Original Article

Evaluation of genetic polymorphism, genomic template stability, condition factor and hemato-biochemical parameters in response to slow reduction in water level during Nile tilapia (*Oreochromis niloticus*) harvest

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Abstract

The aim of this study was to evaluate the effect of slow reduction in water level during Nile tilapia (*Oreochromis niloticus*) harvest on growth performance, condition factor, hemato-biochemical parameters, genetic polymorphism, and genomic template stability. During tilapia harvest, water level was reduced from 150 to 30 cm in different duration as follows: group one (control), in 72 hours (h); group two, in 48 h; and group three, in 24 h. There was a significant correlation between length and weight of fish among all experimental groups. Condition factor showed significant differences ($P<0.05$) among all experimental groups. Hemato-biochemical parameters showed no significant differences in PCV % and lymphocyte/RBC ratio. Moreover, significant differences were recorded in total protein, albumin, globulin, A/G ratio, cholesterol and triglycerides among the experimental groups. To evaluate genetic polymorphism, eleven RAPD primers generated a total of 67 DNA bands, out of which 31 (46.27%) were polymorphic. Cluster analysis and Principal Coordinate Analysis indicated high dissimilarity degree between the control and treatment groups. Genetic fingerprint for all experimental groups was performed by DNA barcoding, which revealed that the number of bands differed among all experimental groups with an average of 52.3 bands. Percentages of genomic template stability were 56.5 and 54.3 for the 48 and 24 h treatment groups, respectively. RAPD technique identified some specific unique markers for the treatment of water level reduction in Nile tilapia harvest and indicated that the rapid reduction (24 h) could reduce the bad effects of crowding stress and improve tilapia welfare during harvest.

Keywords: condition factor, fish harvesting, genetic polymorphism, genomic template stability, *Oreochromis niloticus*

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Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important warm water fishes used for aquaculture production in Egypt because of its fast growth and resistance against unforgiving conditions (Yonas, 2006). Tilapia is reared mainly in earthen ponds in a semi extensive rearing system. Following a period of fasting in the end of rearing period, tilapia is netted and placed on ice, in water tanks or in dry crates, for transportation to markets or point of slaughter (Poli, 2009). Crowding is the first stage in most gathering, harvesting and transporting operations. It is achieved by reducing the level of water and using a special net or by moving partitions or trellises (Mohamed et al., 2015). Nowadays, the interest in fish welfare, including harvesting procedures, becomes important from both ethical and product quality points of view not only to producers but also to consumers. Distress and painful conditions of fish are caused by crowding, which takes the longest time during the catching practice (Grutter and Pankhurst, 2000) and significantly creates conditions of lower level of dissolved oxygen (Wall, 2001). Crowding evokes the onset of stress which can alter fish normal behavior and metabolism (Poli, 2009). The reaction of fish during stress condition is characterized by activation of primary stress responses such as the release of catecholamines, the activation of hypothalo-pituitary-internal axis and the release of cortisol, followed by secondary stress responses which include energy mobilization (decreasing glycogen store) and changes in metabolism (increasing blood glucose, muscle activity and plasma lactate) (Wendelaar Bonga, 1997). In addition, the exposure of fish to prolonged, repeated, unavoidable stress (chronic stress), which meaningfully compromises the well-being of fish, is associated with reduction in body condition and growth, impairment of immune function and suppression of reproductive function (Huntingford et al., 2006).

Genetic marker techniques are useful in fish genetic improvement programs for production with good economical traits and adaptation to environmental changes (Rashed et al., 2009). Also, they are fundamental tools for monitoring fish populations (Rashed et al., 2008) and fish species genetic variability (Saad et al., 2009). Therefore, genetic marker techniques should be practiced to obtain information needed for sound management of farming and wild fish stocks (Saad et al., 2009).

Besides the varied techniques using polymorphic DNA markers, random amplified polymorphic DNA (RAPD) marker has become ubiquitous and essential in aquaculture genetics (Khoo et al., 2011; Abd el kader et al., 2013). It is a dominant marker, inherited as Mendelian marker, by which polymorphism is detected by the presence and absence of bands (Chauhan and Rajiv, 2010). The advantage of RAPD to generate molecular characterization is the production of molecular markers without any previous genomic information on the target species. RAPD assays have been used for evaluation of genetic diversity among different fishes (Saad et al., 2013; Saad et al., 2014). Also, RAPD is a quick and effective method which can be applied to generate specific banding patterns with genotypes (Hassan et al., 2014; Ahmed et al., 2014). There are several studies of fish dealing with genetic diversity and genetic improvement (Hassanien et al., 2004). These studies were used successfully in estimation of genetic diversity for fish populations.

Therefore, this investigation aimed to assess water parameters, growth performance, K-factor, hemato-biochemical parameters and molecular DNA changes by monitoring the RAPD profiles induced by different duration of water level reduction (in 72, 48 and 24 h) during fish harvest.

Materials and Methods

Fish, rearing system and management: This study was carried out in a special fish farm at Kafr El-Sheikh governorate (N: 31, 21, 38 and E: 30, 36, 40) and laboratories of Genetics Department, Faculty of Agriculture, Kafrelsheikh University, Egypt. The farm was composed of eight independent ponds, six for breeding and two for brooding. All ponds were irrigated from a single water source but each pond had separate water inlet and outlet. Each breeding pond had surface area of 2 acres and dimension of 150 x 55 x 1.5 m. Fish type was mono-sex Nile tilapia (*Oreochromis niloticus*) (treated with 17 alpha methyl testosterone) with *Mugil cephalus* and *Mugil capito*. The rearing system was semi extensive with average fish density of three fish/m^2. Fish feed was pelleted sinking type (30% crude protein and 3350 kcal/Kg digestible energy), 3 mm in diameter, manufactured by ALEKHWA® feed factory (local Egyptian fish feed factory). Feeding rate ranged from 4% of the fish biomass at the beginning of rearing period to 1.5% at the end of rearing period, twice daily. The rearing period was 7 months, starting in April 2014, with 20 gm fingerlings.

Management of water level: During the rearing period of fish, the level of water in the breeding ponds ranged from 130 to 150 cm and was not less than 145 cm during the last month of the rearing period. As a common practice for gathering and harvesting tilapia in Egypt, the water level is reduced gradually until reaching the lowest level (30 cm). In this experiment, the water level was reduced from 150 to 30 cm in different duration as follows: group one (control), in 72 hours (h); group two, in 48 h; and group three, in 24 h.

Water sampling and analysis: Water sampling was done around time of fishing. Five water samples were collected from each pond by inverting 250 ml sterilized glass bottle 15 cm below the pond water surface. All samples were transferred to the lab on icebox. Analysis was initiated within 2 h of sample collection. Physico-chemical analysis of the water samples was carried out to determine temperature, dissolved oxygen (DO), pH, total ammonia (NH_3), electrical conductivity (EC) and total dissolved salts (TDS) according to Eaton et al. (2008).

