The effect of nonylphenol on spermiogram, hematological properties and reproductive ability of comet goldfish (*Carassius auratus*)

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Abstract. The degradation product of nonylphenoletoxilate (NPEO), nonylphenol (NP), had adverse effects on the reproduction of several species of male fish. In this study, spermatological properties of comet goldfish (*Carrasius auratus*) after a 30 day exposure with 0.03-0.30 mg NP L^{-1} were investigated. Mature *C. auratus* (weight: ~6.87 g; length: ~10.06 cm) were reared in 15 pairs of glass tank. Thirty days after exposure, significant dose-dependent effects of NP in the treated male fish groups were observed such as reduction in number of sperm, change in semen parameters, and suppressed reproductive behavior. High number of sperm was microscopically founded present in the semen control fish and fish treated by 0.03 mg NP/L, but was scarce in the 0.12 mg NP L^{-1} or higher concentration. Change of semen parameters (pH, color and sperm motility) were observed from fish treated by NP compared to control specimens. A breeding test of treated male fish paired with female matured normal fish revealed that NP suppressed reproductive behavior of male individuals. There were female normal fish spawned and egg hatched when paired with control male fish and treated by 0.03 mg NP L^{-1} but were remarkably no female fish spawned when paired with male fish treated by 0.12 mg NP L^{-1} or higher concentration. The result of this study provided the evidence that NP was suppressed male reproduction, as indicated by the reduced quantity and quality of sperm, and suppressed reproductive ability of male *C. auratus.*
Key Words: blood glucose, fertility*,* nonylphenol, sperm, semen properties, hatching rate.

Introduction. Nonylphenol polyethoxylates (NPEO) has been commonly used for more than 50 years in household laundry detergents. Recently, NPEO is widely used in varied industrial applications in the world such as washing and processing of textiles (wetting and rubbing), paper pulp and paper, paint formulations and resin, recovery of oil and gas, steel manufacturing, pest control and power generation (Staples et al 1998; Bettinetti et al 2002; Kannan et al 2003; US-EPA 2005). NPEO, a non-ionic surfactant, is used as raw material in the industry due to its strong ability to act as an emulsifier and stabilizer of water, effectively work at high temperatures, with very good solubility, low odor, excellent saturation, and low foam production.

NPEO that enters to water comes from industrial disposal which are not properly treated to remove the substance. The substance was further degraded into simpler compounds which are more toxic to organism's metabolism and more persistent in waters such as nonylphenol (NP), a short chain mono-triexthoxylates (Ying et al 2002; Ahel et al 1996; Potter et al 1999). Use of widely large quantities of NPEO and inadequate handling of wastes lead to water pollution which actually was detected in some public water in various countries in the world (Mao et al 2012). The substances has been detected in the water or sediment such as in Kaohsiung Harbor, Taiwan of 101 ng g^{-1} dw (Dong et al 2015), Goro Lagoon, Italy of 372.7 to 464.9 ng g^{-1} dw (Casatta et al 2015), the Pearl River Delta, China of 14.2 to 95.2 ng g^{-1} dw (Chen et al 2014), Cikamasan River, Cisarua-

Bogor, Indonesia of 39.3 to 238.5 ng L^{-1} (Duong et al 2010). Beside water and sediments, NP has also been found in air, soil, and biota (Ying et al 2002).

On the other hand, is considered that NP has the ability to interfere with development and reproduction of aquatic organism which was identified as priority hazardous substances for surface water in the Water Framework Directive (European Parliament, Council of the European Union, 2013). According to Mariel et al (2014) NP in fish tissue can persist and can be biologically active for longer period than endogenous estrogen (Nimrod & Benson 1996). Moreover, some studies showed that NP interfered with reproductive organs, larval development, and growth of fish, amphibians and mammals exposed (Nagao et al 2001; Lukáčová et al 2013; Lee et al 2015). Nonylphenol was reported to inhibit growth and development on larvae of South American toad, *Rhinella arenarum* (Mariel et al 2014), change somatic indices (HSI and GSI), in *Oreochromis niloticus* (Ali et al 2017), histological alteration of testis, liver and kidney in *O. niloticus* (Ismail et al 2016), and disrupted spermiation process in *Acanthopagrus schlegelii* and *Xiphophorus maculatus* (Chang et al 1995; Kinnberg et al 2000). However, there are only scarce information regarding the effect of NP particularly on fish semen and reproductive ability of goldfish.

