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Title of Manuscripts: "Effect of dietary carotenoids on lipoperoxidation in mature sea urchins Loxechinus albus (Echinodermata: Echinoidea)"

Dear Dr **Analía F. Pérez,**

The article entitled **"Effect of dietary carotenoids on lipoperoxidation in mature sea urchins Loxechinus albus (Echinodermata: Echinoidea)"(authours: Analía F. Pérez, María Eugenia Lattuca, Cyntia Fraysse & Gabriela Malanga)**had been accepted for publication in Indian Journal of Marine Sciences subjected to minor corrections and editorial modifications. It is expected to appear in the June 2015 issue of Indian Journal of Marine Sciences.

With Regards
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Thanks

Effect of dietary carotenoids on lipoperoxidation in mature sea urchins *Loxechinus albus* (Echinodermata: Echinoidea)

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Present study consists the effects of dietary carotenoids on the gonad and gut index, colour of gonads, carotenoid pigments and lipid peroxidation in the gonads and gut of the sea urchin *Loxechinus albus*. Individuals were fed for 8 weeks with the following diets: (A) fresh algae, (B) and (C) enriched with 0.02% and 0.045% all trans β-carotene, respectively. After treatment, no significant differences were found in total weight, test diameter, gonad and gut indexes but gonads of *L. albus* fed on Diet C showed a better color than those fed on other diets. Histological analysis showed differences in the gonad reproductive condition among treatments. Content of β-carotene in gonads and gut showed no significant differences among treatments, neither between organs. The content of echinenone in gonads was significantly higher than in gut for Diet C and the field control. Lipid radical content was lower in gonads than in gut for Diet C. The content of α-tocopherol in gonads was higher in sea urchins fed on Diet C and in the field control, than in sea urchins fed on Diet A and B.

[**Keywords**: β-carotene, α-tocopherol, echinenone, reproduction]

Introduction

Carotenoids have an important biological role in growth, survival and reproductive cycles[1]. Particularly, dietary carotenoids are considered essential in sea urchin aquaculture because of their effect on gonad colour[2], which is a relevant commercial characteristic. The optimal colour is bright yellow-orange[3]. Gonad colour can be related to the reproductive condition in some sea urchins; mature gonads show brighter colour while recovering, growing or premature stages show less desirable colour[3,4]. Moreover, palatability can be associated with colour; bright yellow gonads are sour and those with a slight greenish are bitter[3].

The mechanisms by which carotenoids exert their benefits are not completely understood, but may be due in part to their antioxidant activities, by quenching singlet oxygen, scavenging radicals to terminate chain reactions and avoiding lipid peroxidation[5]. In this regard, peculiarities of membrane lipids in marine organisms, particularly high contents of unsaturated fatty acids[6], suggest a special pattern for lipid peroxidation and a complex system of antioxidants operative with regulatory purposes[7]. Changes on reactive oxygen species (ROS) production on aquatic organisms has been awarded to ambient changes (hypoxia, temperature differences, intoxications, UV radiation, food quality and availability) or physiological changes (age, seasonality/reproductive cycle)[8].

Loxechinus albus (Molina, 1782) is a sea urchin species with a wide geographic distribution, from Ecuador (6°S) to the Beagle Channel, south of Tierra del Fuego (54°S)[9]. It is considered one of the most economically significant resources from the littoral-benthic systems in the South Pacific Ocean. Natural stocks on Chilean coasts were reduced or depleted due to excessive harvest for commercial purposes. This situation provides opportunities for sea urchin aquaculture and roe enhancement. High gonadal production by feeding with artificial diets has been reported for *L. albus*[10] among others.

Several studies focused on the evaluation of the effect of food on gonadosomatic index, yield and quality of sea urchins. However, little is known about the effect carotenoids additioned to artificial diets may have on the lipids of sea urchins. Particularly, Liyana-Pathirana *et al*[11] demonstrated the importance of feed supplied with carotenoids in the lipid composition of sea urchin gonads. Although most of the studies dealing with gonad enhancement are usually carried out during pre-spawning periods, when gonads are naturally increasing[10,12], some other works showed that the gonads of post-spawned urchins may also be enhanced in the laboratory[13,14].

In this study, we propose that it is possible to reduce the process of lipoperoxidation in mature sea urchins through the administration of artificial diets enriched with carotenoids. This reduction will lead to the enhancement of gonadal organoleptic traits as well as to the prolongation of the harvesting season, through a delay in the onset of spawning. To test this hypothesis we investigate the effects of dietary carotenoids on the gonad and gut index, gonad colour, carotenoid pigments and lipid peroxidation in the gonad and gut of mature *Loxechinus albus*.

Materials and Methods

Sea urchin collection and maintenance

Thirty adults of *Loxechinus albus* (70-80 mm test diameter and 160-200 mg total weight) (Fig. 1) were collected by SCUBA divers on July 27th 2005 from the rocky cobble bottoms of Bridges Islands (Tierra del Fuego, Argentina, 54º 52’S, 68º 14’W).

Sea urchins were carried to the laboratory in plastic boxes with sea water within 2 h of collection. Each sea urchin was weighed (± 0.01 g), and maximum diameter (through the madreporic plate) and height were measured using an electronic calliper (± 0.1 mm). Thirty individuals were placed in plastic tanks with sea water at 7ºC for 20 days. The quality of the water was carefully controlled, measuring the concentration of nitrates and nitrites. Water was changed every 3 days. Photoperiodic conditions were: 12h light/12h dark.

In order to standardise relative hunger levels, sea urchins were starved for twenty days prior to the beginning of the experiment. No sea urchins died during this period.

