

Influence of Maternal and Larval Immunisation against *Lactococcus garviae* Infection in Rainbow Trout *Oncorhynchus mykiss* (Walaum) Lysozyme Activity and IgM Level

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Abstract

This study evaluated efficacy of maternal and larval immunisation against *Lactococcus garviae* infection and on the lysozyme and immunoglobulin (IgM) levels in rainbow trout *Oncorhynchus mykiss* (Walaum). Forty-eight-day-old larvae (mean weight 96 mg) originating from injected weekly with letrozole and immunised, only immunised and non-immunised parents were experimentally infected with the *L. garviae*, and the mortality rate was recorded daily. Larvae were vaccinated by immersion at 58 days post hatch with live *L. garviae* (10^9 cells/mL) for 15 min. Every third day post larvae vaccination, two larvae from each group were collected for analysis lysozyme (by a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus*) and IgM (by enzyme-linked immunosorbent assay (ELISA)) parameters. Vaccinated and control larvae were tested for protection against *L. garviae* 30 days post larvae immunization when the larvae were 88 days old. Larvae were challenged by bath exposure with live *L. garviae* (10^9 cells/mL) for 2 min and monitored for mortality for at least 10 days following challenge. The challenge experiment with *L. garviae* showed a significant reduction in larvae from immunised ($54.44\% \pm 0.64\%$) and injected weekly with letrozole and immunised fish ($52.96\% \pm 0.97\%$) compared to larvae from control fish ($62.96\% \pm 2.22\%$). Vaccinated larvae originated from injected weekly with letrozole and immunised parents showed significantly higher lysozyme activity compared to other fish groups. Vaccinated larvae showed significantly less mortality compared to controls. The rela-

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tive percent survival (RPS) values of larvae from only immunised, injected weekly with letrozole and immunised and non-immunised parents vaccinated with *L. garvieae* were $67.36\% \pm 0.9\%$, $68.05\% \pm 0.66\%$ and $48.27\% \pm 2.79\%$ respectively. The results indicate that the effect of maternal immunization rainbow trout against *L. garvieae* infection by eliciting the immune responses as indicated by an increase in the IgM level and lysozyme activity.

Keywords

Immunisation, Relative Percent of Survival (RPS), Rainbow Trout Larvae, *Lactococcus garvieae*, Immunoglobulin

1. Introduction

Lactococcus garvieae is considered the most important risk factor for the rainbow trout and marine fish industry [1]. Also the rapidly expanding aquaculture industry incurs a substantial economic loss due to lactococcosis caused by *L. garvieae* resulting in more than 50% mortality over a period of 3 - 7 days [2]. In a study in 2002 in Iran from 235 cases of streptococcosis among rainbow trout collected from farms of Fars province [3], streptococci were isolated from 99 cases. These isolates were biochemically allocated to *Streptococcus iniae* and *L. garvieae*. Also *L. garvieae* outbreaks in farmed rainbow trout are described from different parts of Iran [4] [5]. Therapeutic measures are generally ineffective and therefore, development of vaccines is essential to control the disease [4] [6].

There have been a variety of studies analyzing the effectiveness of immunisation in preventing Lactococcosis in rainbow trout [7]. To prevent rainbow trout lactococcosis caused by *L. garvieae*, different vaccine formulations have been administered by intraperitoneal injection, showing good initial levels of protection but very short duration of immunity and the RPS values obtained with the aqueous vaccine was 40% [7]. Petrie-Hanson and Ainsworth [8] carried out field trials with this type of vaccines in Spanish fish farms and observed mortalities of 1.7% in vaccinated fish compared to 6.5% in non-vaccinated fish. The optimal time for vaccination is when fish weight approximately 50 g.

Larvae vaccinated with *Photobacterium damsel* subsp. *piscicida* (*phdp*) and lipopolysaccharides (LPS) in sea bream (*Sparus auratus*) showed significantly higher lysozyme activity and total immunoglobulin compared to the controls. Vaccinated larvae from both groups showed significantly less mortality compared to the respective controls and RPS values of larvae from immunised parents vaccinated with *phdp* and LPS was 95.83% and 72.22% respectively [9]. Significant increase of the survival in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae was reported after exposure to 50 and 100 µg/mL of LPS from day 20 to 96 and throughout the yolk sac period as compared to untreated larvae [10].

Letrozole (CGS 20264), with commonly used brand name Femara, is a non-steroidal triazole derivation and one of the most potent aromatase inhibitors yet developed [11]. It has been approved for the first line therapy for hormone receptor positive, metastatic breast cancer in postmenopausal women [11] [12]. Letrozole has potential for use to prevent the conversion of androgenic steroids to estrogens [12]. Also it is capable of inhibiting aromatase 98% - 99% and reducing serum concentrations of estrone and E₂ beyond the limit of detection in patients [11] [12].