In this study, we used comet goldfish (*Carassius auratus*), a freshwater fish in the family Cyprinidae. According to FAO (2012) goldfish together with crucian carp had the 6th highest production of freshwater species from aquaculture in 2010. Comet goldfish or comet-tailed goldfish is one of the most popular of fancy goldfish and being one of the important ornamental fish in the world. This comet goldfish, like other goldfish varieties, has various color variations such as yellow, orange, red, white, and red-and-white coloration. *C. auratus* is an easy fish to maintain because its ability to adapt in many environmental conditions, easily to eat most feed offered and very peaceful towards other species. *C. auratus* is prolific and able to breed including in outdoor ponds or in aquariums as well. Ovulating females and ripe males of the fish can be breeded naturally by placing in captivity or artificially by using stripping method.

The present study was carried out to determine the effect of NP on semen properties and sperm production, hematological properties, and reproductive ability of male *C. auratus*.

Material and Method. This study used chemicals such as technical grade nonylphenol, mixture of ring and chain isomers (Sigma CAS No. 84852-15-3), ethanol absolute for analysis (Merck Em. 1.00983.2500) and ethanol technical grade (96%), formol saline solution (125 mL formaldehyde solution 37%; 5.41 g sodium chloride; 6.19 g di-sodium hydrogen phosphate dehydrate; 3.54 g potassium di-hydrogen phosphate; 875 mL distilled water.

The study was conducted at the Research and Development Institute for Ornamental Fish (RDIOF), Depok, Indonesia, whereas sperm observations were conducted at the Laboratory of Reproductive Rehabilitation Unit (URR), Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, Indonesia.

Before being used, nonylphenol technical grade (density of 937 g L^{-1}) was prepared by serially diluting stock solutions with ethanol. At the beginning, technical-grade nonylphenol was diluted in absolute ethanol to a concentration of 64 g L^{-1} (stock-1). The stock 1 was then diluted in technical ethanol until the concentration 3.of 2 g L^{-1} (stock-2). Both of the stock solution was kept at the temperature of -4^{o} C until use. Stock-2 solution was then added to reservoir tank according to desired concentration and stirred.

Evaluation of the effects of NP to *C. auratus* was carried out in few stages that were range finding test (RFT), acute test and sub-chronic test. Experiment were designed in a pair of glass tanks (size: 50 x 40 x 40 cm) consisting of a reservoir tank and a rearing tank. Before being used in experiment, adult and mature *C. auratus* obtained from local traders were adapted in net cage (size: 2 x 2 x 1 m) in pond until the fish were healthy and ready for treatment. Before the treatment started, the fish were adapted in the rearing tank filled with 40 L of ground water. During adaptation, fish were fed once a day by commercial feed to satiation. Experiment was begun when all fish had adapted to the new environment such as swam actively and ate regularly.

Concentration of NP was adjusted in reservoir tank by adding stock 2 of NP solution in 80 liters of ground water, and then flowed to rearing tank. During the experiment, fish were fed with commercial feed until satiation. Feces and remains feed were evacuated to maintain good water quality.

Acute test. Acute test was carried out to find out LC_{50} value or concentration where 50% of test fish died during the exposure time. Acute test was designed in completely randomized design (CRD) 5 treatments with 3 replications. Levels of NP concentration in the acute test were 0.00, 0.20, 0.30, 0.45, 0.67, and 1.00 mg L^{-1}. Prior to acute test, a range finding test (RFT) was performed to determine highest and lowest limit concentration which would be used in acute test. The highest concentration limit was concentration caused died 100 percent of test fish in 24 hours exposure (LC_{100}-24 hours) while the lowest concentration limit was the concentration where there were no test fish died in the same exposure time (LC_0-24 hours). Concentration level of NP in RFT were 0.00 (control), 0.01, 0.1, and 1.0 mg NP L^{-1}.

