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POSITIVE CORRELATION OF BODY LENGTH WITH INNATE IMMUNE STATUS AND RESISTANCE TO *AEROMONAS HYDROPHILA* INFECTION IN ROHU, *LABEO ROHITA*

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Full-sib families of rohu (*Labeo rohita*) of four year-classes *viz.*, 2003 (906 numbers of fish), 2004 (1208 nos.), 2008 (2000 nos.) and 2009 (1355 nos.) were subjected to intraperitoneal *Aeromonas hydrophila* challenge. The fingerlings were tagged individually with passive integrated transponder (PIT) tag and total length was measured prior to challenge. The mortality was recorded on an hourly basis for up to 24 h and later over a period of 10 days post-challenge. Mean body length of survived and dead fish were significantly different ($p<0.05$) in the year-classes 2004, 2008 and 2009, whereas for 2003 there was no marked difference. Positive correlations of 0.004, 0.115 and 0.108 were found between length and survival for 2004, 2008 and 2009 year-classes, respectively. However, the length of 2003 year-class showed a negligible negative correlation (-0.065) with survival. The serum immune parameters *viz.*, haemolysin titre, ceruloplasmin and myeloperoxidase activities analyzed from naïve individuals of each family showed positive correlations with body length of the same family (0.135, 0.290 and 0.109, respectively) in three year-classes i.e., 2003, 2004 and 2009. The positive correlation of length with survival and immune response indicated its possibility to be used as a co-trait for selection for improved resistance to *A. hydrophila* infection.

INTRODUCTION

Recently selective breeding for quantitative traits like growth, meat quality, reduced fat content and disease resistance is getting worldwide importance in aquaculture breeding programs. Selecting multiple traits such as growth and disease resistance simultaneously could lead to improved quality stock with better production efficiency and ultimately resulting in reduced fish loss due to disease. Several studies have reported a relationship between growth trait (weight and length measures) and survival. In many fishes such as common carp (Tran and Nguyen, 1993), tilapia (Kamel *et al.*, 2008), Atlantic salmon (Gjedrem, 1979), rainbow trout (Donaldson and Olson, 1957) and channel catfish (Dunham, 2006), body growth positively influences pond survival. When it comes to survival against diseases, this picture is not quite clear. A positive genetic correlation between growth and disease resistance has been established in Atlantic halibut against *Vibrio anguillarum* (Imsland *et al.*, 2002). However, genetic correlation between growth and disease resistance has not been detected in few studies (Standal and Gjerde, 1987; Beacham and Evelyn, 1992; Barroso *et al.*, 2008; Silverstein *et al.*, 2009) and also few species showed negative correlations between resistance and body weight (Robison and Luempert, 1984; Argue *et al.*, 2002; Henryon *et al.*, 2002). Another big challenge is to introduce an additional trait into an already existing selection program. When animals are selected for improved weight or length, alleles for these traits get accumulated within strains, if the population is not under selective pressure to retain all the diverse alleles. This reduction in genetic diversity due to the selection process may limit the influence of another trait such as resistance of animals to pathogens (Overturf *et al.*, 2010). Therefore, it is important to mention that any correlation between disease resistance and growth is probably specific with regard to species, stock of fish and the pathogen being tested (Overturf *et al.*, 2010).

Since growth is a measure of length and weight, reports relating to individual relationship of these two traits with disease survival are available. Significant positive correlations between harvest body weight and resistance to infectious haematopoietic necrosis (IHN) and, for early body weight and resistance to bacterial coldwater disease (BCWD) in rainbow trout were suggested (Overturf *et al.*, 2010). Low positive genetic correlation between survival time against furunculosis exposure and weight in O+ brook charr (*Salvelinus fontinalis*) has also been reported (Perry *et al.*, 2004). However, in field conditions, measurement of body length has partial advantage over body weight. The body length can be measured accurately in a few seconds as compared to body weight. There is also a strong genetic correlation (0.96-0.99) between body weight and length of fish (Navarro *et al.*, 2009). Hence, it is sufficient to resort to recording either of the two traits as evidenced by the high genetic correlation. A study of genetic correlation between body length of gilthead sea bream (*Sparus aurata* L.) and survival to fish pasteurellosis was found positive and significant ($r = 0.61 \pm 0.16$) (Antonello *et al.*, 2009).

