SNRU Journal of Science and Technology

Journal home page: snrujst.snru.ac.th

Effects of omaga-3 fatty acids (from krill oil) on sperm quality of mahseer barb, *Neolissochilus stracheyi*, brooders reared in captivity

Ekachai Duangjai[1,*], Sutthida Punya-in[1], Auttapon A-ta[1], Settawut Arin[1], Anuwat Sangkamruang[1], Pramod Sresutham[1], Jittra Punroob[2]

[1]Faculty of Science and Agriculture Technology, Rajamangala University of Technology Lanna, Nan, 55000 Thailand

[2]Faculty of Business Administration and Liberal Arts, Rajamangala University of Technology Lanna Nan, 55000 Thailand

[*]**Corresponding Author:** mr1fisheries@hotmail.com

Received: 5 October 2016; **Revised:** 31 March 2017; **Accepted:** 2 April 2017; **Available online:** 1 August 2017

Abstract

The effects of feeding mahseer barb male brooders with different levels of omaga-3 fatty acids (krill oil) on the sperm quality were investigated. All experimental fish at Initial body weight about 130.25±12.50 g bw and Initial body length about 22.85±2.18 cm were randomly distributed in four treatments at stocking density of 3 fish m^{-2}. During study from August to October, 2014, Fish were fed with commercial diet (3% g bw^{-1}) which supplemen-ting omaga-3 fatty acids at 0.00, 0.08, 0.24 and 0.4 g (w v^{-1}) kg^{-1}diet for Cont., Trt_1, Trt_2 and Trt_3, respectively. At the end of culturing, ten fish from each group were randomly selected for milt and blood collection. The sperm quality of male brooders was evaluated by either traditional techniques or comet assay techniques. While, blood $PGF_{2\alpha}$ levels were determined by RT-HPLC. The results were evaluated by the traditional techniques demonstrated that supplementing with 0.4 g kg^{-1}diet of omaga-3 fatty acids (Trt_3) had significantly different ($p<0.05$) milt volume, the total sperm counts, and percentages of abnormal sperm than the other groups. Besides the results observed comet assay techniques showed that supplemented with 0.4 g kg^{-1} diet of omaga-3 fatty acids had significantly lower ($p<0.05$) comet length, tail length, %DNA in tail, and tail moment than the other groups. The blood $PGF_{2\alpha}$ levels were the highest in Trt_3 and was not significantly different with other groups ($p<0.05$). This study showed that supplementing of 0.4 g kg^{-1}diet of omaga-3 fatty acids together with commercial diet was found to be the best diet for male *N. stracheyi* brooders that provided adequate required nutrients for the formation of spermatogenesis. This knowledge can be used for enhance sperm quality of fish male brooders in captivity by manipulating proper types and levels of nutrition at the most appropriate times.

Keywords: *Neolissochilus stracheyi*; Sperm quality; Omaga-3 fatty acids diets

©2017 Sakon Nakhon Rajabhat University reserved

1. Introduction

Mahseer barb or hill-stream fishes is one of domesticated freshwater fish species from the cyprinidae family [1]. Fish was reported as upstream or hill-stream fish that wide distribution from China to Indonesia [2]. The spawning season of this species ranges from early July to late November while the fecundity of female fish is very low compared to other mahseer group [3]. Today, the gonad quality, sperm quality, of freshwater fish male brooders from the natural stocks in reservoir of Thailand have declined by reason of environmental perturbations especially dumping of agro-chemicals and destructive fishing practices in the aquatic ecosystem [4]. In most vertebrates, during spermatogenesis affected by several factors especially nutrition changes due to the season and environmental variation for example, in common barbell [5] and Eurasian perch [6]. They were reported that dietary containing fatty acids affected sperm quality because of fatty acids are the major sources of the n-3 long chain PUFA, EPA, and DHA, and these fatty acids as precursor of reproductive hormones such as progesterone prostaglandins that reported in the study of male European eel [7]. Previous research in freshwater fish reproduction mostly were involved during final maturation and spawning in female fish while information about males gonadal development is less compared with those performed for oogenesis in fish [8] An understanding of this process remains still unknown, but it is as important as quality of sperm quality of males fish. To receive best male brooders, fish required nutrients is a key factor Controlling gonadal development and fecundity of male brooders in captivity [9]. Dahlgren [10] reported that increased dietary lipid Content also resulted in increased gonadal development and fecundity in the guppy. According to Alavi et al., [5] an external morphological examination (traditional techniques) of spermatozoa such as milt volume, motility and fertilizing capacity, as well as composition of the seminal plasma are use to assess sperm quality in fish. However, these traditional monitoring are not able to detect at DNA level of fish sperm. Comet assay, deoxyribonucleic acid quality examination, is generally a technique for DNA damage examination from pollutants impacts on organisms [11]. Today these techniques are increased in popularity as a standard technique for evaluation of DNA damage of aquatic animal [12]. Therefore, the objectives of present study aimed to determine the effects of omaga-3 fatty acids on sperm quality of *N. stracheyi* male brooders reared into captivity, concrete tank.