Sub-chronic test. Sub-chronic test was meant to evaluate effects of chemical to organs, tissues or cells. Level concentration of NP in sub-chronic test was in the range of 5-50% of LC_{50}-96 hours. The treatments were varying concentration of NP which were designed in a completely randomized design (CRD) 5 treatments and 3 replications. Three hundred adult and *C. auratus* (weight: ~6.87 g; length: ~10.06 cm) were cultured in 15 glass tank for 30 days (20 fish $tanks^{-1}$). At the end of the experiment, series of observations were carried out to evaluate profile of blood and semen.

Parameters for blood properties included glucose, total erythrocyte, total leukocyte, hemoglobin and hematocrit, and parameters for semen quality included color, pH, density of sperm ($x10^6$ mL), motility (%), duration of motility of sperm (seconds). Semen volume was not measured due to its low volume to be analyzed.

Semen. Before taking semen, fish were fasted a day. Two male fish per tank were selected and milked by gently press of the abdomen. Only fish that produce semen were brought to laboratory for further analysis of semen. Prior to striping, abdomen and urogenital dried with a tissue. The obtained semen directly placed in the glass object to be analyzed.

After placing semen in a glass slide, semen color was determined visually while semen pH was determined using pH special indicator paper (range 6.4-8).

Evaluation of sperm motility and duration performed immediately after the semen was taken out and put in glass slides. Semen on glass slides (1.2 mm thickness 1.0) were added 1 drop of activation solution (water) covered with a glass object. Immediately after activation, sperm were observed under a microscope (400x magnification) to determine percentage of motile sperm and duration of sperm motility. Sperm that were moving slightly regarded as not mobile sperm. Duration of sperm motility was begun by adding activator in semen until the movement stopped. Observations were carried out at room temperature.

Calculation of sperm number used haemacytometer. Before calculating, semen was diluted (1/500) in formal saline solution (125 mL formaldehyde solution 37%, 5.41 g sodium chloride, 6.19 g di-sodium hydrogen phosphate dehydrate, 3.54 g potassium di-hydrogen phosphate, 875 mL distilled water) and observed under microscope (400x magnification).

Blood and somatic indices. After 30 days exposure, male fish were selected, weighed, and blood was taken from the caudal artery using heparinized spuit. Analysis of total erythrocytes, total leukocytes, hemoglobin, and hematocrit were performed immediately after blood was harvested. Blood plasma was separated by centrifugation (4 min at 9000 rpm at 4°C) and used to analysis of blood glucose.

Reproductive test. Two male fish from each treatment which were prior exposed to NP in sub-chronic test for 30 days were selected for reproductive test. The selected male fish were then paired to normal sexually mature female fish and reared in glass tanks 20 x 20 x 40 cm. All tanks were prior filled with NP-free ground water and added palm fiber as shelter, and aerated. During mating period of a week, fish were no fed. Total number of eggs, fertilized eggs, and larva were recorded.

Statistical analyses. Data were analyzed using MiniTab statistical software. LC_{50} value was analyzed through probit analysis. Number of sperm, blood glucose and hematological properties were analyzed through one-way analyses of variance (ANOVAs). The analysis was continued with Tukey's test at a 95% confidence level if there were significant differences among treatments. Data of spermiogram and reproductive test were analyzed descriptively.

Results. Lethal toxicity of NP exposed to sexually mature *C. auratus*, was time-dependent. Value of LC_{50} NP on *C. auratus* for exposure time of 2-96 hours is shown in Figure 1. Most acute toxicity increased from a LC_{50}-2 h of 1.6 mg NP L^{-1} to LC_{50}-12 h of 0.71 mg NP L^{-1} wherein the highest toxicity occurred at the LC_{50}-2 h to LC_{50}-4 h (0.02 mg NP L^{-1}) and gradually decreased with the time to LC_{50}-72 h of 0.54 mg NP L^{-1}. After 72 h, All fish were died exposed to highest concentration 1.0 mg NP L^{-1}, in contrast no mortality was recorded at exposure of 0.2 mg NP L^{-1}. There were no differences toxicity founded when exposure was extended to 96 h. The LC_{50} pattern during exposed time was following the equation $y = 1{,}4134x^{-0{,}231}$ ($R^2 = 0{,}8537$).

