# Fish health assessment

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# Dietary Supplements for the Health and Quality of Cultured Fish

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# 2 Fish Health Assessment

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## 1 Importance of Nutrition on Fish Health

### 1.1 Background

Infectious disease is a major contributor to economic loss in intensive fish culture. Some antibiotics have been used to reduce death due to bacterial infections. Recently, food-importing countries, such as the EU, the USA and Japan, require exporting countries to meet various food sanitation controls. Many consumers are aware that residues of antibiotics and other chemotherapeutants in fish flesh are an important risk for human health. To produce safe farmed fish, it is necessary to prevent outbreaks of fish diseases without using antibiotics. Vaccination has come into wide use for disease prevention, but the effectiveness of vaccination decreases depending on fish condition. In addition to good management and vaccination schedules, nutritional prophylaxis will ensure beneficial disease control in farming systems.

Diet should both achieve rapid fish growth and maintain health. Health maintenance based on adequate nutrition and feeding practices has acquired greater importance in intensive fish culture. The efficiency of diet should be evaluated not only by growth but also by the impact on fish health. Therefore, suitable indices are needed for evaluating dietary efficiency linked to fish health.

Since major disease outbreaks typically occur when the balance between the fish (host), the etiologic agent and the environment is upset (Snieszko, 1974), the maintenance of fish health should lead to reduced risks of disease outbreaks. External factors, such as quality of diet, management of feeding and environment, and internal factors such as inherited quality would affect the health condition of fish. It is reasonable to assume that the levels and ratios of available dietary nutrients may influence the susceptibility of fish to

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diseases (Blazer and Wolke, 1984). Nutritional strategies that could be adopted to manage fish health include adjustment of specific nutrition levels in the diet, manipulation of nutritional condition through feeding regimes and administration of non-nutrient immunostimulants in the diet.

## 1.2 Fish health and nutritional studies

Adequate nutrition has long been acknowledged as crucial for maintaining animal health and disease resistance. Various immune deficiencies have been reported in humans and animals that do not receive sufficient amounts of essential nutrients (Waagbø, 1994). Evidence supporting the importance of nutrients in maintaining normal immune function and disease resistance is increasing. This research first appeared during the early 1980s (Blazer, 1982), but gained momentum only during the 1990s. Landolt (1989), Blazer (1992), Lall and Oliver (1993), Waagbø (1994), Sealey and Gatlin III (1999) have all published reviews in this area of research. Much of the early work was focused on vitamins C and E, but more recently fatty acids and amino acids are also being studied.

A schematic drawing depicting the relationship between the amount of nutrients in a diet and animal health is shown in Fig. 2.1. The health of fish fed on diets with excess or inadequate amounts of certain nutrients will decline. These health effects are recognized as nutritional diseases, sufficient to cause mortality in the fish. Dietary supplements fed within safety limits will result in beneficial effects for the animals.

Specific nutrient requirements are usually determined under standard, well-defined and favourable environmental conditions. However, such ideal conditions are not always present in commercial farms, where animals are often stressed, confronted with pathogens or other unfavourable environmental conditions. Under these conditions, the requirements for

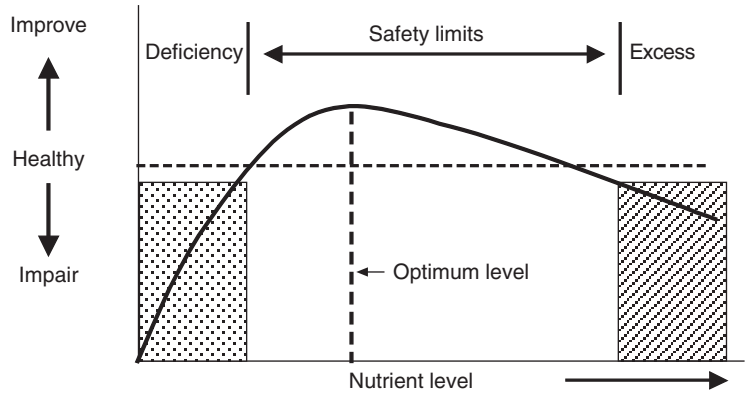


**Fig. 2.1.** Schematic drawing of the relationship between the level of a certain nutrient in a diet and animal health.

certain nutrients are likely to increase, due to greater activity of the immune system and physiological activity. In addition to changes in nutritional requirements, physical damage of digestive tracts, infection with parasites and decreased absorption due to the physical aspects of the diet may cause malnutrition. The deterioration of nutritional status will lead to the loss of disease resistance in fish (Blazer, 1992). Benedich (1988) observed a decline in various immune functions prior to the observation of symptoms of nutrient deficiency in mammals. Thus, it is obvious that insufficient nutrients result in animals susceptible to infectious disease.

## 2 'Fish Health' and Health Assessment

Fish culturists can become aware of abnormalities in fish through changes of body colour, abnormal swimming, increases in rate of mortality, reduction of appetite, etc. However, in the early stages of impaired health, fish may be easily infected with pathogens even if their appearance is normal. These fish that have a higher susceptibility to infectious disease should be recognized as 'compromised hosts' and the loss of disease resistance should be suspected at the earliest opportunity in order to either prevent or reduce the severity of epizootics. It is therefore important to establish evaluation procedures and indices of health condition for fish in order to detect the 'compromised hosts'. Besides sensitivity and precision, ease of application, rapidness and cost-effectiveness are also required for the methods of fish health assessment.

Various parameters have been used to assess the physiological changes or health condition of fish due to the effects of nutrients. Indices of fish health condition are also necessary to understand how fish health is affected by diet and to enhance disease resistance through dietary modification.

Health indices should correlate with factors that accelerate mortality due to infectious diseases. It is considered that various factors, such as imbalance of nutrients, quality of diet and feeding regimes may affect the function of various organs such as the liver and/or cause malnutrition, eventually accelerating mortality.

## 3 Methods of Fish Health Assessment

Fish health can be assessed using morphological, haematological and immunological examination as well as experimental disease challenge.

### 3.1 Morphological examination

Evaluation of fish health condition by observations and measurements of external characteristics and internal organs is rather simple. The fish health

condition profile (HCP) was originally developed for trout fish hatcheries in Utah (Goede, 1988). Novotny and Beeman (1990) reported on the health assessment of juvenile Chinook salmon by HCP. According to the report, abnormality of the thymus and increased abdominal fat were affected by rearing density. However, correlation between those abnormalities and disease resistance of fish was not obvious. While scored observations, scoring selections, and descriptions for each category of the HCP have been shown in detail (Goede, 1988), the external or internal observations are rather subjective. The effects of nutrients on the HCP are also unknown. Thus, evaluation of fish health by morphological examination is rather limited in sensitivity and precision.

