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Grass carp which survive *Dactylogyrus ctenopharyngodonid* infection also gain partial immunity against *Ichthyophthirius multifiliis*

Jian-Pei Li[1,*], Yao-Wu Fu[1,*], Qi-Zhong Zhang[1,], De-Hai Xu[2], Yan-Meng Liu[1], Sheng-Yu Zhou[1], De-Jie Lin[1]**

[1]Institute of Hydrobiology, Jinan University, Engineering Research Center of Tropical and Subtropical Aquatic Ecological Engineering Ministry of Education, Guangzhou 510632, PR China

[2]United States Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Unit, 990 Wire Road, Auburn, AL 36832-4352, USA

ABSTRACT: *Dactylogyrus ctenopharyngodonid* and *Ichthyophthirius multifiliis* are 2 important ectoparasites of fish. Both parasites can induce an immune response in fish that leads to a decrease in parasitic infection intensity and the development of resistance against parasitic reinfection. The present study evaluated whether grass carp *Ctenopharyngodon idella* that survived a *D. ctenopharyngodonid* infection could develop immunity against infection by *D. ctenopharyngodonid* and *I. multifiliis*. The results demonstrated that when grass carp were infected with *D. ctenopharyngodonid*, the number of red blood cells and the percentages of thrombocytes, monocytes, and neutrophils in the white blood cells increased significantly in the early stage of infection. The percentage of lymphocytes increased over time following parasitic infection. The mean infection intensity of *D. ctenopharyngodonid* decreased to 0 on Day 28. The activities of serum acid phosphatase, alkaline phosphatase, lysozyme, and superoxide dismutase increased significantly after *D. ctenopharyngodonid* infection. In addition, the grass carp that survived a previous *D. ctenopharyngodonid* infection could completely resist *D. ctenopharyngodonid* reinfection and partially resist *I. multifiliis* infection.

KEY WORDS: Carp · Fish farming · White spot disease · Monogenean · Ciliate protozoan · Immune response · Reinfection

INTRODUCTION

Fish parasites cause fish lesions, reduce host growth, lead to poor health in fish, and consequently reduce production (Panjvini et al. 2016). Monogeneans are a group of parasites with a series of hooks that attach to the gills, skin, or fins of a fish, and these parasites lead to significant injury or death of the fish (Rastiannasab et al. 2016). *Dactylogyrus ctenopharyngodonid* is an oviparous monogenean parasite that is often found in co-infections with other parasites, bacteria, fungi, or viruses, and causes serious damage to the hosts (Lu et al. 2013).

Previous studies have demonstrated that monogenean infections can induce a fish immune response and that the infected fish can develop resistance against reinfection with the same monogenean parasites (Buchmann 1998, Buchmann & Bresciani 1999). Monogenean parasites attach to mucus-cell-rich areas in the skin and cause cytokine production, mucus secretion, and peptide release (Buchmann 1999). These nonspecific responses in the epidermis

*These authors contributed equally to this work
**Corresponding author: zhangqzdr@126.com

create a microenvironment that is hostile to the parasites and either lead to the death of the monogeneans or force their escape to less hostile surfaces (Buchmann 1999). After primary infection with monogeneans, fish can develop significant resistance to reinfection by the same parasite species (Rubio-Godoy & Tinsley 2004). In addition, rainbow trout *Oncorhynchus mykiss* that were previously immunized with the monogenean *Gyrodactylus derjavini* demonstrated partial cross-protection against the ciliate protozoan *Ichthyophthirius multifiliis* (Buchmann et al. 1999).

I. multifiliis is a ciliated parasite that presents a threat to both wild and cultured fish and is globally distributed (Matthews 2005, Dickerson & Findly 2014). Fish surviving natural and experimental *I. multifiliis* infections are able to acquire protective immunity; however, it is still not known whether these fish can resist *I. multifiliis* infection. The present study evaluated whether grass carp *Ctenopharyngodon idella* that survived a *D. ctenopharyngodonid* infection (hereafter referred to as 'survivors') can develop protective immunity against *D. ctenopharyngodonid* and *I. multifiliis* infection. The hematological response of grass carp infected with *D. ctenopharyngodonid* was also evaluated.

