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Growth Performance and Physiological Characteristics of Diploid and Triploid Marine Medaka *Oryzias dancena*

Advisor: Prof. In-Seok PARK

by

Tae Ho LEE

A dissertation submitted in partial fulfillment of the requirements

for the degree of

Master of Science

In the Department of Marine Bioscience and Environment,

the Graduate School of Korea Maritime and Ocean University, Korea

February 2017

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November 2016

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Growth performance and physiological characteristics of diploid and triploid marine medaka, *Oryzias dancena*

by

Tae Ho LEE

Submitted to

The Department of Marine Bioscience and Environment

Graduate School of Korea Maritime and Ocean University, Korea

(Supervised by In-Seok PARK, Ph. D.)

ABSTRACT

The aim of this study is to determine the growth performance, hematological characteristics, stress responses of various parameters and respiratory function among ploidy, stage and sex in marine medaka, *Oryzias dancena* between diploid and triploid.

The gonadosomatic index (GSI) was measured for 12 months. GSI of triploid was lower than diploid, and the GSI for females in each ploidy group were higher than that for the males. For both groups of GSI were measured the highest values at 4 months of age, and decreased thereafter to 12 months. In comparison of growth and sexual hormone values, male of diploid and triploid had higher values than female values in thyroid stimulating hormone and thyroxine were not significantly different in all groups ($P<0.05$), and the sexual hormone analyses showed that the diploids were higher than triploids in testosterone and estradiol concentrations regardless of sex ($P<0.05$).

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Triploids showed large sizes and decreased numbers of red blood cell and nucleus than diploid. Mean corpuscular volume and mean corpuscular hemoglobin of triploid were higher than those of diploid.

Stress parameters for comparison between diploid and triploid marine medaka were temperature (25→15°C); salinity (15→0 ppt, 15→30 ppt); DO (7.0 mg/L→5.5 mg/L); ammonia (0.01→0.26 ppm); nitric acid (1.8±0.14 ppm →2.7±0.59 ppm) and nitrous acid (0.01→0.07 ppm). In all experimental groups, changed rates of all measurement factors increased until 12 hours, and decreased gradually until 48 hours. Respiratory function and metabolic rate of all experimental groups decreased during experimental period. All measurement factors of female and juvenile in diploid and triploid group were higher than those of male and egg, respectively (P<0.05). All measurement factors of diploid were higher than those of triploid in male and female groups (P<0.05). However, oxygen consumption of egg and juvenile groups were not significantly different between diploid and triploid.

In all stress parameters, plasma cortisol concentrations of diploid and triploid groups had lowest plasma cortisol in 0 h and 48 h. From 6 h to 12 h, plasma cortisol increased gradually, and plasma cortisol decreased drastically from 12 h to 48 h, and diploid's plasma cortisol concentrations were higher than triploid while measurement time. Plasma cortisol responses of diploid were higher than those of triploid.

Analysis of the gonads of one-year-old triploid fish suggested that induction of triploid fish change a every for gonadal development to somatic growth; this experiment was apparently showed the characteristics of diploid and triploid, and provide the basis for the development of unique models for studying reproductive confinement in transgenic fish.

Key words: diploid, hematological parameter, marine medaka, respiratory function, stress response; triploid

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Approved as qualified thesis of **Tae Ho Lee** for the degree of **Master of Philosophy** by the Evaluation Committee in November 2016.

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KOREAN ABSTRACT
(국 문 요 약)

해산송사리, *Oryzias dancena* 2배체와 3배체의 성장과 생리학적 특성 비교

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한국해양대학교 대학원

해양생명환경학과

(지도교수 수산학박사 박인석)

본 연구는 해산송사리, *Oryzias dancena*의 2배체와 3배체의 암·수별 및 크기별로 상대적인 성장패턴과 생리학적 특성을 규명하고, 이를 토대로 2배체와 3배체 어류간의 나타나는 특징에 관련된 기초연구 자료로 이용되고자 실험을 수행하였다. 2배체와 3배체 해산송사리에 von Bertalanffy 성장 방정식을 non-linear regression 방법으로 측정한 결과, 3배체는 L_{∞}=30.9 mm, K=3.08/year로 나타났고 2배체는 L_{∞}=30.2 mm, K=3.22/year로 측정되었다. Gonadosomatic index (GSI)을 측정한 결과 3배체의 GSI가 2배체 보다 낮게 나타났고, 각 배수체에서는 암컷의 GSI가 수컷 보다 높게 나타났다(P<0.05). 즉, GSI는 2배체, 3배체 및 암수 모두 부화 후 4개월에 가장 높았으며 그 후로 12개월 까지는 지속적으로 감소하였다.

성장 및 성 호르몬(growth and sexual hormone)에서는 3배체가 2배체 보다 성장 호르몬(thyroid stimulating hormone and thyroxine)의 수치는 높게 나타났고, 성 호르몬(testosterone and estradiol)은 생식소의 불임으로 인해 3배체가 2배체 보다 낮게 나타났다(P<0.05).

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혈액학적 특징(hematological parameter)에서는 적혈구크기(size of erythrocyte)와 적혈구수(erythrocyte count)를 비교하였는 바, 적혈구 크기에서는 3배체가 2배체 보다 장축은 1.57배, 단축은 1.17배 크게 나타난 반면, 적혈구수(erythrocyte count)는 2배체가 3배체 보다 1.92배 크게 나타났다($P<0.05$). 즉, 즉혈구 크기는 증가하지만 수가 감소함에 따라 3배체의 거대화(gigantism)은 일어나지 않음을 시사한다.

48시간 동안 6시간 간격으로 밀폐된 용기에서 산소소모(oxygen consumption), 호흡횟수(respiratory frequency), pH, CO_2 및 NH_4^+를 측정한 결과, 2배체는 3배체 보다 산소소모와 호흡횟수가 많이 나타났고, 이에 따른 밀폐용기의 pH, CO_2 및 NH_4^+의 농도도 2배체가 3배체 보다 높게 나타났다($P<0.05$), 그러나 2배체와 3배체의 치어와 수정란에서는 두 배수체간에 유의한 차이가 나타나지 않았다($P>0.05$).

환경변화에 따른 스트레스 변수(stress parameter)에서는 수온(25→15°C); 염분(15→0 ppt, 15→30 ppt); DO (7.0 mg/L→5.5 mg/L); ammonia (0.01→0.26 ppm); nitric acid (1.8±0.14 ppm→2.7±0.59 ppm) 및 nitrous acid (0.01→0.07 ppm)을 변화시켰을 때 48시간 동안 이에 따른 plsama cortisol 농도를 비교하였다. 2배체와 3배체 모두 환경변화를 받았을 때 0시간과 48시간에 Cortisol 농도가 가장 낮았고, 6시간 후부터 12시간 까지 농도가 계속 증가하여 12시간 째 농도가 가장 높게 나타났으며($P<0.05$), 12시간 이후로는 48시간까지는 점차적으로 감소하였다($P<0.05$). Cortisol 농도차이는 2배체가 3배체 보다 높은 농도를 보이는 것으로 보아 3배체에 비해 2배체가 환경변화에 더 민감하다는 것을 알 수 있었다($P<0.05$).

본 연구의 결과 파악된 2배체와 3배체 해산송사리 비교 연구는 여타 어종에서의 배수체 연구에 도움이 될 것이고, 해산송사리와 유사한 크기가 작은 관상용 어류 및 여타 실험동물 어류에 유용한 정보가 될 것이라 사료된다

주제어: 2배체, 3배체, 스트레스 반응, 해산송사리, 혈액학적 특징, 호흡능력

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Introduction

The marine medaka, *Oryzias dancena*, is a euryhaline and eurythermal fish that it can live in both fresh and sea water (Nam *et al*., 2010; Park *et al*., 2011). Marine medaka can spawn 60 days after hatching, producing a short interval between generations, and classification of sex in this species is possible by anal fin ray (Kim *et al*., 2009). Triploidization is a technique used to generate sterile aquatic animals by taking advantage of the incompatibility in pairing the three homologous chromosomes during meiosis I (Thorgaard, 1986; Benfey, 1999). This technique has also been used to enhance the productivity of several fish species because of its assumed ability to increase yield by channeling the energy required from gonadal development to somatic growth (Thorgaard, 1986; Benfey, 1999). More importantly, it generates fish that are unable to breed and contribute to the local gene pool if they were to accidentally escape from confinement. By conferring sterility of exotic fish for a limited purpose, triploid could serve as an effective method for reducing or eliminating the environmental risks of genetically modified organisms (Thorgaard, 1986; Benfey, 1999).

Numerous studies have demonstrated that erythrocyte cellular and nuclear

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dimensions are increased, and number of erythrocytes are decreased in triploids (Thorgaard, 1986; Benfey, 1999). Therefore, it is easy to distinguish between diploid and triploid fish by assessing the sizes and numbers of erythrocytes, which are reduced in proportion to the erythrocyte sizes (Thorgaard, 1986; Benfey, 1999). In sweetfish, *Plecoglossus altivelis*, triploid specimens had larger erythrocytes and lower erythrocytes numbers than diploid specimens, and also showed higher hematological parameters (mean corpuscular volume and mean content of haemoglobin), and the oxygen consumption was higher in triploids than diploids (Aliah *et al*., 1991). In ploidy researches, polyploid associated with cell morphology, hematological parameters and physiological behavior (Thorgaard, 1986; Benfey, 1999), which adjust to ecological fitness of individual metabolism. Comparisons of the hematological parameters between diploid and triploid were performed in Salmonids, European sea bass, *Lateolabrax japonicas*, mud loach, *Misgurnus anguillicaudatus* and shi drum, *Umbrina cirrosa* (Cogswell *et al*., 2001; Ballarin *et al*., 2004; Stefano *et al*., 2005; Wang *et al*., 2007). It observed that triploid fish may be more sensitive to the stress parameters, and showed to a considerable change in value rather than diploid (Ballarin *et al*., 2004; Stefano *et al*., 2005; Wang *et al*., 2007).

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The marine medaka has been shown to have better tolerance for hyperosmotic environments than japanese medaka *O. latipes*, including increased survival rates of adult fish and hatching rates of oosperm (Inoue & Takei 2003; Kim *et al*., 2009; Nam *et al*., 2010). Due to the marine medaka was not indigenous and unauthorized fish in Korea, The Institute of Marine Living Modified Organisms (*i*MLMO) selected this species for a living modified organism evaluation project in 2009 (Ordinance of Agriculture, Food and Fisheries, No. 1) and is imported legally from Indonesia (Kim *et al*., 2009). Nam *et al*. (2010) examined the tolerance to salinity changes of this species and found that the marine medaka was highly capable of hyper-osmoregulation as well as hypo-osmoregulation, showing complete switches during transferring from freshwater to 40 ppt, and from 70 ppt to 0 ppt. Recently, the anesthetic effects of clove oil and lidocaine-HCl on marine medaka were reported by Park *et al*. (2011). Anesthetic effects on experimental groups of different sizes of marine medaka were observed, and both anesthesia time and recovery time for the juvenile group were shorter than those of the adult group.

