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ORIGINAL PAPER

Concentration of ascorbic acid and antioxidant response in early life stages of *Engraulis ringens* and zooplankton during the spawning seasons of 2006–2009 off central Chile

M. C. Krautz · L. R. Castro · M. González · A. Llanos-Rivera · I. Montes · H. González · R. R. González · J. C. Vera

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Abstract This study reports changes in ascorbic acid (AA) in anchoveta eggs, copepods and zooplankton during the 2006, 2007 and 2009 main spawning seasons in the coastal area of the central Humboldt Current System, Chile. Anchoveta eggs, copepods and total zooplankton community shared a seasonal variation and an increasing trend in AA concentration from winter through spring which was associated with the spring diatom bloom. The lineal relationship observed between AA concentration in anchoveta eggs, chlorophyll *a* and Sea Surface Temperature (SST) suggests that the increase in phytoplankton abundance could also increase the amount of AA in the spawning female anchoveta incorporated through tissue, thus increasing the concentration in their eggs. Ascorbic acid concentrations in copepods presented size (weight) dependence. Small copepods (e.g. *Acartia*, *Oithona*) had AA concentrations two orders of magnitude higher than the heavier weight class copepods (e.g. *Calanus*, *Rhincalanus*). Results of the determination of glutathione and the antioxidant potential showed a similar trend in interannual variations, suggesting that cold SST conditions observed in the 2007 spawning season could increase the consumption of antioxidants in early stages. Potential connections between AA concentration in the food web on anchoveta reproduction and egg hatching and embryo malformations are discussed.

Introduction

Engraulis ringens (anchoveta) is an iteroparous fish species, endemic to the coastal zone of the Humboldt Current system and one of the most representative Chilean coastal fisheries. Several somatic and biochemical changes in eggs and gonads of this species have been documented during its main reproductive season, from the end of the austral winter (July) to early spring (September) in the central-

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M. C. Krautz (✉) · I. Montes
Programa de Postgrados en Oceanografía, Departamento de Oceanografía, Universidad de Concepción, Concepción, Chile
e-mail: ckrautz@udec.cl; cristina.krautz@gmail.com

M. C. Krautz · L. R. Castro
Laboratorio de Oceanografía Pesquera y Ecología Larval (LOPEL), Departamento de Oceanografía, Universidad de Concepción, Concepción, Chile

L. R. Castro · H. González · R. R. González
Centro FONDAP- COPAS, COPAS Sur-Austral, Universidad de Concepción, Concepción, Chile

M. González
Departamento de Bioquímica Clínica e Inmunología, Facultad de Farmacia, Universidad de Concepción, Concepción, Chile

A. Llanos-Rivera · R. R. González
Unidad de Biotecnología Marina, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile

I. Montes
Helmoholtz Center for Oceanic Research, Kiel, Germany

H. González
Instituto de Ciencias Marinas y Limnológicas, Universidad Austral de Chile, Valdivia, Chile

J. C. Vera
Departamento de Fisiopatología, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile

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southern Chilean spawning zone (Cubillos et al. 1999). Latitudinal variations in egg volume (Llanos-Rivera and Castro 2004), total lipid and triacylglycerol concentration have been documented to occur during the spawning season (Castro et al. 2009), as well as a high consumption of free aminoacids and proteins during in the egg and early larval stages of *E. ringens* have also been reported (Krautz et al. 2010).

Morphological changes in ovarian tissues during oocyte maturation could induce an increasing production of reactive oxygen species (ROS) in fish tissues and a higher consumption of antioxidant molecules (e.g. ascorbic acid and glutathione) in both female and embryo (egg) tissues (Blom and Dabrowski 1995). It has reported in adult fish that environmental variations or stressful conditions such as increased water temperature, strong changes in the water column, oxygen concentration or salinity, increased pollution, food deprivation or changes in diet could stimulate ROS production, lipid peroxidation and changes in enzymatic and non-enzymatic antioxidants (Leggatt et al. 2007; Martinez-Alvarez et al. 2005). During the anchoveta spawning season, changes in temperature, turbulence, oxygen concentration and salinity in the water column (Sobarzo et al. 2007) from winter to spring could induce an increase in the consumption of antioxidants in fish tissues. In addition, the seasonal variations in the food web are expected to affect the availability of essential molecules such as vitamins for fish (Hapette et al. 1991).

Ascorbic acid (vitamin C) and glutathione (GSH) are recognized as key antioxidant molecules. Ascorbic acid is a low molecular weight molecule and an essential fish and crustacean micronutrient (see Brown and Lavens 2001 for a review). It is synthesized by phytoplankton and transferred to the rest of the trophic web through herbivorous/omnivorous zooplankton (Happette and Poulet 1990; Poulet et al. 1989). Concentrations of AA in the range of 2–16 mg g dry weight^{-1} have been detected in the chain-forming diatoms of *Skeletonema*, *Thalassiosira* and *Chaetoceros* and several nanoplanktonic species (e.g. *Nannochloris atomus* or *Nannochloropsis* sp, Brown and Miller 1992). Experimentally, the transfer efficiency of AA among primary producers and copepods has been estimated at 40–60 % (Happette and Poulet 1990). Ascorbic acid requirements for aquacultured fish species have been suggested to be around 20–50 mg kg^{-1} (see Brown and Lavens 2001 for a review), whereas this supply must increase to 350–400 mg AA kg^{-1} to saturate ovaries and optimize reproduction (Gabaudan and Verlhac 2001). Some authors have observed that AA concentrated in female gonads is transferred to the oocyte during maturation and then quickly consumed during the first days of embryonic growth (Blom and Dabrowski 1995). The effect of AA availability on reproduction and early stages survival has been shown through the increase of fecundity, egg survival and the hatching success for the offspring of females fed diets supplemented with ascorbyl phosphate, whereas high mortality and developmental abnormalities have been detected when females have been fed diets with low AA concentrations (Dabrowski and Blom 1994; Blom and Dabrowski 1995).

Glutathione (L-γ-glutamyl-cysteinyl-glycine, GSH) is another key molecule in the cellular redox balance. It is a small thiol molecule with antioxidant functions involved in the reduction of H_2O_2 and lipid hydroperoxides in association with several glutathione peroxidases enzymes. Glutathione interacts with other non-enzymatic antioxidants such as AA or vitamin E and is involved in their recycling (Hamre et al. 1997). From the egg to the larval stage, a progressive consumption of GSH and ascorbic acid during the development has been observed, contrasting with the increase of lipid peroxidation and the antioxidant enzymes activity (Kalaimani et al. 2008). In spite of the main role of GSH in antioxidant function in all stages of fish development, there is little information relative to its natural variability in early stages of development under wild environmental conditions.

