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Role of oxidative stress in seasonal and daily vertical migration of three krill species in the Gulf of California

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Abstract

Vertical distribution and abundance of three numerically dominant krill species (*Nyctiphanes simplex*, *Nematoscelis difficilis*, and *Euphausia eximia*) were surveyed in the Gulf of California to understand the role of oxidative stress in their daily vertical migration (DVM) and zoogeographic patterns. Superoxide radical production, lipid peroxidation, and antioxidant enzyme activities were analyzed from krill collected with stratified nets from the surface down to 200 m during January, July, and October 2007. The upper boundary of the oxygen minimum zone (OMZ) was significantly shallower during October than during January. *N. simplex* was always distributed above the hypoxic layers, mostly in coastal upwelling areas. *Ne. difficilis* and *E. eximia* were relatively abundant during January, but detected mostly during their ascending migration. *N. simplex* was the most sensitive species to high temperatures and low oxygen concentrations, showing evidence of oxidative stress during summer (100 times more lipid peroxidation and 30 times more antioxidant enzyme activities than in winter). *Ne. difficilis* had higher glutathione peroxidase activity than *N. simplex*, which could facilitate its larger DVM. Low abundance of *Ne. difficilis* at 100 m during summer suggests that high temperature was also an environmental limiting factor. Oxidative stress indicators could explain the absence of *N. simplex* and *Ne. difficilis* in the eastern tropical Pacific and the ability of *E. eximia* to live in the OMZ and the eastern tropical Pacific. The latter had higher superoxide radical production and smaller lipid peroxidation during October. This suggests that *E. eximia* antioxidant enzyme activities are enough to avoid oxidative damage when exposed to hypoxic conditions during DVM.

The intermediate-depth low-oxygen zone in the eastern tropical Atlantic and equatorial Pacific has apparently expanded to higher latitudes in the last 50 yr (Stramma et al. 2008). In the eastern tropical Pacific, the oxygen minimum zone (OMZ), where oxygen concentration $<$ 1 mL O_2 L^{-1} represents its upper boundary, can be as shallow as 60 m from the surface at the mouth of the Gulf of California (Fiedler and Talley 2006). Juvenile and adult krill migrate daily through vertical gradients of temperature, density, dissolved oxygen, and food concentrations. During this daily vertical migration (DVM), krill and many other zooplankton species experience fast and dynamic physiological adjustments, particularly when they are exposed to low dissolved oxygen concentrations in deeper layers. Studies of in situ tolerance of tropical and subtropical krill for hypoxic conditions are particularly relevant because numerous zooplanktonic organisms cannot survive under prolonged exposure to hypoxic conditions and tend to adapt to small seasonal variability throughout the year (Spicer et al. 1999; Fernández-Álamo and Färber-Lorda 2006).

We studied physiological changes in three numerically dominant krill species (*Nyctiphanes simplex*, *Nematoscelis difficilis*, and *Euphausia eximia*) in the central and northern part of the Gulf of California collected at distinct depth layers (0–200-m depth). Then we measured and compared seven oxidative stress indicators to evaluate their hypoxic tolerance. These three krill species have distinct DVM ranges and zoogeographic affinities (Brinton 1962, 1979), allowing us to compare their physiological responses during their DVM in the central and northern part of the Gulf of California. *N. simplex* is a small ($<$ 19 mm total length), subtropical, neritic species with a DVM that extends from the surface to about 200-m depth (Brinton 1979; Lavaniegos 1996). *Ne. difficilis* is a medium-size ($<$ 25 mm total length), transitional North Pacific krill species that presumably inhabits the Gulf of California as a relict species (Brinton and Townsend 1980). The adults concentrate migrating mostly between 100 and 200 m with a maximum DVM of about 400-m depth (Brinton 1962). *E. eximia* is a large ($<$ 30 mm total length), endemic, eastern tropical Pacific krill species with a DVM that extends from the surface to depths greater than 400 m (Brinton 1979; Lavaniegos 1996). *E. eximia* has a considerably large gills : cephalothorax surface ratio among the species of the genus *Euphausia*, interpreted as a morphological adaptation to living under the hypoxic conditions (Antezana 2002) prevailing in relatively shallow water depths ($<$ 60 m) in the eastern tropical Pacific (Fiedler and Talley 2006).

As far as we know, the antioxidant responses of zooplankton and/or krill associated with their DVM or their potential exposure to hypoxic conditions have not been previously studied. However, the anaerobic metabolism has been studied in a few krill species under natural

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and laboratory conditions. Spicer et al. (1999) reported that lactate concentration of the northern krill *Meganyctiphanes norvegica*, a North Atlantic temperate species, significantly increased when the environmental oxygen concentration was hypoxic; but *M. norvegica* did not survive if individuals were exposed for prolonged periods to hypoxic conditions ($>$ 12 h). This explains why *M. norvegica* is not typically distributed in zones with low oxygen concentrations. A study of the euphausiid *Euphausia mucronata*, which regularly migrates into the OMZ of the upwelling region off Peru and Chile, and the copepod *Calanus chilensis*, which is always distributed above the OMZ, showed that *E. mucronata* had significantly higher lactate dehydrogenase (LDH) activity than *C. chilensis* (González and Quiñones 2002). LDH is an indicator of anaerobic capacity; thus the authors interpreted that higher LDH in *E. mucronata* is a physiological adaptation to crossing and inhabiting the shallow OMZ of the Humboldt Current system. However, because this last study sampled zooplankton with a bongo net (oblique integrated tows), it was unknown at which specific depth and under which oxygen concentration conditions both crustacean species were collected. A study of vertical distribution with stratified zooplankton nets recently confirmed that *C. chilensis* did not enter the OMZ, being distributed mostly between the surface and 60-m depth, and that *E. mucronata* performed extensive DVM between the surface and the core of the OMZ (200 m), even crossing it (Escribano et al. 2009).

In the central and northern part of the Gulf of California, we collected krill with opening–closing stratified nets at specific depth layers to obtain measurements of their antioxidant activities associated with the in situ environmental conditions that prevailed at each depth on day–night and seasonal time scales. In aerobic organisms, the oxidative stress occurs when reactive oxygen species (ROS) cause damage that cannot be balanced by the organism's antioxidant defense system. ROS are molecules derived from oxygen, such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). ROS are mostly formed in mitochondria during O_2 reduction in the electron transport chain. Each cell of an organism generates about 0.1% ROS from the consumed oxygen molecules (Fridovich 2004). ROS can damage bio-macromolecules, leading to functional alterations in cells and tissues. Many molecules, like glutathione, vitamins C and E, heat shock proteins, transferrins, haptoglobin, and antioxidant enzymes, can eliminate or change the molecular configuration of ROS. The main antioxidant enzymes are superoxide dismutase (SOD), which transforms $O_2^{\bullet -}$ to H_2O_2; catalase (CAT) and glutathione peroxidase (GPx), which remove H_2O_2 and inhibit its accumulation in cells and tissues; glutathione-S-transferase (GST), which, in association with reduced glutathione (GSH), transforms xenobiotics into other conjugates as part of a detoxification route; and glutathione reductase (GR), which furnishes cells with GSH.

Although ROS formation under elevated temperature is well documented, it is still controversial under hypoxic conditions and not enough studied in crustaceans. High oxygen tensions lead to elevated ROS formation, but as a result from changes in mitochondrial redox state at low oxygen supply, ROS could also increase (Schumacker 2003). Based on these metabolic responses previously observed in aerobic organisms that are frequently exposed to hypoxia and reoxygenation processes (Hochachka and Lutz 2001; Hermes-Lima and Zenteno-Savín 2002), we proposed the hypothesis that krill may have two physiological responses when they are exposed to the OMZ conditions during the descending migration in their DVM: ROS generation could increase just before their exposure to hypoxic conditions during their daytime descent to stimulate factors implied in the mitochondrial biogenesis, and krill could generate antioxidant defenses when exposed to hypoxic conditions to avoid oxidative stress potentially caused by the reoxygenation during their night upward migration. Krill species with distinct DVM patterns in regions with relatively shallow OMZ should display different antioxidant defenses that contribute to shaping their long-term zoogeographic patterns. In theory, the shorter migrant *N. simplex* should have less antioxidant enzyme activity than the two other species, because *E. eximia* and *Ne. difficilis* frequently pass across the upper limit of the OMZ during their DVM. Also, as the Gulf of California exhibits strong seasonal changes in environmental conditions (Hidalgo-González and Álvarez-Borrego 2004), we expected that vertical distribution and oxidative stress indicators of each krill species would change seasonally, being strongly influenced by the presence and depth of the thermocline and by the exposition of each species to hypoxic conditions prevailing in the OMZ. Because *N. simplex* tends to form dense swarms that concentrate high biomass available to zooplanktivorous predators, this species constitutes a significant component in the pelagic food web of the northwest coast of Mexico (Gendron 1992; Sampson et al. 2010). A change in the krill daily vertical distribution range caused by an OMZ extension to higher latitudes (Stramma et al. 2008) could have significant consequences on the zooplankton and micronekton community structure and biomass production in the Gulf of California. Physiological responses of krill (or any other pelagic organisms) to oxygen concentrations should be studied in tropical and transitional regions.