Experimental protocol

The experiment was conducted for 8 weeks (August-October 2005) between the period of maximum gonadal development and the onset of spawning. Three different feeds were used: Diet A, (fresh kelp, *Macrocystis pyrifera*) and 2 artificial diets, Diet B (low β-carotene concentration, 0.02% dry weigth) and Diet C (high β-carotene concentration, 0.045% dry weigth). Food was supplied *ad libitum* and removed every 3 days.

Animals were assigned to one of 3 experimental groups, so that each group had equal number of individuals (n= 10). Groups were then randomly assigned to one of the three feeding treatments. Within each treatment, 10 replicates were established with one sea urchin per replicate. Sea urchins were placed in individual containers with independent supply of seawater, and a mesh at the bottom which retained the food but allowed faeces to pass through into an outer plastic bucket. No significant differences were found among individuals asigned to different treatment with respect to weigth and test diameter (One way ANOVA, *P*> 0.05 for both). After 8 weeks all experimental individuals were dissected. In order to establish a field control, 10 sea urchins were collected and dissected.

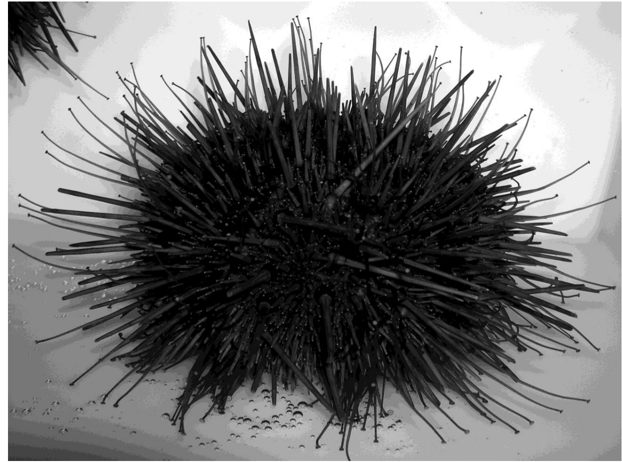

Figure 1. Adult individual of *Loxechinus albus*.

Diet preparations and algae collection

Fresh algae (*Macrocystis pyrifera*) were collected weekly, from mid-August to early October, at the same place where sea urchins were captured. Algae were carried to the lab immediately after collection, and as many epibionts as possible were removed.

Ingredients used in diets are shown in table. Kelp meal (*M. pyrifera*) was produced in our lab. Soybean meal, corn starch, wheat, lecithin, gelatin and corn oil were purchased from a local supermarket. Magnesium oxide, dicalcium phosphate, Vitamin C, ethoxyquin and β-carotene were purchased from Sigma. For vitamins and minerals mix, and fish meal see acknowledgements. Dry ingredients were mixed with hot freshwater (~100ºC) and extruded by a manual machine as a moist pellet (0.5 x 15 mm). These pellets were then air dried at ~15ºC and stored at ambient temperature in hermetic plastic bags.

Table . Ingredients used in artificial diets. Data are expressed as % of dry weight.

Ingredients	Diet B	Diet C
Kelpl meal (*Macrocystis pyrifera*)	14.00	14.00
Soybean meal	13.00	13.00
Corn starch	24.53	24.505
Wheat	23.00	23.00
Fish meal	12.00	12.00
β-carotene	0.02	0.045
Lecithin	2.00	2.00
Corn oil	4.00	4.00
Dicalcium phosphate	1.80	1.80
Ethoxyquin	0.20	0.20
Vitamin C	0.10	0.10
Vitamins/minerals *	0.10	0.10
Magnesium oxide	0.25	0.25
Gelatin	5.00	5.00

* The mixture is made up of (expressed in mg or UI kg^{-1} of feed): tocopherol acetate: 70.8UI, ascorbic acid: 283mg, thiamin: 7.1mg, riboflavin: 7.6mg, pyridoxine: 9.4mg, cyanobalanine: 0.014mg, biotine: 0.47mg, Folic acid: 1.89mg, calcium pantothenate: 23.6mg, vit A: 710UI, vit D: 700UI, niacin: 14.6mg, $CaCO_3$: 2.1mg, $CuSO_4$: 9.4mg, $FeSO_4$: 4.7mg, $MgCO_3$:·174mg, $MnSO_4$: 18.9mg, $CaHPO_4$: 75.5mg, $ZnSO_4$: 7.7mg.

Gonad and gut index

Sea urchins were allowed to drip on a paper towel for approximately one minute before the whole animal was weighed (wet weight). Animals were dissected and gonad and gut (voided of food content) weighed separately (□ 0.01 g). Gonad and gut indexes (IG and IGut) were calculated as organ wet weight (g) x 100/total wet weight (g).

Reproductive conditions and gonad colour

For histological observations, gonads were sectioned at 5 µm and stained with Groat's hematoxylin and eosin. Sections were examined microscopically and gonad stages were assigned following the maturity scale described by Pérez *et al*[15]. For commercial purposes is unnecessary separated by sex.

