

1 **HEMAGGLUTINATION ANTIBODIES TITER IN PACU, *Piaractus mesopotamicus*,**  
2 **AS AN INDICATOR OF ACQUIRED IMMUNITY.**

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4 TÍTULO DE ANTICORPOS HEMAGLUTINANTES DE PACU, *Piaractus mesopotamicus*,  
5 COMO INDICADOR DE IMUNIDADE ADQUIRIDA  
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8 **SUMMARY**

9 The evaluation of the hemagglutination titer is an option to evaluate the response of acquired  
10 immune system in order to assess the immunocompetence for antibodies production. The  
11 study was carried out in order to standardize the antibody titer against rabbit red blood cell in  
12 immunized fish and fed diets with levamisole (0, 250 e 500 mg.kg<sup>-1</sup> of levamisole). As a  
13 result a cell cluster agglutination can be observed by naked eye. Fish fed 250 mg.kg<sup>-1</sup> of  
14 levamisole have shown the highest hemagglutination antibodies titer, however fish fed 500  
15 mg.kg<sup>-1</sup> of levamisole have revealed titers equivalent to control group fed diet levamisole  
16 free. This study has validated the methodology for determination of hemagglutination  
17 antibodies titer of immunized fish and has found an increase in antibodies titer after  
18 administration of 250 mg.kg<sup>-1</sup> of levamisole during 10 days.

19 **KEY-WORDS:** acquired immune system, antibody, immunostimulant, methodology.

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21 **RESUMO**

22 A determinação do título de hemaglutinação é uma alternativa para avaliar as respostas do  
23 sistema imune adquirido, ou seja, analisar a capacidade de produção de anticorpos circulantes  
24 do organismo. O estudo foi realizado a fim de padronizar a titulação de anticorpos produzidos  
25 contra hemácias de coelho em peixes previamente imunizados e submetidos a dietas com  
26 diferentes concentrações de levamisol (0, 250 e 500 mg.kg<sup>-1</sup> de levamisol). O resultado é um  
27 aglomerado celular que pode ser visualizado a olho nu. Peixes do presente estudo alimentados  
28 com 250 mg.kg<sup>-1</sup> de levamisol apresentaram maiores títulos de anticorpos hemaglutinantes,  
29 entretanto os alimentados com 500 mg.kg<sup>-1</sup> não apresentaram títulos diferentes do grupo  
30 controle, alimentado com dieta sem levamisol. Este estudo validou a metodologia para  
31 determinação do título de hemaglutinação do soro de peixe nativo imunizados, após

32 administração de levamisol e verificou um aumento da concentração de anticorpos  
33 hemaglutinantes após administração de 250 mg.kg<sup>-1</sup> de levamisol por 10 dias.

34 **PALAVRAS-CHAVE:** sistema imune adquirido, anticorpo, imunoestimulante, metodologia.

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## INTRODUCTION

37 The evaluation of the hemagglutination titer is an option to evaluate the response of  
38 acquired immune system in order to assess the immunocompetence for antibodies production  
39 (KUMARI & SAHOO, 2005, 2006).

40 Fish immune system is divided into innate and acquired defense mechanism, both  
41 consisting of cell-mediated and humoral defense. The innate system is considered the first  
42 barrier against foreign agents, which acts through several compounds such as the complement  
43 system, antimicrobial enzyme system besides nonspecific mediators such as interferon,  
44 interleukins and defense cells, such as granulocytes, monocytes, macrophages and natural  
45 killer cells. On the other hand, the acquired system requires the presence of antigen to trigger  
46 reactions that will increase specific antibodies production and promote immune memory  
47 (BAYNE, 2001; ELLIS, 2001).

48 Antibodies and lymphocytes comprise the humoral and cell-mediated mechanisms of  
49 the acquired immunity, respectively. The antibodies bind to microorganisms so as to activate  
50 phagocytosis besides to promote neutralization and opsonization of the agent, as well to  
51 prompt complement system and cell cytotoxicity (ELLIS, 2001).