Growth performance, length-weight relationship and condition factor of Nile tilapia (Oreochromis niloticus): A total of 108 fish were randomly sampled

from different locations in production ponds, 36 fish per each treatment. When the water level was reduced to the lowest level (0.3 m), the fish were harvested using 0.5 cm mesh size net and placed in separate polypropylene containers before transporting to the laboratory. The fish were dried using a filter paper to remove excess water from the body before measuring. The fish were weighed using a digital balance (PW Balance, Adam Equipment Co., USA). Length, width and thickness of the fish were measured using a measuring board (Lagler, 1970). The length was measured as the distance from the snout to the tip of the caudal fin. The length and weight of the fish were recorded to the nearest 1 mm and 0.1 g, respectively. Length-weight relationship was calculated according to Pauly (1983) and condition factor was calculated according to Lizama et al. (1999) and Gomiero and Braga (2005).

Hemato-biochemical parameters

Blood collection and serum separation: Blood was drawn from the caudal peduncle region (v. caudalis) of 72 fish, 24 fish per each treatment, using sterile syringes. The blood was transferred to sterile 2 ml test tubes with EDETA for hematological assay and 2 ml plain Eppendorf tubes for serum separation. The blood was left to clot at 4°C for 60 min, after that, the tubes were centrifuged at 3000 rpm using an Eppendorf centrifuge for 10 min for serum separation. The serum was separated in Eppendorf tubes, frozen and stored at -40°C until analyses.

Hematological parameters: Measured parameters included erythrocyte count (RBC), packed cell volume (PCV) and lymphocyte count (Anderson and Siwicki, 1995).

Biochemical parameters: Total protein was quantified by colorimetric method using commercial kits. Albumin was quantified using Bromocresol green binding method (Doumas et al., 1971). Globulin was calculated by subtracting albumin values from total protein. Albumin/globulin (A/G) ratio was calculated by dividing albumin values by globulin values. Triglycerides and cholesterol were measured according to the method described by Stein (1986).

Molecular analysis

Genomic DNA extraction: Genomic DNA was extracted by using i-genomic CTB DNA extraction mini kit (iNtRON Biotechnology, cat. No. 17341) from liver tissues for both treatment groups in addition to the control group. Quantity and quality of the extracted DNA of each sample were estimated by comparing band intensities against standard DNA ladder on 1.5% agarose gel. The DNA samples were diluted to a final concentration of 40 ng/µl before PCR amplification. The DNA was stored at -20°C until use.

Primer selection: A set of 16 random 10-mer primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-06, OPA-07, OPA-08, OPA-09, OPA-10, OPB-05, OPB-07, OPB-10, OPB-11, OPB-12, and OPB-14) from Operon Technologies was used to detect DNA changes among the two treatment groups (48 h and 24 h) during the reduction in water level application compared to their respective control group (72 h). The sequence of primers is shown in Table 3.

RAPD analysis and PCR conditions: The conditions of DNA amplification of RAPD assay were carried out in a total volume of 20 µl containing 1 µl of 40 ng genomic DNA, 1 µl of 10 µM primer, 10 µl master mix [2x *Taq* PCR MasterMix (TIANGEN), cat. No. KT201], and 8 µl dd H_2O. Genomic DNA amplification was carried out in a thermal cycler (Perkin Elmer Cetus) programmed as follows: the reaction mixture was denatured at 94°C for 3 min, followed by 35 cycles consisting of denaturation step for 30 sec at 94°C, primer annealing step for 30-60 sec at 30-34°C (annealing step was optimized for each primer), and extension step for 1 min at 72°C. The amplification was completed with a final extension period (72°C) for 5 min. Mixtures were stored at 4°C until use.

The obtained products were analyzed using electrophoresis in a 1.5% agarose gel stained with ethidium bromide. The amplified fragments were detected on Benchtop UV-transilluminator and photographed using photoDoc-It™ Imaging System. Molecular size of the amplified fragments was determined against 1 Kb plus DNA ladder (TIANGEN, cat. No. MD113).

Scoring and data analysis of DNA profiles: DNA banding patterns generated from each RAPD primer were analyzed by GelAnalyzer 3 program. These bands were scored as present (1) or absent (0) for each primer and entered in the form of a binary data matrix. From this matrix, molecular distances (MD) were estimated using Nei & Li coefficients (Nei and Li, 1979) by computational package MVSP 3.1. Also, depending on this matrix, cluster analysis and Principal Coordinate Analysis (PCoA) were performed using the same program.

To measure the performance of the RAPD markers to differentiate between different treatment, Polymorphic Information Content (PIC) and Resolving Power (RP) were calculated. The PIC value for each locus was calculated as proposed by Roldan-Ruiz et al. (2000) as follows: $PIC_i = 2f_i (1 - fi)$, where PIC_i is the PIC of the locus i, fi is the frequency of the amplified fragments (band present), and 1 - fi is the frequency of non-amplified fragments (band absent). The PIC of each primer was calculated using average PIC values from all loci of each primer. Resolving power (RP) of each primer was calculated according to Prevost and Wilkinson (1999) as follows: $RP = \sum I_b$, where I_b represents the informative fragments. I_b was given on a scale from 0-1 according to the following formula: $I_b = 1 - [2 \times (0.5 - p)]$, where p is the proportion of treatments containing the band.

Estimation of genomic template stability (GTS): Each separate DNA effect observed in the RAPD-PCR profiles (disappearance of normal bands, appearance of new bands and variation in band intensities in comparison with control profiles) was considered in order to assess any DNA damage and genomic template stability percentage (GTS %) was calculated for each experimental group of fish with the chosen

primers. GTS % was calculated as follows: GTS % = (1 - a / n) × 100 , where "a" is the number of RAPD polymorphic profiles detected in each treatment and "n" is the number of total bands in the control. Polymorphism observed in the RAPD profile included disappearance of a normal band and appearance of a new band in comparison with control profile (Atienzar et al., 1999). To compare sensitivity of each parameter (genomic template stability), changes in these values were calculated as a percentage of their control (set to 100%).

Statistical analysis: Data were tested for distribution normality, linearity and homogeneity of variance. Data were analyzed using Graph Pad™ Prism 5. Results are presented as means ± SEM. Data were compared by one-way ANOVA and Tukey's multiple comparison test was used as a post hoc test when appropriate. The length-weight relationship was calculated using linear regression and regression coefficient (R^2) was calculated for each treatment. Level of significance was set at $P<0.05$.