Though disease survival rate is an ultimate method of testing resistance, there exist reports regarding the relationship between immune response and disease survival. Serum IgM level has been shown in cod (*Gadus morhua*) (Magnadottir *et al.*, 1999) and benni (*Barbus sharpeyi*) (Ansari *et al.*, 2011) to increase with increasing weight. However, the specific immunological response depends upon the pathogen, the age of fish at the time of infection and how the immune system responds to the pathogen. There are differences in immunological responses at different growing periods of a fish (Manning and Tatner, 1985) and, there are reports of association between nutritional and energetic cost with the activity and maintenance of an efficient immune system (Lochmiller and Deerenberg, 2000).

Among the three Indian major carp species, rohu, *Labeo rohita* is an important freshwater aquaculture species in South Asia (FAO, 2010). There exists a report that *Aeromonas hydrophila* infections in rohu gave rise to hemorrhagic septicemia that caused a cumulative mortality of nearly 2% per day in a polyculture farm in Odisha (Mohanty *et al.*, 2008). In the selective breeding program at the Central Institute of Freshwater Aquaculture, Bhubaneswar, India, rohu have been selected for higher growth (both weight and length) for over eight generations. However, the relationship between growth and disease resistance could not be estimated due to inconsistent results (Mahapatra *et al.*, 2008).

In the present study, we tried to correlate growth and resistance to *A. hydrophila* in rohu, *L. rohita* by taking body length as a measurable trait for growth.

MATERIALS AND METHODS

Fish

The rohu fingerlings used for challenge study were collected from four different year-classes over three generations generated under the selective breeding program for rohu carp initiated at the Central Institute of Freshwater Aquaculture (CIFA), India, in 1992 under an Indo-Norwegian collaboration between CIFA and Institute of Aquaculture Research (AKVAFORSK), Norway. Four year-classes (2003, 2004, 2008 and 2009) were included in the analysis. In the selective breeding program, the year-classes of 2003 and 2004 represented generation five, while year-classes 2008 and 2009 represented generation seven and eight, respectively. The full-sib families of each year-class were initially reared in separate 100 m^2 earthen ponds at CIFA with a water temperature of 22 to 30 °C. At a size of about 25-45 g fish from each family were brought to 700 L ferro-cement tanks in indoor wet laboratory. The fingerlings were tagged individually with passive integrated transponder (PIT) tags prior to challenge testing.

Challenge test and measurement of body length

In total, 906 fish (27 families) from 2003, 1208 fish (34 families) from 2004, 2000 fish (12 families) from 2008 and 1355 fish (22 families) from 2009 year-classes were challenged separately over four different years with an optimum LD_{50} dose of *Aeromonas hydrophila*. Only PBS was injected to the control group. The challenge tests were carried out in the month of April and May each year (2003, 2004, 2008 and 2009). Fishes were collected from the nursery ponds during each year and kept in indoor cement tanks for one week prior to challenge for acclimatization under standard rearing conditions. An overnight culture of *A. hydrophila* was grown in tryptone soya broth at 30°C for 20 h. The LD_{50} bacterial dose required was determined prior to the experiments with a representative sample of fingerlings collected from all families. Individual fish were injected intraperitoneally with LD_{50} dose of *A. hydrophila* (5×10^6 cfu 0.1 ml^{-1} in 2003, 2004 and 2009; 2×10^6 cfu 0.1 ml^{-1} in 2008), and were kept in duplicate cement tanks of 50 m^2. Length of each fish was recorded prior to challenge. Hourly reading of mortality for each individual in each family was recorded for up to 24 h and the overall mortalities were recorded up to 10th day post-challenge.

Blood collection

Fish were collected from 27 families from 2003 year-class, 33 families from 2004 year-class and 22 families of 2009 year-class (10-25 numbers from each family) from the nursery ponds and kept in 700 l cement tanks separately. Unfortunately, blood samples from 2008 year-class fingerlings could not be collected due to mixing of stock with sudden natural calamity. After one week of acclimatization, blood samples were collected from the caudal vein of fish after anaesthetization, using 2 ml plastic syringe. The blood samples were allowed to clot at room temperature for an hour and then stored at 4°C for 2-3 h. Serum samples were collected from the clotted blood by centrifugation with 2810 x g for 5 min and stored at -30°C till further analysis.

Measurement of immune parameters

Three different serum immune parameters *viz.*; haemolysin titre, ceruloplasmin level and myeloperoxidase activities were studied from 79, 41 and 22 numbers of families, respectively.

Haemolysin titre

Haemolytic activity of unheated serum was determined by serial dilution with PBS and using rabbit RBC (Kumari and Sahoo, 2005). Haemolysin titre was defined as the last dilution of serum showing positive haemolysis.

