EFFECTS OF ASTAXANTHIN ON SPAWNING PERFORMANCE OF THE SCALLOP *Nodipecten nodosus* (LINNAEUS, 1759)*

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ABSTRACT

In worldwide aquaculture, scallops are important bivalve mollusks with high economic and nutritional value. In many countries scallop culture relies on seeds obtained from hatcheries. For this reason, broodstock conditioning is an important step for the production of spats. The experiment was conducted with an astaxanthin-enriched diet and a control diet. Accumulation of astaxanthin and sexual stage of the *Nodipecten nodosus* females gonads before and after spawning and larvae survival were evaluated. The astaxanthin-enriched diet showed a higher astaxanthin accumulation in the scallop gonad (18.73 ± 1.42 µg mL^{-1}) and 100% of the animals in advanced pre-spawning stage before spawning. A positive effect on the spawning process of the scallop, as well as D-larvae (34.96 ± 1.55%) and pediveliger (4.28 ± 0.42%) survival for animals treated with astaxanthin-enriched diet was observed.

Keywords: maturation; carotenoids; sexual stage; diet; larvae

EFEITO DA ASTAXANTINA NA DESOVA DA VIEIRA *Nodipecten nodosus* (LINNAEUS, 1759)

RESUMO

Na aquicultura mundial, as vieiras são importantes moluscos bivalves com alto valor econômico e excelente fonte nutricional. Em muitos países, a sua produção está baseada na obtenção de sementes em laboratório. Neste sentido, o condicionamento de reprodutores é um passo importante para a produção de sementes. O experimento foi realizado com dieta enriquecida com astaxantina e uma dieta controle. Foi avaliado o acúmulo de astaxantina e o estágio sexual da gônada feminina de reprodutores de *Nodipecten nodosus* antes e após a eliminação de gametas, o desempenho na desova, além da sobrevivência das larvas. A dieta enriquecida mostrou maior acúmulo de astaxantina na gonada das vieiras (18,73 ± 1,42 µg mL^{-1}) e 100% dos animais em estágio de pré-desova avançada antes da desova. Foi observado um efeito positivo no processo de eliminação de gametas da vieira, bem como na sobrevivência de larvas D (34,96 ± 1,55%) e pediveliger (4,28 ± 0,42%) para os animais tratados com a dieta enriquecida com astaxantina.

Palavras chave: maturação; carotenoides; estágio sexual; dieta; larva

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INTRODUCTION

The scallop *Nodipecten nodosus* (Linnaeus, 1758) is a prominent species of bivalve mollusks of the Pectinidae family, especially important in Venezuela and Brazil. Due to the high commercial value and intense fishing, this species has been the focus of numerous research studies.

Along the Brazilian coast, the seed supply for *N. nodosus* culture is provided by hatchery production, since capture of seeds from natural population of this species are insignificant (MANZONI *et al.*, 1996; URIARTE *et al.*, 2001). Nowadays, only two hatcheries provide seeds in Brazil: one in Rio de Janeiro and the other in Santa Catarina State.

The broodstock conditioning process in the hatchery for *N. nodosus*, as for other mollusks, can be optimized by controlling temperature and food quality. Additionally, the bivalve feeding process in hatchery can be improved by using different species of algae (TREMBLAYA *et al.*, 2007; RONQUILLO *et al.*, 2012), by including additives such as fatty acids (SÜHNEL *et al.*, 2012). Studies reported by SÜHNEL *et al.* (2014) observed a positive effect of diets enriched with carotenoids in the maturation process.

For aquatic animals, the carotenoids are important pigments responsible for the color of the meat and gonads. They are also involved in different metabolic processes such as gonad maturation and embryo development in some marine invertebrates (LIÑÁN-CABELLO *et al.*, 2002).

