



Temperature effects on immune response and hematological parameters of channel catfish *Ictalurus punctatus* vaccinated with live theronts of *Ichthyophthirius multifiliis*

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ABSTRACT

This study evaluated the influence of temperature on the immune responses and hematological parameters in channel catfish *Ictalurus punctatus* immunized via intraperitoneal injection with live theronts of *Ichthyophthirius multifiliis*. Fish were distributed in 18 aquaria and received 9 treatments: 4 groups of fish were vaccinated with live theronts and maintained at constant temperature 15 °C, 20 °C, 25 °C and 30 °C; 3 groups of fish vaccinated and subjected to cycling temperature regime from 15–25 °C, 20–25 °C and 20–30 °C, changed 5 °C each day; 2 groups of fish were not vaccinated and served as controls at 25 °C, one with Ich challenge and the other without challenge. Non vaccinated fish and those vaccinated at 15 °C or 15–25 °C did not show anti-Ich antibodies in the serum 14 and 21 days post-immunization. The antibody levels were significantly higher from fish vaccinated at 25 °C, 30 °C, 20–25 °C and 20–30 °C compared to fish at 15 °C, 20 °C and 15–25 °C both 14 and 21 days post-immunization. At constant water temperature, fish vaccinated at 15 °C showed significantly higher mortality rate (67.8%, $P < 0.05$) than those vaccinated at 20 °C, 25 °C, and 30 °C (0–10.7% mortalities). At cycling water temperature, fish vaccinated at 15–25 °C showed significantly higher mortality rate (67.8%) than those vaccinated at 20–25 °C and 20–30 °C ($P < 0.05$). Twenty days after immunization fish vaccinated at 30 °C and 20–30 °C showed significant increase in the red blood cells, white blood cells, thrombocytes and monocytes. Six days after challenge with *I. multifiliis* theronts the fish showed decreased white blood cells, thrombocytes and monocytes. This study suggests that vaccinated catfish were severely impacted by low temperature, either at 15 °C constant temperature or at 15–25 °C cycling temperature. The fish showed no anti-Ich antibodies and suffered high mortality similar to non vaccinated control fish.

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1. Introduction

In intensive aquaculture, fish are constantly exposed not only to stressors but also alterations in the aquatic environment. Under these conditions disease may occur when pathogens find an adequate environment for reproduction [1]. Temperature plays an important role in cold-blooded animals and temperatures below or above the thermal limit can induce alterations to the fish immune response [2–4]. The adaptive immune response of channel catfish *Ictalurus punctatus* to T-dependent antigens is suppressed by low temperature (17 °C) [3]. The innate immune response is also

influenced by low temperature (10 °C) as phagocytic activity of channel catfish neutrophils was reduced [4].

Ichthyophthirius multifiliis is responsible for significant losses in cultured fish. Young fry or fingerlings are more severely affected than adult fish. Immunization studies have noted enhanced fish resistance against this ciliate protozoan [5–9]. Effective immunization against *I. multifiliis* depends on the route of application. Fish vaccinated with live theronts developed better resistance than those vaccinated by immersion or intraperitoneal injection with killed parasites [5]. High antibody titers appeared in rainbow trout *Oncorhynchus mykiss* 34 and 43 days after *I. multifiliis* infection [10]. Channel catfish immunized with live theronts showed higher antibody titer than those immunized with formalin inactivated or frozen trophonts that persisted for 50 days after immunization [7]. Intraperitoneal injection of live theronts may be somewhat

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artificial, however, it has been shown to consistently induce protection against gill and skin infection, thus linking the mucosal and systemic response to *I. multifiliis* [5,11,12].

Vaccination conditions, including water temperature, however, can greatly affect the effectiveness of immunization against Ich in fish [6]. The highest immobilization titer of theronts was found in rainbow trout vaccinated with live theronts at 20 °C two and four weeks post-vaccination [6]. A delayed antibody response against *I. multifiliis* was demonstrated in rainbow trout maintained at low temperature (5 °C) compared to those maintained at 12 °C and 20 °C [13].

Several studies have also shown the influence of temperature on hematological parameters and disease resistance. An increase in the white blood cell count was found in rainbow trout [14] and carp *Cyprinus carpio* [15] at high temperature and after cold shock, respectively. Increased number of neutrophils in Atlantic salmon *Salmo salar* maintained at high temperature (18 °C) was also reported [16]. In contrast, no alteration in red blood cells was found in Atlantic salmon exposed to 5 and 15 °C [17]. Lowering the water temperature from 23 °C to 11 °C in a period of 24 h resulted in anemia but no alteration in neutrophil population in the blood of channel catfish [18]. Tilapia *Oreochromis mossambicus* were maintained at 27 °C and then transferred to 19 and 23 °C to evaluate the resistance against infection by *Streptococcus iniae* [19]. Lower mortality was observed in tilapia maintained at 27 °C than those maintained at 19 °C and 35 °C. Fish transferred to low and high temperatures also showed decreased white blood cell count [19]. Natural fluctuations in water temperature may not only affect Ich immunization efficacy but other immune parameters as well.

Due to environmental fluctuations in rearing environment of pond-raised catfish, particularly water temperature, we determined to evaluate the influence of temperature alterations on the immunological and hematological parameters in channel catfish immunized with live theronts of *I. multifiliis*.