Methods

Krill were collected during three oceanographic cruises carried out in 2007 in winter (12–31 January) and summer (17 July–03 August) in the northern and central parts of the Gulf of California (Fig. 1A,B), and in late summer (14–23 October) in the southern part of the gulf (near Bahía de La Paz; Fig. 1C). Each cruise represents a distinct season, according with criteria defined in previous studies (Hidalgo-González and Álvarez-Borrego 2004). The January cruise represents the winter season, with the well-mixed water column and cold conditions that typically prevail between December and May. The July and October cruises represent summer conditions, when a seasonal strong stratification of the water column occurs beside a shallower OMZ.

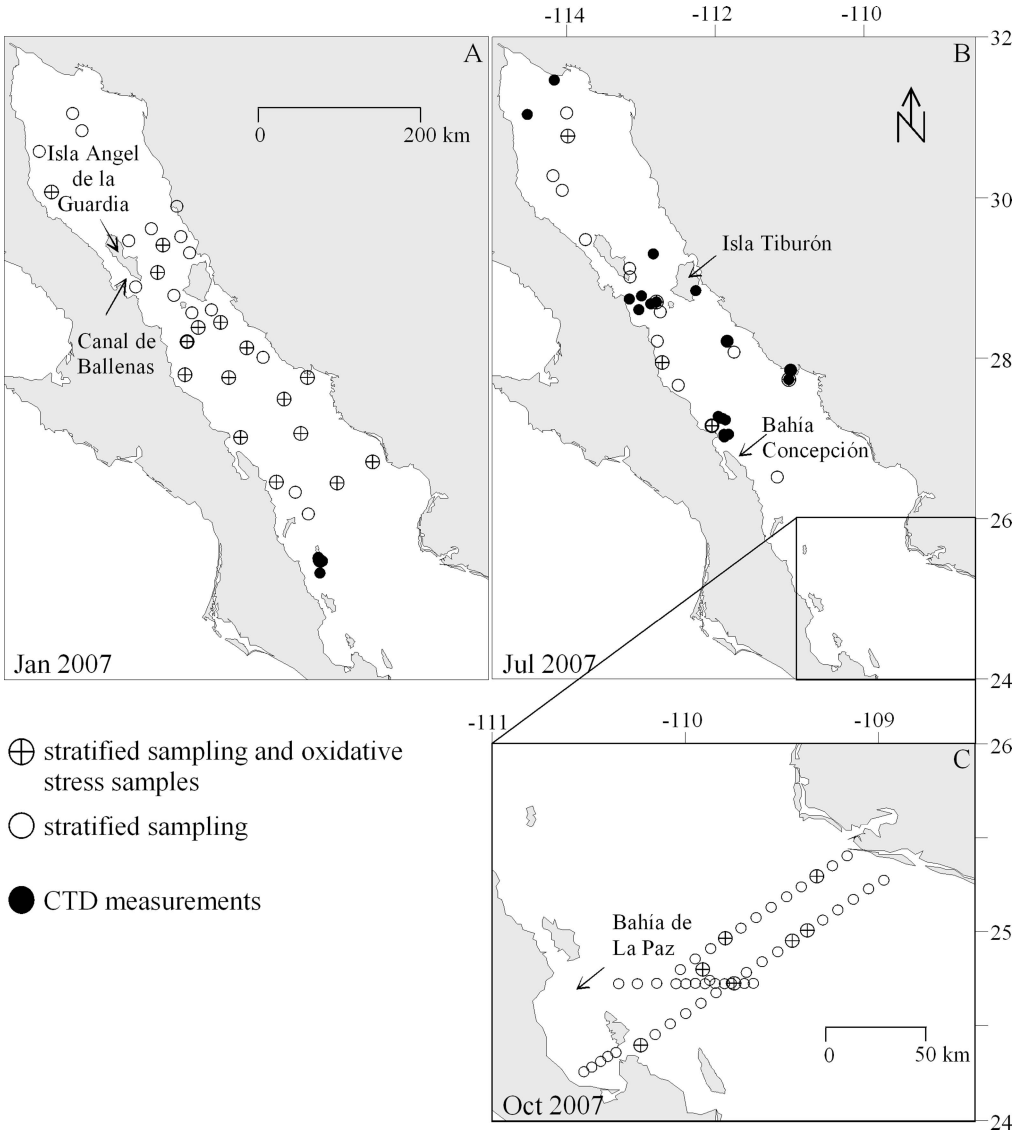

Fig. 1. Location of the zooplankton and CTD oceanographic sampling stations for sampling carried out during (A) January, (B) July, and (C) October 2007 along the northern and central part of the Gulf of California, Mexico.

Environmental data collection—Vertical profiles of temperature and salinity were recorded with a Seabird SB09 conductivity, temperature, depth (CTD) system in the three oceanographic cruises. Dissolved oxygen concentration was recorded at discrete depths (0, 5, 10, 25, 50, 75, 100, 150, and 200 m) with a Yellow Spring Instrument multi-sensor (YSI-1556) in January 2007 and throughout the column water with a Sealab oximeter attached to the CTD in July and October 2007. Each profile was plotted to detect the depth of the thermocline, where the temperature gradient in a 10-m layer was $> 1^{\circ}$C less than the layer above it (Hidalgo-González and Álvarez-Borrego 2004), and the upper boundary of the OMZ was identified at the depth where dissolved oxygen concentration was < 1 mL O_2 L^{-1} (Fiedler and Talley 2006). However, because previous studies considered hypoxic conditions to be when oxygen concentrations drop between 1.4 and 2.75 mL O_2 L^{-1} (Gray et al. 2002), we decided to establish our hypoxic criteria at concentration < 1.5 mL O_2 L^{-1}.

Collection of krill—Zooplankton samples were collected at specific water depths, typically 0–50-, 50–100-, 100–150-, and 150–200-m depth, using four opening–closing conical nets manually operated with metallic messengers (0.5-m mouth diameter, 300-μm mesh). After the vertically stratified nets were obliquely towed and recovered, an additional identical conical net was towed near the surface (1–2-m depth) for 10 min at a speed of 6 km h^{-1} to collect neustonic krill swarms in January and July. All the nets were equipped with digital flowmeters to estimate their filtering volume. Immediately after collection of each zooplankton sample, live krill were sorted out with a plastic spoon and observed on board with a Carl Zeiss SV11 stereoscope to identify the species, sex, ontogenetic

phase (juvenile or adult), and female gonad development stage (Gómez-Gutiérrez et al. 2010). Each healthy krill (transparent and exhibiting energetic swimming) was immediately rinsed with distilled water to eliminate seawater salts and quickly frozen in liquid nitrogen within < 30 s. Samples were stored at -80°C until they were analyzed, which typically was done within 1 month after each oceanographic cruise.

Oxidative stress laboratory assays required a homogenous solution of at least 100 mg of krill biomass per sample (drained wet weight). We carefully added several specimens of the same sex and stage and similar total length from the same sampling depth into single vials to obtain the biomass threshold for each biochemical assay. Dead or moribund krill individuals (with evident physical damage or not transparent) were fixed in formaldehyde (4%) neutralized with saturated sodium borate with the rest of the collected zooplankton for estimating species abundance (ind. 1000 m^{-3}) at each sampling depth.