The aim of this study is to determine whether the natural changes in availability of the natural dietary components (diatoms, zooplankton and copepods) induced by changes in the physical environment could have an effect on the variation in concentration of the micronutrients such as ascorbic acid and stimulate changes in the antioxidant response in *E. ringens* eggs in the coastal zone of central Chile during spawning season. We also discuss the potential importance of antioxidants in anchoveta reproduction and survival of early stages.

Methods

Description of study area

The study area was defined within the anchoveta fishery zone off the coast of Central South Chile. In this coastal area, we obtained zooplankton and fish egg samples from three stations located in the Coliumo Bay area (36.5°S, 72.9°W). Physical data were obtained from satellite information available from October 2006 to December 2009 and from the FONDAP-COPAS time series (Fig. 1). Monthly average data of Sea Surface Temperature (SST) and integrated chlorophyll *a* concentration (mg m^{-3}) were estimated from daily satellite data produced by Moderate Resolution Imaging Spectroradiometer (MODIS, http://gdata1.sci.gsfc.nasa.gov/daac-bin/G3/gui.cgi?instance_id=MODIS_MONTHLY_L3) encompassing the anchoveta fishery zone located between the Itata River mouth (36°S) and Mocha Island (38.5°S) and from the coast to 35 nautical miles (~65 km) offshore.

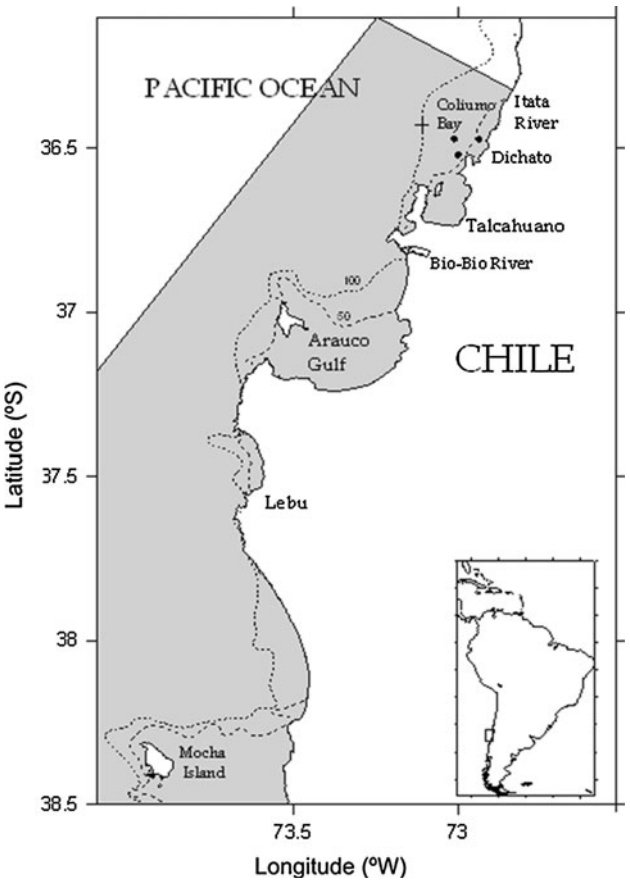

Fig. 1 Study zone showing the MODIS product area where of Chlorophyll *a* and SST were averaged (shaded area), three stations of zooplankton sampling and the location of the COPAS-times series station (+, station 18 nm)

Egg and zooplankton samples

Anchoveta eggs were collected monthly during the anchoveta 2006, 2007 and 2009 main spawning seasons. Zooplankton samples were collected from on board the R/V Kay Kay II (University of Concepción) by gentle tows using a bongo net (60 cm diameter, 300-µm mesh). A group of samples were preserved with formaldehyde 5 % buffered with borax. These samples were used to determine the anchoveta eggs (complete series of samples) and copepod (only 2007 samples were available) abundances in the field.

A second group of samples were placed in plastic containers and rapidly transported to the Dichato Marine Biology Station (University of Concepción, Chile) for egg identification, sorting for biochemical determinations and rearing experiments. In the laboratory, fresh total zooplankton samples were filtered through a 150-µm mesh sieve. Fractions of the samples were placed in cryovials, weighted and stored in liquid nitrogen. Another set of tow samples were used to obtain anchoveta eggs and copepods, which were identified and counted, weighted and frozen in liquid nitrogen until biochemical analysis could be carried out. In parallel, healthy anchoveta eggs were separated from the samples and incubated at 12 °C (temperature controlled chamber) in 1-L glass containers with 100–110 eggs in each. After hatching, new yolk sac larvae, eggs not hatched and abnormal eggs were counted. Anchoveta egg samples for biochemical analysis were obtained throughout the study period (spawning seasons 2006, 2007 and 2009), and copepods samples were obtained mainly in 2007 but some complementary samples (intermediate and the heaviest weight classes, see results section) were obtained during 2008 and 2009. Finally, because the lower egg abundances on the field, egg samples for incubation and zooplankton for AA analysis were obtained in 2007 only. Missing data/repliques in biochemical determination series (eggs, copepods and total zooplankton) correspond to losses occurred during the earthquake and tsunami 2010 in Chile. Details about number of samples included in each analysis were included in the results section and the legend of tables and figures.

For biochemical determinations, groups of 100–200 eggs, 100–200 copepods and 300–500 mg of zooplankton were homogenized on ice with a mechanic tissue homogenizer in phosphate buffer (10 mM, pH 7.0). Homogenized samples were centrifuged at 10,300*g* (4 °C), and the supernatant was maintained on ice until analysis of AA, GSH and antioxidant potential assays (FRAP) could be carried out.

Ascorbic acid (AA) determination

Ascorbic acid concentration in eggs, copepods and total zooplankton community was determined according to Badrakhan et al. (2004) and Moeslinger et al. (1994, 1995) protocols. Briefly, 54 µl of a sample was incubated with 10 µl of ascorbate oxidase for 5 min at room temperature. Then, 210 µl of cold ice methanol was added. The tubes were centrifuged at 10,300*g* per 1.5 min, and the supernatant was removed and transferred to a new tube. Finally, 270 µl of phosphate buffer (37 °C) was added, and the absorbance at 346 nm was continuously registered for 5 min. Concentration of AA in samples was calculated interpolating in a calibration curve elaborated with standard AA (Sigma). Results were expressed in µg per g^{-1} wet weight of tissue.

Copepods were classified in three weight classes (see results section), and results were reported as concentration of AA (µg AA g^{-1} wet weight) and as total potential AA supply (µg AA m^{-3}) by each weight class. Total potential AA supply was obtained during 2007 only. This potential value was estimated as the product among the AA mean concentration (µg AA g^{-1}), the individual mean weight (g $copepod^{-1}$) in each copepod weight class and the number of copepods per 1 m^3 of seawater in the area.