Gonad colours were determined using 25 different paint-card samples (designed by University of Maine, USA) that were later converted to a category from 1-4[12]. Colour was always assessed under standardised light conditions and quantified as follows:

Gonad colour: subjectively by eye with paint samples (category 1-4)

Category 1= bright yellow or orange

Category 2= pale yellow or orange, mustard

Category 3= yellow-brown, orange-brown, red-brown, cream

Category 4= any other colour (*e.g.,* dark brown, grey)

Pigments and α-tocopherol

Total carotenoid content was determined by the method of McBeth[16]. Carotenoids from each gonad and gut sample were extracted with a total of 1 ml acetone for 30 min at 4ºC. Then 1.2 ml of hexane and 3 ml of 5% sodium chloride solution were added at the homogenized samples. After centrifugation at 600×g for 10 min, the hexane phase was transferred into a 15 ml volumetric flask and made up to volume. The absorption spectrum was recorded at 400-600 nm, using a Beckmann DU Serie 7400, diode array spectrophotometer. The total carotenoids present in 100 g of tissue were calculated using the following equation:

$$\text{mg carotenoid/100 g tissue} = (A \times V \times 10^3)/(\square \times W)$$

where, A= absorbance at $\square_{max}$; V= total volume of the sample (ml); □= molar extinction coefficient and W= weight of the tissue (g). Since the crude extracts usually contained a variety of carotenoids, an average coefficient of 2500 was used in the calculations.

The content of α-tocopherol, β-carotene and echinenone in gonad and gut were 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 0.6V[8]. Samples were extracted with 1 ml of ethanol and 4 ml of hexane. After centrifugation at 600×g for 10 min, the hexane phase was removed and evaporated to dryness under N_2. Extracts were dissolved in

methanol/ethanol (1:1) and injected for HPLC analysis[8]. D,L α-Tocopherol (Sigma), echinenone (CaroteNature GmbH), and β-carotene (Sigma) were used as standards. The content of pigment and α-tocopherol were expressed by wet weight (WW).

Content of thiobarbituric acid reactive substances (TBARS)

The homogenates 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*[8].

Lipid radical content

Lipid radicals were detected by a spin trapping technique using N t butil α phenyl nitrone (PBN). A 40 mM PBN stock solution was prepared in DMSO immediately prior to use. The homogenates 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 fiels, 3480 G; modulation frecuency, 50 kHz; and receiver gain, 2 10^4. Quantification was performed according to Kotake[17].

Statistical analyses

The effects of diets on gonad and gut indices and content of total carotenoids, β-carotene, echinenone, TBARS, lipid radical and α-tocopherol were analyzed using an analysis of variance (One-way ANOVA). The assumptions of normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) were tested. When necessary, logaritmic transformations were applied.
The content of total carotenoids, β-carotene, echinenone, TBARS, lipid radical and α-tocopherol in gonads and gut of individuals for each diet and field control were analyzed using paired t test. Statistical analyses were performed with Statistica 6.0.

Results

Survival rates were 100% for Diet A and Diet B (low β-carotene concentration) and 90% for Diet C (high β-carotene concentration). Gonad index (mean= 9.31, SD= 3.31, n= 40) and Gut Index (mean= 2.80, SD= 0.30, n= 40) did not vary significantly among the diet treatments and the field control (One way ANOVA, $P> 0.05$ for both) (Fig. 2a,b). Photomicrographs of gonad stages were indicated in Figure 3. Histological analysis showed differences in the frequency of gonad reproductive condition among treatments (Fig. 4). While immature sea urchins (Fig. 3a) were only found in Diet A and B, mature (Fig. 3b) and spawned (Fig. 3c) *L. albus* were represented in all treatments. Mature and spawned sea urchins showed the highest percentage in Diet C and field control respectively. The mean contents of total carotenoids were 1.65 ± 1.86 mg 100 g^{-1} WW (n= 20) in gonads and 11.27 ± 4.14 mg 100 g^{-1} WW (n= 20) in gut. They did not show significant differences among treatments (gonads One way ANOVA, $P> 0.05$ for both) (Fig. 5a). Total carotenoids in gut were significanthy higher than those in gonads for Diet B, Diet C and the field control (Paired t test, $P< 0.01$; $P< 0.01$ and $P< 0.05$, respectively). Values for Diet A were not significantly different between organs (Paired t test, $P> 0.05$) (Fig. 5a). Mean contents of β-carotene were 0.015 ± 0.011 nmol mg^{-1} WW (n= 20) in gonads and 0.011 ± 0.018 nmol mg^{-1} WW (n= 20) in gut. Neither gonads nor gut contents of β-carotene showed significant differences among treatments (One way ANOVA, $P> 0.05$ for both) (Fig. 4b). For each diet (Diet A, B, C and field control), the content of β-carotene was not significantly different between organs (Paired t test, $P> 0.05$ for both) (Fig. 5b).
Mean contents of echinenone were 0.10 ± 0.06 nmol mg^{-1} WW (n= 20) in gonads and 0.014 ± 0.018 nmol mg^{-1} WW (n= 20) in gut. Gonad and gut contents of echinenone were not significantly different among treatments (One way ANOVA, $P> 0.05$ for both) (Fig. 5c). Gonad content of echinenone was significantly higher than in gut for Diet C and the field control (Paired t test, $P< 0.05$ for both). No significant differences were found for Diet A and B (Paired t test, $P> 0.05$ for both) (Fig. 5c).
Three categories of gonad colours were found in this study (Fig. 6). Gonads of *L. albus* fed on Diet C showed a better color than those fed on other diets, with 30% of category 2, 40% of category 3 and 30% of category 4. Gonads of *L. albus* fed on Diet A and B produced unacceptable gonads for the commercial roe industry, and gonad colours of the field control were the worst, with 100% of the gonads in category 4 (Fig. 6). Lipid peroxidation in gonads and gut were estimated, both as the content

