REPRODUCTIVE CONDITIONS ASSOCIATED TO CHANGES IN THE LIPID-SOLUBLE ANTIOXIDANT CAPACITY AND THE DAMAGE TO LIPIDS IN THE SEA URCHINS *LOXECHINUS ALBUS* (ECHINODERMATA: ECHINOIDEA)

CONDICIÓN REPRODUCTIVA ASOCIADA A CAMBIOS EN LA CAPACIDAD ANTIOXIDANTE LIPOSOLUBLE Y EL DAÑO A LÍPIDOS EN EL ERIZO DE MAR *LOXECHINUS ALBUS* (ECHINODERMATA: ECHINOIDEA)

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ABSTRACT

The hypothesis of this study was that oxidative damage in the lipid phase of the gonad of the sea urchin *Loxechinus albus* increased during gametogenesis. Six different male and female reproductive conditions (reproductive stages) were described: (a) immature, (b) growing, (c) premature I, (d) premature II, (e) mature, and (f) spawned. The content of the lipid-soluble antioxidants, as well as the damage to lipids (assessed as the content of 2-thiobarbituric acid reactive substances (TBARS) and the content of lipid radicals) were analyzed. The content of the lipid-soluble antioxidants α-tocopherol (α-TH), β-carotene, and echinenone decreased during gametogenesis. In contrast, the content of both TBARS and lipid radicals increased. However, the consumption of lipid-soluble antioxidants was not sufficient to efficiently control lipid damage since the ratio of TBARS content/α-TH content, taken as an index of damage/protection ratio, significantly increased during the mature stage. Therefore, an active food intake to incorporate antioxidants to the diet is required to adequately prepare the gonad for the next reproductive cycle.

Keywords: α-tocopherol, β-carotene, echinenone, lipid peroxidation, reproductive conditions.

RESUMEN

La hipótesis de este estudio fue que el daño oxidativo en la fase lipídica de las gónadas en el erizo *Loxechinus albus* se incrementa durante la gametogénesis. Se describieron seis condiciones reproductivas (estadios reproductivos) en machos y hembras: (a) inmaduros, (b) crecimiento, (c) premaduro I, (d) premaduro II, (e) maduro y (f) desove. Fueron analizados el contenido de los antioxidantes liposolubles, así como el daño a lípidos (estimados como el contenido de sustancias reactivas al ácido 2-tiobarbitúrico (TBARS) y el contenido de radicales lipídicos). El contenido de los antioxidantes liposolubles α-tocoferol (α-TH), β-caroteno y equinenona disminuyó durante la gametogénesis. En contraste, tanto el contenido de TBARS como el de los radicales lipídicos aumentó. Sin embargo, el consumo de antioxidantes liposolubles no resultó suficiente para controlar en forma eficiente el daño a lípidos, ya que el índice contenido de TBARS/contenido de α-TH, tomado como un indicador de la relación daño/protección, aumentó significativamente durante la madurez gonadal. Por lo tanto, se requiere una activa alimentación que permita la incorporación de antioxidantes mediante la dieta para preparar adecuadamente a la gónada para el próximo ciclo reproductivo.

Palabras claves: α-tocoferol, β-caroteno, equinenona, peroxidación de lípidos, condición reproductiva.

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INTRODUCTION

Reproduction can be one of the most physiologically demanding periods in an animal's life span (Speakman, 2008), and even variations in the environmental conditions could be reflected in the reproductive cycle (Pérez *et al.* 2010) and in the allocation of energy (Pérez *et al.* 2008) of *Loxechinus albus.* The generation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (-OH), takes place continuously in living cells, mainly as a by-product of respiration (Halliwell & Gutteridge, 1989). Once produced, ROS may damage cellular components and tissues, particularly targeting proteins, lipids and nucleic acids, often leading to cumulative organ injury (Lushchak & Bagnyukova, 2006). There is little direct evidence on the connection between oxidative stress and reproduction, even though free radical-dependent reactions are known to play an important role in cell differentiation (Brewster & Wheldrake, 1989). Regarding antioxidant defences, endogenous antioxidants are synthesized by the animal, but exogenous antioxidants, such as lipid-soluble α-tocopherol (α-TH) and carotenoids (β-carotene, echinenone, astaxanthin) are obtained from food (Miller *et al.* 1996; Tummeleht *et al.* 2006). During reproduction of zebra finches, the concentration of specific antioxidants (Wiersma *et al.* 2004) or the overall antioxidant capacity of the blood decreased (Alonso-Álvarez *et al.* 2004, 2006; Bertrand *et al.* 2006). Alonso-Álvarez *et al.* (2004) suggested that reproduction might generate oxidative stress, which conversely could reduce reproductive potential. In addition, it was reported that as male mammals get older they produce more ROS in their testicular tissues and have lower antioxidant defenses. Thus, age-related increases in the rate of oxidative damage and consequent reductions in steroid production were observed (reviewed by Martin & Grotewiel, 2006). Moreover, oocyte quality in females also declines with age, possibly due to oxidative stress (Martin & Grotewiel, 2006).