Reduced glutathione (GSH) determination

To determine GSH concentrations in anchoveta eggs, samples were treated with sulfosalicylic acid 5 % and centrifuged at 10,300 × *g* per 5 min. Total GSH was determined on the supernatants using the recycling enzymatic method (Griffith 1980) standardized to microplates. In this method, GSH is oxidized by 5 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to give glutathione disulfide (GSSG) with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). Glutathione disulfide is reduced to GSH by the action of 18 µL of glutathione reductase (EC 1.6.4.2), in phosphate buffer (0.1 M, pH 7.4) and 5 µL NADPH (6 mM in Tris 10 mM, pH 9.0). The rate of TNB formation was followed at 25 °C and 412 nm and is proportional to total GSH concentration in the sample interpolated in a calibration curve of GSH standard (Sigma). Results were expressed in nmol per g^{-1} wet weight of tissue.

Protein concentration in anchoveta eggs was determined spectrophotometrically with a commercial Biuret kit. Protein concentration in the sample can be estimated using a standard curve of bovine serum albumin (BSA).

Antioxidant potential assay

Antioxidant potential in anchoveta eggs was determined by the ferric reducing/antioxidant potential (FRAP) assay. The FRAP assay involves the reduction in a yellow complex Fe^{III} and 2,4,6-tripyridyl-s-triazine (TPTZ) to a blue-colored Fe^{II}-TPTZ by biological antioxidants and chemical reductants presented in the sample.

This method was based on Benzie and Strain (1996) modified by Griffin and Bhagoolib (2004). Working FRAP reagent was made by mixing of 300 mM Acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3 \times 6H_2O$ in a proportion of 3:1:1. Twenty microliters of sample were added to each well in a 96-well microtiter plate, and then, 150 µL of FRAP working reagent was added. Change in absorbance at 593 nm was registered each 1 min for 16 min (37 °C). Antioxidant potential was expressed in µM $FeSO_4$ per µg protein^{-1}.

Statistical analysis

Kruskal–Wallis tests were performed to assess seasonal differences in ascorbic acid concentrations, in GSH concentration and in antioxidant potential (FRAP, Hammer et al. 2001). Lineal regression was utilized to determine possible relationships between SST and ascorbic acid and between chlorophyll concentration and ascorbic acid. The potential relationship between GSH and FRAP was also assessed with lineal correlations. The number of samples considered in each analysis was included in figures and tables.

Results

MODIS monthly average chlorophyll *a* and SST in the study area

The monthly average SST showed seasonal and interannual variations in the study area (Fig. 2). Significant seasonal differences were found in SST during 2006–2007 (spring 2006 and autumn–winter 2007, Kruskal–Wallis test, $p = 0.025$) and autumn–winter 2007 and spring 2007 ($p = 0.025$). No differences were found between spring and winter 2009 (Kruskal–Wallis test, $p = 0.083$, Fig. 2). The monthly average SST data also showed the occurrence of a El Niño warm phase during the spring season 2006, with an average SST of 13.5 °C, followed by a cold phase ("La Niña") characterized by a decrease of ~1 °C in the average SST during winter and spring 2007. During spring 2009, our data showed the return to warm conditions with a spring SST average of 12.8 °C.

MODIS monthly data showed a seasonal variation of average surface chlorophyll *a*, with lower values in winter and higher values during spring. Maximum values occurred mainly during December (Fig. 2). Kruskal–Wallis tests did not show any significant differences in chlorophyll *a* among the spring of 2006, 2007 and 2009.

Phytoplankton and microplankton abundance from 2006 to 2009 in the study area

Total abundance of diatoms (Fig. 3a) showed seasonal variability, low abundances in winter and an increasing trend in abundance with the onset of spring. The 2006 spawning season showed lower abundances than the 2007 and 2008 spawning seasons. During 2007 spawning season, increasing abundances (spring bloom) were observed from September onwards and maximum abundances of total diatoms were observed in October (4.0×10^6 cells L^{-1}). During the 2008 spawning season, the start of spring bloom

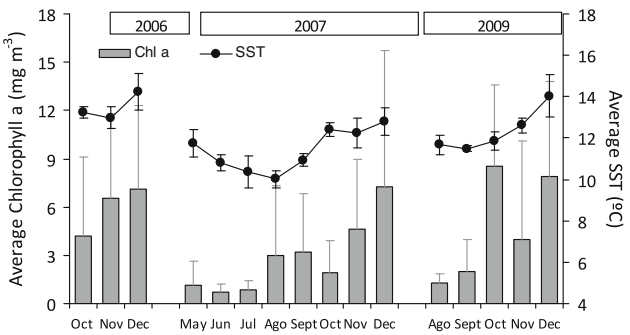

Fig. 2 MODIS Chlorophyll a concentrations (monthly mean ± SD) and Sea Surface Temperature (SST, monthly mean ± SD) in the study area