Results

Water analysis: Water analysis of the experimental fishponds (Table 1) revealed that there were significant differences ($P<0.05$) among the analyzed water parameters, except for water temperature and ammonia. For PH and EC, there was no significant difference between the integral (1.5 m) and reduced (0.3 m) water levels in different duration of time (72, 48, and 24 h). Moreover, there were no significant differences between the 72 and 48 h groups, but they significantly differed ($P<0.05$) from the 24 h group. For TDS, there was a significant difference ($P<0.05$) between the integral (1.5 m) and reduced (0.3 m) water levels in different duration of time (72, 48, and 24 h). However, there was no significant difference between the 72 and 24 h groups, but they significantly differed from the 48 h group. For NH_3, the lowest concentration was recorded in the integral water level (1.5 m) followed by the reduced water level (0.3 m) in the 24, 48 and 72 h groups.

Table 1 Effect of reduced water level before harvest on water parameters of Nile tilapia (*Oreochromis niloticus*) ponds

	Water level of 1.5 m	Time spent in reducing water level from 1.5 m to 0.3 m			*P*-value
		Group one (72 h)	Group two (48 h)	Group three (24 h)	
Water temp. (°C)	12±0.32	11.60±0.4	12±0.32	12.20±0.37	0.683
DO(mg/L)	6.240±0.23^{cd}	4.930±0.146^{ab}	5.340±0133^{b}	5.860±0.187^{c}	0.012
PH	7.618±0.169a	7.436±0.106ac	7.284±0.279ac	8.246±0.045ab	0.019
NH3 (ppm)	16.24±0.56b	21.84±0.90a	20.72±2.10ab	18.88±1.37ab	0.049
UIA (ppm)	0.140±0.005^{c}	0.118±0.004^{b}	0.070±0.003^{a}	0.627±0.004^{d}	0.005
TDS (g/L)	1.473±0.007c	1.558±0.005b	1.618±0.019a	1.532±0.004b	0.009
EC (dSm-1)	3.062±0.007a	3.100±0.001ab	3.240±0.036ab	2.868±0.108ac	0.003

Means within the same row with different superscript letters are significantly different at $P<0.05$.

Growth performance: The growth performance of Nile tilapia (Table 2) showed significant differences ($P<0.05$) in all measured parameters, except for fish width ($P>0.05$) of which group two (48 h) recorded the highest numerical value followed by group three (24 h) and group one (72 h). The lowest values of final body weight and thickness of fish were recorded in group one (72 h) followed by group two (48 h) while the highest values were recorded in group three (24 h). In addition, the fish in group two recorded the highest value of fish length followed by group three, but the values were not significantly different. The lowest value of fish length was recorded in group one (72 h), which was found to be significantly different ($P<0.05$) from those of groups two and three.

Table 2 Body weight, Length, width, thickness, condition factor and hemato-biochemical parameters of Nile tilapia (*Oreochromis niloticus*) under different duration of water level reduction

		Time spent in reducing water level from 1.5 m to 0.3 m			Reference range (Mauel et al., 2007)	*P*-value
		Group one (72 h)	Group two (48 h)	Group three (24 h)		
Body weight (g)		214.7±1.843^{b}	228.8±4.929^{ab}	243.3±8.024^{a}	------	0.0022
Length (cm)		22.88±0.1375^{b}	23.67±0.1960^{a}	23.51±0.2299^{a}	------	0.002
Condition factor	Min	1.740	1.720	1.780	------	-----
	Max	1.820	1.784	1.940	------	-----
	Mean	1.779±0.003^{b}	1.748±0.002^{c}	1.875± 0.007^{a}	------	0.0001
Width (cm)		9.208±0.161	9.533±0.079	9.508±0.154	------	0.1796
Thickness (cm)		2.725±0.051^{b}	2.946±0.045^{a}	2.967±0.079^{a}	------	0.0095
PCV (%)		30.93±0.79	30.90±0.61	30.13±0.51	22–45	0.61
Lymph/RBC ratio		50.32±0.92	50.16±1.53	48.67±1.25	-----	0.60
Total protein (g/dL)		4.42±0.16a	3.85±0.05b	3.67±0.05b	2.7–5.0	0.0001
Albumin (g/dL)		2.74±0.09a	2.34±0.02b	1.77±0.04c	0.8–1.9	0.0001
Globulin (g/dL)		1.692±0.09a	1.525±0.04a	1.904±0.02b	1.3–3.1	0.0001
A/G ratio		1.682±0.06b	1.572±0.04b	0.930±0.02a	0.4–0.8	0.0001
Cholesterol (mg/dL)		232.4±4.76a	205.6±11.78a	151.4±12.76b	88–228	0.0001
Triglycerides (mg/dL)		244.5±8.24a	230.9±8.56a	175.5±7.73b	-----	0.0001

Means within the same row with different superscript letters are significantly different at $P< 0.05$.

Length-weight relationship and condition factor: The Length-weight relationship (LWR) data and values of determination coefficients (R^2) are demonstrated in Figure 1. The values of regression coefficient obtained from the LWR were 0.2635, 0.2113 and 0.8885 for the fish in groups one, two and three, respectively. There was a significant correlation between length and weight among all experimental groups. The condition factor of Nile tilapia showed significant differences ($P<0.05$) among all experimental groups. The highest value of condition factor was found in group three followed by group one while the lowest value was recorded in group two (Table 2).

