Effect of protein level and stocking density on growth performance, feed utilization and resistance of Nile tilapia (Oreochromis niloticus) to infection against aeromonas septicemia (Aeromonas hydrophila)

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SUMMARY - A fourteen week laboratory feeding trial was conducted to ascertain the effect of two dietary crude protein levels (30 and 40 CP%) and stocking densities (10, 30 and 40 fish/105 litre aquarium) on growth performance, feed and nutrient utilization, and the resistance of Nile tilapia (*Oreochromis niloticus*) to infection with *Aeromonas hydrophila.* Fish were reared within indoor glass aquaria (100x30x40 cm) containing dechlorinated water supplemented with aeration and temperature controlled at 28°C $\pm$ 1 for the duration of the experiment. The results showed that growth performance of tilapia fingerlings was significantly (P<0.05) reduced at the higher dietary protein level and stocking densities. Feed and nutrient efficiency was also significantly (P<0.05) reduced at the higher dietary protein and stocking densities tested. Moreover, at the end of the 14-week experiment, the immune response of tilapia after immunization within formalin killed *A. hydrophila* bacteria (FKB) was studied; the results of antibody titer of the immunized fish after 28 days differing significantly (P<0.05) between individual treatments. The highest titer was obtained with fish stocked at the lowest stocking density (10 fish per aquarium) and fed the lowest protein diet (30% CP). By contrast, the lowest antibody titer was found in fish reared at the highest stocking density (40 fish per aquarium) and fed the lowest level of dietary protein (30% CP). Percentage survival rate after bacterial challenge was also highest for fish reared at the lowest stocking density (10 fish/aquarium) and fed the lowest dietary protein level (30% CP). The results clearly showed that fish growth was best at the lowest stocking density and low dietary protein level tested, and that these parameters significantly affected the capability of fish to resist infection against Aeromonas septicemia.

Key words: Protein level, stocking density, tilapia, growth, feed and nutrient utilization, Aeromonas infection, resistance.

RESUME - *"Effet du niveau de protéine et de la densité de peuplement sur les performances de croissance, l'utilisation alimentaire et la résistance de tilapia nilotica (*Oreochromis niloticus*) à l'infection septicémique par Aeromonas (*Aeromonas hydrophila*)". Un essai d'alimentation en laboratoire de quatorze semaines a été mené afin de vérifier l'effet de deux niveaux de protéine brute dans le régime (30 et 40% PB) et des densités de peuplement (10, 30 et 40 poissons/aquarium de 105 l) sur la croissance, l'utilisation des aliments et nutriments, et la résistance de tilapia nilotica (*Oreochromis niloticus*) à l'infection par*

[1] Paper presented by Prof. Dr. A.M.Nour during the Workshop

Aeromonas hydrophila. *Les poissons ont été élevés en intérieur dans des aquariums en verre de dimensions (100x30x40 cm) contenant de l'eau sans chlore, avec une aération continue et une température de 28 ºC ± 1 contrôlée par thermostat pendant toute la durée de l'expérience. Les résultats ont montré que la croissance des alevins de tilapia était significativement réduite (P<0,05) avec les niveaux les plus élevés de protéine et de densité de peuplement. L'efficacité alimentaire et nutritionnelle était également réduite de façon significative (P<0,05) avec les niveaux les plus hauts de protéine et de densité de peuplement. A la fin de cette expérience de 14 semaines, on a étudié la réponse immunitaire du tilapia après immunisation avec des bactéries de* A. hydrophila *neutralisées par formaline ; les résultats du titrage des anticorps des poissons immunisés après 28 jours, ont varié de façon significative (P<0,05) entre les traitements individuels. Le titrage le plus élevé a été obtenu avec des poissons ayant la plus faible densité de peuplement (10 poissons par aquarium) et alimentés avec le niveau le plus faible en protéine (30% PB). D'autre part, le titrage le plus faible en anticorps a été obtenu chez les poissons élevés selon la plus forte densité de peuplement (40 poissons par aquarium) et qui recevaient le régime le plus faible en protéine brute (30% PB). Le taux de survie après confrontation bactérienne était également le plus élevé chez les poissons à la plus faible densité (10 poissons/aquarium) et recevant le niveau le plus faible de protéine (30% PB). Les résultats montrent que la meilleure croissance des poissons était obtenue avec la densité et le niveau les plus faibles de protéine dans le régime, et que ces paramètres affectent de manière significative la capacité des poissons pour résister à l'infection septicémique par Aeromonas.*

Mots-clés *: Niveau de protéine, densité de peuplement, tilapia, croissance, utilisation des aliments et nutriments, infection par Aeromonas, résistance.*

INTRODUCTION

Tilapia are among the most successful cultured finfish species in the world . This has been largely because of their fast growth rate and ability to feed low on the aquatic food chain. Moreover, tilapia are easy to reproduce and have good resistance to disease and handling; tilapia having good tolerance to wide range of environmental conditions and being found in over 100 countries (Balarin and Hatton, 1979).

