Med.J.,Giza. Vol.55, No.2. (2007):523-535.

TUDY ON THE EFFECTS OF DIFFERENT FEEDING REGIM ON HE IMMUNE RESPONSE OF CULTURED MARONE LABRAX INGERLINGS) TO YERSINIA RUCKERI VACCINE.

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Received: 1. 3. 2007
Accepted: 13. 3. 2007

SUMMARY

The effect of different daily feeding frequencies of one time (1X), 2 times (2 X) and 4 times (4 X) on total leucocytes, differential leucocytic counts, phagocytic assay, total proteins, albumin, globulin, Al/Gl ratio and antibody titers before and after immunization by intraperitoneally injection (IP) of formalin killed Yersinia ruckri vaccine were studied as well as histopathological changes. The results proved that (2 X) feeding frequency causes a marked immunopotentiating effects more than (1X) or (4X) feeding frequencies as well as control groups (1% feeding rates) after IP vaccinations. The immunopotentiating effects of feeding frequencies covered both, the humoral and the cell-mediated immunity response and a marked rise in the antibody titers at day 28 after vaccination which reached to (8.0 ± 0.0) in (2X) feeding frequencies after IP. vaccination , compared to the vaccinated control group (4.5 ± 0.5; 9.0 ± 0.0 and 7.0 ± 0.5) in (1X) time feeding frequency, (2X) and (4X) times respectively. Also, lymphocytes, phagocytic assay, total protein and globulin obtained from fish, fed with 2 % feeding rates as well as control ones specially at 2X and 4X frequencies were significantly increased than fish fed with 1% feeding rate in all its treatments. The relative survival percentages after IP. vaccination was 60 %, 50 % in 2 % feeding rates and control groups respectively during 2 X feeding frequencies, compared to the control group where it was 23 %, 30 % and 30 % in 1 X, 2 X and 4 X respectively.

INTRODUCTION

A poor growth rate implies that the immune responsiveness would be markedly impaired (Peters and Hong, 1985). Few studies, however, have been concerned with the effect of nutrients and

feeding regime on the immune response. A dietary defects or deficiency lead to immunosuppression in fish (Soltan et al., 2000).

nce, there is an urgent need to look for diseases preventive measures to promote sustainable culture of Marone labrax. In order to reduce the risk of disease, the level of resistance to infection in the cultured organisms should be increased by selective feeding program for higher disease resistance (Khalil et al., 2001). Few data are available on the effects of feeding frequencies on the immune response of Marone labrax (Shimeno et al., 1997). The shortage of literature concerning the effect of these factors on the immunity enforced us to study their effects on leucocytic counts, phagocytic assay, total protein, globulin in Marone labrax, as well as their effects on the immune response to Yersinia ruckeri vaccine.

MATERIALS AND METHODS

Experimental fish and facilities: A total number of 450 Marone labrax fingerlings (provided by special farms in El- Kaseem K.S.A) with a mean weight of 30 g were stocked in 18 experimental units of 160 liter capacity containing dechlorinated filtered tap water at a temperature of 28.6 - 29.9°C, pH 6.96 - 7.3, dissolved oxygen 4.5 - 6.4 mg/L, salinities 38 - 38.2 ppt, unionized ammonia (NH3) 0.09 - 0.03 mg/L and nitrite (NO2) 0.08 - 0.25 mg/L. Each experimental unit was provided with aeration.

Experimental design: Three feeding frequencies of one time (1 x), two times (2 x) and four times (4 x) daily will be used as treatments in this study. feeding frequency is the number of feedings will be given daily.

The fish were divided into 3 equal treatments combinations of the two factors (2% and 1% feeding rates) and (1% act as control) with each treatment replicated three times (1x, 2x and 3x feeding frequiencies) for a total of 225 fingerlings, whereas 225 fingerlings divided into 9 equal groups (25 Marone labrax each) and kept as control in the treatments (satiation group). The feed used in the experiment contained 48 % protein. The size of the feed to be used ranges from 1.00 to 2.00 mm in crumble form.

Histopathological examination: Fish samples from, kidney , liver, spleen and intestine were collected for histopathological sections which were stained by hematoxyline and eosin (H & E) according to the method described by (Roberts, 1989).

Blood samples: At the end of the 1st, 2nd, 3rd , 4th week of the experimental period (28 days), a whole blood was collected on anticoagulant (0.1ml of heparin solution / 1 ml blood) for determination of total leucocytic count and differential leucocytic counts according to (Ellis, 1977) as well as determination of phagocytic assay according to (Kawahara et al., 1991). The blood serum

was separated by centrifugation for measuring total proteins and albumin were determined according to (Doumas et al., 1981), while serum globulin was calculated as the difference between total protein and albumin (Coles, 1974).