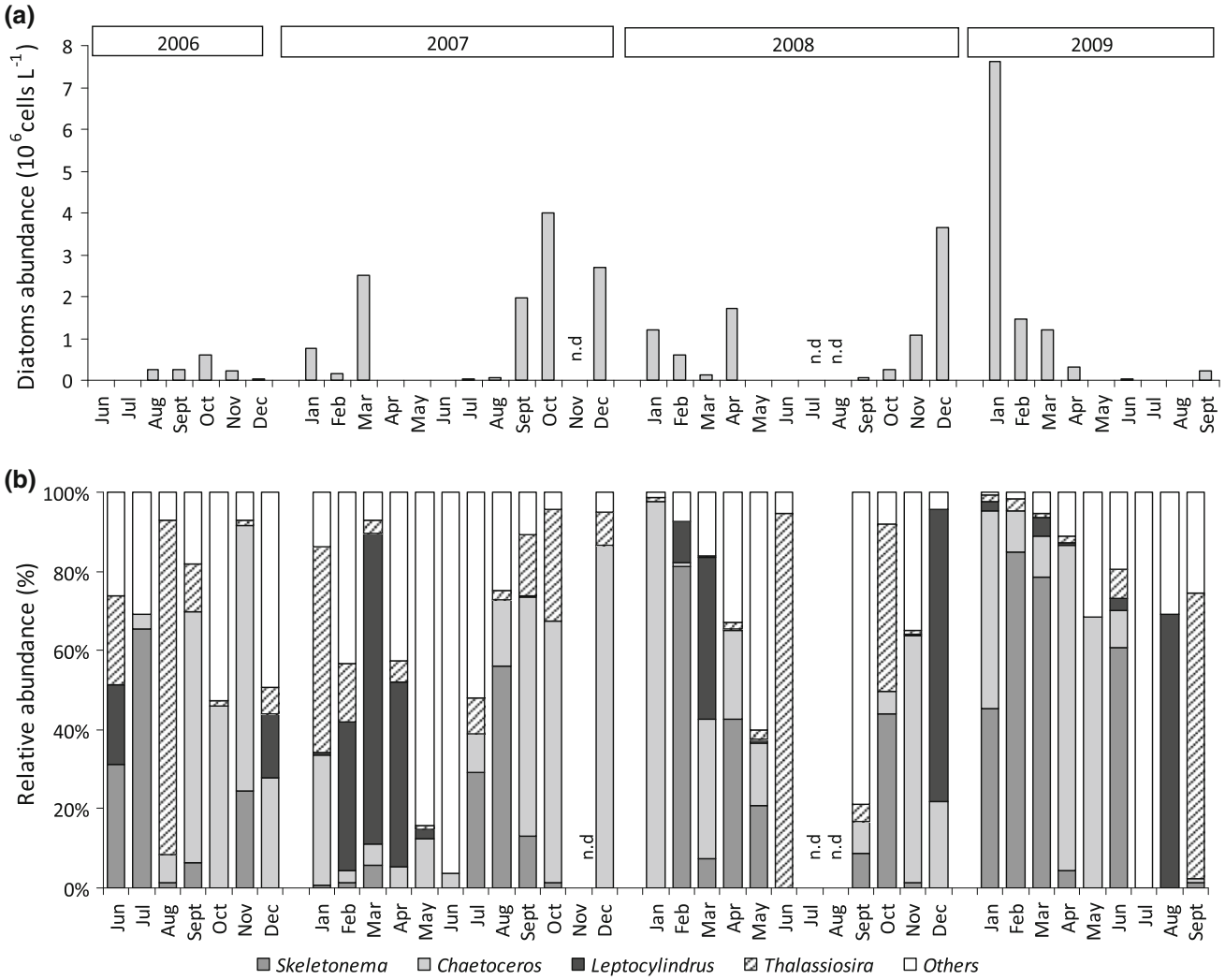

Fig. 3 **a** Total abundance of diatoms and **b** main genera composition (relative abundance %) in diatoms community in the study area

was more gradual and delayed, and the maximum number was reached during December (3.6×10^6 cells L^{-1}). The initial dominant species during 2006 and 2007 spawning seasons was *Skeletonema* sp and then *Chaetoceros* sp or *Thalassiosira* sp. During the spawning seasons of 2008 and 2009, an alternance of several species was observed in which every month a different taxa outnumbered the others two or threefold (Fig. 3b).

Total abundance and composition of microplankton (Fig. 4a) showed seasonal and interannual differences. The total abundance (all groups pooled: dinoflagellates, tintinnids, alloricated ciliates, radiolaria, foraminifers, copepod eggs and nauplii) showed a primary peak during early summer and a secondary during later summer or autumn. This seasonal variation was due primarily to the remarkable increase in abundance of dinoflagellates from winter to spring in all years. Tintinnids, the second group in abundance, did not show a clear seasonality except during autumn–winter 2009 when they became the dominant group within the microplankton community. Inter-annually, lower abundances in total microplankton occurred during the cold spawning season 2007, contrasting with higher values observed during 2008 and 2009. During these two warmer seasons, dinoflagellates showed a notorious increase which is depicted in Fig. 4b.

Copepod abundance during 2007

Copepods presented maximum abundance in spring 2007, reaching a peak in December. The main Copepod genera identified in 2007 (Fig. 5a) were *Calanus*, *Acartia* and *Paracalanus*. *Oncaea* showed lower abundance and reached its maximum abundance in autumn (May). Large copepods (*Rhincalanus and Calanus*) and mid weight copepods (*Paracalanus*) showed low abundances during winter and maximum abundances through spring. Small

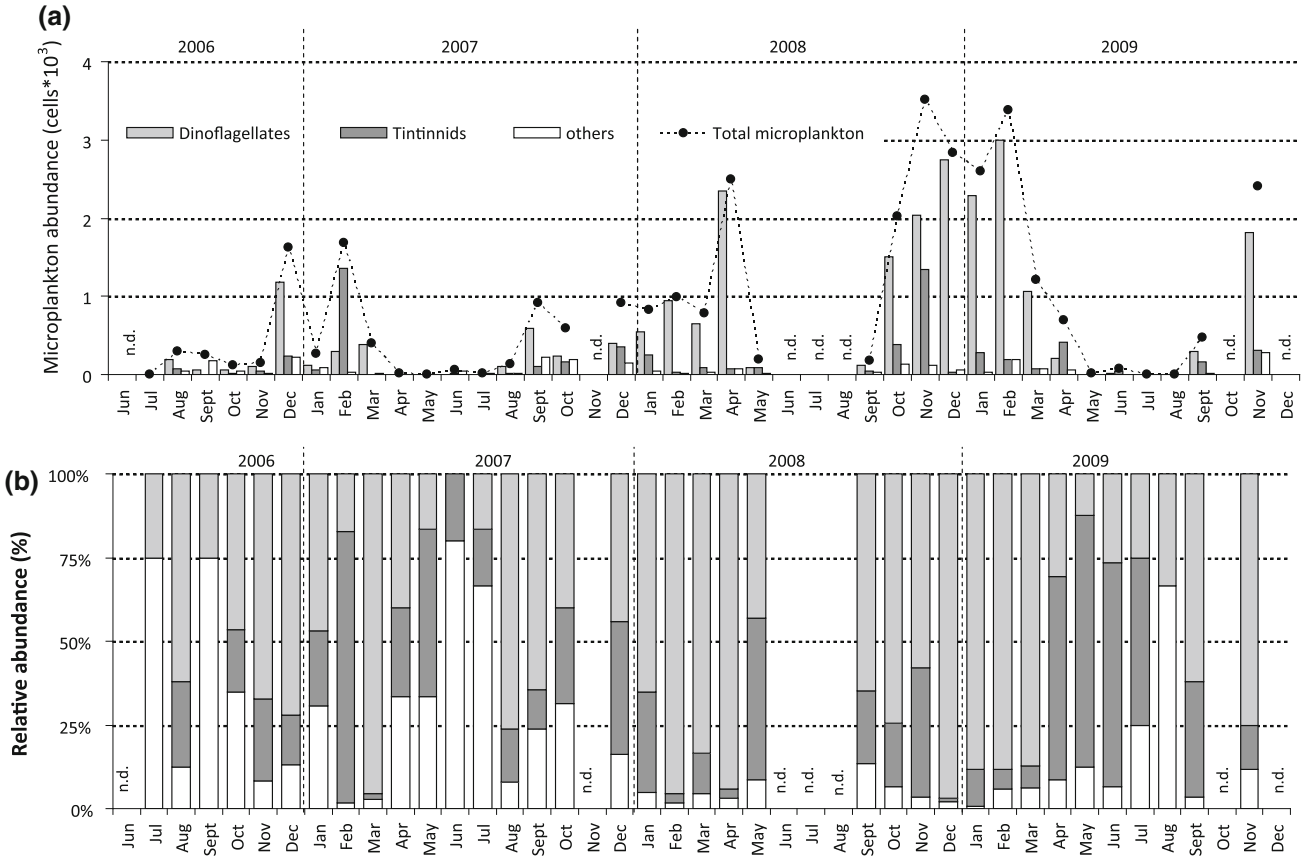

Fig. 4 Microplankton **a** abundance and **b** composition (%) at 10 m depth in the study area during 2006–2009