2. Materials and methods

Experimental design

The present study was carried out at Faculty of Sciences and Agriculture Technology, Rajamangala University of Technology Lanna, Nan province. One hundred and twenty of male *N. stracheyi* brooders (130.25±12.50 g.bw, live body weight of 2 year olds approximately and an average total length of 22.85±2.18 cm) were collected on June, 2014 from earthen pond located in Amphoe Bo Kluea, Nan province.They were carefully collected by nylon net from earth pond which is cultured under polyculture system with the average water depth at 1.50 m and 20 °C of the average water temperature. The aerated containers were used for transport fish sample to the experimental station. Before sample released into each experimental tank, total bodyweight and total length of fish were measured and recorded then transferring into the experimental take and allowed to feed with commercial diets for

a week. A complete randomized design was used in an experiment which consists of four treatments, with two replications per treatment. Twenty seven fish males were randomly distributed in four treatments at the stocking density of 3 fish m^{-2}. Experimental diets enriched with krill oil with the dosage of 0.00, 0.08, 0.24 and 0.40 g kg^{-1} diet for Cont., Trt_1, Trt_2 and Trt_3 respectively. During study from August to October, 2014, fish were fed with diet at 3% g bw^{-1} once a day (08.00 am). Water changes at the rate of one hundred percent of total volume weekly.

Sample collection

At the end of culturing, before sample collected, the fish were fasted for two days. Ten males fish of treatments were collected following anesthetized by 50 ppm peppermint oil. Milt was collected immediately for sperm evaluation and a blood sample of individual male fish was withdrawn from the caudal vein using a 24 Gx1 syringe (NIPRO) and stored at –80 °C for $PGF_{2\alpha}$ hormone extraction. Some of blood sample were centrifuged at 3,000 g for 15 minutes following supernatant collection and stored at –30 °C for protein concentration determi-nation according to the method described by the Bradford method [13]. The sperm quality of males fish were collected for use to evaluate the quality of spermatozoa. One milliliter of milt per fish was collected follow dilute to 10^5 cell ml^{-1} sperm density with 0.85% NaCl solution and stored at room temperature until further evaluate.

Data collection and analytical methods

Evaluation of sperm quality by an external morphological examination

The gross examination was considered for one hundred spermatozoa per fish which were adaptations based on an external morphological observation includes : milt volume by pipetting milt in 1.50 microcentrifuge tube and the level was calculated in milliliters. The microscopic examination was carried out using light microscope (100x) to assess other parameters such as morphological feature (normal and abnormal sperm) and dead sperm cells according to the method described by Kime et al., [14] as follow : to determine the percentage of normal and abnormal sperm cells, cells were stained with a solution 0.40% Trypan blue at ratio of 1:10 (solution 0.40% trypan blue: calcium-free saline solution). After four hours, dead sperm (blue cells) was counted under light microscopy as well as gross morphology of live sperm was examined. Sperm with a spherical body were considered normal. Abnormal sperm were distinguished from normal by malformed bodies or by a bent, short, or missing tail.