of thiobarbituric acid-reactive substances (Fig. 7a), and as the content of lipid radicals assessed by EPR (Fig. 7b). Mean contents of TBARS were 16.85 ± 4.47 nmol mg^{-1} WW (n= 20) in gonads and 25.91 ± 7.90 nmol mg^{-1} WW (n= 20) in gut. Evaluations of oxidative damage of lipids through TBARS content in gonads and gut did not show significant differences among treatments (One way ANOVA, $P > 0.05$ for both) (Fig. 7a). For each diet (Diet A, B, C and field control), the content of TBARS was not significantly different between organs (Paired t test, $P > 0.05$ for all) (Fig. 7a). Lipid radicals in the samples combined with the spin trap PBN resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of a_N= 15.56 G and a_H= 2.79 G, in concordance with computer spectral simulated signals obtained using the overall mentioned parameters. PBN itself was examined and no PBN spin adduct was observed. The mean content of lipid radicals in gonads was 0.56 ± 0.35 pmol mg^{-1} WW (n= 20) and in gut was 1.14 ± 0.94 nmol mg^{-1} WW (n= 20). When oxidative damage of lipids was evaluated by EPR, the lipid radical content did not show significant differences among treatments, neither for gonads nor for gut (One way ANOVA, $P > 0.05$, for both). For Diet C, the lipid radical content was significantly lower in gonads than in gut (Paired t test, $P < 0.05$). For Diets A, B and the field control, no significant differences were found (Paired t test, $P > 0.05$ for all) (Fig. 7b).

Mean contents of α tocopherol were 0.27 ± 0.15 nmol mg^{-1} WW (n= 20) in gonads and 7.7 10^{-3} ± 9.2 10^{-3} nmol mg^{-1} WW (n= 20) in gut. Gonad and gut contents of α tocopherol were not significantly different among treatments (One way ANOVA, $P >$ 0.05, for both) (Fig. 8). For Diet C and the field control, α-tocopherol was significanthy higher in gonads than in gut (Paired t test,$P < 0.01$, for both), and for Diets A and B no significant differences were found (Paired t test, $P > 0.05$, for both) (Fig. 8).

Discussion

Commonly, the dynamics of commercial markets have not been in phase with the life cycle of sea urchins, or the harvest period short[14,15]. One method of addressing the discordance between gonadal cycles and market demand is to enhance gonads artificially. Gonad enhancement can be done in the laboratory or in the field using artificial or natural diets[18]. As can be seen from our results, none of the employed diets, with or without added β-carotene, affected growth parameters and indexes (Fig. 2) during the period of maximum gonadal development and the onset of spawning. These results agree with the observations of Plank *et al*[2] and Shpigel *et al*[19], who showed that carotenoids were not essential for gonad enhanced in the sea urchins *Lytechinus variegatus* and *Paracentrotus lividus*, respectively. As individuals were sampled in winter only three reproductive stage were found[15]. The changes observed in the reproductive stages of individuals fed on Diet C (Fig. 4) suggest that carotenoids have a role in the reproductive condition, showing a high percentage of mature individuals and thus, delay the onset of spawning. Matsuno & Tsushima[20] showed that carotenoids are involved in the production of nutritive phagocytes during the growing state, or in the production of gametes during the gametogenic stage. The gonads of sea urchins have nutritive phagocytes that store the nutrients used in gametogenesis[21]. Carotenoids probably are first stored in the nutritive phagocytes and then transferred to the developing ova[2]. Adult *Strongylocentrotus droebachiensis* fed on an artificial diet containing β-carotene produced eggs with higher fertilization rates and higher larval survival than individuals fed on a diet without it[22]. Carotenoids in the diet increased gamete production, ova size, and larval survival to metamorphosis in *L. variegatus*[23].

Total carotenoid concentrations were higher in gut when sea urchins were fed on Diets B, C, and in the field control (Fig. 5a). Similar results were found by Plank[2] in the sea urchin *L. variegatus*. Contents of β-carotene were similar in gonads and gut (Fig. 5b). The ratio mean β-carotene content/mean echinenone content in the gut was usually approximately 1:1, suggesting that β-carotene is converted into echinenone there, in agreement with previous observations for other sea urchins[2]. Echinenone contents in *L. albus* were higher in gonads than in gut (Fig. 5c). This agrees with others reports where echinenone was the most important carotenoid in gonads of regular and irregular echinoids[8,20]. Echinenone seems to be the terminal carotenoid in the organs of these sea urchins. The presence of echinenone in such high levels plays an important role in gamete production, and it may also influence egg and embryonic development[4].

Low as well as high concentrations of β-carotene in diets (Diets B and C, respectively) were not

sufficient to increase widely the yellow color in the gonads of *L. albus*, probably due to low food intake (data no showed) (Fig. 6). This low rate of consumption suggests that few carotenoids were entering the gonads[3]. Furthermore, some species of sea urchins can suffer a depression in feeding rate during the spawning period[24], and a similar pattern is shown by *S. intermedius* under experimental conditions[25]. In captivity, *L. albus* showed a low rate of natural food ingestion during the reproductive season[26]. These observations could explain our results indeed.

There are several good reasons for adding pigments to dietary preparations for sea urchins. Aside from the obvious commercial interests that reside around higher profit levels for better quality gonads, pigments within actively growing tissues are important as antioxidants. They are involved in the stabilization of proteins, they constitute an integral component of egg and larval development, and provide ultraviolet protection for sensitive tissues[27]. Carotenoids are potent antioxidants in in vitro membrane models and work synergistically with α-tocopherol[28].