With the intensification of culture methods for tilapia species during recent years, it has become necessary to provide complete rations to meet their dietary nutrient requirements. Numerous studies have been conduct so as to ascertain the dietary protein requirements of tilapia (*Oreochromis niloticus*) with reported dietary protein requirements ranging from 30% CP in the case of pond reared fish and juveniles (Omar 1994a; Siddiqui *et al.* 1982) to over 45% CP in the case of fry and fingerlings reared within indoor aquaria (Omar, 1994b; El-Sayed and Teshima, 1992).

Studies concerning the relationship between stocking density and growth of tilapia have shown that optimal stocking density for obtaining the highest possible fish yields depend upon the amount and the quality of food available (Zonneveld and Fadholi, 1991). However, the use of high stocking density as a technique to maximize water usage and thus increase stock production has also been shown to have an adverse effect on growth (Andrews, *et al.*,1971; Refstie, 1977; Trzebiatowski, *et al.* 1981; Schreck, *et al.*,1985; Essa and Nour, 1988). Furthermore, it is still not clear how high stocking density negatively influences fish growth.

Finally, although fish reared under changeable environmental conditions and often subjected to a high degree of stress (i.e. through water quality deterioration, competition between fish, physical handling, changes in ambient temperature and crowding), little information exists concerning mechanisms underlying stress-related disease susceptibility in cultured fish (Pickering, 1988). For example, it is generally believed that stress leads to the suppression of the immune system in fish with a consequent increase in disease susceptibility.

The objective of the present study was therefore to evaluate the effect of protein level and stocking density on growth performance, feed and nutrient utilization, and the resistance of Nile tilapia (*O. niloticus*) to infection with *A. hydrophila* after immunization with formalin killed bacteria (FKB).

MATERIALS AND METHODS

Apparently healthy Nile tilapia (*O. niloticus*) fingerlings (mixed sexes of 13.55$\pm$1.5 g/fish) were obtained from the Experimental Fish Farm of the Faculty of Agriculture, Alexandria University, in June 1993. Fish were held in a 105-litre glass aquaria filled with dechlorinated tap water for 10 days and fed a diet containing 30% crude protein (CP; Table 1). Fourteen fish were killed at the start of the experiment and frozen for subsequent proximate analyses of body chemical composition.

Batches of fingerlings were stocked at 10, 30 and 40 fish per each glass aquarium (105-litre capacity) with 8 aquaria per stocking density. Each aquarium was equipped with an air stone for continuous aeration, thermostatically controlled temperature (28 ^{o}C$\pm$1), and subjected to a 12 h photoperiod using fluorescent lamps. Faeces and uneaten feed residues were siphoned out of the tank together with about one third of the water volume of the aquarium each day and replaced with fresh dechlorinated tap water before the morning feed.

Two experimental diets containing 30% and 40% CP were formulated based on the use of fish meal, soybean meal, yellow corn, corn oil, vitamins and minerals (Table 1). The diets were prepared by first thoroughly mixing the dry ingredients and lipids in a plastic container. Warm water (50^{o} C) was then slowly added until a moist mash was obtained, and the moist mash then passed through a commercial meat grinder to form moist pellets, and then subsequently dried and stored at -20^{0} C before used. All experimental diets were fed to satiation to two duplicate fish groups (each diet fed at three stocking densities: 10, 30 and 40 fish/aquarium with 4 aquaria per stocking density) two times daily at 8.00 and 16.00 h, 6 days per week for 98 days. The amount of feed consumed by the fish within each aquarium was recorded daily. At the end of the feeding experiment representative fish from each aquarium were sacrificed and frozen for subsequent proximate analyses of the fish body composition. After sampling, the remaining live fish from similar treatments were mixed together so as maintain stocking densities of 10,30 and 40 fish/aquarium with the total number of aquaria being reduced from 24 to 12; the fish thereafter fed on one of two dietary protein levels (30% and 40% CP).