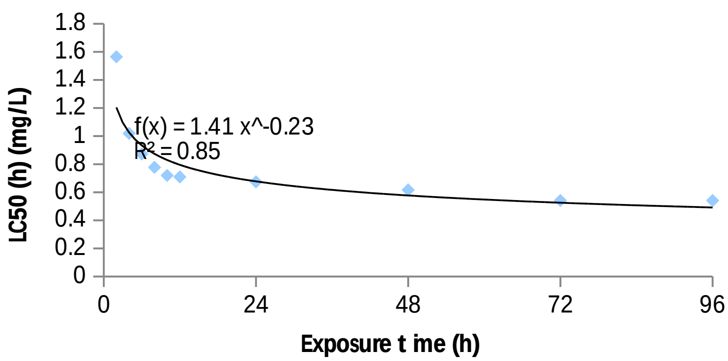

Figure 1. Lethal concentration of 50 (LC_{50}) of nonylphenol in *Carassius auratus* for exposure period of 2-96 hours.

Spermiogram. Spermiogram of *C. auratus* after being exposed to several concentrations of NP for 30 days is shown in Table 1.

Table 1

Spermiogram of *Carassius auratus* after 30 days exposed to several concentration of nonylphenol

Concentration (mg L^{-1})	*Color*	*Consistency*	*pH*	*Motility (%)*	*Motility duration (s)*
0.00	White	Moderate	7.2 (7.2)	85.0 (80-90)	60-120
0.03	White	Moderate	7.0 (6.7-7.2)	88.3 (85-90)	60-120
0.12	Clear white-white	Moderate	7.0 (6.7-7.3)	71.7 (60-90)	60-80
0.21	Clear white-white	Moderate	6.9 (6.7-7.4)	76.7 (70-85)	60-160
0.30	Clear white-white	Moderate	6.9 (6.7-7.7)	76.7 (75-80)	90-120

Particular concentration of NP was changed semen and sperm parameters, such as color, pH, sperm motility, duration of sperm motility. Generally, color, pH and sperm motility of semen in control fish were white, 7.2, and 80-90% respectively, but were clear to cloudy white, 6.7-7.7 and 75-80% respectively in treated male fish particularly exposed to 0.12 mg NP L^{-1} or higher.

Sperm density of *C. auratus* after exposed to several concentrations of NP for 30 days is shown in Figure 2. Compared to control, sperm density of *C. auratus* exposed to 0.03 mg NP L^{-1} for 30 days was significantly higher ($p<0.05$), however were decrease significantly ($p<0.05$) in fish exposed to higher concentrations of NP (0.12–0.30 mg NP L^{-1}).

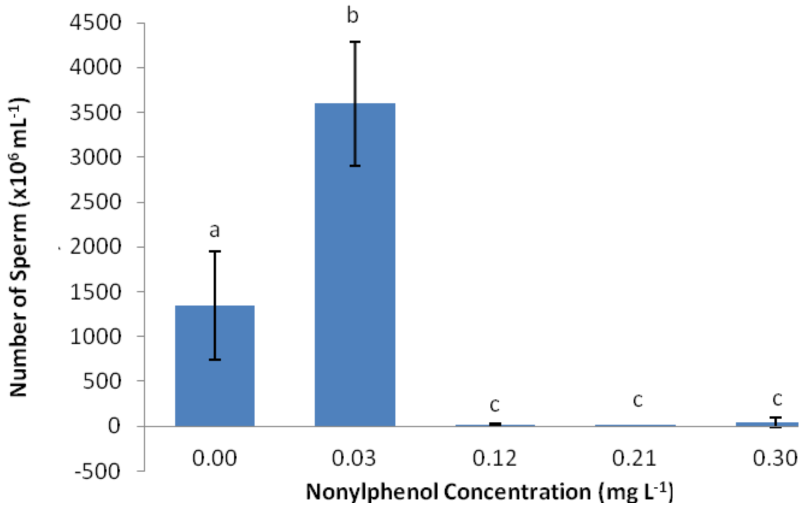

Figure 2. Average number of *Carassius auratus* sperm after 30 days exposed to several concentrations of nonylphenol. Different letters in each nonylphenol concentration show significant differences among treatments ($p<0.05$).