MATERIALS AND METHODS

Fish and parasites

Seven hundred grass carp were obtained from 2 different fish farms belonging to the same company at Huadu, Guangzhou City, Guangdong Province. Fish from one farm weighed 22 ± 1.4 g (mean ± SD) and those from the other weighed 21.4 ± 1.1 g. Ten fish were randomly sampled from each fish farm to examine their parasites and determine the infection intensity. All grass carp from one fish farm (n = 340) were found to be parasitized by *Dactylogyrus ctenopharyngodonid* only and those fish were designated 'infected' fish. All grass carp from the other fish farm (n = 360) were not parasitized by any parasites and were designated 'no parasite' fish. The fish were treated with 5 mg l^{-1} potassium permanganate for 30 min prior to being cultured in the laboratory to ensure that they had no parasites. All fish were acclimated in the laboratory for 2 wk prior to the trial, and the water temperature was controlled at 23.0 ± 0.3°C. Fish were fed twice daily at 09:30 and 17:30 h with commercial granule feed to apparent satiation. *Ichthyophthirius multifiliis* was isolated from goldfish obtained from a local ornamental fish market and maintained by a serial transmission on grass carp. The grass carp infected with *D. ctenopharyngodonid* and *I. multifiliis* were co-housed with no-parasite grass carp to obtain the coinfected fish with 2 parasites; grass carp infected with *D. ctenopharyngodonid* or *I. multifiliis* were co-housed with no-parasite grass carp to obtain the mono-infected fish with one of the 2 parasites.

Experimental design and fish sampling

Initial parasite challenge (infection experiment)

The grass carp infected with *D. ctenopharyngodonid* were randomly distributed into six 150 l tanks (50 individuals per tank). Another 6 tanks each contained 50 no-parasite fish (hereafter referred to as 'control' fish). During the trial, fish were fed twice daily at 09:30 and 17:30 h and the water temperature was controlled at 23.0 ± 0.3°C. The water in each tank was replaced with fresh water daily for 28 d. Three fish were randomly sampled from each tank on Days 0, 5, 14, 21, and 28. The survival of grass carp was determined for each tank on the sampling day.

The sampled fish were anesthetized with 150 mg l^{-1} tricaine methanesulfonate (MS-222) and blood was collected from the fish tail vein using a 1 ml sterile syringe with a drop of 10% EDTA. An aliquot of the blood was used to evaluate hematological parameters and the remaining blood was centrifuged at 956 × *g* for 5 min at 4°C. The serum was collected and stored at −20°C to analyze the immune-related enzyme activities. The gills of sampled fish were removed, and the body surface was gently scraped to prepare wet mounts following the blood sampling. The numbers of *D. ctenopharyngodonid* were counted under a microscope at 4× magnification, and the mean intensity was determined as described by Bush et al. (1997) and Martins et al. (2011).

Hematological parameters

Blood smears were prepared as described by Li et al. (2012). Five replicates were prepared from each fish, and 100 white blood cells (WBCs) were counted from each slide under a microscope (100×). The percentages of lymphocytes, monocytes, neutrophils, and thrombocytes were calculated according to the equation 100 × *A*/100, where *A* was the number of

WBCs. In addition, a 5 µl blood sample was diluted with 995 µl of 0.9% saline to quantify the total number of red blood cells (RBCs) in a hemocytometer, with 5 replicates for each fish.

Immune-related enzyme activities

The serum enzyme activities of acid phosphatase (ACP), alkaline phosphatase (AKP), lysozyme (LZM), and superoxide dismutase (SOD) were determined using assay kits (Nanjing Jiancheng Bioengineering Institute) following the kit protocol.

Parasite rechallenge (cohabitation experiment)

To measure the protective effect, survivors were challenged with either *D. ctenopharyngodonid*, *I. multifiliis* or both parasites. Five groups of fish received the following treatments in the trial. Group 1 contained a mix of control fish and fish coinfected with *D. ctenopharyngodonid* and *I. multifiliis*. Group 2 contained a mix of control fish and fish infected with *I. multifiliis*. Group 3 contained a mix of survivors and fish infected with *I. multifiliis*. Group 4 contained a mix of survivors and fish infected with *D. ctenopharyngodonid*. Group 5 contained a mix of survivors and fish coinfected with *D. ctenopharyngodonid* and *I. multifiliis*. Each group had triplicate tanks with 20 survivors or 20 control fish mixed with 6 infected grass carp per tank. The infected grass carp were infected with either *D. ctenopharyngodonid,* with a mean intensity (number of parasites per infected fish) of 42.2 ± 5.0, or *I. multifiliis* (mean intensity of 79.6 ± 8.4), or both parasites (mean intensities of 54.6 ± 6.3 for *D. ctenopharyngodonid* and 65.0 ± 8.5 for *I. multifiliis*). Before being added to tanks, each infected fish was marked by a cut in the caudal fin. Three survivors or control fish were randomly sampled from each cohabitation tank on Days 7, 11, and 14. The mean parasite intensity and survivors were determined for each tank on each sampling day.