Loxechinus albus (Molina, 1782) is a species with a wide geographic distribution, from Ecuador (6º S) to the Beagle Channel, south of Tierra del Fuego (54º S) (Bernasconi, 1953; Dayton, 1985). The Beagle Channel population represents the southernmost extension of this species, exposed to temperature variations (5-10ºC, winter-summer average temperatures), day-length variations (7 to 18 h), and marked seasonal fluctuation in primary productivity (Hernando, 2006). Among other factors, seasonal variations in the tissue metabolic rate are assumed to affect the formation of ROS in marine ectotherms (Abele & Puntarulo, 2004), including various mussel species (Viarengo *et al.* 1991; Power & Sheehan, 1996; Wilhelm Filho *et al*. 2001; Malanga *et al.* 2007) as well as in *L. albus* (Malanga *et al*. 2009).

The hypothesis of this study was that oxidative damage in the lipid phase of the gonad of the sea urchin *L. albus* increased during gametogenesis. The content of the lipid-soluble antioxidants α-TH, β-carotene, and echinenone, as well as the damage to lipids were analyzed as a fluction of the described reproductive conditions.

MATERIALS AND METHODS

Study site and sampling

A sample of 30 adult specimens of the sea urchin *Loxechinus albus* (Echinodermata: Echinoidea) was collected once a month by SCUBA divers off the Bridges Islands, Beagle Channel (54° 52' S, 68° 11' W) from May 2004 to May 2005. Specimens from 65 to 85 mm test diameter were selected to minimize the variation in reproductive parameters due to differences in body size (Gonor, 1972). The sea urchins were transported to the Eco-physiology Laboratory of CADIC, and kept in sea water at 7°C for 24 h.

Histological determinations

One gonad from each specimen was fixed in Bouin's solution over 12 h, water washed and transferred to 70% (v/v) alcohol. A cross-section block was dehydrated in alcohol series, cleared in benzene, embedded in Paraplast, sectioned at 5 μm and stained with Groat's hematoxylin and eosin. Sections were examined microscopically and each individual was assigned to one of six male and female reproductive conditions: (a) immature, (b) growing, (c) premature I, (d) premature II, (e) mature, and (f) spawned according to Pérez *et al.* (2010) and grouped for subsequent analysis.

Content of lipid-soluble antioxidants

The content of α-TH, β-carotene and echinenone in the gonads was quantified by reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems LC-4C amperometric detector with a glassy carbon working electrode at an applied oxidation potential of 0.6 V (Desai, 1984). D,L-α-TH and β-carotene (Sigma) and echinenone (CaroteNature GmbH) were used as standard.

Content of 2-thiobarbituric acid reactive substances (TBARS)

Homogenates (150 mg wet weight (WW)/ml) were treated with 30% (w/v) trichloroacetic acid and 50 mM potassium phosphate buffer (pH 7.0). After centrifugation, the content of TBARS was determined in the supernatant according to Malanga *et al.* (2004).

Determination of lipid radical content by Electron Paramagnetic Resonance (EPR)-spin trapping

Lipid radical content was detected by EPR employing a spin trapping technique using N-t-butyl-a-phenyl nitrone (PBN). A 40 mM PBN stock solution was prepared in DMSO immediately prior to use. The homogenates (20 mg WW/ml) were prepared in DMSO-PBN (stock solution). EPR spectra were obtained at room temperature using a Bruker spectrometer ECS 106, operating at 9.81 GHz with 50 kHz modulation frequency. EPR instrument settings for the spin trapping experiments were: microwave power, 20 mW; modulation amplitude, 1.194 G; time constant, 81.92 ms; scans number, 5; center fields, 3480 G; modulation frequency, 50 kHz; and receiver gain, 2 10^4 (Lai *et al.* 1986). Quantification was performed according to Kotake *et al.* (1996).

Statistical analysis

Differences in the content of α-TH, β-carotene and echinenone in the gonads among reproductive conditions were analyzed using a non-parametric test (Kruskal-Wallis). Pairwise differences were analyzed using the unplanned Dunn's multiple comparisons test. Differences in lipid radical content between mature and immature stages were tested using the unpaired *t* test. Differences in the TBARS content and in

the TBARS content/α-TH content ratio among the tested reproductive conditions were analyzed using a one-way analysis of variance (ANOVA). The assumptions of normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene´s test) were also tested. Significant differences were analyzed using the unplanned Tukey-Kramer multiple comparisons test (Sokal & Rohlf, 1995). Statistical analyses were performed with Statistica 6.0 and GraphPad InStat packages.

