International Journal of Agricultural Technology 2017 Vol. 13(7.1): 991-1003
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

# Seed Production of Nile Tilapia (*Oreochromis niloticus* L.) as Affected by the Breeders' Stress-Coping Style

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Adrian Deil C. Manliclic and Emmanuel M. Vera Cruz (2017). Seed Production of Nile Tilapia (*Oreochromis niloticus* L.) as Affected by the Breeders' Stress-Coping Style. International Journal of Agricultural Technology 13(7.1): 991-1003.

Physiological and behavioral responses to stress can form distinct profiles in a wide range of animals. These profiles are widely known as proactive and reactive profiles or stress-coping styles. This study determined the effect of stress-coping style on the reproductive performance of *Oreochromis niloticus*. The stress-coping style of the breeders was determined through the changes of their eye color pattern (ECP) after the two-day isolation period. The proactive breeders (PB) were those individuals that manifested shorter period of adjustment in the new environment as indicated by the lower ECP values (0 to 3) at the end of the isolation period; whereas, the reactive breeders (RB) were those individuals that exhibited longer period of adjustment in the new environment as manifested by the higher ECP values (5 to 8) at the end of the isolation period. Different combination of breeders were tested: T1 (PB♂ PB♀); T2 (RB♂ RB♀); T3 (PB♂ RB♀); and T4 (RB♂ PB♀). Breeding was carried-out using twelve (1 x 2 x 1m) hapas installed in a pond. The sex ratio was one male: three females with stocking density of $8/m^2$. Collection of egg and fry was done after fourteen days of breeding. Results showed that the sperm quality of proactive male was significantly different ($P$<0.05) to the sperm quality of reactive male. In terms of sperm motility, PB had significantly higher ($P$<0.05) motility of 9.2±0.577 than the RB (7.0±0.854). On the other hand, in terms of sperm density, PB also had significantly higher ($P$<0.05) sperm density (2.025x109±2.481x108) than the RB (9.688x108 ±2.11x108). On spawning success and seed production per female that spawned, the four treatments showed homogeinity. In total seed production, however, it was found that T1 (PB♂ PB♀) had significantly higher ($P$<0.05) total seed production of 1442.33±80.41 than the other treatments. The total seed production of Treatments 2 (RB♂ RB), 3 (PB♂ RB♀) and 4 (RB♂ PB♀) were found comparable to each other with only 658.00±144.78, 900.00±20.00 and 597.00±170.66 seeds, respectively. Results of the study demonstrated that the stress-coping style as determined by ECP changes during isolation, can influence the reproductive performance of *O. niloticus*. These differences of proactive breeders and reactive breeders in terms of sperm quality and seed production, demonstrated that the determination of stress-coping style by observing the changes in ECP during the two-day isolation was an effective tool in the determination of the breeding quality of the fish.

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**Keywords:** Stress coping style, proactive breeders, reactive breeders, Nile tilapia, breeding, seed production

## Introduction

Fish repeatedly experience stressful situations under experimental and aquaculture conditions, even in their natural habitat (Koakoski *et al.*, 2013). In response to these stressors, fish and other vertebrates experience a group of behavioral and physiological changes in order to maintain or re-establish homeostasis (Crino, 2010; Barton *et al.* 2002). A consistent finding across species is that whenever environmental stressors are too demanding and the individual cannot cope, its health is in danger. For this reason, it is important to understand the mechanisms and factors underlying the individual's capacity to cope with environmental challenges (Koolhaas *et al.*, 1999).

The stress response and its associated behaviors vary greatly between individuals in many taxa and these behaviors coupled with stress responsiveness have been termed as stress-coping style (Crino, 2010). A coping style is defined in terms of the ability of individuals to cope with stress and new information, and closely linked to the growth and welfare of animals (Koolhaas *et al.*, 1999). Based on behavioral studies in several vertebrate species, two coping styles can be distinguished - the proactive and reactive strategies (Campbell *et al.*, 2003; Koolhaas *et al.*, 1999; Koolhaas, 2008). In general, proactive and reactive strategies are associated with a set of physiological and behavioral characteristics. Proactive animals behave more aggressively, are more active, and readily form routines compared to reactive animals when exposed to stress (Backström *et al.*, 2014). Similarly, proactive animals display a slight cortisol increase in response to stress whereas reactive animals have large cortisol increase and respond passively (e.g. low-level aggression and immobility) to remedy the stressful condition (Barreto and Volpato, 2011).