Biochemical analyses—Each 100-mg sample of krill was homogenized (Polytron PT 1300 D, Brinkmann Instruments) in two volumes of phosphate buffer solution (50 mmol L^{-1}, pH 7.5; ethylenediaminetetraacetic acid 50 mmol L^{-1}; phenylmethanesulfonyl fluoride 1 mmol L^{-1}) and centrifuged at $1500 \times g$ for 20 min at 4°C. The supernatant was analyzed in triplicate. Total activity of SOD was estimated with xanthine–xanthine oxidase as a superoxide radical generating system and nitroblue tetrazolium as a detector (Suzuki 2000). CAT activity was analyzed by measuring the decrease in H_2O_2 concentration at 240 nm (Aebi 1984). GPx activity was measured by monitoring the continuous decrease in concentration of nicotinamide adenine dinucleotide phosphate (NADPH) using H_2O_2 as a substrate (Ahmad and Pardini 1988). GR activity was measured by the decrease in absorbance during oxidation of NADPH (Goldberg and Spooner 1987). GST activity was estimated by detecting the formation of the thioether product from the reaction between GSH and 1-chloro-2,4-dinitrobenzene (Habig and Jakoby 1981). Soluble protein content in homogenized samples was measured using the Bio-Rad© kit (500-0006) with bovine serum albumin as standard (Bradford 1976). All enzyme activities were expressed in activity units (U) mg $proteins^{-1}$. The endogenous superoxide radical ($O_2^{\bullet -}$) production was measured following the reduction of cytochrome *c* by the $O_2^{\bullet -}$ during 15 min (Markert et al. 1984; Drossos et al. 1995) and was expressed in nmol L^{-1} $O_2^{\bullet -}$ min^{-1} mg $proteins^{-1}$. Thiobarbituric acid–reactive substances (TBARS) were measured to estimate endogenous lipid peroxidation in the supernatant (Persky et al. 2000) and expressed as nmol TBARS mg $proteins^{-1}$.

The statistical tests Kruskal-Wallis (*KS*), Mann-Whitney (*U*), and Dunn post hoc were performed to test for significant differences of oxidative stress indicators among the three krill species, life stages (sex and reproductive stage), depth of sampling, and seasons (cruises). To identify the main sources of krill interspecific and intraspecific variability of oxidative stress indicators, a nonmetric multidimensional scaling (NMDS) analysis was done using Bray-Curtis distance (McCune et al. 2002) and a configuration in three axes calculated with PC-ORD (version 4.41) software for multivariate analysis of ecological data. The analysis included at each sampling station the sampling depth and environmental conditions (temperature, salinity, dissolved oxygen concentration, thermocline depth, and OMZ depth). All the oxidative stress indicators from all the individuals of each species, sampling station, and depth were averaged to get only one value per krill species, per sampling station, and per sampling depth.

Once the environmental variables that have a major influence on the variability of the oxidative stress indicators of each species were identified with the NMDS analysis, we tested several null hypotheses (H_0), using a multi-response permutation procedure (MRPP; McCune et al. 2002). For example, we statistically tested the null hypothesis that no significant difference exists in the oxidative stress indicators among the three krill species (interspecific variability) and/or among the three oceanographic cruises for each krill species (seasonal intraspecific variability). The MRPP analysis was calculated using the Bray-Curtis distance, the weighting option [*n*/sum (*n*)], and a rank transformation of the matrix distances of the environmental variables. The NMDS and MRPP are two nonparametric statistical techniques that do not require the assumption that the oxidative stress indicators measured have normal distribution or variance homogeneity (McCune et al. 2002). When a null hypothesis H_0 was statistically rejected by the MRPP analysis, we used the indicator species analysis (ISA; McCune et al. 2002) to identify which oxidative stress indicator had the greater influence within a group of seasons and sampling depth strata. The highest ISA value of each oxidative stress indicator was contrasted with the Monte Carlo technique to test its significance (Dufrene and Legendre 1997). The ISA values vary from zero (where no indication exists) to 100 (where a perfect indication exists) expressed as a percentage. The ISA values estimate the degree to which each oxidative stress indicator is detected in a specific group of oceanographic sampling stations and/or sampling depths associated with particular environmental characteristics. These multivariate statistical methods (NMDS, MRPP, and ISA) have been previously used in ecological studies of several krill species (Gómez-Gutiérrez et al. 2005).

Results

Seasonal vertical distributions of the three species—Stratified samples were collected at 21 oceanographic stations in January, 20 stations in July, and 12 stations in October 2007 (Fig. 1A–C). We show results of vertical distribution and oxidative stress indicators of only adult krill because we collected a relatively small number of juveniles. Figure 2 shows the vertical profiles of temperature, salinity, dissolved oxygen concentration, and integrated abundance of the three krill species as a function of the local sampling time during January, July, and October 2007. In January, the water column was cold ($< 17^{\circ}$C) and mixed. Salinity varied from 35.3 to 35.7 in the first 150 m of the water column and was more stable around 35 in the

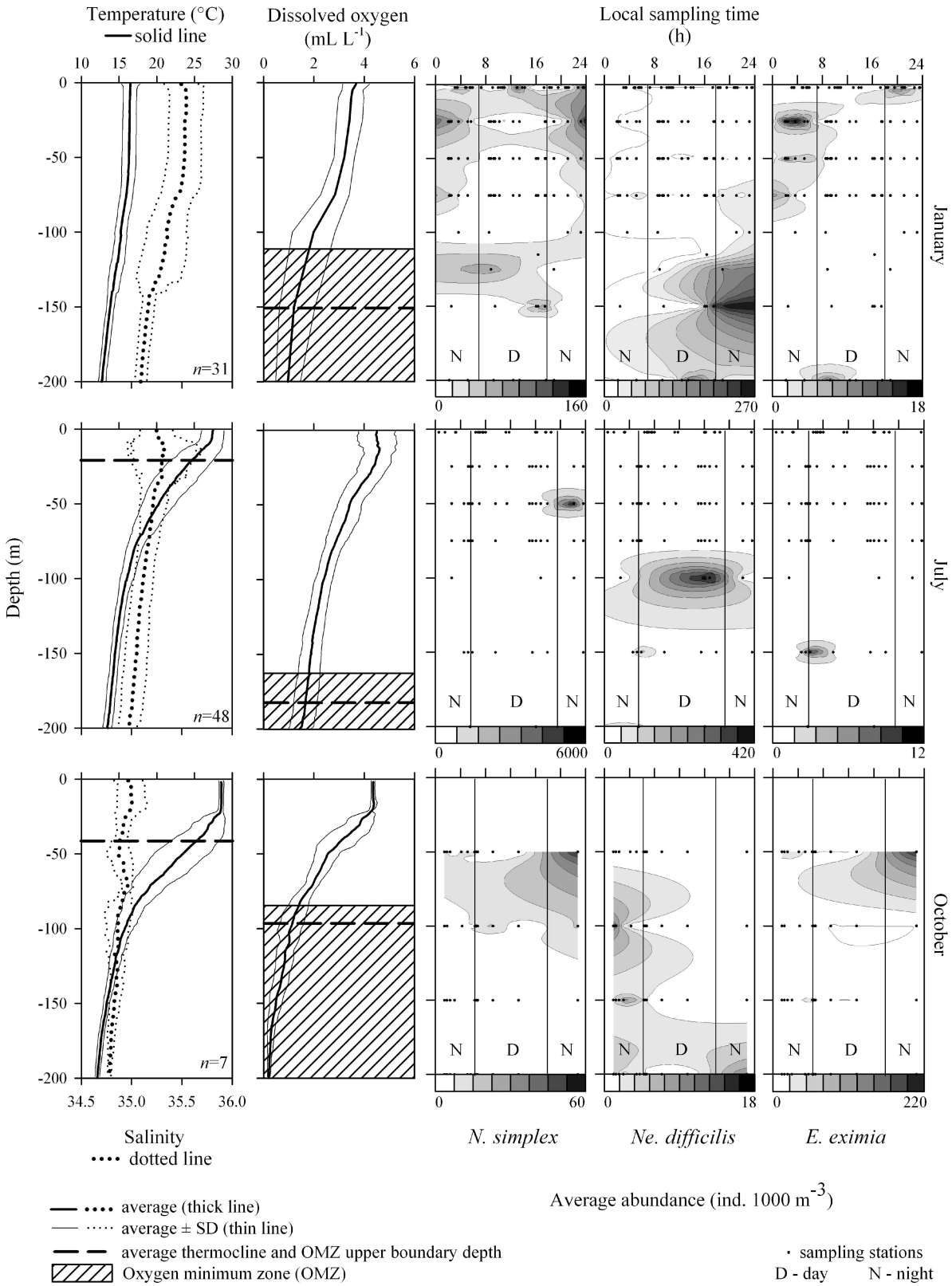

Fig. 2. Vertical profiles of temperature (°C), salinity, dissolved oxygen concentration (mL O_2 L^{-1}), and average abundance (ind. 1000 m^{-3}) of *N. simplex*, *Ne. difficilis*, and *E. eximia* as a function of local sampling time during January, July, and October 2007 in the Gulf of California, Mexico.

