Morpho-behavioral and hematological responses of vaccinated proactive and reactive Nile tilapia (*Oreochromis niloticus*) subjected to handling stress

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Abstract. The stress-coping style of fish can be categorized through the evaluation of morphological and behavioral responses. Proactive individuals respond to stress with bold behavior and are described to be aggressive, while reactive individuals are characterized as shy and subordinate. This study was conducted to evaluate the morphological, behavioral and hematological parameters of vaccinated Nile tilapia and determine which stress-coping style and which sex of the fish will provide better responses when subjected to handling stress. Proactive and reactive fish were determined morphologically using eye color pattern (ECP). Experimental fish were vaccinated using formalin-killed *Aeromonas hydrophila* and handling stress was administered. Results in morpho-behavioral responses revealed that proactive individuals had lower ECP values than reactive individuals. Eye darkening signifying a higher degree of stress was significantly higher in reactive individuals than in proactive individuals. In terms of ventilation rate, comparable results were recorded through time. Hematological responses suggest that the immunological function of white blood cells and lymphocytes was stimulated in response to handling stress, without neglecting the effect of vaccination. Red blood cell components were affected by the increasing level of white blood cells and tended to decrease as time progressed. The study concludes that proactive Nile tilapia performed better as a stress-coping style in response to handling stress. Screening of Nile tilapia by determining the proactive individuals is a potential method to reduce or eliminate the influence of handling stress in the culture process.
Key Words: *Oreochromis niloticus*, proactive, reactive, stress response.

Introduction. Nile tilapia (*Oreochromis niloticus*) have become an excellent choice for aquaculture (El-Sayed 2006). Nile tilapia is an important species for freshwater aquaculture and improvement of its culture techniques and disease resistance is a major challenge that fish farmers face (Abdel-Tawwab et al 2008). Investigations of the effects of various aquaculture procedures on the stress responses of fish have proven very valuable for developing culture techniques that minimize stress and, thereby, enhance production (Pickering 1981; Pickering 1982; Adams 1990; Barton & Iwama 1991).

Handling is a common and inescapable part of the farmed fish life, whether it occurs during cultural operations or during experimental work in laboratories (Barton & Iwama 1991). Acute handling stress is one of the causative occurrences that can mainly be associated to changes in the physiological function of fish (Pickering 1982).

The hematological parameters are important tools that reveal the state of the health of fish (Blaxhall 1972; Rehulka 2002; Martins et al 2004). They can be used as health indicators in aquatic medicine following different stress conditions (Thrall et al 2004).

Hematological responses can be useful for the diagnosis of diseases and for monitoring the physiological status of fish (Stoskopf 1993).

Behavioral profiles, such as proactive and reactive coping styles, were initially characterized in mammals (Koolhaas et al 1999), but they were later extended to other vertebrates, including fish and birds (Schjolden et al 2005). Based on these behavioral traits, individuals within a population often show a bimodal distribution along with a shy-bold continuum, or, in stress-coping styles, they are termed as proactive and reactive (Koolhaas et al 1999). So-called "bold" or "proactive" individuals are characterized as more aggressive when confronted with social challenges. They are more active in their attempt to reduce the effect of aversive stimuli and more willing to investigate unfamiliar objects compared to "shy" or "reactive" individuals. They develop routines more easily as a way to deal with different demands, but respond to the same challenge with immobility and lack of initiative (Koolhaas et al 1999; Bohus et al 1987; Koolhaas et al 2001).

The objective of the study was to evaluate the morpho-behavioral and hematological responses of vaccinated proactive and reactive, as well as male and female Nile tilapia when subjected to handling stress and which stress coping-style group and sex of tilapia presented better responses. It also aims to determine the effectiveness of vaccination if handling stress is applied and assess the progression of response through time.

Material and Method

Experimental fish and set-up. The selected strain of tilapia (FaST) was obtained from the Freshwater Aquaculture Center (FAC), from Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines. The study was conducted at the Wet Laboratory and Fish Pathology Laboratory of the College of Fisheries - Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines from September to December 2017. A total of 120 Nile tilapia were used, with a size ranging from 70 to 80 g. Fish were individually stocked in a 10x10x10 cm aquarium. The four sides of each aquarium were covered with paper to avoid the isolated fish from seeing each other and to eliminate the stress associated to visual and social interaction. The experimental fish were provided with supplemental feed and water quality parameters were maintained in desired conditions.