Knowledge of the oxygen consumption rate of a species is of great interest in aquaculture, since it represents an indication of the metabolic

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expenditure of animals to maintain their vital functions through an aerobic metabolism (Swarup, 1959; Kazakov & Khalyapina, 1981; Tran *et al*., 2008). In the specific case of crustaceans, oxygen consumption is influenced by environmental factors such as oxygen concentration, temperature, salinity or the light–dark cycle, as well as by intrinsic factors such as bodyweight, activity level, feeding state, moulting cycle or biological rhythms (Stillwell & Benfey, 1996; Radford *et al*., 2004; Perera *et al*., 2007). In addition, manipulation and the type of feed supplied may provoke significant changes in oxygen consumption (Hewitt & Irving, 1990). Oxygen consumption per unit weight is an objective and versatile characteristic describing the level of metabolism. Rate of gas exchange gives an indication of energy expenditure on the life-supporting functions associated with growth, feeding and reproduction. It is correlated with such important economic criteria as survival, productivity and growth rate (Kazakov & Khalyapina, 1981).

In intensive culture systems fish are continuously exposed to stress, including increased density, inadequate nutrition, poor sanitation, injury during handling, high water temperature or low water temperature. Stress responses can include physiological changes such as oxygen uptake and transfer, metabolic and hematological changes, mobilization of energy

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substrates, reallocation of energy away from growth and reproduction, and suppressive effects on immune functions (Barton & Iwama, 1991; Thompson *et al*., 1993; Pottinger *et al*., 2002). These changes can increase disease susceptibility leading to increased mortality and subsequent economic losses.

The physiological response of fish under stress can be sorted by first, second, and third responses (Barton & Iwama, 1991). The first response is to increase internal secretion activities by promoting the secretion of catecholamine and glucocorticoid, thus inducing the second response where the fish then undergoes metabolic and hematological changes which subsequently induce the final and third response by which time the fish starts to exhibit obvious signs of stress and discomfort (Thompson *et al*., 1993).

Previous research on marine medaka has no investigated comparative analysis of respiratory function between ploid and sex, and has no determined the stress response between ploid and sex by changing various parameters. Thus, respiratory function, hematological characteristics and stress response among ploid, stage and sex in marine medaka were determined in this study. That is, the aim of this study is to ascertain if respiratory function between male and female has different and to ascertain if triploid induces respiratory function change and to inform the stress response by various parameters.

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Materials and Methods

1. Fish breeding and maintenance

Experimental group of diploid marine medaka, *Oryzias dancena* in this study were reared by methods of Park *et al.* (2011). On 24 September 2015, the one hundred fishes were quarantined by the male and female categories and habituated in the 100 L glass aquariums for 3 days. The sex ratio of males and females were 60 males and 40 females. The culture water was dechlorinated and 30% of water in the aquarium was exchanged every day. Artemia collected from the cultured aquarium were provided to fish every day. For collecting eggs, the fish whose standard length were over 25 mm used in this experiment and 35 males and 15 females of marine medaka were placed in each of two aquariums, and 1,000 fertilized eggs were collected immediately by net. The fertilized eggs of diploid experimental group (n=500) were reared in 100 L glass aquarium.

2. Induction of triploid

The fertilized eggs of triploid experimental group (n=500) were left to fertilize for five minutes, and were subjected to cold-shock treatment (4℃)

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for 60 minutes in order to prevent the onset of the second polar body. The treatment eggs were reared in 100 L glass aquarium. In 2 months after hatched out samples were anesthetized the 100 ppm clove oil, sample tissues for analysis were removed from all individuals of tail fin (Park *et al*., 2011), flowcytometric analysis was performed to estimate the average celluar DNA content of all individuals from triploid marine medaka. One million of tail fin cells were collected and stained using a high-resolution DNA staining kit (Partec GmbH, Germany) under dark conditions at room temperature for 15 minutes. Stained samples were analyzed on Partec PA-II flowcytometer (Partec GmbH, Germany) to determine the relative DNA content. The red blood cells (2.8 pg DNA/nucleus) of mud loach *Misgurnus mizolepis* were used as a standard reference. The mean amounts of DNA in the diploid and triploid group were 1.64±0.019 pg/nucleus and 2.45±0.026 pg/nucleus, respectively. Individual each of sample determined triploid was quarantined in a 30 L glass aquarium for experiment.

3. Measurement of condition factor and GSI

In 12 months after hatched out samples were measured the standard length, body weight, condition factor, and gonadosomatic index (GSI) in

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order to investigate the growth and maturation of diploid and triploid fish. The condition factor was determined using the equation: condition factor = (body weight × 100)/(body length)^3, and the GSI was determined using the equation: GSI = (gonad weight/body weight) × 100.

4. Analysis of gonadal and growth hormone

Sex and growth hormones in the plasma of each ploidy group at 120 days after hatched out samples were measured following centrifugation. The estradiol and testosterone concentrations were measured using fluorophotometry (i-Chroma, Sun Kyung Medical, Korea) over 48 hrs from 8 hrs. To measure the estradiol and testosterone concentrations, whole body samples were used. To investigate changes in growth hormone levels, the concentrations of thyroid stimulating hormone and thyroxine in samples of each group were measured using fluorophotometry during the 12 months following hatching.

5. Comparison of erythrocyte

For the erythrocyte and hormone measurements, 270 days after hatched out six diploid and six triploid fish (three of each gender per ploidy group) at

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were selected. Blood samples were taken by cutting the caudal fin and collecting the blood, or by inserting a heparin-coated syringe (Sigma, USA) into the heart and withdrawing blood. The major and minor axes of each cell and nucleus of at least 30 erythrocytes per fish were measured using a micrometer. The surface area (= 1/4 × abπ) and volume (= 4/3 × π(a/2) × $(b/2)^2$) were calculated; in these formulae, 'a' is the major axis of the cell or nucleus, and 'b' is the minor axis of the cell or nucleus (Lemoine and Smith, 1980)

6. Hematological measurements

For observation of live red blood cells, 270 days after hatched out whole blood of samples were diluted with 1:10 of phosphate-buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4) and a drop of cell suspension was placed in the centre of slide glass, which was then covered with a coverslip and observed by an optical microscope (Axiostar plus, Carl Zeiss, Germany). Hematological measurements were erythrocyte count (EC), hematocrit(HCT), mean corpuscular volume (MCV), total hemoglobin content (THC), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), and there were determined

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using standard hematological techniques (Seol *et al*., 2008). Hematological measurements were calculated according to the following formulas (Seol *et al*., 2008).

MCV (μm^3) = Hct/RBCC (10^6 μl^{-1}), RBCC= Red blood cell count

MCH (pg) = [Hb (g dl^{-1}) × 10] /RBCC (10^6 μl^{-1})

MCHC (g l^{-1}) = [Hb (g dl^{-1}) × 10] / Hct.

7. Analysis of respiratory functions

On 20 May 2016, total samples at 270 days after hatched out of 100 marine medaka, 50 males and 50 females in diploid and triploid were experimented, respectively. Mean standard length of used samples were 3.21 ± 0.49 cm, and mean body weight of used samples were 584.1 ± 11.67 mg. During the experiment, to avoid sampling fish with metabolism that were changed by large quantities of food, fish were starved for 24 hrs before experiment (Park *et al*., 2011). The respirometer chamber was utilized by a simple sealed container. The respirometer chamber was comprised of an acrylic resin box with a thickness of 8mm; the overall dimensions of the box were 10 cm (width) × 50 cm (length) × 10 cm (height). The hose of inflow water was equipped with a temperature controller and 10 μm and 3 μm

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cartridge filters equipped for the exclusion of particles. The flow-through UV lamp was utilized for the reduction of oxygen consumption by microbes. Water from the respirometer chamber flowed into an oxygen measurement chamber. Prepared respirometer chambers were categorized by measurement time and ploidy and sex.

After experiment started, measurement times were chosen at 6 hrs intervals over 48 hrs. Before measuring dissolved oxygen, pH, ammonium (NH_4^+) concentration and carbon dioxide (CO_2) concentration, respiratory frequency (gill cover movement) were measured using a counter and a digital timer. NH_4^+ and CO2 concentrations were measured using spectrophotometer (DR2800, HACH, Loveland, Colorado, USA). Dissolved oxygen and pH were measured using an oxygen measurement electrode and a multi-data logger system (Oxyguard, Denmark). Measurements of oxygen and oxygen consumption rates at each experimental group were saved by the multi-data logger, as described Cech (1990).

Oxygen consumption rate (mg O_2 / h) = (C_{O2} (A) - C_{O2} (B))×V/T;

C_{O2} (A)=Dissolved oxygen concentration of water at the start of the measurement period, mg/L; C_{O2} (B)=Dissolved oxygen concentration of water at the end of the measurement period, mg/L; V=Volume of

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respirometer, L; T =Time elapsed during measurement period.

8. Analysis of stress responses

After the hatched out of 270 days fishes were quarantined in a 30 L glass aquarium for experiment. Samples were exposed to a stress parameter known to evaluate plasma cortisol values. These fish were subjected to handling and were quarantined while 48 hrs. Temperature change (25℃ to 15℃), salinity change (hypo: Hypo: 15 ppt → 0 ppt; Hyper: 15 ppt → 30 ppt), DO change (7.6→5.5 mg/L) and water quality items change (Table 1) were induced for confirm the stress responses. We conducted this experiment to observe the effects of stress on the whole-body cortisol and glucose levels of fish under heat and cold anesthesia.

The stress responses of the experimental fish were measured at 0, 6, 12, 24 and 48 hrs. For these measurements, 20 fish were used in each group, and no distinction was made between male and female fish. We measured the whole-body cortisol levels of the control fish before the experiment. Individual fish were blotted onto paper towels to remove excess water, immediately frozen in liquid nitrogen for 10–30 seconds, and placed in individual 5.0-mL plastic screwcap centrifuge tubes. The samples were stored

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Table 1. Changes of water quality items for this experiment on marine medaka, *Oryzias dancena*[*]

Test parameters	Condition	
	Initial	Final
Temperature (℃)	26 ± 0.5	15 ± 0.5
pH	7.1 ± 0.65	5.1±1.02
DO (dissolved oxygen; mg/L; saturated concentration in 25℃)	7.6	5.5
Ammonia (ppm)	0.01	0.26
Nitric acid (ppm)	1.8 ± 0.14	2.7±0.59
Nitrous acid (ppm)	0.01	0.07

*Test parameters of initial condition were analyzed before marine medaka rearing. Test parameters of change condition were analyzed at 10 days after marine medaka reared. Temperature, pH, dissolved oxygen and salinity were measured using an oxygen measurement electrode and a multi-data logger system (Oxyguard, Denmark). Ammonia, nitric acid and nitrous acid were measured using spectrophotometer (DR2800, HACH, Loveland, Colorado, USA). The values are means of triplicate groups (n=20).

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at –80℃ until we extracted the cortisol. The term “whole-body cortisol” is used to describe the portion of corticosteroid extracted and measured with a cortisol-specific radioimmunoassay (RIA; Pottinger *et al*., 2002).

9. Statistical analysis

Data were analyzed by one- and two-way ANOVA with the SPSS statistical package (SPSS 9.0, SPSS Inc., USA). All experiments were performed in triplicate. Multiple comparisons were performed using Duncan’s multiple range test ($P < 0.05$; Duncan, 1955).

