Dietary nucleotide mixture effects on reproductive and performance, ovary fatty acid profile and biochemical parameters of female Pacific shrimp *Litopenaeus vannamei*

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Abstract

The effects of different dietary nucleotide mixture (NT) levels (0, 0.2, 0.4 and 0.6 %) was investigated on the reproductive performance, fatty acid profile and biochemical parameters in *Litopenaeus vannamei* female that were co-fed with fresh food (2 times daily) and experimental diets (2 times daily) for 30 days. Sampling was carried out at the first day of the trial, before eyestalk ablation (ESA) (day 21) and after ESA (day 30). Reproductive performance parameters including the hepatopancreatic index, absolute fecundity, egg diameter and latency period were higher in the NT supplemented groups than the control group ($P < 0.05$). Total n–3 polyunsaturated fatty acids in the ovaries, especially eicosapentaenoic acid, were higher in the NT supplemented groups than in the control (222.6 ± 7.3 *vs.* 204.7 ± 3.0 mg g^{-1} extracted lipid) ($P < 0.05$). Total hemocyte count, granular cells, plasma glucose (Glu), total protein (TP), calcium (Ca^{2+}), total cholesterol (Chol), triglyceride (Tg) and high density lipoprotein (HDL) levels were significantly increased in the NT supplemented groups in comparison with the control group ($P < 0.05$). After ESA, the concentration of plasma Glu, TP, Chol and Tg significantly decreased, but HDL levels increased in all groups ($P < 0.05$). These results suggested that the application of dietary NT may present a novel strategy for increasing reproductive performance and health status in *L. vannamei*.

Keywords*:* *Litopenaeus vannamei*, nucleotide, hemocyte; reproductive performance, fatty acid profile, plasma biochemical indices

Introduction

The Pacific shrimp (*Litopenaeus vannamei*, Boone 1931) is known for its high survival rates, high tolerance to environmental changes and rapid growth in intensive culture systems, which makes it an important world-wide cultured crustacean species (Lem 2006). Its propagation is currently based on domesticated populations kept under biosecure conditions (Ceballos-Vázquez *et al.* 2010; Thitamadee *et al.* 2016). However, pond-reared brooders are generally unpredictable in their reproductive performance, which could be attributed to their dietary deficiencies and/or general rearing conditions (Palacios *et al.* 2000).

Eyestalk ablation (ESA) in female *Penaeid* spp. broodstock is used in shrimp hatcheries to induce ovary maturation and improve the production of eggs and larvae. In fact, eyestalk ablated female shrimp cannot rely solely on a gradual accumulation of energy and essential nutrients during the rapid period of vitellogenesis; thus, these should be dietary supplemented. In addition, broodstocks in hatcheries are normally under stress conditions due to high stocking densities and handling stress, which may made them more susceptible to pathogenic outbreaks and high mortalities (Govahi *et al.* 2014). Thus, brooders nutritional conditions influence the quality and performance of the off-spring; and consequently, the development of high performing artificial dry diets for shrimp broodstocks is a research priority (Andrino *et al.* 2012; Guo *et al.* 2016).

Increasing the availability of NT in broodstock diets may have a beneficial effect on egg development (González-Vecino *et al.* 2004). As Li & Gatlin (2006) demonstrated, NT as dietary component has a beneficial impact on gastrointestinal tract through improving physiological, morphological and microbiological influences that can increase assimilation and utilization of the other nutrients, which are essential for vitellogenesis and reproductive performance. Moreover, during times of extraordinary stress, such as reproduction, additional NT must be readily available for cell proliferation (Hoffmann 2008). Recent research on the use of NT as dietary additives

indicated that these compounds may improve growth, as well as enhance immune response and disease resistance, increase stress tolerance (Burrells *et al*. 2001a, 2001b; Leonardi *et al*. 2003; Lin *et al*. 2009), improve intestinal morphology (*i.e.* development of intestinal lining, increased intestinal folding, enterocyte and microvilli height) (Borda *et al*. 2003; Cheng *et al.* 2011; Peng *et al*. 2013), elevate antioxidant status and lipid metabolism (Mohebbi *et al*. 2013), modify muscle proteome (Keyvanshokooh & Tahmasebi-Kohyani 2012), as well as increase the spawning quality in different fish species (González-Vecino *et al.* 2004). To date, no work has been published on the effects of NT supplementation of broodstock diets in shrimp; thus, the present study aims to examine the effects of dietary administration of a mixture of NT on the reproductive performance, the fatty acid profile of the ovary and hematological parameters in Pacific shrimp females before and after ESA.

Materials and Methods

Diet formulation and preparation

Four experimental diets formulated to contain approximately 550 g kg^{-1} crude protein, 120 g kg^{-1} crude lipid, 130 g kg^{-1} ash, 40 g kg^{-1} fiber, 100 g kg^{-1} moisture and 18.7 MJ kg^{-1} of digestible energy was formulated in order to meet the known nutritional requirements for *L. vannamei* broodstock (Goimier et al. 2006). In this sense, diets were formulated using Lindo software 6.1 (USA) (Table 1). Experimental diets were supplemented with a mixture of NT disodium salts (UMP: disodium uridine-5´-monophosphate; CMP: cytidine-5´-monophosphate; IMP: disodium inosine-5´-monophosphate; AMP: adenosine-5´-monophosphate; GMP: disodium guanidine-5´–monophosphate) (Chemoforma Agust, Switzerland) at the expense of cellulose (Merck, Germany)

at a concentration of 0 (control diet), 0.2, 0.4 and 0.6%. Dry ingredients including NT mixture were mixed together for 30 min and sufficient distilled water were added to form a soft dough that was mechanically extruded by using an electric grinder to obtain pellets of 3 mm in size. Pellets were dried in a convection oven at 50 °C for 24 h. These diets were prepared only once, at the beginning of experiment then stored in re-sealable plastic bags at -20 °C until their use.