In invertebrate marine tissues that are rich in polyunsaturated fatty acids (PUFA) that are readily oxidized[6], the antioxidant activity of carotenoieds may be of particular importance. The TBARS content was similar among treatments and organs (Fig. 7a), however, the lipid radical content was lower in gonads than in gut for Diet C (Fig. 7b). Even though EPR detection of lipid radicals could be considered a fingerprint of radical presence, spin trapping studies cannot really distinguish among peroxyl (ROO•), alcohoxyl (RO•) and alkyl (R•) adducts owing to the similarity 403 of the corresponding coupling constants[29]. Low levels of lipoperoxidation (lipid radical content) in gonads can be associated with a well developed antioxidant system (Fig. 7b). Previous studies established that the activity of both enzymatic and non-enzymatic antioxidants in marine invertebrate contribute to decrease lipid peroxidation by scavenging of superoxide radical and hydrogen peroxide[8]. In addition, α-tocopherol acts as a chain-breaking antioxidant by donating its phenolic hydrogen to the chain-propagating lipid peroxyl radical forming a less reactive α-tocopheroxyl radical, which can further react with another lipid peroxyl radical to stop the propagation of lipid peroxidation[30].The lipid-soluble component of the antioxidant system, α-tocopherol, is located in a fraction of neutral lipids. Concentrations of this substance in gonads were higher in sea urchins fed on Diet C and the field control than in sea urchins fed on Diet A and B (Fig. 8). The content of α-tocopherol seems to be sufficient for the protection of sea-urchin membrane lipids from oxidation in animals fed with Diet C. It is also known that interactions between carotenoids and α-tocopherol can occur in vitro by mechanisms that are still not completely understood. Various compounds show synergism in their antioxidant capacity, thus permitting mixtures to promote more effective antioxidant responses than when the compounds are applied individually to the substrate[31].

Conclusion

No significant differences were found among diets regarding to carotenoid and α-tocopherol contents and lipid peroxidation level in both organs. However, animals fed on diet C showed a significantly higher content of antioxidants and a lower lipid peroxidation in the gonads than in the gut. These animals also showed a delay in the onset of the spawning, showing a high percentage of mature individuals. Taking into account that the sea urchins' gonads are only acceptable for the market during a seasonal window of a few months, these results show the posibility of prolonging the harvest season in order to meet the market demands in southern Patagonia.

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Figure captions

Figure 1. Adult individual of *Loxechinus albus*.

Figure 2. Gonad (a) and Gut (b) indexes (%) for *Loxechinus albus* fed on 3 experimental diets and the field control. Data are showed as means ± S.D. of 10 independent replicates.

Figure 3. Photomicrographs of gonad stages of *Loxechinus albus*. Immature stage (a), mature stage (b) and spawned stage (c). Scale bar 50 µm. F: female, M: male.

Figure 4. Frecuency of gonad reproductive condition of *Loxechinus albus* fed on 3 experimental diets and the field control (n= 10 per treatment).

Figure 5. Gut and gonad content for (a) total carotenoids (mg $100g^{-1}$ WW); (b) β-carotene (nmol mg^{-1} WW); and (c) echinenone (nmol mg^{-1} WW) for *Loxechinus albus* fed on 3 experimental diets and the field control. Data are showed as means ± S.D. of 5 independent replicates.

Figure 6. Percentage of each colour category in gonads of *Loxechinus albus* fed on 3 experimental diets (n= 10 per treatment) and the field control.

Figure 7. Gut and gonad content for (a) TBARS (nmol mg^{-1} WW) and (b) lipid radical (pmol mg^{-1} WW) for *Loxechinus albus* fed on 3 experimental diets and the field control. Data are showed as means ± S.D. of 5 independent replicates.

Figure 8. α tocopherol contents (nmol mg^{-1} WW) in gonads and gut of *Loxechinus albus* fed on 3 experimental diets and the field control. Data are showed as means ± S.D. of 5 independent replicates.

