Growth and Hepatic Insulin-Like Growth Factor-I Gene Expression in Dominant and Subordinate *Oreochromis niloticus* L Under Limited Period of Physical Interaction

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**Abstract.** Social stress is known to regulate several aspects of the teleost physiology. This study explored the influence of limited period of physical interaction on growth and hepatic insulin-like growth factor-1 (IGF-I) messenger RNA (mRNA) expression of the fish. Twenty all-male *Oreochromis niloticus* were isolated for 10 days and were used in a social pair study. After the social interaction was settled, dominant and subordinate individuals in a pair were reared separately in one aquarium separated by glass divider. The fish were fed at the same rate daily to remove the possible effect of nutrition. The glass divider was removed 10 min daily for social interaction. Weight was monitored on Days 2, 7 and 14 during the experimental period, then hepatic IGF-I mRNA expression was quantified. During the 14 days social experiment, mean specific growth rate of dominant fish (1.6%/day) was significantly higher ($P<0.01$) than that of the subordinate fish (0.9%/day). Dominant fish also had 2.5 fold significantly higher ($P<0.05$) mean IGF-I mRNA expression than that of subordinate fish. These indicate that even under limited period of physical contact but with period of visual communication, social status regulates growth and hepatic IGF-I gene expression in this species of fish. There was also a significant positive correlation ($r = 0.52; P<0.01$) between growth and IGF-I mRNA level which supports the previous studies that hepatic IGF-I gene expression has a potential utility as an instantaneous growth rate indicator for *O. niloticus*.

1. Introduction
Somatic growth in fishes is regulated by several factors which include nutrition and environmental conditions. These factors in turn control the synthesis and release of hormones which regulate growth through autocrine, paracrine and/or endocrine modes. In cultured fish populations, heterogeneous growth is a common phenomenon [1, 2]. Variation in individual growth rates of fish within a population may be attributed in part to social aggression and the establishment of feeding hierarchies [3, 4]. Aside from the effect on growth elicited by changes in food intake, growth variation may also be due to changes in metabolism of subordinate and dominant fish [5, 6, 7].

The growth hormone (GH) – insulin-like growth factor-I (IGF-I) axis is central in the hormonal control of fish growth [8]. The growth promoting actions of GH are mediated through induction of IGF-I synthesis [8, 9]. The GH is naturally synthesized in the pituitary gland and is released in the...
blood stream and it induces the production of circulating IGF-I which is predominantly synthesized in the liver [10]. Most of the circulating IGF-I are bounded to IGF binding proteins (IGFBP) [9]. When released by these IGFBPs, free IGF-1s interact with IGF-I receptors in the cell membrane of the target cells. It is in this mechanism that mediates the majority of IGF-I actions.

Previous study has shown that social rank influenced IGF-I gene expression and growth in fish [4]. However, fish were reared in small aquarium (64 L) where subordinate fish had limited space to stay away from the dominant fish. Thus, this study evaluated the influence of social rank under limited period of physical interaction on the two previously mentioned parameters. This study also attempted to confirm results of previous studies [3, 4, 11, 12] on the use of hepatic IGF-I mRNA as instantaneous indicator of growth in O. niloticus.

2. Methodology

2.1 Experimental protocols

Twenty all-male juveniles of Nile tilapia (Oreochromis niloticus) from the Freshwater Aquaculture Center, Central Luzon State University, Philippines (15°44’8.9”N, 120°56’49.2”E) were randomly isolated in 30cm x 15cm x 30cm aerated glass aquaria for 10 days. To remove the possible effect of size and previous social history during the social interaction, the fish used were of similar size and with no apparent differences in social history. For the aquaria used in the isolation of fish, the three sides of each aquarium were covered by paper board to prevent the isolated fish from viewing other isolated fish in the nearby aquaria. After the isolation period, competing pairs were identified. For the purpose of identifying the fish during the physical interaction, each fish in a competing pair was individually marked by a small cut on either the lower or upper part of the caudal fin. In the social pair study, the two competing fish were introduced into a new aquarium at the same time, thus preventing the effect of place familiarity. After the social interaction was settled, dominant and subordinate individuals were identified. During the first day of pairing and after the social interaction was settled, subordinate fish were identified as those with less aggression and staying away from the dominant fish. They also exhibited dark body and eye coloration and with higher ventilation rate (i.e. opercular or buccal beat rate per unit time) and more frequent movement of fins. On the other hand, dominant fish were those observed having pale skin and eye coloration and increased levels of aggression. After the establishment of hierarchy, the opponent fish were reared separately in one aquarium divided by glass separator for 14 days. Every morning the glass separating the two fish was removed for 10 minutes for the fish to interact. After daily interaction, the glass separator was replaced and both fish were fed at the same rate to possibly remove the effect of nutrition. Individual weight of each fish was monitored on Day 2 and weekly after the first day of interaction. After the second week, all fish were sacrificed for quantification of hepatic IGF-I mRNA.