Experimental design

A third generation family of *L. vannamei* from an imported strain (SPF_3, domesticated in Hawaii) was transferred from ponds (surface: 0.1 Acre) in a private shrimp farm (Bandar Rig, Boshehr, Iran) to a private hatchery in Genaveh (Seydan Jonoub, Genaveh, Boshehr, Iran). Upon arrival, animals were treated against filamentous bacteria, fungal and protozoan infections with formalin (100 ppm for 30 sec) according to Alday-Sanz (2010), and then acclimated to the experimental conditions for a week. A total of 120 females and males (30.4 ± 2.0 g) at a ratio of 1.5:1 were randomly allotted into 12 black circular tanks (250 l) at a density of 10 shrimp $tank^{-1}$. Tanks were connected to an open-flow system with filtered running seawater (100 % daily water exchange). Average values for water temperature, salinity, dissolved oxygen and pH were 27.8 ± 1.2 °C, 31.8 ± 3.1 ‰, 5.8 ± 0.4 mg l^{-1} and 7.9 ± 0.2, respectively, and photoperiod was 10L:14D (light: darkness). Shrimp were co-fed with fresh food [squid (40%), polychaetes (20%) and chicken liver (40%); two times daily (0600 and 1800 h) accounting for a total daily supply of 20% of wet weight biomass] and experimental diets [two times daily (1200 and 2300 h) accounting for a total daily supply of 5% of wet weight biomass] 4 times per day.

Eyestalk ablation and shrimp sampling

Before each sampling, shrimp were placed in chilled (4 °C) and aerated seawater for 10 min in order to reduce their stress, metabolic activity and minimize any potential manipulation effect. After 21 days of feeding shrimps with experimental diets, females (n = 18 per diet) were ablated unilaterally (left eyestalk only) by cutting the eye stalk under water at the base of the peduncle and applying burn to the wound to minimize fluid loss and help coagulation. Gonad development was assessed daily with a lamp by observing the size and color of the gonad through the exoskeleton (Alday-Sanz 2010). Female shrimps were sampled at the beginning of the trial (day 0), before ESA (day 21) and after ESA (day 30). The hepatopancreas (before and after ESA) and ovaries (only after ESA) were dissected and weighed (n = 3 samples per dietary treatment), then transferred into liquid nitrogen for further analysis. Moreover, haemolymph was obtained from the base of the pleopod at the first abdominal segment near the genital pore (n = 3 samples per replicate, n = 9 samples per diet) at days 0, 21 and 30, using a 1 mL syringe containing 0.4 mL pre-cooled anticoagulant (10 mM Tris-HCl, 250 mM sucrose, 100 mM sodium citrate, pH 7.6, 4 °C) and it was aliquoted into two parts. An aliquot of haemolymph was stored in ice (0-4 °C) and transferred to the laboratory for evaluating immunological factors, and the other aliquot centrifuged (6000 *g*, 10 min, 4 °C) and plasma separated. Then, vials containing plasma samples were transferred into liquid nitrogen and stored at −80 °C until their analysis.

Growth and reproductive performance assessments

Body weight (BW, g) and hepatopancreas weight (g) were measured to the nearest 0.1 g, whereas total body length (TL, mm) was measured to the nearest 1 mm. Standard formulae were used to assess growth performance parameters: SGR (% BW day^{-1}) = [(ln BW_f – ln BW_i) / t] ×100, where t is experimental period = 30 days; weight gain (WG, %), WG = (BW_f – BW_i) / BW_i; survival (S,

%), S = (number of specimens in each group remaining on day 30 / initial number of specimens) × 100; condition factor (K), K = (BW_f (g) / TL_f (cm^3) × 100. The reproductive performance was evaluated in terms of the hepatopancreatic (HPI) and gonadosomatic (GSI) indexes, the absolute fecundity calculated as the number of eggs per female body weight, egg diameter and the latency period, defined as the time interval between the ESA and the first spawning. Standard formula were used to determine HPI and GSI: hepatopancreatic index (HPI, %), HPI = (hepatopancreas weight / BW) × 100; gonadosomatic index (GSI, %), GSI = (gonad weight / BW) × 100. For determining absolute fecundity values, ovary samples (n= 3 per replicate from head, mid and hind parts of ovaries) were collected and weighted (0.1 g), then transferred in Gilson's fluid (5 ml for two months, Simpson 1951). Egg diameter (mm) was determined by means of a light microscope with an adapted micrometer (Nikon, Japan).

Lipid and fatty acid ovary analyses

Total lipids in the ovaries (n = 1 sample per replicate, n= 3 samples per treatment) was extracted using chloroform: methanol (2:1, v/v) according to Folch *et al.* (1957). Fatty acid (FA) methyl esters were prepared by acidic methanolysis of lipid extracts using BF_3 in methanol. The FA methyl esters were recovered with n-hexane according to Metcalfe & Schmitz (1961) and analyzed using a gas chromatograph (model: CP3800 Varian, Walnut Creek, CA, USA) with a flame ionization detector, equipped with a capillary column (BPX70 SGM; 120 m × 0.25 mm i.d., film thickness 0.25 mm, Victoria, Australia). Injector and detector temperatures were 210 and 240 °C respectively. The column temperature was programmed from 160 to 180 °C at a rate of 2 °C min^{-1}, helium was used as the carrier gas, and the total run time was 85 min per sample. Fatty acid peaks were integrated using VARIANSTAR chromatography software (version6.41) and

identification was carried out with reference to known standards (Sigma-Aldrich, Munich, Germany).

Haematological parameters

Total haemocyte count (THC) was measured with a haemocytometer cell counting method (× 400) (Song *et al.* 2003). To determine differential haemocyte count, the number of hyaline cells (HC), semigranular cells (SGC) and granular cells (GC) were enumerated according to protocol described by Song *et al.* (2003), after the following stages: i) preparation and drying of haemolymph extension at room temperature (25 °C), ii) fixation in methanol for 1 min, iii) staining with May-Grun-Wald-Giemsa method, and iv) counting with a light microscope (× 1000).

Plasma biochemical parameters were analyzed by means of an autoanalyzer (Mindray BS-200, China) using commercial clinical investigation kits (Pars Azmoon Kit, Tehran, Iran). Biochemical measurements were conducted for glucose (Glu), total protein (TP), calcium (Ca^{2+}), total cholesterol (Chol), high-density lipoprotein (HDL) and triglyceride (Tg).

Statistical analysis

Data were analyzed using the SPSS ver. 19.0 (Chicago, Illinois, USA). All the data are presented as means ± standard error of the mean. Arcsine transformations were conducted on all percentage data to achieve homogeneity of variance before statistical analysis. A one-way ANOVA was

performed to check differences among treatments for morphometric and reproductive parameters as well as fatty acid composition of ovaries (data from the end of the study), whereas the effects of NT level and ESA and their interactions on immunological and plasma biochemical parameters were analyzed using a two-way ANOVA (data from different sampling times related to ESA); if effects of NT level or ESA were significant, then these effects were evaluated separately by a one-way ANOVA. Duncan test was performed when significant differences were found among them. The level of significant difference was set at $P < 0.05$ for all statistical tests. The Pearson product moment correlation test was used to determine any correlation between dietary NT levels and physiological parameters, and in all cases, $P < 0.05$ was considered as significant.