deeper strata (150–200 m; Fig. 2). The upper boundary of the OMZ depth was detected between 110 and 200 m with an average depth of 150 m. *N. simplex* and *E. eximia* had a typical DVM pattern, being more abundant near the surface at night, whereas *Ne. difficilis* was concentrated between 100 m at night and 200 m during the day without showing any evident day–night vertical migration pattern (Fig. 2). *N. simplex* formed diurnal surface swarms during January and was not distributed below the average OMZ depth in any of the oceanographic stations (Fig. 2). In July, the average sea surface temperature was 27°C and the mean depth of the seasonal thermocline was 24 m (Fig. 2). The greater variability of the salinity was situated around the thermocline, with values that oscillated between 34.8 and 35.7 (Fig. 2). The upper boundary of the OMZ depth was detected between 160 and 200 m, with an average depth of 183 m (Fig. 2), as almost all the sampling stations were concentrated along the peninsular coast of the gulf where upwelling events were detected (Fig. 1B). In July, the three species did not seem to migrate vertically, having the core of their abundance at specific depth layers. *N. simplex* was mostly concentrated in large densities and distributed below the seasonal thermocline and above the OMZ, mainly during the first hours of the night, having no clear DVM pattern as detected in January (Fig. 2). *Ne. difficilis* concentrated between 75- and 125-m depth, and *E. eximia* was detected in low densities at 150-m depth (Fig. 2). *N. simplex* and *Ne. difficilis* were more abundant in January and July than in October. In October, the average depth of the seasonal thermocline was 40 m, twice as deep as in July (Fig. 2). The average salinity (range 34.6–35.2) recorded for this oceanographic cruise was lower than in January and July (Fig. 2). The upper boundary of the OMZ was located between 65 and 200 m with an average depth of 90 m, being significantly shallower that the OMZ detected in January ($U = 283.5$, $p = 0.006$; Fig. 2). Because the October cruise covered only the southern part of the Gulf of California (near Bahía de La Paz; Fig. 1C), *E. eximia* was the most abundant of the three krill species studied. *E. eximia* and *N. simplex* were collected just after sunset between 25- and 50-m depth, likely during their ascending vertical migration (Fig. 2). During the day, *N. simplex* was always $<$ 75-m depth. *Ne. difficilis* was scarce in the October cruise, being collected only in the first 100 m just before dawn (Fig. 2) presumably during its descending DVM.

Dissolved oxygen concentration maps at each sampling depth (sea surface and 50, 100, and 200 m) for the January and July oceanographic cruises showed that the lower dissolved oxygen concentrations at sea surface and 50-m depth were detected around Isla Angel de la Guardia and Isla Tiburón, possibly associated with wind-induced upwelling events (Fig. 3A–D). The upper boundary of the hypoxic conditions was detected at 100-m depth, being geographically more extended in January than in July (Fig. 3E,F). In both seasons almost all the Gulf of California had hypoxic conditions ($<$ 1.5 mL O_2 L^{-1}) at 200 m (Fig. 3G,H).

Oxidative stress indicators among species and seasons—Samples for oxidative stress indicator analysis were collected at 18 oceanographic stations in January, at 6 stations in July, and at 5 stations in October 2007 (Fig. 1A–C). In total, 84 krill samples were analyzed for the winter season (*N. simplex*, $n = 18$; *Ne. difficilis*, $n = 57$; *E. eximia*, $n = 9$) and 56 samples for the summer season, combining the July and October oceanographic cruises (*N. simplex*, $n = 17$; *Ne. difficilis*, $n = 12$; *E. eximia*, $n = 27$; Table 1). The average and standard error values of the oxidative stress indicators measured for January, July, and October 2007 (Fig. 4A–G) showed that during January, superoxide radical production and TBARS levels were not significantly different among the three krill species, suggesting that no one of the krill species studied was exposed to unfavorable environmental conditions (Fig. 4A,B). *Ne. difficilis* had significantly higher GPx activity than *N. simplex* ($KS = 9.634$, $p = 0.008$; Fig. 4G).

The superoxide radical production of *N. simplex* could not be measured for the July cruise because of equipment failure during the analysis. TBARS levels ($KS = 22.192$, $p = 0.000$) and enzyme activities of SOD ($KS = 21.107$, $p = 0.000$), CAT ($KS = 22.573$, $p = 0.000$), GR ($KS = 21.170$, $p = 0.000$), and GPx ($KS = 9.995$, $p = 0.007$) were significantly higher for *N. simplex* than for the other two krill species during July (Fig. 4B–D,F,G). During July, TBARS levels of *N. simplex* were 100 times higher and all enzyme activities were about 30 times higher than in specimens collected in January (Fig. 4B–G). The superoxide radical production of *Ne. difficilis* and *E. eximia* was significantly higher during July and October than during January ($U = 0.000$ and $p = 0.000$), but TBARS levels in both krill species were significantly lower than during January ($U = 540$, $p = 0.000$, and $U = 189$, $p = 0.000$, respectively; Fig. 4A, B). GST activity was significantly higher for *Ne. difficilis* in July and October than in January ($U = 135$, $p = 0.001$; Fig. 4E). For *E. eximia*, no significant differences were found in the antioxidant enzyme activities between January and October, except for GPx activity, which was significantly higher in October ($U = 22$, $p = 0.023$; Fig. 4G).

Krill oxidative stress indicators associated with vertical stratified environmental conditions—The NMDS analysis showed that each krill species from each oceanographic cruise occupied a distinct multidimensional position in the krill oxidative stress indicators space ordered by sampling stations and sampling depth layers, suggesting significant interspecific differences in the physiological responses related to environmental conditions (Fig. 5). The associations between ordination distances and original n-dimensional space distances for the environmental variables of the first and third axes had determination coefficients (r^2) of 0.238 and 0.339 respectively, explaining about 57.7% of total variability (Table 2). To evaluate whether NMDS was extracting stronger axes than expected by chance, we did a randomization Monte Carlo test, which yielded a probability < 0.0099 that the final stress level of 10.6 could have been obtained by coincidence. In the first axis, the oxidative stress indicators of the three species were negatively associated with the depth of the OMZ (Table 2). This suggests that oxidative stress indicators tended to increase