Experimental treatment. The study used three factors: the stress-coping style [proactive (P) or reactive individuals (R)], sex of the fish [male (M) or female (F)] and the application of a stressor [without handling stress (c) or with handling stress (hs)]. The treatments were replicated thrice and named PMc, PMhs, PFc, PFhs, RMc, RMhs, RFc and RFhs.

Identification of proactive and reactive individuals (Vera Cruz & Tauli 2015). The experimental fish were isolated individually for 7 days to determine the proactive and reactive individuals by eye color pattern (ECP) evaluation. ECP was determined by quantifying the eye darkening of the fish and converting it into an ECP score with a value from 0 (no darkening) to 8 (total darkening). Fish with ECP scores of less than or equal to 4 after the isolation period of 7 days were classified as proactive individuals, while those with ECP scores of more than 4 were classified as reactive individuals.

Preparation of formalin-killed Aeromonas hydrophila vaccine. The *A. hydrophila* was mass produced in tryptic soy broth at 30°C for 48 h. Bacterial cells were collected by centrifugation at 6500 rpm for 15 minutes and washed three times with phosphate buffer saline (PBS). The *A. hydrophila* was re-suspended in PBS at 10^{10} cells mL^{-1}. Formalin (37% w/v) was used and added to the bacterial cell suspension at a final concentration of 0.4% (V/V). The final concentration (0.4% formalin) was derived by diluting 10 mL of 37%

formalin with 90 mL of distilled water to produce 3.7% formalin. The suspension was incubated for 3 hours at room temperature and then at 4°C overnight. The dilutions were adjusted to a turbidity score at $1x10^8$ cells mL^{-1} following the McFarland scale. The bacteria were tested for their sterility (free from the living cells) by streaking them onto newly prepared nutrient agar. Non-appearance of white colonies means no bacterial growth.

Vaccination and implementation of handling stress. A 300 µL formalin-killed *A. hydrophila* vaccine was injected to individual fish two weeks prior to the monitoring of morpho-behavioral responses and blood parameters. The vaccine was administered intramuscularly. The influence of handling stress was done by keeping the fish out of the water for three minutes using scooping nets prior to stocking.

Morpho-behavioral responses

Eye color pattern and ventilation rates. The ECP and ventilation rate (VR) were adapted from the study of Vera Cruz & Tauli (2015). The ECP value was determined by dividing the eye into eight equal parts using four imaginary diameter lines. Darkening of each division of the iris and sclera around the pupil was observed and for each measurement, a value ranging from 0 (no darkening) to 8 (total darkening) was recorded. The VR was estimated by counting the time (seconds) for 20 successive opercular or buccal movements. Daily VR measurements were recorded three times per fish in a period of 3 consecutive minutes. The three VR readings were averaged for each fish. Data was recorded before and after the handling stress was administered and the succeeding time period of 1 hour, 2 hours, 6 hours, 12 hours, 24 hours and from the second day until the 15^{th} day of observation.

Blood collection and analysis. Blood samples were obtained from experimental fish through cardiac puncture using a sterile syringe. Blood sampling was conducted in a series of collections: initially (after handling stress was administered), on the 5^{th} day, 10^{th} day and 15^{th} day. The blood was preserved in a microtainer EDTA test tube and kept at 4°C temperature until subjected for blood analysis. Blood testing was conducted at the Hi-Precision Diagnostic in Cabanatuan City, Nueva Ecija, Philippines.

Statistical analyses. The relationships of the ECP, VR and blood parameters were determined using the Pearson correlation coefficient and Linear Regression. Means were analyzed using factorial, one-way ANOVA, T-test and were compared using Duncan Multiple Range Test. The analyses were carried out using the SPSS version 16.0.