Results and discussion

Growth and reproductive performance

The results of the present study showed that the survival and growth performance of shrimp females were not improved by the inclusion of dietary NT (Table 2; $P > 0.05$). These results are in disagreement with previous studies that reported an improvement in growth performance of *L. vannamei* juveniles fed NT-supplemented diets (Li & Gatlin 2006; Li *et al*. 2007; Oujifard *et al.* 2008; Murthy *et al*. 2009; Andrino *et al.* 2012). Moreover, other studies also reported that inclusion of the dietary NT led to an increase in growth performance of other decapod species in juvenile stage such as black tiger shrimp (*Penaeus monodon*; BW_i = 3.8 g; dietary NT tested = 0.5% DM; Huu *et al.* 2013), freshwater prawn (*Macrobrachium rosenbergii*; BW_i = 0.3 g; dietary NT tested = 0.15–0.3% DM; Shankar *et al.* 2012) and narrow clawed crayfish (*Astacus leptodactylus leptodactylus*; BW_i = 8.3 g; dietary NT tested = 0.05–0.25% DM; Safari *et al.* 2014). However,

the disagreement between available literature on shrimp juveniles and present results might be attributed to the higher body weight of brooders and also their priority for gonad development rather than somatic growth.

Regarding the reproductive performance parameters measured, in spite of the tendency in increasing in absolute fecundity and egg diameter values in female shrimps fed supplemented NT diets, these parameters were not statistically different ($P > 0.05$). Before the ESA, female shrimps fed the fresh food supplemented with the 0.4% NT diet had the highest HPI value, whereas females fed the control and 0.6% NT diets had the lowest HPI values ($P < 0.05$). Moreover, ESA led to a significant decrease in the HPI in all groups, as a consequence of the mobilization of lipids from the hepatopancreas to the ovaries, which may be attributed to the concomitant reduction in the levels of gonad inhibiting hormone produced by the sinus gland (Sainz-Hernández *et al.* 2008). This result was also supported by the higher plasma HDL concentration in all groups after the ESA, which indicated an increased lipid mobilization from the hepatopancreas to the ovaries. Furthermore, shrimps fed the 0.4% NT diet had a shorter latency period in comparison to the other dietary groups ($P < 0.05$). These results indicated that the exogenous NT supplementation contributed to the NT pools in the ovaries and improved the reproductive performance in shrimp females fed these diets. Since oogenesis is a process of intensive cell division with high nucleic acid formation and a concomitant high requirement for NT, supplementation of broodstock diets with NT was beneficial for their reproductive performance (Gonzalez–Vecino 2005). In this context, Gonzalez–Vecino (2005) reported that both finfish species like halibut (*Hippoglossus hippoglossus*) and haddock (*Melanogrammus aeglefinus*) broodstocks fed diets enriched with NT had a higher fecundity and egg quality than the control fish.

Ovary FA profile

The results of the FA profile in the ovaries fed different experimental diets showed that female shrimps fed NT supplemented diets had higher total saturated fatty acids levels, mainly palmitic acid (16:0), than the control group (Table 3). On the other hand, female shrimps fed NT supplemented diets had higher total n–3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA, 20:5n–3), than the control group ($P < 0.05$). Marine shrimps are believed to have a limited ability to synthesize n–3 LC-PUFAs from their precursor the α-linolenic acid (18:3n–3) (Gonzalez-Felix *et al.* 2002); thus, their increase in shrimp ovaries may be provided by other factors. These results may indicate a positive effect of dietary NT on the FA profile of shrimp ovaries, since it has been reported that there is a positive relationship between eggs and larval quality and their EPA content (Palacios *et al.* 2001), although the mechanism by which dietary NT affect lipid metabolism and FA profile remains unknown. However, different hypothesis may explain the above-mentioned results; thus, dietary NT may result in changes in the intestinal microbiota that may affect long chain PUFA levels, because bacteria possess the necessary enzymes for fatty acid elongation and desaturation (Fontana *et al.* 1998) that may be released into the gut lumen by microbiota and be available for the host. In this context, Abtahi *et al*. (2013) reported that NT (dietary NT tested = 0.15–0.5% DM; fish fed 5 times daily) significantly increased docosapentaenoic acid (22:5n–3) in beluga sturgeon (*Huso Huso*) juveniles (BW_i = 12.6 g). The results of the current study may be indicative that, providing exogenous NT supplementation increase the absorption and utilization of n-3 LC-PUFA from the diet and their accumulation in the ovaries.

Plasma biochemical parameters

Plasma biochemical parameters were significantly affected by either dietary NT supplementation or ESA (Table 4). Plasma TP and Glu are regarded as good indicators of the nutritional status of shrimp and also diet quality, because both are highly sensitive and reflect the regulation of other processes that exceed those involved only in diet catabolism (Pascual *et al.* 2003). In this study, Glu levels were within the normal baseline range values reported for this species (Racotta & Palacios 1998; Palacios *et al.* 1999; Arcos *et al.* 2003; Pascual *et al.* 2003; Sainz-Hernández *et al.* 2008) suggesting an optimal physiological condition of shrimps fed different experimental diets. In particular, female shrimps fed the control and 0.4% NT diets had the lowest and highest plasma Glu levels at day 30, respectively ($P < 0.05$). There was also a significant and positive correlation among dietary NT level and plasma Glu values ($r = 0.756$; $P = 0.004$). Plasma Glu significantly decreased after ESA (day 30) in all groups ($P < 0.05$). Similarly, Sainz-Hernández *et al.* (2008) reported that unilateral and bilateral ESA led to a significant decrease in plasma Glu in *L. vannamei* females. In addition, it should be mentioned that ESA increased energy expenditure in female shrimps that are in the process of reproduction (Racotta & Palacios 1998), which may lead to a reduction in plasma Glu. Thus, it seems that NT diet supplementation had a positive effect on the nutritional status of female shrimps.