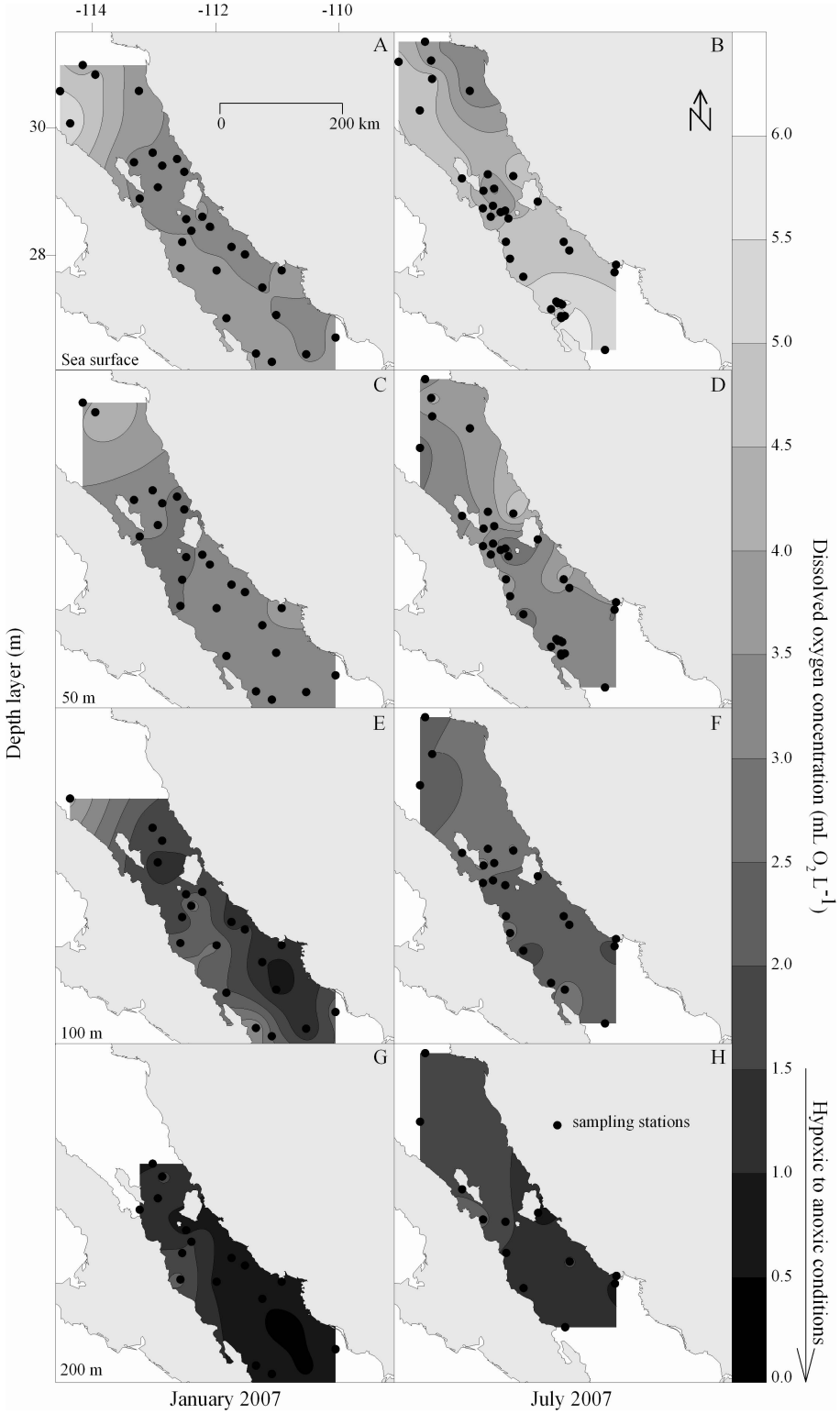

Fig. 3. Dissolved oxygen concentration (mL L^{-1}) recorded at discrete sampling depths: (A, B) sea surface, (C, D) 50 m, (E, F) 100 m, and (G, H) 200 m. Hypoxic conditions were considered at $\leq$ 1.5 mL O_2 L^{-1}.

when the upper boundary of the OMZ was shallower in the water column. The third axis was negatively related to depth of the thermocline and the salinity, and weakly associated with temperature and dissolved oxygen concentration (Table 2). Thus, the depths of the thermocline and the OMZ seem to be strongly associated with the oxidative stress indicators of the three krill species. Most of the oceanographic stations where *N. simplex* was present (clustered at the right side of the NMDS ordination) were directly associated with the OMZ depth vector, indicating that this species inhabits mostly the well-oxygenated column layers (Fig. 5). The tropical krill species *E. eximia* was clearly segregated at the left of the ordination, with an inverse association with the depth of the OMZ along axis 1 and the depth of the thermocline along axis 3 (Fig. 5). Thus, *E. eximia* oxidative stress indicators were typically high when the OMZ and the thermocline were relatively shallow. *Ne. difficilis* oxidative stress indicators had a positive association with the depth of the mixed layer, especially during January. *Ne. difficilis* seemed not to be affected by the depth of the OMZ in any of the oceanographic cruises, showing all its oxidative stress indicator positions centered close to the origin of the OMZ depth vector (Fig. 5).

The MRPP showed significant interspecific differences in the oxidative stress indicators among the three krill species ($p < 0.0001$) and in the seasonal intraspecific variability among the three oceanographic cruises ($p < 0.0001$; Table 3). The MRPP also showed significant vertical variability in the oxidative stress indicators as a function of the sampling depths ($p = 0.020$), particularly in krill collected above and below the OMZ depth ($p = 0.006$), and above and below the thermocline ($p = 0.004$; Table 3). The ISA demonstrated that all oxidative stress indicators of each krill species were significant ecophysiological indicators to characterize each krill species (Table 4). Endogenous superoxide radical production of *N. simplex* had a significant ISA value for the January cruise (winter season; Table 4). The comparison of oxidative stress indicators among sampling depth layers also showed that only the superoxide radical production of *N. simplex* had a significant ISA value for the surface layer (2 m; Table 4). All the other oxidative stress indicators of *N. simplex* had significant ISA values for the July cruise. The ISA test supports the idea that *N. simplex* was facing considerable oxidative stress during July. Also, all *N. simplex* oxidative stress indicators had significant high ISA values for environmental conditions above the OMZ layer, whereas only superoxide radical production was a significant indicator of conditions above the thermocline (Table 4). Superoxide radical production and GPx activity of *E. eximia* had significant ISA values for conditions below the OMZ and the thermocline, and all the oxidative stress indicators of *E. eximia* characterized the October cruise, except TBARS levels when the OMZ and thermocline were shallow (Table 4).

Table 1. Number of krill samples (*n*, groups of krill individuals to attain > 100 mg of wet weight) to analyze oxidative stress indicators per krill species, depth layer, and oceanographic cruise.

Krill species	Depth layer (m)	Month of the oceanographic cruise (2007)		
		Jan	Jul	Oct
N. simplex	0–50	15		
	50–100		8	
	100–150	3	9	
Ne. difficilis	0–50	5		2
	50–100	22	1	3
	100–150	9		3
	150–200	21	2	1
E. eximia	0–50	6		13
	50–100	2		7
	100–150			4
	150–200	1		3

Discussion

We demonstrated distinct inter- and intraspecific DVM behavioral response associated with physiological requirements evidenced with oxidative stress indicators as a function of the physical, chemical, and biological environmental features prevailing in the strata where each krill species was collected. We concluded that *E. eximia* and *Ne. difficilis* can penetrate the OMZ and that *N. simplex* cannot do it in the Gulf of California, likely because of their physiological responses and their antioxidant systems. The krill response to the thermocline is considerably less clear, but *N. simplex* tends to avoid water layers with high temperatures (> 23°C) and low dissolved oxygen concentrations. *N. simplex*, *Ne. difficilis*, and *E. eximia* had distinct interspecific patterns of DVM that support previous vertical distribution studies done in the Gulf of California and along the west coast of the Baja California peninsula (Brinton 1979; Lavaniegos 1996). However, the ultimate causes of the interspecific and intraspecific DVM differences were then only speculated. Brinton (1979) and Lavaniegos (1996) included only three sampling stations in May–June (transition from spring to summer season) studying the association of distribution, abundance, and environmental conditions. Thus, these studies did not explore interspecific and intraspecific DVM patterns on seasonal or regional scales or their response to environmental vertical gradients.

In January 2007, *N. simplex* had a typical DVM pattern, migrating toward deeper strata during the day and near the surface (0–50 m) during the night, even if a small portion of the population remained at deeper layers during the night, similar to the *N. simplex* population in the California Current region (Brinton 1967). The diurnal surface swarms of *N. simplex* detected in January were likely associated with reproductive events (Gendron 1992). Superoxide radical production of *N. simplex* had a significant ISA value in January for conditions above the OMZ (Table 4). Superoxide radical production of *N. simplex* at the surface was significantly lower than in the 100–150-m depth layer (Tremblay et al. in press). The antioxidant capacity of *N. simplex* near the surface decreases superoxide radical production during its diurnal surface swarms, allowing this species to minimize oxidative damage. This is sustained in our results that *N. simplex* TBARS levels were not