Results and Discussion

Morpho-behavioral responses

Eye color pattern. Proactive individuals (1.93±1.35) had a significantly lower ($p<0.05$) mean of the ECP values than reactive (4.45±0.48) individuals. The ECP values in proactive fish (Figure 1) were initially high in the first succeeding hours of observation due to the influence of handling stress applied. As time and days progressed, a decrease on the ECP values was observed, which could signify the adaptation of the fish to the stressful handling. Furthermore, starting on the third day of observation, the ECP scores of proactive individuals tend to be consistent, ranging from 0 to 2 with a minimal decrease in value. Statistically, a negative correlation ($r=-0.414$, $p<0.000$, $n=84$) occurred between the ECP values of proactive individuals and time, suggesting a significant reduction of the ECP through time.

The ECP values in reactive fish (Figure 1) were also initially high, as they were influenced by the handling stress applied. The values eventually decreased in time, particularly from the 12th hour to the succeeding hours and days. The ECP values were within the interval of 3-7, significantly higher compared with the values from proactive fish. A negative correlation had also occurred between the ECP values of reactive individuals ($r=-0.831$, $p<0.00$) and time.

The comparison of the ECP values when considering the sex of the fish revealed that the mean ECP value of male fish (3.16±1.80) was comparable with that of the female fish (3.21±1.74). Figure 2 shows the trend of the ECP values of male and female fish as time progressed.

Ventilation rate (VR). Proactive (0.97±0.01 beat/sec) and reactive (0.95±0.01 beat/sec) individuals showed comparable results in terms of the VR. Furthermore, male (0.96±0.01 beat/sec) and female (0.96±0.01 beat/sec) Nile tilapia were also not significantly different regarding the VR. Based on the behavioral response within 15 days of observation (Figure 3), the mean VR had minimal fluctuations as time progressed and appeared to have negligible variations. However, considering the relationship of VR and time, the result revealed that a significant positive correlation ($r=0.548$, $p=0.00$) occurred, suggesting that VR increased as time progressed.

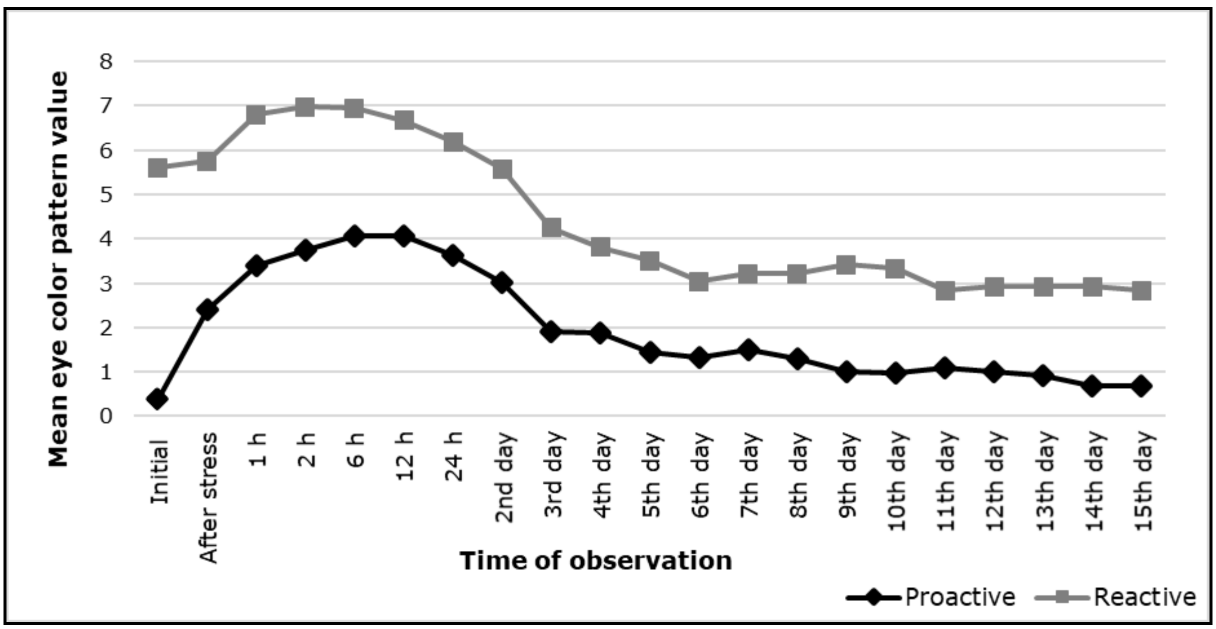

Figure 1. Eye color pattern trend of proactive and reactive individuals as time progressed.