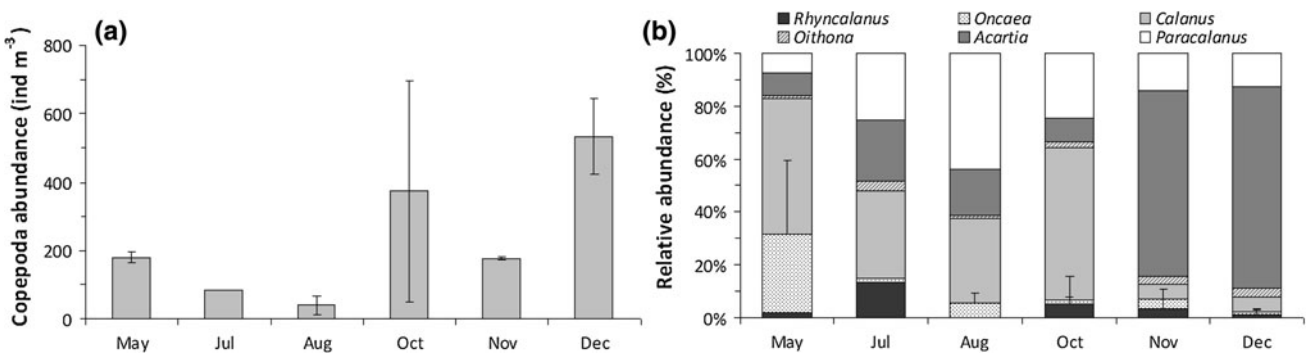

Fig. 5 **a** Mean abundance (individuals $m^{-3} \pm$ SD) and **b** the relative abundance (%) of the main copepod groups present at two coastal stations in the study area during the anchoveta 2007 main spawning season

weight copepods such as *Acartia* increased in abundance during spring and reached their maximum abundance at the end of the season (December) (Fig. 5b).

Ascorbic acid in copepods and total zooplankton community in 2007

Copepods were classified in three weight classes: small (53.2 ± 12.7 µg ind^{-1}, composed mainly by *Acartia*), intermediate (383 ± 63.9 µg ind^{-1}, composed by *Paracalanus*) and large ($2{,}386 \pm 884.5$ µg ind^{-1}, composed by *Rhincalanus* and *Calanus*) copepods. Differences in AA concentrations occurred depending on the copepod weight (Fig. 6a). The highest concentrations were observed in small weight copepods reaching maximum values during the spring 2007 (November). Intermediate weight copepods showed up to one order of magnitude lower concentration than small copepods and up to one order or

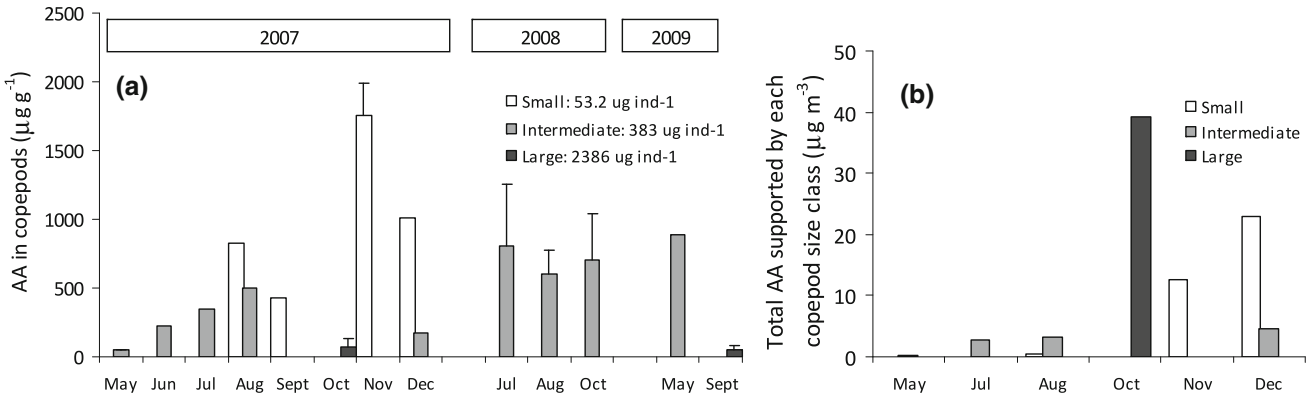

Fig. 6 **a** Ascorbic acid concentration (Mean ± SD) in three weight classes of coastal copepods during 2007–2009. Samples of three weight classes were obtained during 2007, and additional samples of intermediate weight class were obtained during 2008 (3 months) and 2009 (1 months). Samples of the heaviest weight class were obtained during 2007 (October) and 2009 (September). **b** Potential AA supply provided by each weight class of coastal copepods during 2007 spawning season

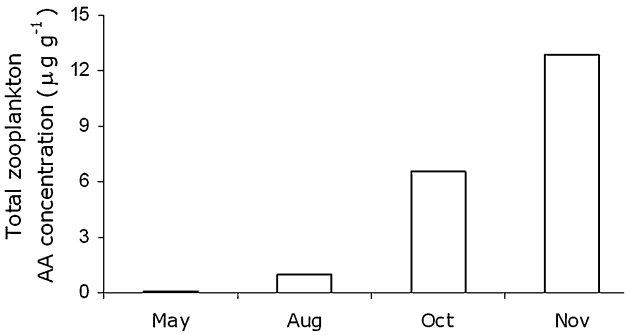

Fig. 7 Ascorbic acid concentration in the total zooplankton community ($n = 1$) in the study area during 2007

magnitude higher than the large size class. During 2007, intermediate copepod size class showed increasing AA concentration from autumn (May) to the winter (August), but lower concentrations than spring 2008 and autumn 2009. The largest (heaviest) copepods showed the lowest AA concentration than the other two groups, reaching 70.3 µg g^{-1} in spring (October) 2007 and 50.3 µg g^{-1} during 2009 (September). Despite the data limitations, our overall results suggest a trend of higher copepod AA contents in spring than winter during 2007. These results were consistent with the increased trend observed in the samples containing the entire zooplankton community (Fig. 7) in which the lowest concentrations of AA occurred in autumn (May, 0.108 µg g^{-1}) and the highest in late spring (November, 12.9 µg g^{-1}).