2.2 RNA extraction and purification and complementary DNA synthesis

Livers were collected from dissected fish and were immediately frozen in liquid nitrogen. Trizol® (Invitrogen™ Carlsbad, California, USA) was used to isolate and purify total hepatic RNA. Glycogen in the samples was precipitated using high salt precipitation solutions (0.8 M sodium citrate and 1.2 M sodium chloride). Possible genomic DNA contamination were removed by treating the isolated RNA with a DNase I kit (DNA-free™, Ambion®, Austin, Texas, USA) in two separate reactions. The A260 : A280 ratio was used to assess the purity of the isolated RNA, which ranged from 1.6-2.0, with most readings ranging from 1.9-2.0. Normalization of samples was performed against total RNA concentration [13, 14]. The addition of 1 μg total RNA template in the reactions was based on the amount of RNA (ng·μL⁻¹) in the sample. First strand complementary DNA was generated in 20 μl RT reactions consisting of 1 μg total RNA template, 10X RT buffer, reverse transcriptase (Omniscript®), 5 μM dNTP, RNase inhibitor (RNasin®, Promega® Madison, Wisconsin, USA) and 10 μM oligo-dT primer (Promega®). Samples were reverse transcribed by 60 min incubation at 37 °C.

2.3 Quantification of hepatic IGF-I mRNA
The TaqMan real time quantitative reverse transcriptase – polymerase chain reaction assay described in [3] was performed on Lightcycler® 480 II (Roche Ltd., Basel, Switzerland) to quantify the hepatic IGF-I mRNA from the samples.

2.4 Data analysis
All statistical analyses were performed using SPSS v. 16. Percentage data were transformed using arcsine square root transformation prior to statistical analysis. The Student’s T-test was performed to assess the effect of social stress on the growth rate [i.e. specific growth rate (SGR)] and IGF-I gene expression of the fish. The bivariate Pearson correlation was used to measure the linear correlation between growth and absolute abundance of IGF-I mRNA.

3. Results and Discussion
The mean body weights of the experimental fish in the two social groups during the start, and on the 2nd, 7th and 14th day of the interaction period are presented in Table 1. Prior to social interaction there was no significant difference on the mean body weight between the competing individuals. During and after the interaction period, dominant individuals had significantly higher mean body weights than those of the opponents (P<0.05). It is important to note that the subordinate fish were observed not consuming food when fed after the first day of interaction and a day after. The mean SGRs of the fish from Day 7 to 14 and the mean IGF-I mRNA levels after the experiment are shown in Figures 1 and 2, respectively. Dominant fish had a mean SGR of 1.61±0.22% day\(^{-1}\) which was significantly higher than that in the subordinate fish (0.93±0.16% day\(^{-1}\)) (P<0.01). On mean IGF-I mRNA concentration, dominant fish (14.52±3.23 ng\(\mu\)l\(^{-1}\)) also had a significantly higher value than that in the subordinate fish (5.86±1.84 ng\(\mu\)l\(^{-1}\)) (P<0.05). Generally, fish with faster growth rate (i.e. higher SGR), had higher IGF-I mRNA gene expression. In addition, there was a significant positive correlation (r = 0.56) between IGF-I mRNA level and SGR (P<0.01; Fig. 3).

| Social group | Mean body weight ±SE (g) |
|--------------|--------------------------|
| Dominant     | Initial                  | Day 2          | Day 7          | Day 14         |
|              | 24.15\(a\) (±0.81)       | 24.70\(a\) (±0.76) | 28.53\(b\) (±1.30) | 31.62\(b\) (±1.26) |
| Subordinate  | 24.04\(a\) (±0.80)       | 23.77\(b\) (±0.60) | 26.45\(b\) (±0.66) | 28.26\(b\) (±0.71) |

Mean values under each social interaction period superscripted with different letters are significantly different at 5% level of significance.