RESULTS

A standard mix of non-enzymatic antioxidants α-TH, β-carotene and echinenone was analyzed by HPLC, and a typical chromatogram is shown in Fig. 1. Identical profile of elution was observed in samples from *L. albus* gonads. The concentration of lipid-soluble antioxidants was assessed in each group of the sea urchin gonads. Data shown in Fig. 2 indicated that the concentration of α-TH and echinenone varied significantly among the

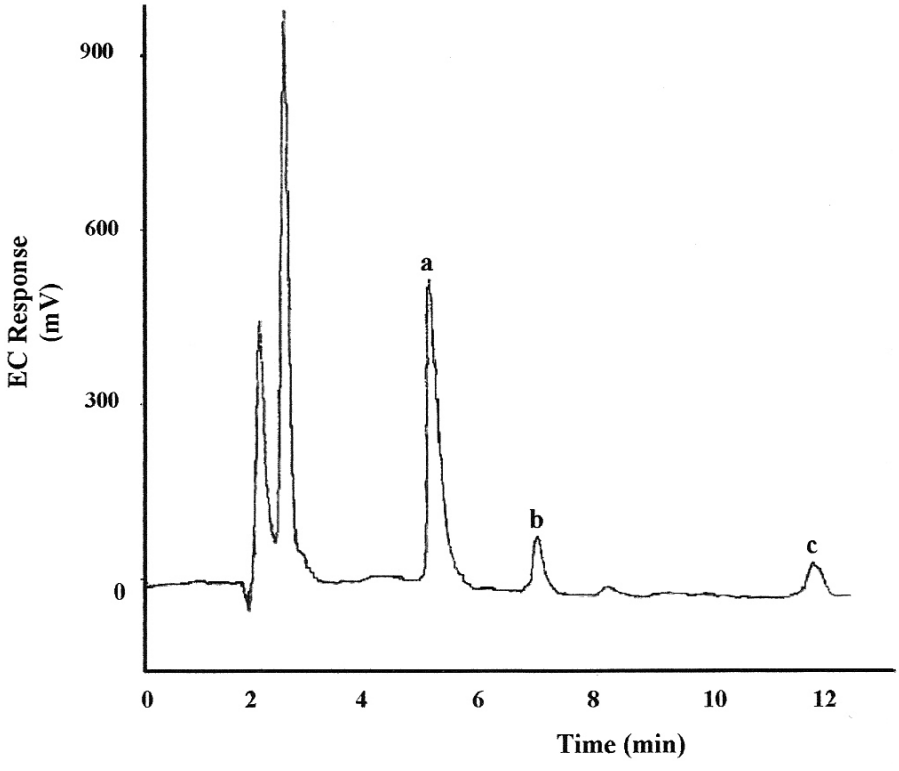

Fig. 1. Determination of the lipid-soluble antioxidants content by the high-performance liquid chromatography (HPLC) method. Elution time for the tested standards were: 5.5 min for α-TH (a), 7 min for echinenone (b), and 12 min for β-carotene (c)

Fig. 1. Determinación del contenido de antioxidantes liposolubles por cromatografía líquida de alta resolución (HPLC). Los tiempos de elución para los estándares fueron para: α-TH 5.5 min (a), equinenona 7 min (b) y β-caroteno 12 min (c)

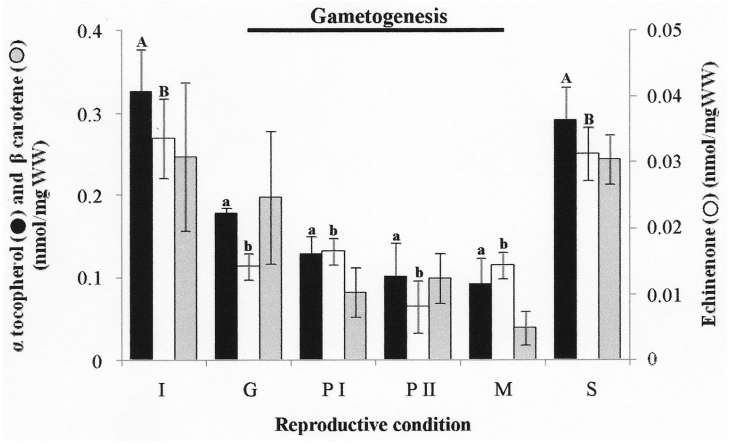

Fig. 2. Content of lipid-soluble antioxidants: α-TH, β-carotene, and echinenone in gonads from *L. albus* during gametogenesis. Data are expressed as median ± S.E.M.; K-W α-TH $P = 0.0017$; echinenone $P = 0.012$. Significant differences in the content of each antioxidant are indicated by the same capital and small letter

Reproductive conditions: I (immature), G (growing), P I (premature I), P II (premature II), M (mature), and S (spawned)

Fig. 2. Contenido de los antioxidantes liposolubles: α-TH, β-caroteno y equinenona en gónadas de *L. albus* durante la gametogénesis. Los datos son expresados como media ± S.E.M.; K-W α-TH $P = 0.0017$; equinenona $P = 0.012$. Las diferencias significativas en el contenido de cada antioxidante son indicadas con la misma letra en mayúscula y minúscula