### 3.2 Blood examination

Measurement of peripheral blood parameters and plasma constituent levels generally constitute blood examination. The advantage of blood examination is that it is easy to measure using commercial kits or equipment, and several samples can be analysed in a short period. In addition, it generally can be carried out without killing the fish. Therefore, blood examination is a reliable way of monitoring the health condition of fish.

Various factors including differences in species, age, sex, water quality, water temperature and handling methods may contribute to variability in haematological and biochemical data that is difficult to interpret. For this reason it is difficult to compare results from different studies or set 'normal ranges' and it has been suggested that reference intervals for each set of culture conditions may need to be determined.

Haematology is used as an index of fish health status in a number of fish species to detect physiological changes following different stress conditions such as exposure to pollutants, diseases, metals, hypoxia, etc. (Blaxhall, 1972; Duthie and Tort, 1985). Haematological characteristics such as haematocrit value (Ht), haemoglobin concentration (Hb) and red blood cell counts (RBC) also have been examined to evaluate the requirement of certain dietary micronutrients, and the quality of feed or feeding strategies. Mean corpuscular volume (MCV), the average size of erythrocytes, is calculated as (Ht $\times$ 10)/RBC. Mean corpuscular haemoglobin (MCH), the weight of haemoglobin in the average RBC, is determined as (Hb $\times$ 10)/RBC. Mean corpuscular haemoglobin concentration (MCHC), the relationship between the size of erythrocytes and their haemoglobin content, is calculated as (Hb $\times$ 10)/Ht. These blood indices (MCV, MCH and MCHC) need to be analysed together with erythrocyte morphology, because they provide complementary information.

It is well known that deficiency of various vitamins, minerals and malnutrition (starvation), as well as other feed-related factors often cause anaemia in fish (Smith *et al*., 1974; Plumb *et al*., 1986; Soliman and Wilson, 1992; Mohamed, 2001). A hypochromic microcytic anaemia, where the Ht,

Hb and MCV in the blood are reduced below normal, has been observed in fish fed iron-deficient diets (Sakamoto and Yone, 1978; Gatlin and Wilson, 1985). A macrocytic anaemia that showed marked reduction in Ht, RBC and an increase in MCV of red blood cells also was observed in Indian catfish, *Heteropneustes fossilis*, deprived of pyridoxine (Mohamed, 2001). The development of macrocytic anaemia that is defined by an increase of MCV has been reported for channel catfish (Lim and Lovell, 1978), rainbow trout (Hilton *et al*., 1978) and hybrid tilapia (Shiau and Jan, 1992) fed ascorbic acid deficient diets.

Decreases in Ht, Hb and RBC below the normal ranges are all signs of anaemia. Anaemic fish are sensitive to certain secondary pathogens and show a decreased tolerance to oxygen depletion (Piacentini *et al*., 1989; Haney *et al*., 1992; Rios *et al*., 2005). Maita *et al.* (1996) suggested that anaemia caused reduced ability to produce energy in the gill and kidney of Coho salmon and thus reduced tolerance to secondary stressors. Thus, haematological examination of fish to diagnose anaemia is recommended as a general and rather simple means of health assessment.

### *3.2.1 Parameters of blood smears*

Observations obtained from examination of blood smears are also able to provide much information about the physiological status of fish. For example the percentage of erythroblasts, structural abnormality of erythrocytes, composition of leucocytes and leucocyte counts can be obtained from blood smears. Blood smears are commonly air-dried and stained using the May–Grünwald–Giemsa method and the proportion of each cell type is determined. Total number of leucocytes is estimated in relation to the number of red blood cells (obtained with a haemocytometer), and the proportion of each cell type observed in the blood smear.

Erythroblast abundance is observed as a result of the acceleration of erythropoiesis in different physiological conditions (Ueda *et al*., 2001). Decreases of erythroblast and lymphocyte counts were observed in starved *Hoplias malabaricus* (Rios *et al*., 2005), indicating that reduced erythropoiesis was caused by malnutrition. Rainbow trout deprived of folic acid have been shown to enucleate erythrocytes or possess erythrocytes with segmented or fragmented nuclei (Smith, 1968; Kawatsu, 1975). Appearance of poikilocytes (degenerating and segmented red blood cells) in blood smears provides evidence of nutrient imbalance or insufficient nourishment, and these malformed red blood cells may be removed from circulation, keeping the fish anaemic (Rios *et al*., 2005).

The primary consequence of a low lymphocyte count is immuno-suppression, resulting in an increased susceptibility to disease (Wedemeyer *et al*., 1990). Malnutrition could be a factor leading to a low lymphocyte count as mentioned above. Circulating monocytes/macrophages (represented as a percentage of total leucocytes) was significantly elevated in tilapia fed bacterial-derived β-1,3 glucan (Cain *et al*., 2003). Klinger *et al.* (1996) found that dietary lipid may affect not only the thrombocyte count, but also the

function of these cells. Lee *et al.* (2004) reported that leukocyte numbers were increased and survival was significantly improved by dietary supplementation of maca tuber meal in rainbow trout alevins.

### *3.2.2 Parameters of clinical biochemistry*

The levels of different constituents in plasma have been used as indices for evaluating the physiological and health condition of fish. While examination of the blood may be useful for research on nutrient requirements, new diet ingredients, additives, etc., immunological indices, however, may serve as better indicators when examining the effects of immunostimulants because blood parameters do not easily reflect alteration. Among the various plasma components, the levels of plasma total protein, urea nitrogen, glucose, total cholesterol and triglyceride, as well as activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST; former GOT), and alanine aminotransferase (ALT; former GPT) seem to be useful indices for health assessment in fish.

Plasma total protein level has been measured frequently as an indicator of physiological condition in the studies of fish nutrition (Smith *et al*., 1974; El-Mowafi *et al*., 1997; Lygren *et al*., 1999; Bagni *et al*., 2000; Nakagawa *et al*., 2000; Farhangi and Carter, 2001; Watanabe *et al*., 2001; Harikrishnan *et al*., 2003). Total protein in plasma is the most stable component, and few dietary factors have been reported to affect the levels in fish. Decreases of plasma total protein accompanied with anaemia were reported in fish influenced by the effects of a low fishmeal diet (Takagi *et al*., 2001) or supplementation of gossypol (Yildirima *et al*., 2003). In these cases, as body moisture and protein contents were also lowered in fish showing low plasma total protein levels, a decrease in plasma total protein levels might be accounted for by blood dilution and disturbance of protein metabolism. On the other hand, Dügenci *et al*. (2003) reported that plasma total protein levels in rainbow trout fed diets supplemented with some medicinal plants were significantly elevated. In this case, it is considered that plasma immunoglobulin content should be measured.