Determination of phagocytic activity and phagocytic index:

Phagocytic activity was determined according to Kawahara et al. (1991).

$$\text{Phagocytic activity (PA)} = \text{Percentage of phagocytic cells containing yeast cells.}$$

$$\text{Phagocytic index (PI)} = \frac{\text{Number of yeast cells phagocytized}}{\text{Number of phagocytic cells}}$$

Antibody titration against Yersinia ruckeri bacterin and challenge test:

Detection of immune response to Yersinia ruckeri was evaluated by micro-agglutination (MA) test. Agglutination titers were expressed as Log2 of the highest serum dilution still giving a clear agglutination according to(Eurell et al., 1979).

A virulent strain of Y. Ruckeri (Kindly provided by Fish disease and hygiene dep. Fac. Of vet. Med. Alex. Univ.) was inactivated by formalin according to (Sakai et al., 1984). The inactivated Y. Ruckeri was tested for safety and sterility according to (Anderson et al., 1970). After the antibody titration the survival fish were IP challenged with 0.1 ml / fish containing 9 X 10^7 cells of the virulent Y. Ruckeri after 28 days from vaccination. Daily morbidity and mortality were recorded

$$\text{Relative protection level (RPL)} = \frac{\text{Vaccinated mortality \%}}{\text{Control mortality}} \times 100$$

Statistical analysis:- Statistical analysis of the obtained data was performed using the Statistical Analysis System (SAS, 1987).

RESULTS

The results of the effect of different feeding frequencies (one time, two time and four times daily) on the immune status of Marone Labrax fingerlings cultured in 38-38.2 ppt, saline water was evaluated by measuring the total leucocytes, differential leucocytic counts (Table, 1), phagocytic assay (Table, 2) and amounts of globulin (Table, 3) as well as immunopotentiating effect on the antibody production accompanied by the calculation of relative survival percentage (Table 4 & 5). *No significant differences of total leucocytes in Control (1 % feeding rate) supplemented groups in all feeding frequencies was observed (table, 1). Also, from Table (1), noted that significant differences in total leucocytes between 1 X, 2 X and 4 X feeding frequency supplemented groups allover the period of experiment (4 weeks). Total leucocytes were observed in lower count in all replicates of control group (1 % feeding rate) than in 2 % feeding rate replicates specially at the end of the experiment. *From Table (1), it is clear that the number of lymphocytes were very decreased in 1 % feeding rate supplemented group if compared

with 2 % feeding rate supplemented groups in all types of feeding frequencies all over the periods of experiments. On the other hand, the monocytopenia appeared only in 1 X feeding frequencies accompanied with 4 feeding frequencies and accompanied with 2 feeding frequencies (Table, 1).The results of phagocytic activity and phagocytic index of this study are presented in (Table 2, Fig.1). From the results it can be notice that the phagocytioc activity (PA) and phagocytic index (PI) was significantly decreased after 3 weeks in control (1 % feeding rate supplemented groups), but these assay was significantly increased in 2 % feeding rates supplemented groups in all its replicates allover the periods of experiment. Moreover, the PA and PI appeared in high level in all replicates of 2 % feeding rate if compared to control group (1 % feeding rate) which came in second rank. The total proteins (TP) and globulin especially in the last 3-weeks of 2 and 4 times frequencies of 2 % feeding rate supplemented groups were significantly increased compared to control (1 % feeding rate) in its all feeding frequencies (1 X, 2 X and 4 X). Table (3), showed no significant differences of TP and globulin in control (1 % feeding rate) in its feeding frequencies which came in the second rank. Moreover, the results revealed that hypoalbuminaemia in all treatments of control (1% feeding rate) and 2, 4 times feeding frequencies than in 2 % feeding rate supplemented groups. It is worthy to note that the hypoglobulinaemia was reported in control (1 % feeding rate) supplemented group in all its feeding frequencies. Injection vaccination intraperitoneally (IP) in all groups of study have a significant immunopotentiating effect on the antibody production of Marone labrax . The antibody response in fish supplemented by 2 % feeding ratio accompanied by 2 X and 4 X feeding frequencies was (7.5 ± 0.5) and (8.0 ± 0.0) respectively . In the control group (1 % feeding rate) supplemented group, the IP application of Yersinia ruckeri vaccine elicited lowest mean of antibody titers (3.5 ± 0.5, 3.5 ±0.0 and 4.0 ± 0.5) in 1 X, 2 X and 4 X feeding frequencies, respectively. (Table, 4). The results indicated that the low feeding rate of fish reduced the level of antibody titers compared to fish supplemented by high feeding rate. Moreover, the results revealed that the 2 X and 4 X feeding supplemented groups were able to respond with level of antibodies titers after immunization with Yersinia ruckeri bacterin higher than all experimental groups.