Condiciones reproductivas: I (inmaduros), G (crecimiento), P I (premaduro I), P II (premaduro II), M (maduro) y S (desove)

reproductive conditions (Kruskal-Wallis $H = 19.27$, $P = 0.0017$ and H: 14.6, $P = 0.012$, respectively). Values were higher in immature and spawned stages (Dunn's Multiple Comparisons Test $P < 0.05$), as compared to the other stages. No significant differences were found between immature and spawned stages ($P > 0.05$). The minimum values were obtained for growing, premature I, premature II, and mature stages ($P < 0.05$), and no significant differences were found among them ($P > 0.05$). The concentration of β-carotene did not vary significantly among the reproductive conditions (Kruskal Wallis H: 3.72, $P = 0.58$), although the described profile was similar to that shown by the other lipid-soluble antioxidants.

The content of TBARS is the most currently used parameter as index of lipid peroxidation (Lattuca *et al.* 2009). TBARS content in the gonads was significantly increased during gametogenesis (One-way Analysis of Variance F: 2.41, $P = 0.049$), showing a higher value in the mature stage as compared to the immature stage (Tukey-Kramer Multiple Comparisons Test $P < 0.05$) (Fig. 3). Lipid radicals from the isolated gonads combined with the spin trap PBN resulted in adducts that gave a characteristic EPR spectrum (Fig. 4, traces b and c) in agreement with computer spectral simulated signals obtained using the same EPR parameters (Fig. 4 trace d). No spin adduct was observed

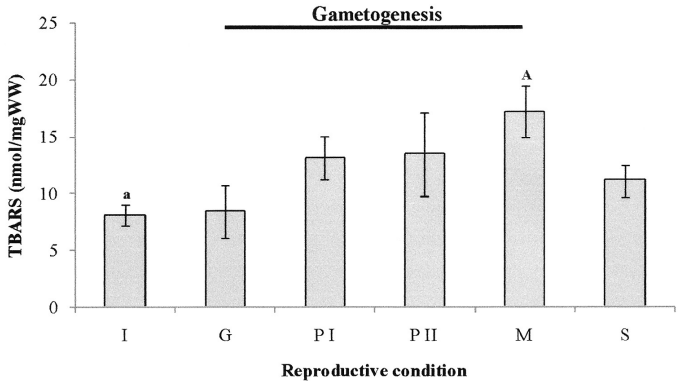

Fig. 3. Content of TBARS in gonads from *L. albus* during gametogenesis. Data are expressed as median ± S.E.M.; ANOVA, $P = 0.04$. Significant differences in the content of each antioxidant are indicated by the same capital and small letter

Reproductive conditions: I (immature), G (growing), P I (premature I), P II (premature II), M (mature), and S (spawned)

Fig. 3. Contenido de TBARS en gónadas de *L. albus* durante la gametogénesis. Los datos son expresados como media ± S.E.M.; ANOVA, $P = 0.04$. Las diferencias significativas en el contenido de cada antioxidante son indicadas con la misma letra en mayúscula y minúscula

Condiciones reproductivas: I (inmaduros), G (crecimiento), P I (premaduro I), P II (premaduro II), M (maduro), y S (desove)

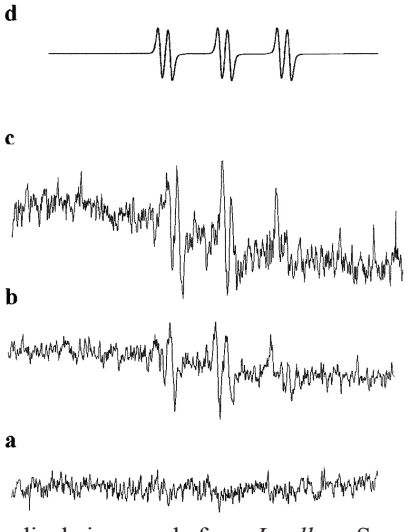

Fig. 4. EPR detection of lipid radicals in gonads from *L. albus*. Spectra in the presence of PBN of: (a) DMSO by itself, (b) immature gonads, (c) mature gonads, and (d) computer-simulated for PBN-lipid radical adduct exhibiting hyperfine splittings $a_N = 15.56$ G and $a_H = 2.79$ G, are shown

Fig. 4. Detección por EPR de radicales lipídicos en gónadas de *L. albus*. Espectros en presencia de PBN de: (a) DMSO solo, (b) gónadas inmaduras, (c) gónadas maduras, y (d) espectro simulado del aducto por computadora caracterizado por $a_N = 15.56$ G y $a_H = 2.79$ G

in the presence of DMSO-PBN (Fig. 4, trace a). Even though EPR signal-constants could be assigned to lipid radicals (Buettner, 1987), spin trapping studies cannot readily distinguish between peroxyl-, alkoxyl- and alkyl-radical adducts, owing to the similarity of the corresponding coupling constants (Buettner, 1987). In agreement with the observed increase on the TBARS content, the lipid radical content was significantly higher in mature gonads (2.1±0.4 pmol/mg WW) as compared to values in immature gonads (1.2±0.1 pmol/mg WW) (Unpaired t test *t*: 2.91, *P*= 0.017).