The levels of glucose, urea nitrogen, total cholesterol and triglyceride have been reported to reflect the nutritional status of several fish species. Significant decreases in urea nitrogen and total cholesterol levels, and increases in triglycerides were observed in yellowtail starved for a short period (approximately 10 days) (Maita, unpublished data). Although plasma triglycerides and glucose showed a positive correlation with average daily food intake, total cholesterol levels did not show a significant correlation with average daily food intake in European sea bass (Kavadias *et al*., 2003). The positive influence of feeding rate on plasma cholesterol concentration was reported for sea bass (Lemaire *et al*., 1991) under laboratory conditions. On the other hand, in adult farmed Atlantic salmon, plasma total cholesterol levels showed a negative correlation with feeding rate (Sandnes *et al*., 1988). It is well known that the level of plasma glucose is also a good indicator of a stress response in fish.

Plasma urea nitrogen level of rainbow trout was elevated by an increase of dietary arginine (Kaushik *et al.*, 1988) because of the increase in arginine catabolism through the urea cycle. In Coho salmon affected by kidney disease, elevation of plasma urea nitrogen indicated the loss of renal function (Wedemeyer and Ross, 1973).

It is well known that increases in AST and ALT activities indicate injury of liver cells caused by various chemicals or lipid peroxidation. Elevated plasma ALP activity measured in sea bass corresponded to an inflammatory reaction of the bile ducts induced by the diet (Lemaire *et al.*, 1991). It was reported that enzymatic activity of plasma AST increased in a linear fashion, as acclimation temperature increased; however, ALP activity was not related to acclimation temperature in rainbow trout (Miller *et al.*, 1983).

### *3.2.3 Correlation between plasma constituent level and disease resistance*

It is considered that indices of plasma biochemistry to assess fish health condition should be selected based on the relationship between the levels in plasma and disease resistance. Maita *et al.* (1998a, b) have attempted to correlate some blood parameters and mortality due to the bacterial infection in yellowtail *Seriola quinqueradiata* in order to select suitable indices for fish health assessment.

Table 2.1 shows plasma constituent levels in a normal fish population reared under common conditions. Yellowtail were marked individually by placement of a PIT tag in their peritoneal cavity and plasma component levels were measured prior to artificial challenge, and in surviving and dead fish after challenge. Plasma cholesterol levels of fish that died following artificial infection with *Lactococcus garvieae* were significantly lower than that of surviving fish. Significant differences between surviving and dead fish were not observed in other plasma constituents. There was a significant correlation between mortality due to the artificial infection with *L. garvieae* and plasma cholesterol level ($r = -0.951$, $P < 0.01$). The mortality of the fish with low plasma cholesterol levels (lower than 250 mg/100 ml)

**Table 2.1.** Comparison of plasma constituent levels in yellowtail following artificial infection with *L. garvieae*.[a,b]

| | Survived ($n = 23$) | Dead ($n = 11$) |
|---|---|---|
| Body weight (g) | 349 ± 48 | 345 ± 55 |
| Total cholesterol (mg/100 ml) | 274 ± 51 | 238 ± 36[c] |
| Triglyceride (mg/100 ml) | 72 ± 35 | 63 ± 29 |
| Alkaline phosphatase (IU/L) | 137 ± 28 | 145 ± 31 |
| Total protein (g/100 ml) | 2.7 ± 0.3 | 2.5 ± 0.4 |
| Glucose (mg/100 ml) | 110 ± 26 | 110 ± 28 |
| Urea nitrogen (mg/100 ml) | 10.2 ± 1.6 | 9.6 ± 2.1 |

[a] Data shown are mean values ± standard deviation.
[b] Fish were marked individually by PIT tag in their peritoneal cavity.
[c] Significant difference between two categories by *t*-test ($P < 0.05$).

was significantly higher than that of the fish with high cholesterol levels (higher than 275 mg/100 ml) ($P < 0.05$). In the case of rainbow trout, a significant correlation ($r = -0.924$, $P < 0.01$) was established between the plasma cholesterol level and the mortality due to the artificial infection with *Vibrio anguillarum*. These results suggest that fish with a low plasma cholesterol level had lowered disease resistance compared to fish with a high plasma cholesterol level, and the 'compromised host' could be identified by the levels of their plasma cholesterol.

An experiment was conducted to examine the effects of spoiled sardine and oxidized oil on disease resistance in yellowtail. Experimental diets were prepared as follows: a combination of frozen sardine and fresh oil (group 1); frozen sardine and oxidized oil (group 2); spoiled sardine and fresh oil (group 3); and spoiled sardine and oxidized oil (group 4). The effect of spoiled sardine was observed by comparing groups 1 and 3 and the effect of oxidized oil was observed by comparing groups 1 and 2. Table 2.2 shows the mortality due to natural and artificial infection with *L. garvieae* and the plasma constituent levels of yellowtail fed the moist diets. The mortality due to both natural infection and artificial infection with *L. garvieae* in fish fed spoiled sardine (groups 3 and 4) was higher than that in fish fed frozen sardine (groups 1 and 2). Fish fed spoiled sardine and oxidized oil had reduced disease resistance. A significant decline in plasma total cholesterol and urea nitrogen levels was observed in fish fed spoiled sardines. It would be suspected that fish health condition would be worsened by the decrease in plasma cholesterol and urea nitrogen. In group 2, mortality due to natural infection was similar to the control group, however, mortality due to the artificial infection was increased. This suggests that fish fed oxidized oil would be latently fragile. The effects of oxidized oil were observed in elevation of plasma triglyceride and glucose levels.