Several studies investigated the various stress-coping strategies to further improve fish's welfare (Clement *et al.*, 2005; Barreto and Volpato, 2011; Brelin, 2008; Meager *et al.*, 2012). For example, in rainbow trout (*Oncorhynchus mykiss*), it has been found that stress-coping style (i.e. feeding response) predicts aggression and social dominance (Øverli *et al.*, 2004). These findings support the view that distinct behavioral–physiological stress coping styles are present in teleost fish, and coping characteristics influence both social rank and levels of aggression (Øverli *et al.*, 2004; Øverli *et al.*, 2006).

Under aquaculture conditions, physiological stress is one of the primary contributing factors of fish disease and mortality (Rottman *et al.*, 1992). It can also affect fish reproduction in a variety of ways depending on the nature and severity of the stressor (Schreck, 2010). In fact, evidence suggests that stress can inhibit the reproductive function of fish (Pankhurst, 2001). Thus, it is important to identify individuals with better stress-coping abilities before breeding since stress can negatively affect fish health and welfare, and therefore production. Further, knowledge on individual coping style may also be of great importance to improve the welfare and performance of the fish not only during breeding, but also during grow-out operation.

Nile tilapia (*O. niloticus*) has become an excellent candidate for aquaculture, especially in tropical and subtropical regions (Fitzsimmons, 2008). Any technology that can further improve its production can be of great importance since this can contribute in achieving food security and can be a source of additional income for farmers especially in developing countries. In recent a study, eye color pattern (ECP) was proven reliable, easy and inexpensive indicator of stress-coping style in *O. niloticus*. (Vera Cruz and Tauli, 2015). This study used ECP to identify stress-coping style in *O. niloticus* and investigated the potential of this approach in the selection of broodstock with great reproductive advantage. The results of the study can contribute to easier identification of potential broodstocks and can reduce the problem of laborious and time-consuming period of identification of proactive and reactive breeders prior to breeding.

**Objectives**: The main objective of the study was to investigate the effects of stress-coping style (i.e. ECP) on seed production of *O. niloticus*. The study specifically aimed to determine:

1. the best breeding combination of proactive and/or reactive breeders that showed the highest production of seed after the breeding periods;
2. the sperm quality of a male breeder such as motility and density in relation to its stress-coping style as indicated by its ECP after isolation; and
3. the spawning success, total seed production, seed production per female that spawned in each breeding combination.

## Materials and methods

### *Experimental fish*

Male and female Freshwater Aquaculture Center Selected Tilapia (FaST also known as IDRC strain) breeders (387.545±6.75 g) were used in the study. They were acquired from Freshwater Aquaculture Center, Central Luzon State University, Science City of Muñoz, Nueva Ecija (15º44’8.9”N, 120º56’49.2”E). The breeders were maintained in 2 x 1 x 1 m hapas (with mesh size 17) for two weeks, at 6-10 fish·m$^{-2}$ before and after isolation. They were fed daily with commercial diet (30% crude protein) at 3% of their body weight.

### *Isolation and Identification of Social Grouping*

Rectangular aquaria with dimensions of 30 x 15 x 30 cm were used as isolation chambers. During isolation, the sides of the aquarium were covered with white plastic boards to avoid the isolated fish from seeing other fish in the adjacent isolation chamber. A total of 279 breeders, consisting of 115 males and 164 females were isolated at random for two days in the aquaria. These breeders were fed once a day, at 1% of their body weight. Aeration was provided in each unit for the continuous supply of dissolved oxygen and water exchange was done whenever necessary for the maintenance of water quality. The ECP of each fish was monitored daily during the 2-day isolation period following the procedure of Volpato et al., 2003. In determining the ECP values, the circular area of the eye was divided into eight equal parts using four imaginary lines. The ECP values were marked by fractional changes of the color of the iris and sclera around the pupil which was transformed into scores ranging from 0 (no darkening) to 8 (total darkening).

After the isolation period, the breeders were separated into two social groups, namely: proactive breeders (PB) and reactive breeders (RB), in relation to their final ECP during the two-day isolation period (adapted and modified from Vera Cruz and Tauli, 2015). The PB were those individuals that manifested shorter period of adjustment in the new environment as indicated by the lower ECP values (0 to 3) at the end of the isolation period; whereas, the RB were those individuals that exhibited longer period of adjustment in the new environment as manifested by the higher ECP values (5 to 8) at the end of the isolation period. Males and females in each social group had mean body weights of 401.983±8.69 g (PB males), 397.03±9.06 (RB males), 394.96±15.28 (PB females) and 372.16±8.30 (RB females).