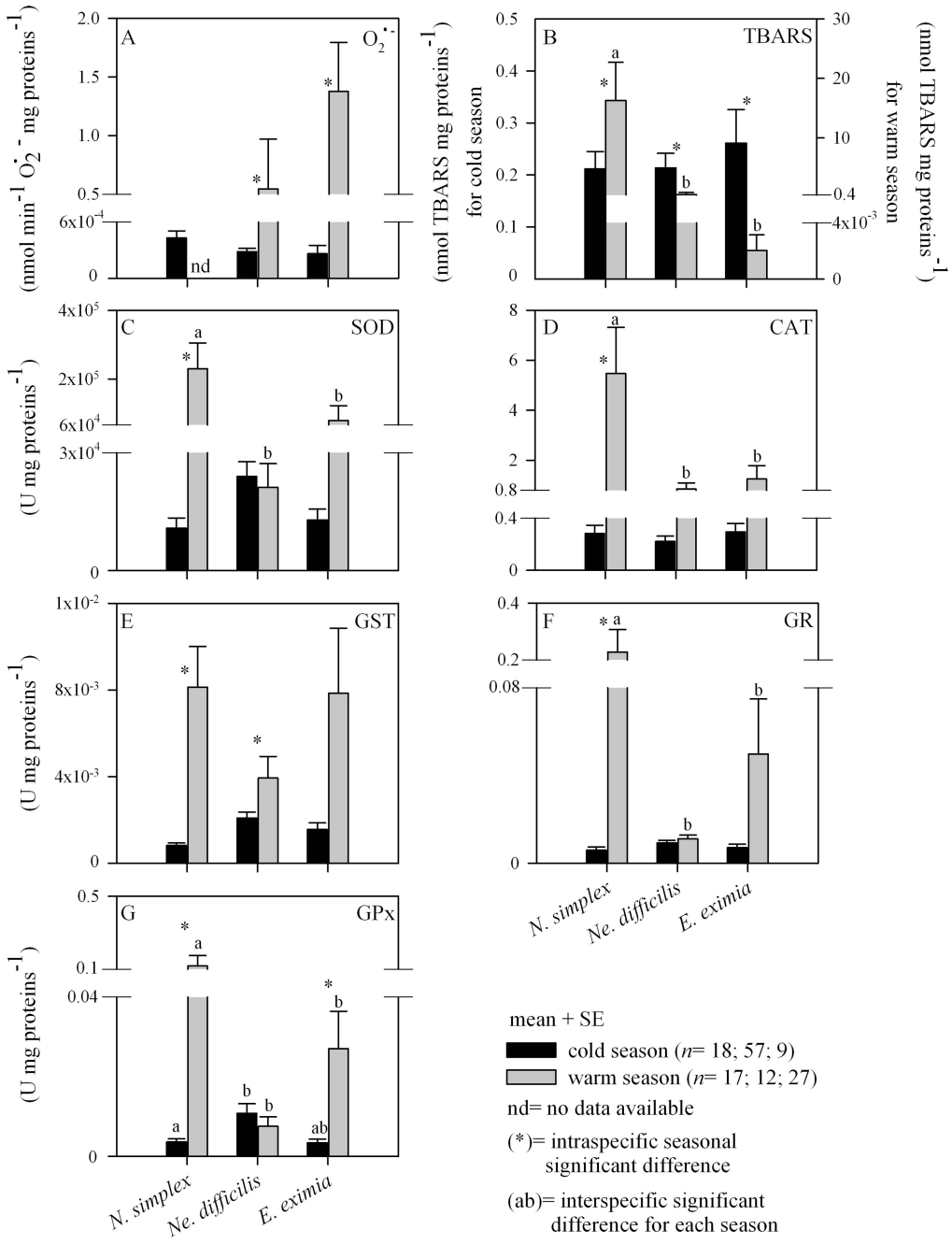

Fig. 4. Average and standard errors of oxidative stress indicators of *N. simplex*, *Ne. difficilis*, and *E. eximia* during the winter (January) and summer (July and October) season during 2007. The oxidative stress indicators are: (A) superoxide radical production (nmol $O_2^{\bullet-}$ min^{-1} mg $proteins^{-1}$), (B) lipid peroxidation (nmol TBARS mg $proteins^{-1}$), and activities of (C) total SOD, (D) CAT, (E) GST, (F) GR, and (G) GPx (U mg $proteins^{-1}$).

significantly higher than those measured for *E. eximia* and *Ne. difficilis* during January. *N. simplex* is the only krill species in the region that forms dense daytime surface swarms (Gendron 1992). This is a significant ecological adaptation because it is known that other krill species are sensitive to solar radiation, particularly to UVB rays, which cause significant deoxyribonucleic acid (DNA) damage in the Antarctic krill *Euphausia superba* (Jarman et al. 1999). Therefore, DNA oxidative damage should also be evaluated in the future to find out if diurnal surface swarms may negatively affect *N. simplex*. Surface swarms were observed only during January, probably because the temperature in summer was $> 22^{\circ}C$ in the first 50 m of depth. Those warm conditions would be unfavorable for the *N. simplex* ontogenic development of early larvae (Gómez-Gutiérrez 1996) and its brood sizes (Gómez-Gutiérrez et al. 2010).

N. simplex was highly abundant south of Isla Tiburón in January and south of Isla Angel de la Guardia in July 2007, where cold water and a relatively well-mixed water column indicated the occurrence of upwelling events (Gómez-

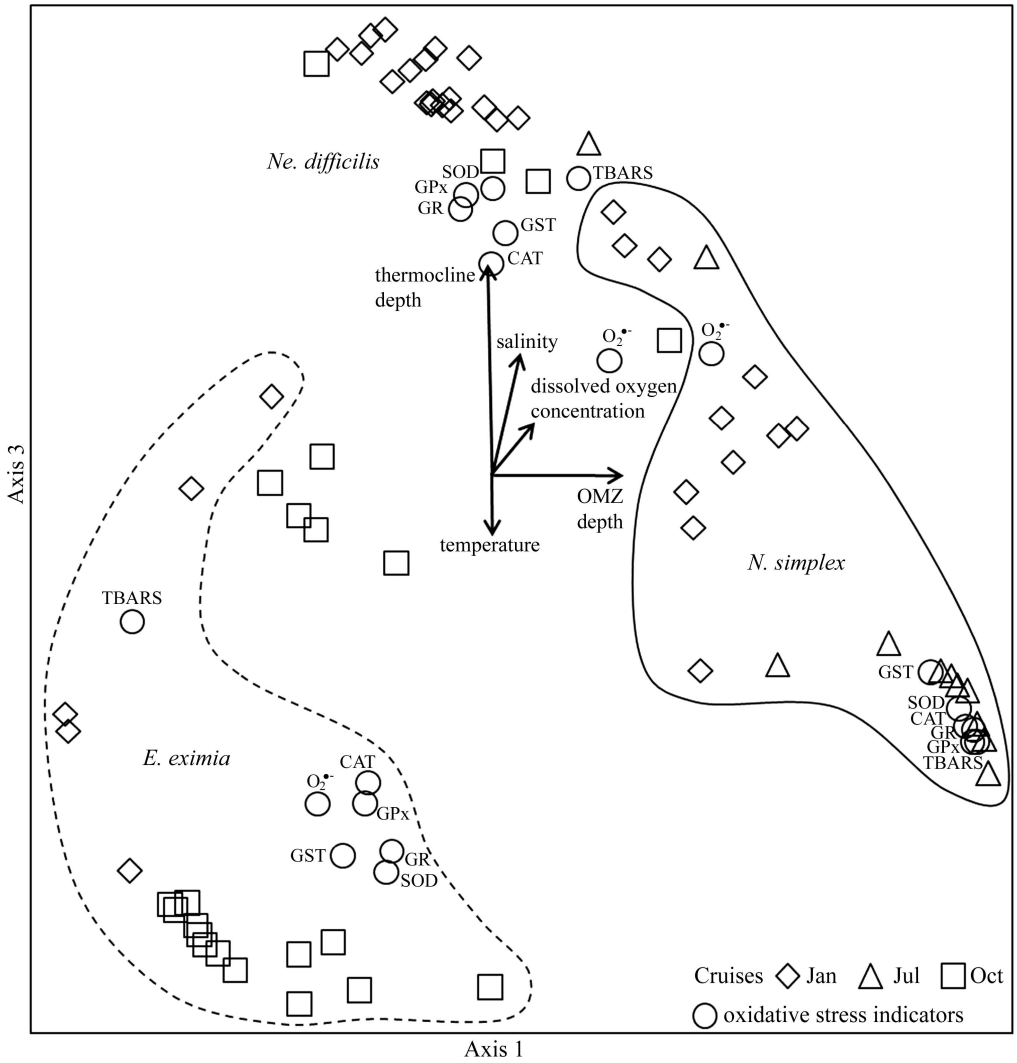

Fig. 5. Nonmetric multidimensional scaling analysis showing the oceanographic stations categorized by sampling month in the oxidative stress indicators space for *N. simplex*, *Ne. difficilis*, and *E. eximia*. The arrows indicate the direction and magnitude of the correlation vectors with the oceanographic stations and the percentage of variance in the oxidative stress indicators explained by each environmental variable.