As vitellogenin (Vtg) is a highly phosphorylated and calcium-rich protein, the determination of plasma TP and Ca^{2+} can be used as indirect techniques to evaluate plasma Vtg levels (Verslycke *et al.* 2002). In the present study, plasma TP increased with increasing dietary NT levels ($P < 0.05$). Moreover, plasma TP concentration significantly decreased in female shrimps fed the control and 0.2% NT supplemented diets after ESA, which may be as a consequence of the stressful condition resulting from ESA. Low levels of plasma TP can be explained by their utilization as energy supply in a situation of increased energy demands such as

stress (Racotta & Palacios 1998) and/or reduction of the proteins participating in the immune response (*i.e.* penaeidins, crustins and agglutinins), which could have adverse consequences for immune capacity (Perazzolo *et al.* 2002). In this context, Perazzolo *et al.* (2002) reported that plasma TP levels decreased by 50% in unilaterally ablated *Farfantepenaeus paulensis* females. In the present study, female shrimps fed the 0.4 and 0.6% NT supplemented diets had higher plasma TP levels ($P > 0.05$), whereas its concentration did not change after ESA, which indicated a higher resistance and immunocompetence of these groups in stressful conditions. On the other hand, female shrimps fed NT supplemented diets had higher plasma TP ($r = 0.917$; $P = 0.001$) and Ca^{2+} concentrations than the control group, which may indirectly indicate a higher hemolymph Vtg concentration in these groups.

The results of the current study showed that plasma Chol and Trg concentrations significantly increased with increasing dietary NT levels ($P < 0.05$), which may indicate a positive effect of dietary NT in shrimp reproductive performance. There was also a significant and positive correlation between dietary NT levels and plasma Chol ($r = 0.872$; $P = 0.001$) and Tg ($r = 0.658$; $P = 0.02$) values. Moreover, the concentrations of lipid components gradually increased up to day 21 (before ESA), which was in agreement with an increasing maturity stage caused by substrate mobilization to the ovary, as it was previously reported by Racotta & Palacios (1998). In addition, plasma Chol and Trg significantly decreased in all groups after ESA as it was also reported in other studies (Wouters *et al.* 2001; Arcos *et al.* 2003), as a consequence of an accelerated transfer of nutrients to maturing ovaries, in which lipids are mainly accumulated. The general effects of ESA on reproduction, including mobilization of lipids from the hepatopancreas to the gonad, are mainly attributed to the concomitant reduction in the levels of gonad inhibiting hormone produced by the X-organ-sinus glands (Sainz-Hernández *et al.* 2008). Similarly, Sainz-Hernández *et al.* (2008)

reported that unilateral ESA significantly decreased plasma Tg, but not Chol levels in *L. vannamei* females. In the present study, female shrimps fed NT supplemented diets had higher hemolymph HDL levels than the control group. There was also a significant and positive correlation between dietary NT levels and plasma HDL values (r = 0.706; P = 0.01). Studies on human infants have shown that NT supplementation enhance plasma HDL levels by enhancing the protein as well as apolipoproteins synthesis in the liver (Sanchez-Pozo *et al.* 1995). In this context, Mohebbi *et al.* (2013) reported that dietary NT supplementation led to an increase in plasma HDL in rainbow trout (*Oncorhynchus mykiss*). Moreover, after ESA hemolymph HDL concentration significantly increased in all groups might be as a consequence of increasing mobilization of lipids from the hepatopancreas to the ovaries.

Hemolymph immunological parameters

It is recognized that an exogenous source of NT not only optimizes the functions of rapidly dividing cells, such as those of the immune system that lack the capacity to synthesize NT, but also saves energy expenses for their synthesis (Li & Gatlin 2006). In the current study, THC (r = 0.775; P = 0.003) and GC (r = 0.797; P = 0.002) in shrimp fed NT supplemented diets were significantly higher than in the control group; however, HC (r = -0.797; P = 0.002) was lower in groups fed NT supplemented diets than in the control group ($P < 0.05$; Table 5). The high number of THC in shrimp in the treatments showed that NT is capable of enhancing both cellular and humoral immune responses. In this sense, several studies have reported that dietary NT supplementation led to an increase in cellular (mainly THC) and humoral immune responses (*i.e.* superoxide onion, superoxide dismutase activity, phenoloxidase activity) and disease resistance in *L. vannamei* juveniles (Li *et al.* 2007; Oujifard *et al.* 2008; Murthy *et al.* 2009; Andrino *et al.* 2012;

Guo *et al.* 2015), black tiger shrimp (*Penaeus monodon*) (Huu *et al*. 2012), freshwater prawn (Parmar *et al.* 2011; Shankar *et al.* 2012) and juvenile narrow clawed crayfish (Safari *et al.* 2014). Only HC was affected by the ESA, and its values significantly decreased after ESA in all groups (P = 0.001). These results might be linked to the incorporation of NT into the cells and their subsequent higher proliferation rate; however, the underlying mechanisms are still not completely understood and require further studies.

In conclusion, the results of the present study showed that the application of dietary NT did not have any significant effect on growth performance of female brooders of *L. vannamei*; however, it had positive effects on some reproductive parameters (HPI and latency period), the fatty acid profile of ovaries, hemocyte counts as well as plasma biochemical indices (Glu, TP, Chol and HDL). Thus, it may be assumed that dietary NT supplementation can promote health status in shrimp brooders in stressful conditions such as the ESA.

References

Abtahi, B., Yousefi, M. & Kenari, A.A. (2013) Influence of dietary nucleotides supplementation on growth, body composition and fatty acid profile of Beluga sturgeon juveniles (*Huso huso*). Aquac. Res. **44**, 254–260.

Alday-Sanz, V. (2010) The shrimp book. U. K. Nottingham University Press. 899.

Andrino, K.G.S., Augusto, E., Serrano, J., Valeriano, L. & Corre, J. (2012) Effects of dietary nucleotides on the immune response and growth of juvenile Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931). Asian Fish. Soc. **25**,180–192. Arcos, F. G., Ibarra, A. M., Palacios, E., Vazquez-Boucard, C. & Racotta, I. S. (2003). Feasible predictive criteria for reproductive performance of white shrimp *Litopenaeus vannamei*: egg quality and female physiological condition. Aquaculture **228**, 335–349.

Borda, E., Martinez-Puig, D. & Cordoba, X. (2003) A balanced nucleotide supply makes sense. Feed Mix **11**, 24–26.

Burrells, C., William, P.D. & Forno, P.F. (2001a) Dietary nucleotides: a novel supplement in fish feeds 1. Effects on resistance to diseases in salmonids. Aquaculture **199**, 159–169.

Burrells, C., William, P.D., Southage, P.J. & Wadsworth, S.L. (2001b) Dietary nucleotides: a novel supplement in fish feeds 2. Effects on vaccination, salt water transfer, growth rate and physiology of Atlantic salmon. Aquaculture. **199**, 171–184.