Ceruloplasmin activity

Ceruloplasmin activity in serum was measured as p-phenylenediamine (PPD) oxidase activity as described by Pelgrom *et al.* (1995) and Dautremepuits *et al.* (2004) with minor modifications. Briefly, 25 µl of serum was mixed with 0.5 mL acetate buffer (1.2 M, pH 5.0) containing 0.1% PPD as substrate. At the same time a blank was taken by adding 0.5 mL 0.5% sodium azide (NaN_3) to 25 µl of same serum sample. Both the mixtures were incubated for 30 min at 30°C. The reaction was stopped by addition of 0.5 mL 0.5% NaN_3. One unit of ceruloplasmin was defined as the amount of oxidase that catalyzed a decrease in absorbance of 0.001 min^{-1} at 550 nm.

Myeloperoxidase activity

The myeloperoxidase assay was carried out as described by Quade and Roth (1997) with a partial modification as described by Sahoo *et al.* (2005). Briefly, 10 µl of serum was diluted in 90 µl of Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well micro titre plate to which 35 µl of 1:20 diluted 3, 3′, 5, 5′ tetramethylbenzidine hydrochloride (TMB) (Genei, India) with H_2O_2 was added. The plate was incubated for 2 min at RT and 35 µl of 4 M sulphuric acid was added to stop the reaction. The optical density was read at 450 nm in a microtitre plate reader (Anthos 2010, Austria).

Statistical analysis

For total length, a t-test was carried out for all the survived and dead individuals in four different year-classes separately. Pearson's correlation was estimated between body length of all challenge-tested fish with disease survival separately in four different year-class and from the pooled data as well, through a linear model to observe binary response at median time (24 hours post challenge) where (y_i=0 if the fish died post challenge and y_i=1 if it survived post challenge) in PASW statistics 18 software of SPSS. Bootstrap test was conducted to test the significance and standard error of the estimated correlations. Correlation between length and survival was estimated in individual year-class and by taking pooled data of all four year-classes. Similarly, correlations of the mean of all the immune parameters tested were analyzed with the respective length data of that family combining all the year-classes.

RESULTS

The mortality started after first hour of challenge and recorded from 2nd hour onwards. At the end of 10 days the percentage survival records were 47.13, 56.71, 29.83 and 31.61 for 2003, 2004, 2008 and 2009 year-class, respectively (Fig. 1). No mortality was observed in the control group. The details of dead and survived individuals are given in Table 1. The statistical analysis revealed significant difference ($p<0.05$) between the mean length of survived and dead individuals in 2004, 2008 and 2009 year-classes, whereas in 2003 year-class, no significant difference was found in the length of dead and survived individuals. Correlations between length and survival calculated over three generations for 2004, 2008 and 2009 year-classes were found to be 0.004 ± 0.027, 0.115 ± 0.022 and 0.108 ± 0.026, respectively (Table 2). However, the length of 2003 year-class fish showed a negligible negative correlation (-0.065 ± 0.032) with survival. The correlations of 2008 and 2009 year-classes were found to be significant at the level of $p<0.01$ (2-tailed). The overall correlation of length in all the year-classes to their survival records was found to be 0.008 ± 0.014 and not significant.

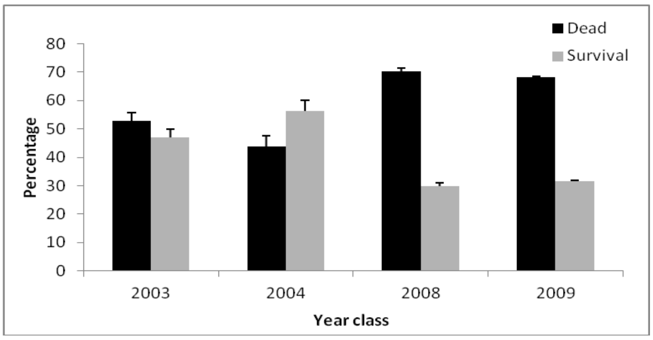

Fig. 1. Percentage dead and survival results of rohu during *Aeromonas hydrophila* challenge in four different year-classes

Table 1. Details of dead and survived individuals of rohu during *Aeromonas hydrophila* challenge test in four different year-classes studied in duplicate tanks

Year class	Tank 1			Tank 2		
	Total no. of fish challenged	Dead	Survived	Total no. of fish challenged	Dead	Survived
2003	441	246	195	465	233	232
2004	616	246	370	592	281	311
2008	987	704	283	1013	699	314
2009	649	445	204	706	481	225

Table 2. Mean length of dead and survived fish of different year-classes used in challenge test and Pearson's correlation measured between length and survival of fish of a particular year-class