Hematological properties and blood glucose. Hematological properties of *C. auratus* exposed to several concentrations of NP are shown in Table 2. The total average erythrocytes, total leukocytes, hemoglobin, hematocrit of *C. auratus* did not significantly differ between control and treated fish groups.

Table 2

Hematological properties of *Carassius auratus* after 30 days exposure to several concentration of nonylphenol

Nonylphenol concentration (mg L^{-1})	*Total erythrocyte (mm^3)*	*Total leukocyte (mm^3)*	*Hemoglobin (g)*	*Hematocrit (g)*
0	1.65±0.28[a]	3.71±1.85[a]	6.47±0.70[a]	37.90±4.52[a]
0.03	1.77±0.51[a]	3.85±1.60[a]	7.00±0.92[a]	32.00±2.36[a]
0.12	1.54±0.23[a]	2.51±0.33[a]	6.60±0.20[a]	31.73±7.57[a]
0.21	1.71±0.21[a]	2.36±0.50[a]	7.27±0.12[a]	36.43±2.67[a]
0.30	1.96±0.26[a]	2.98±1.13[a]	7.03±0.32[a]	36.00±3.11[a]

Means±standard deviations in the same column with different superscript letters indicate significantly different results ($p<0.05$).

Concentration of blood glucose of male *C. auratus* after 30 days exposure to several concentrations of NP is shown in Figure 3. Generally, concentration of blood glucose in *C. auratus* exposed to several level concentration of NP was significantly lower compared to the control ones. *C. auratus* exposed to 0.03 mg NP L^{-1} have significantly lower blood glucose concentration than the control ones ($p<0.05$). Moreover, in *C. auratus* exposed to 0.3 mg NP L^{-1}, the concentration of blood glucose was significantly low compared to fish exposed to 0.03 mg NP L^{-1} ($p<0.05$). This result indicated that increasing concentration of NP in medium lead to decrease of blood glucose levels in exposed fish.

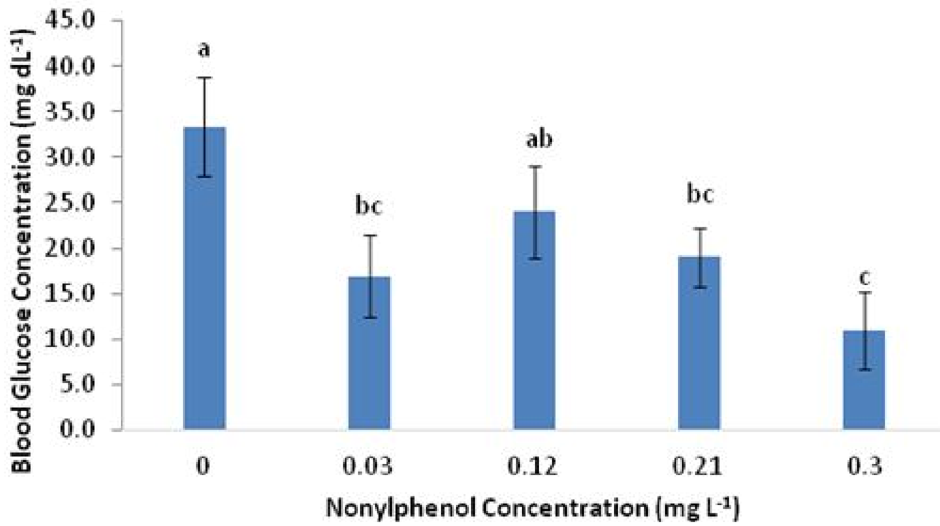

Figure 3. Concentration of blood glucose of *Carassius auratus* after 30 days exposure to several concentrations of nonylphenol. Different letters in each nonylphenol concentration show significant differences among treatments ($p<0.05$).