Gutiérrez et al. 2010). Brinton and Townsend (1980) concluded that *N. simplex* is always abundant around the Grandes Islas region throughout the year because it is the coldest region of the Gulf of California. The flow of deep cold water along the Canal de Ballenas, intense tidal currents, and island effect promote colder waters near the surface (Hidalgo-González and Álvarez-Borrego 2004). The Grandes Islas area is also characterized by low oxygen

Table 2. NMDS analysis of the oxidative stress indicators of the krill species *Nyctiphanes simplex, Nematoscelis difficilis*, and *Euphausia eximia* showing the determination coefficient (r^2) and the Pearson and Kendall correlations (r) for the association between ordination distances and original n-dimensional space distances for the available environmental variables. Pearson and Kendall correlations ($r > 0.40$) are shown in bold.

Determination coefficient by axis	Axis 1 ($r^2 = 0.238$)	Axis 2 ($r^2 = 0.050$)	Axis 3 ($r^2 = 0.339$)
Temperature (°C)	−0.013	−0.066	0.337
Salinity	−0.158	0.379	**−0.490**
Dissolved oxygen concentration (mL O_2 L^{-1})	−0.251	0.322	−0.332
OMZ depth (m)	**−0.533**	0.026	−0.100
Thermocline depth (m)	0.222	0.376	**−0.577**

Table 3. MRPP analysis showing the oxidative stress indicators and environmental conditions comparison among the three krill species (*Nyctiphanes simplex, Nematoscelis difficilis*, and *Euphausia eximia*) in the Gulf of California during Jan, Jul, and Oct 2007. A max = 1 when all items are identical within groups (delta = 0), $A = 0$ when heterogeneity within groups equal expectations by chance, and $A < 0$ with more heterogeneity within groups than expected by chance.

Null hypotheses (H_0) tested for the following group of oceanographic stations as a function of each variable	Matrix size	t statistic	A	p
Comparison among species				
N. simplex (n=17) vs. *Ne. difficilis* (n=23) vs. *E. eximia* (n=16)	56×21	−37.27	0.631	0.000
N. simplex (n=17) vs. *Ne. difficilis* (n=23)	40×14	−25.77	0.457	0.000
Ne. difficilis (n=23) vs. *E. eximia* (n=16)	39×14	−25.16	0.449	0.000
N. simplex (n=17) vs. *E. eximia* (n=16)	33×14	−20.93	0.486	0.000
Comparison among oceanographic cruises				
Jan (n=34) vs. Jul (n=11) vs. Oct (n=22)	67×21	−18.18	0.261	0.000
Jan (n=34) vs. Jul (n=11)	45×21	−9.66	0.149	0.000
Jan (n=34) vs. Oct (n=22)	56×21	−13.67	0.162	0.000
Jul (n=11) vs. Oct (n=22)	33×21	−15.22	0.320	0.000
Comparison among sampling depths (m)				
2 (n=4) vs. 25 (n=9) vs. 50 (n=14) vs. 75 (n=7) vs. 100 (n=10) vs. 125 (n=2) vs. 150 (n=12) vs. 200 (n=9)	67×21	−2.30	0.067	0.021
OMZ depth (m)				
Above the OMZ (n=43) vs. below the OMZ (n=24)	67×21	−3.90	0.039	0.006
Thermocline depth (m)				
Above the thermocline (n=23) vs. below the thermocline (n=44)	67×21	−4.18	0.042	0.005

concentration near the surface throughout the year, but according to this study it never reaches hypoxic or anoxic conditions at layers above 200-m depth (Fig. 3E,F). According with Brinton (1979) and Lavaniegos (1996), who associated krill DVM with oxygen concentration $>$ 1 mL O_2 L^{-1}, the short DVM of *N. simplex* in the Gulf of California could be explained by the presence of hypoxic conditions detected from 100 m during January in almost all the area of study (Fig. 3E). Shipboard respiration rate experiments indicated that oxygen concentration $<$ 2.5 mL O_2 L^{-1} was lethal for *N. simplex*, providing strong evidence that this species cannot enter or inhabit hypoxic conditions under field conditions (Tremblay et al. in press). A similar response was detected in the northern krill *M. norvegica*, which did not survive when it was under prolonged exposure to hypoxic conditions ($>$ 12 h; Spicer et al. 1999).

In July, *N. simplex* was concentrated in the seasonal upwelling zone located between the Canal de Ballenas and Bahía Concepción, the relatively colder region detected during summer (Fig. 1B; Gómez-Gutiérrez et al. 2010). Lavaniegos (1996) proposed that the seasonal thermocline acts as an environmental barrier to the DVM ascent phase of *N. simplex* juveniles and adults and other krill species in the western region of the Gulf of California. Thus, *N. simplex* maintained its vertical distribution range below the seasonal thermocline at night compared with the DVM detected in January. We showed evidence that the high temperatures detected near the surface in July and October negatively affect the homeostasis of *N. simplex*, significantly increasing its oxidative stress indicators. In July, the *N. simplex* TBARS levels were 100 times higher and its antioxidant enzymatic activities were about 30 times greater than in January 2007. The MRPP and ISA analyses also confirmed that *N. simplex* tended to avoid oxygen concentrations within the OMZ and temperature conditions above the thermocline in the Gulf of California, particularly in July. This physiological disturbance could be associated with the increase of water temperature and a longer daylight exposure because of an increase of the seasonal photoperiod in the gulf from $\sim$ 10 h 45 min d^{-1} in January to 15 h d^{-1} in July (Navy observatory, http://www.usno.navy.mil/USNO/astronomical-applications). All these pronounced seasonal environmental changes may cause a seasonal decrease in the abundance of *N. simplex* from an average density of 6156 ind. 1000 m^{-3} in January 2007 to 315 ind. 1000 m^{-3} in July 2007 according to the additional information obtained from the bongo net (not the stratified vertical nets) samplings done during these two oceanographic cruises (Gómez-Gutiérrez et al. 2010), and also the lower growth, molting, and egg production rates measured in shipboard incubations in July compared to January 2007 (S. Martínez-Gómez pers. comm.). Similar physiological, oxidative stress, and abundance changes have been detected in other marine benthic invertebrates (Polychaeta and Decapoda) associated with in situ changes of environmental temperature and salinity (Buchner et al. 1996; Kong et al. 2008). SOD and CAT activities of the polychaete *Arenicola marina* in sand flats of the German Wadden Sea increased significantly in response to an increase of temperature during summer and high concentration of H_2O_2 in the seafloor sediments (Buchner et al. 1996). In the decapod *Scylla serrata*, the GPx activities and TBARS levels also increased significantly during summer, demonstrating that the enzymatic activity was not sufficient to

Table 4. Results of the indicator species analysis (ISA) including all the oxidative stress indicators measured for each krill species using only comparisons with significant differences in the MRPP null hypotheses tested. The oxidative stress indicators were superoxide radical production ($O_2^{\bullet-}$; nmol $O_2^{\bullet-}$ min^{-1} mg $proteins^{-1}$), lipid peroxidation (TBARS; nmol TBARS mg $proteins^{-1}$), and enzyme activities of total superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) (units (U) mg $proteins^{-1}$).