Year class	Mean length of survival (mm)	Mean length of dead (mm)	Pearson's correlation
2003	$147.69^a\pm1.51$	$151.99^a\pm1.00$	-0.065±0.032
2004	$124.17^a\pm1.31$	$115.91^b\pm1.07$	0.004±0.027
2008	$98.93^a\pm0.49$	$95.42^b\pm0.47$	0.115±0.022*
2009	$132.72^a\pm1.64$	$124.66^b\pm1.14$	0.108±0.026*

Means bearing different superscript within a row (between survival and dead) are significantly different at $p<0.05$ level.
*Correlation is significant at $p<0.01$ level (2-tailed)

The immune parameter studied here showed a wide variation among families (Fig. 2). The family means of each of the three immune parameters studied over two generations of 2003, 2004 and 2009 year-classes showed a positive correlation with mean length of the respective family i.e., 0.135 ± 0.104 for haemolysin titre, 0.290 ± 0.147 for ceruloplasmin activity, and 0.109 ± 0.103 for myeloperoxidase activity (Table 3). However, the correlations were not significant.

Table 3. Pearson's correlation between length and innate immune response of fish measured over three year-classes (2003, 2004 & 2009)

Immune parameters	Correlation with body length
Heamolysin titre	0.135±0.104
Ceruloplasmin level	0.290±0.147
Myeloperoxidase assay	0.109±0.103