Statistical analysis

All experimental data are expressed as the mean ± SD. Assumptions of normality and homogeneity of variances were confirmed with the Shapiro-Wilk test and Levene's test, respectively. For the infection experiment, we compared parasitic intensity, fish survival, infection incidence, hematological parameters, and enzyme activity in grass carp among the 5 sampling days (in both the control and infected groups) using 1-way ANOVA, and between the control and infected groups (for each sampling day) using independent-samples *t*-tests (see Tables 1–3). For the cohabitation experiment, we compared parasitic intensity and fish mortality among the 5 groups using 1-way ANOVA, or independent-samples *t*-tests when only 2 groups were compared (see Tables 5 & 6). Student-Newman-Keul's (SNK) test was used for multiple comparisons when ANOVA showed significant differences. The results were considered statistically significant at $p < 0.05$. All analyses were performed in SPSS 19.0.

RESULTS

Parasitic intensity and fish survival

The mean intensity of *Dactylogyrus ctenopharyngodonid* in the infected groups was significantly higher on Days 5 and 14 than on Days 0 and 21, and it finally dropped to 0 on Day 28 (see Table 1 and the summary data in Table 4 below; SNK test). During the

Table 1. Mean intensity, infection incidence, and survival rate of grass carp infected with *Dactylogyrus ctenopharyngodonid* ('infected') compared with those not previously infected by the parasite ('control') over the course of a 28 d laboratory experiment. Within a given column for mean intensity, means followed by different lowercase superscripts are significantly different (*t*-test, $p < 0.05$). Within a given row, values with different uppercase superscripts are significantly different (SNK test, $p < 0.05$). Mean intensity was calculated as the number of parasites on fish divided by the number of infected fish

	Group	Day 0	Day 5	Day 14	Day 21	Day 28
Mean intensity	Control	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	Infected	56.5 ± 6.8^{bA}	142.7 ± 21.7^{bB}	83.3 ± 53.3^{bB}	1.4 ± 2.9^{aC}	0.0 ± 0.0^{aC}
Survival (%)	Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
	Infected	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Infection incidence (%)	Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Infected	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0

trial, the infection incidence was 100% in the infected group. The survival rates of grass carp were 100% in both the control and infected groups (Table 1).

Hematological parameters

The percentage of lymphocytes in the WBCs was lower in the infected group than in the control group on Day 0 (Table 2), but the percentages of monocytes and neutrophils were higher in the infected group than in the control group (Table 2). The percentage of lymphocytes significantly increased over time following *D. ctenopharyngodonid* infection, but the thrombocyte and neutrophil percentages were significantly decreased in the infected group (Tables 2 & 4; SNK test). The numbers of RBCs were higher in the infected group than in the control group during the infection trial (Table 2).

Enzyme activities

ACP, AKP, and SOD activities were significantly higher in the infected group than in the control group on Days 0, 5, and 14 (Table 3; *t*-test). AKP and SOD activities decreased significantly from Day 21 to 28 (Tables 3 & 4; SNK test). LZM activity increased from 146.4 U ml^{-1} on Day 0 to 194.4 U ml^{-1} on Day 14 and then decreased from Day 21 to 28 (Table 3).

Parasite challenge

When the survivors were challenged with *D. ctenopharyngodonid*, *Ichthyophthirius multifiliis* or both parasites, the mean intensities of *D. ctenopharyngodonid* for survivors were 0 in Groups 4 and 5 and significantly lower than that observed for the control fish (Tables 5 & 6; SNK test). The mean intensities of

Table 2. Hematological parameters of grass carp infected with *Dactylogyrus ctenopharyngodonid* ('infected') compared with those not previously infected by the parasite ('control'), showing presence of lymphocytes, neutrophils, monocytes, and thrombocytes as percentages of total white blood cells, and number of red blood cells (RBCs) over the course of a 28 d laboratory experiment. Within a given column, means followed by different lowercase superscripts between control and infected groups are significantly different (*t*-test, $p < 0.05$). Within a given row, values with different uppercase superscripts are significantly different (SNK test, $p < 0.05$)