Groups	Oxidative stress indicators	ISA value	Monte Carlo test *p*-value
Species			
N. simplex	$O_2^{\bullet-}$	41.2	0.0010
	TBARS	82.4	0.0010
	SOD	100.0	0.0010
	CAT	100.0	0.0010
	GPx	94.1	0.0010
	GR	100.0	0.0010
	GST	100.0	0.0010
Ne. difficilis	$O_2^{\bullet-}$	87.0	0.0010
	TBARS	91.3	0.0010
	SOD	100.0	0.0010
	CAT	95.7	0.0010
	GPx	100.0	0.0010
	GR	95.7	0.0010
	GST	100.0	0.0010
E. eximia	$O_2^{\bullet-}$	75.0	0.0010
	TBARS	93.7	0.0010
	SOD	100.0	0.0010
	CAT	93.7	0.0010
	GPx	100.0	0.0010
	GR	87.5	0.0010
	GST	100.0	0.0010
Cruise			
Jan, *N. simplex*	$O_2^{\bullet-}$	29.4	0.0081
Jul, *N. simplex*	TBARS	63.4	0.0001
	SOD	89.5	0.0000
	CAT	89.6	0.0000
	GPx	90.1	0.0000
	GR	90.3	0.0000
	GST	87.3	0.0000
Oct, *E. eximia*	$O_2^{\bullet-}$	63.6	0.0001
	SOD	75.0	0.0000
	CAT	70.5	0.0000
	GPx	74.5	0.0000
	GR	68.5	0.0001
	GST	79.9	0.0000
Sampling depth			
2 m, *N. simplex*	$O_2^{\bullet-}$	37.1	0.0234
Oxygen Minimum Zone (OMZ)			
Above OMZ, *N. simplex*	$O_2^{\bullet-}$	23.3	0.0209
	TBARS	30.3	0.0430
	SOD	40.2	0.0119
	CAT	40.2	0.0142
	GPx	36.8	0.0390
	GR	39.5	0.0175
	GST	33.9	0.0367
Below OMZ, *E. eximia*	$O_2^{\bullet-}$	34.3	0.0289
	GPx	38.3	0.0324
Thermocline			
Above, *N. simplex*	$O_2^{\bullet-}$	25.9	0.0071
Below, *E. eximia*	$O_2^{\bullet-}$	34.1	0.0292
	GPx	40.4	0.0180

avoid oxidative stress (Kong et al. 2008). Thus, we concluded that *N. simplex*'s seasonal decrease in population abundance and the narrowing of its DVM extension during summer is strongly influenced by the temperature increase near the surface ($> 22^{\circ}C$) and the shallower upper boundary of hypoxic conditions in the OMZ. This physiological limitation explains why *N. simplex* is not distributed in the eastern tropical Pacific, where high temperatures and a typically shallow OMZ prevail throughout the year (Fiedler and Talley 2006). *N. simplex* zoogeographic distribution is currently separated by the eastern tropical Pacific, having two populations in each hemisphere (Brinton 1962, 1979).

Our study provides the first observations of DVM of *Ne. difficilis* and *E. eximia* in the Gulf of California on a seasonal time scale. Apparently these species do not clearly modify their DVM pattern among seasons, as *N. simplex* does. In January, *Ne. difficilis* was abundant within the 100- to 200-m depth layer, but only from 16:00 h to midnight. Outside of this period, it is possible that *Ne. difficilis* was distributed deeper, because this species has been frequently detected up to 400 m (Brinton 1967, 1979; Lavaniegos 1996). The NMDS analysis showed that during January *Ne. difficilis* was not influenced by the depth of the upper boundary of the OMZ. The greater GPx activity of *Ne. difficilis* compared to that of *N. simplex* during the winter season could indicate its physiological ability to do deeper DVM. The metabolic function of GPx is to eliminate H_2O_2, an ROS that can be produced as a by-product of oxygen metabolism (Halliwell and Gutteridge 2002). High respiration rates could be the cause of higher H_2O_2 production during the DVM descent phase of *Ne. difficilis* when entering hypoxic conditions. Tremblay et al. (in press) measured the respiration rates of *Ne. difficilis* under distinct dissolved oxygen concentrations (from 2 to 4.5 mL O_2 L^{-1}) and observed that this species increased its respiration rate with decreasing environmental oxygen concentration. Dissolved oxygen concentrations $<$ 2.5 mL L^{-1} were not lethal for *Ne. difficilis* under laboratory conditions as observed for *N. simplex*. Lushchak et al. (2001) detected that GPx activity in the brain of goldfish *Carassius auratus* increased about 79% after 8 h exposure under anoxic conditions and remained 59% higher than in the experimental controls after 14 h of reoxygenation. Because TBARS levels of *Ne. difficilis* were not significantly different from those of the other two krill species during January, we conclude that this species was not under oxidative stress when migrating between 100- and 200-m depth. Thus, the role of GPx to decrease oxidative stress should be significant for mesopelagic krill species that tolerate low oxygen concentration, compared, for example, with short-migratory krill species, such as *N. simplex*.

Brinton (1979) proposed that the presence of hypoxic conditions ($<$ 1 mL O_2 L^{-1}) restricted the DVM of *Ne. difficilis* in the mouth of the Gulf of California, near Cabo San Lucas (22°27′N). In July, *Ne. difficilis* was detected at 100-m depth, mostly during the day, suggesting that it did not migrate to deeper waters. Its superoxide radical production was higher in July and October than in

January, but TBARS levels were significantly lower, perhaps because of the significantly higher GST activity. *Ne. difficilis* is a krill species that usually stays within or below the thermocline (Lavaniegos 1996). We propose the hypothesis that warm temperature is harmful for this species because superoxide radical production seems to increase under high temperature conditions but is less affected by hypoxic cold conditions. This might explain why this species has never been reported in the eastern tropical Pacific, where *Nematoscelis gracilis* is the only species of the genus recorded in the Mexican tropical region (Brinton 1962, 1979).

E. eximia was observed at greater abundance near the surface in January, mainly at dawn and around 20:00 h, likely as part of its DVM ascent. *E. eximia* was collected in its lowest densities during the day, similar to previous daily vertical distribution studies (Brinton 1979). Without doubt, this indicates that *E. eximia* migrates deeper than our maximum sampling depth (200 m), likely facilitated by its morphological large gills : cephalothorax surface ratio (Antezana 2002). However, in July, *E. eximia* was not abundant enough to detect any pattern of its DVM. *E. eximia* has its highest densities along the cold margins of the eastern tropical Pacific (Brinton 1979); thus, it is not expected to be highly abundant in the Gulf of California. In October, a greater abundance of *E. eximia* was observed because the sampling area was located in the southern part of the Gulf of California, near Bahía de La Paz (Fig. 1C). As in January, *E. eximia* was collected only during the night, inhabiting a deeper layer than 200 m during the day. In October, all its antioxidant enzyme activities were higher than in January, but only GPx activity was significantly higher. As with *Ne. difficilis*, the significant increase of superoxide radical production was balanced by antioxidant enzymatic defenses, as TBARS levels were significantly lower in October than in January. Superoxide radical production and GPx activity of *E. eximia* had significant ISA value for conditions below the OMZ, so this may indicate that these two indicators are closely associated. This means that if superoxide radical production increases, GPx activity also increases. The antioxidant enzyme system of *E. eximia* seems to exert a significant role in its tolerance for inhabiting regions with low dissolved oxygen concentrations.

N. simplex was the only krill species in our study that showed clear evidence of oxidative damage when it was exposed to hypoxic and warm conditions. We conclude that *N. simplex* does not inhabit regions with high temperature and low oxygen concentrations, like the conditions that prevail along the eastern tropical Pacific, because these environmental conditions, particularly during summer, induce significant oxidative stress in this species. The observed metabolic responses explain its zoogeographic affinity for living in high population densities along neritic regions with frequent wind-inducing coastal upwelling activity in the southern part of the California Current, in the Gulf of California, and in the northern region of the Humboldt Current system (Brinton 1962). *N. simplex* is the most abundant krill of the Gulf of California in biomass and number, being a key species in the food web of this region because it is preyed upon by large predators such as the blue whale *Balaenoptera musculus*, the fin whale *Balaenoptera physalus* (Gendron 1992), the smooth-tail mobula *Mobula thurstoni*, and the spinetail mobula *Mobula japanica* (Sampson et al. 2010). In a future hypothetical scenario of global warming and likely an expansion of the OMZ to higher latitudes (Stramma et al. 2008), *N. simplex* distribution and abundance might shift northward or be exposed to perhaps longer and more pronounced warm periods in the Gulf of California. This could modify the DVM, horizontal distribution patterns, and/or productivity rates of *N. simplex* and its predators, having significant ecological implications (tropicalization of the zooplankton fauna with lower biomass production) and possibly undesirable economic consequences for the society that inhabits both coasts of the Gulf of California. *Ne. difficilis* appears to deal more efficiently with low oxygen concentrations than *N. simplex*, being distributed at deeper layers but avoiding exposure to warmer temperatures. *E. eximia* as a tropical species seems to tolerate well low dissolved oxygen concentration and high temperature, even if these conditions occur simultaneously, partly because of its larger gills : cephalothorax surface ratio (Antezana 2002) and, as suggested in the present study, its effective antioxidant defense. Studying biochemical, e.g., oxidative stress, indicators of key zooplankton species, rather than only their abundance, as a function of their space and temporal environmental gradients can provide valuable information about how species could respond in their zoogeographic distribution patterns and population dynamics now and in the future under distinct climatic scenarios forced by the current global climatic change.

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