The potential AA supply provided by each copepod weight class (µg AA m^{-3}) during 2007 showed seasonal differences (Fig. 6b). Between small and intermediate weight copepods, the largest potential AA supply in winter came from the intermediate weight group, while during spring, this was supported by the small copepod weight class. An increase was observed in the potential AA supply for the low weight copepods from winter to spring, whereas the intermediate weight class showed similar AA supplies during both seasons. In the only month in which we count with values of large copepods (October 2007), the heaviest copepod group showed the highest AA potential supply among all groups and all months.

Anchoveta egg abundance and hatching success

Anchoveta egg abundances were low in austral winter, and higher abundances were observed during spring (September to December, Fig. 8). Maximum abundances were observed during the 2007 spawning season.

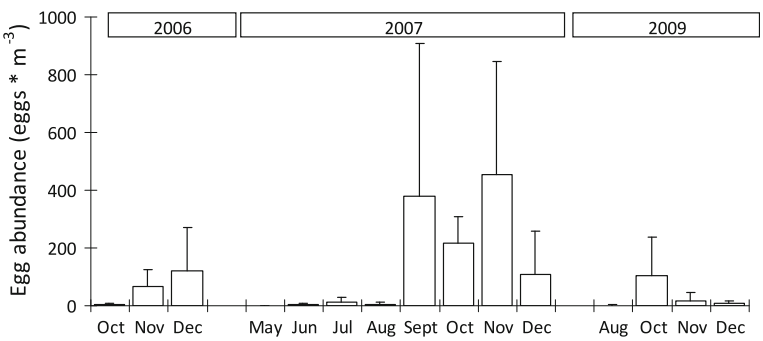

Fig. 8 Anchoveta eggs abundance (Mean ± SD) in three coastal stations at the study area during the 2006–2009 main spawning periods

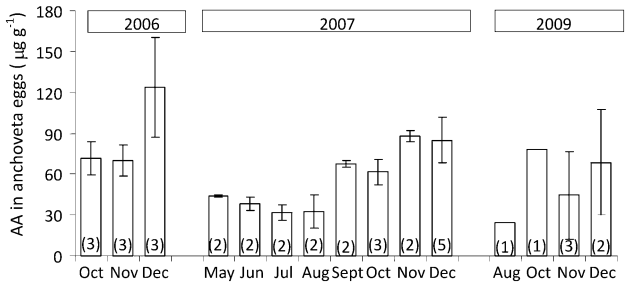

Fig. 9 Ascorbic acid concentration (µg g^{-1} eggs, mean ± SD) in planktonic anchoveta eggs during the 2006, 2007 and 2009 main spawning periods in the study area. Number of samples was indicated among parenthesis

During winter, mean hatching success ranged between 88.1 ± 1.4 and 91.9 ± 4.2 %, whereas maximum hatching percentage was observed at the end of spring (November and December, 93 and 93.5 %). Egg mortality ranged between 1.5 ± 2.1 % in July to 8.8 ± 7.6 % in October experiments. In the same month of spring, we observed the lowest hatching rates, averaging 63.5 % (Table 1; Fig. 11). Presence of abnormal development (e.g. eggs with notochord malformations in later stages of embryo development) was only reported during winter averaging 2 %.

Ascorbic acid in anchoveta eggs

Mean monthly AA concentrations in anchoveta eggs ranged between 24.3 and 123.3 µg g eggs^{-1} and showed a seasonal variation pattern. Observed concentrations were higher in spring than in winter during the 3 years considered in the study (Fig. 9), whereas higher variability was observed during warmer (2006 and 2009) than the colder (2007) spawning seasons. Significant differences were observed between AA concentration detected in eggs collected in spring 2006 and autumn–winter seasons in 2007 (Kruskal–Wallis, $p = 0.00035$) and between autumn–winter 2007 and spring 2007 (Kruskal–Wallis test, $p = 0.00012$).

The mean concentration of AA in anchoveta eggs showed a significant linear relationship with monthly average chlorophyll *a* concentration in the study area (Fig. 10a, $R^2 = 0.53$, $p = 0.0021$) and between AA concentration in eggs and SST (Fig. 10b, $R^2 = 0.71$, $p = 0.0027$).

Figure 11 shows a comparison between average AA concentrations with experimental data of hatching success data carried out with eggs collected during the same sampling dates. Two particular periods were observed. The winter period (July and August) showed high hatching rates (>88 % survival), low AA concentrations (<35 µg g^{-1}) and the presence of abnormal eggs (i.e. eggs with embryo in advanced stages of development with some degree of notochord torsion, Table 1). The spring period (September to December), showed higher AA concentrations (>60 µg g^{-1}), and an increase in hatching success occurring with increasing concentrations of AA observed in eggs. No abnormal eggs were observed during these later incubations.

Glutathione (GSH), proteins and antioxidant potential (FRAP assays) in anchoveta eggs

Total glutathione in eggs (Table 2) showed high variability between months. The highest concentrations were detected in autumn (May) 2007 and spring mid (October) 2009, and the lowest values were observed in late spring (November) 2007 and 2009. Mean concentrations of GSH were significantly higher in 2006 than 2007 (all data, Kruskal–Wallis, $p = 0.012$) and significantly lower GSH concentrations were observed in winter-spring 2007 than in 2009 (June to December, Kruskal–Wallis, $p = 0.039$).

Protein concentration ranged between 35.2 and 62.5 mg g^{-1} eggs (Table 2). Average concentration showed seasonal variability, with slightly lower values during the winter season and higher values during spring months. Concentrations were significantly higher during spring 2006 than spring 2009 (Kruskal–Wallis, $p = 0.019$).