2. Materials and methods

2.1. Fish and water quality

A total of 432 channel catfish with 10.3 g (standard error (SE) = 0.4) mean body weight and 10.6 cm (SE = 0.1) total length, from the same spawn were maintained in a holding tank supplied with 25 ± 2 °C dechlorinated municipal water from a centralized heater at a flow rate of 0.5–1.0 L per min at the US Department of Agriculture–Agriculture Research Service (USDA–ARS), Aquatic Animal Health Research Unit (AAHRU), Auburn, Alabama. The fish were reared at the AAHRU and not exposed to Ich infection previously. Prior to the trial, fish were prophylactically treated with potassium permanganate at 5 mg/l for a 1 h bath. No parasites were observed from the skin and gills of 10 fish from the holding tank. Fish were then distributed to 9 tanks and acclimated for 10 days post treatment and fed ~3–4% body weight daily (Aquamax Grower 400 with 45% crude protein, PMI Nutrition International, LLC, Brentwood, MO).

During the experiment, total ammonia nitrogen, nitrite, alkalinity, pH, hardness and dissolved oxygen were measured once a week in random tanks. Water temperature was measured every day and adjusted with the aid of heaters (Marineland Visitherm VTH 300, Blacksburg, VA). Dissolved oxygen was measured with a Sper Scientific 840041 oxygen meter (Sper Scientific Ltd, Scottsdale, AZ) and the pH with a Corning 540 pH meter (Corning Incorporated, Corning, NY). Ammonia, nitrite, nitrate, hardness and alkalinity were determined using CEL/890 Advanced Portable Laboratory (Hach, Loveland, CO).

2.2. Experimental design and water temperature monitoring

Seven days after acclimation, fish were distributed in eighteen 57 l glass aquaria as 9 treatments with 2 replicates as follows: 1-vaccinated and maintained at 15 °C (Vaccinated 15), 2-vaccinated and maintained at 20 °C (Vaccinated 20), 3-vaccinated and maintained at 25 °C (Vaccinated 25), 4-vaccinated and maintained at 30 °C (Vaccinated 30), 5-vaccinated and subjected to cycling temperatures 15–25 °C, changed 5 °C each day (Vaccinated 15–25), 6-vaccinated and subjected to cycling temperature from 20 to 25 °C (Vaccinated 20–25), 7-vaccinated and changed 5 °C each day from 20 to 30 °C (Vaccinated 20–30). Two other treatments were 8-non vaccinated fish that received no theront challenge at 25 °C (Non vaccinated No Ich) and 9-non vaccinated fish infected with theronts (Non vaccinated Ich) at 25 °C. The cycling water temperature was changed 5 °C each day. For example, the temperature in tank with 15–25 °C was increased 5 °C each day from 15 °C, 20 °C to 25 °C and then decreased 5 °C daily from 25 °C, 20 °C to 15 °C until day of challenge.

2.3. Live theront immunization

For immunization, fish infected with maturing trophonts were anesthetized with 200 mg/l MS-222, rinsed with water and the skin was gently scraped to dislodge the parasites similar to [20]. Trophonts were harvested by filtering through a US standard sieve with 425 µm pore size (W.S. Tyler Incorporated, Ohio) to remove fish skin and surface debris. The collected trophonts were placed in petri dishes and allowed to attach. After replacing the water in the Petri dishes with fresh dechlorinated tank water to remove contaminating mucus, the trophonts were incubated for 18 h at 24 °C. Theronts were harvested by pouring through a sieve with a pore size of 45 microns to remove debris. For intraperitoneal injection, theronts were concentrated by centrifugation at 100 × g for 5 min. Theronts were counted with the aid of a Sedgewick-Rafter cell and adjusted to 100,000 theronts/ml. Fish were vaccinated with 10,000 live Ich theronts per fish by intraperitoneal injection. After vaccination, the fish were returned to each tank with the designated temperature. Water temperature was adjusted and recorded daily. After theront challenge, the water temperature was maintained ~25 °C in all tanks, the optimum temperature for *I. multifiliis* [21], until the end of the trial.

2.4. Challenge and infection level determination

For the Ich theront challenge, the water temperature was adjusted to 25 °C in each tank. Water inflow was ceased, and water volume was lowered to 10 l in each tank. Live Ich theronts were added to each tank, and fish were challenged with theronts at 20,000/fish for 1 h [22]. At the end of the challenge, water inflow in each tank was resumed, and temperature was maintained at 25 °C. Mortality of fish was recorded for 21 days after theront challenge.

Six days after theront challenge, 4 fish were sampled from each treatment for parasitic evaluation. Smears were made from 2 × 3 cm² area of the body surface in left side of each fish on microscope slides to evaluate the parasite infection levels.