Figures 1

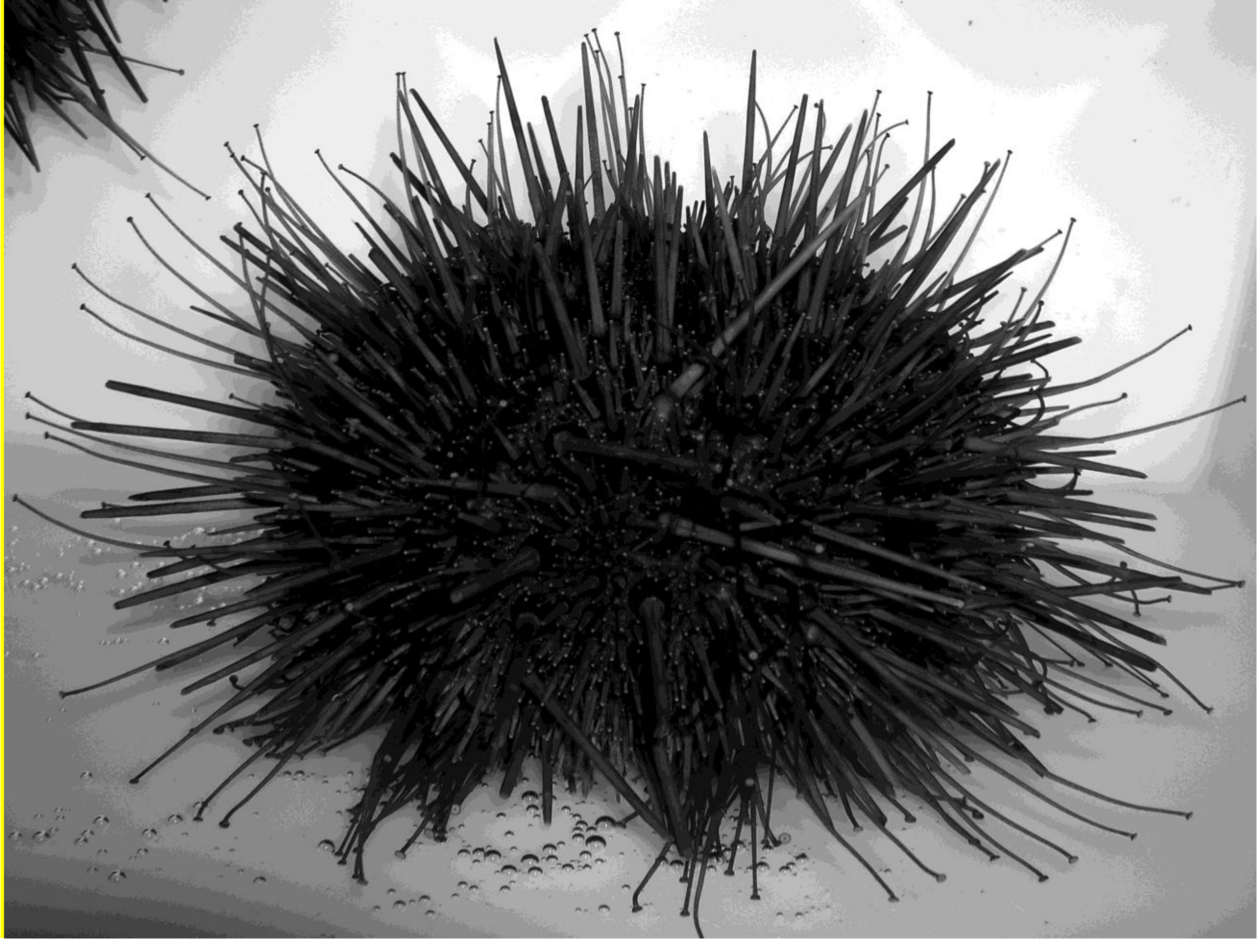

Figure 2

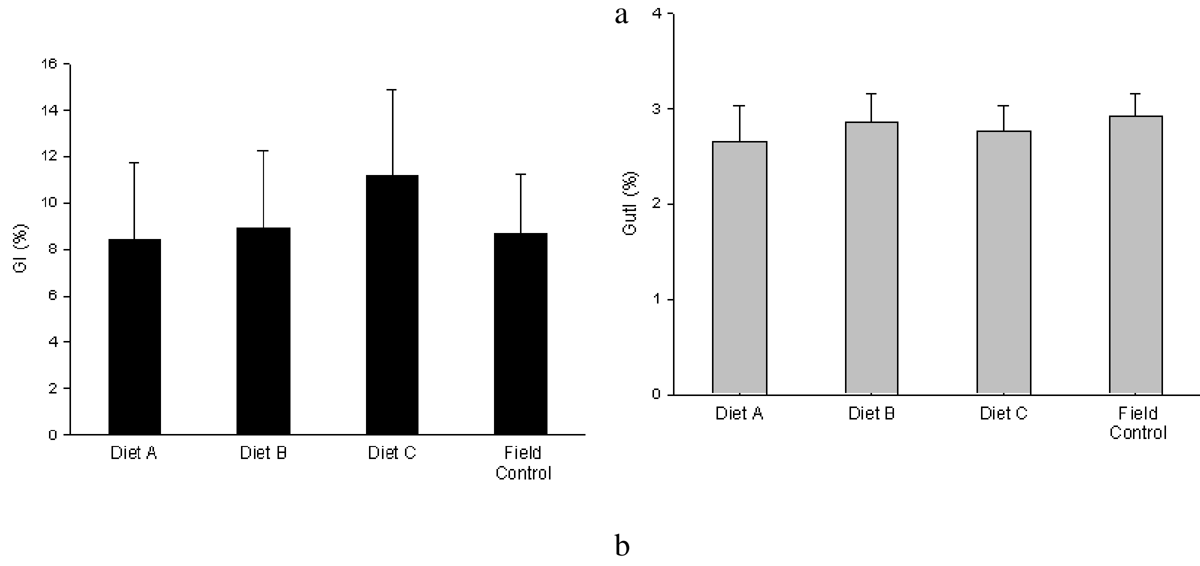

Figure 3

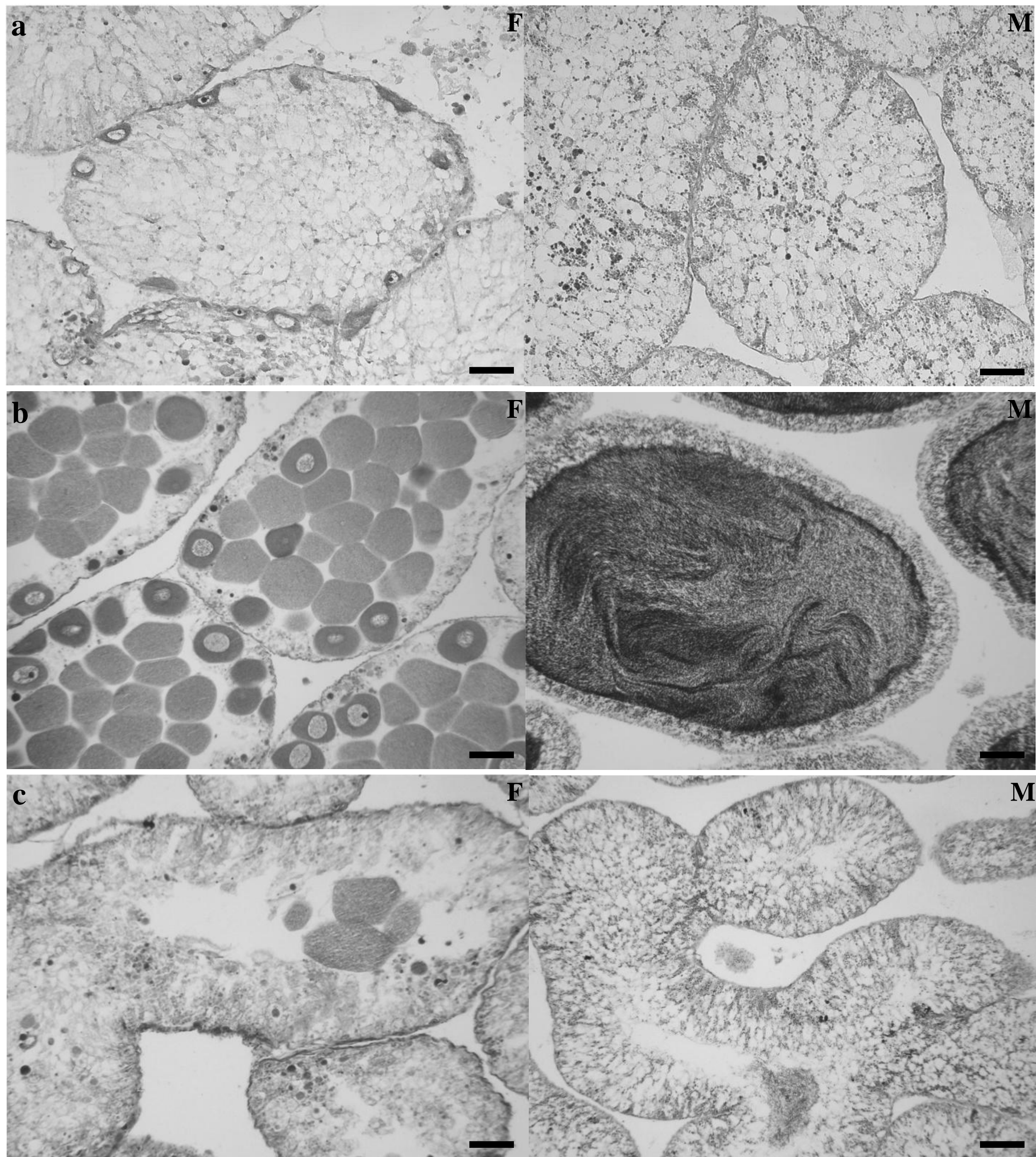