Evaluation of DNA quality by single cell gel electrophoresis analysis

The deoxyribonucleic acid quality of fish spermatozoa were examined by comet assay techniques according to the method described by Singh et al., [12] as follow: step 1 : target cells were embedded by adding 250 µl of 1 x 10^5 cells ml^{-1} of sperm solution into the tube containing 250 µl of 1% low melting-point agarose in 1xPBS at 37 °C and mixed by tapping the tube, then 100 µl aliquot of warm mixture was dropped on coated slide, covered immediately by coverslip and left for 30 minutes at 4 °C to solidify. The coverslip was removed after solidification. Step 2 : the slide was transferred into a black chamber containing 50 ml of ice-cooled lysis solution for 30 minutes to allow DNA to unwind. Step 3 : 500 ml of electrophoresis buffer was added into an electrophoresis chamber. The solidified slide without coverslip was placed carefully into the chamber and electrophoresis is performed using volt constant at 25

V (300 mA) for 20 minutes. After electrophoresis, all slides were neutralized by rinsing 3 times in neutralization buffer and immersing in 70% ethanol for 30 minutes before staining with EtBr. step 4 : DNA damage was visualized using an Olympus BX 50 microscope epifluorescence, Japan, under excitation filter 510-560 nm UV light, barrier filter 590 nm (40x) using an ocular micrometer. Fifty comets cell per slide were randomly selected and subjected to head and tail measurements. Subsequently, five slides were considered per replication. The migration of DNA damage was used to determine the quality of sperm cell according to the method described by Singh et al., [12] as follow:

Comet length:

$$\text{Comet length } (\mu m) = \text{Tail Length} + \text{Head Length} \quad (1)$$

Tail Length:

$$\text{Tail Length } (\mu m) = \text{Tail Extent (Tail from Center) Tail Extent} + \text{Head Extent}/2 \quad (2)$$

% DNA in tail:

$$\text{\% DNA in tail} = 100 - \text{Head \% DNA}. \quad (3)$$

Tail moment:

$$\text{Tail moment} = \text{Tail Length } (\mu m) \times \text{Tail DNA } [\%] \quad (4)$$

ProstaglandinF$_{2\alpha}$ (PGF$_{2\alpha}$) extraction and analysis

The blood $PGF_{2\alpha}$ extraction and analysis were carried out in department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok. A blood samples of fish was extracted according to the method described by Tahara and Yano [15]. as follow : one milliliter of blood samples was extracted into glass homogenizer tubes containing 1 ml of extracted solution at ratio of 1:4 (DI water : ethanol solution, into 50 µl acetic acid). The homogenate was mixed and immediately centrifuged at 3,000 rpm for 5 minutes at 4 °C following supernatant collection. The blood $PGF_{2\alpha}$ levels in an extract solution was quantified using a Sep Pak(C_{18}) mini column (Alltech, USA). The eluted solution was evaporated under the vacuum dried drying and re-dissolved in twenty microliter of methanol. The supernatant was then injected onto a Prevail C_{18} column (4.60 mm x 15 cm I.D.) (Alltech, MD, USA) and run on an isocratic solvent of 30% acetonitrile (CH_3CN) per 70% HPLC water (3:7, v v^{-1}) (pH 2 by 17 mM phosphoric acid) at a flow rate of 1 ml min^{-1}. The $PGF_{2\alpha}$ peak of the sample was measured at 200 nm. The blood $PGF_{2\alpha}$ levels was calculated and compared with the $PGF_{2\alpha}$ standard curve.

Statistical analyses

All data was expressed as mean±standard deviation (S.D.). A statistical difference was estimated by one-way analysis of variance (ANOVA). The treatment analysis to determine the differences between group means was estimated by Duncan's multiple range test at $P<0.05$.

3. Results

Sperm quality evaluated of males N. stracheyi brooders by an external morphological examination

Males bodyweight (g) and males bodylength (cm) between treatments were not significantly different. Milt volume was reported to be lowest in fish males fed only commercial fish diets control group (Cont.) and was significantly different from the other treatment groups (Trt_1, Trt_2 and Trt_3) (Table 1). It was not significantly different between the treatment of 1, 2 and 3. Evaluation of the average total sperm counts of fish were presented as sperm cell g bw^{-1}. It was demonstrated that fish of Trt_3 had higher the average total sperm counts which was significantly different from other treatment groups ($p<0.05$) and it was lower in control group (Table 1). Percentages of dead sperm cells was found significantly higher in Cont. than other treatment groups ($p<0.05$) (Table 1). Moreover, percentages of dead sperm cells of fish in Trt_3 showed significantly lower than other treatment groups ($p<0.05$) but not significantly different from Trt_1 and Trt_2 ($p>0.05$). In addition, percentages of abnormal sperm were not significant different between treatment groups ($p>0.05$) (Table 1).

Table 1 Evaluation on some external morphological and some sperm quality characteristics of males *N. stracheyi* brooders between groups of treatments (n = 10).