More than 750 structural variants of carotenoids were reported (LORENZ, 2000; MAOKA, 2009). For aquatic species, one important carotenoid structural variant is astaxanthin. In scallops, astaxanthin was reported in *Partinopecten yessoensis, Chlamys nipponensis akazara* and *Pecten albicans* (MIKI *et al.*, 1982), *Chlamys nobilis* (MIKI *et al.*, 1982; ZHENG *et al.*, 2010; 2012), *Argopecten ventricosus* (ESCARRIA *et al.*, 1989) and *Nodipecten nodosus* (SÜHNEL *et al.*, 2009; 2014). Different carotenoids were also studied in other mollusks, such as in the mussel *Mytilus edulis* (TANAKA and KATAYAMA, 1979; HERTZBERG *et al.*, 1988), oysters *Crassostrea gigas* (FUJIWARA *et al.*, 1992; MAOKA *et al.*, 2001) and *Ostrea edulis* (CZECZUGA, 1980), clams *Ruditapes philippinarum* and *Meretrix petechialis* (MAOKA *et al.*, 2010), *Anadara inaequivalvis* (GOSTYUKHINA *et al.*, 2013; SOLDATOV *et al.*, 2013), sea hare *Aplysia kurodai* (YAMASHITA and MATSUNO, 1990), gastropod *Rapana venosa* (BORODINA *et al.*, 2013), and spindle shell *Fusinus perplexus* (TSUSHIMA *et al.*, 2001).

Bivalve mollusks cannot produce astaxanthin endogenously and need to acquire it through their diet, typically by the ingestion of organisms that synthesize this pigment (JOHNSON and SCHROEDER, 1995), as microalgae. According to LORENZ and CYSEQSKI (2000) and PÉREZ-LOPEZ *et al.* (2014), the unicellular green alga *Haematococcus pluvialis* produces large amounts of astaxanthin. Besides its pigmentation function in marine organisms, astaxanthin is also known for its antioxidant role in cellular metabolism (KOBAYASHI and SAKAMOTO, 1999; SCHLEDER *et al.*, 2008; CHANG *et al.*, 2013; NIU *et al.*, 2014), as vitamin A precursor (LIÑÁN-CABELLO *et al.*, 2002), and as a photoprotector (HAIRSTON, 1981; HANSSON, 2000).

As a contribution to knowledge about the role of carotenoids in bivalves, the present work evaluated the effects of an astaxanthin-enriched diet on astaxanthin content, sexual stage of the female gonad before and after spawning, oocyte release, and larvae survival.

MATERIALS AND METHODS

Experimental design

The broodstock conditioning experiment and the successive larviculture was performed at the Laboratory of Marine Mollusks (LMM) (Federal University of Santa Catarina-UFSC), located in Barra da Lagoa, Florianópolis, Brazil. The astaxanthin analysis was performed at the Laboratory of Morphogenesis (UFSC) and the histology at the Nucleus of Aquatic Pathology Studies (UFSC).

The conditioning experiment was performed in a completely randomized design with three replications, during 15 days, testing a dry microalgae *H. pluvialis*, commercially produced by NatuRose® (Cyanotech; composition in 100 g according the manufacturer: 1,5% of astaxanthin; 24% of protein; 38% of carbohydrates; 14% of fat; 14% of ash; 6-9% moisture) as a source of astaxanthin. To evaluate the astaxanthin source,

two diets were tested: i) control: containing 100% of standard diet (mix of live microalgae species: *Isochrysis galbana*, variety *Tahit* (CCMP 1324, T-Iso), *Chaetoceros muelleri* (CCMP 1316, Cm) and *Skeletonema* sp. (CCMP 795, Sk), in the ratios 50:25:25, respectively); and ii) treated: containing 50% of NatuRose® and 50% of a standard diet. The choice of 50% concentration of the astaxanthin source for the treated diet was selected from a previously study (SÜHNEL *et al.,* 2014) that showed best results for this concentration.