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Results

As shown in Fig. 1, the von Bertalanffy growth parameters estimated by the non-linear regression method for diploid and triploid marine medaka, *Oryzias dancena*. The von Bertalanffy growth equations were $L_t = 30.2(1-e^{-3.22(t-0.01)})$ and $L_t = 30.9(1-e^{-3.08(t-0.02)})$ in diploid and triploid, respectively. The growth coefficients (K) of diploid and triploid are estimated to be 3.22/year and 3.08/year, respectively. The asymptotic maximum length (L_∞) of diploid and triploid are estimated to be 30.2 mm and 30.9 mm, respectively, and the theoretical age at zero length (t_0) are estimated to be -0.01 and -0.02 in diploid and triploid, respectively. For every measured characteristic, significant differences in growth were found among ploidy and sex ($P < 0.05$).

Changes of body weight on diploid and triploid are shown in Fig. 2. During experimental period, growth pattern of diploid was similar to triploid in female and male groups. In female and male group, diploid and triploid samples were grow rapidly from 3 months to 4 months, and were grow slowly from 8 months to 12 months. In female group, triploid samples were larger than diploid during experimental period, and trend of male group were similar to result of female group. In diploid and triploid, body weight of male samples were heavier than female samples.

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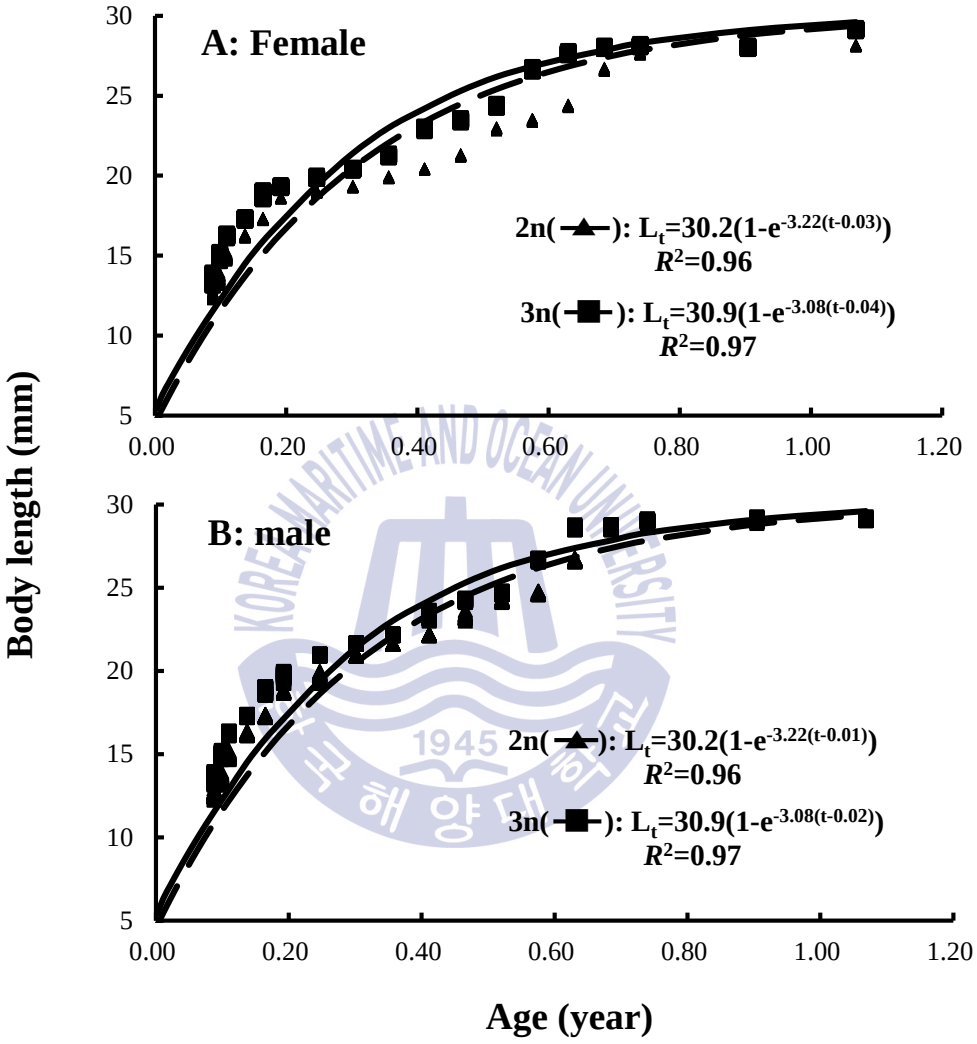

Fig. 1. The von Bertalanffy growth curve on diploid and triploid marine medaka, *Oryzias dancena* in this experiment. Each values are means ± SD of triplicate experiment. A: female; B: male.

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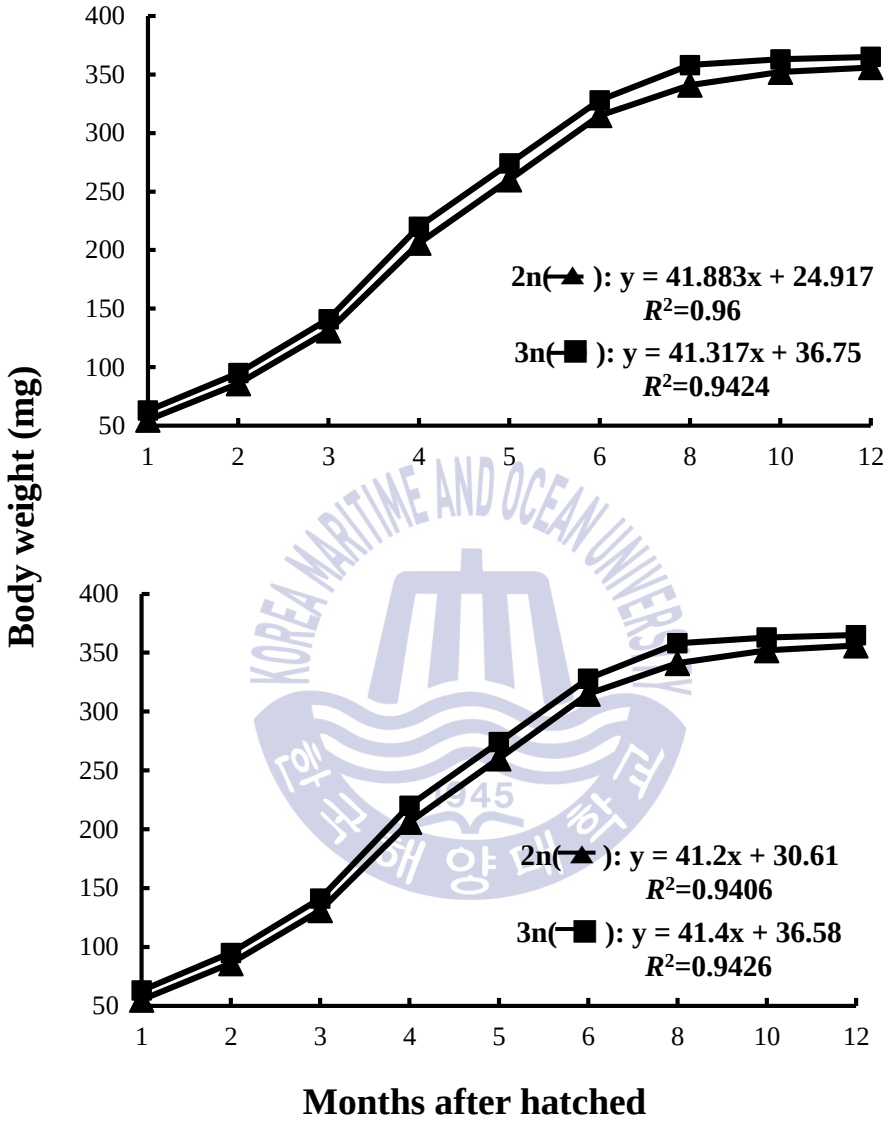

Fig. 2. Change of body weight on diploid and triploid marine medaka, *Oryzias dancena* during this experiment. Each values are means ± SD of triplicate experiment. A: female; B: male.

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Condition factors were affected by ploid, but was not affected by sex (Table 2; $P < 0.05$). Condition factors of all groups decreased rapidly from 1 month to 2 months. During 4 months, condition fafctor of male and female groups were significantly different between diploid and triploid, and condition factor of diploid in female and male groups were higher than those of triploid. However, condition factor of male and female groups were not significantly different between diploid and triploid after 4 months. During experimental period, condition factor of diploid and triploid groups were not significantly different between female and male.

Changes of GSI on diploid and triploid are shown in Fig. 3. In female and male groups, GSI of triploid were lower than those of diploid, and GSI of female in each ploidy were higher than those of male. Change patterns of GSI were similar in all groups. GSI of triploid female group increased from 3.17 % at 1 month after hatched to 20.45% at 4 months ($P < 0.05$), and decreased to 15.43% at 12 months. GSI of triploid male group increased from 1.56% at 1 month after hatched to 9.05% at 4 months ($P < 0.05$), and decreased to 7.18% at 12 months. GSI of all groups were the highest at 4 months, and decreased from 4 months to 12 months.

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Table 2. Change of condition factor on diploid and triploid marine medaka, *Oryzias dancena* during this experiment*

Time (months after hatch)	Condition factor			
	Diploid		Triploid	
	Male	Female	Male	Female
1	51.2[a]	50.2[a]	39.6[b]	39.0[b]
2	6.32[a]	6.25[a]	5.67[b]	5.78[a]
3	3.07[a]	2.98[a]	2.80[b]	2.82[b]
4	2.42[a]	2.40[a]	2.26[b]	2.24[b]
5	2.07[a]	2.04[a]	1.90[a]	1.93[a]
6	1.95[a]	1.92[a]	1.82[a]	1.83[a]
8	1.58[a]	1.58[a]	1.52[a]	1.58[a]
10	1.47[a]	1.47[a]	1.47[a]	1.47[a]
12	1.39[a]	1.40[a]	1.40[a]	1.41[a]

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	30512.581	7783.904	259.349	<0.0001
Sex	3	254.017	14.127	1.448	<0.9589
Interaction	7	4144.312	1038.084	11.874	<0.0942

*Each values are means ± SD of triplicate experiment. Condition factor=(Body weight×100)/(Body length)^3. The different superscripts of each value are significantly different between ploid and sex ($P < 0.05$).

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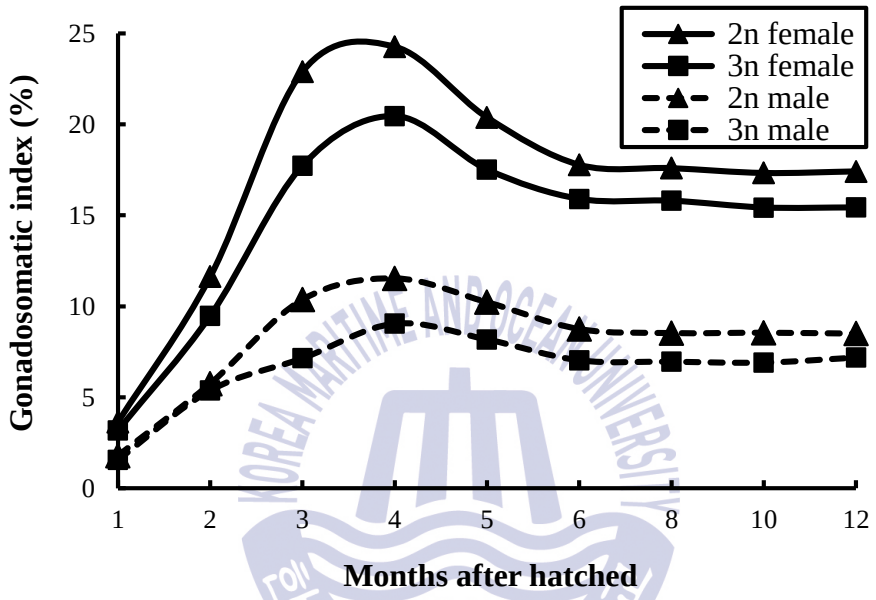

Fig. 3. Change of gonadosomatic index (GSI) on diploid and triploid marine medaka, *Oryzias dancena* during this experiment. GSI=(gonad weight/body weight)×100. Each values are means ± SD of triplicate experiment.