2.5. Immobilization assay

The immobilization assay was conducted in 96 well, flat-bottomed microtiter plates (Corning Costar) as described by [7]. Fish serum was 2-fold serially diluted with phosphate buffered saline (PBS, pH 7.2) starting from 1:100 in 50 µl per well. A 50 µl theront solution with 400–500 theronts was added to each well and incubated at room temperature for 30 min. The immobilization titer was the inverse of the highest dilution in which all theronts were immobilized.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The anti-Ich antibodies were determined using an ELISA assay [23]. Each well of 96 well microtitre plates was coated with 100 µl coating buffer (pH 9.6) that contained sonicated Ich theront antigen at 22 °C for 1 h. The plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T) using an automatic plate washer (Tecan US Inc., Durham, NC, USA). Two-fold serial dilutions were made for each culture fluid sample with PBS-T and incubated for 1 h. After washing as described above, 100 µl mouse anti-catfish immunoglobulin (Ig) heavy chain monoclonal antibody [24] at a concentration of 1:100 was added to each well and incubated for 30 min. A 100 µl goat anti-mouse IgG conjugated with horse radish peroxidase (Pierce, Rockford, IL, USA) at a dilution of 1:2000 was added to each well and incubated for 30 min. Then, 100 µl of O-phenylenediamine substrate (OPD, Sigma) was added to each well of the plate, and the reaction was stopped with 50 µl 3 m H₂SO₄ after 15 min incubation. The plate was read at 490 nm using an ELISA-reader (Dynatech, Chantilly, VA, USA). Optical density in the immune sample two times greater than the OD readings in control was considered positive. The ELISA titer was the inverse of the highest dilution in which the sample was positive.

2.7. Hematological analysis and serum collection

Blood samples were taken from 6 fish per treatment 20 days after immunization and 4 fish per treatment 6 days after theront challenge. After fish were anesthetized by immersion in 100 mg/l MS-222 solution, 200 µl of blood was withdrawn from the caudal vasculature using a 1.0 ml syringe with a drop of 10% EDTA to make blood smears. The smears were stained with Giemsa/MayGrunwald [25] for differential counting of leucocytes. An aliquot was used to determine hematocrit [26]. The remaining sample was stored in tubes on ice to quantify the total number of red blood cells (RBC) in a hemocytometer. The total number of blood thrombocytes and leucocytes were counted by the indirect method [27].

For evaluating antibody levels against Ich, blood samples were taken from 4 fish per treatment 14 and 21 days after immunization. At cycling temperature, fish were collected at 20, 25, and 25 °C from 15 to 25 °C, 20 to 25 °C, and 20 to 30 °C groups, respectively when sampled 14 days post-immunization. Fish were sampled at 15, 20, and 20 °C from 15 to 25 °C, 20 to 25 °C, and 20 to 30 °C groups, respectively when sampled 21 days post-immunization. For each anesthetized fish 200 µl of blood was withdrawn, kept refrigerated overnight, and centrifuged at 6000 g for 5 min. The sera were collected and kept at –20 °C for immobilization and ELISA assays.

2.8. Statistical analysis

The data was analyzed using SAS software. Mortality, median days to death, immobilization and ELISA antibody titers, and hematological parameters of different treatments were analyzed with Duncan's multiple range test of the general linear model (GLM) procedure [28]. *P*-values of 0.05 or less were considered statistically significant. Measured parameters are reported as means ± standard error (SE).

3. Results

3.1. Water quality

Water temperatures during the acclimation and immunization period are presented in Table 1. Degree days were calculated by multiplying the mean daily water temperature with total number of days measured [29]. After immunization, water quality

Table 1

Planned and measured water temperature (± standard error) in each treatment during 10 days of fish acclimation and 21 days immunization after vaccination with live theronts in channel catfish. Degree days are calculated by multiplying the mean daily water temperature with total number of days measured.

| Treatments | Planned temperature (°C) | Measured temperature (°C) | Degree days |
|-----------------------|--------------------------|---------------------------|-------------|
| Non vaccinated No Ich | 25 | 24.8 ± 0.1 | 767.3 |
| Non vaccinated Ich | 25 | 24.6 ± 0.1 | 762.3 |
| Vaccinated 15 | 15 | 15.2 ± 0.1 | 471.8 |
| Vaccinated 20 | 20 | 19.9 ± 0.1 | 616.6 |
| Vaccinated 25 | 25 | 24.7 ± 0.2 | 765.4 |
| Vaccinated 30 | 30 | 29.6 ± 0.2 | 917.3 |
| Vaccinated 15–25 | 15–25 | 15.2, 19.9 & 24.7 | 609.1 |
| Vaccinated 20–25 | 20–25 | 19.9 & 24.6 | 687.3 |
| Vaccinated 20–30 | 20–30 | 19.8, 24.7 & 29.9 | 758.0 |

Non vaccinated No Ich – fish were not vaccinated and not challenged by *I. multifiliis* theronts maintained at 25 °C; Non vaccinated Ich – fish were not vaccinated and challenged by *I. multifiliis* theronts maintained at 25 °C; Vaccinated 15 – fish were vaccinated and maintained at 15 °C; Vaccinated 15–25 – fish were vaccinated and maintained at cycling water temperature from 15 to 25 °C, 5 °C per day; Vaccinated 20–25 – fish were vaccinated and maintained at cycling water temperature from 20 to 25 °C, 5 °C per day; Vaccinated 20–30 – fish were vaccinated and maintained at cycling water temperature from 20 to 30 °C, 5 °C per day.

parameters were: pH 6.33 (SE = 0.04), ammonia 0.33 (SE = 0.05) mg/l, hardness 150.10 (SE = 4.70) mg/l, nitrite 0.17 (SE = 0.10) mg/l, nitrate 0.35 (SE = 0.04) mg/l, alkalinity 76.0 (SE = 4.1) mg/l, dissolved oxygen 7.75 (SE = 0.23) mg/l. After theront challenge, the water temperature was maintained at 24.71 (SE = 0.04)°C until the end of the trial in all tanks.