52 Fish immune system can be triggered by some molecules, such as levamisole. This  
53 compound is a synthetic anthelmintic applied in mammals, which features a powerful action  
54 on innate and specific immune systems of fish (KIRON et al., 2012). This immunostimulant  
55 promotes increase of some parameters such as cytotoxic activity of leukocytes (CUESTA et  
56 al., 2002), number of phagocytes (MULERO et al., 1998; FINDLAY & MUNDAY, 2000),  
57 respiratory activity of macrophages (SIWICKI, 1989; MULERO et al., 1998), besides the

58 improvement of some acquired immune responses (JENEY and ANDERSON, 1993;  
59 CUESTA et al., 2004).

60 The pacu, *Piaractus mesopotamicus* is an important farmed teleost fish with special  
61 features, such as rapid growth rate and well-known artificial reproduction (OLIVEIRA et al.,  
62 2004; QUEIROZ et al., 2005). However, for this species, there is a lack of validated methods  
63 in order to evaluate the responses of immune system, especially the antibodies production  
64 (BILLER et al, 2013). Accordingly, the aim of this study was to standardize a simplified  
65 assay based on the use of rabbit red blood cells as antigen for induction of antibodies  
66 production in pacu fed diets with levamisole.

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## MATERIAL E METHODS

### 69 **Animals, experimental design and sampling**

70 A total of 180 pacu, *Piaractus mesopotamicus*, with  $218.92 \pm 45.74$  g;  $21.36 \pm 2.15$  cm  
71 was distributed in 18 100 L tanks (10 fish per tank) with a continuous water flow system and  
72 aerated with compressed air diffused through air stones. Fish remained in these conditions  
73 during 20 days for acclimatization, being fed to apparent satiation twice a day with  
74 commercial diet (28% protein, 3% fat, 1% fiber, levamisole free). After that, fish have  
75 received the experimental diet until the apparent satiety in two daily meals. The experimental  
76 diets were prepared with commercial diet in which was added 0, 250 and 500 mg.kg<sup>-1</sup> of  
77 levamisole. The parameters of water quality were monitored daily and were within the values  
78 described for the species (URBINATI et al. 2010): temperature  $28.82 \pm 0.67^{\circ}\text{C}$ ; dissolved  
79 oxygen  $5.96 \pm 0.89$  mg.L<sup>-1</sup>, NH<sub>4</sub>  $0.41 \pm 0.22$  mg.L<sup>-1</sup> and pH  $7.09 \pm 0.11$ .

80 Fish were randomly distributed into three groups, each one in six tanks, and were fed  
81 during ten days with their respectively experimental diets (0, 250 and 500 mg.kg<sup>-1</sup> of  
82 levamisole), subsequently fish were inoculated with 10% rabbit red blood cell suspension.

83 Fifteen days afterward, two fish from each tank (each treatment with n=12) were anesthetized  
84 in benzocaine (0.1 g.L<sup>-1</sup>) and the blood was drawn for serum extraction so as to assess the  
85 hemagglutination titer. Sampled fish were not reusable and they were taken out of the  
86 experiment.

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### 88 **Hemagglutination antibodies titer**

89 A serum agglutination reaction was carried out in order to titer the antibodies produced  
90 against rabbit red blood cell. The reaction is a cell flocculation response, in which the antigen  
91 consists of stable cells and as a result a cell cluster agglutination can be observed by naked  
92 eye.

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### 94 **Rabbit red blood cell suspension and inoculation**

95 Rabbit whole blood was added in an equal volume of Alsever's solution (pH 6.1  
96 anticoagulant) and the resulting suspension was filtered in sterile gauze. Then, the red blood  
97 cells were washed and centrifuged at 3000 g by three minutes in sterile phosphate buffer  
98 solution (PBS) (NaCl, 0.137 M; KCl, 2.7 mM; KH<sub>2</sub>PO<sub>4</sub>, 1.5 mM; Na<sub>2</sub>HPO<sub>4</sub>, 8.1 mM; CaCl<sub>2</sub>,  
99 0.9 mM; MgCl<sub>2</sub>, 0.49 mM in 1 l Milli-Q distilled water), with pH 7.4. The suspension was  
100 diluted twice, at 1% (optical density between 0.8 and 0.9, at 700 nm) employed in microplates  
101 assay and at 10% employed for fish inoculation.