Parameters	Cont.	Trt_1	Trt_2	Trt_3
Males bodyweight (g)	172.15 ± 15.36^{a}	170.34 ± 10.31^{a}	179.54 ± 21.55^{a}	171.36 ± 23.54^{a}
Males body length (cm)	24.35 ± 8.36^{a}	24.98 ± 6.54^{a}	24.05 ± 8.66^{a}	24.69 ± 4.91^{a}
Milt volume (ml.)	1.69 ± 0.65^{b}	3.92 ± 0.91^{a}	3.58 ± 0.12^{a}	3.85 ± 0.54^{a}
Total sperm counts ($x10^4$) (cell g bw^{-1})	12.65 ± 5.44^{b}	28.45 ± 9.32^{a}	32.65 ± 7.34^{a}	38.66 ± 10.66^{a}
Percentages of dead sperm cells	78.26 ± 12.88^{a}	21.54 ± 8.33^{b}	15.95 ± 2.02^{b}	15.22 ± 2.66^{b}
Percentages of abnormal sperm	29.44 ± 8.99^{a}	25.79 ± 5.31^{a}	21.75 ± 6.47^{a}	22.46 ± 4.12^{a}

Different letters in the same row indicate significant comparisons. Significant differences ($P<0.05$) in the value of all parameters (mean±SD, n = 10 fish, 50 sperm cells $fish^{-1}$) of *N. stracheyi* brooders between treatment group and the control groups are indicated with letters (a, b and c).

DNA quality evaluated of males N. stracheyi brooders by comet assay techniques

The result of DNA quality examination by comet assay techniques were shown in Table 2. The degrees of DNA damage of spermatozoa were estimated in the terms of comet length, tail length, % DNA in tail, and tail moment. The values of comet length of control group had significant differences from other treatment groups ($p<0.05$). The values of tail length was highest in Cont. (89.36±24.55 μm) followed by Trt_1 (73.14±37.01 μm) and Trt_2 (54.46±19.60 μm). The values of tail length was lowest in Trt_3 (32.64±16.28 μm). The values of % DNA in Tail were highest in Cont. (12.59±2.69%) and they were not significantly different between the treatment groups of 1, 2 and 3. The values of tail moment was highest in cont. (13.48±2.87) followed by Trt_1 (7.30±1.26) and Trt_2 (4.62±1.89). The values of tail length was lowest in Trt_3 (2.64±1.13).

Table 2 Evaluation for sperm quality of males *N. stracheyi* brooders of Cont., Trt_1, Trt_2, and Trt_3 by comet assay techniques. (n = 10 fish, 50 sperm cells $fish^{-1}$).

Parameters	Cont.	Trt_1	Trt_2	Trt_3
Comet length (μm)	164.88 ± 88.32^{a}	123.26 ± 83.23^{b}	109.16 ± 46.44^{b}	86.74 ± 19.69^{c}
Tail length (μm)	89.36 ± 24.55^{a}	73.14 ± 37.01^{a}	54.46 ± 19.60^{b}	32.64 ± 16.28^{c}
DNA in tail (%)	12.59 ± 2.69^{a}	6.05 ± 1.22^{b}	7.97 ± 2.97^{b}	5.84 ± 1.52^{b}
Tail moment	13.48 ± 2.87^{a}	7.30 ± 1.26^{b}	4.62 ± 1.89^{c}	2.64 ± 1.13^{c}

Different letters in the same row indicate significant comparisons. Significant differences (P<0.05) in the value of all parameters (mean±SD, n = 10 fish, 50 sperm cells $fish^{-1}$) of the sperm cell between treatment group (Trt) and the control groups (Cont.) are indicated with letters (a, b and c).