### *Experimental procedure*

A total of 96 breeders (24 males, 72 females) were used for breeding. They were divided and paired based on the treatments and were stocked randomly in twelve 2 x 1 x 1 m fine mesh net enclosures installed in a 1000 $m^2$ earthen pond. Different combination of breeders were tested: T1 (PB♂ PB♀); T2 (RB♂ RB♀); T3 (PB♂ RB♀); and T4 (RB♂ PB♀). Each treatment was replicated thrice and the study was laid-out in Completely Randomized Design (CRD). Each group of breeders had eight fish consisting of two males and six females. Breeders were fed with commercial feed at 2% of their body weight. Dissolved oxygen and water temperature were monitored every other day between 0800-0900 h and between 1400-1500 h using a DO meter.

After 14 days of breeding, collection of eggs and fry was done. Eggs were artificially incubated in downwelling incubation system receiving continuous water supply since this can increase hatching and survival rates (Watson and Chapman, 1996). Spawning success was determined following the equation: Spawning rate (%) = (number of females that spawned/ number of stocked females) x 100. Total seed production was determined by adding the total number of fry collected in each hapa and the total number of fry from hatched eggs. Seed production per female that spawned was also determined by dividing total seed production by the number of females that spawned. In the case of sperm quality analyses, the quality of sperm of male PB and RB was evaluated through sperm motility and sperm density following the standard procedure of Bureau of Fisheries and Aquatic Resources-National Freshwater Fisheries Technology Center (BFAR–NFFTC). The sperm motility in all good samples was scored on a subjective rating scale of 0 to 10. A rating of 10 denotes that 100% of the spermatozoa under observation are motile and moving actively, while zero (0) rating indicates that no sperms are moving after activation. On the other hand, the sperm density was estimated using a Neubauer slide counter (Haemacytometer, 0.1 mm. 1/4 mm2, Weber Scientific, England). A dilution was done by taking 10 µl of sperm sample added in 490 µl of diluent (Modified Fish Ringer, MFR), making a total of 500 µl sperm suspension in microcap vial. From the first dilution, 10 µl was drawn and added in the 2nd tube with 90 µl MFR making a dilution of 1/10. A small sample from the 2nd dilution was dropped on the Neubauer slide for counting. The slide was left for approximately 10 minutes to allow the sperm to settle into one plane. Subsequently, sperms were counted from the five random large squares of the Neubauer slide using a microscope. Sperm density was determined following the formula: Sperm Density = no. of sperms counted in five squares x 500 x 50,000.

***Statistical analyses***

Treatments data were subjected to analysis of variance (ANOVA) and the least significant difference (LSD) test was used to determine differences among individual treatment means. In the case of sperm quality analyses, t-test was used to compare treatment means. For data which are expressed in percentage, they were transformed first to their arcsine values before subjecting them to data analysis. Statistical analyses were done using SPSS 17 for windows.

## Results

### *Determination of Stress-Coping Style*

The trends of mean ECP changes during the two-day isolation period of male and female breeders of the two social groups were shown in Figure 1. During the first day of isolation, the ECP values ranged from 0 to 8 with the mean of 6.23±0.11. Most breeders had ECP values greater than 4 (n=222), four with values of 4 and 53 with values less than 4. At the end of the isolation period, 92 individuals, consisting of 37 males and 55 females, had been identified as proactive breeders as indicated by their lower ECP values (0 to 3) (Vera Cruz and Tauli, 2015). Thirteen of these breeders had ECP values equal to the initial value on Day 1, eleven with increased ECP values and 68 with decreased ECP values compared to the initial value. On the other hand, 187 individuals, consisting of 78 males and 109 females, had been identified as reactive breeders as indicated by their higher ECP (5 to 8) (Vera Cruz and Tauli, 2015). Fifty-two of these breeders had ECP values equal to the initial value on Day 1, thirty-two with increased ECP values and 103 with decreased ECP values compared to the initial value.