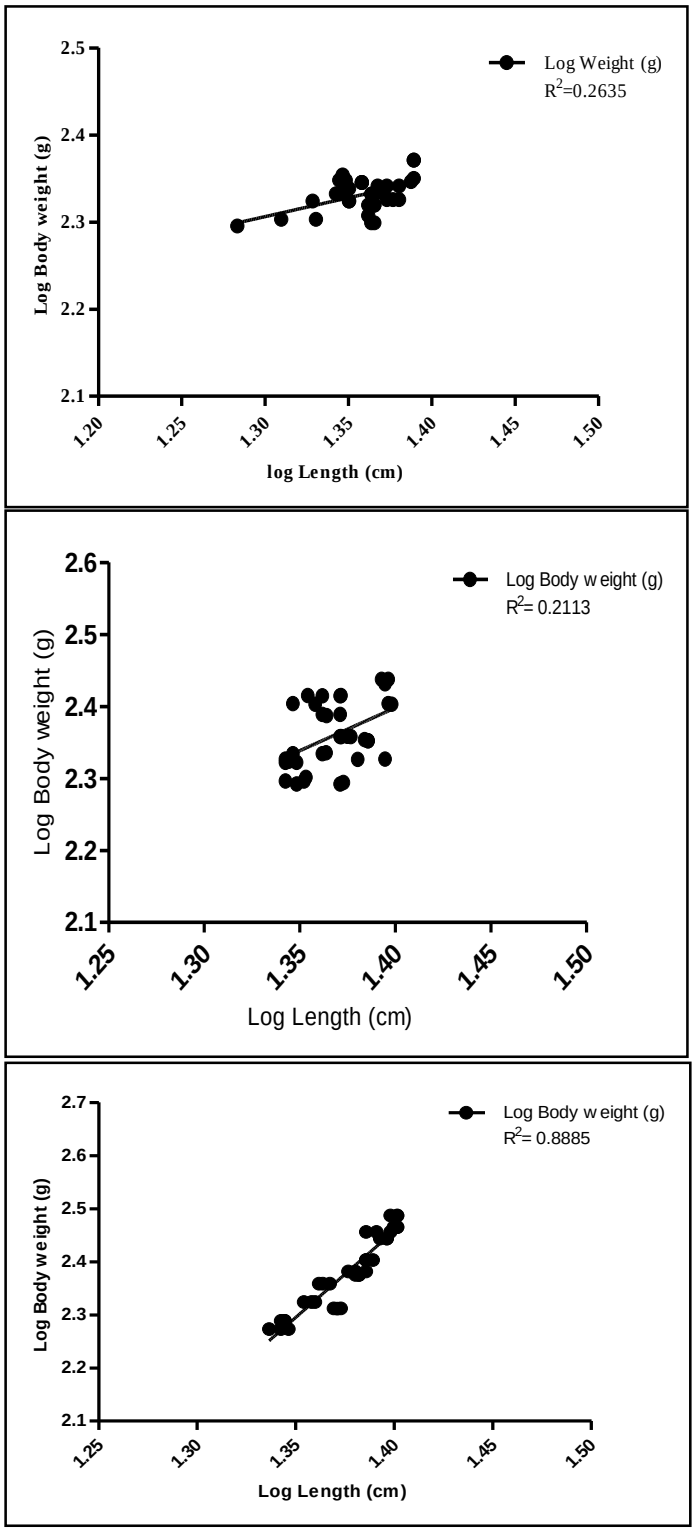

Figure 1 Length-weight relationship of Nile tilapia (*Oreochromis niloticus*) in (A) group one (72 h), (B) group two (48 h) and (C) group three (24 h)

Hemato-biochemical parameters: The results in Table 2 shows the hemato-biochemical parameters of Nile tilapia. There were no significant differences in PCV % and lymphocyte/RBC ratio in all experimental groups. The highest value was recorded in group one (72 h) followed by group two (48 h) while the lowest value

was recorded in group three (24 h). However, significant differences ($P<0.05$) were recorded in total protein, albumin, globulin, A/G ratio, cholesterol and triglycerides among all experimental groups. For total protein, group one (the highest value) showed a significant difference ($P<0.05$) from both groups two and three (the lowest value). The albumin values were significantly different ($P<0.05$) between all experimental groups and the highest value was recorded in group one followed by group two and group three. For globulin, there were no significant differences between groups one and two (the lowest value), but they were significantly different ($P<0.05$) from group three (the highest value). There were no significant differences between groups one (the highest values) and two, but they were significantly different ($P<0.05$) from group three (the lowest values), for A/G ratio, cholesterol and triglycerides.

Assessment of DNA changes using molecular analysis

RAPD marker analysis: In this study, 16 RAPD primers were used to analyze DNA changes of the two treatment groups and the control group which were caused by water level reduction in Nile tilapia. Only 11 primers were successful in generating reproducible and reliable bands as shown in Table 3 and only nine out of the eleven primers produced polymorphic bands and generated different percentages of polymorphism which ranged from 20.0% (OPA-04 and OPB-14) to 91.67% (OPB-07) as presented in Table 3 and Figure 2. The number of amplification products by each of the used primers varied from one (OPA-02) to 12 (OPB-05 and OPB-07). A total of 67 fragments were generated by the 11 RAPD primers across the three experimental groups with an average of 6.09 per primer and the fragment sizes ranged from 168 to 1505 bp. Of these bands, 31 (46.27%) were polymorphic, of which 13 (19.4%) were positive unique markers.

Table 3 Primer name, primer sequence, marker size, percentage of polymorphism, polymorphic information content and resolving power detected by 11 RAPD markers in Nile tilapia (*Oreochromis niloticus*) under different duration of water level reduction

Primer name	Primer sequence (5´→ 3´)	Molecular size range (bp) of amplified fragments	Monomorphic bands	Polymorphic bands				Total number of bands	Polymorphism %	Polymorphic information content (PIC)	Resolving power (RP)
				Polymorphic without unique…	Positive unique markers						
					Number	Size (bp)	treatment				
OPA-01	CAGGCCCTTC	319-1095	5	0	0	-	-	5	0.00	0.00	10.00
OPA-02	TGCCGAGCTG	587	1	0	0	-	-	1	0.00	0.00	2.00
OPA-03	AGTCAGCCAC	719-1349	3	0	1	1349	24 h	4	25.0	0.11	6.67
OPA-04	AATCGGGCTG	469-907	4	1	0	-	-	5	20.0	0.09	9.33
OPA-08	GTGACGTAGG	392-1290	3	2	2	1021 & 1290	24 h	7	57.14	0.25	10.00
OPB-05	TGCGCCCTTC	247-1449	7	2	3	784 1307 & 1449	72 h 24 h	12	41.67	0.19	18.67
OPB-O7	GGTGACGCAG	175-1505	1	6	5	889 & 1157&1505 710 & 972	48 h 24 h	12	91.67	0.41	13.33
OPB-10	CTGCTGGGAC	224-705	2	2	2	390 & 489	24 h	6	66.67	0.30	8.00
OPB-11	GTAGACCCGT	188-1076	4	2	0	-	-	6	33.33	0.15	10.67
OPB-12	CCTTGACGCA	168-547	2	2	0	-	-	4	50.0	0.22	6.67
OPB-14	TCCGCTCTGG	225-742	4	1	0	-	-	5	20.0	0.09	9.33
Total			**36**	**18**	**13**			**67**	**46.27**	**1.81**	**104.7**
Average			**3.27**	**1.64**	**1.18**			**6.09**		**0.16**	**9.52**

The information on genetic profile of each treatment obtained using the 11 RAPD primers was used to assess the marker performance through evaluation of polymorphic information content (PIC) and resolving power (RP) parameters. The PIC values of each RAPD primer was determined by analyzing the mean of PIC values for all loci of each primer. The range of PIC for the 67 loci was 0.00-0.41, 0.16 on average (Table 3). RP is a parameter that indicates the discriminatory potential of the primers chosen. The average RP was 9.52 per RAPD primer (Table 3). The highest RP value was observed with the primer OPB-05 (18.67) and the lowest with the primer OPA-02 (2.0).

Cluster analysis and Principal Coordinate Analysis (PCoA) based on RAPD data: The results in Figure 3 shows the molecular distance (MD) and UPGMA clustering dendrogram based on RAPD analysis. The MD ranged from 0.189 to 0.208 for the three experimental groups. The lowest MD value (0.189) was reported between the 48 and 24 h groups as they were localized in the same cluster (Figure 3) while the highest MD value (0.208) was observed between the 48 and 72 h groups as they were separated into two different clusters. Also, these results indicated that the three experimental groups were divided into two groups with different degrees of MD. The first group

(A) comprised the 72 h group alone and the second group (B) comprised the two groups of 24 and 48 h. The mean value of MD recorded between all combinations of these two groups was 0.195 while the mean value of MD between groups A and B was 0.202 and between the 24 and 48 h groups was 0.189.