3.2. Effect of temperature on anti-Ich antibody

Non vaccinated fish and those vaccinated at 15 °C did not show immobilization of theronts in serum from both 14 and 21 days after immunization (Table 2). Significantly lower immobilization titers were found in fish vaccinated at 20 °C at both 14 and 21 day sampling times when compared to those vaccinated at 25 °C and 30 °C. Fish vaccinated at 25 °C and 30 °C showed significantly higher values of immobilization titer 14 and 21 days after immunization with no difference between the two temperatures (Table 2).

For fish subjected to cycling temperatures, vaccination at 15–25 °C did not show immobilization of theronts in serum at

Table 2

Immobilization titer (± standard error) in serum of channel catfish 14 and 21 days after immunization with live theronts of *Ichthyophthirius multifiliis*. Means followed by different superscript letters are statistically different (*P* < 0.05).

| Treatments | Day 14 after vaccine | Day 21 after vaccine |
|-----------------------|------------------------------|-------------------------------|
| Non vaccinated No Ich | 0 ^a | 0 ^a |
| Non vaccinated Ich | 0 ^a | 0 ^a |
| Vaccinated 15 | 0 ^a | 0 ^a |
| Vaccinated 20 | 103.3 ± 49.1 ^a | 26.7 ± 4.2 ^a |
| Vaccinated 25 | 2133.3 ± 269.8 ^b | 10250.0 ± 6473.0 ^b |
| Vaccinated 30 | 2186.7 ± 683.4 ^b | 7380.0 ± 6719.0 ^b |
| Vaccinated 15–25 | 0 ^a | 0 ^a |
| Vaccinated 20–25 | 333.3 ± 214.8 ^a | 13663.3 ± 6822.0 ^b |
| Vaccinated 20–30 | 2996.7 ± 1021.0 ^b | 20486.7 ± 9155.0 ^c |

Non vaccinated No Ich – fish were not vaccinated and not challenged by *I. multifiliis* theronts maintained at 25 °C; Non vaccinated Ich – fish were not vaccinated and challenged by *I. multifiliis* theronts maintained at 25 °C; Vaccinated 15 – fish were vaccinated and maintained at 15 °C; Vaccinated 15–25 – fish were vaccinated and maintained at cycling water temperature from 15 to 25 °C, 5 °C per day; Vaccinated 20–25 – fish were vaccinated and maintained at cycling water temperature from 20 to 25 °C, 5 °C per day; Vaccinated 20–30 – fish were vaccinated and maintained at cycling water temperature from 20 to 30 °C, 5 °C per day.

either 14 or 21 days after immunization (Table 2). The vaccinated fish reared at 20–30 °C showed the highest immobilization titers 14 days (2996.7 SE = 1021) and 21 days (20,486.7 SE = 9155) post-immunization. The immobilization titers from fish at 20–30 °C were significantly higher than vaccinated fish at 20–25 °C or 15–25 °C 14 and 21 days after immunization.

Serum anti-Ich antibodies were also determined by ELISA. Similar to the immobilization assay results, non vaccinated fish and those vaccinated at 15 °C or 15–25 °C did not show ELISA titer in the serum 14 and 21 days after immunization (Table 3). The antibody levels were significantly higher in fish vaccinated at 25 °C, 30 °C, 20–25 °C and 20–30 °C compared to other groups of fish 14 and 21 days after immunization.

3.3. Parasitic infection level and fish mortality

Six days after *I. multifiliis* theront challenge, heavy infection (>100 trophonts per fish) was found in non vaccinated fish and vaccinated fish at 15 °C, 20 °C, and 15–25 °C. Fish vaccinated at 25 °C and 30 °C as well as those from the group vaccinated at 20–30 °C showed light infection. No parasite was observed in non vaccinated fish that were not challenged with Ich.

At constant water temperature, fish vaccinated at 15 °C (67.8% SE = 32.1) showed significantly higher mortality when compared to those vaccinated at 20 °C (10.7% SE = 3.6), 25 °C (no mortality) and 30 °C (3.6 SE = 3.6%). Fish vaccinated at 15 °C showed higher mortality than those non-vaccinated but challenged with theronts (Table 4). The non vaccinated challenged fish were maintained at 25 °C for the duration of the trial. At cycling water temperatures, fish vaccinated at 15–25 °C showed a significantly higher mortality rate (67.8% SE = 32.1) than vaccinated fish reared at 20–25 °C (39.3 SE = 3.6%) and 20–30 °C (no mortality) (Table 4). Interestingly, no differences in mortality occurred between vaccinated groups maintained at constant and cycling temperatures in the same temperature range: 15 °C and 15–25 °C; 20 °C and 20–25 °C; and between 25 °C, 30 °C and 20–30 °C (Table 4).