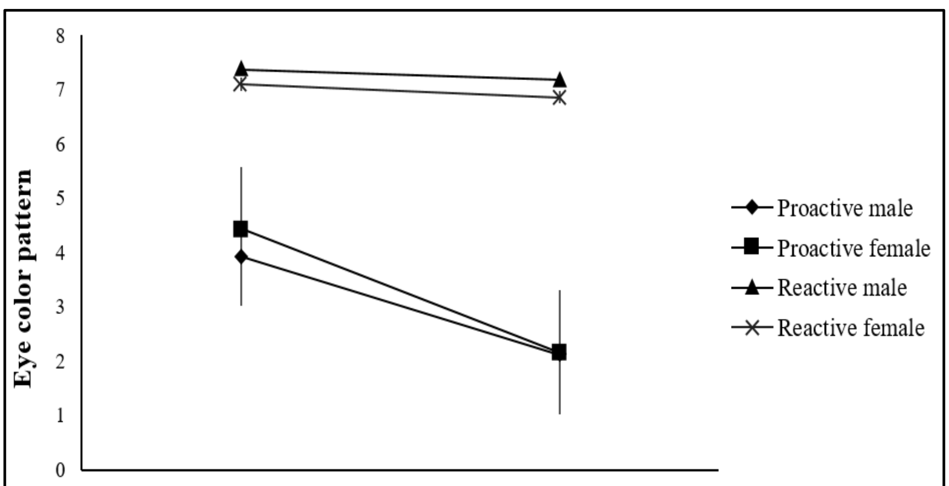


**Figure** 1. Trend of mean ECP values during the isolation period of male and female breeders of the two social groups.

### *Sperm quality analyses*

The mean motility scores and sperm densities of male PB and RB are presented in Table 1. In motility scoring, results showed that the sperm motility scores of PB (9.2±0.577) were higher than the motility scores of RB (7.0±0.854). In terms of sperm density, it was found that the sperm densities of PB ($2.03x10^9$ ±$2.48x10^8$) were also higher than the sperm densities of RB ($9.69x10^8$ ±$2.11x10^8$). Data analysis further revealed that motility scores and sperm density of male PB were significantly higher ($P$<0.05) than the male RB.

**Table 1**. Motility scores and sperm density (sperm/ml) of male PB and RB.

| Male | Mean | |
|---|---|---|
| | Motility Score | Sperm Density (sperm/ml) |
| **PB** | 9.2±0.577[a] | 2.03x109 ± $2.48x10^8$ (a) |
| **RB** | 7.0±0.854[b] | 9.69x108 ± $2.11x10^8$ (b) |

Note: Means with different superscript letters within the column are significantly different at 5% level of significance.

### *Seed production evaluation*

The spawning rate, seed production per female that spawned, and total seed production during the study are presented in Table 2. On spawning rate, the highest was found in T1 (PB♂ PB♀) with 50.00%, followed by T3 (PB♂ RB♀) with 41.67%, and T2 (RB♂ RB♀) with 33.33%, while the lowest was found in T4 (RB♂ PB♀) with only 27.78% spawning success. However, data analysis revealed no significant differences on mean spawning success among the treatments. On seed production per female that spawned, T1 (PB♂ PB♀) attained the highest with 511.36±79.58 fry. This was followed by T4 (RB♂ PB♀) with 409.50±153.08 fry, and T3 (PB♂ RB♀) with 373.33±66.67 fry, while the lowest was found in T2 (RB♂ RB♀) with only 366.44±89.19 fry. Similarly, statistical analysis revealed no significant difference on the means of seed production per female that spawned among the treatments. On total seed production, T1 (PB♂ PB♀) obtained the highest with 1442.33±80.41 fry. This was followed by T3 (PB♂ RB♀), T2 (RB♂ RB♀) and T4 (RB♂ PB♀) with 900.00±20.00, 658.00±144.78, and 597.00±170.66 total seed produced, respectively. Statistical analysis revealed that the total seed production of T1 (PB♂ PB♀) was significantly higher ($P$<0.05) than the rest of the treatments. Treatment 2, 3 and 4 were found comparable to each other.

**Table 2**. Spawning rate (%), seed production per female (number of fry), and total seed production (number of fry) of the treatments during the study.

| Treatment | Mean | | |
|---|---|---|---|
| | Spawning Rate (%) | Seed Production per Female (number of fry) | Total Seed Production (number of fry) |
| **1 (PB♂ PB♀)** | 50.00 ±9.62[a] | 511.36 ±79.58[a] | 1442.33 ±80.41[a] |
| **2 (RB♂ RB♀)** | 33.33 ±9.62[a] | 366.44 ±89.19[a] | 658.00 ±144.78[b] |
| **3 (PB♂ RB♀)** | 41.67 ±8.33[a] | 373.33 ±66.67[a] | 900.00 ±20.00[b] |
| **4 (RB♂ PB♀)** | 27.78 ±5.55[a] | 409.50 ±153.08[a] | 597.00 ±170.66[b] |

Note: Means with different superscript letters within the column are significantly different at 5% level of significance.