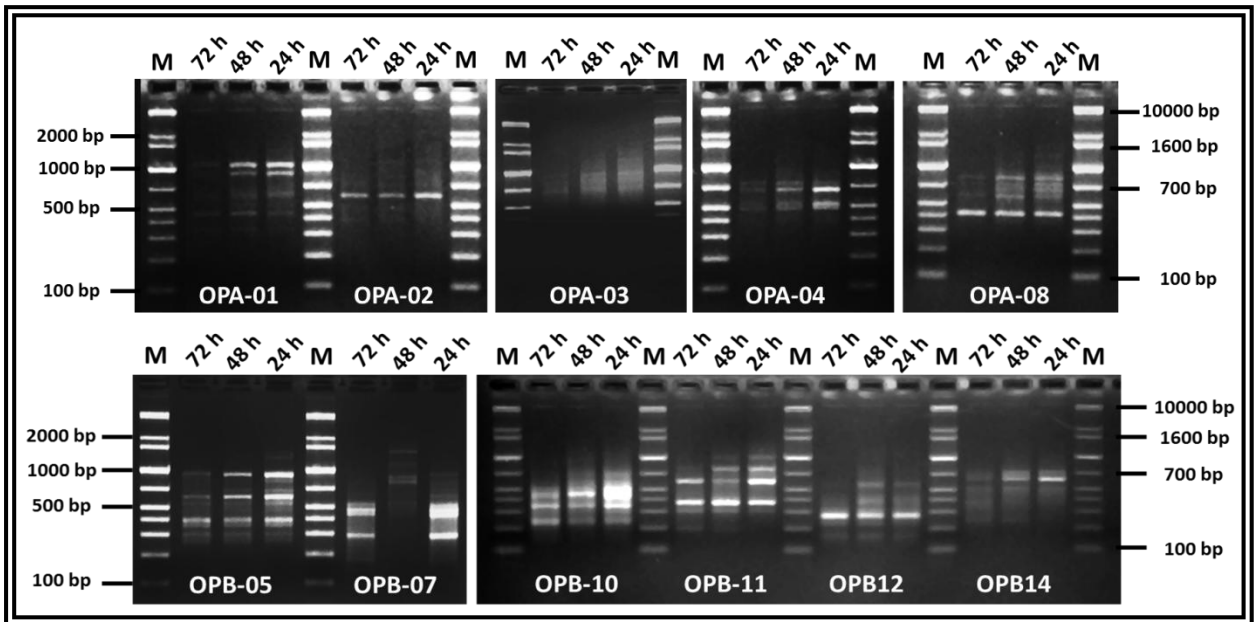

Figure 2 RAPD fingerprints of Nile tilapia (*Oreochromis niloticus*) under different duration of water level reduction generated by 11 RAPD primers. Lanes M: 1Kb plus DNA ladder; 72 h, 48 h and 24 h: time spent in water level reduction from 150 to 30 cm.

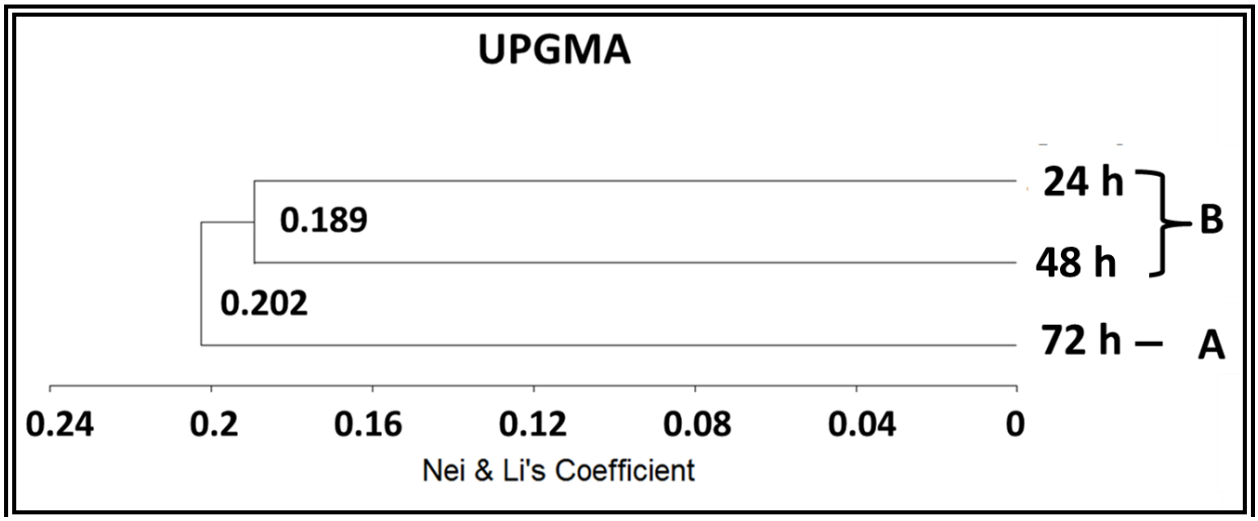

Figure 3 Dendrogram based on UPGMA cluster analysis showing molecular distance among the Nile tilapia (*Oreochromis niloticus*) under different duration of water level reduction using RAPD marker analysis

Accordingly, the Principal Coordinate Analysis (PCoA) for the three different duration of water level reduction during Nile tilapia harvest was performed based on the relative genetic distances from the obtained RAPD data (Figure 4). The PCo analysis determines the consistency of differentiation among different treatment defined by the cluster analysis (Adhikari et al., 2015). The cluster analysis indicated that the genetic distances between the three experimental groups were very high, indicating that the degree of similarity was low between the groups. Nevertheless, the PCo analysis managed to divide these groups into three groups, each group containing one of the studied types of treatment. The first eigenvector (PCo axis1) accounted for 53.28% of the RAPD data variation among the groups. This value indicates that the PCo analysis succeeded in the assessment of genetic diversity and description of heterogeneity within the studied types of treatment (Sonja et al., 2008).

DNA barcoding: It became clear that the RAPD molecular technique was efficient in terms of assessing genetic diversity among the studied Nile tilapia, suggesting the possibility of using results of this technique in signing genetic fingerprinting for the different types of treatment. Therefore, these genetic fingerprints for the Nile tilapia treated with three duration of water level reduction were performed by DNA barcoding diagram (Figure 5) based on 67 fragments (loci) obtained using the RAPD technique. This barcoding showed that the fragments per

treatment varied from 46 for the 72 h group (control) to 61 for the 24 h group with average number of 52.33. In addition, the three experimental groups showed 13 positive unique marker loci, nine of which were found in the 24 h group, one in the 72 h group and three in the 48 h group. All experimental groups differed from each other. These results indicate that DNA barcoding diagram is also a useful tool for molecular identification in Nile tilapia.