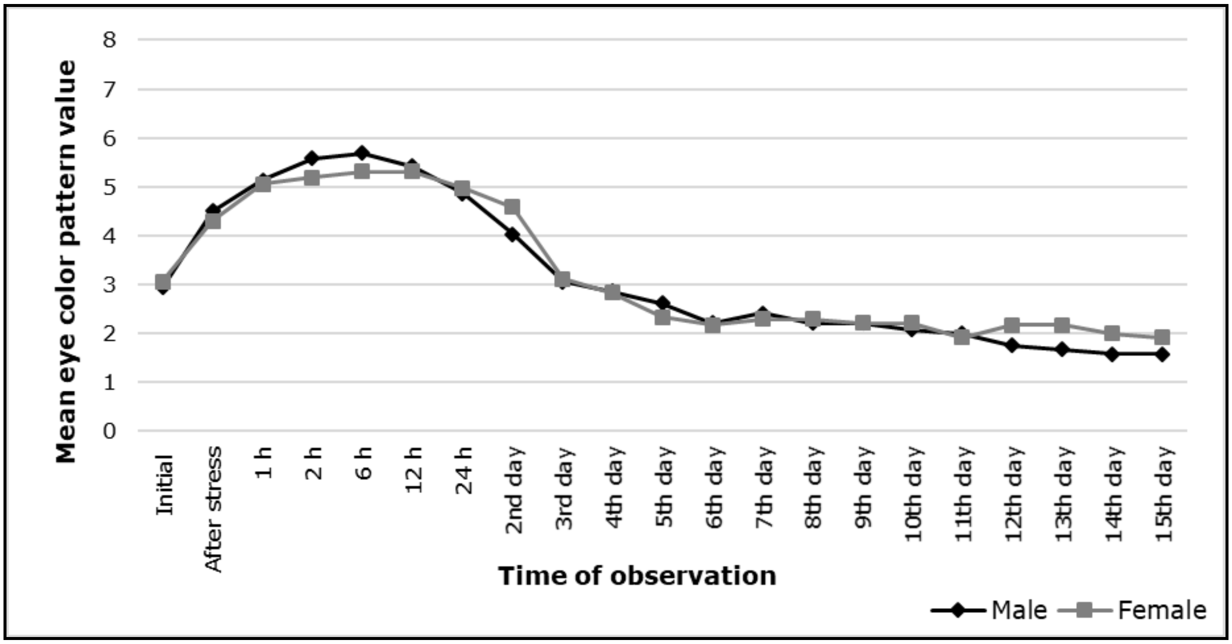

Figure 2. Eye color pattern trend of male and female individuals as time progressed.