Cells	Group	Day 0	Day 5	Day 14	Day 21	Day 28
Lymphocytes (%)	Control	82.50 ± 0.71^{aA}	81.50 ± 2.12^{aA}	81.50 ± 2.12^{aA}	79.50 ± 4.95^{aA}	83.50 ± 3.54^{aA}
	Infected	64.75 ± 6.65^{bA}	78.75 ± 3.59^{aB}	85.75 ± 5.56^{aBC}	82.00 ± 5.66^{aBC}	89.00 ± 2.65^{aC}
Neutrophils (%)	Control	2.00 ± 0.71^{aA}	4.00 ± 0.0^{aA}	3.00 ± 1.41^{aA}	4.00 ± 0.00^{aA}	2.00 ± 1.41^{aA}
	Infected	14.75 ± 3.86^{bA}	5.75 ± 0.96^{bBC}	7.25 ± 3.95^{bB}	6.00 ± 1.41^{bBC}	1.67 ± 1.15^{aC}
Monocytes (%)	Control	3.50 ± 0.71^{aA}	3.50 ± 0.71^{aA}	3.00 ± 0.00^{aA}	5.00 ± 1.41^{aA}	4.50 ± 0.71^{aA}
	Infected	9.50 ± 2.52^{bA}	5.00 ± 0.82^{aA}	5.50 ± 2.52^{aA}	7.25 ± 5.74^{aA}	6.0 ± 3.46^{aA}
Thrombocytes (%)	Control	11.50 ± 0.71^{aA}	11.50 ± 2.12^{aA}	12.50 ± 0.71^{aA}	12.00 ± 2.83^{aA}	10.00 ± 2.83^{aA}
	Infected	10.75 ± 1.26^{aA}	10.50 ± 1.91^{aA}	1.50 ± 1.73^{bB}	4.75 ± 1.71^{bC}	3.00 ± 2.00^{bBC}
RBCs (10^9 ml^{-1})	Control	1.43 ± 0.18^{aA}	1.38 ± 0.11^{aA}	1.48 ± 0.11^{aA}	1.39 ± 0.16^{aA}	1.38 ± 0.12^{aA}
	Infected	2.48 ± 0.30^{bA}	2.39 ± 0.21^{bA}	1.83 ± 0.18^{aA}	1.52 ± 0.34^{aA}	1.57 ± 0.20^{aA}

Table 3. Enzyme activity in grass carp infected with *Dactylogyrus ctenopharyngodonid* ('infected') compared with those not previously infected by the parasite ('control') over the course of a 28 d laboratory experiment. Within a given column, means followed by different lowercase superscripts between control and infected groups are significantly different (*t*-test, $p < 0.05$). Within a given row, values with different uppercase superscripts are significantly different (SNK test, $p < 0.05$). ACP: serum acid phosphatase; AKP: alkaline phosphatase; LZM: lysozyme; SOD: superoxide dismutase; 1 King unit per 100 ml = 7.14 U l^{-1}

Enzyme	Group	Day 0	Day 5	Day 14	Day 21	Day 28
ACP (King unit per 100 ml)	Control	2.75 ± 0.15^{aA}	2.68 ± 0.15^{aA}	2.75 ± 0.05^{aA}	2.78 ± 0.11^{aA}	2.68 ± 0.15^{aA}
	Infected	3.52 ± 0.12^{bA}	3.43 ± 0.95^{bA}	3.50 ± 0.75^{bA}	2.80 ± 0.32^{aA}	2.71 ± 0.17^{aA}
AKP (King unit per 100 ml)	Control	0.19 ± 0.01^{aA}	0.17 ± 0.01^{aA}	0.18 ± 0.04^{aA}	0.16 ± 0.03^{aA}	0.17 ± 0.02^{aA}
	Infected	0.36 ± 0.02^{bAB}	0.38 ± 0.03^{bA}	0.31 ± 0.08^{bB}	0.21 ± 0.02^{aC}	0.19 ± 0.04^{aC}
LZM (U ml^{-1})	Control	111.2 ± 6.7^{aA}	116.7 ± 7.3^{aA}	108.2 ± 8.5^{aA}	106.4 ± 2.4^{aA}	114.6 ± 3.0^{aA}
	Infected	146.4 ± 3.0^{bA}	182.0 ± 1.2^{bB}	194.4 ± 5.5^{bB}	155.8 ± 1.8^{bA}	122.7 ± 10.9^{aC}
SOD (U ml^{-1})	Control	12.8 ± 0.2^{aA}	12.6 ± 0.4^{aA}	12.6 ± 0.3^{aA}	13.0 ± 0.5^{aA}	13.1 ± 0.4^{aA}
	Infected	16.1 ± 0.1^{bA}	16.1 ± 0.1^{bA}	15.2 ± 0.4^{bB}	13.8 ± 0.3^{aC}	14.0 ± 0.2^{aD}

Table 4. One-way ANOVA results for response variables (see Tables 1–3 for details and abbreviations) measured in grass carp infected with *Dactylogyrus ctenopharyngodonid* (*Dc*; 'infected') compared with those not previously infected by the parasite ('control'), sampled at 0, 5, 14, 21, and 28 d after infection. Within-groups mean square (MS) values not shown; where *F*- and p-values are not given (–), these cannot be computed because the within-groups MS value is 0. The 'groups' in this Table are the response variables on the different sampling days