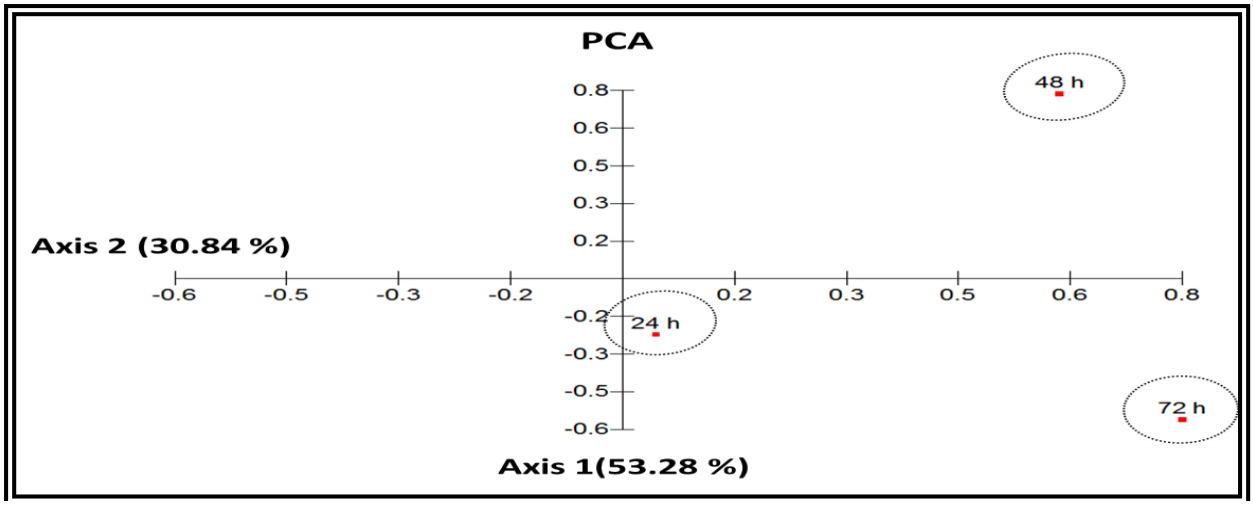

Figure 4 Principal Coordinate Analysis (PCoA) based on genetic fingerprint from RAPD analysis of the Nile tilapia (*Oreochromis niloticus*) under different duration of water level reduction. The first axis accounts for 53.28% of the genetic variation while the second axis accounts for 30.84%.

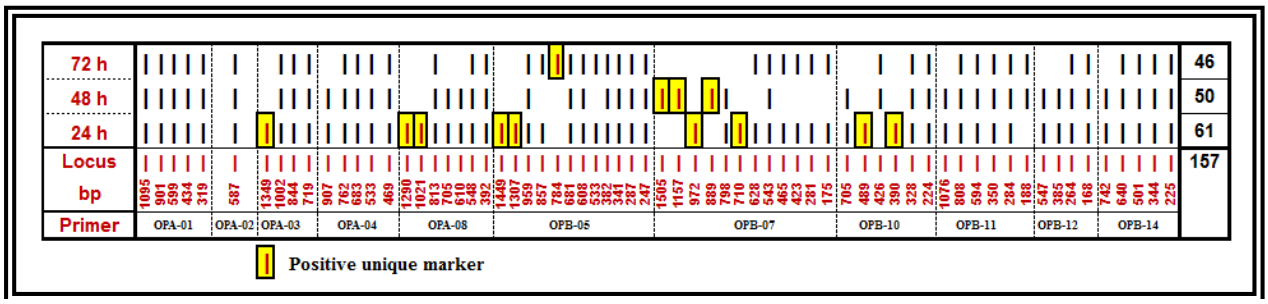

Figure 5 DNA barcoding presentation of RAPD fingerprint of Nile tilapia (*Oreochromis niloticus*) under different duration of water level reduction based on 67 fragments, 13 of which were positive unique marker loci according to Adhikari et al. (2015)

Genomic template stability percentage (GTS %): The polymorphic patterns obtained by RAPD-PCR were used to evaluate the percentage of genomic template stability (GTS %) in Nile tilapia from liver cells as shown in Table 4. DNA changes induced by the slow water level reduction from 72 h to 48 h and 24 h using RAPD markers as described by the absence or presence of new bands and the increase or decrease in band intensity for each treatment are presented in Table 4. Data showed that eleven random primers generated a total of 46 bands in the control group. Band number ranged from one band for primer OPA-02 to 10 bands for primer OPB-05. The eleven tested primers gave specific and stable results, with apparent changes in the number and intensity of amplified DNA bands. The two treatment groups, 48 h and 24 h, gave variable bands, compared to the control group, reflected by the changes in appearance of new bands, disappearance of normal bands and band intensity (increase/decrease).

The increase in band intensity was the major event arising in the patterns generated from Nile tilapia DNA under 24 h water level reduction treatment (increase in 25 bands intensity) while the 48 h water level reduction treatment showed increased intensity in 15 bands compared with the control group.

On the other hand, the two treatment groups increased the appearance of new bands compared with the control group. These changes in apparent new bands were increased as water level reduction time decreased. Also, the disappearance of normal bands was decreased with the decrease in time of water level reduction. The highest changes in band numbers (both appearance of new bands and disappearance of normal bands) was shown in the Nile tilapia treated with 24 h water level reduction (18 and 3 bands, respectively). The numbers of appeared and lost RAPD bands for the 48 h group were 12 and 8, respectively, compared with the control group. The duration of water level reduction from 72 h to 48 h and 24 h produced 56.5% and 54.3% reduction in genomic stability, respectively.