Half of the fish in each aquarium were immunized against *Aeromonas hydrophila* by intrapritoneal injection with 0.2 ml of formalin-killed bacteria(FKB) (approximately 2.8X10^{8} cell/fish) and after 15 days fish received another 0.2 ml of FKB as a booster dose. The remaining fish in the aquarium served as control and were injected with 0.2 ml sterile saline solution according to Navarre and Halver (1980). Formalin-killed bacterium was prepared according to the method of Baba *et al.* (1988); the *A. hydrophila* strain 0228 previously isolated from the liver of tilapia *(O. niloticus*) and identified according to Hsu *et al.* (1985).

Table 1. Formulation and composition of the experimental diets.

Item	International Feed Number	Dietary protein level % 30	40
Feed Ingredient %			
Fish meal	5-02-009	25	33
Soybean meal	5-04-604	33	44
Yellow corn	4-02-935	38	19
corn oil	4-07-882	2	2
Vitamin premix*		1	1
Mineral premix**		1	1
Nutrient % (% as DM basis)			
Crude protein (CP)		30.33	40.37
Ether extract (EE)		5.44	5.39
Crude fibre (CF)		3.84	3.35
Ash		6.35	5.32
Nitrogen free extract (NFE)		54.04	45.57
Calculated gross energy (kcal GE/ gDM)***		4.43	4.66
Calculated energy to protein ratio (kcal GE/g CP)		13.30	11.54

*Vitamin mixture/Kg premix containing the following: 33000 IU vitamin A, 3300 IU vitamin D3, 410 IU vitamin E, 2660 mg Vitamin B1, 133 mg vitamin B2, 580 mg vitamin B6, 410 mg vitamin B12, 50 mg biotin, 9330 mg coline chloride, 4000 mg vitamin C, 2660 mg Inositol, 330 mg para-amino benzoic acid, 9330 mg niacin, 26.60 mg pantothenic acid.

**Mineral mixture/kg premix containing the following : 325 mg Manganese, 200 mg Iron, 25 mg Copper, 5 mg Iodine, 5 mg Cobalt.

***Gross energy (GE kcal/g diet)calculated according to NCR(1993) using the following calorific values: 5.64 , 9.44 ,and 4.11 kcal/g diet of protein, fat and carbohydrate, respectively.

Blood samples were collected from the caudal vein after 28 days post inoculation from three fishes of the control group and three fish from the immunized group in each aquarium using a plastic syringe coated with 0.1 ml of sodium citrate according to Hardie et al. (1990). Agglutination titers were determined according to the method of Antipa and Amend (1977).

After 28 days, all fish (including immunized and control groups) were challenged with approximately $1.4x10^6$ cells/fish of a 0228 hot strain of *A. hydrophila*. Fish mortalities were subsequently counted for 10 days after challenge and the resolution of *A. hydrophila* attempted from infected liver and kidney tissues using RS media (Rimler and Shotts, 1973) to verify the cause of death.

The relative level of protection (RLP) afforded by the FKB was calculated using the formula of Newman and Mainarich (1982), as follows :

$$RLP = 1 - \frac{(\%\ \text{mortality of immunized fish})}{\%\ \text{mortality of control}} \times 100\ ,$$

Proximate chemical analyses of the experimental diets and fish body composition were carried out according to the official methods of analysis of the Association of Official Analytical Chemists (AOAC, 1984). Mean fish weight, average gain, average daily gain (ADG), specific growth rate % (SGR%/fish/day), feed conversion ratio (feed/gain ratio), protein utilization (protein efficiency ratio, PER and productive protein value, PPV%) and energy utilization (EU%) were calculated as described by Omar (1984). Statistical analyses of the results were conducted according to Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

The effect of the interaction between dietary protein levels and stocking density on the growth performance of tilapia is shown in Table 2. From the data presented it can be seen that increasing protein level from 30 to 40% CP and increasing stocking density from 10 to 40 fish /aquarium resulted in a significant ($P<0.05$) reduction in growth performance. However, analyses of variance on the interaction between dietary protein level and stocking density per aquaria were not statistically significant.

During the present experimental conditions the study showed that the 30% CP diet produced the best growth performance of *O. niloticus* fingerlings. Similar results were obtained by Omar (1994a), Siddiqui *et al.* (1988), Wang (1985), and De Silva and Perera (1985). Similarly, Magouz (1990) reported that the dietary protein level for *O. niloticus* could be reduced from 40% to 33% without any significant effect on growth of fish. On the other hand Santiago *et al.* (1982) reported that the optimum dietary crude protein level for *O. niloticus* fry was between 35 to 40% crude protein.