During the last decade, a number of studies have shown that in addition to their classically described reproductive functions, estrogens and androgens also regulate the immune system in teleost [13].

The reproductive-immune interactions studied in fishes indicate that sexual maturation (*i.e.*, gonadal maturity and reproductive activities) can potentially affect both the innate and adaptive immune responses [13]. In fish, the reproductive cycle is marked by seasonal variation in plasma sex-steroids and by changes in immune parameters which render them more sensitive to the presence of pathogens [14]. Furthermore, immune biomarkers may prove useful for the identification of contamination in the wild [13].

Other studies showed that estrogens may affect the immune system directly or more likely indirectly through several tissues and may modulate the immune system by altering the patterns of cytokines and apoptosis [13] [14]. For example, in *O. mykiss*, and gilthead seabream, shown that the rise of sex hormones can suppress the plasma IgM and IgM secreting cells during spawning period leading to immunosuppressive condition although

the suppressive effects of E₂ on IgM production are not observed in carp and zebra fish (*Danio rerio*) [15].

Elevation of sex steroids can make fish more vulnerable to several microbial infections during the spawning season and subsequent mortality following the spawning phase. Therefore the overall health and immune status of brood fish is very important not only for breeding performances but also for the health condition of offspring [10] [14] [16]. In this paper, we investigated the effects of endocrine disrupter component (EDC) letrozole on lysozyme and IgM levels after female immunisation of rainbow trout

This study was aimed to determine the effect of rainbow trout maternal and larval immunisation and injected of non-steroidal compound, letrozole, two months before spawning season on the susceptibility by *L. garvieae* and on lysozyme activity and IgM levels.

2. Materials and Methods

2.1. Bacteria

L. garvieae (EU727199) used in this study was isolated from an outbreak of Lactococcosis in Fars province, Iran. This strain was selected on the basis of its antigenic characteristics that were determined in previous work [17].

2.2. Media and Culture Conditions

Inoculate of *L. garvieae* were prepared by growing cells overnight on blood agar (BioMerieux, France) at 25°C for 24 - 48 h. Bacterial cells were collected by centrifugation at 6500 r/30 min at 4°C and washed three times with phosphate buffered saline (PBS:pH 7.4) and then were re-suspended in PBS at a final concentration of 1.2×10^8 cells/mL. Cultures were checked for purity and the number of colony forming units (CFU) estimated by plate counting [18].

2.3. Preparation of the Bacteria

A formaline-killed vaccine was prepared as previously described [18]. The selected strain of *L. garvieae* was grown in Trypticase Soy Broth (TSB, Difco) for 48 h, obtaining a final concentration of 1×10^9 cells/mL. Then cells were killed by addition of formalin to achieve a final concentration of 0.3% and incubated for 3 h at 25°C and then 4°C overnight.

2.4. Fish and Rearing Conditions

Adult rainbow trout (mean weight approximately 850 g) were maintained in 800 L concrete pond, under natural day length, at the Dalkhan Fisheries Research center of Shiraz, Iran. The water temperature was maintained at 12°C - 15°C, with oxygen 5 mg/L and pH 7.5. The total capacity of the system was 9 m³ and the exchange of 25% water occurred every day. The levels of ammonia, nitrites and nitrates were monitored daily. Fish were fed 2 times per day with pellets a commercial feed at 1% of body weight.

2.5. Vaccination and Injection of Letrozole of Brood Fish

Thirty six adult rainbow trout (*Oncorhynchus mykiss*) females were obtained in mid-September 2011 from Dalkhan Fisheries Research Center of Shiraz, Iran and held outdoors in a 10 m² concrete pond, which was constantly with through-flowing river water. The fish were kept in well-aerated water at $15.8^\circ\text{C} \pm 0.5^\circ\text{C}$, dissolved oxygen 5.5 ± 0.1 mg/L and pH 7.8 ± 0.18 . Fish were initially weighted (854 ± 0.1 g) under anesthesia (150 mg/L clove oil). After 7 days of acclimation, they randomly divided in three groups and each group was kept in a 2 m² concrete pond with a water depth of 50 cm supplied with through-flowing river. During the experiment, the fish were feed with commercial salmon food (Beyza Feed Mill (BFM) Co., Ltd., Iran). The non-steroidal aromatase inhibitor Letrozole (CGS 20267) was obtained as a gift from Iran hormone venture pharmaceutical technology development Co., Ltd., Iran was dissolved in the vehicle dichloromethane [19]. One of groups was injected with 2.5 mg letrozole/kg weekly, and other groups were only injected with vehicle dichloromethane. After three weeks, the group injected weekly with letrozole and twelve females injected with dichloromethane were intraperitoneally (ip) immunised with 1.0 mL of formalin (3%) inactivated *Lactococcus garvieae* (10^9 cells/mL and twelve females of control group was ip immunised only with 1.0 mL sterile PBS (phosphate-buffered saline 0.1 mol/L, pH 7.2).