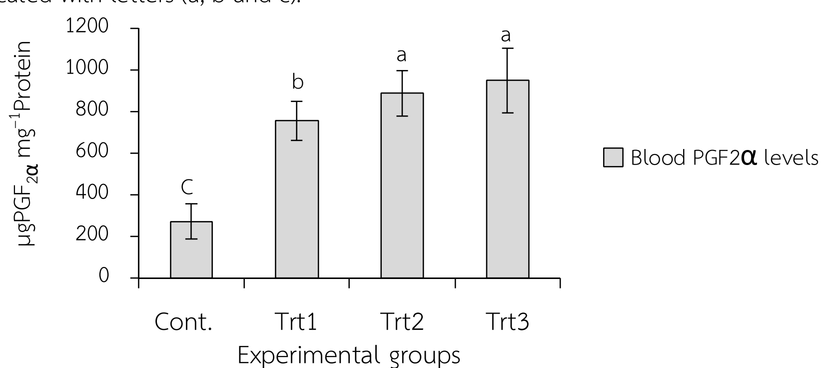

Fig. 1 Blood $PGF_{2\alpha}$ levels of males *N. stracheyi* brooders received fed with omaga-3 fatty acids diet represented in Cont., Trt_1, Trt_2, and Trt_3, respectively. Different letters in the same bar indicate significant comparisons. Significant differences (P<0.05) in the value of blood $PGF_{2\alpha}$ levels (mean±SD, n = 10 fish) of fish brooders between treatment groups and the control groups (Cont.) are indicated with letters (a, b, and c).

The blood $PGF_{2\alpha}$ levels of males N. stracheyi brooders

The result of the blood $PGF_{2\alpha}$ levels of males *N. stracheyi* brooders as shown in Fig. 1. The blood $PGF_{2\alpha}$ levels were estimated in the terms of μg$PGF_{2\alpha}$ mg^{-1}protein. The blood $PGF_{2\alpha}$ levels of Trt_3 (950.67±153.91 μg$PGF_{2\alpha}$ mg^{-1}protein) has significant differences compared with cont. (271.62±85.15 μg$PGF_{2\alpha}$ mg^{-1}protein) ($p<0.05$) and Trt_1 (755.28±95.33 μg$PGF_{2\alpha}$ mg^{-1}protein), however, there are not significant different between Trt_3 and Trt_2 (886.88±108.82 μg$PGF_{2\alpha}$ mg^{-1}protein).

4. Discussion

An understanding of the nutrient requirements of male fish brooders during spawning season is important because it allow us to establish the reproductive tools for controlling the quality of sperm production. According to Kime et al., [16] gonadal development in male freshwater fish is affected by several factors such as water rise, temperature, rainfall and photoperiod as well as feeding. These factors are directly affected on male brooders

health which is resulted in the quality of sperm production. Considering the existing previous research, we found that dietary containing fatty acids directly affects on sperm quality for example; in common barbell, *Barbus barbus*, dietary containing fatty acids significantly affected sperm morphology (except for anterior and posterior parts of the mid piece) and sperm velocity ($P<0.05$) [14]. Based on the studied of African catfish, *Clarias gariepinus*, by Nyina-Wamwiza et al., [17], the results suggest a positive impact of short chain n-6 fatty acids on androgen synthesis and sperm quality. In Eurasian perch, *Perca fluviatilis*, Henrotte et al., [6] reported that the dietary n-3/n-6 ratio affects the lipid composition of perch semen but not the indicators of sperm quality. Similar the study in European eel, *Anguilla anguilla*, Baeza et al., [7] demonstrates that the progression of spermiation is influenced by water temperature and demonstrate the importance of EPA, ARA and DHA in European eel reproduction. In the present study, the result has clearly shown that sperm of males *N. stracheyi* brooders gained better quality in the terms of milt volume, motility duration, percentage of sperm diameter (≥3 µm) and percentages of normal sperm when fed with commercial diet together with 0.40 g of omaga-3 fatty acids (Trt_3). These fatty acids especially arachidonic acid function as precursor for reproductive hormones synthesis such as prostaglandins effect on gonad development of aquatic animal for example the study in African catfish, Nyina-Wamwiza et al. [18] reported that dietary plant products have a positive impact on sex steroid level production which is associated with a significant improvement of sperm quality. In an Indian major carp, Nandi et al., [19] studied on the effect of dietary supplementation of n-3 and n-6 polyunsaturated fatty acids (PUFA) on reproductive performances in both sexes of an Indian major carp over a consecutive 2-year period. The result shown that sperm count and spermatocrit value were in the range of 3.2 – 3.8 x 10^7 ml^{-1} and 80 – 82%, respectively in fish fed the PUFA- enriched test diet and significantly higher than in fish fed control diet (2.3 – 2.5 x 10^7 ml^{-1} and 64 – 70%. In contrast, our result of sperm quality of group 1 which fed with none supplementing of omaga-3 fatty acids had significantly lower than other groups. This result of an external morphological examination was related to the lower amount of omaga-3 fatty acids in commercial diet, because it has been demonstrated that omaga-3 fatty acids are not abundant in commercial diet. Previous research in freshwater fish reproduction, they were suggest that lower fatty acids in feeding are implicated in physiological functions linked to sperm activity by negative effect on membrane structure and also inducers of sex steroid, eicosanoid, and prostaglandins hormone synthesis [20]. In addition, one of the objectives of this study was to assess the quality of the sperm by single cell gel electrophoresis analysis which displayed in the terms of comet mean intensity, head mean intensity, tail mean intensity and tail length and tail moment. According to Speit et al., [21] the single cell gel electrophoresis performed on spermatozoa of a freshwater invertebrate, because of the highly compact structure of chromatin in spermatozoa, additional steps to allow migration of DNA are currently applied by authors working with sperm. In this study, values of all parameters were recorded from study by using the different techniques had lower in the Trt_3 and were significantly different from cont., Trt_1 and Trt_2 ($p<0.05$). By the reason that the single cell gel electrophoresis analysis which is use to assess the DNA quality [12], this may be related to the function of fatty acids in membrane structure of spermatozoa when fed with commercial diet in conjunction with omaga-3 fatty acids [22]. Similar, the reported of Tinoco [23] that sperm phospholipids contain a high proportion of polyunsaturated fatty acids, and male reproductive tissue together with neural tissue has the