**Table 2.2.** Mortality and plasma constituent levels[a] of yellowtail fed various moist diets.[b]

| | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| Mortality (%)[c] | 4 | 6 | 13 | 11 |
| Mortality (%)[d] | 5 | 30 | 35 | 35 |
| Total cholesterol (mg/dl) | 218 ± 60 | 195 ± 15 | 163 ± 23 | 142 ± 42 |
| Triglyceride (mg/dl) | 70 ± 33 | 122 ± 25 | 93 ± 28 | 107 ± 25 |
| Alkaline phosphatase (K-U) | 0.7 ± 0.2 | 0.3 ± 0.2 | 0.3 ± 0.3 | 0.8 ± 0.3 |
| Total protein (g/dl) | 3.2 ± 0.1 | 3.7 ± 0.2 | 3.0 ± 0.4 | 3.2 ± 0.4 |
| Glucose (mg/dl) | 86 ± 3 | 104 ± 6 | 92 ± 5 | 150 ± 85 |
| Urea nitrogen (mg/dl) | 26.8 ± 4.7 | 20.7 ± 2.4 | 16.9 ± 3.3 | 14.5 ± 3.5 |

[a] Data shown are mean values ± standard deviation for five fish.
[b] Mash : Sardine : Oil = 47.5 : 47.5 : 5; Experimental diets were: Group 1, a combination of frozen sardine and fresh oil; Group 2, frozen sardine and oxidized oil; Group 3, spoiled sardine and fresh oil; and Group 4, spoiled sardine and oxidized oil.
[c] Mortality due to natural infection with *L. garvieae*.
[d] Mortality due to artificial infection with *L. garvieae*.

In cases where plasma cholesterol levels were lowered by various external factors, such as impaired diet quality, raw sardine feeding in yellowtail (Nakagawa *et al*., 1984) and supplementation of excessive vitamin E in ayu, *Plecoglossus altivelis* (Maita and Lee, 2001), there was a corresponding increase in mortality due to natural infection with pathogens. In red sea bream reared in high-density conditions, the level of plasma cholesterol was significantly lowered and the mortality due to Iridovirus infection was increased (Tanaka and Inoue, 2005). These studies all demonstrate that a reduction in the plasma total cholesterol level is linked to impaired disease resistance in fish.

In yellowtail, feeding of a non-fishmeal diet caused anaemia, hypocholesterolemia and a decrease in disease resistance (Maita *et al*., 1998a). It was suggested that the cause of malnutrition due to the feeding of the non-fishmeal diet was a deficiency of taurine (Goto *et al*., 2001).

The plasma constituent levels and mortality due to natural infection with pseudotuberculosis were compared between two groups of yellowtail fed a non-fishmeal diet manufactured under different processing conditions. Although the compositions of the two experimental diets were identical, diet 1 was manufactured by a large-sized twin-screw extruder, and diet 2 was made by a small-sized single-screw extruder (Fig. 2.2). The two experimental groups were reared in neighbouring net cages and each group were fed the experimental diet at the same feeding rate. Fish fed diet 2 had a shorter gut retention time, significantly lower plasma total cholesterol levels, while levels of triglycerides, urea nitrogen and alkaline phosphatase activity were significantly higher than those of fish fed diet 1 (Table 2.3). These changes suggest that fish fed diet 2 were malnourished compared to fish fed diet 1. It is stressed that these results were obtained at the time when there were no differences in growth performance or mortality between the two groups of fish. Mortality due to natural infection with pseudotuberculosis in fish fed diet 2 was significantly higher than that

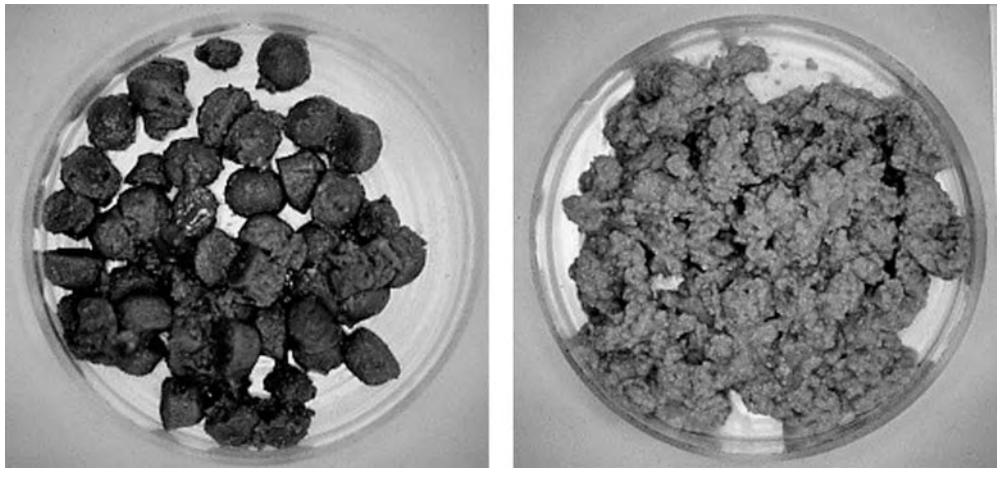

**Fig. 2.2.** Appearance of stomach contents 5 hours after feeding.

**Table 2.3.** Comparison of plasma constituent levels in yellowtail fed experimental diets with different gut retention times.[a]

| | Diet 1 ($n$ = 5) | Diet 2 ($n$ = 5) |
|---|---|---|
| Condition factor (%)[b] | 14.8 ± 0.6 | 15.0 ± 0.7 |
| Total cholesterol (mg/dl) | 240 ± 25 | 193 ± 16[c] |
| Triglyceride (mg/dl) | 81 ± 20 | 133 ± 26[c] |
| Alkaline phosphatase (IU/L) | 137 ± 12 | 168 ± 17[c] |
| Total protein (g/dl) | 3.6 ± 0.1 | 3.4 ± 0.2 |
| Glucose (mg/dl) | 173 ± 33 | 153 ± 30 |
| Urea nitrogen (mg/dl) | 20.6 ± 1.2 | 23.9 ± 1.2[c] |

[a] Data shown are mean values ± standard deviation.
[b] Condition factor (%) = body weight (g)/{fish length (cm)} 3 × 100.
[c] Significant difference between two categories by t-test ($P$ < 0.01).

of fish in the other treatment (Fig. 2.3). Apparently, nutrients were not absorbed sufficiently because of the shorter retention time in the stomach and thus the fish were suffering from malnutrition and were more susceptible to pathogens.

