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Short communication

# Effect of crowding stress on the immune response in turbot (*Scophthalmus maximus*) vaccinated with attenuated *Edwardsiella tarda*

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ABSTRACT

The aim of this study was to evaluate the immune responses in turbot, *Scophthalmus maximus*, treated with $1 \times 10^7$ cfu/ml attenuated *Edwardsiella tarda* (0.1 ml/fish) under low density (LD; ~5.25–5.13 kg/m$^2$, initial to final density), medium density (MD; ~10.41–13.95 kg/m$^2$), and high density (HD; ~20.53 –30.77 kg/m$^2$) conditions for 8 weeks. The results showed that there was a peak value in the percentage of sIg+ (surface immunoglobulin-positive) cells in blood leucocytes (BL), spleen leucocytes (SL), and pronephros leucocytes (PL) during the sixth week in the HD, which was delayed by week compared with the other groups. The specific immunoglobulin M (IgM) antibody levels increased from the first week in all groups and reached a peak in the fifth week in the LD and MD groups, but in the sixth week in the HD group. The serum cortisol levels were greater in the HD group compared with the other groups in the last 3 or 4 weeks. These results show that stocking turbot at a LD obtained the most effective immunization, and thus we conclude that crowding stress may reduce the ability to deal with immune challenge.


## 1. Introduction

In commercial aquaculture, overcrowding has negative effects on the growth, health, welfare, and immune response in farmed fish [1,2]. As a chronic stressor in teleosts, overcrowding may increase the incidence of physical injuries and susceptibility to diseases [3], as well as suppressing the immune response [4,5]. A high stocking density can elevate the level of cortisol which appears to be effective as an immunosuppressive agent with complex actions on immune cells [6]. It was shown that treating carp with cortisol (200 mg/kg) increased granulocyte proportion and decreased the proportion of lymphocytes among blood leucocytes [7].

Edwardsiellosis is a devastating fish disease that causes extensive losses in turbot (*S. maximus*) and the feasibility of using a live attenuated vaccine against edwardsiellosis has been demonstrated in turbot [8]. However, if fish undergo stress in adverse aquatic settings, vaccinations may attenuate the effects of immunoprophylaxis, and even recover their virulence [9]. Thus, it is necessary to confirm whether a live attenuated vaccine is still efficacious under crowding stress conditions. The aim of this study was to evaluate the effects of crowding stress on the immune response in turbot when exposed to attenuated *Edwardsiella tarda* in this study.

## 2. Materials and methods

### 2.1. Fish and rearing conditions

Healthy turbot weighing 150 ± 10 g were obtained from the farm of Shandong Oriental Ocean Sci-Tech Co. Ltd (Shandong, China), where the study was conducted. The fish were acclimated to the flow-through system which consisted of nine 3-m$^2$ tanks for 15 days at a same density. When the experiment began, all of the fish were randomly assigned to three different stocking density treatments: low density (LD) with 100 fish per tank (5.25 ± 0.02 kg/m$^2$), medium density (MD) with 200 fish per tank (10.41 ± 0.04 kg/m$^2$), and high density (HD) with 400 fish per tank (20.53 ± 0.05 kg/

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$m^2$). Each density treatment was tested with four replicates under immunization and control.

The fish were fed twice daily with commercial turbot feed (Ningbo Tech-Bank Co. Ltd, Zhejiang, China) and the daily ration was 1% of their body weight. During the test period, the water temperature was maintained at 16 ± 0.5 °C, salinity at 28.2 ± 3‰, dissolved oxygen at 8.0 ± 0.5 mg/l, and pH at 7.5 ± 0.3. The photoperiod was 12-h light and 12-h dark. No mortality was recorded during the experiment.

### 2.2. Vaccination

The strain of attenuated *E. tarda* was provided by Professor Yuanxing Zhang and it was described previously by Xiao et al. [8]. The bacteria were suspended in 0.9% physiological saline at a concentration of approximately $1 \times 10^7$ colony forming units (cfu) per ml and stored at 4 °C until use for vaccination. The experimental fish were injected with 100 μl attenuated *E. tarda* and the control fish received 100 μl of 0.9% sterile physiological saline.

### 2.3. Sampling

The fish were sampled after fasting for 24 h. The first samples were collected on the day before immunization. Five fish were randomly netted from each immunized and control density group immediately after anesthetization with 0.05% tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO, USA). Samples were collected once each week after immunization until the eighth week.

