006) comparisons between the Atlantic and the Adriatic Sea ($F_{st}$ 0.52 and 0.51, respectively) and the Atlantic and Black Sea ($F_{st}$ 0.51; value not reported by Unal et al., 2005). Yebra et al. (2011) performed a similar population structure analysis but it was based on a different marker (mitochondrial 16s), and like Papadopoulos et al. (2005) and Unal et al. (2006), they did not include samples from the Western Mediterranean (their westernmost sample is from the west coast of Italy where they collected 12 individuals). They show the greatest genetic differences to be between the European Atlantic coast and eastern Mediterranean and Black Sea ($F_{st}$ 0.50 and 0.57, respectively). Both values are in the same range as those reported by Papadopoulos et al. (2005) and Unal et al. (2006), but much higher than those found in our present study. The smaller genetic differentiation demonstrated by our analyses between the Balearic Sea and the Atlantic when compared to that between other regions of the Mediterranean and the Atlantic, and the high proportion of individuals in the Balearic Sea with the same dominant haplotype found in the Northeast Atlantic is consistent with the Atlantic origin of much of the deep Mediterranean fauna (Pérès, 1985) and with present oceanographic connectivity through the strait of Gibraltar which facilitates dispersal between the two basins.

Though the general pattern of relative genetic differentiation of \textit{C. helgolandicus} is largely similar between our present study and that of previous research (Papadopoulos et al., 2005; Unal et al., 2006; Yebra et al., 2011); our study shows a notable exception of almost no differentiation between the population of \textit{C. helgolandicus} in the Balearic Sea and that of \textit{C. euxinus} in the Black Sea. In their study, Papadopoulos et al. (2005) and Unal et al. (2006) found that the smallest population divergence is between the Adriatic and Black Sea ($F_{st}$ 0.2 and 0.32, respectively). Yebra et al. (2011) reports a similar level of differentiation between Black Sea and Eastern Mediterranean ($F_{st}$ 0.312). Our analyses show smaller differences between the Adriatic Sea and The Black Sea ($F_{st}$ 0.13) and almost no difference between the Balearic Sea and the Black Sea ($F_{st}$ 0.06). Our findings of a close genetic connection between the Balearic Sea and Black Sea suggests that there are populations of \textit{C. helgolandicus} within the Mediterranean that are virtually identical in genetic makeup to that of \textit{C. euxinus} in the Black Sea. This unexpected genetic similarity and the general pattern of lower genetic differentiation than that observed in previous studies can be explained by the ontogenetic distribution of \textit{C. helgolandicus} and the circulation of water masses across the Mediterranean.

Our research shows that in the Balearic Sea, CV copepodites of \textit{Calanus helgolandicus} are found in high abundance (3,921 copepods/m$^3$) near the seafloor at depths of 2,000–2,170 m. They are the dominant taxa, and represent 92% of the copepod abundance at these depths. These abundances are similar to those reported from previous research across the Mediterranean (Weikert et al., 2001; Bonnet et al., 2005). The distribution of \textit{C. helgolandicus}, including vertical profiles in the Mediterranean, is reviewed by Bonnet et al. (2005). In the Levantine Sea (Eastern Mediterranean) \textit{C. helgolandicus} inhabits the whole water column, from surface to $>4,000$ m (Weikert et al., 2001) with a deep peak of higher abundance at depths of 2,000 m to 3,000 m (Weikert et al., 2001; Bon-
In the Eastern Mediterranean, *C. helgolandicus* is also present close to the sea bottom, in the Cretan Sea, Sporades Basin or Caso Strait (Bonnet et al., 2005) at bottom depths between 1,250–2,260 m. This large abundance of *Calanus helgolandicus* in deep-sea waters of the Mediterranean also makes them the dominant zooplankton at those depths. Weikert et al. (2001) show that *C. helgolandicus* comprises between 40–60% of all zooplankton by numbers. Furthermore, their samples show that most of the *C. helgolandicus* at these depths are CV copepodids (other copepodid stages are absent), reaching abundances of 9,000 individuals per 1,000 m$^3$ while adults (females) have abundances of less than 80 individuals per 1,000 m$^3$. Our samples are in general agreement with these abundances.

The high abundance of CV copepodites of *C. helgolandicus* near the seafloor is a result of their ontogenetic vertical migration (Williams and Conway, 1984). In the North Atlantic, CV stages of *C. helgolandicus* overwinter at depth, near the seafloor, feeding on detritus (Williams and Conway, 1984). By early spring, these copepodites mature and reproduce, and females migrate to the surface where they release their eggs (Williams and Conway, 1984). Early copepodite stages peak in early spring, after the spring bloom, while CV and adults reach a peak between late spring and late summer (Williams and Conway, 1984). A similar ontogenetic pattern has been observed in the northwestern Mediterranean, Adriatic Sea, and Levantine Sea (Hure and Scotto di Carlo, 1968; Gaudy, 1971; Boucher et al., 1987; Weikert et al., 2001; Bonnet et al., 2005). Though details on the length of diapause in *C. helgolandicus* is not known, it is well studied in other species of *Calanus* and can last between 2–9 months (e.g., Johnson and Checkley, 2004; Saumweber and Durbin, 2006). If *C. helgolandicus* is able to diapause for similar lengths of time, then we can assume that it can survive long distance transport by deep-ocean currents.