Another experiment was conducted to examine the effects of supplementing taurine and cholesterol in non-fishmeal diets on haematological parameters and disease resistance at the end of 30 and 60 days. The diets were as follows: a control fishmeal diet (FM), a non-fishmeal diet (N), and non-fishmeal diets supplemented with cholesterol (C), or with taurine (T), or with both cholesterol and taurine (CT). The levels of Ht in the control and CT group were significantly higher than that in the other groups after 30 days feeding. After 60 days feeding, the level of Ht in group T was similar to the control and the CT group (Fig. 2.4). Cumulative mortality upon artificial infection with *L. garvieae* in the control and CT groups was significantly

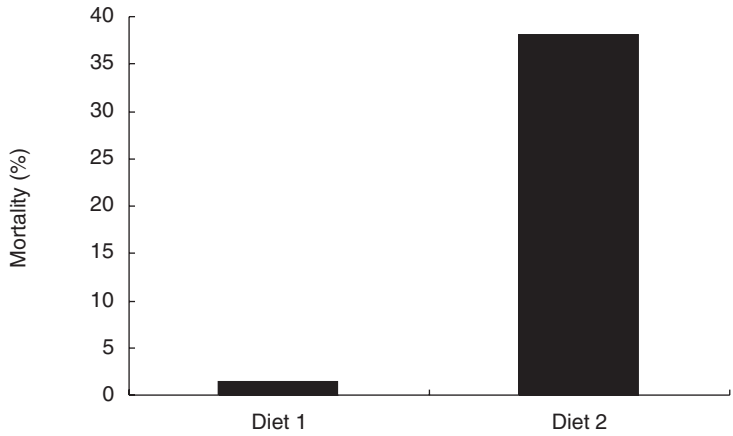


**Fig. 2.3.** Mortality due to natural infection with pseudotuberculosis in yellowtail fed experimental diets that had different gut retention times.

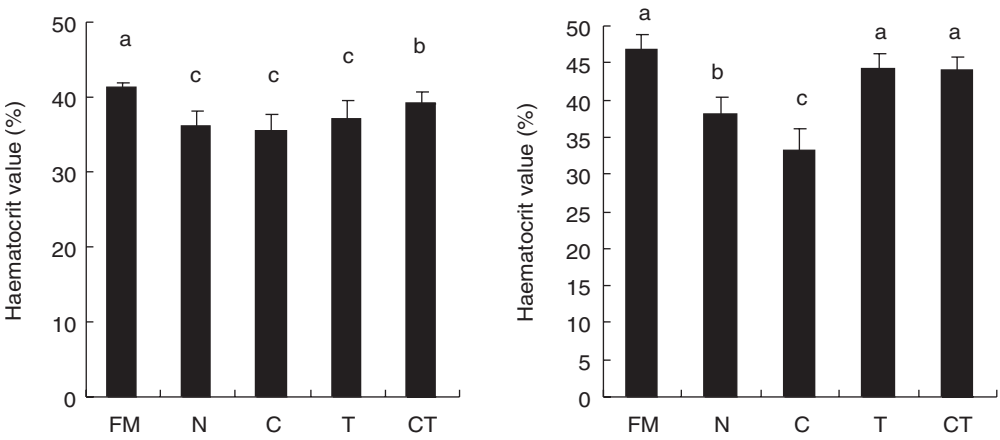


**Fig. 2.4.** The effects on haematocrit value of supplementing taurine and cholesterol in non-fishmeal diets. Data are shown as means and standard deviation for six fish in each group. FM, control diet containing fishmeal; N, non-fishmeal diet; C, non-fishmeal diet supplemented with cholesterol; T, non-fishmeal diet supplemented with taurine; CT, non-fishmeal diet supplemented with both cholesterol and taurine. Different superscripts are significantly different at $P < 0.05$.

lower compared to other groups at day 30; however, mortality in the control, T and CT groups was significantly lower than that of groups N and C in which Ht level remained low (Fig. 2.5). Mortality due to artificial infection was decreased only by both supplementation of cholesterol and taurine at day 30, but improved by the supplementation of taurine alone at day 60. The handling easily killed the fish belonging to groups N and C. Plasma cholesterol levels were increased by supplementation of dietary cholesterol or taurine, which improved lipid metabolism (Fig. 2.6). These results suggest that anaemia is one of the pathophysiological changes that may decrease disease resistance, and dietary taurine and cholesterol may improve lipid metabolism. It is well known that cholesterol is metabolized to bile salts, and bile salts conjugate with taurine and is excreted in the bile. It is considered that feeding a non-fishmeal diet (i.e. deficiency of dietary taurine) could derange lipid metabolism and the derangement may be related to a decrease in disease resistance.

Elevation of plasma cholesterol is observed to accompany steatosis. Accumulation of excessive fat is correlated with an alteration of lipid metabolism, secondary to a deficiency in essential fatty acids (Lemaire *et al.*, 1991). Satoh *et al.* (1996) reported that disease resistance of yellowtail was lowered by an excess dose of dietary lipid. In these cases, increased plasma cholesterol levels do not reflect increased disease resistance.

Changes in plasma cholesterol level as affected by various factors of dietary origin are divided into several patterns in Fig. 2.7. The plasma cholesterol level is affected by dietary cholesterol (exogenous cholesterol) and synthesized cholesterol in the liver (endogenous cholesterol). Pattern A is lowered plasma cholesterol level caused by a shortage of dietary

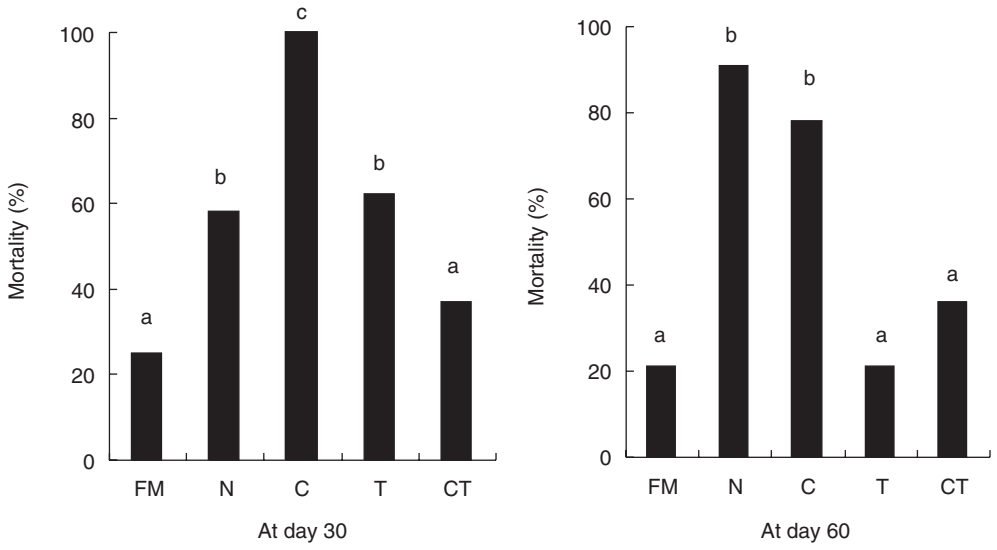


**Fig. 2.5.** The effects of supplementing taurine and cholesterol in a non-fishmeal diet on mortality due to artificial infection with *L. garvieae.* Different superscripts are significantly different at $P < 0.05$. Codes used are as described in Fig. 2.4.