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Thyroid stimulating hormone and thyroxine were affected by ploid, but were not affected by sex (Table 3; $P < 0.05$). Thyroid stimulating hormone and thyroxine of all groups increased slowly while experimental period. In diploid and triploid groups, two hormones of female were lower than those of male. Thyroid stimulating hormone and thyroxine of triploid in male and female groups were higher than those of diploid. As shown in Fig. 4, testosterone values in diploid gradually decreased after the experiment. At 24 hrs, values of testosterone restored initial value, and at 48 hrs of testosterone, values showed similar to the values at 0 hour and 24 hrs. However, in induced triploid, there had low testosterone data than diploid because of lowest GSI numerical value ($P < 0.05$; Fig. 4). Estradiol of diploid showed a tendency to gradually decrease from 6 to 12 hrs (Fig. 5). However at 24 hrs and 48 hrs, values of estradiol the initial value of 0 hour restored. However, estradiol of induced triploid was not changed and hormone concentrations of testosterone and estradiol in induced triploid than those in diploid ($P < 0.05$; Fig. 5).

Overall, the erythrocyte of induced triploid appear to be larger than diploid (Table 4). Ratios of erythrocyte in diploid and induced triploid are measured that major axis of triploid had 1.57 times larger than diploid and

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Table 3. Comparative analysis of thyroid stimulating hormone and thyroxine between ploid and sex on marine medaka, *Oryzias dancena* during this experiment*

Time (months after hatch)	Thyroid stimulating hormone (μIU/L)				Thyroxine (μg/dL)			
	Diploid		Triploid		Diploid		Triploid	
	Male	Female	Male	Female	Male	Female	Male	Female
1	3.1^{a}	2.8^{a}	3.9^{b}	3.4^{b}	3.8^{1}	3.7^{1}	4.3^{2}	4.2^{2}
2	3.2^{a}	2.9^{a}	4.0^{b}	3.5^{b}	4.2^{1}	4.1^{1}	4.6^{2}	4.5^{2}
3	3.4^{a}	3.0^{a}	4.1^{b}	3.7^{b}	4.7^{1}	4.7^{1}	5.0^{2}	5.1^{2}
4	3.6^{a}	3.2^{a}	4.3^{b}	3.9^{b}	5.1^{1}	4.9^{1}	5.3^{2}	5.3^{2}
5	3.9^{a}	3.5^{a}	4.6^{b}	4.3^{b}	5.2^{1}	5.1^{1}	5.6^{2}	5.7^{2}
6	4.1^{a}	3.7^{a}	4.8^{b}	4.6^{b}	5.7^{1}	5.7^{1}	6.3^{2}	6.3^{2}
8	4.6^{a}	4.1^{a}	5.3^{b}	4.9^{b}	6.1^{1}	5.9^{1}	6.5^{2}	6.4^{2}
10	4.6^{a}	4.2^{a}	5.1^{db}	5.0^{b}	6.3^{1}	6.2^{1}	7.6^{2}	7.4^{2}
12	4.6^{a}	4.1^{a}	5.2^{b}	5.1^{b}	6.3^{1}	6.3^{1}	8.7^{2}	8.6^{2}

Throid stimulating hormone

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	30475.440	7618.860	248.055	<0.0001
Sex	3	438.095	54.049	7.612	<0.9024
Interaction	7	5762.451	1382.093	29.588	<0.0459

Thyroxine

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	34259.1	67591.4	548.4	< 0.0001
Sex	3	5711.0	896.1	16.2	< 0.7841
Interaction	7	45867.5	12438.1	121.5	< 0.0446

*Each values are means ± SD of triplicate experiment. Differences between ploid and sex/stage are significant at this level ($P < 0.05$). The different superscripts of each value are significantly different between ploid and sex ($P < 0.05$).

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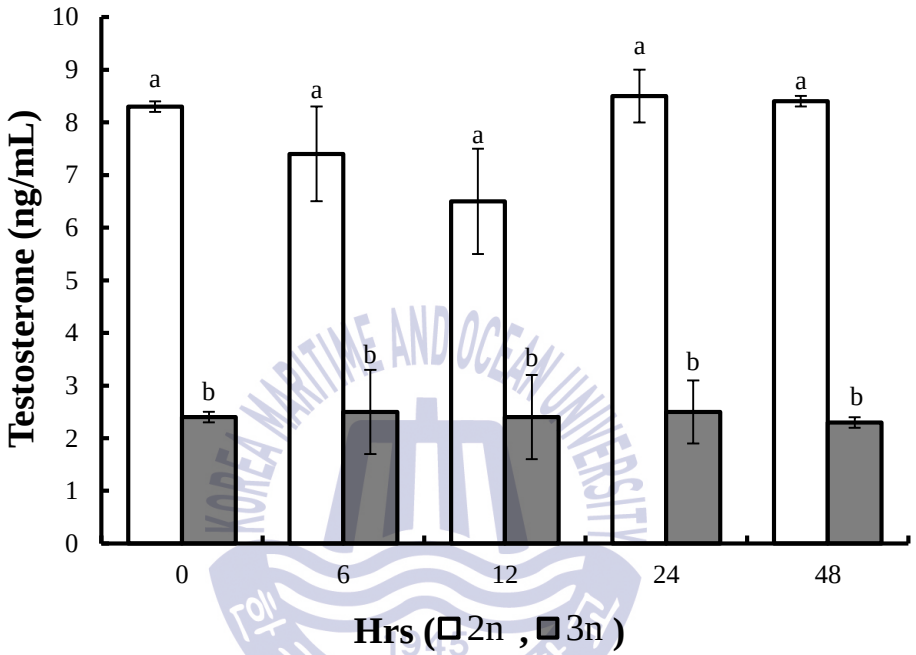

Fig. 4. Change of testosterone in diploid and triploid male marine medaka, *Oryzias dancena* while 48 hrs. Each values are means ± SD of triplicate experiment. Different letters on error bars are significantly different for each group ($P < 0.05$).

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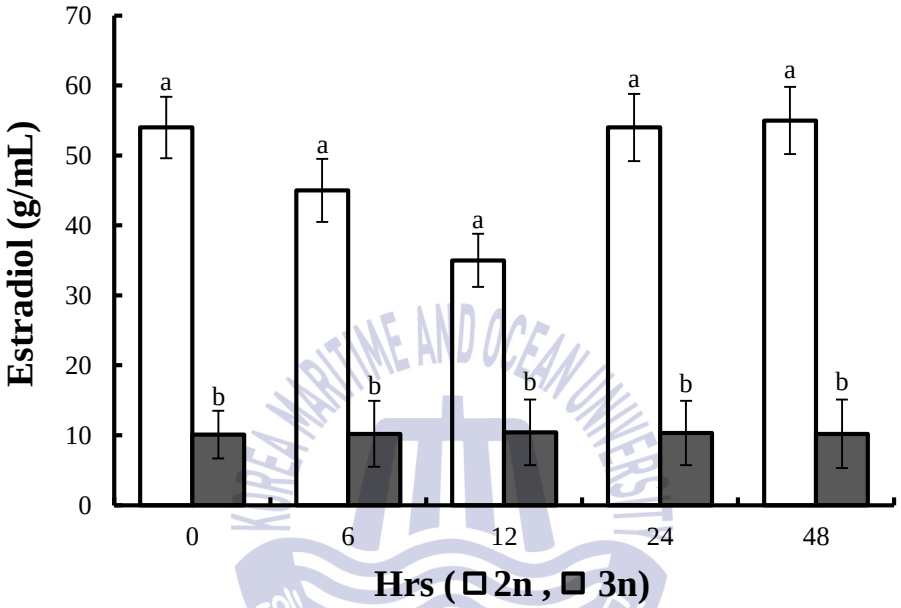

Fig. 5. Change of estradiol in diploid and triploid male marine medaka, *Oryzias dancena* while 48 hrs. Each values are means ± SD of triplicate experiment. Different letters on error bars are significantly different for each group ($P < 0.05$).

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Table 4. Size of erythrocyte and nucleus in diploid and triploid marine medaka, *Oryzias dancena**

	Diploid	Triploid	Ratios of means
Erythrocyte**			
Major axis (μm)	7.5±0.82[a]	11.8±0.86[b]	1.57
Minor axis (μm)	5.5±0.75[a]	6.5±0.51[b]	1.17
Surface area (μm^2)	33.5±6.33[a]	61.5±6.50[b]	1.82
Volume (μm^3)	125.5±35.97[a]	271.2±44.28[b]	2.10
Nucleus of erythrocyte**			
Major axis (μm)	2.7±0.37[a]	4.8±0.50[b]	1.78
Minor axis (μm)	1.7±0.33[a]	2.3±0.34[b]	1.42
Surface area (μm^2)	3.5±0.81[a]	9.0±1.71[b]	2.57
Volume (μm^3)	4.0±1.48[a]	14.5±4.52[b]	3.60

* Each values are the means±standard deviation of triplicated groups. Means in rows with the different superscript letter are significantly different ($P < 0.05$), Ratios of means = triploid/diploid.

**Surface area= $1/4 \times ab\pi$ and volume = $4/3 \times \pi(a/2) \times (b/2)^2$ (where a = the major axis of a cell or nucleus; b = the minor axis of a cell or nucleus; after Park *et al*., 2015).

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minor axis 1.17 times larger, respectively. Surface and volume based on major axis and minor axis were 1.78 times and 1.42 times each. Similarly, ratios of nucleus for erythrocyte in diploid and induced triploid were major axis and minor axis with 1.78 times and 1.42 times each. Surface and volume nucleus of erythrocyte based on major axis and minor axis are 1.78 times and 1.42 times each (Table 4). Induced triploids with erythrocyte and erythrocyte nucleus are significantly larger than diploid ($P < 0.05$).

As shown in Table 5, the EC of diploid were doubled to triploid, and HC were not significantly different between diploid and triploid ($P > 0.05$). MCV values of diploid and triploid were inverse proportionate to values of EC, and triploid's MCV was higher than diploid's MCV ($P < 0.05$). THC and MCHC were not significantly different between diploid and triploid ($P > 0.05$), but MCH of triploid was 60% higher than diploid ($P < 0.05$).

In Table 6, Figs. 6 and 7, the two-way ANOVA test and measurement results of oxygen consumption rate of male, female, egg and juvenile groups in each diploid and triploid marine medaka are presented. In diploid groups, oxygen consumption rates has significant different between male and female (Table 2, Fig. 6), and oxygen consumption rates in triploid groups has significant different between male and female ($P < 0.05$).