2.6. Larvae Rearing Conditions

The completion of yolk sac absorption was achieved very early at day 14. Larvae were fed 8 times per day with pellets from a commercial feed at 5% of body weight. After weaning they were transferred to Department of Aquatic Animal Health Unit, School of Veterinary Medicine, and Shiraz University, Iran at day 42 of age. Larvae were kept in a tank system. The water temperature was maintained 12°C - 15°C, oxygen 5 mg/L and pH 7.8. The water was aerated with the aid of an air pump with capacity of 1 m³/h. Each tank had 500 L holding capacity, the total capacity of system was 6 m³ and the water exchange occurred every 1 h. The levels of ammonia, nitrites and nitrates were monitored daily. Larvae were fed two times per day with pellets from a commercial feed at 1% of body weight. Experimentally infected larvae were kept in a static water aquaria system and fed two times a day. All other conditions were as described previously.

2.7. Susceptibility Test of Rainbow Trout Larvae from Injected Weekly with Letrozole and Immunised, Only Immunised and Non-Immunised Parents

A virulent bath challenge was used to evaluate differences in protective immunity in larvae from vaccinated and non-vaccinated parents when larvae were 48 days old. The challenge baths were prepared by cultivating the bacteria in Trypticase Soy Broth (TSB; Difco) for 48 h with continuous shaking. The cultures were washed twice in phosphate-buffered saline (PBS, pH 7.2). After final centrifugation, the suspension was diluted to a density giving 50% transmission at 520 nm in a spectrophotometer, corresponding to approximately 1.2×10^8 cells/mL. For the challenge experiments, triplicate groups of forty-eight days old fry originating from non-immunised (NIM), immunised (IM) only and injected with letrozole and immunised (Let.IM) broodstock were immersion for 2 min with dilutions of 1.2×10^8 bacterial cells/mL. Control non infected larvae (CNI) of each group received exactly the same treatment with sterile sea water. Thirty larvae were used in each sub group. Mortalities were monitored for period of 10 days and fish were tested with sampling of kidney and viewing it by microscope to confirm the cause of morbidity.

2.8. Bath Vaccination Experiments of Larvae from Immunised and Non-Immunised Fish

For the bath vaccination experiments triplicate groups of fifty-eight days old larvae originating from non immunized (NIM), immunized (IM) only and injected with letrozole and immunized (Let.IM) females were vaccinated by immersion with 1.0 mL of formalin (3%) inactivated *L. garvieae* (10^9 cells/mL). Also some fifty-eight days old larvae originating from NIM, IM and Let.IM females were vaccinated only with phosphate buffered saline (PBS) as control non immunized (CNI) larvae of each group.

2.9. Collection of Larvae after Vaccination, Weight and Homogenates

Every third day post-larvae vaccination, two larvae from each group were collected for analysis of lysozyme activity and total immunoglobulin. Larvae were washed two times with sterile PBS, pH 7.2, homogenized with 1 volume of phosphate-buffered saline and centrifuged at 3000 r/10 min at 4°C. The supernatants were collected, centrifuged once at 3000 g for 5 min and stored at -20°C. Samples from the different larval rearing were collected, rinsed with distilled water, and their wet body weight measured and recorded. Weight was recorded in triplicate groups.

2.10. Susceptibility Test of Vaccinated Larvae from Injected Weekly with Letrozole and Immunised, Only Immunised and Non-Immunised Parents

Vaccinated and control larvae were tested for protection against *Lactococcus garvieae* 30 days post immunisation when the larvae were 88 days old. Larvae were challenged by bath exposure as described above with the previously dose (1.2×10^8 cells/mL). Thirty larvae were used in each group and the experiment was run in triplicate. Mortality was recorded for 10 days and the RPS was calculated:

$$\text{RPS} = (1 - \% \text{mortality of immunised larvae} / \% \text{mortality of control non-immunised larvae}) \times 100 \text{ [20].}$$

2.11. Lysozyme Activity

The lysozyme activity of samples (larval homogenates) was measured using a method based on the ability of

lysozyme to lyse the bacterium *Micrococcus lysodeikticus* [21]. In a 96-well microplate, 250 μ L of larval homogenates in four twofold serial dilutions in PBS (0.05 M, PH 7.2) were mixed with 250 μ L of 0.7 mg/mL suspension of *Micrococcus lysodeikticus* (Sigma) in phosphate buffer (175 mL). The micro plate was incubated at 24°C and O.D. was read at 450 nm at 0, 15, 30 and 60 min. For a positive and a control, serum was replaced by hen egg white lysozyme (serial dilutions starting at 1.6 μ g/mL) and buffer respectively. Data were expressed either as μ g/mL.