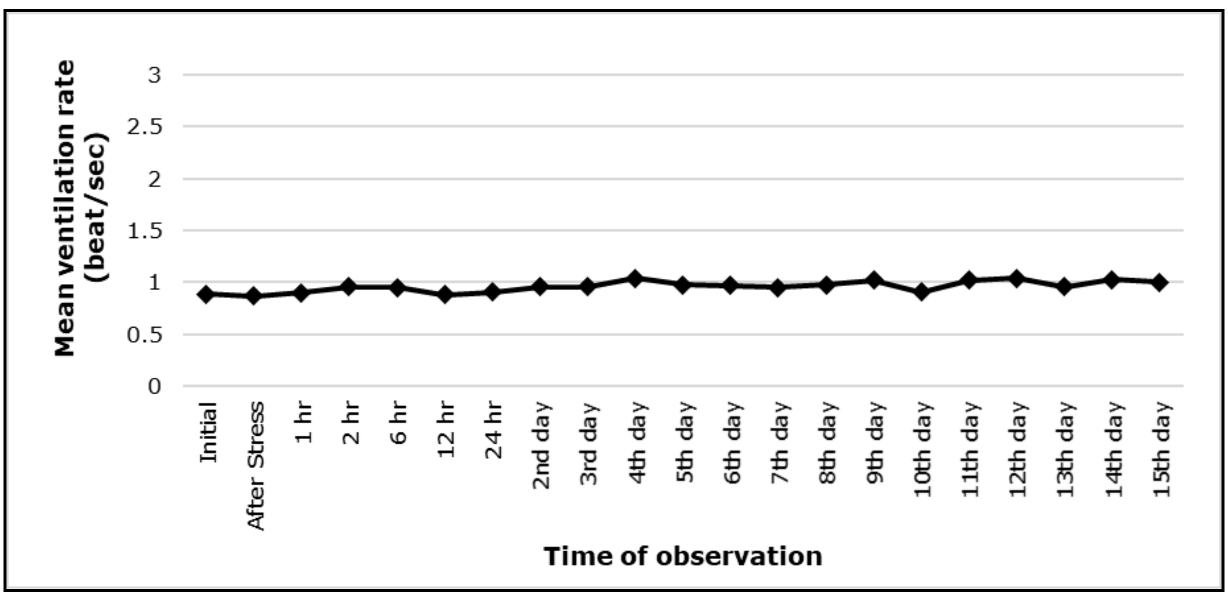

Figure 3. Trend of the mean ventilation rate during the 15 days of observation.

Hematological analysis. Table 1 shows the means of blood parameter values in the different treatments and the comparison of their levels with a reference range of values representing the normal blood parameter levels in fish.

Comparing the results of blood parameter levels to the reference values suggested by Hrubec et al (2000), blood levels of white blood cells, lymphocytes and neutrophils were within the reference values. This means that blood parameters were within normal values. However decreased levels of red blood cells, hematocrit and hemoglobin were observed.

The mean white blood cell counts of proactive and reactive fish (Figure 4A) are $29.47x10^9\ L^{-1}$ (±12.27) and $26.67x10^9\ L^{-1}$ (±8.37), respectively. In terms of the sex of the fish (Figure 4B), the mean white blood cell count in males was $28.13x10^9\ L^{-1}$ (±9.45) and in females the value was $28x10^9\ L^{-1}$ (±10.46). The values appeared to be insignificantly different from each other. The white blood cell count values of stressed and unstressed fish were insignificantly different, $29.6x10^9\ L^{-1}$ (±14.73) and $26.54x10^9\ L^{-1}$ (±8.87), respectively. No significant difference in mean lymphocyte count values was observed between individuals without handling stress with a value of $77.76x10^9\ L^{-1}$ (±7.83) and with stress with $66.81x10^9\ L^{-1}$ (±0.21).

In terms of the red blood cell count values, proactive and reactive individuals (Figure 4C) attained comparable mean values of $1.54x10^{12}\ L^{-1}$ (±0.17) and $1.42x10^{12}\ L^{-1}$ (±0.22), respectively. Similarly, male and female mean red blood cell count values with $1.53x10^{12}\ L^{-1}$ (± 0.32) and $1.43x10^{12}\ L^{-1}$ (± 0.16), respectively, were not significantly different (Figure 4D).

Results in terms of white blood cell count through time revealed a positive correlation ($r=0.595$, $p<0.041$). Likewise, lymphocyte values and time have a positive correlation ($r=0.574$, $p<0.051$). These blood parameter values increased as time progressed. Contrary, a negative correlation with time was revealed for the red blood cell count ($r=-0.004$, $p<0.989$), hemoglobin ($r=-0.074$, $p<0.819$) and hematocrit ($r=-0.029$, $p<0.928$). In this case, it can be generalized that, as time progressed, a reduction of these blood parameters was notable.