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Table 5. Comparative analysis of hematological parameter between diploid and triploid marine medaka, *Oryzias dancena*[*]

	Ploidy[**]		t-test
	Diploid	Triploid	
Erythrocyte count (cells/μL)	2.3±0.13	1.2±0.03	*
Hematocrit (%)	30.7±2.01	31.0±1.74	NS
Mean corpuscular volume (μm^3)	121.3±4.11	174.1±6.77	*
Total hemoglobin content (g/100mL)	8.3±0.44	8.4±0.37	NS
Mean corpuscular hemoglobin (pg)	25.6±5.11	44.7±6.89	*
Mean corpuscular hemoglobin concentration (%)	20.6±1.54	20.1±1.44	NS

* The values are means±SD (*n*=50). Data of each experimental group were analyzed using t-test on data transformed to the arcsine of the square root. NS: no significant; *: indicate statistical significance between morphometric distances ($P < 0.05$).

**All parameters of each group were measured at 90 days after hatched.

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Table 6. Results of the two-way ANOVA for differences in oxygen consumption rate between ploid and sex/stage on marine medaka, *Oryzias dancena**

	Oxygen consumption rate ($O_2mg^{-1}kg^{-1}hr^{-1}$)				
	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	376723.1	188361.5	190.2	< 0.0001
Sex	3	1489907.0	297981.3	300.9	< 0.0001
Interaction	7	1568742.2	306342.1	312.4	< 0.0001
	Oxygen consumption rate ($O_2mg^{-1}kg^{-1}hr^{-1}$)				
	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	2368.0	349.7	6.7	< 0.8573
Stage	3	15671.0	7163.9	300.9	< 0.0001
Interaction	7	2881.3	391.8	26.9	< 0.0412

*Each values are means ± SD of triplicate experiment. Results of oxygen consumption rate between ploid and sex were shown in Fig. 6, and oxygen consumption rates between ploid and stage were shown in Fig. 7.

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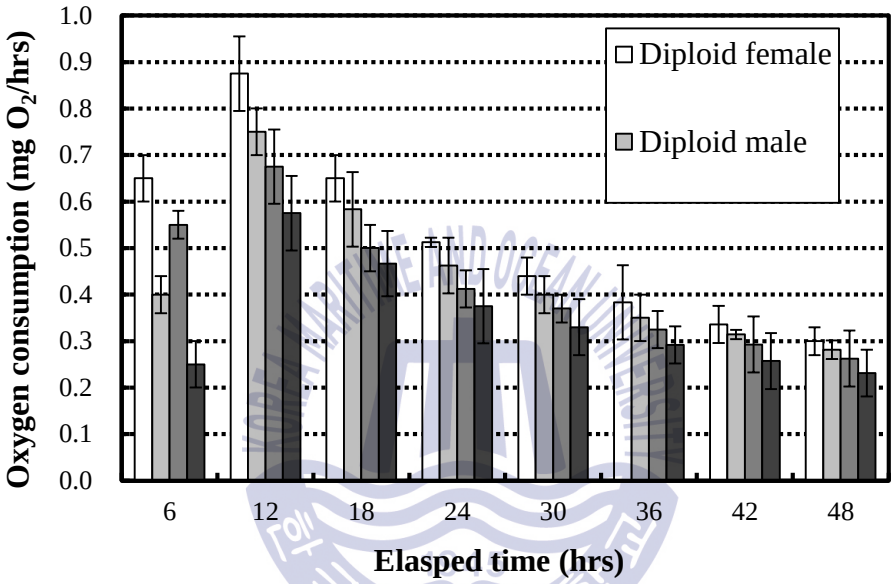

Fig. 6. Oxygen consumption between ploid and sex on marine medaka, *Oryzias dancena*. Each values are means ± SD of triplicate experiment. Different letters on error bars are significantly different for each group ($P < 0.05$).

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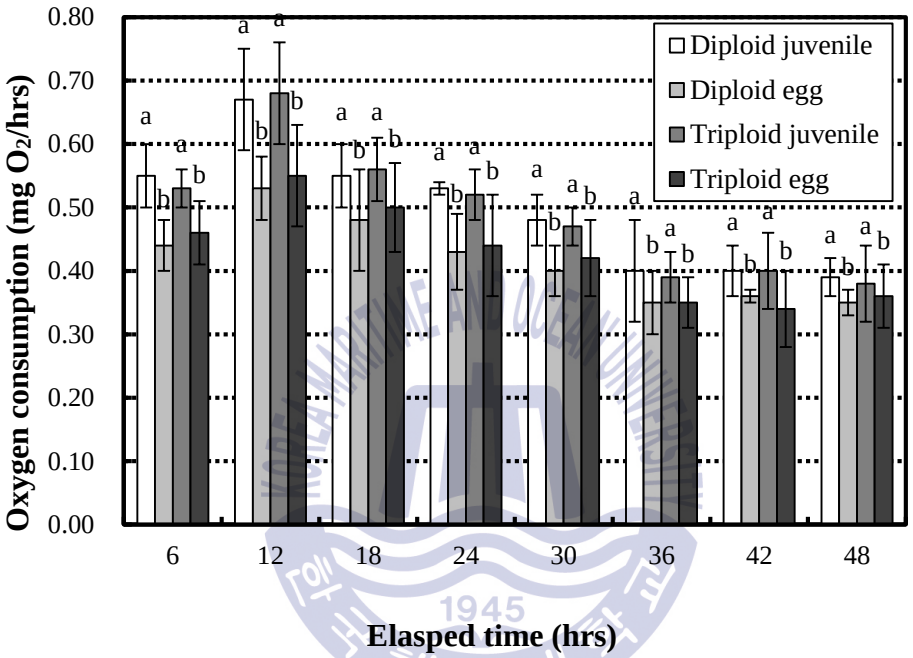

Fig. 7. Oxygen consumption between ploid and stage on marine medaka, *Oryzias dancena*. Each values are means ± SD of triplicate experiment. Different letters on error bars are significantly different for each group ($P < 0.05$).

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In all measurement times, oxygen consumption rates of diploid female were higher than those of diploid male, and oxygen consumption rates of triploid female in all measurement times were higher than those of triploid male. That is, respiratory function of female was higher than that of male. In male groups, oxygen consumption rates have significant different between diploid and triploid (Table 6 and Fig. 6), and oxygen consumption rates in female groups has significant different between diploid and triploid ($P < 0.05$). In male and female groups, oxygen consumption rates of diploid in all measurement times were higher than those of triploid. That is, respiratory function of diploid was higher than that of triploid.

Oxygen consumption rates of all experimental groups increased until 12 hrs, and decreased gradually until 48 hrs. Respiratory frequency also increased until 12 hrs, and decreased gradually until 48 hrs (Table 7). That is, respiratory function of all experimental groups decreased while experimental period. pH decreased drastically until 12 hrs, and decreased gradually until 48 hrs (Table 8). CO_2 and NH_4^+ concentrations increased drastically until 12 hrs, and decreased gradually until 48 hrs as shown in Tables 9 and 10. Comparing with oxygen consumption rate, pH, CO_2 and NH_4^+ concentrations showed different trend, but rates of pH, CO_2 and NH_4^+ concentrations change

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Table 7. Respiratory frequency (gill cover movement) between ploid and sex on marine medaka, *Oryzias dancena**

Time (hrs)	Respiratory frequency (min^{-1})			
	Diploid		Triploid	
	Male	Female	Male	Female
6	102^{3}	128^{1}	95^{4}	118^{2}
12	110^{3}	135^{1}	104^{4}	127^{2}
18	100^{3}	124^{1}	92^{4}	114^{2}
24	72^{3}	91^{1}	65^{4}	84^{2}
30	71^{3}	91^{1}	64^{4}	82^{2}
36	67^{3}	88^{1}	63^{4}	81^{2}
42	66^{3}	80^{1}	62^{4}	76^{2}
48	65^{3}	77^{1}	60^{4}	70^{2}

Respiratory frequency (min^{-1})

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	74451.0	37225.5	229.3	< 0.0001
Sex	3	344669.7	68933.9	554.3	< 0.0001
Interaction	7	41357.9	14135.7	133.2	< 0.0001

*Each values are means ± SD of triplicate experiment. Respiratory frequency was measured while 1 minute. Difference between ploid and sex is significant at this level ($P < 0.05$).

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Table 8. pH values between ploid and sex/stage on marine medaka, *Oryzias dancena**

Time (hrs)	pH							
	Diploid		Triploid		Diploid		Triploid	
	Male	Female	Male	Female	Egg	Juvenile	Egg	Juvenile
Pre-experiment	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6
6	6.4^{c}	6.8^{a}	7.1^{d}	7.2^{b}	7.2^{1}	7.3^{1}	7.1^{1}	7.2^{1}
12	4.6^{c}	4.1^{a}	5.3^{d}	4.9^{b}	6.5^{2}	6.1^{1}	6.4^{2}	5.9^{1}
18	4.1^{c}	3.7^{a}	4.8^{d}	4.6^{b}	6.3^{2}	5.7^{1}	6.3^{2}	5.7^{1}
24	3.9^{c}	3.5^{a}	4.6^{d}	4.3^{b}	5.6^{2}	5.2^{1}	5.7^{2}	5.1^{1}
30	3.6^{c}	3.2^{a}	4.3^{d}	3.9^{b}	5.3^{2}	5.1^{1}	5.3^{2}	4.9^{1}
36	3.4^{c}	3.0^{a}	4.1^{d}	3.7^{b}	5.0^{2}	4.7^{1}	5.1^{2}	4.7^{1}
42	3.2^{c}	2.9^{a}	4.0^{d}	3.5^{b}	4.6^{2}	4.2^{1}	4.5^{2}	4.1^{1}
48	3.1^{c}	2.8^{a}	3.9^{d}	3.4^{b}	4.3^{2}	3.8^{1}	4.3^{2}	3.8^{1}

pH

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	30475.440	7618.860	248.055	<0.0001
Sex	3	618919.0	154729.7	529.150	<0.0001
Interaction	7	12833.400	4802.087	219.743	<0.0001

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	5711.0	896.1	16.2	< 0.7841
Stage	3	34259.1	67591.4	548.4	< 0.0001
Interaction	7	45867.5	12438.1	121.5	< 0.0446

*Each values are means ± SD of triplicate experiment. Differences between ploid and sex/stage are significant at this level ($P < 0.05$).