Ceballos-Vázquez, B.P., Palacios, E., Aguilar-Villavicencio, J. & Racotta, I.S. (2010) Gonadal development in male and female domesticated whiteleg shrimp, *Litopenaeus vannamei*, in relation to age and weight. Aquaculture **308**, 116–123.

Cheng, Z., Buentello, A. & Gatlin, D.M. (2011) Dietary nucleotides influence immune responses and intestinal morphology of red drum *Sciaenops ocellatus*. Fish. Shellfish Immunol. **30**, 143–147.

Folch, j., Less, M. & Stainley, G.H.S. (1957) A simple method for the isolation and purfication of total lipids from animal tissue. J. Biol. Chem. **226**, 497–509.

Fontana, L., Moreira, E., Torres, M.I., Fernández, I., Ríos A., Sánchez de Medina, F. & Gil, A. (1998) Dietary nucleotides correct plasma and liver microsomal fatty acid alterations in rats with liver cirrhosis induced by oral intake of thioacetamide. J. Hepatol. **28**, 662–669.

Goimier, Y., Pascual, C., Sanchez, A., Gaxiola, G., Sanchez, A. & Rosas, C. (2006) Relation between reproductive, physiological, and immunological condition of *Litopenaeus setiferus* pre-adult males fed different dietary protein levels (Crustacea; Penaeidae). J. Anim. Rep. Science. **92**, 193–208.

Gonzalez-Felix, M.L., Gatlin, D.M., Lawrence, A.L. & Perez-Velazquez, M. (2002) Effect of dietary phospholipid on essential fatty acid requirements and tissue lipid composition of *Litopenaeus vannamei* juveniles. Aquaculture **207**, 151–167.

Gonzalez-Vecino, J.L., Cutts, C.J., Batty, R.S., Greenhaff, P.L. & Wadsworth, S. (2004) The effects of nucleotide enriched broodstock diet on first feeding success and survival of Hadock (*Melanogrammus aeglefinus* L.) larvae. 11th International Symposium on Nutrition and Feeding in Fish 2-7 May, Phuket, Thailand. 121–122.

González Vecino J.L. (2005) Nucleotide enhancement of diets, fish reproduction and egg quality. PhD Thesis. Open University, UHI Millennium Institute, Scottish Association for Marine Science, UK.

Govahi, M., Afsharnasb, M., Motalbei, M.A.A. Haghighi, A. (2014) Multiple infections in shrimp *Litopenaeus vannamei* broodstock in commercial hatcheries in Khouzestan Province. Iran. J. Fish. Sci. **13,** 869–885.

Guo, J., Guo, B., Zhang, H., Xu, W., Zhang, W. & Mai, K. (2016) Effects of nucleotides on growth performance, immune response, disease resistance and intestinal morphology in shrimp *Litopenaeus vannamei* fed with a low fish meal diet. Aquacult. Inter. **24**, 1077-1023.

Hoffmann, K. (2008) Nucleotides-essential nutrients of fish farming. Infofish International, June issue 8–11.

Huu, H.D., Tabrett, S., Hoffmann, K., Köppel P, Lucas, J.S. & Barnes, A.C. (2012) Dietary nucleotides are semi-essential nutrients for optimal growth of black tiger shrimp (*Penaeus monodon*). Aquaculture **366–367**, 115–121.

Keyvanshokooh, S. & Tahmasebi-Kohyani, A (2012) Proteome modifications of fingerling rainbow trout (*Oncorhynchus mykiss*) muscle as an effect of dietary nucleotides. Aquaculture. **324–325**, 79–84.

Lem A. (2006) An overview of global shrimp markets and trade. In: Shrimp Culture: Economics, Market and Trade (Leung, P. & Engle, C. eds), pp.3–10. Blackwell Publishing, Ames, Iowa, USA.

Li, P., Lewis, D.H. & Gatlin III, D.M. (2004) Dietary oligonucleotide from yeast RNA influences immune responses and resistance of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) to *Streptococcus iniae* infection. Fish Shellfish. Immunol. **16**, 561–569.

Li, P. & Gatlin, D.M. III (2006) Nucleotide nutrition in fish: current knowledge and future applications. Aquaculture **251**, 141–152.

Li, P., Lawrence, A.L., Gastille, F.L. & Gatlin, D.M. (2007) Preliminary evaluation of a purified nucleotide mixture as a dietary supplement for Pacific white shrimp *Litopenaeus vannamei* (Boone). Aquacult. Res. **38**, 887–890.

Lin, Y.H., Wang, H. & Shiau, S.Y. (2009) Dietary nucleotide supplementation enhances growth and immune responses of grouper (*Epinephelus malabaricus*). Aquac. Nutr. **15,** 117–122.

Leonardi, M., Sandino, A.M. & Klempau, A. (2003) Effect of a nucleotide-enriched diet on the immune system, plasma cortisol levels and resistance to infectious pancreatic necrosis (IPN) in juvenile rainbow trout (*Oncorhynchus mykiss*). Bull. Eur. Assoc. Fish Pathol. **23**, 52–59.

Mohebbi, A., Nematollahi, A., Gholamhoseini, A., Tahmasebi-Kohyani, A. & Keyvanshokooh, S. (2013) Effects of dietary nucleotide on the antioxidant status serum lipids of rainbow trout (*Oncorhynchus mykiss*). Aquacult. Nutr. **19**, 506–514.

Murthy, H.S., Li, P., Lawrence, A.L. & Gatlin D.M. III. (2009) Dietaryb-glucan and nucleotide effects on growth and immune responses of Pacific white shrimp *Litopenaeus vannamei*. J. App. Aquacult. **21**, 160–168.

Oujifard, A., Abedian, A.H., Nafisi Bahabadi, M., Ghaednia, B. & Mahmoudi N.A. (2008) Effect of dietary nucleotide on the growth performance, survival and some hemolymph parameters of pacific white shrimp (*Litopenaeus vannamei* Boone, 1931). J. Mar. Sci. Tech. **7**, 21–30.

Palacios, E., Ibarra, A.M. & Racotta, I.S. (2000) Tissue biochemical composition in relation to multiple spawning in wild and pond-reared *Penaeus vannamei* broodstock. Aquaculture **185**, 353–371.