The body composition of the tilapia varied with stocking density (Table 3). The results showed that increasing the stocking density above 10 fish/aquarium resulted in a significant ($P<0.05$) lower dry matter, crude protein and lipid content. Furthermore, an increase in crude protein level from 30 to 40% CP resulted in significantly increased ($P<0.05$) body dry matter and decreased body crude protein content.

The observed dietary feed intake was highest for fish reared at the lowest density (Table 4); these results being in agreement with those reported by Clark *et al.* (1990) and Zonneveld and Fadholi (1991) for red tilapia. The reduced growth rates observed for fish reared at high density were attributed to the lower feed intake of these fish .

Table 2. Growth* performance of fish fed the experimental diets for 14 weeks

Protein level%	Stocking Density (fish/ aquarium) 10	30	40	Mean**
	Initial Fish Weight (g/fish)			
30	13.65	12.95	13.47	13.34
40	13.62	13.33	13.38	13.44
Average**	13.64	13.14	13.43	
	Final Weight (g/fish)			
30	41.47	28.17	22.17	30.60[a]
40	34.26	23.82	19.25	25.78[b]
Average**	37.87[a]	25.99[b]	20.71[c]	
	Weight Gain (g/ fish)			
30	27.82	15.22	8.77	17.27[a]
40	20.64	10.49	5.87	12.33[b]
Average**	24.23[a]	12.86[b]	7.32[c]	
	Average Daily Gain (g/fish/day)			
30	0.284	0.155	0.089	0.176[a]
40	0.211	0.107	0.060	0.126[b]
Average**	0.247[a]	0.131[b]	0.075[c]	
	Specific Growth Rate (SGR %)			
30	1.13	0.78	0.51	0.81[a]
40	0.96	0.59	0.38	0.64[b]
Average**	1.05[a]	0.69[b]	0.45[c]	

*each value was the mean of four replicates.
** means in the same row with different superscripts are significantly (P<0.05) different.

Table 3. Body composition* of fish fed the experimental diets

Protein level	Stocking Density (fish /aquarium) 10	30	40	Mean**
	Dry Matter (DM%)			
30	22.54	22.08	22.15	22.26[b]
40	23.41	23.17	23.07	23.22[a]
Average**	22.98[a]	22.63[b]	22.61[b]	
	Crude Protein (CP%)***			
30	62.95	61.53	60.74	61.74
40	61.74	61.04	60.60	61.13
Average**	62.35[a]	61.29[b]	60.67[b]	
	Ether Extract (EE%)***			
30	21.09	21.39	21.44	21.31
40	21.79	21.22	21.57	21.53
Average**	21.44	21.31	21.51	
	Ash %***			
30	16.27	17.09	17.82	17.06
40	16.48	17.74	17.83	17.35
Average**	16.38	17.42	17.83	

*Chemical composition of fish at the start of the experiment was as follows; 20.23, DM %, and on DM basis containing 58.60% CP, 18.29% EE, and 23.10% ash each value being the mean of four replicates).
** means in the same row with different superscripts are significantly (P< 0.05) different.
*** % on dry matter basis.

Feed utilization efficiency also decreased with increasing stocking density. (Table 4); the best FCR observed with fish reared at the lowest stocking density and fed the 30% protein diet. These results are similar to those reported by Papoutsoqlou *et al.* (1987); Essa and Nour (1988); Cruz and Ridha (1991) and Zaki (1993). Similarly, protein and energy utilization efficiency were also found to be affected by dietary crude protein level and fish stocking density; fish feed the lowest protein level and reared at the lowest stocking density displaying the best protein efficiency ratio (PER), protein productive value (PPV%), and energy utilization efficiency (EU%). These results are also in agreement with those reported by Jauncey (1982); Magouz (1990); Zonneveld and Fadholi (1991) and Mazid *et al.* (1979) .