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Table 9. Carbon dioxide (CO_2) concentration between ploid and sex/stage on marine medaka, *Oryzias dancena**

Time (hrs)	CO_2 concentration (mg/L)							
	Diploid		Triploid		Diploid		Triploid	
	Male	Female	Male	Female	Egg	Juvenile	Egg	Juvenile
Pre-experiment	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.1
6	13.6^{c}	14.2^{a}	13.0^{d}	13.4^{b}	5.6^{1}	6.4^{2}	5.5^{1}	6.3^{2}
12	15.2^{c}	17.2^{a}	14.6^{d}	14.8^{b}	6.2^{1}	6.8^{2}	6.3^{1}	6.8^{2}
18	17.6^{c}	18.4^{a}	16.4^{d}	16.8^{b}	6.6^{1}	7.4^{2}	6.5^{1}	7.2^{2}
24	19.2^{c}	20.0^{a}	18.6^{d}	19.8^{b}	6.9^{1}	8.0^{2}	7.0^{1}	7.9^{2}
30	19.8^{c}	20.8^{a}	19.4^{d}	20.2^{b}	7.3^{1}	8.4^{2}	7.4^{1}	8.4^{2}
36	20.4^{c}	21.2^{a}	20.0^{d}	20.6^{b}	7.7^{1}	9.2^{2}	7.7^{1}	9.1^{2}
42	21.0^{c}	21.6^{a}	20.4^{d}	20.8^{b}	8.3^{1}	9.6^{2}	8.4^{1}	9.6^{2}
48	21.4^{c}	22.0^{a}	20.6^{d}	21.0^{b}	8.9^{1}	10.1^{2}	9.0^{1}	10.2^{2}

CO_2 concentration (mg/L)

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	21896.9	21379.8	202.72	< 0.0001
Sex	3	282359.0	141179.8	1011.23	< 0.0001
Interaction	7	38707.9	47870.7	386.23	< 0.0001

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	5711.0	896.1	16.2	< 0.7841
Stage	3	34259.1	67591.4	548.4	< 0.0001
Interaction	7	45867.5	12438.1	121.5	< 0.0446

*Each values are means ± SD of triplicate experiment. Differences between ploid and sex/stage are significant at this level ($P < 0.05$).

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Table 10. Ammonium (NH_4^+) concentration between ploid and sex/stage on marine medaka, *Oryzias dancena**

Time (hrs)	Ammonium (NH_4^+)							
	Diploid		Triploid		Diploid		Triploid	
	Male	Female	Male	Female	Egg	Juvenile	Egg	Juvenile
Pre-experiment	0	0	0	0	0	0	0	0
6	0.11^{c}	0.13^{a}	0.08^{d}	0.10^{c}	0.01^{1}	0.02^{1}	0.01^{1}	0.01^{1}
12	0.14^{c}	0.17^{a}	0.11^{d}	0.12^{c}	0.02^{1}	0.03^{1}	0.01^{1}	0.02^{1}
18	0.17^{c}	0.19^{a}	0.14^{d}	0.15^{c}	0.02^{1}	0.05^{2}	0.02^{1}	0.05^{2}
24	0.18^{c}	0.23^{a}	0.15^{d}	0.17^{c}	0.02^{1}	0.06^{2}	0.02^{1}	0.07^{2}
30	0.20^{c}	0.24^{a}	0.16^{d}	0.20^{c}	0.04^{1}	0.07^{2}	0.04^{1}	0.08^{2}
36	0.22^{c}	0.26^{a}	0.17^{d}	0.22^{c}	0.05^{1}	0.09^{2}	0.05^{1}	0.10^{2}
42	0.24^{c}	0.28^{a}	0.18^{d}	0.23^{c}	0.05^{1}	0.11^{2}	0.06^{1}	0.11^{2}
48	0.25^{c}	0.29^{a}	0.19^{d}	0.24^{c}	0.06^{1}	0.13^{2}	0.06^{1}	0.12^{2}

Ammonium (NH_4^+)

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	48654.896	12163.724	29.521	<0.0001
Sex	3	45557.856	11389.464	23.372	<0.0001
Interaction	7	39551.864	10596.992	19.449	<0.0001

	DF	Anova SS	Mean square	*F*-value	*P*-value
Ploid	1	438.095	54.049	7.612	<0.9024
Stage	3	45557.856	11389.464	125.661	<0.0001
Interaction	7	5762.451	1382.093	29.588	<0.0459

*Each values are means ± SD of triplicate experiment. Differences between ploid and sex/stage are significant at this level ($P < 0.05$).

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were similar to oxygen consumption rate. That is, metabolic rate of allexperimental groups decreased while experimental period.

In egg groups, oxygen consumption rates have not significant different between diploid and triploid ($P > 0.05$; Table 6, Fig. 7), and oxygen consumption rates in juvenile groups have not significant different between diploid and triploid ($P > 0.05$). In egg and juvenile groups, oxygen consumption rates of diploid in all measurement times were higher than those of triploid. In egg and juvenile, respiratory functions of diploid were not significantly different from those of triploid. In diploid groups, oxygen consumption rates have significant different between egg and juvenile (Table 6, Fig. 7), and oxygen consumption rates in triploid groups have significant different between egg and juvenile ($P < 0.05$). In all measurement times, oxygen consumption rates of diploid and triploid juvenile were higher than those of diploid and triploid egg, respectively. That is, respiratory function of juvenile was higher than that of egg.

Respiratory frequency, pH, CO_2 and NH_4^+ in diploid groups has significant different between male and female ($P < 0.05$; Tables 7, 8, 9 and 10). In triploid groups, respiratory frequency, pH, CO_2, NH_4^+ have significant different between male and female ($P < 0.05$), and respiratory frequency, pH,

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CO_2 and NH_4^+ of female in all measurement times were higher than those of female. That is, metabolic rate of female was higher than that of male. In male and female group, the other measurement factors (respiratory frequency, pH, CO_2 and NH_4^+) have significant different between diploid and triploid ($P < 0.05$; Tables 7, 8, 9 and 10). Respiratory frequency, pH, CO_2 and NH_4^+ concentrations of diploid in all measurement times were higher than those of triploid. That is, metabolic rate of diploid was higher than that of triploid. To sum up, respiratory function and metabolic rate of diploid female in all measurement times were highest than those of the other groups. Respiratory function and metabolic rate of triploid male were lowest than those of the other groups.

In egg and juvenile groups, the other measurement factors (pH, CO_2 and NH_4^+) have not significant different between diploid and triploid ($P > 0.05$; Tables 8, 9 and 10). In all measurement times, pH, CO_2 and NH_4^+ concentrations of diploid were not significantly different from those of triploid. That is, metabolic rate of diploid was not significantly different from that of triploid. To sum up, respiratory function and metabolic rate of juvenile in all measurement times were higher than those of egg groups. pH, CO_2 and NH_4^+ in diploid and triploid groups have significant different between egg

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and juvenile ($P < 0.05$; Tables 8, 9 and 10), and pH, CO_2 and NH_4^+ of juvenile in all measurement times were higher than those of egg. That is, metabolic rate of juvenile was higher than that of egg.

Fig. 8 shows results of stress response to temperature change (25→ 15℃). 0 hr and 48 hrs have the lowest cortisol concentrations (0.5 ug/dL) and have not significant difference between diploid and triploid ($P > 0.05$). Compare to plasma cortisol concentrations of 0 hr and 48 hrs, diploid and triploid in 6 hrs, 12 hrs and 24 hrs have conspicuously increased plasma cortisol ($P < 0.05$). Fig. 9 summarizes that established hypo (15→0 ppt) group was similar the tendency of plasma cortisol to hyper (15→30 ppt) group (from 0 hr to 48 hrs). Plasma cortisol concentrations of hypo and hyper salinity changes in diploid and triploid groups have the lowest plasma cortisol in 0 hr and 48 hrs. From 6 hrs to 12 hrs, plasma cortisol increased gradually, and plasma cortisol decreased drastically from 12 hrs to 48 hrs, and diploid's plasma cortisol concentrations were higher than triploid while measurement time. Fig. 10 shows that change of plasma cortisol by DO change (7.0→5.5 mg/L). Plasma cortisol concentrations of diploid and triploid groups have the lowest plasma cortisol in 0 hr. Plasma cortisol concentrations of 6 hrs, 12 hrs and 24 hrs have similar plasma cortisol tendency to cortisol concentrations of

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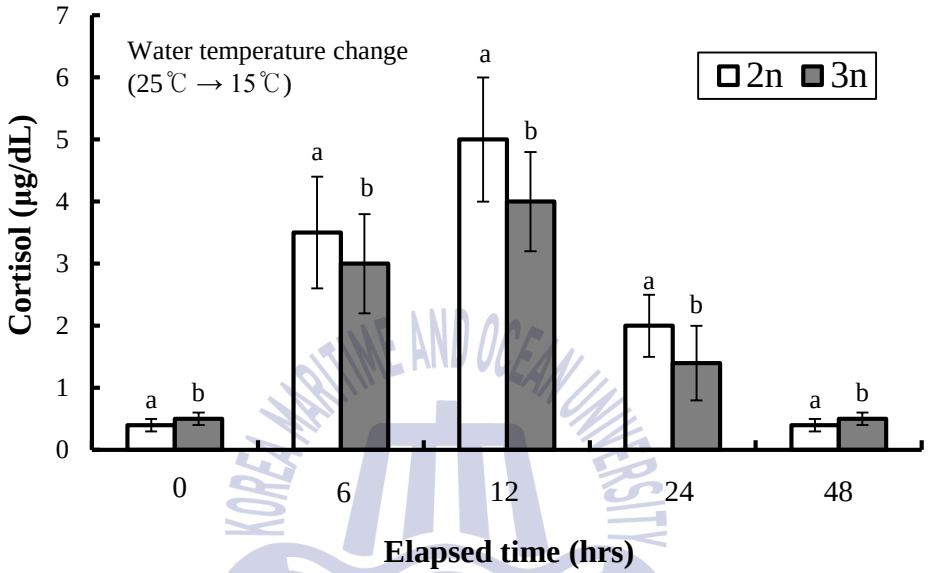

Fig. 8. Change of cortisol in diploid and triploid marine medaka, *Oryzias dancena* while 48 hrs after water temperature changed. Water temperatures of each group were changed from 25℃ to 15℃. Each values are means ± SD of triplicate experiment. Different letters on error bars are significantly different for each group ($P < 0.05$).

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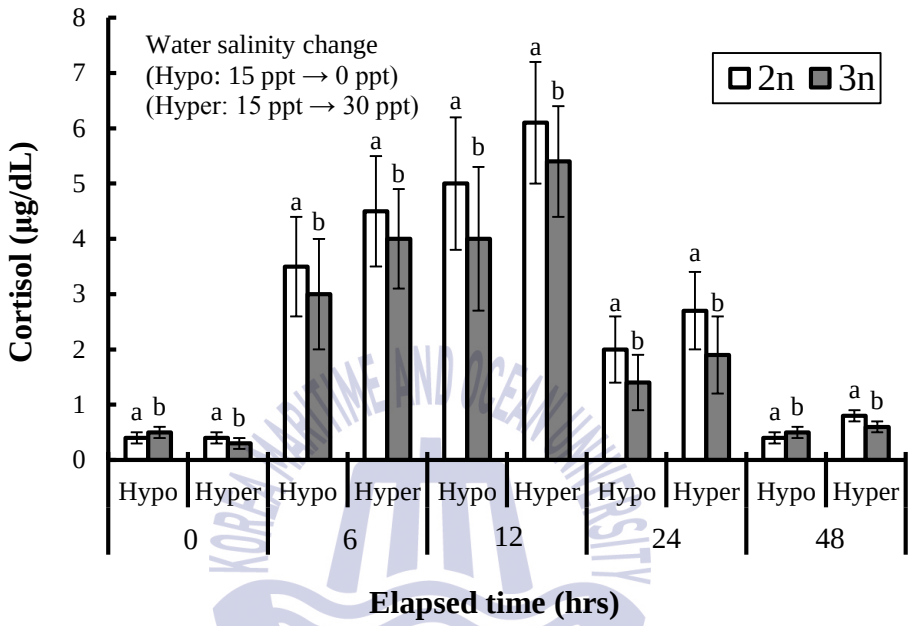

Fig. 9. Change of cortisol in diploid and triploid marine medaka, *Oryzias dancena* while 48 hrs after water salinity changed. Water salinities of hypo-osmoregulation group and hyper-osmoregulation group were changed from 15 ppt to 0 ppt and from 15 ppt to 30 ppt, respectively. Each values are means ± SD of triplicate experiment. Different letters on error bars are significantly different for each group ($P < 0.05$).