Figure 4

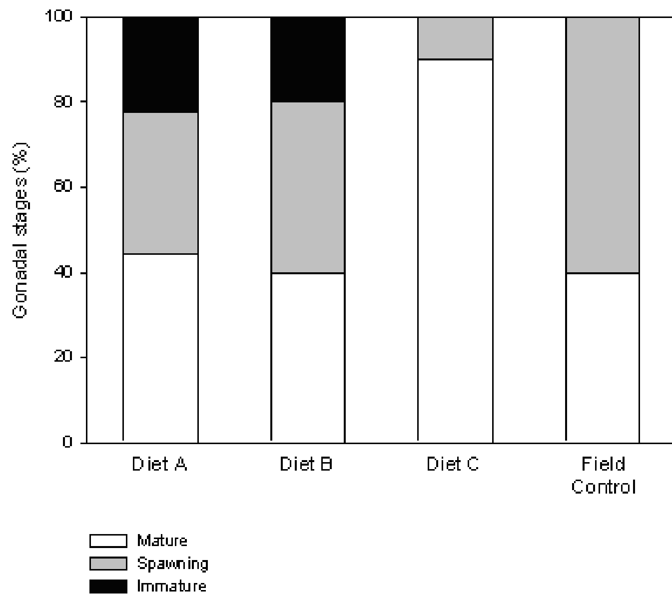
100
80
60
40
20
0
Gonadal stages (%)
Diet A
Diet B
Diet C
Field Control
Mature
Spawning
Immature