3.4. Hematological analysis

Twenty days after immunization, significant hematological alterations were found. Fish vaccinated at high temperatures (30 °C and 30–20 °C) showed the highest RBC count and total thrombocyte number compared to other treatments (Table 5). Consequently,

Table 3

Enzyme-linked immunosorbent assay (ELISA) titer (\pm standard error) of anti-*Ichthyophthirius multifiliis* antibody in serum of channel catfish 14 and 21 days after immunization with live theronts. Means followed by different superscript letters are statistically different ($P < 0.05$).

| Treatments | Day 14 after vaccine | Day 21 after vaccine |
|-----------------------|---------------------------------|---------------------------------|
| Non vaccinated No Ich | 0 ^a | 0 ^a |
| Non vaccinated Ich | 0 ^a | 0 ^a |
| Vaccinated 15 | 0 ^a | 0 ^a |
| Vaccinated 20 | 50.0 \pm 34.1 ^a | 1000.0 \pm 200.0 ^b |
| Vaccinated 25 | 683.3 \pm 296.8 ^b | 1266.7 \pm 217.0 ^b |
| Vaccinated 30 | 1466.7 \pm 133.3 ^c | 933.3 \pm 133.3 ^b |
| Vaccinated 15–25 | 0 ^a | 0 ^a |
| Vaccinated 20–25 | 350.0 \pm 114.7 ^b | 566.7 \pm 108.5 ^c |
| Vaccinated 20–30 | 1466.7 \pm 133.3 ^c | 1366.7 \pm 233.3 ^d |

Non vaccinated No Ich – fish were not vaccinated and not challenged by *I. multifiliis* theronts maintained at 25 °C; Non vaccinated Ich – fish were not vaccinated and challenged by *I. multifiliis* theronts maintained at 25 °C; Vaccinated 15 – fish were vaccinated and maintained at 15 °C; Vaccinated 15–25 – fish were vaccinated and maintained at cycling water temperature from 15 to 25 °C, 5 °C per day; Vaccinated 20–25 – fish were vaccinated and maintained at cycling water temperature from 20 to 25 °C, 5 °C per day; Vaccinated 20–30 – fish were vaccinated and maintained at cycling water temperature from 20 to 30 °C, 5 °C per day.

Table 4

Cumulative mortality and median days to death (MDD) (\pm standard error) of channel catfish vaccinated with 10,000 live theronts and challenged with *Ichthyophthirius multifiliis* (Ich). Fish were held at different water temperatures after immunization to evaluate the temperature influence on fish immune response. Means followed by different superscript letters are statistically different among the treatments ($P < 0.05$).

| Treatments | Mortality | MDD |
|-----------------------|------------------------------|-----------------------------|
| Non vaccinated No Ich | 0 ^a | – |
| Non vaccinated Ich | 42.8 \pm 7.0 ^b | 11.2 \pm 0.6 ^a |
| Vaccinated 15 | 67.8 \pm 32.1 ^c | 13.1 \pm 0.7 ^a |
| Vaccinated 20 | 10.7 \pm 3.6 ^b | 6.0 \pm 1.0 ^b |
| Vaccinated 25 | 0 ^a | – |
| Vaccinated 30 | 3.6 \pm 3.6 ^a | – |
| Vaccinated 15–25 | 67.8 \pm 32.1 ^c | 14.3 \pm 0.3 ^a |
| Vaccinated 20–25 | 39.3 \pm 3.6 ^b | 6.1 \pm 2.6 ^b |
| Vaccinated 20–30 | 0 ^a | – |

Non vaccinated No Ich – fish were not vaccinated and not challenged by *I. multifiliis* theronts maintained at 25 °C; Non vaccinated Ich – fish were not vaccinated and challenged by *I. multifiliis* theronts maintained at 25 °C; Vaccinated 15 – fish were vaccinated and maintained at 15 °C; Vaccinated 15–25 – fish were vaccinated and maintained at cycling water temperature from 15 to 25 °C, 5 °C per day; Vaccinated 20–25 – fish were vaccinated and maintained at cycling water temperature from 20 to 25 °C, 5 °C per day; Vaccinated 20–30 – fish were vaccinated and maintained at cycling water temperature from 20 to 30 °C, 5 °C per day.

increased hematocrits were found in fish vaccinated at high temperatures (25 °C, 30 °C and 20–30 °C). Twenty days after immunization, significant increases in WBC were observed in all vaccinated fish compared to non vaccinated, non-infected fish (Table 5). Thrombocyte numbers increased significantly in fish vaccinated at 20 °C, 30 °C and 20–30 °C. An increase in the monocyte number was also found twenty days post-immunization in fish vaccinated at 20–25 °C, 25 °C, 30 °C and 20–30 °C compared to those vaccinated at lower temperatures and not vaccinated. Fish vaccinated at 20 and 20–25 °C exhibited significantly higher neutrophil numbers compared to other groups (Table 5).

Six days after challenge with theronts, a significant decrease in numbers of RBC, WBC, thrombocytes, and monocytes was seen in fish vaccinated at high temperatures (30 °C and 20–30 °C) (Table 6). A significant decrease in the number of neutrophils in fish vaccinated at 20–25 °C, 25 °C, 30 °C and 20–30 °C was found when compared to fish vaccinated at 20 °C.

Analyzing each parameter within in each sampling time, fish vaccinated at 30 °C, 15–25 °C and 20–30 °C showed a decrease in RBC count 6 days after challenge (Table 6) when compared to 20 days after immunization (Table 5). Twenty days after immunization, fish from all vaccinated treatments showed increases in WBC count and monocyte number compared to non vaccinated fish (Table 5). Infection (six days after challenge) reduced WBC and monocyte numbers in fish vaccinated at 20 °C, 30 °C and 20–30 °C (Table 6).