At time zero (T_0 = before start the experiment) astaxanthin content was evaluated and the sexual stage was determined in the female portion of the gonad of three animals (n = 3). After 15 days (T_{15}) of experiment, for both treated (n = 3) and control groups (n = 3), were evaluated for the same animal the astaxanthin amount and the sexual stage of the female portion of the gonad, calling the control group "CM" (matured) and the treated group with astaxanthin "TMa" (matured plus astaxanthin) (Table 1). After sampling, the matured animals (group CM and TMa) were submitted to spawn, as described below. After spawning were evaluated (Table 1): i) for the same animal the astaxanthin amount and the sexual stage of the female portion of the gonad, calling group control "CS" (spawned) (n = 3) and group treated called "TSa" (spawned plus astaxanthin) (n = 3); and ii) the spawning performance (number of spaned females and oocyte number) of control group (n = 21) and treated group (n = 21). The larvae survival (D-larvae and pediveliger larvae) from treated and control groups also were evaluated, in triplicate.

Table 1. Description of the groups, diets, and number (n) of animals analysed in the histology, astaxanthin quantification and spawning (number of spawned females and released oocites) procedures, where: T_0 = before start the experiment time zero; T_{15} = after fifteen days of experiment; D-larvae = after 24 hours of larviculture; Pediveliger larvae = after 13 days of larviculture; CM = group control matured; TMa = groups treated (with addiction of astaxanthin source) and matured; CS = groups control spawned; TSa = groups treated (with addiction of astaxanthin source) and spawned.

Time	Description	Treatment (group)	Procedure		Larvae survival (n: tank number)
			Histology and Astaxanthin quantification (n: animal number)	Spawning performance (n: animal number)	
T_0	—	—	3	-	-
T_{15}	Before spawning	Control (CM)	3	-	-
		Treated (TMa)	3	-	-
	Spawning	Control	-	21	-
		Treated	-	21	-
	After spawning	Control (CS)	3	-	-
		Treated (TSa)	3	-	-
Larviculture	D-larvae	Control	-	-	3
		Treated	-	-	3
	Pediveliger larvae	Control	-	-	3
		Treated	-	-	3

Conditioning experiment

The broodstock (70-75 mm in height and 18 months old - adults animals) was collected in April of 2007 (according to SÜHNEL *et al*., 2010, animals collected at this time of the year, May, were predominantly in pre-spawning sexual stage), from culture (long-line under water system at a depth of 2 m), located in Porto Belo, Brazil (latitude 27°11'52.96"S and longitude 48°30'39.58"W). After collection, the animals were immediately transported in thermal boxes with seawater at 23 °C to the laboratory.

In the laboratory, the animals (n = 72) were all initially acclimated overnight (14 hours) with the same experimental conditions, as follows: 12 animals were conditioned in each tank (10 L;

6 tanks total; three for treated and three for controls); the seawater temperature in the tanks was maintained at 19.1 ± 0.4 ºC, with continuous flow of seawater at 333 mL min^{-1} per animal and feed at 33 mL min^{-1} per animal; maintained with constant aeration in the tanks. After acclimatation, start the experiment period (T_0).

For both treatments, the feed tanks were cleaned daily and fresh diet was added. After handling, the water and food flow were adjusted according to the values described above. The feed tanks were maintained with constant aeration to avoid microalgae precipitation. The final microalgae concentration for the control (100% of standard diet) and the treated (50% of standard diet plus 50% of astaxanthin source) was fixed in 5 x 10^4 cells mL^{-1} (approximated 3,66 mg L^{-1}). The dry microalgae *H. pluvialis* (NatuRose®) used in the treated was hydrated in a fixed volume of circulating seawater for 12 h at 19 °C and was kept in the dark before use in experiment. The live microalgae (standard diet) used in this study were grown in medium Guillard F/2 (GUILLARD and LORENZEN, 1972), and for the diatoms (*Chaetoceros* and *Skeletonema* genus) added silica to the medium. Sterile filtered water was used with continuous filtered aeration, at a temperature 22 °C and a photoperiod of 24 hours. The microalgae were used in the exponential phase of growth.