For the lipophilic compartment, the TBARS content/α-TH content ratio can be understood as an indicator of the balance between free radical-dependent damage and the antioxidant protection (Galleano *et al.* 2002). This index showed significant differences among the reproductive stages (One-way Analysis of Variance *F*: 6.74, *P* = 0.0005) (Fig. 5). At the mature stage, this index increased significantly as compared to the values recorded in samples collected for immature, growing, premature I and spawned stages (Tukey-Kramer Multiple Comparisons Test $P < 0.01$).

DISCUSSION

Previous data from Malanga *et al.* (2009) suggested a differential behavior between the oxidative-dependent pathways triggered at the lipophilic and hydrophilic milieu in *L. albus* gonads at the last stages of the spawning period. The data reported here is the first evidence

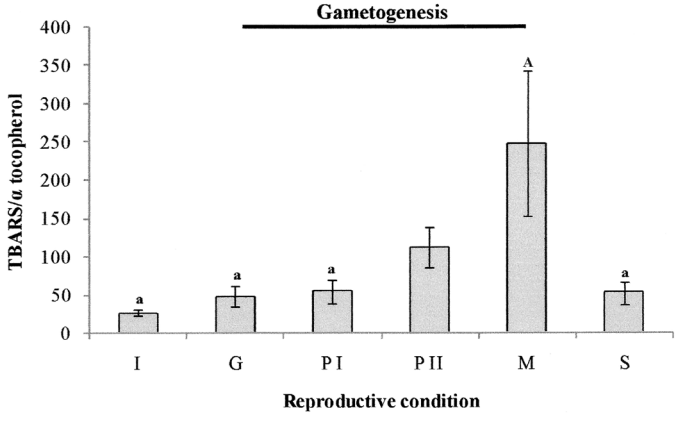

Fig. 5. The TBARS content/α-TH content ratio in gonads from *L. albus* during gametogenesis. Data are expressed as median ± S.E.M.; ANOVA *P* = 0.0005. Significant differences in the content of each antioxidant are indicated by the same capital and small letter
Reproductive conditions: I (immature), G (growing), P I (premature I), P II (premature II), M (mature), and S (spawned)

Fig. 5. Relación contenido de TBARS/contenido de α-TH en gónadas de *L. albus* durante la gametogénesis. Los datos son expresados como media ± S.E.M.; ANOVA, *P* = 0.0005. Las diferencias significativas en el contenido de cada antioxidante son indicadas con la misma letra en mayúscula y minúscula
Condiciones reproductivas: I (inmaduros), G (crecimiento), P I (premaduro I), P II (premaduro II), M (maduro), y S (desove)

in echinoderms, such as *L. albus*, that the content of lipid-soluble antioxidants could be associated with gametogenesis. α-TH is considered one of the most critical factors to control lipid peroxidation in biological membranes (Yamamoto *et al*. 1988), and its concentration has been used in many biological systems as an indicator of the membrane protection ability against harmful oxidants (Malanga *et al.* 2009). The decline in α-TH content over gametogenesis strongly indicates an active generation of ROS over this period leading to the antioxidant consumption. However, carotenoids should also be taken into account. The functions of carotenoids are currently being intensively studied, since they are found among marine invertebrates and are frequently the reason for their coloration (Karnaukhov, 2000). Carotenoid pigments that can bind to singlet oxygen and convert them into less-damaging H_2O_2 (Krinsky, 1989) showed strong influence on the growth and survival of the organisms (Tsushima & Matsuno, 1998; George *et al.* 2001). In addition, the high content of these pigments in the gonads of many species of animals suggested the need for carotenoids in the reproductive cycle (Goodwin, 1980). Tsushima *et al.* (1993) reported that the bioconversion of β,β-carotene into echinenone via b-isocryptoxanthin in sea urchins mainly takes place in the viscera (gut wall), and the resulting echinenone is incorporated into the gonad (ovary and testis), where it may play an important role in the biological antioxidant protection network of the sea urchins (Kawakami *et al*. 1998). Carotenoids are incorporated from the diet in most animals (Buchecker, 1982). In the sea urchins, carotenoids are transferred to the growing oocyte through nutritive phagocytes (Walker *et al*. 2001; Plank *et al*. 2002). The relative amount of each carotenoid varies among species (Matsuno & Tsushima, 2001). Echinenone is the main final carotenoid and the most important in the gonads of many animals (Tsushima & Matsuno, 1990; Tsushima *et al.* 1997; Kawakami *et al.* 1998; Matsuno & Tsushima, 2001), where it showed antioxidant functions (Miller *et al.* 1996; Mortensen *et al*. 1997). The content of carotenoids in *Strongylocentrotus intermedius* is the highest at the spawned stage of gonad maturation for both sexes, meanwhile for *S. nudus*, the content of these pigments is the highest at stages of active gametogenesis and spawning for males and at growing stage for females (Borisovets *et al.* 2002). However, in both *S. intermedius* and *S. nudus*, Borisovets *et al.* (2002) reported that the carotenoid content suddenly decreased after spawning by the loss of pigments through gametes. Eggs from both species are pigmented; however, in males where pigments are not accumulated in the gametes, a decrease in the carotenoid content was observed. Moreover, Lamare & Hoffman (2004) reported that four species of *Strongylocentrotus* increased gonadal concentration of carotenoids after spawning. The data reported here employing *L. albus* clearly showed that the accumulated carotenoids decreased during gametogenesis and increased after spawning. This profile could respond to the fact that the pigment was consumed over the gametogenesis, followed by a phase of accumulation after spawning. Moreover, diet could alter *L. albus* gonads lipid composition and subsequently the nature of lipid radical generation (Brazão *et al.* 2003) and affect the effectiveness of the lipid-soluble antioxidants. Changes in saturation of fatty acids seem to depend also on temperature, and