4. Discussion

Channel catfish has an optimum growing temperature between 26 °C and 32 °C [30]. Optimum growth and the best food conversion ratio was observed from 28 °C to 30 °C in a study on temperature requirements for rearing channel catfish from fingerling to market size [30]. Although considerable work has been conducted on temperature requirements for optimal growth performance in catfish, limited information is available for effects of temperature on the immune response to Ich infection in channel catfish. This study demonstrated that vaccinated channel catfish were severely impacted by low water temperature, either at 15 °C constant temperature or at 15–25 °C cycling temperature. No anti-Ich antibodies were detected from these two groups of fish, and

Table 5
Hematological parameters (\pm standard error) of channel catfish 20 days after vaccine with theronts of *Ichthyophthirius multifiliis*. Different superscript letters indicate significant difference ($P < 0.05$) among the treatments in each hematological parameter. RBC: red blood cells, HTC: hematocrit, WBC: white blood cells, Thro: thrombocytes, Lymph: lymphocytes, Mono: monocytes, Neut: neutrophils.

| Treatments | RBC $\times 10^6/\mu\text{L}$ | HTC % | WBC $\times 10^3/\mu\text{L}$ | Thro $\times 10^3/\mu\text{L}$ | Lymph $\times 10^3/\mu\text{L}$ | Mono $\times 10^3/\mu\text{L}$ | Neut $\times 10^3/\mu\text{L}$ | PAS $\times 10^3/\mu\text{L}$ |
|-----------------------|-------------------------------|-----------------------------|-------------------------------|--------------------------------|---------------------------------|--------------------------------|--------------------------------|-------------------------------|
| Non vaccinated No Ich | 1.7 \pm 0.2 ^a | 53.8 \pm 1.0 ^a | 15.2 \pm 1.9 ^a | 60.4 \pm 8.4 ^a | 8.1 \pm 1.4 ^a | 3.7 \pm 0.5 ^a | 3.1 \pm 0.4 ^a | 0.3 \pm 0.2 ^a |
| Non vaccinated Ich | 2.4 \pm 0.2 ^b | 49.3 \pm 2.2 ^b | 28.7 \pm 4.8 ^b | 79.5 \pm 10.1 ^a | 13.9 \pm 2.9 ^b | 7.9 \pm 1.0 ^b | 6.8 \pm 2.1 ^b | 0 ^a |
| Vaccinated 15 | 2.1 \pm 0.9 ^c | 44.8 \pm 1.7 ^c | 23.1 \pm 3.6 ^b | 65.9 \pm 4.9 ^a | 4.1 \pm 1.0 ^c | 8.2 \pm 1.5 ^b | 10.7 \pm 1.8 ^b | 0.1 \pm 0.1 ^a |
| Vaccinated 20 | 2.4 \pm 0.1 ^b | 51.5 \pm 2.9 ^b | 64.2 \pm 7.5 ^c | 88.3 \pm 13.3 ^b | 15.3 \pm 3.6 ^d | 27.3 \pm 4.2 ^c | 20.3 \pm 7.8 ^c | 1.1 \pm 0.5 ^b |
| Vaccinated 25 | 2.1 \pm 0.1 ^c | 69.0 \pm 2.3 ^d | 26.7 \pm 1.5 ^b | 84.5 \pm 9.6 ^a | 6.7 \pm 0.8 ^e | 13.2 \pm 1.6 ^d | 6.8 \pm 2.5 ^b | 0 ^a |
| Vaccinated 30 | 3.1 \pm 0.1 ^d | 64.7 \pm 5.9 ^e | 33.5 \pm 2.3 ^c | 154.4 \pm 20.7 ^c | 12.4 \pm 2.7 ^b | 15.0 \pm 2.1 ^e | 6.1 \pm 1.5 ^b | 0 ^a |
| Vaccinated 15–25 | 2.2 \pm 0.1 ^c | 39.3 \pm 2.0 ^f | 23.8 \pm 2.8 ^b | 45.5 \pm 3.9 ^d | 7.5 \pm 2.0 ^e | 5.1 \pm 0.8 ^f | 11.1 \pm 1.5 ^b | 0 ^a |
| Vaccinated 20–25 | 2.0 \pm 0.2 ^c | 38.7 \pm 2.3 ^f | 38.4 \pm 9.1 ^c | 71.8 \pm 13.8 ^a | 10.6 \pm 3.1 ^a | 18.6 \pm 4.0 ^g | 29.3 \pm 11.0 ^d | 0.8 \pm 0.5 ^c |
| Vaccinated 20–30 | 2.7 \pm 0.2 ^d | 57.7 \pm 2.6 ^g | 32.1 \pm 3.7 ^c | 125.2 \pm 15.7 ^c | 9.3 \pm 2.2 ^a | 17.2 \pm 4.2 ^g | 5.3 \pm 0.6 ^b | 0.2 \pm 0.1 ^a |

both suffered heavy parasite infection and showed high mortality similar to non vaccinated parasitized fish. Within or near the optimum growing temperature of channel catfish (25 °C, 30 °C, and 20–30 °C groups), good immune response (high antibody titers) and strong host protection (low mortality) were demonstrated. Our results are in agreement with findings which reported a delayed antibody response in fish immunized at low water temperature [13]. Enhanced immune response in fish immunized at high temperature was also reported [6]. The present results showed the highest antibody titer production in fish vaccinated at 30 °C regardless of exposure to cycling 20–30 °C and consequently high survival rate after challenge. High survival rate (100%) and the importance of live theront immunization in fish [5,6,11,12,20] was also supported by this study.