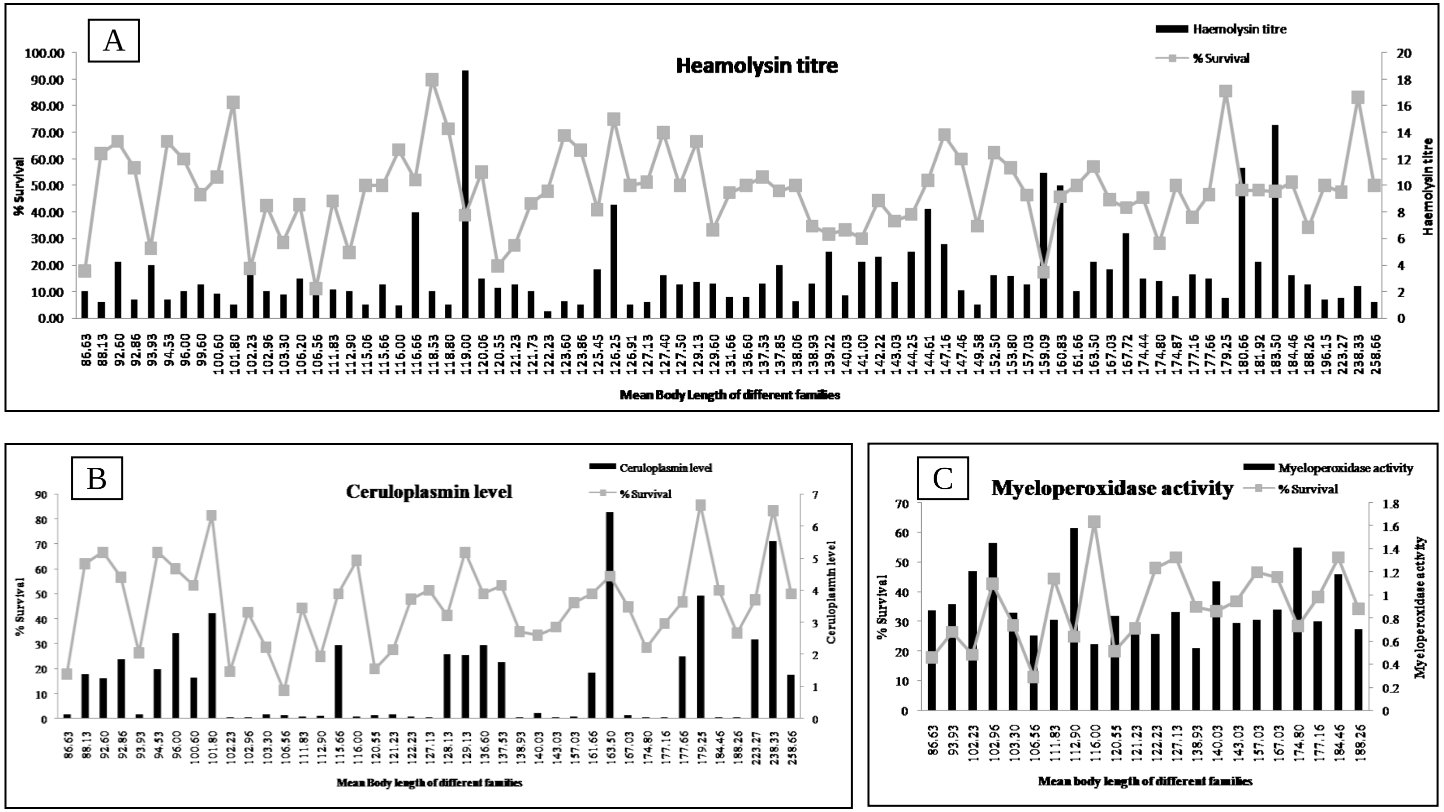

Fig. 2. Graphs showing mean body length (in mm) plotted in X-axis and percent survival to *Aeromonas hydrophila* challenge plotted as primary Y-axis and immune parameters plotted as secondary Y-axis of different families of rohu studied during three year-classes (2003, 2004 and 2009). The natural haemolysin titre (a) was studied in 79 families, the serum ceruloplasmin level (b) studied in 41 families and serum myeloperoxidase activity (c) studied in 22 families of naïve fish.

DISCUSSION

The two commercially important traits, growth and disease resistance in aquaculture research are quantitative and are under the control of hundreds of genes. Resistance to a pathogen may be inferred, not only by survival but also by the surviving individuals' ability to maintain growth after exposure to the pathogen. Initially, the selective breeding program started at CIFA, Bhubaneswar focused on improvement of body growth in rohu. In a subsequent study, wide variation among different families of fish to *A. hydrophila* infection was noticed (Sahoo *et al.*, 2004). However, the relationship between growth (weight and length) and disease resistance could not be estimated (Mahapatra *et al.*, 2008). In 2003 year-class, the average length of both the survived and dead individuals did not differ significantly. However, 2004, 2008 and 2009 year-class families showed higher survival in longer fishes compared to shorter ones. The correlation of length and survival in successive generations (2004, 2008 and 2009) was consistently positive. Though the correlation is not significant in 2004 year-class, it was significantly high in the subsequent generations of 2008 and 2009. Therefore, there is a possibility of improvement in the successive generations as well. Similarly, the genetic correlation between body length and survival to pasteurellosis measured in gilthead sea bream (*Sparus aurata* L.) has been reported to be positive and significant (r=0.61±0.16) (Antonello *et al.*, 2009). This indicated the possibility of selection for disease resistance in breeding programmes in which size is the selected traits.

Further, this study also examined the existence of correlation of body length to innate immune status of fish. Disease resistance can be studied by indirect recordings of indicator traits such as immune parameters. An acute phase protein, ceruloplasmin functions in a variety of defense related activities such as limiting the dispersal of infectious agents, repair tissue damages, killing of microbes and other potential pathogens (Dautremepuits *et al.*, 2004). The ceruloplasmin level revealed association with aeromoniasis survival in rohu (Sahoo *et al.*, 2008) and was significantly higher in case of resistant line than that of susceptible line (Sahoo *et al.*, 2011). In the present study, serum ceruloplasmin revealed a positive correlation (0.29) with body length of all the year-classes. There was a simultaneous increase in disease survival rate and ceruloplasmin level with an increasing body length. The complement pathway recognizes the lipololysaccharide membrane present in the cell wall of Gram-negative bacteria and helps in lysis of bacterial cell membrane through antibody-antigen complex, which can be measured by haemolysin titre. In the present study, the haemolysin titre showed a slightly positive correlation (0.135) with body length. The level of myeloperoxidase can be a strong indicator of immune status since it involved in the production of hypohalites, which is highly bactericidal. Increased body length also showed a slightly positive correlation with myeloperoxidase activity (0.109). The parameters checked here were mostly from 2003 and 2004 year-classes where length was not correlating well with survival. Since among the positively correlated generations only 2009 year-class was being tested, the correlations of immune responses with length were not significant. To our knowledge, this is the first study where genetic correlations between body length and the above important innate immune parameters are estimated. The results indicated the survival rate of the fish in the generations tested is due to strong immunological defense system, which became stronger with increased growth of fish. Similarly, earlier studies have shown serum immunoglobulin M (IgM) concentration being correlated positively with fish size in benni (Ansari *et al.*, 2011) and weight in cod (Magnadottir *et al.*, 1999).

In conclusion, positive correlations were estimated for body length and survival to *A. hydrophila* challenge, and innate immunity parameters of the fish. Body length might be considered as an indirect trait for selective breeding for disease resistance. This may replace challenge tests where large numbers of fish are sacrificed.

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