many authors consider them to be adaptive reactions to support membrane fluidity (Lukyanova & Khotimchenko, 1995). An increase in the oxidative condition in the lipophilic phase could be predicted in the mature stage as a consequence of the increased metabolic activity and the changes in the physiological status. Lipid peroxidation could be postulated as a required physiological event for the gonadal maturation (e.g. release of eggs and sperm from the organ) by making the lipids available and thus, oxidation could contribute to the process. This hypothesis supports the model previously postulated where low temperatures lead to higher degrees of unsaturation in fatty acids (Abele & Puntarulo, 2004). On the other hand, it is known that all synthetic processes slow down in the pre-spawning stage including protein synthesis (Lukyanova & Khotimchenko, 1995) and accordingly, a decrease in lipid peroxidation in this period has been previously described in *M. galloprovincialis* digestive organs (Viarengo *et al.* 1991). After spawning, a period characterized by the absence of gametogenetic activity, reabsorption of unshed gametes and membrane lysis, along with reparation of damaged tissue and formation of new membranes structures has been described (Lukyanova & Khotimchenko, 1995).

Taken as a whole the data presented here confirmed the hypothesis that there is an increase in the oxidative damage in the lipid phase in the gonads of the sea urchin *L. albus*, accompanied by a significant decrease in the content of the lipid-soluble antioxidants (α-TH and echinenone) during gametogenesis. The consumption of the lipid-soluble antioxidants does not seen to be sufficient to efficiently control lipid damage since the TBARS content/α-TH content ratio significantly increased during the mature stage. Overall, this data strongly supports the strict requirement of an active food intake to recover lost antioxidants before starting the next reproductive cycle, suggesting a close interaction between the organism and the nature of the environment.

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REFERENCES

Abele, D. & Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comp. Biochem. Physiol. A., 138*, 405-415.

Alonso-Álvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., & Sorci, G. (2004). Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol. Lett.*, *7*(5), 363-368.

Alonso-Álvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Chastel, O., & Sorci, G. (2006). An experimental manipulation of life history trajectories and resistance to oxidative stress. *Evolution*, *60*(9), 1913-1924.

Bernasconi, I. (1953). *Monografía de los Equinoideos argentinos*. *An. Mus. His. Nat.*, *Montevideo*, 2nd Series *6*(2), 23-25.

Bertrand, S., Alonso-Alvarez, C., Devevey, G., Faivre, B., Prost, J., & Sorci, G. (2006). Carotenoids modulate the

trade-off between egg production and resistance to oxidative stress in zebra finches. *Oecologia*, *147*, 576-584.

Borisovets, E. E., Zadorozhny, P. A., Kalinini, M. V., Lepskaya, N. V., & Yakush, E. V. (2002). Change of major carotenoids in gonads of sea urchins (*Strongylocentrotus intermedius* and *S. nudus*) at maturation. *Comp. Biochem. Physiol. B., 132*(4), 779-790.

Brazão, S., Morais, S., Boaventura, D., Ré, P., Narciso, L., & Hawkins, S. J. (2003). Spatial and temporal variation of the fatty acid composition of *Patella* spp. (Gastropoda: Prosobranchia) soft bodies and gonads. *Comp. Biochem. Physiol. B., 136*, 425-441.

Brewster, N. K. & Wheldrake, J. F. (1989). Free radical scavenging agents during the development of *Dictyostelium discoideum. Biochem. Int., 19*, 439-444.