FRAP assays results (Table 2) showed higher values (>3 µM $FeSO_4$ µg protein^{-1}) during spring 2006 and 2009 than in 2007. Spring 2006 showed antioxidant potential values significantly higher than those in spring 2007 (Kruskal–Wallis, $p = 0.0019$). More homogeneous values

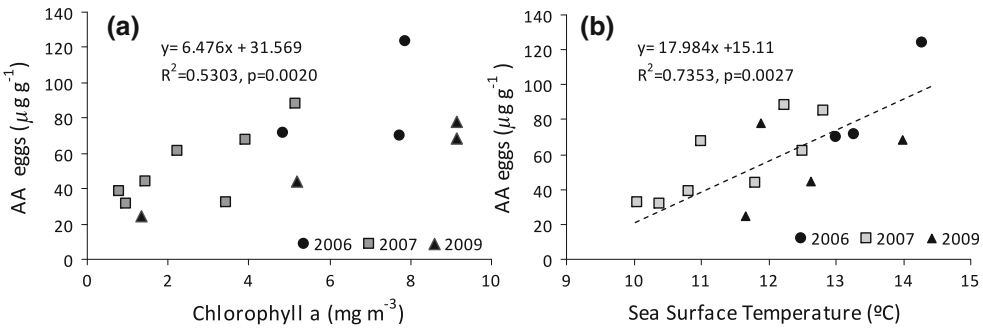

Fig. 10 Lineal relationship between eggs AA concentration and **a** MODIS monthly average chlorophyll a concentration and **b** Sea Surface Temperature (SST) in the period of study

Table 1 Hatching success (%, Mean ± SD) and percentage of abnormal eggs (%, Mean ± SD) during 2007 main spawning season (July to December) in anchoveta egg incubations

Month	*n*	Hatching success (%)	SD	Abnormal (%)	SD	Dead (%)
July	2	91.9	4.2	2.6	3.6	1.5
August	2	88.1	1.4	1.4	0.6	3.9
September	2	85.3	3.6	0.0	0.0	2.9
October	4	65.5	5.7	0.0	0.0	3.4
November	2	93.0	7.1	0.0	0.0	1.9
December	2	93.5	0.7	0.0	0.0	4.0

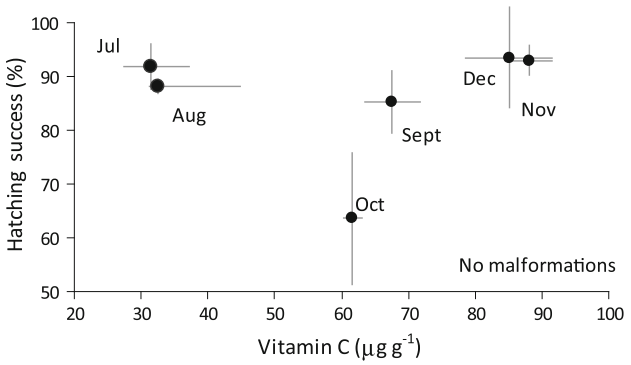

Fig. 11 Concentration of AA in anchoveta eggs (µg g^{-1}, Mean ± SD) and hatching success (%) during the 2007 main spawning period (July to December) in the study area

were observed during 2007 than in the other spawning seasons. Higher variability of all study periods occurred during winter 2009. During 2007, average FRAP values were around 2.3 ± 0.3 µM $FeSO_4$ µg $protein^{-1}$ and higher values were observed in May (2.6 ± 0.3 µM $FeSO_4$ µg $protein^{-1}$ and September (2.8 ± 0.2 µM $FeSO_4$ µg $protein^{-1}$).

Results of GSH concentration and FRAP in anchoveta eggs showed a lineal, positive and significant correlation ($r = 0.64$, $p = 0.0093$).

Discussion

The coastal area of the Humboldt Current system is characterized by a seasonal variation in the wind patterns that modulate the coastal upwelling events affecting productivity, carbon and nutrient fluxes in the area. During the austral spring and summer, winds from the SW quadrant induce active upwelling events, which contribute to increased nutrients in the coastal zone thereby increasing primary productivity (Daneri et al. 2000), favoring the seasonal occurrence of large phytoplankton blooms that constitute an important source of food and micronutrients for the rest the trophic web components. Variations in chlorophyll *a* concentration have been utilized as a proxy for fish and zooplankton food availability for; more recently, the changes in food quality and composition have

Table 2 Reduced glutathione (GSH, Mean ± SD), antioxidant potential (FRAP, Mean ± SD) and total proteins (Mean ± SD) in planktonic anchoveta eggs during 2006, 2007 and 2009 main spawning seasons in the study area

Year	Month	GSH (nmol g^{-1})			FRAP (uM g $protein^{-1}$)			Total protein (mg g^{-1})		
		Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
2006	Oct	97.7	48.0	2	3.1	0.1	2	53.6	0.9	2
	Dec	74.8	48.8	3	3.8	0.8	2	50.1	3.6	3
2007	May	152.4		1	2.6	0.3	1	43.9	3.5	2
	Jun	19.1		1	2.2		1	35.3		1
	Jul	36.7		1	2.4		1	48.7		1
	Aug	26.4	2.6	3	1.9	0.3	3	47.6	3.9	3
	Sept	29.7	9.5	2	2.8	0.2	2	40.1	6.9	2
	Oct	nd	nd		2.4	0.1	2	43.7	1.9	2
	Nov	2.4		1	2.1	0.5	3	50.9	2.6	3
	Dec	7.8	9.0	1	2.1	1.0	3	47.3	5.9	3
2009	Jun	11.7		1	2.3		1	43.6		1
	Aug	65.6		1	2.7		1	32.5		1
	Sept	38.6	39.1	2	1.7	1.1	2	54.8	10.9	2
	Oct	195.9	60.3	4	3.9	1.2	4	40.5	7.1	4
	Nov	8.1	3.6	2	1.1	0.0	2	32.6	3.4	2
	Dec	55.9	6.2	3	3.8	0.9	2	46.0	7.7	3

been considered an important source of variability in the reproductive success of copepods and small pelagic fish in the Humboldt Current System (e.g. Aguilera et al. 2011; Castro et al. 2009). However, no information exists on essential nutrients such as ascorbic acid (vitamin C) that has been shown to have a high importance in many aspects of the physiology of cultured fish such as in immune response and antioxidant defense, nor on the way it is transferred in the trophic web to adult fishes in natural populations and on potential effects due to its variations in early stages. In this study, we report the seasonal variability of vitamin C concentration in anchoveta eggs and mesozooplankton, the major food source for adult small pelagic fish, during three spawning seasons. Complementarily, the antioxidant response in anchoveta eggs was evaluated through the determination of another key molecule, glutathione, and the antioxidant potential (FRAP). Because of its relevance in physiological responses, changes in micronutrients such as ascorbic acid or in key molecules involved in the response against oxidative stress such as glutathione might represent an unexplored mechanism by which the physical environment affects the food web and the final reproductive success in natural populations in productive seasonally varying environments.

In addition to seasonal variability observed in SST and chlorophyll a in the study area, a contrasting environmental scenario was observed through the occurrence of a particularly cold 2007 spawning season contrasted with the warmer 2006 and 2009 spawning seasons (previously reported in Krautz et al. (2012); Castro et al. (2010) from in situ 2007–2008 observations). Observations coincided with the regional trend observed in region Niño 1 + 2 (http://www.cpc.ncep.noaa.gov/data/indices/sstoi.indices). Estimates of monthly average chlorophyll *a* values obtained from MODIS were in the range of in situ values reported in the literature (e.g. Anabalon et al. 2007; Böttjer and Morales 2007) and followed the same trend as the SST (Fig. 2). Interestingly, both SST and chlorophyll a data showed a significant lineal relationship with ascorbic acid concentration in eggs, suggesting that variations in the abundance of primary producers could affect ascorbic acid availability/incorporation into spawning females, and subsequent transfer to their offspring.