Response variable	Treatment	Between-groups MS	df	*F*	p
Intensity of *Dc*	Control	0.000	4,10	–	–
	Infected	14372.138	4,10	21.309	<0.001
Survival	Control	0.000	4,10	–	–
	Infected	0.000	4,10	–	–
Infection incidence	Control	0.000	4,10	–	–
	Infected	6000.000	4,10	–	–
Lymphocytes	Control	4.400	4,10	0.473	0.756
	Infected	331.095	4,10	12.386	<0.001
Neutrophils	Control	1.600	4,10	1.778	0.270
	Infected	83.376	4,10	11.342	<0.001
Monocytes	Control	1.350	4,10	1.929	0.244
	Infected	12.839	4,10	1.104	0.393
Thrombocytes	Control	1.750	4,10	0.407	0.798
	Infected	71.046	4,10	23.967	<0.001
RBCs	Control	0.004	4,10	0.198	0.929
	Infected	0.783	4,10	11.629	0.291
ACP activity	Control	0.04	4,10	0.250	0.893
	Infected	0.323	4,10	1.003	0.485
AKP activity	Control	0.002	4,10	0.667	0.738
	Infected	0.015	4,10	37.5	<0.01
LZM activity	Control	36.981	4,10	1.001	0.485
	Infected	1629.554	4,10	49.950	<0.001
SOD activity	Control	0.109	4,10	0.791	0.578
	Infected	2.481	4,10	41.286	<0.01

Table 5. Mean intensity, calculated as the number of parasites on fish divided by the number of infected fish, of *Dactylogyrus ctenopharyngodonid* (*Dc*) and *Ichthyophthirius multifiliis* (*Im*) in grass carp from different cohabitation groups. Groups containing survivors of a previous *Dc* infection or fish with no previous exposure to the parasites ('control') were housed with fish infected by *Dc*, *Im* or both over the course of a 14 d laboratory experiment. Group 1: control fish coinfected with *Dc* and *Im*; Group 2: control fish infected with *Im*; Group 3: survivors infected with *Im*; Group 4: survivors infected with *Dc*; Group 5: survivors coinfected with *Dc* and *Im*. Within a given column for each parasite, means followed by different lowercase superscripts are significantly different (SNK test for Day 7, *t*-test for Day 11, $p < 0.05$). The symbol '/' indicates that fish were not infected with the parasite, and '–' indicates that all fish died before the sampling day

Group	Day 7		Day 11		Day 14	
	Dc	*Im*	*Dc*	*Im*	*Dc*	*Im*
1	31 ± 6^a	1135 ± 346^a	–	–	–	–
2	/	1057 ± 80^a	/	–	/	–
3	/	223 ± 42^b	/	1258 ± 204^a	/	–
4	0 ± 0^b	/	0 ± 0^a	/	0 ± 0	/
5	0 ± 0^b	266 ± 147^b	0 ± 0^a	1340 ± 495^a	–	–

I. multifiliis for survivors were just 266 in Group 5 and 223 in Group 3 on Day 7 (Table 5). When naïve grass carp were challenged with the same parasites as the survivors, fish showed parasite intensities of 1135 in Group 1 and 1057 in Group 2 on Day 7 (Table 5). The mean intensity of *I. multifiliis* was significantly higher in control fish than in survivors (Tables 5 & 6; SNK test). The mortality rates of control fish infected with *I. multifiliis* were 100% in both Groups 1 and 2 on Day 11 (Table 7). However, the mortality rates of survivors were 30% in Group 3 and 25% in Group 5 on Day 11 when infected with *I. multifiliis* only or by both parasites (Table 7).

DISCUSSION

The present study demonstrated that grass carp could survive a natural *Dactylogyrus ctenopharyngodonid* infection when the infection intensity was low. The mean intensity of *D. ctenopharyngodonid* decreased significantly on Day 21 and reached 0 on Day 28. Previous studies reported that the cellular response of the host probably causes the elimination of the parasite (Buchmann 1999, Nakayasu et al. 2005). In a study of Japanese flounder *Paralichthys olivaceus* infected with the monogenean *Sparicotyle chrysophrii*, a 100% infection incidence was noted on Day 40 and subsequently decreased to 12.5% on Day 61. The cellular response of the fish was demonstrated to destroy the posterior region of the parasite embedded in the tissue, thereby eliminating the parasites (Nakayasu et al. 2005).