After 15 days (T_{15}) of maturation the experiment ended and the animals (from control and treated) were induced to spawning**.**

Spawning and larviculture

The spawning induction technique and oocyte fertilization was following RUPP (1994) and RUPP *et al.* (2004) with adjustments, basically using stress by withdrawal of fouling organisms from the valves, exposing the animals to UV treated water and increasing the seawater temperature from 20 °C to 24 °C. The spawning induction time was fixed at one hour. Once the animals started releasing gametes, they were transferred into 2 L beakers for individual spawning. For each treatment, the female gametes was quantified, fertilized and maintained in separate tanks for larval development.

Larviculture for each treatment (as described above: treated and control) was carried out in triplicate tanks (14 L). The embryos were transferred to the larval tanks at an initial density of 100 oocytes mL^{-1} and after the second day adjusted to 10 larvae mL^{-1} (D-larvae). Larviculture handling was repeated every 48 hours (seawater exchange and larvae screening, for cleaning and discard non growth and dead larvae). The seawater temperature was controlled, ranging from 23 to 24 °C, with constant weak aeration. The daily feeding process with live microalgae (standard diet for larviculture) began on the second day with *I. galbana* (T-Iso) at a concentration of 0.5 x 10^4 cells mL^{-1}. On the third day: *I. galbana*, *Pavlova lutheri* (Pav) and *Chaetoceros calcitrans* (Cc), in the proportion 35:35:30, respectively, at a concentration of 1 x 10^4 cells mL^{-1} was provided. After the fifth day: feeding was changed to T-Iso, Pav and Cc, in the proportion 30:20:50, respectively, at a concentration of 2 x 10^4 cells mL^{-1}. These microalgae were cultured as previously described in the "Conditioning experiment" section. The larvae survival was quantified after 24 hours (D-larvae, related to the initial oocytes number) and 13 days (larvae pediveliger, related to the initial D-larvae number).

Astaxanthin analysis and histology

Astaxanthin accumulation was evaluated in the female portion of the scallop *N. nodosus* (simultaneous hermaphrodite bivalve). The astaxanthin analysis of each sample followed the methodology described by SÜHNEL *et al.* (2009). Briefly, the carotenoid extraction was performed adding 5 mL of acetone extracting solution (Ac=O, Nuclear, P.A.) and *n*-hexane (Hex, TediaBrazil, P.A.) in a proportion of 1:3 respectively, to the sample (50 mg). The organosolvent extract was centrifuged during 5 min in a microcentrifuge (Centrifuge Ministar, with 2.4 mm of ray, fixed rotor angle; 6200 rpm; 103.33 G), filtered (0.22 µm), and an aliquot (10 µL, triplicate analysis) was injected into HPLC (Shimadzu LC-10A) equipped with a C18 (Vydac 218TP54, 250 mm, 4.6 mm Ø, 5 µm, 35 °C) reverse-phase column, fitted to a C18 reversed-phase guard column (4.6 mm Ø, 5 µm) and UV-visible detector (450 nm). Elution consisted in methanol: acetonitrile (90: 10 v/v) at 1 mL min^{-1} flow rate. Identification and quantification of astaxanthin was performed using the retention time and co-chromatography of standard

compound and external standard curve (astaxanthin, Sigma-Aldrich, MO–USA; y = 7044.96x; r^2 = 0.994). All measurements were performed in triplicate and the results were expressed in µg mL^{-1}, as mean ± standard deviation (SD).

The histology and sexual stage identification of each sample followed the methodology described by SÜHNEL *et al*. (2010). Briefly, the gonad tissues were fixed in a Davidson's solution; embedded with paraffin, cut into 5 µm-thick slices and coloured with Harris's Hematoxylin and Eosin. The identification of the reproductive stages involved two independent evaluations with the aid of a microscope, carried out by two different people, without previous identification of the slide, enlarged to 200 and 400x magnification. For this study 6 sexual stages were used, according to SÜHNEL *et al.* (2010): gametogenesis (GA); initial pre-spawning (2A); advanced pre-spawning (2B); initial spawning (3A); advanced spawning (3B); and rest (4).