The dissolved oxygen (DO) concentration during the breeding period ranged from 2.84 to 3.69 mg $L^{-1}$ in the morning and 6.42 to 7.24 mg $L^{-1}$ in the afternoon. On water temperature, the value ranged from 28.83 to 31.37 ˚C in the morning and 31.37 to 34.73 ˚C in the afternoon. In general, the recorded temperature and DO values during the study are suitable for normal growth and reproduction of the fish. (Popma and Masser, 1999; El-Sayed, 2006).

## Discussion

During the isolation period, it was observed that most of breeders had ECP values greater than four. Since these fish were introduced to a new and confined environment, this eye darkening was clearly a stress response. This observation is similar to the results of the study of Freitas *et al.* (2014) where *O. niloticus* induced eye darkening after being exposed to novel and confined environment. Moreover, this eye darkening was previously observed on the subordinate fish exposed to social stress (Volpato *et al.*, 2003; Suter and Huntingford, 2002; Miyai *et al.*, 2011). The subordinate fish displayed darker eyes as it was being stressed more than the dominant ones during social interaction. Further, the breeders with ECPs greater than four (reactive) were observed to have darker body coloration. This change of the body color was presumably the physiological effect of stress. In the study conducted in Atlantic salmon (*Salmo salar*), the overall body coloration tended to darken in the fish that were losing territorial encounters (O'Connor *et al.*, 1999). The darkening was rapid and usually occurred during a period of sustained attacks by the opponent; thus the change of body color may not only be a signal of subordination but may also be caused by stress generated from the opponent's attacks. Conversely, most of the breeders identified as proactive as manifested by their lower ECPs (0 to 3) displayed light body coloration. This implies that there is a clear distinction that separates individuals when coping with stress and different physiological changes (e.g. darker body coloration, etc.) may occur within the same species as individuals have different capacities when coping in stressful conditions.

Sperm motility is one of the most widely used quantification of sperm quality (Alavi *et al.*, 2004; Alavi *et al.*, 2006; Abascal *et al.*, 2007; Rurangwa *et al.*, 2004). In the present study, male PB (9.2 ±0.577) had significantly higher ($P$<0.05) sperm motility scores than the male RB (7.0 ±0.854). It was evident the sperm of PB were very active and fast swimming after activation. This high motility observed on the proactive males is a prerequisite for fertilization and correlates strongly with fertilization success (Rurangwa *et al.*, 2004). On the other hand, in general, not all the sperm of male RB was actively swimming after activation. This decrease in sperm motility found on some of samples of RB could actually reduce fertility. Low sperm motility has a critical influence on successful fertilization, since the spermatozoa must find and enter the egg during external fertilization.

The concentration of sperm in the seminal fluid has been traditionally used for the assessment of sperm quality in fish (Rurangwa *et al.*, 2004). In the study, the sperm density of male PB ($2.03 \times 10^9 \pm 2.48 \times 10^8$) were also significantly higher ($P$<0.05) than the sperm densities of RB ($9.69 \times 10^8 \pm 2.11 \times 10^8$). The lower sperm densities and motility found on some RB samples in this study could be linked to stress since reactive individuals are known to be more negatively affected by stress than proactive individuals (Barreto and Volpato, 2011). According to Koolhaas (2008), proactive individuals tend to do something to prevent or manipulate a stressor whereas reactive individuals tend to passively accept it or react to it. As stress can affect reproduction (Schreck, 2010), that behavior of proactive individuals is vital, particularly during breeding. In addition, the quality of fish gametes depends on the appropriate hormonal environment during development which can be disturbed by stress (Kime and Nash, 1999). In male fish, stress can induce changes in plasma osmolarity which in turn can affect sperm quality (Rurangwa *et al.*, 2004). In rainbow trout (*Oncorhynchus mykiss*), the repeated acute stress during reproductive development prior to spawning significantly resulted in a delay in ovulation and decreased sperm density (Campbell *et al.*, 1992). In the case of striped bass, males produced milt with non-motile sperm under confinement conditions in freshwater (Berlinsky *et al.*, 1997). The non-motility observed in the sperm of male striped bass might be caused by handling stress since the broodstock were captured from the wild during the spawning season and were moved to captivity. Nevertheless, stress has a clear negative effect on the sperm quality of fish particularly during reproductive development. And under aquaculture conditions, stress can come from several factors such as stocking density, malnutrition, handling, selection, transportation and other environmental factors (Francis-Floyd, 1990; Rottman *et al.*, 1992). Since proactive individuals can quickly cope with stress, their reproductive fitness was not greatly affected as compared to the reactive individuals which was manifested JMBN in the present study.