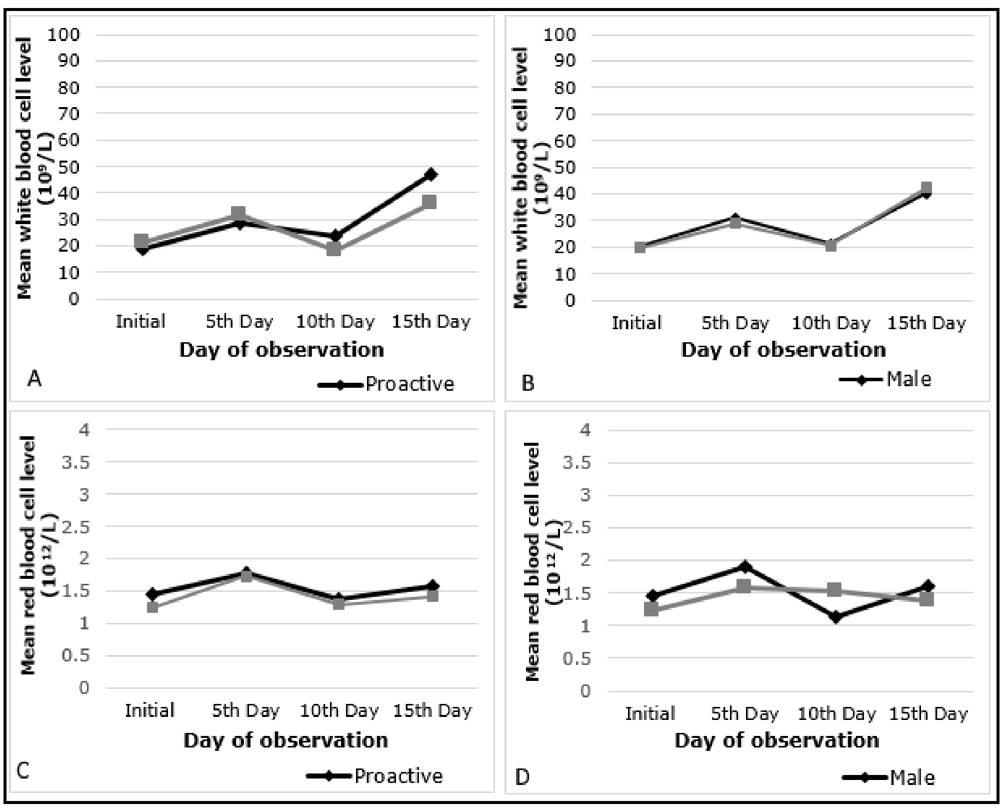

Figure 4. A - mean white blood cell trend of proactive and reactive individuals; B - mean white blood cell trend of male and female individuals; C - mean red blood cell trend of proactive and reactive individuals; D - mean red blood cell trend of male and female individuals.

As categorized by Verbeek et al (2008), characteristics that separate proactive or bold individuals from reactive or shy individuals are their active attempt to reduce the effect of aversive stimuli distinctively occurring when subjected to changes. In the current study, proactive individuals performed better in terms of ECP than reactive individuals, when subjected to handling stress. It can be observed that upon the application of handling stress, proactive individuals attempt to alter the effect of the stressor by actively responding to it, thus, better coping in terms of eye color. As defined by Koolhas et al (2001), immobility and lack of initiative are the associated responses of reactive individuals to stressors. This occurred among reactive individuals; when the stressor was applied, the darkening of the eye was their stress response.

Eye color pattern can be an indicator of the stress level in each individual fish, being related to the increase in salinity levels (Karsi & Yildiz 2004). The changes in the ECP of fish during isolation may reflect fish physiological responses to confinement, and proactive and reactive individuals can be categorized based on eye darkening (Vera Cruz & Tauli 2015). In the study conducted by Vera Cruz & Brown (2007), the response of fish to social encounter as a stressor revealed that dominant fish (associated to proactive individuals) had decreased eye darkening compared with subordinate fish (associated to reactive individuals). Relating to the current study, it can be generalized that the same response occurred when utilizing handling as a stressor in which the level of eye darkening was greater among reactive individuals than among proactive individuals. Furthermore, Freitas et al (2014) utilized air exposure as a way of non-social stress to assess the response of *O. niloticus* and the study concluded that eye darkening is an important indicator in reflecting stress and other adjustment conditions associated with non-social stressors. The current study concluded that proactive individuals had better adjustment to stress (handling stress) than reactive individuals, as revealed by the level of eye darkening.