Weikert et al. (2001) suggest that the distribution of *C. helgolandicus* in the Levantine sea could be explained by the transport of deep-water diapausing CV copepodites from the Aegean Sea into the Levantine Sea by the Eastern Mediterranean Transient (EMT), an outflow of saline deep water (Klein et al., 1999). Tracing the path of this outflow provides a mechanism for transportation of deep-water diapausing *C. helgolandicus* beyond the Levantine Sea and into the western regions of the Mediterranean. When the EMT flows into the Levantine Sea, it becomes part of the Eastern Mediterranean Deep Water which spreads west to the Ionian Sea (Bergamasco and Malanotte-Rizzoli, 2010). Tracking of water masses show that water generated by the EMT can reach the westernmost regions of the Mediterranean, including the Balearic basin (Schröder et al., 2006). Within the Balearic basin, the EMT contributes to the Western Mediterranean Deep Water (Schröder et al., 2006) which is formed in the winter when the water column is homogenized and surface waters can arrive to depths exceeding 2,500 m (Lacombe, 1972; Chiggiato et al., 2016). This deep-sea circulation provides a clear path of dispersal for deep-sea diapausing *C. helgolandicus* across the entire Mediterranean. The Aegean Sea can also mediate dispersal of *C. helgolandicus* between the Mediterranean and the Black Sea through the Bosphorus-Dardanelles Straits. Through these straits, the Black Sea receives a subsurface flux of saltier water from the Mediterranean under surficial water (less saline) outflow (Falina et al., 2017). This water exchange between the Aegean Sea and the Black Sea provides a clear path for dispersal of *C. helgolandicus* between the two basins, and combined with the deep-water circulation across the Mediterranean, it can explain the low genetic differentiation observed between distant regions.

While the potential dispersal of diapausing *C. helgolandicus* copepodites by deep ocean currents helps explain the lower genetic differenti-
ation between distant sites such as the Balearic Sea and the Black Sea; it is at odds with the observation of greater genetic differences between neighboring populations as observed by previous research (Papadopoulos et al., 2005; Unal et al., 2006; Yebra et al., 2011). A plausible explanation for this discrepancy is that there are two types of *C. helgolandicus*, separated from each other by their vertical distribution and proximity to the coast. Previous genetic research has been based solely on specimens of *C. helgolandicus/euxinus* collected in the epipelagic zone along coastal-shallow waters from the Northeast Atlantic, Adriatic Sea and Black Sea (Papadopoulos et al., 2005; Unal et al., 2006; Yebra et al., 2011). These neritic specimens of *C. helgolandicus* have clear genetic structuring corresponding to natural barriers which include the Strait of Gibraltar (between the Atlantic and Mediterranean) and the straits of Dardanelles, Sea of Marmara, and the Bosporus strait (between the Black Sea and the Mediterranean) discussed at length by Papadopoulos et al. (2005), Unal et al. (2006), and Yebra et al. (2011). It may be that *C. helgolandicus* in coastal areas represent marginal populations that are secluded due to local retention effects, while those found in deeper waters represent an oceanic population capable of dispersal throughout the Atlantic Ocean, the Mediterranean Sea, and the Black Sea by deep ocean circulation. There are several studies that show evidence of two coexisting populations of *C. helgolandicus* that are separated by their vertical distribution in the Northeast Atlantic (Nicholls, 1933; Marshall et al., 1934; Ostvedt, 1955; Krause, 1978; Hirche, 1983). These studies show that only the deep population is diapausing with large oil sacs and empty guts, while individuals of *C. helgolandicus* in the near-surface population remain active (Bonnet et al., 2005). Bonnet et al. (2005) review the ecology of *C. helgolandicus* in European waters and conclude that while there is ample evidence that *C. helgolandicus* populations living over deep oceanic waters can diapause at great depths, such evidence is lacking from near-shore populations. Therefore results from past research and our present observations can be reconciled by the presence of two populations of *C. helgolandicus*. A near-shore population with high genetic structure that does not migrate to deep-waters, subject to local retention effects and separated from each other by clear oceanic barriers (Papadopoulos et al., 2005; Unal et al., 2006; Yebra et al., 2011); and an oceanic population with lower genetic differentiation across distant regions that diapauses in deep waters and undergoes long distance transport through deep ocean circulation. To test this hypothesis and further elucidate the genetic structure of *C. helgolandicus* more targeted sampling at other deep sites across the Atlantic and Mediterranean is needed along with concurrent sampling of neighboring coastal waters including those within the Black Sea. The use of additional markers, including nuclear regions would also be beneficial in elucidating the genetic structure of this copepod.

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**Biosketches:** Diego Figueroa’s research focuses on biodiversity, community structure, and phylogenetics of copepods and deep-sea corals. Joan Cartes conducts research in trophic dynamics and ecology of zooplankton and su-
prabenthos. Nicole Figueroa specializes in molecular methods and analyses for population structure and phylogeography. Author contributions: DFF and JEC designed the study. JEC conducted fieldwork. NJF generated molecular data, conducted analyses, and prepared figures. DFF and JEC led the writing and interpretation of the data.

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**Addresses**

(DFF)(NJF) School of Earth, Environmental, and Marine Sciences, University of Texas Rio Grande Valley, Brownsville, Texas, 78520, USA. (DFF) One West University Boulevard, School of Earth, Environmental, and Marine Sciences, University of Texas Rio Grande Valley, Brownsville, Texas, 78520, USA. (JEC) Institut de Ciències del Mar, ICM-CSIC, Barcelona, Spain

**E-mail address of corresponding author**

(DFF) diego.figueroa@utrgv.edu.