Reproductive test. Nonylphenol could harm and have negative effect on male fertility of *C. auratus*. The result of reproductive test of pairing sexually matured females of *C. auratus* with males fish exposed to various concentrations of nonylphenol for 30 days, are shown in Table 3. The results indicated that, there were no female fish laying eggs when paired with male fish exposed to 12 mg NP L^{-1} or higher concentrations. Instead, normal female fish paired with control male fish, as well as exposed by 0.03 NP mg L^{-1} succeeded to spawn eggs. However, fertility rate (FR) and hatching rate (HR) in female fish paired to control male fish were higher (>95%) than the ones exposed by 0.03 mg NP L^{-1} (<90%).

Table 3

Total number of eggs, hatching rate (HR), fertility rate (FR) of female *Carassius auratus* paired with male *Carassius auratus* exposed to nonylphenol for 30 days

NP concentration (mg /L^{-1})	*Σ eggs*	*Fr*	*Hr*	*Σ larvae*
0.00	435.5^{a}	$97.0\%^{a}$	$96.0\%^{a}$	404.5^{a}
0.03	176.5^{a}	$44.0\%^{a}$	$45.0\%^{a}$	140.5^{a}
0.12	0.0^{b}	$0.0\%^{b}$	0.0^{b}	0.0^{b}
0.21	0.0^{b}	$0.0\%^{b}$	0.0^{b}	0.0^{b}
0.3	0.0^{b}	$0.0\%^{b}$	0.0^{b}	0.0^{b}

In the same column different superscript letters indicate significantly different results ($p<0.05$); FR: fertility rate. HR: hatching rate.

Discussion. In general, the results of this study indicated that the concentration of 1.0 mg NP mL^{-1} or higher would cause sudden death of *C. auratus* while lower concentration caused changes in semen and sperm which suppressed the male fish reproduction. This phenomenon was shown from the observation of several parameters, such as acute test, spermiogram test, gonadosomatic index and reproductive test.

This study revealed that LC_{50}-96 hour of NP on *C. auratus* was 0.54 mg NP L^{-1}. This value is in range of LC_{50}-96 h of NP of most other fish species which are between 0.13 to 1.4 mg L^{-1} (Naylor 1995). Moreover, the value indicated that toxicity level (LC_{50}-96 h) of NP was higher than other surfactants such as linear alkylbenzena sulfonate (LAS) to tilapia larval (*O. niloticus*) of 8.523 mg L^{-1} (Suparjo 2010), kuruma shrimp juvenile (*Penaeus japonicus*) of 6.00 mg L^{-1} (Supriyono et al 1998) and alkyl sulfate (AS) to tiger shrimp post larvae (PL10) of 22.8 mg L^{-1} (Supriyono et al 2008). Furthermore, 100% mortality occurred when *C. auratus* were exposed to 1.0 mg NP L^{-1} for 72 hours (LC_{100}-72 hours) indicating that the toxicity of NP reaching a maximum of 72 hours. Moreover, in a test with higher concentration (≥1.0 to 2.0 mg NP L^{-1}) resulted 100% fish mortality reached in less than 24 hours (data not shown).

Nonylphenol toxicity in *C. auratus* is affected by concentration and duration of exposure (Hoang et al 2007). The effect of concentration on *C. auratus* mortality rate could be seen in the mortality rate of fish exposed to 1.0 mg NP L^{-1} for 10 hours is 5 times higher than rate mortality in 0.45 mg NP L^{-1}. Furthermore, the effect of exposure duration to *C. auratus* mortality could be seen in trend of LC_{50} value which is 2 times lower compared to LC_{50}-2 hours in 10 hour exposure to 3 times lower in 96 hours exposure. These results are in line with Mariel et al (2014) which stated that toxicity of NP in *R. arenarum* was influenced by duration of exposure.

The sudden death of *C. auratus* exposed to toxic concentration of NP might be caused by excessive mucus production in gill surface which was disrupted oxygen absorption and gas exchange process. As a result, fish were suffocated by lack of oxygen. Due to visual observations, the fish were died with mouth wide open and gill opercula raised as results of over production of mucus on the surface of the gills. This is in line with Chitra & Mohan (2014) who reported that NP lead to increased levels of mucus in exposed tilapia.