The results of the challenge test in immunized experimental fish as well as the control one after IP injection with 0.2 ml (approximately 10^8 cells) of Yersinia ruckeri are shown in (Table 5). After one-week post-injection mortality rates of 80 % and 100 % were observed in the case of groups of fish supplemented with control (1 % feeding rate in both vaccinated and non-vaccinated treatments respectively in all types of feeding frequencies. It is worthy to note that the mortality rates wer

Table (1):

Feeding rate	Feeding frequencies	Lymphocytes				Monocytes				Leucocytes (White Blood cells X 10^3)			
		1st Week	2nd Week	3rd Week	4th Week	1st Week	2nd Week	3rd Week	4th Week	1st Week	2nd Week	3rd Week	4th Week
2%	1X	Aa 50.2±0.98	Ba 52.4±0.99	Ba 54.3±0.93	Ba 54.2±0.91	Bb 3.1±0.07	Bb 2.8±0.07	Cb 3.3±0.09	Cc 2.2±0.07	Bc 24.7±0.28	Bc 26.0±0.31	Bc 25.3±0.25	Bb 34.3±0.28
	2X	Aa 49.3±0.92	Ba 51.6±0.95	Ba 51.5±0.94	Ba 52.1±0.93	Bb 3.5±0.07	Ab 3.8±0.06	Aa 5.3±0.09	Ab 4.1±0.06	Bc 27.3±0.22	Bc 27.3±0.19	Ab 32.0±0.19	Aa 39.3±0.23
	4X	Aa 50.6±0.91	Ba 52.7±0.93	Ba 51.3±0.76	Ca 52.6±0.91	Cc 2.9±0.06	Bc 2.8±0.07	Bb 3.7±0.47	Cc 2.7±0.2	Ab 32.7±0.21	Aa 36.7±0.23	Aa 38.0±0.25	Aa 41.3±0.26
1% Satiation (Control)	1X	Aa 47.91±	Cb 41±0.93	Ca 45±1.34	Ca 46.7±1.32	Cc 1.7±0.08	Bb 3.3±0.09	Bb 4.3±0.09	Bb 3.7±0.08	Bc 23±0.71	Bb 25±0.68	Bb 26±0.59	Cb 27±0.63
	2X	Ab 52±0.81	Aa 58±0.8	Aa 59.7±0.33	Aa 61±0.75	Bc 3±0.05	Ab 4±0.05	Bb 3.7±0.06	Aa 5.3±0.07	Bd 26±0.53	Bc 27.9±0.46	Ac 30.9±0.31	Ab 36.5±0.31
	4X	Ab 48.7±0.91	Bb 48.3±0.83	Ba 54.3±0.39	Ba 56.3±0.91	Ab 4±0.05	Aa 4.7±0.05	Bb 4±0.03	Bc 3.3±0.02	Bc 25.3±0.56	Bc 28.2±0.51	Ab 33.6±0.51	Bb 33.2±0.53

Table (2):

Feeding rate	Feeding frequencies	Phagocytic activity				Phagocytic index			
		1st Week	2nd Week	3rd Week	4th Week	1st Week	2nd Week	3rd Week	4th Week
2 %	1 X	Ac 25.66±1.09	Ac 28.0±0.51	Bb 32.66±0.62	Ab 33.66±0.83	Ab 137.33±0.42	Ab 141±0.53	Aa 158.33±0.78	Aa 153.33±0.78
	2 X	Ac 25.66±1.05	Ac 28.33±0.91	Bb 31.33±0.86	Ab 34.33±0.52	Ac 139±0.53	Ac 143.33±0.62	Aa 168±0.91	Ab 155.66±0.67
	3 X	Ad 22.33±1.02	Ac 29.0±0.46	Aa 37.0±0.47	Ab 35.0±0.62	Bd 118.66±0.45	Ab 151±0.43	Aa 166.33±0.53	Ab 156.66±0.57
1% Satiation	1X	Ca 15.22±1.23	Ca 16.11±1.12	Da 14.33±1.23	Da 15.2±1.12	Da 81.2±1.13	Ea 86.33±0.95	Da 79±0.93	Ea 83.33±0.96
	2X	Aa 21.2±0.72	Ba 22.3±26.6	Ca 21.2±0.94	Ca 22.3±0.45	Aa 132.5±0.31	Ba 132.33±0.27	Ba 122.6±0.51	Ba 126±0.36
	4X	Aa 23.7±0.89	Aa 26.6±0.93	Ba 29.3±0.91	Ba 29.1±0.86	Ba 121.33±0.59	Ba 130.33±0.98	Ba 121.33±0.83	Ca 119.7±0.92