Palacios, E., Racotta, I.S., Heras, H., Marty, Y., Moal, J. & Samain, J.F. (2001) The relation between lipid and fatty acid composition of eggs and larval survival in white pacific shrimp (*Penaeus vannamei*, Boone, 1931). Aquac. Int. **96**, 531–543.

Parmar, P.V., Murthy, H.S., Tejpal, C.S. & Kumar, B.T.N. (2011) Effect of brewer's yeast on immune response of giant freshwater prawn, *Macrobrachium rosenbergii*, and its resistance to white muscle disease. Aquacult. Inter. **20**, 951–964.

Pascual, C., Gaxiola, G. & Rosas, C. (2003) Blood metabolites and hemocyanin of the white shrimp, *Litopenaeus vannamei*: the effect of culture conditions and a comparison with other crustacean species. Mar. Biol. **142**, 735–745.

Peng, M., Xu, W., Ai, Q., Mai, K., Liufu. & Zhang, K. (2013) Effects of nucleotide supplementation on growth, immune responses and intestinal morphology in juvenile turbot fed diets with graded levels of soybean meal (*Scophthalmus maximus* L). Aquaculture **367**, 51–58.

Perazzolo, L.M., Gargioni, R., Ogliari, P. & Barraco, M.A.A. (2002) Evaluation of some hemato-immunological parameters in the shrimp *Farfantepenaeus paulensis* submitted to environmental and physiological stress. Aquaculture **214**, 19–33.

Racotta, I.S. & Palacios, E. 1998. Hemolymph metabolic variables in response to experimental manipulation stress and serotonin injection in Penaeus vannamei. J. World Aquac. Soc. **29**, 351–356.

Safari, O., Shahsavani, D., Paolucci, M. & Mehraban, S.A.M. (2014) The effects of dietary nucleotide content on the growth performance, digestibility and immune responses of juvenile narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz. Aquacult. Res. **420–421**, 211–218.

Sainz-Hernández, J.C., Racotta, I.S., Dumas, S. & Hernández-López, J. (2008) Effect of unilateral and bilateral eyestalk ablation in *Litopenaeus vannamei* male and female on several metabolic and immunologic variables. Aquaculture **283**, 188–193.

Sanchez-Pozo, A., Morillas, J., Molto, L., Robles, R. & Gil, A. (1994) Dietary nucleotides influence lipoprotein metabolism in newborn infants. Pediatr. Res. **35**, 112–116.

Shankar, R., Murthy, H.S., Sujatha, H., Jayaraj, E., Tejpal, C. & Chinthamani, V. (2012) Effect of nucleotide on growth, immune responses and resistance of *Macrobrachium rosenbergii* (De Man) to *Macrobrachium rosenbergii nodavirus* (MrNV) and extra small virus (XSV) and *Aeromonas hydrophila* infection. Aquac. Int. **20**, 1–12.

Simpson, A.C. (1951) The fecundity of the plaice. Fisheries Invest. Lond. **17**, 1–27.

Song, L., Yu, I., Lien, W. & Huang, C. (2003) Haemolymph parameters of *Litopenaeus vanname*i infected with Taura syndrome virus. Fish. Shellfish Immunol. **14**, 317–331.

Verslycke, T., Vandenbergh, G.F., Versonnen, B., Arijs, K. & Janssen, C.R. (2002) Induction of vitellogenesis in 17a-ethinylestradiol-exposed rainbow trout (*Oncorhynchus mykiss*): a method comparison. Comp. Biochem. Physiol. **132C**, 483–492.

Wouters, R., Nieto, J. & Sorgeloos, P. (2000) Artificial Diets for Penaeid Shrimp. The Advocate, Global Aquaculture Alliance. April: 61–62.

Wouters, R., Molina, C., Lavens, P. & Calderon J. (2001) Lipid composition and vitamin content of wild female *Litopenaeus vannamei*. Different stages of sexual maturation. Aquaculture **198**, 307–323.

Wouters, R., Zambrano, B., Espin, M., Calderon, J., Lavens, P. & Sorgeloos, P. (2002) Experimental broodstock diets as partial fresh food substitutes in white shrimp *Litopenaeus vannamei*. Aquacult. Nutr. **8**, 249–256.

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Table 1

Composition of experimental diets.

		Different levels of nucleotide (%)		
Ingredients (g kg^{-1})[1]	control	0.2	0.4	0.6
Fish meal[2]	650	650	650	650
Shrimp meal[3]	150	150	150	150
Wheat middling	70.5	70.5	70.5	70.5
Fish oil[2]	25.9	25.9	25.9	25.9
Soybean oil[4]	25.9	25.9	25.9	25.9
Soy lecithin[4]	10	10	10	10
Vitamin premix[5]	10	10	10	10
Mineral premix[6]	10	10	10	10
Cholesterol[7]	5	5	5	5
Anti fungus[8]	2.5	2.5	2.5	2.5
Antioxidant[9]	0.2	0.2	0.2	0.2
Di calcium phosphate[3]	15	15	15	15
Binder[3]	15	15	15	15
Cellulose[7]	10	8	6	4
Nucleotide mix[10]	-	2	4	6

[1] Composition of ingredients as % Dry-weight basis [fish meal (710 g kg^{-1} crude protein, 80 g kg^{-1} crude lipid); shrimp meal (510 g kg^{-1} crude protein, 70 g kg^{-1} crude lipid); wheat middling (120 g kg^{-1} crude protein, 30 g kg^{-1} crude lipid)]

[2] Pars kilka (Mazandaran, Iran).

[3] Havorash (boshehr, Iran).

[4] Behpak Industrial Company, Behshahr, Mazandaran, Iran.

[5] Vitamin premix U kg^{-1} of diet: vitamin A 8000000 IU, vitamin D_3 2000000 IU, vitamin E 150 mg, vitamin K_3 50 mg, vitamin H 1.5 mg, vitamin C 500 mg, Inositol 500 mg, vitamin B_1 50 mg, vitamin B_2 40 mg, vitamin B_3 150 mg, vitamin B_5 200 mg, vitamin B_6 80 mg, vitamin B_9 15 mg, vitamin B_{12} 0.05 mg, career up to 1 kg.

[6] Mineral premix U kg^{-1} of diet: manganese 2600 mg, copper 600 mg, iron 6000 mg, zinc 600 mg, selenium 50 mg, iodine 100 mg, cobalt 50 mg, choline chloride 100000 mg, career up to 1 kg.

[7] Merck, Germany.

[8] 21 beyza, Shiraz, Iran.

[9] Butylated hydroxy toluene, Garmab Shimi, Iran.