Statistical analysis

First steps in the statistical analysis were an assessment of error normality and variance homogeneity (Barttlet) of astaxanthin accumulation and sexual stage in T_0, control (CM and CS) and treated (TMa and TSa), total amount of spawned oocytes (control and treated), D-larvae and pediveliger survival (control and treated). Next, we applied one way Analysis of Variance (ANOVA) and Tukey Test for comparison between means (ZAR, 1974), using the package Statistica™ (StatSoft). For the results in percentage (sexual stages, D-larval and pediveliger survival) the absolute value was then transformed into arccosine for statistical testing.

RESULTS

Throughout the experimental period (15 days of conditioning) no mortality of adult animals was observed for both tested diets.

Sexual stage

At T_0, 33% of the female broodstock were in initial pre-spawning stage and 66% in advanced pre-spawning (Figure 1). After 15 days of maturation (T_{15}) before spawning, the control (group CM) was not different from T_0, namely they did not change sexual stage, but remained equal to T_0. However, the treated (group TMa) was significantly different (p<0.05) from T_0, showing 100% of the animals in advanced pre-spawning stage. After spawning induction, only 33% of the animals in the control group spawned (CS), significantly different (p<0.05) from the other groups (T_0, CM, TMa and TSa). Yet, in those treated with astaxanthin source (TSa), 100% of the animals spawned, also significantly different (p<0.05) from the other groups (T_0, CM, TMa and CS).

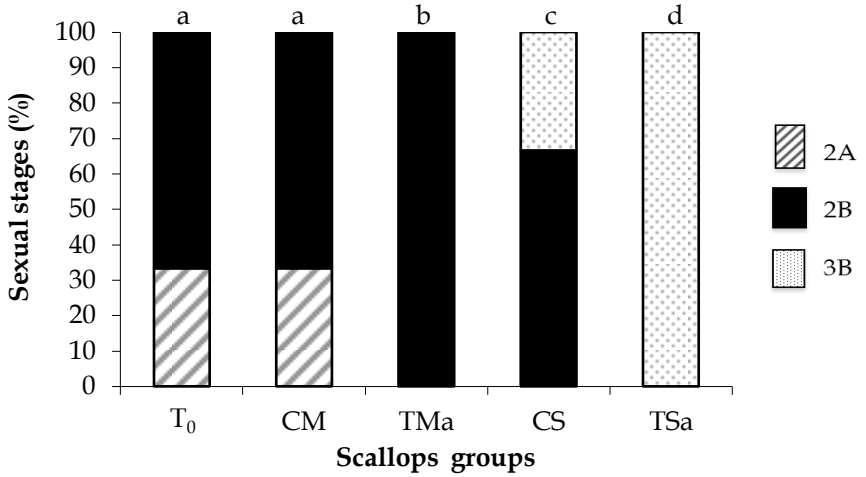

Figure 1. Sexual stages of the scallop *Nodipecten nodosus* female gonad (n = 3) in T_0 and after 15 days of conditioning experiment (T_{15}), before spawning (control CM; treated TMa) and after spawning (control CS; treated TSa) groups, where: (2A) initial pre-spawning; (2B) advanced pre-spawning; (3B) advanced spawning. The lowercase letters represent the statistical differences (p<0.05, Tukey Test).

Astaxanthin amount in the female portion of the gonad

Before spawning, the astaxanthin quantity in the female portion of the broodstock gonad (Figure 2) at T_0 (T_0: 10.39 ± 5.49 µg mL^{-1}) did not differ significantly from controls after 15 days of maturation (CM: 12.05 ± 1.49 µg mL^{-1}), as observed for the treated samples in which the astaxanthin amount was observed to be statistically (p<0.05) higher (TMa: 18.73 ± 1.42 µg mL^{-1}) related to T_0, CM, CS and TSa groups.