Our data suggest that a constant temperature of 20 °C or cycling temperature 20–25 °C was a requirement for channel catfish to develop an immune response against Ich. At cycling temperature, low end temperature had a greater influence on fish immunity especially if the temperature was below 20 °C. At 15–25 °C cycling temperature, fish were subjected to water temperature change 5 °C per day and 4 days per cycling cycle. Within a cycling temperature for 15–25 °C, fish were at water temperature 15 °C for one day, 20 °C for two days and 25 °C for one day, respectively. However, fish immune function was greatly impacted at low temperature (15 °C) even if only for 1/4 of the temperature cycle (1 day). Even when fish were then returned to 20 °C or higher in the other 3/4 temperature cycle (3 days), fish immune function was greatly impaired by the effect of short term exposure to 15 °C. Fish in the 15–25 °C cycling group had 609° days during acclimation and immunization period, similar to the 617° days for fish in 20 °C constant temperature. However, no anti-Ich antibody was detected in fish from 15 to 25 °C cycling group and fish suffered high mortality (67.8%). Clem et al. [3] and Bly and Clem [18] demonstrated that exposure to non-permissive temperature (<17 °C) resulted in immunosuppression. The fish in the 15 °C or 15–25 °C cycling group were acclimated to a non-permissive temperature (<17 °C) in our experiment may

have influenced the non-induction of immunity. The vaccinated fish at 20 °C constant temperature developed an immune response and fish were mostly protected (10.7% mortality). Rain events and/or cold fronts in the early spring and fall could result in similar temperature changes (i.e., ≥ 5 °C per day) in pond aquaculture and thus may be detrimental to catfish health when environmental temperatures are around 15 °C or lower, however, we did not test temperatures below this level.

When temperature was maintained at 20 °C or higher, a change in temperature of 5 °C per day did not impact immunity to Ich. In the 20–30 °C group, fish were at 20 °C for one day, 25 °C for 2 days, and 30 °C for one day during each cycling temperature. The fish were maintained at 25 °C or higher most of the time (3/4 of the cycling temperature). These fish developed a high level of anti-Ich antibodies and were completely protected after Ich theront challenge. No difference was observed in anti-Ich antibody level and fish mortality between fish at 20–30 °C cycling temperature and fish at 25 °C or 30 °C constant temperature. Fish immunized at 25 °C, 30 °C and 20–30 °C in the present study showed higher immobilization titer than that observed in rainbow trout [6,10], as well in channel catfish immunized at 24 °C [7]. Interestingly, when the temperature was increased from 20 °C to 25 °C (Vaccinated 20–25 °C) no significant enhancement in antibody response was noted.

Twenty days after immunization, significant increases in hematocrit and RBC, WBC, thrombocyte, and monocyte numbers were found in fish vaccinated at high temperatures (25 °C, 30 °C, 20–30 °C). Enhanced production of red blood cells in these groups is supported by the high hematocrit values. A positive correlation between these parameters in tropical fish has been demonstrated [31]. Increased RBC may also be related to increased metabolism in response to high temperatures in fish vaccinated at 30 °C and 20–30 °C [32]. Increased WBC count has been found as a consequence of immunization in fish [33,34]. However, decreased WBC counts have been found in hybrid striped bass *Morone chrysops* \times *Morone saxatilis* maintained at high temperature [35]

Table 6
Hematological parameters (\pm standard error) of channel catfish 6 days after challenge with theronts of *Ichthyophthirius multifiliis*. Different superscript letters indicate significant difference ($P < 0.05$) among the treatments in each hematological parameter. RBC: red blood cells, HTC: hematocrit, WBC: white blood cells, Thro: thrombocytes, Lymph: lymphocytes, Mono: monocytes, Neut: neutrophils.