2.12. Enzyme Linked Immunosorbent Assay (ELISA) for Determination of Total Immunoglobulin

The IgM levels of Larval extracts of fish were assessed with ELISA by the method of Bakopoulos, Peric [22] with some modification. 50 μ L of serial dilutions of the sera (1:50) were added to the appropriate micro titer plate wells with a HRP-conjugated IgM and antibody preparation specific for IgM and incubated. Then substrate solutions were added to each well. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm \pm 2 nm. The concentration of steroid in the samples was then determined by comparing the O.D of the samples to the standard curve. All samples were placed in triplicate on the plates and the mean and standard deviation (S.D) were calculated for each sample IgM concentration. Data were expressed either as μ g/g of wet body weight larvae.

2.13. Statistical Analysis

Data were evaluated using one-way analysis of variance (ANOVA). Groups were considered to be significantly different if $P < 0.05$. When a significant F value was obtained for ANOVA the differences between all groups were tested by using Duncan's multiple range test. All statistics were performed using SPSS for windows version 16.

3. Results

3.1. Susceptibility Test of Larvae from Injected Weekly with Letrozole and Immunized (Let.IM), Only Immunized (IM) and Non-Immunized (NIM) Parents

The larvae originating from Let.IM and IM parents showed delayed onset and reduced mortality rates in comparison to the larvae originating from NIM parents. The challenge programmed using with 1.2×10^8 cells/mL of *L. garviae* in the larvae from each group indicated a cumulative mortality rate was significantly lower in the larvae from Let.IM parents (52.96% \pm 0.97%) than the larvae from IM (54.44% \pm 0.64%) and from NIM (62.96% \pm 2.22%) parents. Cumulative mortality rate, in the larvae originated from IM and Let.IM parents had not significantly different (Table 1). The mortality rate was again different between larval groups, with the mortalities of larvae originating from Let.IM and IM broodstock beginning between day3 while for the offspring of NIM parents mortalities started from day 1 (Figure 1).

Microscopic evaluation ($\times 100$) of the kidney tissue confirmed the cause of morbidity (*L. garviae*) (Figure 2).

The RPS values of larvae from only immunised, injected weekly with letrozole and immunised and non-immunised parents were 66.41% \pm 1.20%, 66.42% \pm 0.83%, and 53.88% \pm 0% respectively. Significant difference ($P < 0.05$) was found between IM and NIM, also Let.IM and NIM. No significant difference ($P > 0.05$) was found among IM and Let.IM.

3.2. Weight of Larvae

The wet body weight of vaccinated larvae was recorded in three groups from day 0 to day 30 (Figure 3). A difference in body weight increase was observed in larvae originating from Let.IM, IM and parents, but this difference was non-significant ($P > 0.05$). In vaccinated larvae from immunised (IM), injected weekly with letrozole and immunised (Let.IM) and from non-immunised (NIM) parents vaccinated with *L.garviae*, the body weight was 252.67 \pm 0.66, 250 \pm 1.15 and 251.33 \pm 1.33 mg respectively.

3.3. Lysozyme Activity

Data on lysozyme activity in the larval extracts were shown in Figure 4. From day 0 to day 30 and increase in

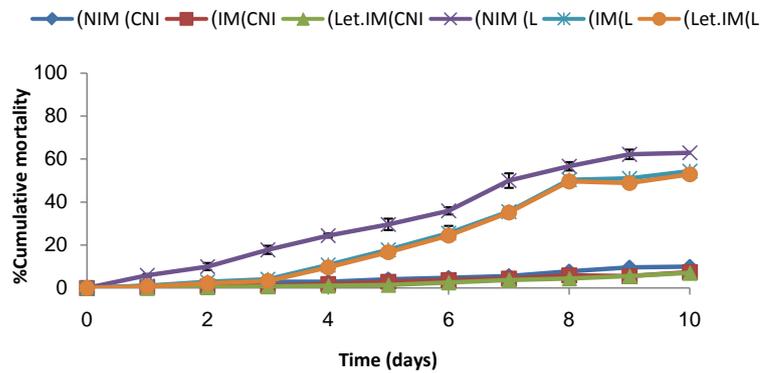


Figure 1. Cumulative percent mortality due of larvae from injected weekly with letrozole and immunised (Let.IM) and only immunised (IM) non-immunised (NIM) broodstock Control Non-infected (CNI). Each data point represents the mean (\pm S.E.) of triplicates.