highest concentration of docosahexaenioc acid. These fatty acids requirements may be related to the formation of spermatogenesis and may be related to the formation of oxygen radicals during steroid hormone biosynthesis as observed in higher vertebrates [24]. Similar the animal fatty acids requirements, Am-in et al. [25] demonstrated that DHA and polyun-saturated fatty acids n–3 were positively correlated with boar sperm quality. Overall the study results suggest that feeding fish brooders with omaga-3 fatty acids have a positive impact on spermatogenesis.

5. Conclusion

Our results confirm the supplementing of omaga-3 fatty acids effect on sperm quality characteristics of fish reared in captivity. According to an external morphological examination and DNA quality examination data presented that, encouraging sperm quality of fish through the use of 0.40 g kg^{-1} diet (Trt_3) is more dependable in male gonadal development potential. The study of males brooders diet is a better way of promoting fish males production with respect to male gonadal development potential in Thailand mahseer barb aquaculture.

6. Acknowledgment

The first author would also like to thank the Research and Development Institute of Rajamangala University of Technology, Lanna, for the financial support used for this research, Hands-on Researcher Large (2014), in title by Effects of omaga-3 fatty acids on sperm quality of mahseer barb (*N. stracheyi*) brooders reared in captivity.

7. References

[1] W.N. Eschmeyer, C.J.J.r. Ferraris, M.D. Hoang, D.J. Long, The catalog of fishes, species of fishes, http://www.calacademy.org/scientists/ichthyology/catalog/intro.html., 19 September 2016.

[2] W.J. Rainboth, Fishes of the Cambodian Mekong. FAO Species Identification Field Guide for Fishery Purposes. FAO, Rome, 1996, pp. 265.

[3] S.C. Mahata, M.G. Hussain, M.A. Mazid, Successful spawning of pond reared mahseer *Tor putitora* for the first time in Bangladesh, Bangladesh J. Environ. Sci. 1 (1995) 74 – 81.

[4] G.J. Piet, Impact of environmental perturbations on a tropical fish community, Can.J. Fish. Aquat. Sci. 55 (1998) 1842 – 1853.

[5] S. Alavi, M. Psenicka, T. Policar, M. Rodina, J. Hamackova, P. Kozak, O. Linhart, Sperm quality in male *Barbus barbus* L. fed different diets during the spawning season, Fish Physiol. Biochem. 35 (2009) 683 – 693.

[6] E. Henrotte, V. Kaspar, M. Rodina, M. Psenicka, O. Linhart, P. Kestemont, Dietary ratio affects the biochemical composition of Eurasian perch semen but not indicators of sperm quality, Aquac. Res. 41 (2010) 31 – 38.

[7] R. Baeza, I. Mazzeo, M.C. Vílchez, V. Gallego, D.S. Peñaranda, L. Pérez, J.F. Asturiano, Effect of thermal regime on fatty acid dynamics in male European eels during hormonally-induced spermatogenesis, Aquaculture. 430 (2014) 86 – 97.