Fish blood cells are considered as an indicator of fish health status and play an important role in innate immunity (Roberts 1978). Previous studies reported that the number of RBCs increased significantly after fish were infected with *D. ctenopharyngodonid*, while the increase in RBCs

Table 6. 1-way ANOVA results for comparisons of mean intensities of parasites *Dactylogyrus ctenopharyngodonid* (*Dc*) and *Ichthyophthirius multifiliis* (*Im*) and mortality in grass carp among the 5 cohabitation groups (see Table 5 legend for details)

Response variable		Between-groups MS	df	*F*	p
Mean parasite intensity	Day 7 (*Dc*)	961.00	2,6	80.08	<0.001
	Day 7 (*Im*)	729042.53	3,8	19.48	<0.001
Grass carp mortality	Day 7	458.733	4,10	19.60	<0.001
	Day 11	6390.00	4,10	319.50	<0.001

Table 7. Mortality of grass carp from different cohabitation groups (see Table 5 legend for details) after challenge with *Dactylogyrus ctenopharyngodonid* and *Ichthyophthirius multifiliis*. Within a given column, means followed by different lowercase superscripts are significantly different (SNK test, $p < 0.05$)

Group	Mortality of grass carp (%)		
	Day 7	Day 11	Day 14
1	25.0 ± 7.1^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0
2	26 ± 5.7^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0
3	6.7 ± 5.7^{b}	30 ± 10^{b}	100.0 ± 0.0
4	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0
5	5 ± 7.1^{b}	25 ± 7.1^{b}	100.0 ± 0.0

made the host resistant to parasite infection (Nakayasu et al. 2005, Shoemaker et al. 2012). When channel catfish were coinfected with *Edwardsiella ictaluri* and *Ichthyophthirius multifiliis*, anemia was detected on Day 8, and a mortality of 71.1% was observed on Day 19 (Shoemaker et al. 2012); anemia caused a more severe infection and even led to fish death. In contrast, a high level of RBCs indicated a low infection intensity of *S. chrysophrii* on *Paralichthys olivaceus* (Nakayasu et al. 2005). In the same study, the increased number of RBCs decreased the infection intensity of *D. ctenopharyngodonid* in grass carp.

WBCs play an important role during infestation (Panjvini et al. 2016). WBCs stimulate the hemopoietic tissues and the immune system to produce antibodies and chemical substances to defend fish against pathogen infection (Lebelo et al. 2001). The current study showed that the percentages of defense cells, i.e. monocytes, neutrophils, and thrombocytes, were significantly higher in the infection group than in the control group during the early stage of infection. The increase of monocytes and neutrophils could promote innate immunity and alleviate bodily damage by phagocytosis (Yakhnenko & Klimenlov 2009). In addition, neutrophils from fish secrete interleukin-1 to affect fish lymphocytes in response to ectoparasite infections (Buchmann 1999). Thus, as the duration post-*D. ctenopharyngodonid* infection increased, the percentage of lymphocytes increased significantly in the fish infected with *D. ctenopharyngodonid*. The percentage of thrombocytes decreased significantly in the infected fish. The low percentage of thrombocytes was probably related to the migration of the cells to the infected site for the phagocytic role of thrombocytes, and/or the enhancement of the other white blood cells. Leukocytes contribute to an increased level of complement factors during the period of host response and create a hostile microenvironment for parasites (Buchmann & Bresciani 1999). This suggests that, in the current study, WBCs might have been directly involved in the cellular immune response against *D. ctenopharyngodonid* infection and contributed to the elimination of *D. ctenopharyngodonid*.

Non-specific molecules in fish, such as lectins, complement factors, acute phase proteins, lysozyme, and anti-microbial peptides, play an important role in the response to monogeneans (Buchmann & Lindenstrom 2002). Lysozymes produced from neutrophils and macrophages have antiviral, antibacterial, and anti-inflammatory properties (Saurabh & Sahoo 2008, Yin et al. 2014), as well as an anti-parasitic effect (Alishahi & Buchmann 2006). Lysozyme activity was significantly higher in the infection group than in the control group in the present study, indicating that the serum lysozyme was involved in the fish resistance against *D. ctenopharyngodonid* infection.

Reactive oxygen species produced by pathogen infection cause damage to cells (Verma et al. 2013). Superoxide dismutase (SOD) is the first line of defense of the antioxidant enzymes and protects cells from oxidization (Metaxa et al. 2006). SOD activity was significantly higher in infected grass carp than in naïve fish in the present study, indicating that the increased SOD activity could protect grass carp from the damage of free radicals produced by *D. ctenopharyngodonid* infection. The enzymes AKP and ACP also participate in the process of resistance against pathogen infections (Cheng & Dougherty 1989, Tang et al. 2010). In a previous study, the activity of AKP and ACP significantly increased when *Epinephelus coioides* were infected with *Cryptocaryon irritans* (Yin et al. 2014). The current study

also showed that the AKP and ACP activities in parasitized fish were higher than those in naïve fish. The above results demonstrated that the nonspecific molecules in fish played an important role in the elimination of *D. ctenopharyngodonid* and protected hosts from the parasitic infection.