Table (3):

Feeding rate	Feeding frequencies	Total protein (g%)				Albumin				Globulin			
		1	2	3	4	1	2	3	4	1	2	3	4
2 %	1 X	Ba 4.94±0.12	Aa 4.95±0.14	Ca 5.12±0.15	Ba 5.15±0.13	Aa 3.82±0.80	Ba 3.51±0.19	Aa 3.71±0.90	Aa 3.26±0.80	Cd 1.16±0.15	Bc 1.44±0.12	Cc 1.41±0.13	Cb 1.89±0.12
	2 X	Ab 5.14±0.13	Ab 5.35±0.13	Aa 5.78±0.17	Aa 5.93±0.12	Ba 3.71±0.90	Ba 3.61±0.15	Ba 3.15±0.14	Bb 2.96±0.15	Bc 1.43±0.11	Bc 1.74±0.13	Ab 2.63±0.12	Aa 2.97±0.14
	3 X	Ab 5.84±0.02	Aa 5.9±0.03	Aa 6.26±0.12	Aa 6.33±0.13	Ba 3.12±0.80	Ca 3.25±0.18	Ba 3.11±0.19	Bb 2.86±0.17	Ac 2.72±0.13	Ac 2.65±0.14	Ab 3.15±0.13	Aa 3.47±0.12
1% Satiation (Control)	1X	Ba 4.12±0.17	Ba 4.13±0.15	Ca 4.25±0.1	Ca 4.3±0.17	Aa 4.8±0.1	Aa 4.1±0.15	Aa 3.96±0.11	Aa 3.89±0.91	Eb 0.4±0.0	Eb 0.3±0.01	Eb 0.29±0.01	Fb 0.41±0.02
	2X	Bb 4.66±0.14	Ba 4.78±0.11	Ca 4.74±0.15	Ca 4.9±0.13	Aa 3.95±0.31	Aa 3.91±0.50	Aa 3.65±0.90	Aa 3.62±0.80	Dd 0.71±0.12	Dd 0.87±0.11	Cc 1.14±0.12	Dc 1.28±0.9
	4X	Bb 4.89±0.12	Ab 4.91±0.13	Ba 5.13±0.14	Ba 5.15±0.15	Aa 3.96±0.17	Aa 3.9±0.15	Aa 3.83±0.17	Aa 3.68±0.17	Ba 1.93±0.11	Cb 1.01±0.15	Cb 1.3±0.14	Cb 1.47±0.15

1 % feeding ratio 2 % feeding ratio 1X = One time/day 2X = One time/day 3X = One time/day

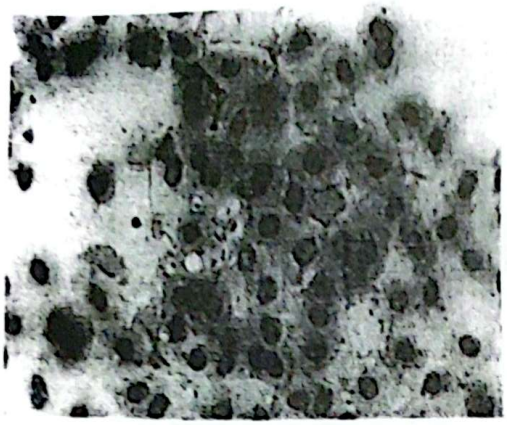
Means with different letters in the same column differ significantly at ($P < 0.05$).
Values receiving the same superscript are statistically not significant ($P > 0.05$).

Table (4): Antibody titers (Log 2) in different vaccinated groups (Means ± Standard error).