Behavioral flexibility reflects the degree to which behavior is guided by stimuli from alterations in the environment and in the living state, and it can be considered an important fundamental and rather stable differential characteristic of coping styles. Furthermore, behavioral flexibility leads to behavioral changes, which can be demonstrated in several other situations where the fish have to switch suddenly from a familiar situation to a new one (Coppens et al 2010). Based on the current study, due to the flexibility of the fish behavior, no notable response was observed in terms of VR. Thus, it can be concluded that VR may not be a subsequent response to consider when handling stress is applied to fish.

Table 1
Mean values (±SD) of different blood parameters in the different treatments

Parameter	*Reference value*	*PMc*	*PMhs*	*PFc*	*PFhs*	*RMc*	*RMhs*	*RFc*	*RFhs*
Red blood cell count (10^{12} L^{-1})	1.91-2.83	1.75[a] (±0.38)	1.36[a] (±0.49)	1.52[a] (±0.21)	1.58[a] (±0.41)	1.53[a] (±0.37)	1.45[a] (±0.23)	1.51[a] (±0.32)	1.17[a] (±0.54)
Hematocrit (Vol. Fraction)	27-37	26.50[a] (±6.39)	19.67[a] (±7.08)	23.67[a] (±2.29)	24.17[a] (±4.51)	22.92[a] (±5.55)	22.67[a] (±2.74)	22.71[a] (±4.56)	18.75[a] (±10.2)
Hemoglobin (g L^{-1})	7.0-9.8	4.83[a] (±0.62)	4.22[a] (±1.30)	4.27[a] (±0.57)	4.94[a] (±0.62)	4.61[a] (±0.87)	4.33[a] (±0.65)	4.20[a] (±1.18)	3.91[a] (±0.89)
White blood cell count (10^9 L^{-1})	21.5-154.7	26.23[a] (±9.28)	33.55[a] (±19.81)	27.56[a] (±22.73)	30.52[a] (±10.44)	23.08[a] (±3.85)	29.64[a] (±12.64)	29.26[a] (±8.95)	24.68[a] (±21.52)
Lymphocytes (10^9 L^{-1})	6.8-13.64	74.83[ab] (±3.34)	72.00[ab] (±6.58)	81.67[b] (±10.83)	77.17[b] (±10.31)	81.42[b] (±6.33)	66.92[ab] (±24.44)	73.13[ab] (±7.78)	51.17[a] (±30.87)
Neutrophils (10^9 L^{-1})	0.5-9.873	13.83[ab] (±1.91)	15.71[ab] (±9.06)	9.38[a] (±6.85)	8.33[a] (±8.00)	7.83[a] (±3.55)	19.82[ab] (±20.3)	10.38[a] (±5.47)	34.04[b] (±3.72)
Eosinophils (10^9 L^{-1})	0.035-1.645	0.00[a] (±0.00)	0.21[a] (±0.00)	0.00[a] (±0.00)	0.75[a] (±0.50)	0.25[a] (±0.00)	0.00[a] (±0.00)	0.00[a] (±0.00)	0.00[a] (±0.00)
Monocytes (10^9 L^{-1})	0.4-4.3	3.67[a] (±0.90)	3.58[a] (±1.77)	4.63[a] (±2.55)	4.63[a] (±2.68)	2.92[a] (±0.32)	4.50[a] (±2.20)	3.58[a] (±1.17)	3.04[a] (±1.53)

Note: means in rows with different superscripts are significantly different (5% level of significance). The source of the reference/normal values of blood parameters is Hrubec et al (2000). PMc - proactive male without handling stress; PMhs - proactive male with handling stress; PFc - proactive female without handling stress; PFhs - proactive female with handling stress; RMc - reactive male without handling stress; RMhs - reactive male with handling stress; RFc - reactive female without handling stress; RFhs - reactive female with handling stress.