The present study also investigated whether survivors could be protected against *D. ctenopharyngodonid* and *I. multifiliis*. The results showed that the survivors demonstrated a robust protective immunity against *D. ctenopharyngodonid* reinfection. As discussed above, it was postulated that the WBCs, specific antibodies, and complement factors protected the fish from *D. ctenopharyngodonid* reinfection. A previous study reported that primary infection with monogenean *Protopolystoma xenopodis* in the frog *Xenopus laevis* could elicit strong, long-lasting protective immunity against reinfection of the parasite (Jackson & Tinsley 2001). Rainbow trout challenged with the monogenean *Discocotyle sagittata* in a primary infection could develop significant partial resistance to parasitic reinfection (Rubio-Godoy & Tinsley 2004). These results showed that both frogs and fish are able to acquire resistance against monogenean reinfection.

Multiple infections are common in natural host populations, and host resistance induced by one parasitic species is effective against another (Christensen et al. 1987). Richards & Chubb (1996) demonstrated that resistance induced by skin parasite *Gyrodactylus* spp. in guppies *Poecilia reticulata* was not species specific. Kidney infections caused by postlarval *Protopolystoma* spp. in *Xenopus* spp. induced weak resistance to heterospecifics (Jackson & Tinsley 2003). In addition, rainbow trout immunized against monogenean *G. derjavini* became less infective and showed lower mortality when exposed to *I. multifiliis* infection compared to naïve fish (Buchmann et al. 1999). The present study demonstrated that the survivors showed significantly lower mean intensity of *I. multifiliis* than naïve grass carp did. Previous studies on the immune response against *I. multifiliis* contributed to a better understanding of the antiparasitic mechanism in fish. Monocytes and neutrophils were the primary response to *I. multifiliis* infection and were directly involved in the fish immune response (Shoemaker et al. 2012, Dickerson & Findly 2014). In addition, complement factors play a role in protection against *I. multifiliis* (Buchmann et al. 1999, Dickerson & Findly 2014). *D. ctenopharyngodonid* infection caused the increment of leukocytes, which in turn led to the higher level of complement factors (Buchmann & Bresciani 1999). Thus, the resistance induced by *D. ctenopharyngodonid* provided partial cross protection against *I. multifiliis* infection.

In conclusion, when grass carp were infected with *D. ctenopharyngodonid*, WBCs, RBCs and activity of ATP, AKP, SOD, and LZM increased significantly to eliminate the *D. ctenopharyngodonid* from the fish. The survivors were completely resistant to *D. ctenopharyngodonid* reinfection and partially protected against *I. multifiliis* infection.

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LITERATURE CITED

Alishahi M, Buchmann K (2006) Temperature-dependent protection against *Ichthyophthirius multifiliis* following immunisation of rainbow trout using live theronts. Dis Aquat Org 72:269–273

Buchmann K (1998) Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). Dis Aquat Org 32:195–200

Buchmann K (1999) Immune mechanisms in fish skin against monogeneans — a model. Folia Parasitol 46:1–9

Buchmann K, Bresciani J (1999) Rainbow trout leucocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini*. Dis Aquat Org 35:13–22

Buchmann K, Lindenstrom T (2002) Interactions between monogenean parasites and their fish hosts. Int J Parasitol 32:309–319

Buchmann K, Lindenstrøm T, Sigh J (1999) Partial cross protection against *Ichthyophthirius multifiliis* in *Gyrodactylus derjavini* immunized rainbow trout. J Helminthol 73: 189–195

Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997) Parasitology meets ecology on its own terms: Margolis et al. revisited. J Parasitol 83:575-583.