Table 4. Feed intake, feed conversion ratio, and protein/energy utilization* of fish fed the experimental diets for 14 weeks

Protein level%	Stocking Density (fish/ aquarium) 10	30	40	Mean**
	Feed Intake(gDM/fish)			
30	50.64	44.59	38.30	44.51[a]
40	46.94	39.96	35.98	40.96[b]
Average**	48.79[a]	42.28[b]	37.14[c]	
	Feed Conversion Ratio (FCR)			
30	1.83	2.93	4.37	3.04[b]
40	2.28	3.95	6.14	4.12[a]
Average**	2.06[c]	3.44[b]	5.26[a]	
	Protein Efficiency Ratio (PER)			
30	1.81	1.13	0.76	1.23[a]
40	1.08	0.63	0.40	0.71[b]
Average**	1.45[a]	0.88[b]	0.58[c]	
	Protein Productive Value (PPV%)			
30	31.04	17.65	12.71	20.47[a]
40	19.32	9.28	8.09	12.23[b]
Average**	25.18[a]	13.47[b]	10.40[c]	
	Energy Utilization (EU%)			
30	18.92	10.94	8.36	12.74[a]
40	15.48	7.74	7.85	10.36[b]
Average**	17.20[a]	9.34[b]	8.11[c]	

* Each value was the mean of four replicates.

** means in the same row with different superscripts are significantly ($P<0.05$) different.

The results of the antibody serum test of immunized fish are shown in Figure 1. The highest titer was recorded with fish stocked at the lowest stocking density and fed the lowest protein level. By contrast, the lowest of antibody titer was found in fish stocked at the highest stocking density and fed the 30% protein diet.

Moreover, from the data presented in Table 5 it can be concluded that the highest survival rate, non-specific resistance to infection, and highest antibody levels were recorded with fish reared at the lowest stocking density and fed the lowest dietary protein level.

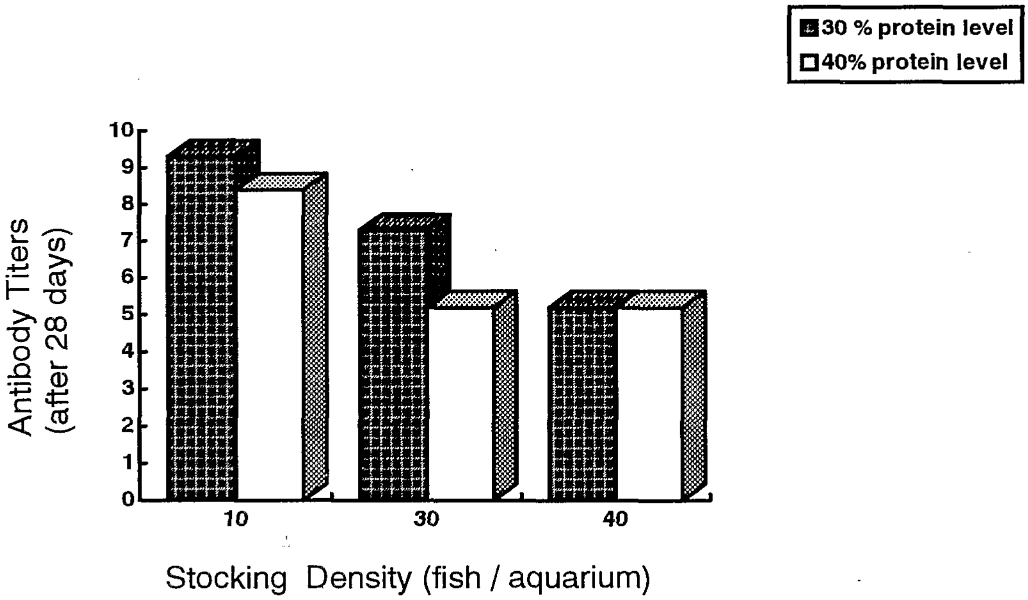

Fig. 1. Antibody titer in the serum obtaned from immunized tilapia previously fed on diets contaning different protein levels and different stocking densities.

Table 5. Survival rate and relative level of protection (RLP)* afforded by virulent *Aeromonas hydrophila* for fish fed the different experimental diets and reared at different stocking densities.

Stocking Density (fish/ aquarium)	Protein Level %	Challenged Fish**	No of Fish with Pathological Symptoms	No of Fish Mortality	Survival Rate %	RLP*%
10	30	9	1	2	78	63.3
10	40	9	1	3	67	45.0
30	30	15	4	5	67	45.0
30	40	15	6	6	60	33.3
40	30	20	8	9	55	25.0
40	30	20	7	9	55	25.0
Control(Saline)		20	8	12	40	0.0

* RLP = 1 - (% Mortality of vaccinated fish / % Mortality of control) x 100
** each value was an average of two duplicates.

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