Table 4 Genomic template stability according to changes in DNA-RAPD fingerprint of Nile tilapia (*Oreochromis niloticus*) under different duration of water level reduction

Primer no.	Primer name	Total number of control (72 h) bands	48 h				24 h			
			a	b	C	d	a	b	c	d
1	**OPA-01**	5	0	0	2	0	0	0	2	0
2	**OPA-02**	1	0	0	0	0	0	0	1	0
3	**OPA-03**	3	0	0	3	0	1	0	3	0
4	**OPA-04**	4	1	0	1	0	1	0	3	0
5	**OPA-08**	3	2	0	2	0	4	0	2	0
6	**OPB-05**	10	0	3	3	0	2	1	5	0
7	**OPB-O7**	6	4	5	0	1	3	0	5	0
8	**OPB-10**	3	1	0	1	1	3	1	1	0
9	**OPB-11**	5	1	0	2	1	1	1	2	1
10	**OPB-12**	2	2	0	0	0	2	0	0	0
11	**OPB-14**	4	1	0	1	0	1	0	1	0
Total		**46**	**12**	**8**	**15**	**3**	**18**	**3**	**25**	**1**
	a+b+c+d		**38**				**47**			
	a+b		**20**				**21**			
	GTS (%)		**56.5 %**				**54.3 %**			

a: appearance of new bands, b: disappearance of normal bands, c: increase in band intensity, d:decrease in band intensity, a+b: polymorphic bands, GTS: genomic template stability

Discussion

Most fish farmers tend to reduce water level of fishpond before the harvesting process. It results in severe overcrowding and very high density that interfere with fish welfare. Therefore, this study was conducted to evaluate the effect of reduced water level during fish harvest on water parameters, growth performance, K-factor, hemato-biochemical parameters and molecular DNA changes by monitoring the RAPD profiles.

Water quality parameters such as temperature, pH, dissolved oxygen (DO) concentrations, ammonia (NH_3) and unionized ammonia (UIA) should be monitored due to the complexity of aquaculture system environment. According to the previous mentioned results, there were significant differences among the analyzed water parameters, except for water temperature and ammonia. For unionized ammonia (UIA), the highest level was found in group three and the lowest in group two. These values of UIA increase with the concomitant pH of the pond water in different groups (Abouelenien et al., 2015 and Mohamed et al., 2015). The increase in pH values with regard to accumulation of ammonia is due to decomposition of nitrogenous compounds by the microbial activities (Erkan and Ozden, 2008). In the current study, the highest UIA in group three could be due to both the rapid decrease in water level (24 h) and the sudden increase in fish activity, which resulted in sudden increase in nitrogenous compound concentration of the pond water (Randall and Tsui, 2002). This increase in nitrogenous compound concentration together with the high water temperature enhanced microbial degradation of these compounds with production of UIA (Farag, 2012). The estimated UIA in all groups of the present study was higher than the optimum concentration, 0.05 mg/l (El-Sherif and EL-Feky, 2008). The optimum growth of tilapia is obtained at concentrations greater than 3 mg/l (Ross, 2000) and it is highly tolerant to low DO concentration, 0.1 mg/l (Magid and Babiker, 1975). In this field study, the measured DO concentration in all groups was lower than that obtained by Rapatsa and Moyo (2013).

Time spent in reducing the water level of fishpond has been found to have a direct effect on the growth performance of Nile tilapia. Tilapia is sensitive fish because their growth can be seriously influenced by the physical and biological changes of the pond environment (Olurin and Aderibigbe, 2006). In the present study, the growth performance of Nile tilapia showed significant differences among all measured parameters (body weight, length and thickness), except for fish width. These differences may be attributed to the prolonged period of overcrowding (72 and 48 h) compared to the shorter period (24 h). The high density during harvest may interfere in a complex way with changes in water quality, especially DO and ammonia altering the growth of fish (Wall, 2001). Mallya et al. (2007) stated that lower DO was necessary for growth and survival of fish and higher NH_3 concentration might provide unfavorable condition for fish growth performance.

The physical and biological changes of the pond environment have a direct effect on fish biology and growth. The length-weight relationship (LWR) can be used as a quantitative parameter in studying fish biology and growth performance because it is used for estimating the weight in relation to a given length for each fish in a certain group and between different groups (Froese, 2006; Pauly, 1993). In this study, there was a positive correlation between weight and length of fish in all experimental groups and the fish showed positive allometric growth indicated by the increase in fish weight which was associated with the increase in fish length. The highest value of the regression coefficient obtained from the LWR was recorded in group three (24 h). It may be attributed to the improved growth conditions associated with good water quality parameters and shorter period of starvation and overcrowding (Bagenal and Tesch, 1978; Pauly, 1993; Mommsen, 1998).

The condition factor (K-factor) is an indicator used for assessment of fish well-being and a quantitative indicator of fish welfare (Blackwell et al., 2000). Higher values of K-factor indicate favorable aquaculture system environment conditions while lower values indicate hostile aquaculture system environment. In the current study, the fish subjected to

rapid reduction in water level (24 h) recorded the highest K-factor value while the fish that suffered from prolonged reduction in water level (48 and 72 h) recorded the lowest value. This may be due to the prolonged period of overcrowding and high stocking density (Blackwell et al., 2000). In general, the values of K-factor were above one in all fish in this experiment, indicating that they were reared under good condition. These results are in agreement with those of Ighwela et al. (2011).

Hematological and biochemical parameters are used as stress indices in Nile tilapia because their reference values are validated and they are very simple to determine. In the current work, the fish in groups one (72 h) and two (48 h), which were subjected to long period of stress and overcrowding, showed higher values of packed cell volume (PCV), lymphocyte/red blood cell (RBC) ratio, total protein (TP), albumin (A), A/G ratio, cholesterol and triglycerides, whereas they showed lower values of globulin (G) compared to group three (24 h). These differences may be due to the long period of overcrowding and unfavorable water parameters associated with the slow reduction in water level. In response to high stocking density, overcrowding and bad water parameter, there was an increase in oxygen demand that resulted in an increase in circulating RBCs and PCV value and a reduction in circulating lymphocytes (Reddy and Leatherland, 1998). As a secondary response of fish to overcrowding stresses, fish increases the mobilization cholesterol and triglycerides to blood streams which acts as extra energy sources required for the vigorous swimming during crowding and compensates the longer period of starvation (Vijayan et al., 1990).

The 11 RAPD markers used for the evaluation of the three types of treatment of water level reduction in Nile tilapia harvest revealed high levels of genetic polymorphism, detecting a total of 67 loci, 31 of which were polymorphic, 2.82 polymorphic loci per primer on average. Our study also evaluated the discriminatory power of RAPD primers for genetic relationship studies through PIC and RP. The highest PIC (0.41) was found for primer OPB-07, which is therefore recommended for genetic analysis. Resolving powers in our study were in the range of 2-18.67 (9.52 on average) per primer. Prevost and Wilkinson (1999) and Fernandez et al. (2002) detected a strong and linear relationship between the ability of a primer to distinguish genotype and resolving power values, suggesting that in our study primer OPB-05, with the highest resolving power (18.67), should be the most informative primer for distinguishing the treatment groups.

RAPDs were proved to be useful as genetic markers and fingerprinting (Ghanem et al., 2012; Ahmed and Rezk, 2015). It can be concluded from this study that RAPD markers are effective in detecting similarity among fish treated differently and are potential tool for studying the intra-species genetic similarity and the establishment of genetic relationships. Molecular markers are widely used to identify lines or strains, define stock diversity, monitor inbreeding, diagnose simply inherited traits and even to improve stocks (Rashed et al., 2009).