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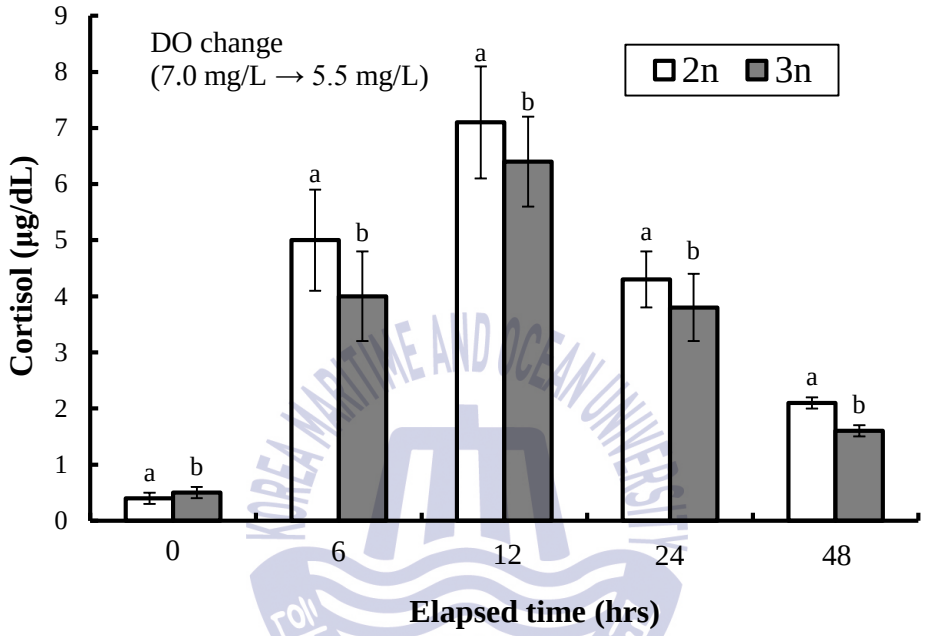

Fig. 10. Change of cortisol in diploid and triploid marine medaka, *Oryzias dancena* while 48 hrs after dissolve oxygen changed. Dissolve oxygen of each group were changed from 7.0 to 5.5. Each values are means ± SD of triplicate experiment. Different letters on error bars are significantly different for each group ($P < 0.05$).

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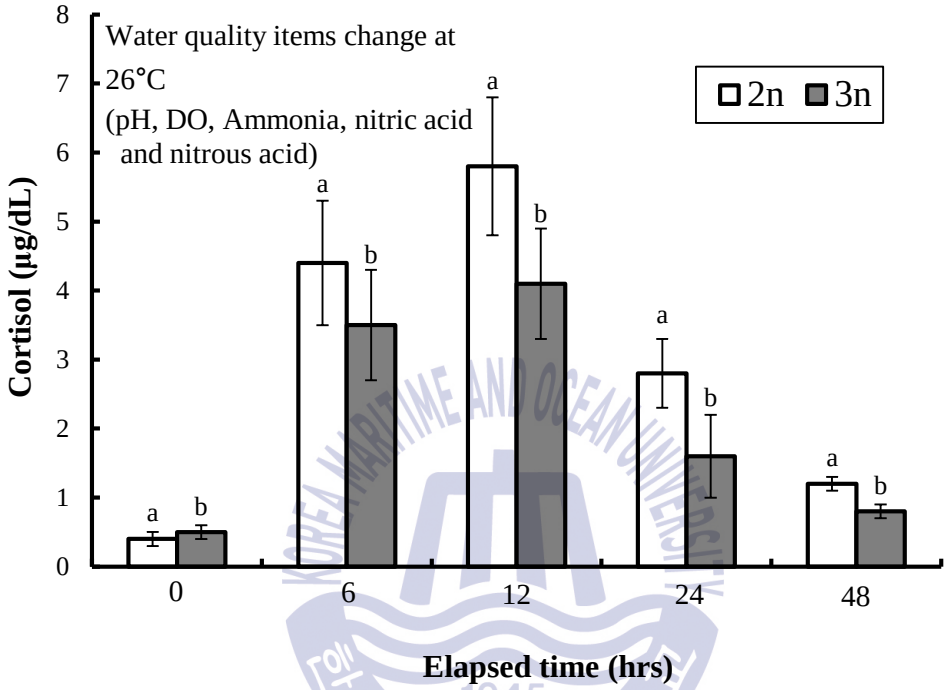

Fig. 11. Change of cortisol in diploid and triploid marine medaka, *Oryzias dancena* while 48 hrs after water quality changed. Change of water quality in each group was mentioned in Table 1. Each values are means ± SD of triplicate experiment. Different letters on error bars are significantly different for each group ($P < 0.05$).

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temperature and salinity stress responses, and diploid's plasma cortisol concentrations were higher than those of triploid in all measurement times. Fig. 11 shows that results of the plasma cortisol by water quality items (temperature, pH, DO, ammonia, nitric acid and nitrous acid) changed. The tendency of plasma cortisol concentrations was similar to the tendency of DO change in diploid and triploid.

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Discussion

The induction of triploidy fish has been used to generate sterility in commercial fish farming and fishery management applications (Thorgaard, 1986; Benfey, 1999). Induced triploidy fish subject to thermal shock show impaired gametogenesis and reduced reproduction, but investment in somatic growth may not be affected by the metabolic costs of sexual maturation (Thorgaard, 1986; Benfey, 1999). Sterility in triploids can also be used to prevent reduced flesh quality associated with sexual maturation, and also addresses concerns regarding the environmental impact of farmed escapes.

Triploid marine medaka, *Oryzias dancena* were shown to grow more rapidly than diploid marine medaka ($P < 0.05$). Prervious study reported that triploid fish was more rapidly growth than diploid. Referred to Nam *et al.* (2001), triploid mud loach, *Misgurnus mizolepis* was displayed growth acceleration 22-25 times that of diploid Triploid marine medaka was larger than diploid, but giantism of triploid marine medaka was not caused in this experiment. Nam *et al.* (2001) and Seol *et al*. (2008) suggested that gigantism of triploid did not appear due to the decreasing cell number of triploid. In this study, male marine medaka in diploid and triploid were larger than female marine medaka ($P < 0.05$). Females are usually larger than males of the same

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age, although in some species, males are larger than females, e.g., the gudgeon, *Gobio gobio* (Mann, 1980). The reasons for these size differences are unclear (Katano, 1998). Several authors have reported that the evolution of a larger body size in males probably results from male–male competition associated with a polygynous mating system (Katano, 1998). Therefore, exploring the nature and extent of sexual dimorphism can extend our understanding of social structure and adaptation, as well as species identification. During 1 month, rapid increase of the length caused the reduction of the condition factor, and condition factor of triploid were lower than those of diploid. For find out the cause in detail, we searched the literature studied in similar parameters as this experiment. Unfortunately, there were no previous studies to investigate rapid decrease of condition factor and difference of condition factor between diploid and triploid in marine medaka and other fish species.

The condition factor of a fish reflects physical and biological circumstances by feeding conditions, parasitic infections and physiological factors (Le Cren, 1951). This also indicates the changes in food reserves and therefore an indicator of the general fish condition. Therefore, information on condition factor can be vital to culture system because they provide the

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producer with information of the specific condition under which organisms are developing (Araneda *et al.,* 2008). According to the Table 2, Figs. 1 and 2, regardless of gender, the triploid marine medaka showed a low level of condition factor to diploid for 6 months after hatching. This means that under the conditions of the same feeding conditions and environment, triploids had more rapid growth than diploid.

Induced triploids had more chromosome numbers are generally believed to be sterile because of chromosome incompatibility during meiosis (Thorgaard, 1986; Benfey, 1999). Triploidy induction in our study resulted in functionally sterile fish having abnormal gametogenesis and gonadal development (Thorgaard, 1986; Benfey, 1999). As shown in Fig 3, gonadosomatic index values of induced triploid marine medaka were less than those of diploid during all through the year ($P < 0.05$).

Sexual hormone including testosterone and estradiol in induced triploid are regular, because testes and ovary of induced triploid doesn't mature (Weatherley & Gill, 1987). Unlike normal gonadal maturation of diploid and induced triploid's gonads are immatured on formal and histological (Lincoln, 1981; Nam *et al*., 2001). As shown in Figs 4 and 5, induced triploid marine medaka showed lower values of sex hormone to diploid, it resulted from

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decreasing hormone secretion caused by immaturity (Lincoln, 1981). Testes of diploid were exhibited normal spermatids and spermatozoa, while a few were seen in induced triploid. Ovary of diploid was full of well development oocyte, those of induced triploid exhibited oogonia (Nam *et al*., 2001).

According to the results of Table 3, thyroid stimulating hormone and thyroxine were observed to be higher in induced triploid during 1 year ($P < 0.05$). Induced triploids can be expected to show better growth compared to the diploid. In diploid and triploid groups, thyroid stimulating hormone and thyroxine of male are higher than female. The followings in previous study were observed: initial survival of freshwater fish, transformation, and the effect of thyroid stimulating hormone and thyroxine on the development and growth (Lam & Sharma, 1985; Weatherley & Gill, 1987).

We compared the erythrocyte of diploids and induced triploids. In a previous study by Seol *et al*. (2008), erythrocyte counts of diploid were higher than that of triploid counts. In the case of triploid, the nucleus of red blood cell had major axis which is 1.33 times larger and the minor axis is 1.26 times larger than the diploid (Seol *et al*., 2008). This fact is well accepted, and the measure of erythrocyte size is frequently used as the sole

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criterion for determining ploidy level in a fish (Benfey, 1999). From the previous study, size of cell and nuclear of induced triploid erythrocyte is approximately 1.3 times larger than diploid erythrocyte in far eastern catfish, *Silurus asotus* (Seol *et al*, 2008). Size of erythrocyte cell for induced triploid sweetfish, *Plecoglossus altivelis* is major axis of 1.26 times and minor axis of 1.27 times larger than diploid with the size of erythrocyte nuclear is major axis of 1.35 times and minor axis of 1.16 times larger than diploid (Aliah *et al*., 1991; Park & Park, 1995; Nam *et al*., 2001; Ballarin *et al*., 2004; Seol *et al*, 2008; Park *et al*., 2015).

Although the triploid fish appeared to be 1.5 times with chromosome increased and bigger cell size phenomenon than diploid, but the body size did not present gigantism. This phenomenon reported by Swarup (1959), the triploid stickleback, *Gasterosteus aculeatus*, cartilage, blood and neuron cell and nuclear size increased as compared to diploid, but these results not affect body size gigantism. Triploid has red blood cells and nuclear sizes bigger than diploids while the triploid number of red blood cells decreased more than diploid, this reason offsets the body size giantism effect, and triploid fish which causes red blood cell enzyme activity reductions, and lowers oxygen transport (Park & Park, 1995).