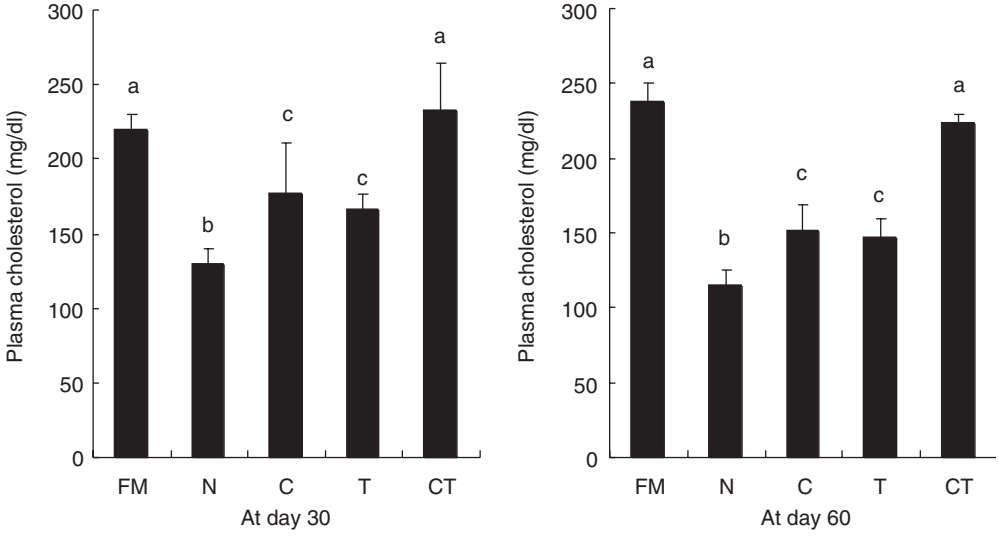


**Fig. 2.6.** The effects on plasma cholesterol levels of supplementing taurine and cholesterol in a non-fishmeal diet. Different superscripts are significantly different at $P < 0.05$. Codes used are as described in Fig. 2.4.

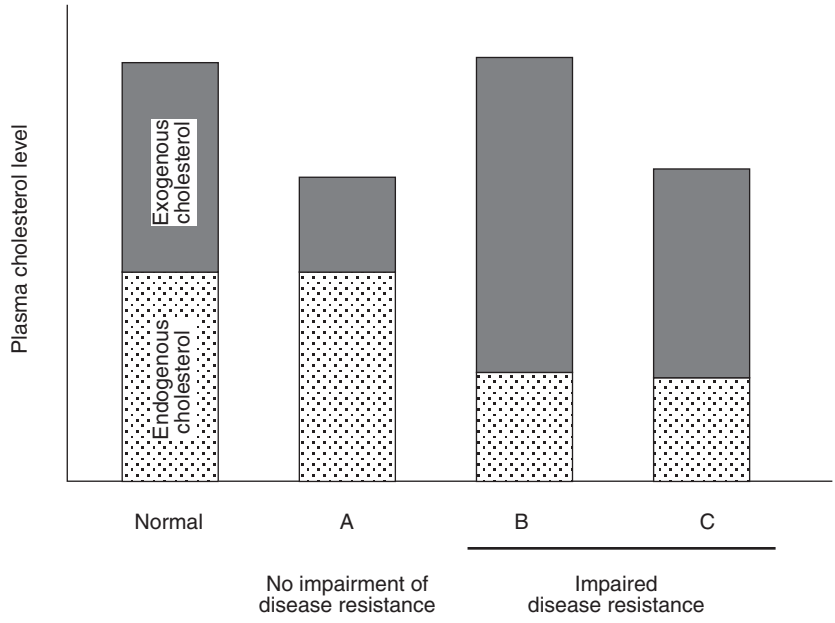


**Fig. 2.7.** Correlation between the level of plasma cholesterol and fish health condition.

cholesterol; however, endogenous cholesterol is maintained at a normal level. Pattern B is observed in the case of an excess dose of dietary cholesterol. The plasma cholesterol level is maintained at a normal level; however, endogenous cholesterol decreases. Pattern C indicates that low plasma cholesterol levels are caused by a decline in cholesterol synthesis, while fish are fed a normal diet. Fish belonging to patterns B and C would have impaired disease resistance.

### 3.3 Examination of immune function

As fish are primitive vertebrates they possess both non-specific defence mechanisms, such as phagocytosis, that are characteristic of invertebrates, as well as specific humoral and cellular responses mediated by lymphocytes, as in higher vertebrates (Table 2.4). They rely more on their non-specific defences, primarily the skin and mucus. When they encounter a pathogen, cellular mechanisms like phagocytosis are initiated, aided by humoral factors like complement and lysozyme. There also are other cells such as the natural killer cells and several other soluble factors that are involved at different stages. If the non-specific defences are not sufficient to ward off the infectious agent, then disease will develop leading to the induction of specific defence mechanisms. The cells functioning here are macrophages that are antigen-presenting cells and T-lymphocytes that participate in cell-mediated immunity and B-lymphocytes that are antibody producers and protect the fish for a certain period. These components of the

immune system in fish influence some responses that fish nutritionists have attempted to monitor in order to describe the role of dietary nutrients in immunity.

A number of researchers have investigated the relationship between nutrients and the immune responses of fish. Various responses of the immune system have been measured to evaluate their functions. However, the methodologies adopted in each study have not always been standardized so that the results cannot be compared and occasionally results from different studies are contradictory. The methodology used to study immune function in fish has been covered by Stolen *et al*. (1990). Table 2.5 summarizes the different immunological assays used in nutritional research and their sensitivity, variability and ease of use from a practical point of view.