The dendrogram separated the three types of treatment of water level reduction in Nile tilapia harvest into two main clusters. The two treatment groups, 48 h and 24 h, were grouped together and separated from the control group (72 h). This study indicate that the RAPD molecular marker technique as well as the analyses based on this technique such as cluster analysis, Principal Coordinate Analysis and DNA barcoding are suitable tools for assessing genetic diversity and signing genetic fingerprinting, and also have potential for identifying specific markers for Nile tilapia. All of these lead to the consideration of this technique as an important tool for breeding and improving Nile tilapia. The effectiveness of RAPD markers in detecting polymorphism in different types of treatment of Nile tilapia, their applicability in population studies and establishment of genetic changes were demonstrated with this study. It is important to mention the fact that data obtained from RAPD marker assay can be extended to further dissect traits in a more refined way to gain exact knowledge on specific genes and molecular genetic pathways. Sequences of the obtained specific polymorphic bands are also of great interest to determine the genes detected by RAPD experiment.

In the present study, the RAPD analysis offered some treatment-specific markers. The numbers of these molecular markers varied from treatment to treatment. These DNA markers will be useful in fish breeding programs, depending on the use of genetic markers as marker-assisted selection to improve the fish performance (Rashed et al., 2009). The number of band products per RAPD primer varied from 1 to 12. A total of eleven RAPD primers generated 67 bands; these results are in agreement with the results obtained by Rashed et al. (2011), who used RAPD markers to detect genetic variations among some Tilapia species. They found that the values of similarity among four studied Tilapia species were high in Sokal and Sneath I, due to the use of shared present and absent fragments between each two estimated Tilapia species. Also, Hassan et al. (2014) used RAPD markers to detect genetic variations among some fish species.

The changes in DNA patterns of Nile tilapia induced by stress during the reduction in water level in different duration were detected based on the estimated genomic template stability percentage (GTS %) as presented in Table 4. The genomic template stability test, performed for the qualitative measurement of changes in RAPD fingerprint, showed considerable effects of the three duration of water level reduction. The changes observed among the RAPD fingerprint obtained from the control group (72 h) and the two treatment groups (48 h and 24 h) may be induced by direct and/or indirect interact with genomic DNA. These changes include loss of one or more nucleotides which can lead to alteration of DNA sequence. The change in the number of bands is associated with alteration in genetic material (Rocco et al., 2012; Noel and Rath, 2006; Liu et al., 2007). The reduction in water level induced a marked decrease in GTS %, which is usually considered one of the earliest molecular responses to DNA damage (Atienzar and Jha, 2006; Rocco et al., 2015). Alteration in the pattern of the RAPD-PCR bands may be related to events such

as oxidative DNA damage, DNA-protein cross-links, mutations and/or complex chromosomal rearrangements induced by genotoxins (Atienzar and Jha, 2006).

Finally, the results of molecular studies confirmed those results obtained by the hemato-biochemical parameters and condition factors. Also, this study confirmed DNA changes induced by stress as a result of water level reduction compared to those from previous studies and demonstrated the potential of RAPD-PCR analyses. In all cases, the RAPD patterns generated by the 48 h and 24 h groups were clearly different from the control group, and exhibited a distinct change with the decrease in the time of water reduction. The differences appeared as a result of using RAPD markers to refer to band intensity, loss of normal bands and appearance of new bands as compared with the control group.

In conclusion, rapid reduction in water level during Nile tilapia harvest could improve the health status of fish and reduce the harmful effects of crowding stress, leading to improvement in fish welfare.

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บทคัดย่อ

การประเมินความหลากหลายทางพันธุกรรม ความเสถียรทางจีโนมิก ปัจจัยสภาพตัวแปรทางชีวเคมี ในการตอบสนองต่อการลดลงของระดับฆ้าในการเก็บจับปลานิล (*Oreochromis niloticus*)

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินผลของการลดระดับน้ำในระหว่างการเก็บจับปลานิล (*Oreochromis niloticus*) ต่อสมรรถภาพการเจริญเติบโต ปัจจัยสภาพ และตัวแปรทางชีวเคมี ความหลากหลายทางพันธุกรรมและความเสถียรทางจีโนม โดยในระหว่างการเก็บจับปลานิลระดับน้ำจะลดลงจาก 150 เหลือ 30 ซม. ในช่วงเวลาที่ต่างกันดังนี้กลุ่มที่ 1 (ควบคุม) ใน 72 ชั่วโมง; กลุ่มที่สองใน 48 ชั่วโมง; และกลุ่มที่สามใน 24 ชั่วโมง ผลการศึกษาพบว่า ความยาวและน้ำหนักของปลาในกลุ่มทดลองทุกกลุ่มมีความสัมพันธ์กันอย่างมีนัยสำคัญทางสถิติ โดยปัจจัยสภาพมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) ในกลุ่มทดลองทุกกลุ่ม ตัวแปรทางชีวเคมี พบว่า PCV% และ lymphocyte/ RBC ไม่มีความแตกต่างอย่างมีนัยสำคัญ แต่พบค่าโปรตีนโปรตีนอัลบูมิน โกบูลิน คอเลสเตอรอลและไตรกลีเซอไรด์ มีความแตกต่างอย่างมีนัยสำคัญ ผลการประเมินความหลากหลายทางพันธุกรรม ด้วยไพรเมอร์ RAPD จำนวน 11 คู่ พบแถบดีเอ็นเอ 67 แถบซึ่งในจำนวนนี้มี 31 แถบ เป็น polymorphic คิดเป็น 46.27% ผลการวิเคราะห์แบบ Cluster analysis และ Principal Coordinate Analysis พบว่ามีความแตกต่างกันระหว่างกลุ่มควบคุมและกลุ่มทดลอง การศึกษาลายพิมพ์รหัสพันธุกรรม ด้วยวิธี DNA barcoding พบจำนวนแถบแตกต่างกันในกลุ่มทดลองโดยเฉลี่ย 52.3 แถบ ผลการวิเคราะห์ความเสถียรทางจีโนม พบเปอร์เซ็นต์ของความคงตัวของยีนจีโนม สำหรับกลุ่มควบคุม 48 และ 24 ชั่วโมง เท่ากับ 56.5 และ 54.3 ตามลำดับ โดยการศึกษาครั้งนี้แสดงให้เห็นว่าวิธี RAPD สามารถระบุเครื่องหมายสำหรับการลดระดับน้ำในการเก็บจับปลานิล และชี้ให้เห็นว่าการลดระดับน้ำอย่างรวดเร็ว (24 ชั่วโมง) อาจลดผลกระทบที่เกิดจากความเครียดและเพิ่มสวัสดิภาพระหว่างการเก็บจับปลานิล

คำสำคัญ: ปัจจัยสภาพ การเก็บรักษา ความหลากหลายทางพันธุกรรม ความเสถียรทางจีโนมิก ***Oreochromis niloticus***

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