Beside killing *C. auratus*, NP could cause sub-lethal effect such as disruption of male fish reproduction and organ development. One of the main effects observed in this study was the change in the semen and reproductive organs of fish exposed to NP. The results of semen parameter showed that NP caused interference with *C. auratus* semen in terms of pH, color, motility, and sperm number. Nonylphenol was significantly reducing number of sperm per milliliter semen especially on fish exposed to NP of 0.12, 0.21 and 0.30 mg NP L^{-1} compared to control and exposed to 0.03 mg NP L^{-1}. Reduction number of sperm in exposed fish might be caused by the ability of NP to increase cell death (apoptosis) in spermatocytes, Sertoli and Leydig-homologue in medaka fish (*Oryzias latipes*) (Weber et al 2002)

The reduction of total number of sperm in male *C. auratus* exposed to NP was also expressed in semen color that was from white in control to clear-white in exposed fish. White color of semen is indicating abundant of sperm while cloudy white is indicating scarcer sperm. These results are in line with Kinnberg et al (2000) who showed that NP and 17β-estradiol caused decreasing number of cysts containing sperm from various stages of development (spermatogenic stage) on testicular structure *X. maculatus*. Spermiation disruption by 17β-estradiol was also reported by Chang et al (1995) who stated that the presence of the hormone disrupt spermiation process in *A. schlegeli*. Spermiation disruption occurred because NP disturbed sertoli and leydig cells which lead to decrease number and motility of sperm.

Nonylphenol had negative effect to sperm motility of *C. auratus* where in concentration 0.12 mg NP L^{-1} could decrease sperm motility compared to control. Disruption of sperm motility was allegedly linked to changes in semen pH where fish exposed to NP had semen pH varied than control pH (7.2) especially in the treatment of 0.12 mg NP L^{-1} or higher. Zhou et al (2015) reported that acidic pH in semen lead to declined Na(+)/K(+)-ATPase activity which is one factor in decreased sperm movement and capacitation.

Blood glucose of *C. auratus* exposed to NP for 30 days was significantly lower than in control group that were from 33.3 mg/dL (control) to 11.0 mg $dL^{-1}$of fish exposed to

0.3 mg NP L^{-1}. Decreasing of blood glucose concentrations in fish exposed to NP in a long time was caused by increasing requirements energy of fish to mitigate metabolic disorders such as increasing of vitelogenin expression. This result is in line with Suto (2000) which stated that blood glucose of rainbow trout, *Oncorhynchus mykiss* exposed to high concentration NP for a long time (2 and 3 weeks) were lower than of controls. In contrast, if the exposure is in a short time (2 hour), the blood glucose were going higher. The same result was also reported by Lorenzon, et al (2004) which stated that the concentrations of glucose blood of *Palaemon elegans* exposed to sub-chronic concentrations of Hg^{2+} and Cu^{2+} were increase in the first 2 hours but going decline with increasing exposure duration.

Reproductive test result of comet male fish exposed to various concentrations of NP paired with normal matured female fish was consistent with sperm observations. Normal adult female fish paired with male exposed to 0.03 mg NP L^{-1} or control male fish succeed to spawn eggs and produce larvae. Instead, female fish paired with male fish exposed to NP 0.12 mg NP L^{-1} or higher leaded to unsuccessful reproduction. The result of no female fish laid eggs paired to exposed male fish was possibly due to NP suppressed sexual behavior of male fish exposed to 0.12 mg NP L^{-1} or higher. The influence of NP and other estrogen compounds in suppressing the reproductive ability of male fish have also been reported (Kang et al 2003; Oshima et al 2002). Kang et al (2003) reported that 4-nonylphenol reduced the ability of *O. latipes* male fish to fertilize female fish. While Oshima et al (2002) reported that 17β-estradiol suppressed fish mating behavior of male *O. latipes* which had impact on the reproductive ability.

Conclusions. The results of this study revealed that *C. auratus* exposed to low concentrations of nonylphenol (0.03 mg NP L^{-1}) could stimulate spermiation. In contrast, higher concentration of nonylphenol (≥0.12 mg L^{-1}) inhibited sperm production. Nonylphenol could change semen properties of *C. auratus* which were pH, color, and motility of sperm. Nonyphenol suppressed sexual behavior of male *C. auratus* which was affected until the reduction of reproductive ability of the fish.

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