The leucocytes used for examination of immunological parameters are usually collected from peripheral blood or head kidney by density gradient

**Table 2.4.** Immunological defence mechanisms in fish.

| Components | Non-specific | Specific |
|---|---|---|
| Natural barriers | Skin and mucus<br>Lysozyme | |
| Cellular defence mechanisms | Phagocytes | Macrophages |
| | Non-specific cytotoxic cells | Lymphocytes (T and B cells) |
| Humoral defence mechanisms | Complement | Antibodies |
| | Lysozyme | Cytokines |
| | Cytokines | |

**Table 2.5.** Immunological assays that have been employed in nutritional studies on fish indicating their level of suitability.

| Assay | Function | Sensitivity | Variability | Ease of use |
|---|---|---|---|---|
| *Non-specific responses* | | | | |
| Phagocytic ability | Phagocytosis | Good | Wide | Fair |
| Oxidative burst | Phagocytosis | Excellent | Medium | Fair |
| T and B cell mitogenesis | Lymphocyte differentiation | Fair | Wide | Fair |
| Blastogenesis (RIA)[a] | Lymphocyte differentiation | Good | Medium | Difficult |
| Natural killer activity (RIA) | Lymphocyte or neutrophil function | Good | Wide | Difficult/Fair |
| Complement activity | Lysis of bacterial cell membrane, chemotaxis of phagocytes, splitr | Good | Wide | Fair |
| Lysozyme | Mucopolysaccharides from bacterial cell wall | Good | Medium | Fair |
| *Specific responses* | | | | |
| Melanomacrophage centre | Haemopoesis | Good | Medium | Moderate |
| Passive haemolytic plaque assay | Antibody production | Good | Narrow | Moderate |
| ELISA | Antibody titre | Excellent | Narrow | Difficult |

[a] RIA, Radio Immuno Assay.

centrifugation (Secombes, 1990; Rowley, 1990; Jeney *et al*., 1997; Sealey and Gatlin, 2002). As the band lying in each density interface is enriched in different leucocyte populations, optimum density should be established and chosen to obtain the appropriate cell population.

Phagocytes are the most important cells in non-specific cellular mechanism and are also helped by several soluble factors such as complement and lysozyme. It is well known that fish treated with immunostimulants show increased phagocytosis as well as respiratory burst activity. Both phagocytosis and respiratory burst are affected by several factors including the nutrients, temperature and pathogens. Fish phagocytic cells are able to engulf bacteria and kill them by generating a superoxide anion ($O_2^-$) and its derivatives such as hydrogen peroxide ($H_2O_2$) and hydroxyl-free radicals ($OH^-$) in the process known as respiratory burst. These reactive oxygen intermediates have potent bactericidal activities. Phagocytic activity (PA) and phagocytic index (PI) are determined by various procedures with minor modifications (Seeley *et al*., 1990; Yoshida *et al*., 1993; Bhatia *et al*., 1994). Congo red stained yeast cells, latex beads, fluorescent latex beads and opsonized zymosan have been used as materials, which are engulfed by the cells. The relatively small differences in phagocytic rates may be a result of the type of material used and the length of time the cells are incubated. Bactericidal activity of phagocytic cells is measured by two methods (detection of extracellular and intracellular activities). The intracellular superoxide anion production is often determined using the procedure of Secombes (1990) or Anderson and Siwicki (1995). Dügenci *et al*. (2003) measured the reduction of ferricytochrome *c* to determine levels of extracellular $O_2^-$. The reactive oxygen intermediates (ROIs) produced by stimulated phagocytes is also quantified using an automatic photoluminometer (Lygren *et al*., 1999; Kim *et al*., 2002). The chemiluminescent response measures the respiratory burst activity of phagocytic cells in which oxygen is converted into ROIs. Flow cytometry is an effective technique recently applied to examine phagocytosis (Thuvander *et al*., 1987; Chilmonczyk and Monge, 1999) and respiratory burst activity (Ortuño *et al*., 2000; Moritomo *et al*., 2003).

Non-specific cytotoxic cells (NCCs) in fish have been reported to resemble natural killer (NK) cells in mammals (Graves *et al*., 1984). NCCs from peripheral blood and head kidney of fish have been found to possess spontaneous cytotoxic activity against a mammalian cell line (Hinuma *et al*., 1980; Evans *et al*., 1984; Morita *et al*., 1989), protozoans (Graves *et al*., 1985) and certain cultured tumour cells (Moody *et al*., 1985). In rainbow trout, the occurrence of NCCs has been reported by Sakai (1984) and later found responsible for non-specific killing of virus-infected cells (Yoshinaga *et al*., 1994). The activity of the NCCs from the head kidney of rainbow trout has been reported to be influenced by the essential fatty acids and minerals such as zinc and manganese (Kiron *et al*., 1993; Inoue *et al*., 1998). The $^{51}Cr$ release and lactic acid dehydrogenase (LDH) release assays are general methods used for measuring the cytotoxic activity of the NCCs.

Lysozyme is an important enzyme in blood that actively lyses bacteria and an increased level of this enzyme has been considered to be a natural protective mechanism in fish (Ingram, 1980). Lysozyme has an antibacterial activity by attacking the peptidoglycan in the cell wall of bacteria, predominantly Gram-positive bacteria, thereby causing lysis and stimulation of phagocytosis of bacteria by phagocytic cells (Ellis, 1990). Neutrophils are thought to be the source of lysozyme, and the enzyme appears to be much more bactericidal than lysozyme of higher vertebrates (Ellis, 2001). Lysozyme levels in biological samples (plasma, serum, mucus) are typically assayed based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus luteus* (*Micrococcus lysodeikticus*) that can be measured turbidimetrically (Siwicki and Anderson, 1993), or on an agarose plate (Osserman and Lawlor, 1966).

The Alternate Complement Pathway (ACP) activity is very high in fish serum as compared with mammals (Yano, 1996), suggesting that this pathway is very important as a defence mechanism of fish (Ellis, 2001). Measurement of serum haemolytic complement activity has been accomplished with the microtitre plate technique as described by Ingram (1990). Nutrients such as lipids, vitamin E, vitamin C, carotenoids, etc. have been found to modulate the complement activity in fish (Blazer and Wolke, 1984; Li and Lovell, 1985; Montero *et al*., 1998; Pearce *et al*., 2003; Amar *et al*., 2004).

Chemotaxis is the major mechanism for defending the host against infection and injury (Imhof *et al*., 1990). Lim and Klesius (1997) determined chemotaxis in channel catfish by a modification of the lower-surface method of Boyden (1962) as described by Klesius and Sealey (1996).