Cheng TC, Dougherty WJ (1989) Ultrastructural evidence for the destruction of *Schistosoma mansoni* sporocysts associated with elevated lysosomal enzyme levels in *Biomphalaria glabrata*. J Parasitol 75:928–941

Christensen NO, Nansen P, Fagbemi BO, Monrad J (1987) Heterologous antagonistic interactions between helminths and between helminths and protozoans in concurrent experimental infection of mammal hosts. Parasitol Res 73:387–410

Dickerson HW, Findly RC (2014) Immunity to *Ichthyophthirius* infections in fish: a synopsis. Dev Comp Immunol 43: 290–299

Jackson JA, Tinsley RC (2001) *Protopolystoma xenopodis* (Monogenea) primary and secondary infections in *Xenopus laevis*. Parasitology 123:455–463

Jackson JA, Tinsley RC (2003) Postlarval *Protopolystoma* spp. kidney infections in incompatible *Xenopus* spp. induce weak resistance to heterospecifics. Parasitol Res 90:429–434

Lebelo SL, Saunders DK, Crawford TG (2001) Observations on blood viscosity in striped bass, *Morone saxatilis* (Walbaum) associated with fish hatchery conditions. Trans Kans Acad Sci 104:183–194

Li C, Zhang Qz, Yang Y, Zhu Ck, Li Ct, Chen X, Luo F (2012) Effects of compound Chinese herbal medicine on growth and blood cell number in grass carp (*Ctenopharyngodon idella*). Fish Sci 31:7–11 (in Chinese with English Abstract)

Lu C, Ling F, Ji J, Kang YJ, Wang GX (2013) Expression of immune-related genes in goldfish gills induced by *Dactylogyrus intermedius* infections. Fish Shellfish Immunol 34:372–377

Martins ML, Shoemaker CA, Xu D, Klesius PH (2011) Effect of parasitism on vaccine efficacy against *Streptococcus iniae* in *Nile tilapia.* Aquaculture 314:18–23

Matthews RA (2005) *Ichthyophthirius multifiliis* Fouquet and ichthyophthiriosis in freshwater teleosts. Adv Parasitol 59:159–241

Metaxa E, Deviller G, Pagand P, Alliaume C, Casellas C, Blancheton JP (2006) High rate algal pond treatment for water reuse in a marine fish recirculation: water purification and fish health. Aquaculture 252:92–101

Nakayasu C, Tsutsumi N, Oseko N, Hasegawa S (2005) Role of cellular response in elimination of the monogenean *Neoheterobothrium hirame* in Japanese flounder *Paralichthys olivaceus.* Dis Aquat Org 64:127–134

Panjvini F, Abarghuei S, Khara H, Parashkoh HM (2016) Parasitic infection alters haematology and immunity parameters of common carp, *Cyprinus carpio*, Linnaeus, 1758. J Parasit Dis 40:1540–1543

Rastiannasab A, Afsharmanesh S, Rahimi R, Sharifian I (2016) Alternations in the liver enzymatic activity of common carp, *Cyprinus carpio* in response to parasites, *Dactylogyrus* spp. and *Gyrodactylus* spp. J Parasit Dis 40:1146–1149

Richards GR, Chubb JC (1996) Host response to initial and challenge infections, following treatment, of *Gyrodactylus bullatarudis* and *G. turnbulli* (Monogenea) on the guppy (*Poecilia reticulata*). Parasitol Res 82:242–247

Roberts RJ (1978) The pathophysiology and systemic pathology of teleosts. In: Fish pathology. Bailliere Tindal, London, p 55–91

Rubio-Godoy M, Tinsley RC (2004) Immunity in rainbow trout, *Oncorhynchus mykiss*, against the monogenean *Discocotyle sagittata* following primary infection. Parasitol Res 92:367–374

Saurabh S, Sahoo PK (2008) Lysozyme: an important defence molecule of fish innate immune system. Aquacult Res 39:223–239

Shoemaker CA, Martins ML, Xu DH, Klesius PH (2012) Effect of *Ichthyophthirius multifiliis* parasitism on the survival, hematology and bacterial load in channel catfish previously exposed to *Edwardsiella ictaluri.* Parasitol Res 111:2223–2228

Tang B, Liu B, Wang X, Yue X, Xiang J (2010) Physiological and immune responses of zhikong scallop *Chlamys farreri* to the acute viral necrobiotic virus infection. Fish Shellfish Immunol 29:42–48

Verma VK, Rani KV, Sehgal N, Prakash O (2013) Immunostimulatory effect of artificial feed supplemented with indigenous plants on *Clarias gariepinus* against *Aeromonas hydrophila.* Fish Shellfish Immunol 35:1924–1931

Yakhnenko VM, Klimenlov IV (2009) Specific features of blood cell composition and structure in fishes from the pelagial and coastal zones of Lake Baikal. Biol Bull Russ Acad Sci 36:37–44

Yin F, Dan XM, Sun P, Shi ZH, Gao QX, Peng SM, Li AX (2014) Growth, feed intake and immune responses of orange-spotted grouper (*Epinephelus coioides*) exposed to low infectious doses of ectoparasite (*Cryptocaryon irritans*). Fish Shellfish Immunol 36:291–298

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