Feeding rates	Feeding frequency	Week post-vaccination			
		1	2	3	4
2 %	1 X	Cd 2.5±0.0	Dd 2.5±0.0	Cc 3.5±0.5	Bb 4.5±0.5
	2 X	Cd 2.5±0.0	Cc 3.0±0.0	Cc 3.5±0.0	Ab 5.0±0.5
	4 X	Bd 3.0±0.0	Bc 4.0±0.0	Bc 4.5±0.0	Ab 6.5±0.5
1% Satiation (Control)	1 X	Cc 2.5±0.0	Cb 3.0±0.0	Cb 3.0±0.5	Cb 3.±0.5
	2 X	Ac 4.5±0.5	Ac 5.0±0.5	Ab 6.0±0.5	Ab 6.5±0.5
	4 X	Ac 4.0±0.0	Ab 5.0±0.0	Ab 5.5±0.5	Aa 6.0±0.0

Table (5):

Feeding rate	Feeding frequency	Treatments	Dead	Survival	Mortality %	Relative Protection level R. P. L
2 %	1 X	Non-vaccinated	27	3	90	10
		Vaccinated	15	15	50	50
	2 X	Non-vaccinated	27	3	90	10
		Vaccinated	12	18	40	60
	4 X	Non-vaccinated	21	9	70	30
		Vaccinated	18	12	60	40
1% Sastiation (Control)	1 X	Non-vaccinated	27	3	90	10
		Vaccinated	21	9	70	30
	2 X	Non-vaccinated	24	6	80	20
		Vaccinated	15	15	50	50
	4 X	Non-vaccinated	27	3	90	10
		Vaccinated	18	12	60	40



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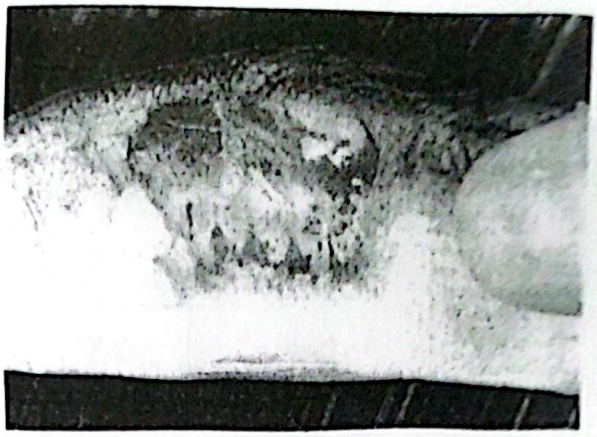

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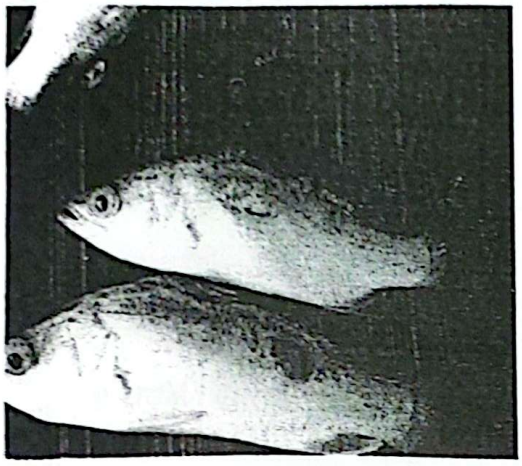

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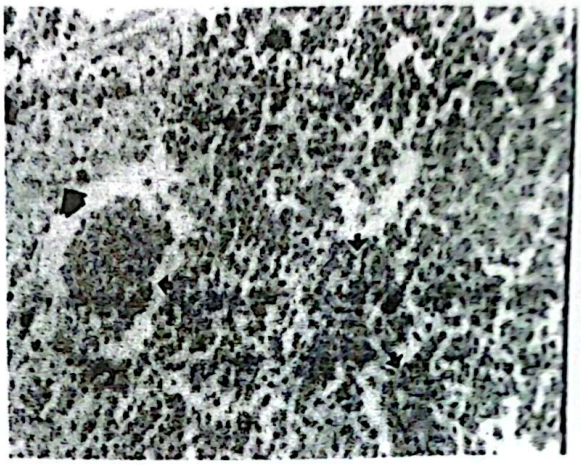

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very low in 2 % feeding rate supplemented groups compared to 1 % feeding rate supplemented groups in all treated groups, whereas , the mortalities in vaccinated groups were 50 % , 40 % and 60 % in 2 % feeding rate and in 1 X, 2 X and 4 X respectively. The results of relative level of protection (RLP) afforded by injected bacterin revealed that RLP values of 60, 50 and 50 were achieved in the case of 2 % feeding rate and 2 X feeding frequencies , 2 % feeding rate and 1 X feeding frequency supplemented groups respectively. On the other hand, the RLP value reached to 50 in the case of control and 2 X feeding frequencies. The clinical signs due to bacterial injection in control fish appeared in the form of hemorrhage, ulceration and congestion of the dorsal musculature (Fig.2,3). Histopathologically the haemopiotic organs mainly affected especially the spleen and kidney showing hyperactivation of melanomacrophage centers and haemopiosis of hemopiotic cells especially in groups supplemented with 2% feeding rates (Fig.4,5 and 6).