Phytoplankton and microplankton showed remarkable seasonal patterns of increase in abundance from winter to spring, despite the differences in absolute values among years. Copepods, the dominant taxon in the mesozooplankton community of the study area commonly reaching up to 40 % of the total zooplankton biomass (Manríquez et al. 2009) also varied during the spawning season in both abundance and taxonomically. During the 2007 spawning season, we observed the most common species of copepods reported in the literature for this area *Paracalanus parvus*, *Oithona similis*, *Acartia tonsa*, *Calanus chilensis* and *Rhincalanus nasutus* (Escribano et al. 2007). The smaller copepod genera (e.g. *Oithona* and *Acartia*) were found all year long and increased their abundance during spring becoming dominant at the end of the season (December). From autumn to the beginning of spring 2007, large calanoids *Calanus* and *Rhincalanus* and intermediate weight copepods (*Paracalanus*), although low in number, were dominant compared with the small copepod size class and showed a peak in abundances in early spring (October), coinciding with the spring diatoms bloom in the area. The large calanoid genera in this area have been commonly associated with upwelling conditions, the availability of large phytoplankton cells and of an increased C:N ratio during the spring season (Manriquez et al. 2009; Escribano et al. 2007). Coincidently, the AA concentrations and potential AA supply of the copepod community increased also from winter to spring despite the differences in relative abundance of the three copepod size classes. The AA concentrations of large and medium size copepod classes are within the ranges reported for other copepod species (Happette and Poulet 1990; Van der Meeren et al. 2008) but the small size copepods show higher AA values than those previously reported. Poulet et al. (1989) and Happette and Poulet (1990) suggested a positive trend between AA concentration and size in adults of *Calanus helgolandicus* (large, herbivore) and *Acartia clausi* (small, omnivore). These authors emphasized that the feeding type (herbivore-omnivore *versus* carnivore) could determine the AA contents better than the species involved. Potential AA supply provided by the copepod community during 2007 showed that the increase in AA sources for anchovetas during the spawning season could come from either small (*Acartia*, *Oithona*) or large copepods (*Calanus, Rhincalanus*) depending on their abundance and biomass while potential AA supply for the intermediate size class shows less variability during the spawning season.

The concentration of AA in anchoveta eggs showed seasonal variability, with an increasing trend from winter to spring season, as was observed in copepods and the total zooplankton samples and was in the range reported in the literature for wild species. Pelagic eggs of *Gadhus morhua* showed AA concentrations between 100 and 450 μg g^{-1} (Sandnes and Braekkan 1981), 200–344 μg g^{-1} in small *Salvelinus alpinus* (Dabrowski 1991) and between 37 (fertilized egg) and 42 μg g^{-1} (eyed eggs) in *Salmo salar* (Cowey et al. 1985). The lineal relationship observed between anchoveta AA concentration in eggs, chlorophyll *a* and SST suggests that the increase in phytoplankton abundance (e.g. large size phytoplankton dominated by of AA rich genera like *Skeletonema, Chaetoceros* and *Thalassiosira* in diatom community during 2007) could increase AA incorporation through the tissue of spawning female anchoveta, finally increasing the concentration in

their eggs. Changes in trophic web characteristics (phytoplankton biomass, copepods size composition) have been suggested to affect AA concentrations in the gonads and liver of female anchoveta during their reproductive main season (Krautz et al. 2012).

Our results showed that in winter, when AA concentrations in anchoveta eggs were low, hatch success was high and a higher proportion of notochord malformations was also observed, although this was within the range reported for this area in the 2007 spawning season (average 5.6 %; Llanos-Rivera et al. unpublished data). In spring, under higher AA concentrations, hatching success continued to be high and no malformations were observed. These results suggest that hatching variations do not seem to be associated with AA deficiency but, instead, suggest that this AA deficiency might contribute to the presence of embryonic malformations early in winter. The mechanisms have not been yet clearly defined but the influence of AA as cofactor on collagen synthesis (see Lall and Lewis-McCrea 2007 for a review) may be a potential way in which AA could structurally affect the embryo development. Other sources of reported malformations indicate several nutritional deficiencies such as in lipids (essential fatty acids, phospholipids, Cahu et al. 2003) and C and D_3 avitaminosis (Darias et al. 2011). However, high lipid concentrations observed in anchoveta eggs during winter 2007 and other spawning seasons (Castro et al. 2009, 2010) suggest that the malformations observed in our study should not be attributed to their deficit or any other lipid-soluble components.

A small time series of variations in the concentration in two key antioxidant molecules, AA and GSH, and the antioxidant potential (FRAP) in anchoveta early stages is reported in this study by the first time. Anchoveta egg GSH concentration showed high inter-annual variability and were in the range reported for fish eggs (19–25 nmol g^{-1} in eggs of *Salmo salar* (Cowey et al. 1995); 36.2 nmol g^{-1} in sturgeon *Acipenser naccarii* eggs (Diaz et al. 2010). Antioxidant potential (FRAP) in anchoveta eggs showed inter-annual differences: higher values in spring 2006, more homogeneous values in 2007 and high variability in 2009 data. The range of FRAP values (1.08–3.90 μM $FeSO_4$ μg $protein^{-1}$) in anchoveta eggs are within the ranges reported for marine organisms (Griffin and Bhagoolib 2004). Results of the determination of these two stress oxidative indicators showed a similar trend in interannual variations, suggesting that the particularly cold conditions of SST observed in the 2007 spawning season affected them simultaneously suggesting a higher consumption of antioxidants in the eggs during cold years. The complex antioxidant demands/responses scenario and the relative importance of these components in natural marine populations is a topic that deserves further research.

This paper documents, for the first time, temporal variations in micronutrient and key antioxidant AA in early stages of small pelagic fish and zooplankton from the highly productive upwelling of the Humboldt Current. Ascorbic acid and GSH molecules have key functions in the redox balance of the cell and could potentially have important effects in the reproduction and survival of the fish and zooplankton. Similar trends observed between variation of AA concentration in anchoveta eggs, AA concentrations in copepods, the zooplankton community and the standing stock of primary producers (chlorophyll a), in addition to the potential effects on early stages success observed through the occurrence of embryonic malformations, suggest a link between food quality available to spawning females and the fate of their eggs. Testing of this hypothesis will require further experimentation along with this preliminary data on the vitamin contents in the upwelling trophic web and changes in the antioxidant response herein established will contribute to elucidate how the physiology of small pelagic fish responds to local and global environmental changes at both the population and community level.

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