Lymphocytes are involved in a variety of immunological functions such as immunoglobulin production and modulation of immune defense (Campbell 1996). Changes observed in the white blood cell profile, particularly in lymphocytes, showed that stress affects the immune potential of fish (Witeska 2005). Relating to the current study, the lymphocyte profile of experimental fish increased, its immunological function in response to the stressor being activated. In addition, comparable results were obtained between treatments with and without handling stress. This is an indication that changes in lymphocyte count were influenced by vaccination, and it possibly contributed in stabilizing the lymphocyte count in response to handling stress. Similar results were obtained by Ramadan et al (1994), where a significant potentiation of lymphocyte response was manifested in vaccinated tilapia as influenced by ascogen. In addition, the study of Bailone et al (2010) revealed that the increase in the number of total leukocytes and lymphocytes in Nile tilapia was influenced by inactivated *A. hydropila* vaccine. In the study conducted by Pasnik et al (2006), it was emphasized that specific anti-*Streptococcus galactiae* antibodies played a primary role in immunity against *S. galactiae* in vaccinated Nile tilapia. Therefore, it was perceived that an increase in white blood cells, specifically lymphocytes, may be due to the influence of formalin-killed *A. hydrophila* vaccine, as a way of improving the immunological function in response to stress.

Stress induces changes in blood cell numbers and activity. In the red blood cell system, changes in hematocrit, cell count and volume, and hemoglobin level usually take place (Houston et al 1996). Another important consideration regarding the red blood cell system under stress is that the haemoconcentration affects the haematocrit, haemoglobin and red blood cell level, as a response related to the oxygen carrying capacity of blood (Montero et al 1995). In the case of the current study, a decreasing count of the red blood cells and their counterparts (hematocrit and haemoglobin) was observed as time progressed. The reduction is not due to the constraint associated to the oxygen-binding capacity of the red blood cell system, since the experimental fish received sufficient oxygen through aeration. However, Kirk (1974) stated that a decrease in the number of erythrocytes and hematocrit percentage suggested that erythrocytes have been destructed by leukocytic activity. This is in accordance with the results obtained in terms of white blood cells from the current study, where the elevated level for immunological functions influenced the decreasing level of red blood cells.

Many researchers used haematocrit as an index of the red blood cell population and, despite a wide variation in its response to stress (Mcleay & Gordon 1977), it seems that an increase in haematocrit in freshwater fish is the most common reaction (Soivio & Oikari 1976). However, interpretation of these observations is complicated because of possible changes in blood volume and the probability of erythrocytic swelling when the fish are stressed (Soivio et al 1977). According to Vosyliene (1999), an increase in hematocrit in stressed fish is an "alarm reaction", and subsequent decrease indicates adaptation. In the case of the current study, it is notable that a decreased level of hematocrit was perceived as time progressed and, thus, it can be concluded that adaptation had occurred in response to stress administered to experimental fish. Furthermore, as stated by Vosyliene (1999), the quantitative red blood cell parameters are rather stable and little sensitive to the stressor due to the considerable compensatory abilities of the fish organism. Red blood cell status seems to be influenced by the type of response and may differ depending on the stressor, the fish species and the duration of the exposure (Hardig et al 1988).

Conclusions. Based on the results of the study, in Nile tilapia, the handling stress affected the effectiveness of vaccination during the first hours and days of application. However, as time progressed, the influence of vaccination was observed in terms of eye color pattern and hematological parameters. The morphological responses of Nile tilapia suggest that proactive individuals attained better eye color pattern than reactive individuals. The behavioral response suggests that the ventilation rate may not be a contributory factor in the response of fish to handling stress. In terms of hematological responses, the study suggests that stressed fish presented increased white blood cells and lymphocytes, as a physiological adaptation against handling stress. Moreover, the immunological function of the white blood cells and lymphocytes was stimulated against handling stress without neglecting the effect of vaccination. The study concluded that male and female Nile tilapia had comparable responses among all the parameters evaluated. However, proactive individuals subjected to handling stress performed better in terms of stress-coping than reactive individuals subjected to handling stress. Screening of Nile tilapia by determining the proactive individuals is a potential method to reduce or eliminate the influence of handling stress in the culture process.

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