DISCUSSION

Specific immunity is based on cell-mediated reactions and on the secretion of antibody molecules by B-cells (Manning, 1994). Teleost immunoglobulins (IgS) have been shown against fish pathogens in aquaculture are based on the secretion of antigen-specific Ig, and this process is studied extensively in the light of protocol by means of vaccination strategies (Riley et al., 1996). In the present study, the results revealed that the 2 % feeding rate accompanied by 2X feeding frequencies potentiates the immune response stronger than control (1 % feeding rates) supplemented groups. The immunoptentiating effects of feeding rates and frequencies cover both the humoral and cell-mediated immune response. 2 % feeding rate and control combination by 2X feeding frequencies increased total leucocytes, lymphocytes and phagocytic assay in tested fish. One possible explanation for these results that increased feeding rates and feeding frequencies may lead to increase growth performance such as specific growth rates, good feed conversion and high survival rate (Shimeno et al., 1997). As it has been shown previously in fish from growth promotion which reflect on activation of immune memory and macrophages by lymphokines which exist in fish and play an important part in the cell-mediated immunity as stated by (Safinaz et al., 2002). Interestingly, by examination of phagocytosis in groups supplemented by 1 % feeding rate (control), it was noticed that the ready to engulf the particles of Candida albicans but failed to do so. These results may suggest stress effects of low feeding rates on Marone labrax which leads to increased levels of serum cortisol. The increase of cortisol levels may lead in turn to suppression of phagocytosis processes . This suppression may be mediated directly via the corticosteroid receptors on macrophages or indirectly through the enhanced production of certain factors by the macrophages themselves which suppress the secretion

of other macrophage products (Jain, 1993). The present findings show an increase in the total protein and globulins in the same groups above mentioned (2 % feeding rates and control with 2X frequencies) these results may be a sequalla of improvement of growth, compared to a decrease of these parameters in other groups (1 % and 4 % feeding rates). In the present work, the results cleared that increase in antibody titers in fish supplemented by 2 % and satiation feeding rates more than 1 % and 2X, 4X feeding frequencies more than 1X as well as a relative level of protection (RLP) induced by injected bacterein. This may be due to increase number of lymphocytes or decrease according to treatment which reflection on the antibody production and RLP by decrease or increase. The low feeding rate 1 % decreased phagocytic activity, antibody titers, total protein, globulin and relative level of protection may be attributed to generalized stress response which lead to increased pituitary-internal activity (Wepener et al., 1992), and secretion of corticosteroids (Ellis, 1981). The reduction of globulin levels in comparison with 2 % feeding rate and control groups suggests that feeding rate could produce stress in fishes, which leads to increase of infections due to immunosuppression effects. The same conclusion was reported by (Khalil, 1988) especially in toxicity conditions. Permmanent stress includes impaired globulin formation and depressed interferon production, which play an important role in decreasing the resistance of fish to various bacterial, parasitic and viral disea es (Wedenyer, 1970). Promising positive resu were obtained. Both the phagocytic assay a globulin were almost equal to those in 2 % a control feeding rate accompanied by 2 X feedi frequencies groups. The same results were al recorded in the case of antibody titers and relati level of protection. A possible explanation f these encouraging results is that both feeding ra and feeding frequencies improved the physiolo cal function of fish and immune response, whi in turn increased the ability of supplemented fi to resist the stress effect of different environme tal condition. The clinical signs are mainly due the effect of bacterial endotoxines and the septic mia which was induced. The histopathological a terations are due to the effect of feeding rat which increase the immune status of fish again disease conditions (El-Gamal 2005). The resul suggest that the most suitable feeding regime f cultured Marone labrax under hypersaline cond tion is 2 % feeding rate or control accompanie by two times feeding frequencies due to a goo defense strategy leading to a reduction in diseas prevalence and a great control of the a sympt matic carriers after an outbreak.

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Tables & Figures

A-List of tables

B-List of figures:-