| Treatments | RBC $\times 10^6/\mu\text{L}$ | HTC % | WBC $\times 10^3/\mu\text{L}$ | Thro $\times 10^3/\mu\text{L}$ | Lymph $\times 10^3/\mu\text{L}$ | Mono $\times 10^3/\mu\text{L}$ | Neut $\times 10^3/\mu\text{L}$ | PAS $\times 10^3/\mu\text{L}$ |
|-----------------------|-------------------------------|-----------------------------|-------------------------------|--------------------------------|---------------------------------|--------------------------------|--------------------------------|-------------------------------|
| Non vaccinated No Ich | 2.1 \pm 0.1 ^a | 49.0 \pm 2.6 ^a | 21.7 \pm 0.2 ^a | 63.0 \pm 10.3 ^a | 10.9 \pm 1.6 ^a | 6.9 \pm 1.1 ^a | 3.9 \pm 0.6 ^a | 0 ^a |
| Non vaccinated Ich | 1.7 \pm 0.5 ^b | 41.5 \pm 3.8 ^b | 26.9 \pm 14.3 ^a | 43.3 \pm 3.9 ^a | 4.2 \pm 1.9 ^b | 12.8 \pm 8.7 ^a | 9.8 \pm 3.7 ^a | 0 ^a |
| Vaccinated 15 | 1.8 \pm 0.4 ^b | 50.0 \pm 3.0 ^a | 26.9 \pm 6.7 ^b | 58.7 \pm 21.0 ^a | 13.0 \pm 3.2 ^c | 5.8 \pm 1.4 ^b | 6.5 \pm 3.0 ^a | 1.5 \pm 1.0 ^b |
| Vaccinated 20 | 2.1 \pm 0.3 ^a | 60.0 \pm 4.8 ^c | 45.4 \pm 10.3 ^b | 80.5 \pm 9.3 ^b | 3.6 \pm 1.1 ^b | 11.1 \pm 2.8 ^a | 29.4 \pm 7.4 ^b | 1.2 \pm 0.2 ^c |
| Vaccinated 25 | 2.5 \pm 0.2 ^c | 64.0 \pm 2.4 ^d | 41.6 \pm 5.9 ^b | 123.0 \pm 16.6 ^c | 19.6 \pm 5.2 ^d | 11.8 \pm 1.5 ^a | 9.5 \pm 0.9 ^a | 0.7 \pm 0.2 ^c |
| Vaccinated 30 | 0.9 \pm 0.05 ^d | 62.0 \pm 6.7 ^c | 13.9 \pm 2.9 ^c | 40.3 \pm 7.4 ^d | 2.7 \pm 0.8 ^e | 6.6 \pm 1.8 ^a | 4.2 \pm 2.0 ^a | 0.2 \pm 0.1 ^a |
| Vaccinated 15–25 | 1.5 \pm 0.3 ^e | 60.0 \pm 5.1 ^c | 27.7 \pm 7.1 ^a | 45.4 \pm 9.2 ^a | 15.1 \pm 5.2 ^f | 8.7 \pm 2.0 ^a | 3.8 \pm 0.8 ^a | 0 ^a |
| Vaccinated 20–25 | 1.4 \pm 0.1 ^e | 65.7 \pm 2.7 ^d | 31.1 \pm 2.9 ^a | 60.1 \pm 8.8 ^a | 2.3 \pm 0.3 ^e | 17.7 \pm 2.8 ^c | 9.4 \pm 2.1 ^a | 0.2 \pm 0.1 ^a |
| Vaccinated 20–30 | 1.2 \pm 0.07 ^e | 53.7 \pm 4.2 ^e | 16.6 \pm 2.6 ^c | 32.6 \pm 4.4 ^d | 7.4 \pm 1.4 ^g | 5.4 \pm 0.8 ^b | 3.3 \pm 0.4 ^a | 0.1 \pm 0.1 ^a |

and in tilapia transferred from 27 °C to 19 °C and 27 °C to 35 °C [19]. These findings showed that immune cellular response can be affected by temperature alterations. White blood cells participate directly in fish cell-mediated immune response and phagocytosis [36], which likely explains their increase after immunization. Enhanced WBC counts found in this study are in agreement with the studies in fish immunized at high temperature [14,15]. Six days after challenge WBC count of these fish in this study decreased significantly. This event may be explained by the leucocyte migration to the site of infection as demonstrated by Olsen et al. [12]. Fish immunized against *I. multifiliis* showed the presence of immunoglobulins IgT on the gill lamellae and IgM positive cells on the gill arterioles and capillaries [12]. Decreased number of WBC might be related to infection response and leukocyte trafficking to the gills or skin [12]. The number of circulating lymphocytes on the leucocyte population was reduced in fish exposed to low temperature and a decrease in the lymphocyte number could be related to apoptosis and redistribution of these cells [15]. Our results support immunosuppression caused by low temperature [3] in fish immunized at 15 °C and 15–25 °C.

Besides participation in hemostasis, thrombocytes are involved both in phagocytic [37] and inflammatory responses [27]. In the present study, hemostasis was enhanced in fish vaccinated at 30 °C and 20–30 °C. A decrease in thrombocyte numbers was observed after challenge and possibly related to migration in response to Ich infection.

Lymphocyte numbers did not vary significantly 20 days after immunization, but an increase in the lymphocyte number was found 6 days after challenge in fish vaccinated at 25 °C compared to those at 20 °C and 15 °C. While a decrease in the lymphocyte number is related to stress conditions and parasitic infections [38], an increase observed in this study may possibly be involved in the immune response [12]. The fish immune response involves activation and interaction of cells which produce humoral factors [36]. In addition to surface antibodies [12] lymphocytes are involved in immune system regulation [36]. Monocyte numbers also increased in fish immunized at high temperatures in the current study. Increased number of monocytes might be associated with an enhanced immune response, phagocytic activity [39] and in presenting antigen to lymphocytes [11,12]. In this study, immunization contributed to enhancement of monocyte in the circulating blood 20 days after immunization. Monocytes play an important role in the non-specific immune response by producing cytokines which can enhance the activity of lymphocytes [11,40]. Immunization at higher temperatures may increase immunity by not only increasing the humoral response but also by enhancing non-specific immune function.

In conclusion, the vaccinated catfish were severely impacted by low water temperature (15 °C or cycling temperature 15–25 °C). The fish at low temperatures showed no anti-Ich antibodies and suffered high mortality after Ich theront challenge. When water temperature was 25 °C or higher, channel catfish developed a strong immune response, had high survival, and showed increases in red blood cells, white blood cells, thrombocytes and monocytes.

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