Buchecker, R. (1982). A chemist's view of animal carotenoids. In G. Britton & T. W. Goodwin (Eds.), *Carotenoid chemistry and biochemistry*. (pp. 175-193). Oxford, England.: Pergamon Press.

Buettner, G. R. (1987). Spin trapping: ESR parameters of spin adducts. *Free Radic. Biol. Med., 3*, 259-303.

Dayton, P. K. (1985). The structure and regulation of some South American kelp communities. *Ecol. Monographs, 55*(4), 447-468.

Desai, I. (1984). Vitamin E analysis methods for animal tissues. *Meth. Enzymol., 105*, 138-147.

Galleano, M., Aimo, L., & Puntarulo, S. (2002). Ascorbyl radical/ascorbate ratio in plasma from iron overloaded rats as oxidative stress indicator. *Toxicol. Lett., 133*(2, 3), 193-201.

George, S. B., Lawrence, J. M., Lawrence, A. L., Smiley, J., & Plank, L. (2001). Carotenoids in the adult diet enhance egg and juvenile production in the sea urchin *Lytechinus Variegatus. Aquaculture, 199*(3, 4), 353-369.

Gonor, J. J. (1972). Gonad growth in the sea urchin *Strongylocentrotus purpuratus* (Stimpson) (Echinodermata: Echinoidea) and the assumptions of gonad index methods. *J. Exp. Mar. Biol. Ecol., 10*(2), 89-103.

Goodwin, T. W. (1980). *The biochemistry of the carotenoids. Vol. I, Plants*. 2nd ed. London, England.: Chapman and Hall.

Halliwell, B. & Gutteridge, J. M. C. (1989). *Free radicals in biology and medicine*. 2nd ed. Oxford.: Clarendon Press.

Hernando, M. P. (2006). *Efectos de la radiación solar sobre el fitoplancton de aguas Antárticas y sub-Antárticas*. Unpublished doctoral thesis, Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina.

Karnaukhov, V. N. (2000). Functions of carotenoids as a subject of biophysical investigation. *Biophysics*, *45*, 355-377.

Kawakami, T., Tsushima, M., Katabami, Y., Mine, M., Ishida, A., & Matsuno, T. (1998). Effect of ß,ß-carotene, ß-echinenone, astaxanthin, fucoxanthin, vitamin A and E on the biological defense of the sea urchin in *Pseudocentrotus depressus*. *J. Exp. Mar. Biol. Ecol.*, *226*(2), 165-174.

Kotake, Y., Tanigawa, T., Tanigawa, M., Ueno, I., Allen, D. R., & Lai, C. (1996). Continuous monitoring of cellular nitric oxide generation by spin trapping with an iron–dithiocarbamate complex. *Biochim. Biophys. Acta, 1289*(3), 362-368.

Krinsky, N. I. (1989). Antioxidant functions of carotenoids. *Free Radic. Biol. Med., 7*(6), 617-635.

Lai, E. K., Crossley, C., Sridhar, R., Misra, H. P., Janzen, E. G., & McCay, P. B. (1986). In vivo spin trapping of free radicals generated in brain, spleen, and liver during gamma radiation of mice. *Arch. Biochem. Biophys., 244*(1), 156-160.

Lamare, M. D. & Hoffman, J. (2004). Natural variation of carotenoids in the eggs and gonads of the echinoid genus, *Strongylocentrotus*: implications for their role in ultraviolet radiation photoprotection. *J. Exp. Mar. Biol. Ecol., 312*(2), 215-233.

Lattuca, M. E., Malanga, G., Aguilar Hurtado, C., Pérez, A. F., Calvo, J., & Puntarulo, S. (2009). Main features of the oxidative metabolism in gills and liver of *Odontesthes nigricans* Richardson (Pisces, Atherinopsidae). *Comp. Biochem. Physiol. B, 154*(4), 406-411.

Lukyanova, O. N. & Khotimchenko, J. S. (1995). Lipid-peroxidation in organs of the scallop *Mizuhopecten yessoensis* and sea-urchin *Strongylocentrotus intermedius* during the reproductive cycle. *Biol. Scien., 110* (2), 371-377.

Lushchak, V. I. & Bagnyukova, T. (2006). Effects of different environmental oxygen levels on free radical processes in fish. *Comp. Biochem. Physiol. B* Biochem Mol Biol*., 144*(3), 283-289.

Malanga, G., Estevez, M. S., Calvo, J., & Puntarulo, S. (2004). Oxidative stress in limpets exposed to different environmental conditions in the Beagle Channel. *Aquat. Toxicol*., *69*(4), 299-309.

Malanga, G., Estevez, M. S., Calvo, J., Abele, D., & Puntarulo S. (2007). The effect of seasonality on oxidative metabolism in *Nacella* (*Patinigera*) *magellanica*. *Comp. Biochem. Physiol. A*, *146*, 551-558.