Findings of the current study confirmed the results obtained in [4] that fish’s social status consistently influence hepatic IGF-I mRNA expression and growth in O. niloticus. Although the competing pairs of fish in the recent study had lesser changes of physical interaction (i.e. only during the period for establishment of social hierarchy on Day 1 and 10 minutes daily starting Day 2) compared to those used in [4], growth of dominant fish in the two studies were comparable. In the recent study the mean SGR of dominant fish was 1.61% day\(^{-1}\) compared to 1.48% day\(^{-1}\) in the earlier study, while there was greater mean SGR difference (i.e. 0.50% day\(^{-1}\)) of subordinate fish between the recent study (0.93% day\(^{-1}\)) and the earlier study (0.43% day\(^{-1}\)). The greater difference between growth rates of subordinate fish in the two studies compared to that of dominant fish may partly be attributed to the possible lesser stress in subordinate fish brought about by the separation of the competing fish after social hierarchy was settled except for 10 minutes interaction daily from Days 2 to 14. In addition, the recent study was performed at longer duration of 14 days compared to 10 days in the earlier study, therefore giving more time for subordinate fish to adapt to the social condition.
Figure 1. Mean specific growth rate (±S.E.; %·day⁻¹) of the dominant and subordinate fish from Day 7 to 14, n = 20. Mean values are significantly different (P < 0.01)

Figure 2. Mean hepatic IGF-I gene expression (±SE; ng·µl⁻¹) in the dominant and subordinate fish, n = 20. Mean values are significantly different (P < 0.05)
Dominance-related social interactions develop as a consequence of competition of conspecifics for limited resources such as space and food. Fish in a social group are ranked based on the capability of the fish (dominant) of out-competing its opponents (subordinate and intermediate) in contests of aggression. The formation of stable dominant hierarchy induces social aggression and physiological stress and this condition is stressful for both subordinate and dominant fish [15]. In the present study, both subordinate and dominant fish were stressed during the social fight as evidenced by the observed darkening of the eyes and body color, increased ventilation rates (VRs) and erected fins in both individuals [16, 17]. The eye coloration and VR are stress-coping styles in fish and they can be easily used and are inexpensive tools for assessing both stress level and alertness [18, 19]. Social aggression was brought about by the limited spaces available leading to the establishment of social hierarchy. This social hierarchy consequently resulted to reduced aggressiveness and appetite of subordinate fish; 9 out of 10 of which displayed reduced weight a day after the start of social interaction. During the first day of pairing and after the social interaction was settled, subordinate fish were observed staying away from the location of the dominant fish and they exhibited higher VR and the movement of fins became more incessant and frequent. Subordinate fish were also observed not consuming food up to the second day. The weight loss of most subordinate fish on Day 2 of social interaction (see Table 1) is therefore, not a reflection of mobilization of stored metabolic reserves for physical activity associated with social stress encountered but it may be more an outcome of reduced food consumption and the consequent emptiness of the stomach as supported by the observed existence of hollow-bellied subordinate fish. Dominant fish, on the other hand, were observed having higher levels of aggression and with pale skin coloration, which are changes in behavior and physical appearance, indicating that they have won the contest [20].