[10] Chemoforma Agust, Switzerland.

Table 2

Morphometric and reproductive parameters of *L. vannamei* female fed diets supplemented with nucleotide mixture (mean ± SEM, n = 3).

	Control	Different levels of nucleotide (%) 0.2	0.4	0.6
Morphometric parameters				
BW_i (g)[a]	29.2 ± 1.9	30.6 ± 2.7	30.1 ± 2.5	31.6 ± 2.3
BW_f(g)[b]	35.4 ± 3.0	38.3 ± 3.2	38.5 ± 1.6	39.6 ± 2.6
TL_i (cm)[c]	15.1 ± 0.8	15.1 ± 0.7	15.2 ± 0.8	15.4 ± 0.3
TL_f (cm)[d]	16.2 ± 0.6	16.4 ± 0.8	16.4 ± 0.2	16.8 ± 0.4
WG (%)	21.3 ± 3.4	26.2 ± 2.3	28.3 ± 4.3	25.4 ± 1.1
SGR (%)	0.5 ± 0.2	0.6 ± 0.2	0.8 ± 0.3	0.7 ± 0.0
S (%)	80.0 ± 1.3	86.7 ± 2.5	86.7 ± 1.2	80.0 ± 2.8
K (%)	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0
Reproductive parameters				
HPI (%) before eye stalk ablation	$^{B}3.2 \pm 0.2^{a}$	$^{B}3.4 \pm 0.2^{ab}$	$^{B}3.5 \pm 0.4^{b}$	$^{B}3.1 \pm 0.4^{a}$
HPI (%) after eye stalk ablation	$^{A}2.8 \pm 0.6^{b}$	$^{A}2.8 \pm 0.4^{b}$	$^{A}2.5 \pm 0.2^{ab}$	$^{A}2.3 \pm 0.3^{a}$
GSI (%)	2.3 ± 1.2	3.9 ± 0.9	4.3 ± 0.6	3.7 ± 0.6
Absolute fecundity ($\times 10^3$)	150.6 ± 13.3	162.9 ± 19.3	163.9 ± 11.7	167.8 ± 12.7
Number of egg ($\times 10^3$) per g of female's body weight	4.25 ± 0.0	4.29 ± 0.6	4.25 ± 0.2	4.28 ± 0.6
Eggs diameter (μm)	105.6 ± 36.7	114.2 ± 41.4	114.9 ± 42.8	118.3 ± 38.6
Latency period (days after ESA to spawning)	9.0 ± 0.6^{a}	8.0 ± 0.6^{a}	7.0 ± 0.5^{b}	8.0 ± 0.6^{a}

Different lower case letters correspond to statistical differences among experimental groups within the same sampling (days) tested by one-way ANOVA, whereas different upper case letters indicate differences between different sampling times tested by one-way ANOVA.

[a] BW_i: initial body weight.

[b] BW_f: final body weight.

[c] TL_i: initial total length.

[d] TL_f: final total length.

Table 3

Fatty acid composition (mg g^{-1} lipid) of ovaries of *L. vannamei* fed diets supplemented with nucleotide mixture (mean ± SEM, n = 3).

		Different levels of nucleotide (%)		
	Control	0.2	0.4	0.6
14:0	23.6 ± 0.5	25.6 ± 1.5	26.3 ± 0.5	23.3 ± 1.5
16:0	240.6 ± 8.6[a]	231.4 ± 8.0[ab]	233.3 ± 11.9[ab]	223.0 ± 10.8[b]
17:0	18.0 ± 1.0	17.0 ± 0.0	17.3 ± 1.5	18.0 ± 0.0
18:0	7.6 ± 0.2	8.1 ± 0.2	7.8 ± 0.9	8.0 ± 0.7
SFA[1]	322.0 ± 9.1[b]	354.6 ± 10.5[a]	354.6± 6.1[a]	344.6 ± 16.0[a]
16:1n–7	105.3 ± 1.5	104.3 ± 1.1	103.0 ± 1.0	104.3 ± 1.1
18:1n–7	28.3 ± 0.5	28.0 ± 1.0	28.0 ± 2.0	28.9 ± 0.9
18:1n–9	141.6 ± 11.2	147.6 ± 9.2	154.3 ± 10.5	152.0 ± 15.7
20:1n–9	16.0 ± 1.7	17.0 ± 2.0	16.3 ± 0.5	17.0 ± 0.0
MUFA[2]	291.3 ± 8.5	297.0 ± 8.7	301.6 ± 9.7	302.2 ± 17.0
18:2n–6	35.0 ± 5.2	34.6 ± 0.5	36.0 ± 3.6	36.0 ± 1.0
20:2n–6	7.6 ± 0.5	7.3 ± 0.5	6.6 ± 1.1	6.6 ± 0.5
20:4n–6, ARA[3]	37.0 ± 3.0	36.6 ± 1.5	37.3 ± 1.5	37.3 ± 3.0
n–6 PUFA[4]	79.6 ± 8.0	78.6 ± 1.5	80.0 ± 4.5	80.0 ± 2.6
18:3n–3,	26.6 ± 2.5	26.6 ± 0.5	28.3 ± 1.1	27.6 ± 0.5
20:5n–3, EPA[5]	103.1 ± 4.2[b]	109.2 ± 2.3[ab]	114.3 ± 5.5[a]	113.3 ± 4.1[a]
22:6n–3, DHA[6]	75.0 ± 7.0	84.1 ± 5.1	80.0 ± 8.9	77.3 ± 2.0
n–3 PUFA	204.7 ± 3.0[b]	219.9 ± 5.4[a]	222.6 ± 7.3[a]	218.2 ± 4.9[a]
n-3 LC-PUFA[7]	178.1 ± 3.0[b]	193.3 ± 4.9[a]	194.3 ± 7.0[a]	190.6 ± 4.7[a]
n-3 LC-PUFA / ARA	4.8 ± 0.1	5.3 ± 0.1	5.2 ± 0.0	5.1 ± 0.0
ARA / EPA	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
DHA / EPA	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.0
n–3 / n–6	2.6 ± 0.2	2.8 ± 0.1	2.8 ± 0.2	2.6 ± 0.1

Different lower case letters correspond to statistical differences among experimental groups tested by one-way ANOVA.

[1] SFA: saturated fatty acids including: 14:0, 16:0, 17:0 and 18:0

[2] MUFA: monounsaturated fatty acids including: 16:1*n*7, 18:1*n*9, 18:1*n*7, 20:1*n*9

[3]ARA: arachidonic acid.