Malanga, G., Pérez, A. F., Calvo, J., & Puntarulo, S. (2009). The effect of seasonality on oxidative metabolism in the sea urchin *Loxechinus albus*. *Mar. Biol., 156*, 763-770.

Martin, I. & Grotewiel, M. S. (2006). Oxidative damage and age-related functional declines. *Mech. Ageing Dev.*, *127*(5), 411-423.

Matsuno, T. & Tsushima, M. (2001). Carotenoids in sea urchins. In J. M. Lawrence (Ed.), *Edible sea urchins: biology and ecology*. (pp. 115-138). Amsterdam, The Netherlands.: Elsevier Science.

Miller, N. J., Sampson, J., Candeias, L. P., Bramley, P. M., & Rice-Evans, C. A. (1996). Antioxidant activities of carotenes and xanthophylls. *FEBS Lett., 384*(3), 240-242.

Mortensen, A., Skibsted, L. H., Sampson, J., Rice-Evans, C. A., & Everett, S. A. (1997). Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. *FEBS Lett., 418*(1, 2), 91-97.

Pérez, A. F., Morriconi, E., Boy, C., & Calvo, J. (2008). Energetic variation of the sea urchin *Loxechinus albus* at the southernmost limit of their distribution range (Beagle Channel, Tierra del Fuego). *Polar Biol., 31*, 443-449.

Pérez, A. F., Boy, C., Morriconi, E., & Calvo, J. (2010). Reproductive cycle and reproductive output of the sea urchin *Loxechinus albus* (Echinodermata: Echinoidea) from Beagle Channel, Tierra del Fuego, Argentina. *Polar Biol., 33*, 271-280.

Plank, L. R., Lawrence, J. M., Lawrence, A. L., & Montoya Olvera, R. (2002). The effect of dietary carotenoids on gonad

production and carotenoid profiles in the sea urchin *Lytechinus variegatus*. *J. World Aquacul. Soc.*, *33*(2), 127-137.

Power, A. & Sheehan, D. (1996). Seasonal variations in the antioxidant defense systems of gill and digestive gland of the blue mussel, *Mytilus edulis*. *Comp. Biochem. Physiol. C, 114*(2), 99-103.

Sokal, R. & Rohlf, F. J. (1995). *Biometry: The principles and practice of statistics in biological research*. New York, USA.: W. H. Freeman and Company.

Speakman, J. R. (2008). The physiological costs of reproduction in small mammals. *Phill. Trans. R. Soc. Lond. B Biol. Sci*., *363*, 375-398.

Tsushima, M. & Matsuno, T. (1990). Comparative biochemical studies of carotenoids in sea urchins-I. *Comp. Biochem. Physiol. B, 96*(4), 801-810.

Tsushima, M., Kawakami, T., & Matsuno, T. (1993). Metabolism of carotenoids in sea urchin *Pseudocentrotus depressus*. *Comp. Biochem. Physiol. B, 106*(3), 737-741.

Tsushima, M., Kawakami, T., Mine, M., & Matsuno, T. (1997). The role of carotenoids in the development of the sea urchin *Pseudocentrotus depressus. Invert. Reprod. Develop., 32*, 149-153.

Tsushima, M. & Matsuno, T. (1998). The role of beta-carotene on growth and survival of juvenile Japanese abalone *Haliotis discus*. *Fish. Sci., 64*(4), 660-661.

Tummeleht, L., Mägi, M., Kilgas, P., Mänd, R., & Hõrak, P. (2006). Antioxidant protection and plasma carotenoids of incubating great tits (*Parus major L.*) in relation to health state and breeding conditions. *Comp. Biochem. Phys. C, 144*(2), 166-172.

Viarengo, A., Canesi, L., Pertica, M., & Livingstone, D. R. (1991). Seasonal variations in the antioxidant defence enzymes and lipid peroxidation of the digestive gland of mussels. *Comp. Biochem. Physiol. C*, *100*, 187-190.

Walker, C. W., Unuma, T., McGinn, N. A., Harrington, L. M., & Lesser, M. P. (2001). Reproduction of sea urchins. In J. M. Lawrence (Ed.), *Edible sea urchins: biology and ecology* (pp. 5-26). Amsterdam, The Netherlands.: Elsevier Science.

Wiersma, P., Selman, C., Speakman, J. R., & Verhulst, S. (2004). Birds sacrifice oxidative protection for reproduction. *Proc. R. Soc. Lond. B Biol.*, *271*, S360-S363.

Wilhelm Filho, D., Tribess, T. B., Gáspari, C., Claudio, F. D., Torres, M. A., & Magalhães, A. R. M. (2001). Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). *Aquaculture, 203*(1, 2), 149-158.

Yamamoto, M., Ishine, M., & Yoshida, M. (1988). Gonadal maduration independient of photic conditions in laboratory-reared sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*. *Zool. Sci. (Tokyo)*, *5*(5), 979-988.