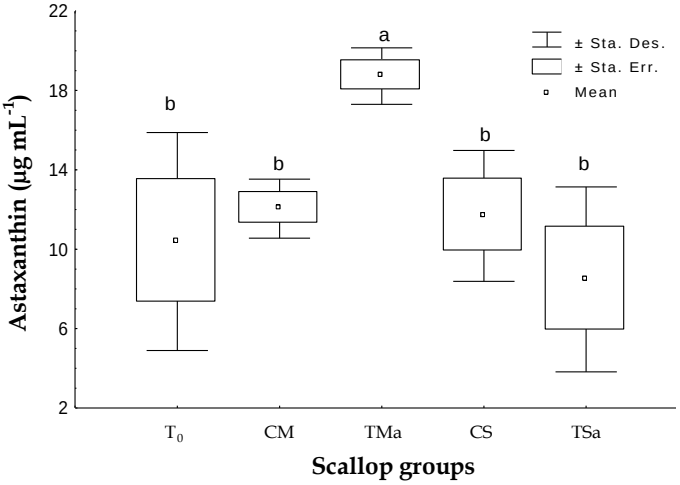

Figure 2. Mean, standard deviation, and standard error of the astaxanthin amount in the *Nodipecten nodosus* female gonad (n = 3) in T_0 and after 15 days of conditioning experiment (T_{15}), before spawning (control CM; treated TMa) and after spawning (control CS; treated TSa) groups. The lowercase letters represent the statistical differences (p<0.05, Tukey Test); Sta. Des. = Standard deviation; Sta. Err. = Standard error.

Spawning and larvae yield

The number of spawned females was 14 females in the treated group and 6 females in the control group. However, the oocyte number per spawned female was significantly (p<0.05) higher for controls with 1,841,661.25 ± 13.89 oocytes per female (Table 2), but with deform cellular division. Moreover, the treated showed good cellular division, with regular shape.

Nevertheless, after 24 hours of fecundation, the treated animals showed a significant difference (p<0.05) in the D-larvae survival (34.96 ± 1.55%) related to controls. After 13 days of larviculture, the treated group showed (p<0.05) higher pediveliger survival (4.28 ± 0.42%) (Table 2) related to the controls. The total number of D-larvae (349,555.56 ± 15,457.23) and pediveliger (5,222.22 ± 509.18) was higher for animals treated with astaxanthin.

Table 2. Total number of spawned female and mean [± standard deviation (SD)] of the oocytes number per spawned female, D-larvae (related to the initial oocytes number) and pediveliger (related to the initial D-larvae number) survival **(± SD)** for the scallop *Nodipecten nodosus* in the control and treated (n = 3). The lowercase letters in the lines represents the statistical differences (p<0.05; Tukey Test).

Parameters	**Diet (Group)**	
	Control (CS)	**Treated (TSa)**
Total number of spawned female (percentage of the total female number)	6 (28.57%)	14 (66.66%)
Number of oocytes per spawned female	1,841,661.25 ± 13.89^a	1,577,351.31 ± 08.71^b
D-larvae survival (%) [mean (±SD) of total number of larvae]	13.93 ± 2.03^a [139,333. 33 ± 20,301.53]	34.96 ± 1.55^b [349,555.56 ± 15,457.23]
Pediveliger larvae survival (%) [mean (±SD) of total]	0.66 ± 0.57^a [924.67 ± 1.15]	4.28 ± 0.42^b [5,222.22 ± 509.18]

DISCUSSION

The dried microalgae rich in astaxanthin used in this study produced high accumulation of this carotenoid in the female gonad. Nevertheless, a diet composed by the three microalgae (*I. galbana*, *C. muelleri* and *Skeletonema* sp.) did not provide enough carotenoids for high astaxanthin accumulation in the female gonad. Studying the scallop *N. nodosus* from farming, SÜHNEL *et al*. (2010) observed in the female portion of the gonad the highest astaxanthin amounts when the animals were in advanced pre-spawning sexual stage. This indicates that the natural diet, composed of different species of microalgae and other organisms provide a good source of astaxanthin to the animals.