[8] I.A.E. Butts, R. Baeza, J.G. Støttrup, M. Krüger-Johnsen, C. Jacobsen, L.Pérez, J. F. Asturiano, J. Tomkiewicz, Impact of dietary fatty acids on muscle composition, liver lipids, milt composition and sperm performance in European eel, Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 183 (2015) 87 – 96.

[9] W.K. Ng, Y. Wang, Inclusion of crude palm oil in the broodstock diets of female Nile tilapia, *Oreochromis niloticus*, resulted in enhanced reproductive performance compared to broodfish fed diets with added fish oil or linseed oil, Aquaculture. 314 (2011) 122 – 131.

[10] B.T. Dahlgren, The effects of three different protein levels on the fecundity in the guppy, J. Fish Biol. 15 (1980) 83 – 97.

[11] C. Lewis, T. Galloway, Reproductive Consequences of Paternal Genotoxin Exposure in Marine Invertebrates, Environ Sci Technol. 43 (2009) 928 – 933.

[12] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp Cell Res. 175 (1988) 184 – 191.

[13] M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding, Anal Biochem. 28 (1976) 350 – 356.

[14] D.E. Kime, M. Ebrahimi, K. Nysten, I. Roelants, E. Rurangwa, H.D.M. Moore, F. Ollevier, Use of computer assisted sperm analysis (CASA) for monitoring the effects of pollution on sperm quality of fish: application to the effects of heavy metals, Aquat. toxicol. 36 (1996) 223 – 237.

[15] D. Tahara, I. Yano, Development of hemolymph prostaglandins assay systems and their concentration variations during ovarian maturation in the kuruma prawn, Aquaculture. 220 (2003) 791 – 800.

[16] D.E. Kime, M. Ebrahimi, K. Nysten, I. Roelants, E. Rurangwa, H.D.M. Moore, F. Olivier, Use of Computer Assisted Sperm Analysis (CASA) for Monitoring the Effects of Pollution on Sperm Quality of Fish, Application to the Effects of Heavy Metals, Aquat Toxicol. 36 (1996) 223 – 237.

[17] L. Nyina-Wamwiza, S. Milla, M.-A. Pierrard, E. Rurangwa, S.N.M. Mandiki, K.J. W. Van Look, P. Kestemont, Partial and total fish meal replacement by agricultural products in the diets improve sperm quality in African catfish (*Clarias gariepinus*), Theriogenology. 77 (2012) 184 – 194.

[18] L. Nyina-Wamwiza, S. Milla, M.-A. Pierrard, E. Rurangwa, S.N.M. Mandiki, K.J.W. Van Look, P. Kestemont, Partial and total fish meal replacement by agricultural products in the diets improve sperm quality in African catfish, Theriogenology. 77 (2012) 184 – 194.

[19] S. Nandi, P.Routray, S.D. Gupta, S.C. Rath, S. Dasgupta, P.K. Meher, P.K. Mukhopadhyay, Reproductive performance of carp, *Catla catla* (Ham.), reared on a formulated diet with PUFA supplementation, J Appl Ichthyol. 23 (2007) 684 – 691.

[20] M.S. Izquierdo, H. Fernandez-Palacios, A.G.J. Tacon, Effect of brooders nutrition on reproductive performance of fish, Aquaculture. 197 (2001) 25 – 42.

[21] G. Speit, M. Vasquez, A. Hartmann, The comet assay as an indicator test for germ cell genotoxicity, Mutat Res Rev Mutat Res. 681 (2009) 3 – 12.

[22] J. Beirao, F. Soares, P. Pousao-Ferreira, P. Diogo, J. Dias, M.T. Dinis, M.P. Herraez, E. Cabrita, The effect of enriched diets on *Solea senegalensis* sperm quality, Aquaculture. 435 (2015) 187 – 194.

[23] J. Tinoco, Dietary requirements and functions of a-linolenic acid in animals, Prog. Lipid Res. 21 (1982) 1 – 45.

[24] R. Rosa, P.R. Costa, M.L. Nunes, Effect of sexual maturation on the tissue biochemical composition of *Octopus vulgaris* and *O. defflippi* (Mollusca : Cephalopoda), Mar Biol. 145 (2004) 563 – 574.

[25] N. Am-in, R.N. Kirkwood, M. Techakumphu, W. Tantasuparuk, Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility, Theriogenology. 75 (2011) 897 – 903.