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The hematological responses of diploid and triploid fish have been studied (Benfey, 1999; Ballarin *et al*., 2004; Stefano *et al*., 2005; Wang *et al*., 2007). Triploidization led to increase the erythrocyte and thrombocyte size and reduce the amount of erythrocyte and thrombocyte (Benfey, 1999; Cogswell *et al*., 2001; Ballarin *et al*., 2004.). In this study, triploids have lack of cell number, increased celluar size and higher DNA content (diploid 1.7 pg/cell, triploid 2.6 pg/cell). Diploid marine medaka (EC, 1.2±0.03 cells/μL) have twice more than triploid (EC, 2.3±0.13 cells/μL) which values were similar tendendy to shi drum, *Umbrina cirrosa* of EC (diploid 5.09±1.17 and triploid 2.22±0.38 cells/μL; $P < 0.05$, Ballarin *et al*., 2004). Hematocrit (Hct) was not significant difference between diploid and triploid, such that the other fishes have similar tendency ($P > 0.05$, Ballarin *et al*., 2004; Wang *et al*., 2007). In mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) of previous study, the values of the triploid were 1.8 times higher than the diploid values ($P < 0.05$, Ballarin *et al*., 2004; Wang *et al*., 2007). Total hemoglobin content (THC) means an ability of respiratory rate (Benfey, 1999; Ballarin *et al*., 2004). Marine medaka's respiratory ability was not significant difference between diploid's THC (8.3±0.44 g/100mL) and triploid (8.4±0.37 g/100mL; $P > 0.05$). Mean corpuscular hemoglobin

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contentration was equivalent to previous experiment, and diploid (20.6±1.54%) and triploid (20.1±1.44%) were similar value ($P < 0.05$; Wang *et al*., 2007). Except for MCV and MCH, the remained haematological parameters are no differences between diploid and triploid. MCV and MCH reported to be higher in polyploids ($P > 0.05$, Benfey, 1999).

As mentioned Kazakov & Khalyapina (1981), rates of oxygen consumption per unit weight were found to be lower in adult Atlantic salmon, *Salmo salar* than in juvenile fish. The rates were higher in males than in females and declined with increasing age in both sexes (Kazakov & Khalyapina, 1981). As mentioned by Tran *et al*. (2008), oxidative metabolisms of female and male oysters, *Crassostrea gigas* were measured separately before and after spawning, and male oysters had higher O_2 consumption than that of female oysters (Tran *et al*., 2008). In pre- and post-spawners, males presented a higher oxidative metabolism than females, independently of gametogenic condition. The increase in oxidative metabolism in the male oyster is thus not directly related to the spawning status. This difference could not be explained by a difference in gametogenic cycles between females and males (Tran *et al*., 2008). A difference in O_2 consumption was also shown to the gametogenic status for both female and

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male individuals (Tran *et al*., 2008). Results of Kazakov & Khalyapina (1981) and Tran *et al*. (2008) showed conflicting trend to results of this study.

As mentioned by Benfey (1999), numerous studies have shown that oxygen consumption rates are similar for triploids and diploids under a variety of experimental conditions (Oliva Teles & Kaushik, 1987; Aliah *et al*., 1991). Using triploid rainbow trout, *Oncorhynchus mykiss* produced in two different ways, Oliva Teles & Kaushik (1987) found triploids originating from retention of the second polar body (the common way of producing triploids: Benfey, 1999) to have higher oxygen consumption rates than diploids and triploids, originating from diploid-tetraploid crosses to have intermediate oxygen consumption rates. On the other hand, similar to our results, triploid brook trout, *Salvelinus fontinalis* to have lower oxygen consumption rates than diploids (Stillwell & Benfey, 1996). According to the Table 6 and 7, and Figs 6 and 7, oxygen consumption and respiratory frequency were showed that diploid female and male had relatively high oxygen consumption and respiratory frequency to triploids ($P < 0.05$). Through the oxygen consumption and respiratory frequency, pH values, NH_4^+ and CO_2 concentrations were showed in Table 8, 9 and 10. Similar to Table 6 and 7, results of pH values, NH_4^+ and CO_2 were showed consistent

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tendency between diploid and triploid.

Most of these studies cannot be compared because of differences in the size and stage of development of the fish used and in their levels of activity both prior to and during oxygen consumption measurements. The importance of controlling for activity, sex, and maturity in studies of triploid hematology and respiratory physiology has been outlined by Stillwell & Benfey (1996). Opercular abduction rate at a given oxygen consumption rate, a measure of respiratory system efficiency, has been shown to the same for triploids and diploids in some studies (Aliah *et al*., 1991; Stillwell & Benfey, 1996), but higher for triploids in many studies (Sezaki *et al*., 1991).

Increased opercular abduction rate may indicate a compensatory mechanism to increase oxygen uptake. Other possible compensatory mechanisms, such as increased opercular abduction volume and/or changes in heart rate and stroke volume, have not been investigated. Presently, it is unclear whether such compensatory mechanisms are even necessary. As mentioned by Seol *et al*. (2008), the oxygen consumption rate did not differ significantly between diploid and triploid far eastern catfish. The respiratory frequency was higher in triploid than in diploid, therefore, respiratory function was higher in triploid than in diploid (Seol *et al*., 2008). Results of

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Seol *et al*. (2008) showed similar trend to results of this study. As mentioned by Park *et al*. (2015), mitosis of diploid in each tissue was more active than those of triploid marine medaka in each tissue, and mitosis of liver tissue and gill tissue in each ploidy was more active than those of tail fin tissue in each ploidy of marine medaka. The period of cell cycle is the period of mitotic cycle, and short mitotic cycle period means a rapid growth and high metabolism (Park *et al*., 2015). Therefore, diploid marine medaka had a rapid growth than triploid marine medaka (Park *et al*., 2015).

The only controlled experiment to have assessed physiological aspects of the stress response in triploid fish is that of Benfey (1999), who found no difference between triploids and diploids in hematocrit and plasma cortisol and glucose profiles after an acute handling stress. In light of abundant anecdotal information that triploids do not cope well with poor water quality, a common source of chronic stress in aquaculture, detailed study of the response of triploids to chronic stress is warranted. Poorer survival due to chronic stress may be reflected in reduced energy stores and/or increased rates of depletion of these stores during stressful conditions. Although substrate utilization during aerobic metabolism does not differ between triploids and diploids, it may be that triploids differ in their ability to

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withstand sustained anaerobic metabolism (Benfey, 1999). But the hormonal responses of cortisol in this study were different from previous study. In this study, plasma cortisol of diploid adult samples were higher than triploid. The result of this study is suggested that it is different stress response between triploids and diploids in plasma cortisol after various stress parameters.

Plasma cortisol is an indicator of stress response that appears when fish receive various stress parameters, and being involved in metabolism and ecologic balance (Mommsen *et al*., 1999). And cortisol affects the body balance, carbohydrates, protein and lipid (Mommsen *et al*., 1999). Both diploid and triploid showed relatively similar tendency of plasma cortisol in water parameter. Cortisol levels of stressed fish suggest that anesthesia effects, salinity, DO, pH, Ammonia and acid procedures can affect fish metabolism and hormone (Mommsen *et al*., 1999). Plasma cortisol concentrations was controlled in Mommsen *et al*. (1999)'s experiment, it means they made the annually changeable temperature, fresh water to sea water, salinity, waste water and DO.

Referred to previous study, it was carried out on sea bass, *Dicentrarchus labrax*, which have examined possible differences in the stress response between diploid and triploid fish (Stefano *et al*., 2005). In our study, similarly

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to Stefano *et al*. (2005) triploid fish showed relatively lower cortisol concentration than diploid.

The results of the plasma cortisol levels of the water parameters shown in Figs 8, 9, 10 and 11, measured plasma cortisol concentrations were ranged from 0 to 8 μg/dL, and observed the highest cortisol concentration in 12 h. That means cortisol concentration was gradually increased to 12 h and decreased to 48 h. Stress response different from the anesthetic effect, anesthetic effect means highest cortisol concentration in 0 h (Park *et al*., 2014).

Thus, this experiment was similar to our results. Although the triploid fish showed lower oxygen consumption and respiratory efficiency as compared to diploid, some triploid fish, triploid Atlantic salmon triploid white crappie, *Pomoxis annularis* and triploid cherry salmon have red blood cell hemoglobin contents and concentrations higher than each diploid, and compensated by few red blood cell of triploid (Thorgaard, 1986; Benfey, 1999). In this study, triploid marine medaka showed increased sizes in red blood cell and nuclear. The increased size of cell and nucleus and the decreased number of cell and nucleus in some tissue phenomenon are useful for indicating the use of ploidy distinctions.

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Considering the result of this research, diploid has much higher red blood cell, faster respiratory frequencies, releasing higher CO_2 and NH_4^+ concentrations and causing faster acidification of water, and oxygen consumption rate was different between diploid and triploid. Diploid were more used oxygen efficiently than triploid (Park & Park, 1995; Cogswell *et al*., 2001; Ballarin *et al*., 2004; Stefano *et al*., 2005). In summary, respiratory function and metabolic abilities of diploids were higher than those of triploids. By stress exposure, hormone responses of diploid were higher than those of triploid. So, diploids were more sensitive for stress response than triploids. In this study, differences of stress responses, metabolic ability, respiratory function and hematological characteristic were determined clearly between diploid and triploid. In addition, relationship between respiratory function and hematological characteristic was determined clearly in diploid and triploid (Swarup, 1959; Park & Park, 1995; Stefano *et al*., 2005). However, relationship between stress response and metabolic ability was not determined clearly in diploid and triploid. Thus, it is necessary to study afresh the difference of physiological response between stress and metabolism in diploid and triploid. So, future investigations in marine medaka should focus on relationship between stress and metabolism and

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comparative physiological reactions between the diploid and the triploid by other stress factor.

For find out the cause in detail, we searched the literature studied in a similar parameters as this experiment. Unfortunately, there were no previous studies to investigate difference of thyroid stimulating hormone and thyroxine between diploid and triploid in other fish species. Although induced triploids are infertile, it is economically feasible because of faster grow than diploid. It thought that can going to stably produce through coming study.

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Acknowledgements

I sincerely thank you for your continuous interest and practical help to complete this thesis.

Special thanks to:

- my supervisor, Dr. **In-Seok PARK**, go sincere thanks not only for his sound technical advice and penetrating criticisms, but also for his availability and limitless supply of patience. Working under his guidance has been an instructive and pleasurable experience.

- a panel of the awarding committee, Drs. **Sung Hwoan CHO** and **Sun Young LIM** and for their practical advices, particularly with enormous calculations and editorial changes in the thesis

- Drs. **Youngwan SEO**, **Jong Woong AHN** and **Cheol Young CHOI** for their kind advice and interests in this thesis.

- my colleagues in the Fishery Genetics & Breeding Sciences Laboratory, **Hyun Woo GIL**, **In Bon GOO** and **Soo Yeon IM** for their friendship, invaluable assistance, cooperation, support, and attention to my research.

- finally, deepest gratitude goes to my mother and my father sharer of adventures, guardian of happiness and sanity on all the long days.

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