**Figure 3.** Correlation between IGF-I mRNA level (ng·µl⁻¹) and SGR (%·day⁻¹) of the fish,\( n = 20, r = 0.52, P < 0.05\)
Behavioral changes during the physical and visual interactions may be related to prior fighting experiences of the fish, in which previous losing experiences decrease the willingness of the fish to enter into a social interaction while winning experiences increase its willingness to enter into a social interaction [21]. Therefore, the difference in body weight between the subordinate and dominant fish during the formation of social hierarchy was mainly attributed to behavioral differences (i.e. appetite) rather than to differences in physical activities (i.e. aggression) which conforms to the findings of several studies [2, 4, 22, 23]. The conflict over social rank may partly be responsible for the decreased in growth rate of subordinate fish [24]. Inhibited food intake appears to be a behavioral mechanism of subordinate fish ensuring that they remain smaller than dominant fish within the hierarchy. This self-imposed growth restraints is beneficial to subordinates in order to free them of the cost of social fight. The inhibited food intake observed in the subordinates may be caused partly by an increase in neuropeptide Y gene expression [25] and/or serotonin [26]. It is also possible that the appetite suppressing effects of stress in subordinate fish was induced by corticotrophin-releasing factor (CRF) as the CFR system was found to mediate the stress-induced feeding inhibition in fishes [27].

Although subordinate fish resumed food consumption on Day 3, the low food intake indicates that they were more stressed than the dominant fish. The stressful condition and low food intake resulted to lower IGF-I synthesis in the liver as indicated by the significantly lower liver mRNA expression of IGF-I in subordinate fish compared to that in dominant fish [8]. Although stress was found to induce the synthesis and release of cortisol, a stress response-mediating hormone, it indirectly depressed IGF-I and GH levels [4, 28, 29]. This lower hepatic IGF-I synthesis resulted to significantly lower SGR value in subordinate than in dominant fish.

Aside from those alterations induced by inhibited food intake, the significantly lower SGR in subordinate fish during the 14-day interaction period compared to that in dominant fish may also partly be due to alterations in metabolism. Weight loss in subordinate fish under prolonged cohabitation or social interaction with dominant fish may partly be due to retention of bile in the gallbladder of subordinate fish [5, 7]. Under this condition, subordinate fish cannot effectively convert energy-rich fatty food into body weight. A study by [6] on the other hand, found out that in dominant fish hepatic pyruvate kinase activity was higher relative to that in subordinate fish which reflects that dominant fish used ingested food for energy. Meanwhile, in the same study, phosphoenolpyruvate carboxy kinase activity in the liver was higher in subordinate fish compared to that in dominant fish which implies the reliance of subordinate fish to the stored metabolic reserves.

These possible metabolic changes and physiological stress in subordinate fish even under limited physical contact with the dominant fish may partly be due to the effect of visual communication. It may be possible that the subordinate fish were stressed even during periods that they were separated from the dominant fish because they received visual signals from and/or saw the dominant fish through the glass barrier. Visual communication was found to stimulate reproduction in *O. niloticus* [30]. On the other hand, fish may assess the competitive ability of neighbors by watching social interactions [31]. Therefore, metabolic changes and physiological stress under limited physical contact but with visualization of the dominant conspecific may be considered as causes of the reduced growth in the subordinate individuals [32].

The results of this study are important in aquaculture as competing fish in a population will result to the formation of hierarchy, with dominant, intermediate and subordinate fish. Formation of social hierarchies may lead to decreased feeding behavior [4], changes in metabolism [5, 6, 7], decreased growth rate in subordinate and intermediate individuals and faster growth rate in dominant individuals [4], and chronic social stress [33]. So in the harvested population, dominant fish will be the largest, followed by intermediate and the smallest are the subordinate population.

On the other hand, the significantly positive association between SGR and IGF-1 level confirmed the results of previous studies [3, 4, 11, 12, 34] and may be interpreted as strongly supportive of the idea that liver mRNA expression of IGF-I can be used as instantaneous growth indicator for *O. niloticus*. This positive association of growth to IGF-I mRNA level was also observed in coho salmon (*O. kisutch*) [35] and Chinook salmon (*Oncorhynchus tshawytscha*) [36].
4. Conclusion
Social stress underlies several behavioral and physiological changes in fish. The study indicated that even under reduced period of physical contact but with chances of visual communication, social rank regulated IGF-I mRNA expression and growth in Nile tilapia. Behavioral changes such as reduced appetite and maybe metabolic changes in subordinate fish resulted to a reduced growth rate. In addition, social dominancy instantaneously increased liver-derived IGF-I mRNA level while social and physiological stresses reduced it.

Acknowledgment
This report formed part of the “Hormone Gene Expression as Growth Indicator for Nile Tilapia Project” funded by grant from Philippine Commission on Higher Education to E M Vera Cruz.

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