[4] PUFA: polyunsaturated fatty acids.

[5] EPA: eicosapentaenoic acid.

[6] DHA: docosahexaenoic acid.

[7] n-3 LC-PUFA = EPA+DHA

Table 4

Plasma biochemical parameters of *L. vannamei* female fed diets supplemented with nucleotide mixture before and after of ESA (mean ± SEM, n = 3).

		Different levels of nucleotide (%)				Two-Way ANOVA		
	Sampling time	Control	0.2	0.4	0.6	NT Level	ESA	NT level × ESA
Glu (mg dl^{-1})	initial	11.2 ± 0.3	11.2 ± 0.3	11.2 ± 0.3	11.2 ± 0.3	0.001	0.001	0.09
	before ESA	12.8 ± 0.3^{a}	13.7 ± 0.3^{b}	14.4 ± 0.5^{c}	14.1 ± 0.2^{bc}			
	after ESA	7.9 ± 0.3^{a}	9.2 ± 0.4^{b}	10.7 ± 0.5^{c}	9.8 ± 0.3^{c}			
TP(g dl^{-1})	initial	3.6 ± 0.1	3.6 ± 0.1	3.6 ± 0.1	3.6 ± 0.1	0.001	0.001	0.455
	before ESA	4.3 ± 0.1^{a}	4.7 ± 0.1^{b}	5.4 ± 0.1^{c}	5.7 ± 0.1^{d}			
	after ESA	3.7 ± 0.0^{a}	4.3 ± 0.1^{b}	5.3 ± 0.0^{c}	5.7 ± 0.1^{d}			
Ca^{+2} (mg dl^{-1})	initial	57.3 ± 1.5	57.3 ± 1.5	57.3 ± 1.5	57.3 ± 1.5	0.278	0.001	0.094
	before ESA	58.0 ± 1.8	62.0 ± 1.3	61.7 ± 2.6	61.6 ± 2.0			
	after ESA	56.0 ± 4.4	58.1 ± 1.7	59.1 ± 2.9	60.2 ± 2.4			
Chol (mg dl^{-1})	Initial	57.4 ± 0.6	57.4 ± 0.6	57.4 ± 0.6	57.4 ± 0.6	0.001	0.001	0.922
	before ESA	75.9 ± 1.0^{a}	78.1 ± 1.2^{ab}	80.3 ± 1.8^{bc}	81.2 ± 0.6^{c}			
	after ESA	56.0 ± 1.2^{a}	59.0 ± 1.5^{b}	60.7 ± 1.2^{bc}	62.3 ± 1.1^{c}			
Tg (mg dl^{-1})	Initial	37.7 ± 2.5	37.7 ± 2.5	37.7 ± 2.5	37.7 ± 2.5	0.001	0.001	0.205
	before ESA	49.0 ± 2.0^{a}	52.7 ± 1.5^{ab}	58.7 ± 1.5^{c}	55.0 ± 2.0^{bc}			
	after ESA	40.7 ± 1.5^{a}	45.0 ± 0.2^{a}	46.7 ± 1.5^{b}	43.7 ± 2.1^{ab}			
HDL (mg dl^{-1})	Initial	5.7 ± 1.5	5.7 ± 1.5	5.7 ± 1.5	5.7 ± 1.5	0.012	0.001	0.441
	before ESA	6.7 ± 1.5^{a}	8.7 ± 1.8^{ab}	11.3 ± 1.3^{b}	10.3 ± 1.4^{b}			
	after ESA	14.7 ± 1.5	15.0 ± 0.1	17.0 ± 2.0	15.3 ± 2.1			

Different lower case letters correspond to statistical differences among experimental groups tested by one-way ANOVA. The significance of the two main effects (NT level and ESA) and interaction (NT level × ESA) were analyzed using two-way ANOVA.

Table 5

Total hemocyte count and differential hemocyte count of *L. vannamei* female fed diets supplemented with nucleotide mixture before and after ESA (mean ± SEM, n = 3).

			Different levels of nucleotide (%)			Two-Way ANOVA		
	Sampling time	Control	0.2	0.4	0.6	NT Level	ESA	NT level × ESA
THC ($\times 10^5$ cell ml^{-1})[1]	initial	108.9 ± 11.0	108.9 ± 11.0	108.9 ± 11.0	108.9 ± 11.0	0.001	0.146	1.0
	before ESA	114.9 ± 6.9^{a}	150.8 ± 6.0^{b}	152.1 ± 4.1^{b}	153.5 ± 6.7^{b}			
	after ESA	110.5 ± 6.0^{a}	146.8 ± 8.3^{b}	148.5 ± 4.6^{b}	149.5 ± 8.1^{b}			
HC (%)[2]	initial	18.5 ± 2.5	18.5 ± 2.5	18.5 ± 2.5	18.5 ± 2.5	0.011	0.001	0.929
	before ESA	19.5 ± 1.2^{b}	15.0 ± 2.1^{a}	14.0 ± 1.5^{a}	13.4 ± 1.3^{a}			
	after ESA	17.5 ± 3.1^{c}	13.5 ± 2.5^{b}	11.5 ± 2.0^{ab}	10.5 ± 1.0^{a}			
SGC (%)[3]	initial	60.0 ± 3.8	60.0 ± 3.8	60.0 ± 3.8	60.0 ± 3.8	0.232	0.612	0.912
	before ESA	59.0 ± 3.9	55.2 ± 2.6	54.9 ± 3.7	54.2 ± 2.4			
	after ESA	58.5 ± 4.3	55.5 ± 3.1	55.8 ± 3.8	56.4 ± 2.9			
GC (%)[4]	initial	21.5 ± 2.7	21.5 ± 2.7	21.5 ± 2.7	21.5 ± 2.7	0.001	0.144	0.916
	before ESA	21.5 ± 2.0^{a}	29.9 ± 2.8^{b}	31.2 ± 1.5^{b}	31.5 ± 1.8^{b}			
	after ESA	24.0 ± 2.6^{a}	31.0 ± 1.7^{b}	32.8 ± 2.1^{bc}	33.2 ± 1.3^{c}			

Different lower case letters correspond to statistical differences among experimental groups tested by one-way ANOVA. The significance of the two main effects (NT level and ESA) and interaction (NT level × ESA) were analyzed using two-way ANOVA.

[1] THC: total hemocyte count.

[2] HC: hyaline cells.

[3] SGC: semigranular cells.

[4] GC: granular cells.

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