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### 103 **Serum agglutination reaction**

104 Initially, fish hyperimmune serum were sampled after 15 days of rabbit red blood cells  
105 inoculation at 10%, and then were inactivated at 56°C during 20 minutes in order to achieve  
106 the denaturation of termolabiles proteins from complement system due to its ability to lyse red  
107 blood cells.

108 The hemagglutination titer was established in a 96-well microtiter plate with round  
109 bottom wells. The assay was initiated with a dilution of 1:1 (50  $\mu$ L of phosphate buffer: 50  $\mu$ L  
110 of serum) and consequently a two-fold serial serum dilutions were made by adding 50  $\mu$ L of  
111 diluted serum into the remaining wells with 50  $\mu$ L of PBS. As a result the serum dilutions  
112 were 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and 1/2048. Thereafter, 50  $\mu$ L  
113 of rabbit red blood cell suspension at 1% was added to each well and then micro plates were  
114 covered with plastic film and incubated at room temperature for 16–18 h. The agglutination  
115 end point was established as the last serum dilution where agglutination was visible.  
116 Agglutination antibodies titers were expressed as log<sub>10</sub> of the reciprocal of the highest serum  
117 dilution showing visible agglutination. The last well was used as a negative control which has  
118 consisted of 50  $\mu$ L PBS buffer only.

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#### 120 **Experimental design and statistical analysis**

121 Data were submitted to one-way ANOVA. If results were significant, Tukey test was  
122 applied for means comparison. Differences were considered significant at  $P < 0.05$ .

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### 124 **RESULTS AND DISCUSSION**

125 Fish fed 250 mg.kg<sup>-1</sup> of levamisole have shown the highest hemagglutination antibodies  
126 titer, however fish fed 500 mg.kg<sup>-1</sup> of levamisole have revealed titers equivalent to control  
127 group which were fed diet levamisole free. The immunostimulant has been evaluated in  
128 several species and Cuesta et al. (2004) have found in *Sparus aurata* elevated IgM  
129 concentrations after two weeks of levamisole administration and the effect has persisted for  
130 more than six weeks. As well Hung et al. (1997) that have reported in Japanese eel, increased  
131 immunoglobulins concentrations with peaks between three and four weeks after  
132 immunization.

133 As a result, levamisole has improved the resistance against various etiological agents,  
134 such as *Vibrio anguillarum* (KAJITA et al., 1990), *Aeromonas hydrophila* (BABA et al.,  
135 1993), *Paramoeba sp.* (FINDLAY et al., 2000; MUNDAY & ZILBERG, 2003), *Edwardsiella*  
136 *tarda* (SAHOO & MUKHERJEE, 2002), *Photobacterium damsela* (LEANO et al. 2003) and  
137 nematodes as *Anguillicola crassus* (GEETS et al., 1992). However, the immunomodulatory  
138 effect of levamisole on antibody production is dependent on the dose and time of  
139 administration, and it is necessary to investigate these parameters so as to evaluate their action  
140 on the immune system (KIRON et al., 2012).

141 The hemagglutination titer is a reaction between particulate antigen, as bacteria  
142 suspension or erythrocytes, and serum of previously immunized fish (TIZARD, 2002;  
143 KUMARI & SAHOO, 2005, 2006). The antibody production by immunized fish is prompt  
144 after antigen recognition, initially by the innate system, through antigen-presenting cells  
145 (macrophages or dendritic cells), that process the antigen and trigger proliferation immune  
146 responses and secondly, in conjunction with specific defense compounds, trigger memory  
147 response (ABBAS & LICHMAN, 2004). Cellular and humoral factors of innate and acquired  
148 systems operate together in order to promote an increase in circulating antibodies (IWANA &  
149 NAKANISHI, 1996; MAGNADOTTIR et al., 2011).