The proliferative response of head kidney lymphocytes was determined by the MTT [3-(4,5-Dimethyl thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide] colorimetric assay method described by Mosmann (1983) as modified for use with fish lymphocytes by Siwicki *et al*. (1996). Total plasma immunoglobulin (total Ig) levels in serum have been determined by a colorimetric assay (Anderson and Siwicki, 1995). To measure the potential killing activity of head kidney macrophages, the method of Rook *et al*. (1985) as modified by Siwicki and Anderson (1993) has been used.

### 3.4 Experimental challenge test

Because current methodology to comprehensively investigate immunity and disease resistance of fish is still limited, an effective biomarker for disease resistance of fish has been difficult to identify (Li and Gatlin III, 2006). Recently, Aoshima *et al*. (2005) reported that increased non-specific defence responses *in vitro* do not always reflect increased disease resistance. Therefore, the ultimate integrated responses of various immune mechanisms should be tested with a properly planned and standardized artificial challenge with an infectious agent.

The majority of reports have described increased resistance to mainly bacterial infections in the disease challenges. However, a few reports have

shown the effectiveness of nutrients on the resistance to parasitic and viral pathogens. There are some reports indicating dietary modification or feed additives may affect the resistance of fish to challenge with infectious hematopoietic necrosis (IHN) virus (LaPatra *et al*., 1998). Burrells *et al*. (2001) reported beneficial effects of dietary nucleotides when challenging salmonids with infectious salmon anaemia virus, *V. anguillarum* and a rickettsial organism (*Piscirickettsia salmonis*).

One of two different methods are usually adopted for a challenge with a pathogenic organism and these are a waterborne challenge or intraperitoneal injection. It seems that waterborne challenge more closely resembles natural infection in comparison with intraperitoneal challenge. If fish are immersed in aquaria containing a pathogen, and each group of fish is then returned to their respective aquarium, at least three replicate tanks per treatment should be used because mortality may vary considerably among the replicate tanks.

Immersion (bath) challenge, in which experimental fish are immersed in water containing a certain amount of bacteria for a specified period of time, has often been used as a means of providing a waterborne challenge. The disadvantage with this method is that the fish may be affected by handling stress. An alternative method is cohabitation of experimental fish (recipients) with diseased fish (donor) in the same tank. Fukuda *et al*. (1997) adopted a method in which a fixed bacterial suspension was introduced into a tank by use of a peristaltic pump. These two means of challenge will minimize handling stress during exposure to the pathogen.

The pathogenicity of the disease-causing agent and dose should be adjusted to detect differences in disease resistance of fish. If fish are overexposed to pathogenic organisms, their cumulative mortality may be elevated regardless of their health condition. Barros *et al*. (2002) examined the $LC_{50}$ (lethal concentration which causes 50% mortality in exposed fish) of *Edwardsiella ictaluri* prior to experimental challenge to determine the optimum bacterial cell concentration to use. This is a reasonable method to determine the effective dose of pathogen.

Hypothetically, an experimental challenge that more closely mimics natural infection should provide a truer indication of a fish's disease resistance. Lygren *et al*. (1999) conducted an experimental challenge by the following protocol. Fish were marked according to dietary group using a Panjet (Wright Dental Group, Kingsway West, UK). Fish from each tank were randomly selected and moved to the challenge facilities. The fish were redistributed in three tanks with all the dietary groups represented in equal numbers. After an acclimatization period of 1 week, the fish were challenged using 12 cohabitants/tank injected intraperitoneally with 0.2 ml of a $2.1 \times 10^5$ colony-forming units (CFU)/ml suspension of *Aeromonas salmonicida* ssp. This protocol would closely mimic natural infection and eliminate variation among replicate tanks, but would preclude continuing to feed experimental diets during the disease exposure.

Regarding the effects of glucan on disease resistance, increases in non-specific resistance have been demonstrated in response to challenge

infections with *V. anguillarum*, *Vibrio salmonicida* or *Yersinia ruckeri* (Robertsen *et al*., 1990), *V. anguillarum* and *V. salmonicida* (Raa *et al*., 1992) and *A. salmonicida* (Nikl *et al*., 1993; Siwicki *et al*., 1994). However, no such benefit has been shown against *A. salmonicida* and *V. salmonicida* (Dalmo *et al*., 1998), *Enterococcus* species in turbot (Toranzo *et al*., 1995) or *V. anguillarum* in turbot (Ogier de Baulny *et al*., 1996). The results of previous studies on the effect of ascorbic acid on disease resistance in channel catfish are not consistent (Lim *et al*., 2000). In addition, information on the effects of ascorbic acid on disease resistance is also contradictory to that of salmonids. Thus, the differences between the results of those studies may be related to differences in species, strain, size and nutritional status of the fish used, pathogenicity of the bacteria and/or methods of disease challenge. Therefore, optimization and standardization of these methods should be carefully established. The proper procedures for a challenge test in the field of fish nutrition may be different from that in fish pathology. A standardized protocol for experimental challenge suitable for the studies of fish nutrition and a data validation system among laboratories should be developed in order to evaluate disease resistance in fish.

The size and sexual maturity of the experimental fish as well as the stressors of the rearing system affect the observations discussed in this section. Hence these points have to be given due consideration in interpreting the results from any study. It is difficult to evaluate fish health condition by a single indicator because the individual indicator may vary in sensitivity and precision. In addition, each indicator often shows wide individual variation among fish. A combination of measuring several parameters as well as using a standardized challenge test is recommended to assess fish health more comprehensively (Wedemeyer and McLeay, 1981; Sandnes *et al*., 1988).

## 4 Maintenance of Fish Health by Effective Feeding Regimes

The relationship between nutrition and fish health requires careful attention be paid to the effects of feeding regimes on fish health. Kim and Lovell (1995) first reported the effect of restricted feeding on disease resistance in channel catfish. They showed that plasma antibody titre and the survival rate in young fish (1 year old) following artificial infection with *E. ictaluri* were significantly higher in fish fed every day than those fed every other day or not fed at all. No differences, however, were observed between the fish fed every day and every other day in adult fish (2 years old). According to another report, it is suggested that humoral and cellular defences are not affected by starvation but disease resistance was improved in catfish (Okwoche and Lovell, 1997). In larval and juvenile Japanese flounder and red sea bream, excessive feeding caused lethal histopathological alterations, and increased mortality (Mobin *et al*., 2000, 2001). Self-feeding systems reduce stress and boost immune defences, as indicated by an increase in anticoagulant titre and lymphocyte counts (Endo *et al*., 2002).

Management of fish health by improving nutritional status and adopting effective feeding regimes will become more important in the future. However, many problems remain to be solved.

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