On spawing rate, no significant difference among the treatments were observed. Treatment 1, however, generated the highest spawning success with 50.00 $\pm$ 9.62 % after the breeding period. On the other hand, the low spawning success found on T4 (RB♂ PB♀) (27.78%) was not expected since this treatment was composed of proactive females. Treatment 2, which was composed of reactive females, obtained the second lowest spawning success with only 33.33%. The posible reason behind this lower spawning success found in T4 (RB♂ PB♀) and T2 (RB♂ RB♀) could be the short breeding period (14 days) and some individuals may require longer period in order to spawn. Nevertheless, no significant differences were found among the four treatments.

Meanwhile, it is worthy to mention that all the females in one of the replicate of T3 (PB♂ RB♀) did not spawn at all. According to Schreck (2010), stress can affect reproduction in various ways, depending on when in the life cycle it is experienced and the severity and duration of the stressor. In tilapia, delayed ovulation occurred when the fish were exposed with stressors (disturbance and agitation) during early ovarian development, while those stressed during late vitellogenesis spawned immediately (Contreras-Sanchez, unpublished data as cited by Schreck *et al.,* 2001). This was most likely what happened in some of the female breeders in T3 (PB♂ RB♀). The fish experienced stress that completely delayed their ovulation. Additionally, T3 (PB♂ RB♀) was composed of reactive females, and reactive individuals are known to have lesser ability to cope with stress.

On total seed production, results showed that total seed production of T1 (PB♂ PB♀) (1442.33 ± 80.41) was significantly higher ($P$<0.05) than the total seed production of the other treatments. These findings revealed that breeding individuals with the same stress-coping style of proactive could result to significant increase in total seed production. In the analysis of sperm quality, proactive individuals showed promising results as compared to the reactive individuals. Significantly higher sperm motility and density were found in proactive males than the reactive males. This difference in sperm quality presumably contributed to the greater seed production found in T1 (PB♂ PB♀) since the good sperm quality (i.e. high motility) correlates strongly with fertilization success (Rurangwa *et al.*, 2004). Additionally, T3 (PB♂ RB♀) which was also composed of proactive males, obtained greater seed production than the treatments with reactive males [T2 (RB♂ RB♀) and T4 (RB♂ PB♀)]. Moreover, it was observed that the total seed production was affected by the number of female breeders that spawned. In T1 (PB♂ PB♀), the spawning success was 50%. This means that an average of three females have spawned in this group while in Treatments 2 (RB♂ RB♀), 3 (PB♂ RB♀) and 4 (RB♂ PB♀), lower number of females have spawned. In addition, mean seed production per female that spawned was also highest in T1 (PB♂ PB♀) (511.36±79.58 fry) as compared with the other treatments. This higher seed production per female that spawned found in T1 (PB♂ PB♀) agrees with the results of Abella (unpublished), where the highest number of egg produced per female was obtained by the breeders with low stress response (proactive individuals).

In *O. niloticus*, eye color can indicate fish's relative position in the social hierarchy. In the study of Volpato *et al.* (2003), the subordinate's eye had more 80% of its area darkened, while in the dominant's eye had 25% of its area darkened during social interaction. In the present study, the subordinate's eye color reflected the breeders identified as reactive while the dominant's eye color reflected the breeders identified as proactive. In previous studies, it was found out that social rank had profound impacts on the individual's reproductive behavior and physiology. According to Maruska (2014), dominance is associated with increased reproductive opportunities and improved fitness compared to subordinate individuals. This further explains why T1 (PB♂ PB♀) produced significantly greater number of seeds than the other group since this particular group of breeders were all considered as dominants and had greater reproductive advantage than the other groups.

## Acknowledgement

The authors would like thank the Philippine Department of Science and Technology – Accelerated Science and Technology Human Resource Development Program for funding this research.

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(Received 23 October 2017, accepted 25 November 2017)