Leucocytes were isolated from the blood, spleen, and pronephros using the methods described by Xu et al. [10], with slight modifications. Briefly, blood was collected from the caudal peduncle in syringes and diluted with four volumes of 65% RPMI-1640 (containing 20 IU/ml heparin and 1% bovine serum albumin; BSA). After maintaining at 4 °C for 1 h, the red cells were separated by centrifugation at 100 × *g* for 10 min. The spleen and pronephros were ground gently through a 100 μm nylon gauze filter and cell suspensions were prepared in 65% RPMI-1640. The cell suspensions were layered over a discontinuous Percoll gradient containing two Percoll densities of 1.070 g/$cm^3$ and 1.020 g/$cm^3$. Before layering, the tubes were washed with newborn calf serum. The leucocyte layer was obtained from the 1.020–1.070 g/$cm^3$ interface after centrifugation at 840 × *g* for 30 min. The leucocytes were washed three times with phosphate-buffered saline (PBS) to remove the Percoll, centrifuged at 640 × *g* for 5 min, and then subjected to flow cytometry analysis.

The remaining blood samples were collected in centrifuge tubes and stored overnight at 4 °C. The blood samples were then centrifuged for 20 min at 3000 × *g* to collect the serum and stored at —80 °C until use.

### 2.4. Flow cytometry analysis

The number of leucocytes in PBS was adjusted to $10^6$ cells/ml before incubating for 1 h at 37 °C with 1:50 diluted mouse-anti-turbot IgM monoclonal antibody (MATI-MAb; ADL, Stryn,UK), where the mouse IgG culture supernatant was used as the negative control. Subsequently, the cells were washed three times with PBS containing 5% (v/v) newborn calf serum for 5 min at 640 × *g*. After washing, the cells were incubated with goat-anti-mouse Ig-FITC (Fluorescein isothiocyanate) (1:256, Sigma) for 45 min at 37 °C and washed again. Next, the leucocytes were analyzed by flow cytometry (BD Accuri C6, Franklin lakes, New Jersey, USA).

### 2.5. Specific antibody and serum cortisol

Enzyme-linked immunosorbent assays (ELISA) were performed to detect the specific antibody according to the method described by Xiao et al. [8], with minor modifications. Turbot serum was diluted 20-fold in PBS and used in the ELISA assays. Microtiter plate wells (96-well plates, Costar) were coated with *E. tarda* ($10^{-6}$ cfu/well) in 100 μl of carbonate-bicarbonate buffer in triplicate, and then incubated overnight at 4 °C. The wells were washed three times to remove any excess cells with PBS-T (phosphate buffer saline with 0.05% tween-20) and then blocked with 200 μl of PBS containing 3% BSA for 2 h at room temperature. Each plate was washed as described above and the wells were incubated with 100 μl of diluted serum for 2 h at 37 °C. After incubation, the plates were washed and 100 μl of mouse anti-turbot IgM (Aquatic Diagnostic, Stirling, UK) diluted 1:30 in PBS was added, before incubating for 1 h at 37 °C. The plates were washed three more times and incubated for 1 h at 37 °C with 100 μl of goat-anti-mouse IgG peroxidase conjugate diluted 1:4000 in PBS. After three more washes, 100 μl of 3,3′,5,5′-tetramethylbenzidine (Sigma) was added to each well and the plates were incubated for 5 min at room-temperature in the dark. After adding 50 μl $H_2SO_4$ (2 M) to each well to terminate the reaction, the absorbance was measured at 450 nm with a microplate reader. Serum cortisol was determined using a commercially available radioimmunoassay kit (Mlbio, Shanghai, China) according to the manufacturer's instructions. The 50 μl samples and standards were respectively incubated in 100 μl monoclonal antibody-coated tubes with 100 μl $^{125}$I-labelled cortisol tracer. After shaking well, the tubes were incubated at 37 °C for 1 h. Then, 500 μl immune separating agents were added to each well and incubated for 15 min at room-temperature. The suspensions were discarded by centrifugation at 1000g for 20 min. The radioactivity bound to the antibody was measured. Each sample and standard was tested in duplicate.

### 2.6. Statistics

SPSS version 18.0 for windows was used to perform the statistical analyses. The data were expressed as the mean ± standard error of the mean. One-way analysis of variance and Tukey's test were used to evaluate the significant differences between groups. Differences in the results obtained from the experiments were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Flow cytometry analysis

Leucocytes isolated from the blood (BL), spleen (SL), and pronephros (PL) were analyzed based on their forward scatter (FS) and sideward scatter (SS) patterns, where the FS and SS parameters represented the cell size and granularity, respectively. Dead cells and debris were excluded from the analyses by gating. The gated leucocytes and three representative BL, SL, and PL samples from the LD groups in the fifth week after immunization are shown in Fig. 1. The fluorescence histogram showed that the negative control only contained a single peak with weak self-fluorescence (L1) whereas the positive sample exhibited two peaks, where the second peak indicated the subpopulation of $sIg^+$ lymphocytes (L2).

### 3.2. Kinetics of $sIg^+$ cells in isolated BL, SL, and PL

The kinetics of the proportions of $sIg^+$ cells in BL from fish reared in the three density groups are shown in Fig. 2. The proportions of $sIg^+$ cells were relatively stable in the controls