Figure 5

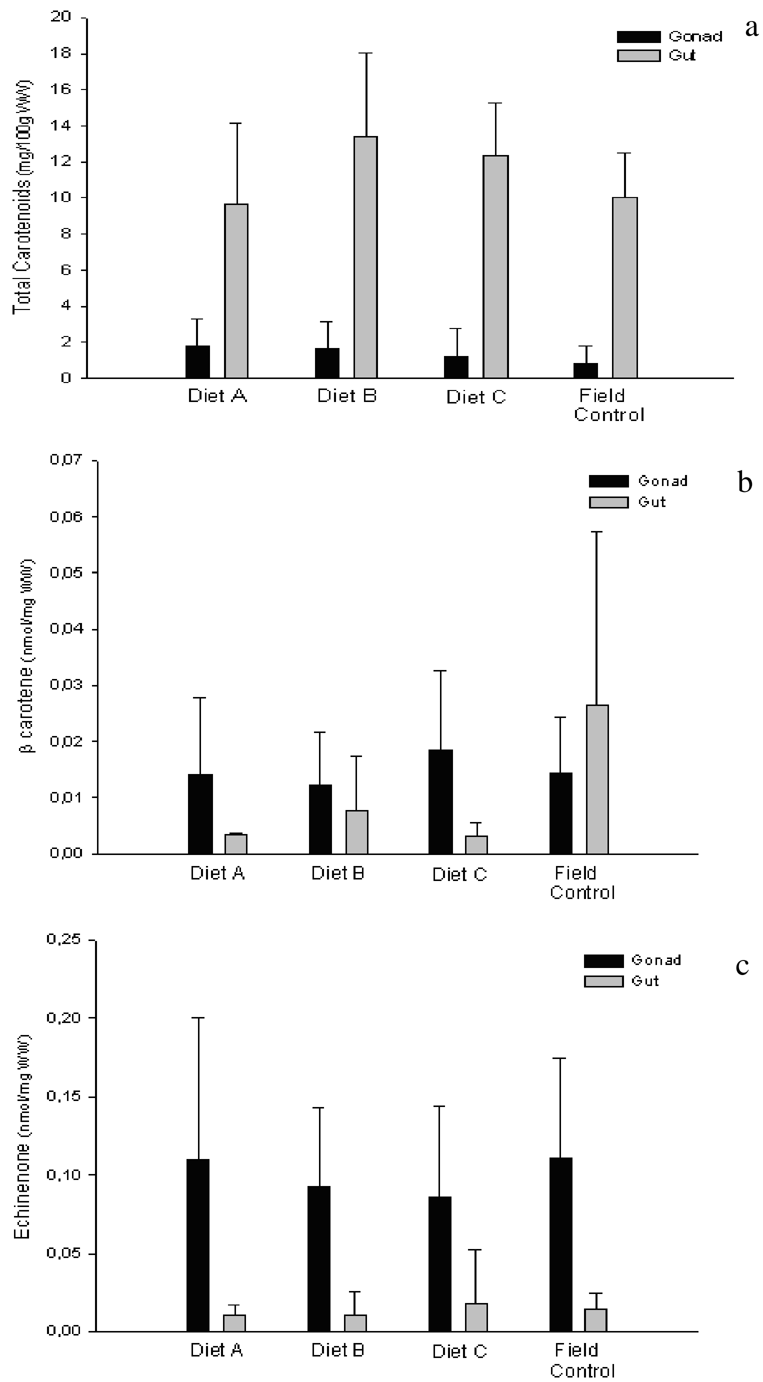

Figure 6

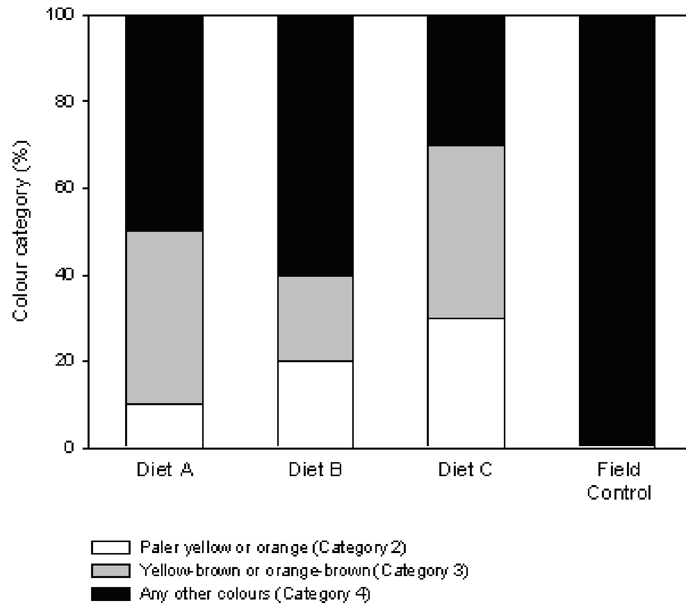

Figure 7

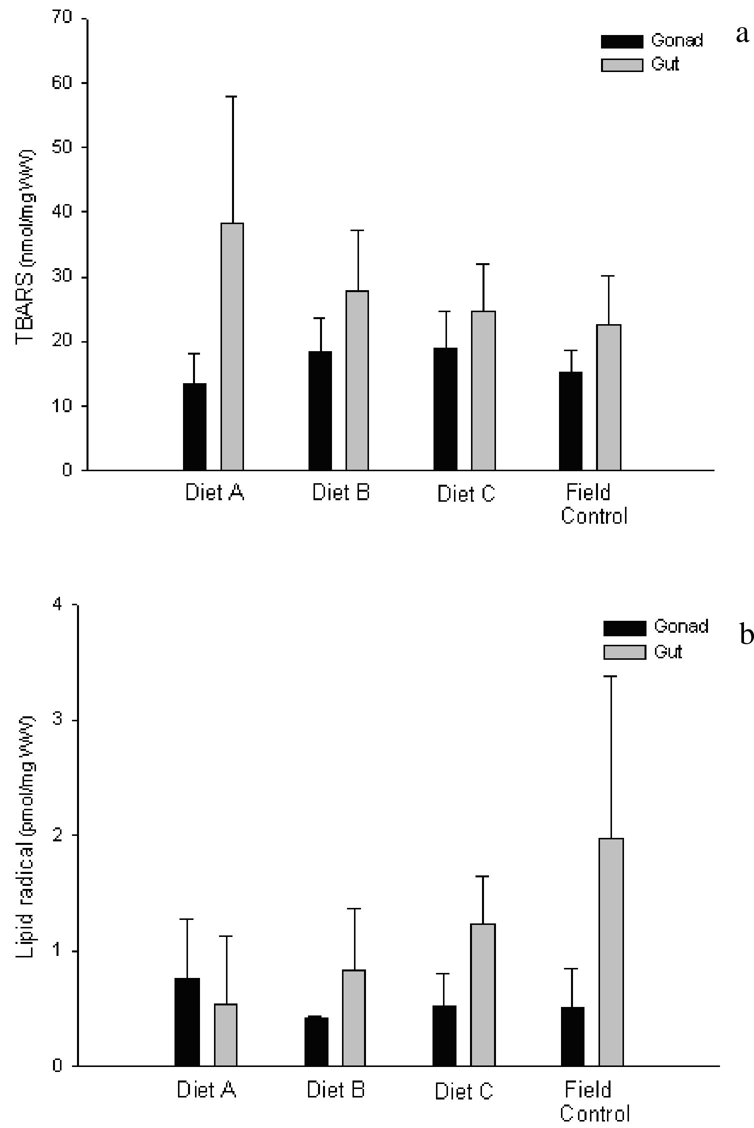

Figure 8