150 The evaluation of the antibodies production is very important due to its critical function  
151 on pathogens recognition and destruction, as well as immune memory. Diseases outbreak,  
152 despite of defense system, can occur mainly in situations of excessive organic matter in water,  
153 stressful handling or in the incidence of parasites (POST, 1987). In Brazilian fish farms, large  
154 economic losses take place as a result of microorganisms spread, including bacteria, fungi and  
155 parasites and further the indiscriminate antibiotics administration at sub-therapeutic doses  
156 results in increased resistance of bacteria to antibiotics (SUHET et al., 2011; VIEIRA, 2003;  
157 VIVEKANANDHAN et al., 2002).

158 Currently, the defense against microorganisms can be stimulated through immunization  
159 and immunostimulant utilization, with consequent increase of circulating antibodies,  
160 improvements in survival and in growth performance (POUEY et al., 2011; SUHET et al.,  
161 2011; TIZARD, 2002). In aquaculture, immunization can be an alternative to the antibiotics  
162 use once the disease prevention is fundamental for the development of this economical  
163 activity (ROMANO & MEJÍA, 2003; PLANT & LAPATRA, 2011).

164 Consequently, methods to evaluate immunocompetence employing only a single sample  
165 are useful in comparative studies, besides the method validated in this work analyzes the  
166 ability of antibody production by immunized fish. However, in order to avoid losses in the  
167 determination of the hemagglutination titer, the denaturation of proteins from complement  
168 system is an important step, since these proteins have natural affinity for red blood cells and  
169 consequently may promote cell lysis (BILLER et al, 2012).

170 The hemagglutination is a reaction with particulate antigens and occurs only with  
171 antigens on the cell surface, and often this identification can initiate naturally, without prior  
172 immunization, as verified by Kumari & Sahoo (2005), Sahoo (2005) and Dash et al. (1993).  
173 The hemagglutinating antibodies are usually multivalent as Ig-M that promotes elevated  
174 agglutination activity. Since fish have higher concentration of IgM the hemagglutination  
175 reaction can be observed very effectively (BILLER et al., 2014; DASH et al., 1993).

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## CONCLUSIONS

178 The standardization of techniques in order to evaluate indicators of native fish immunity  
179 denotes a great importance for national survey. This study has validated the methodology for  
180 determination of hemagglutination antibodies titer of immunized fish and has found an  
181 increase in antibodies titer after administration of 250 mg.kg<sup>-1</sup> of levamisole during 10 days.

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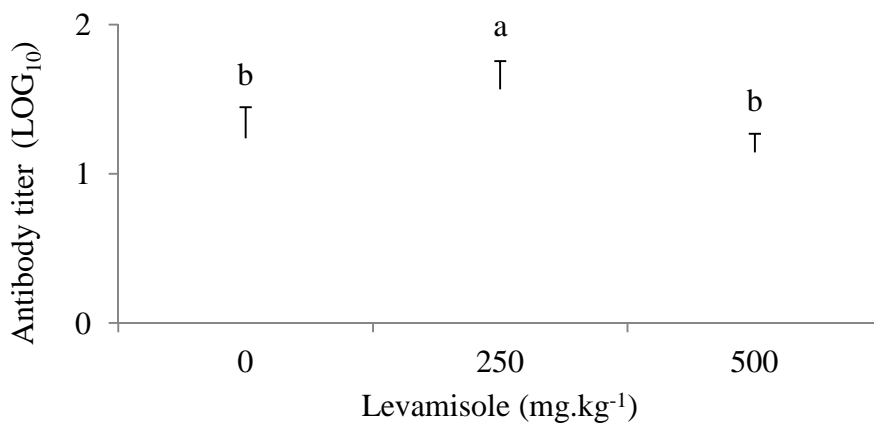
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332 **Figure 1.** Hemagglutinating antibodies titer against rabbit red blood cell of immunized pacu,  
333 *Piaractus mesopotamicus* (mean  $\pm$  sd). Significant differences are indicated by different  
334 letters (P<0.05).  
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