RUPP *et al*. (1997) feeding the animals with three microalgae species (*I. galbana*, *C. muelleri* and *Thalassiosina pseudonata*) observed only 66% of the *N. nodosus* broodstock matured, after 8 days of conditioning at 17-25 °C. Also, RUPP (1994) using four microalgae species (*I. galbana*, *C. muelleri, C. calcitrans* and *Tetraselmis tetrabele*) reported that only 60% of the broodstock matured after 35 days of conditioning at 24-27 °C. Nevertheless, the astaxanthin source (treated group) afforded better female maturation in the present study, where after 15 days all animals were in advanced pre-spawning sexual stage (matured). These results showed that the astaxanthin present in the dried microalgae used in the treated diet has an important role in the broodstock gonadal development.

The advanced maturation observed in those animals treated with an astaxanthin-enriched diet also showed a better performance in gamete release. The analysis after spawning (group TSa) showed that all treated animals spawned and the subsequent gametes exhibited good cellular division resulting in a successfully fertilization. The lower astaxanthin amount observed in the animals after spawning (group Sa) corroborate the results reported by SÜHNEL *et al.* (2010), where the animals in spawning sexual stage present a lower quantity of astaxanthin.

The largest number of D-larvae observed for those animals treated with an astaxanthin-enriched diet is not related to higher number of gametes, but with the oocyte quality at spawning. The astaxanthin amount in the female portion of the gonads before spawning can contribute to the D-larvae survival. These results corroborate the findings of SÜHNEL *et al.* (2014) using a different commercial dried diet and live microalgae as an astaxanthin source.

The carotenoid effect on reproductive processes in other marine animals is reported just by few studies. For fish, MEYERS (1994), observed that salmon larvae and juveniles from broodstocks fed without sources of carotenoids had a lower survival rate (<15%) when compared with broodstocks fed with a diet supplemented with carotenoids. In the same way, but for shrimp (*Penaeus* sp.) LORENZ (1998) and HOWELL and MATTHEWS (1991), observed that broodstock fed with a carotenoid deficient diet result in deformity and low survival of larvae. Also, CUZON *et al.* (2004) reported that the use of astaxanthin in the broodstock feed for shrimp *Litopennaeus vannamei* provided greater nauplii larvae survival.

In addition to the advanced maturation and better larvae development, the higher astaxanthin accumulation in the female gonads of the scallops observed in the astaxanthin-enriched diet (treated) group is important to explain the results observed by SCHLEDER *et al.* (2008), which used a group of animals from the present study (control and treated before and after spawning), sampled at T_0 and at T_{15}, for immunological studies. SCHLEDER *et al.* (2008) observed a protective effect in the oxidative stress with the treated animals with astaxanthin source (NatuRose). Also, under oxygen depletion stress, NIU *et al.* (2014) mentioned that carotenoids protect the polyunsaturated fatty acids of shrimp tissues from the detrimental effects of oxidation. The antioxidant property of carotenoids is due to conjugated double bonds, where free radical deactivation and the scavenging of reactive oxygen species (ROS) occurs (MIKI, 1991). Enhancement of oxidative stress protection in scallops could improve their defense ability, leading to a better broodstock and larvae immunological response.

The results of the present study show that astaxanthin-enriched diets can provide an enhanced broodstock performance and consequently increased larval survival in hatchery conditions, especially to achieve higher

performance in commercial scallop seeds production. Considering that the microalgae production in hatcheries is expensive and a critical factor, the possibility of use dried diet, specially enriched in astaxanthin, can contribute increasing nutritional value of molluscs diet, reduce costs of production and be a option of food in absence of live microalgae.

CONCLUSION

The present study demonstrates that use of enriched microalgae with astaxanthin during the conditioning contribute to the spawning of scallop *N. nodosus* in hatchery. The animals treated with astaxanthin-enriched diet showed higher amounts of astaxanthin in the female gonad of *N. nodosus* and 100% of the animals matured before spawning. Also, the treated broodstock exhibited better spawning performance and subsequent larval survival.

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