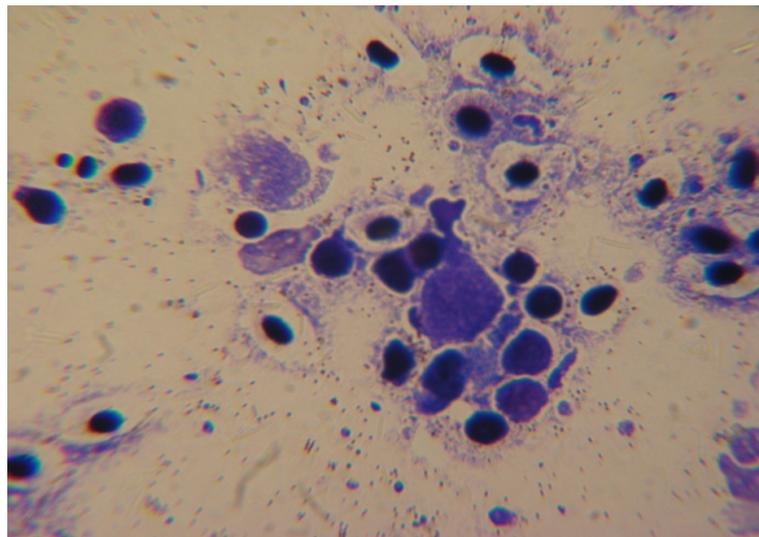


Figure 2. Microscopic evaluation ($\times 100$) of the kidney tissue to confirm the cause of morbidity.

Table 1. Difference tested by one-way ANOVA for Cumulative mortality (%) in larvae* of experimental groups.

Groups/days	1	2	3	4	5	6	7	8	9	10
NIM (CNI)	A	BCD	AB	A	B	A	A	A	B	A
IM (CNI)	A	AB	A	A	AB	A	A	A	A	A
Let.IM (CNI)	A	A	A	A	A	A	A	A	A	A
NIM (L)	B	D	C	C	D	C	C	C	D	C
IM (L)	A	C	B	B	C	B	B	B	C	B
Let.IM (L)	A	BC	AB	B	C	B	B	B	C	B

*Larvae from immunised (IM), injected weekly with letrozole and immunised (Let.IM) and non-immunised (NIM) parents Control Non-infected (NIC). Each data point represents the mean (\pm S.E.) of triplicates..different letters indicate a significant difference ($P < 0.05$).

lysozyme activities was observed. Significant difference ($P < 0.05$) was found among activities in IM, Let.IM and NIM groups (Figure 4).

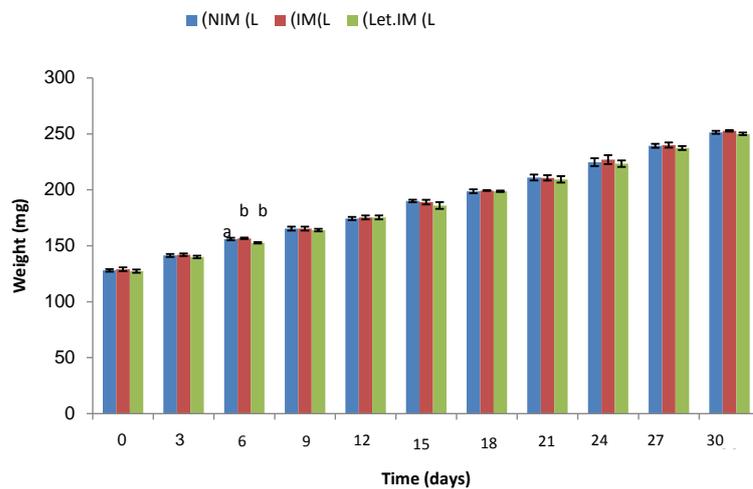


Figure 3. The wet body weight of vaccinated larvae from injected weekly with letrozole and immunized (Let.IM), only immunised (IM) and non-immunised (NIM) broodstock. Each data point represents the mean (\pm S.E) of triplicates. Similar values among the groups are identified by the same super-script letter ($P > 0.05$).

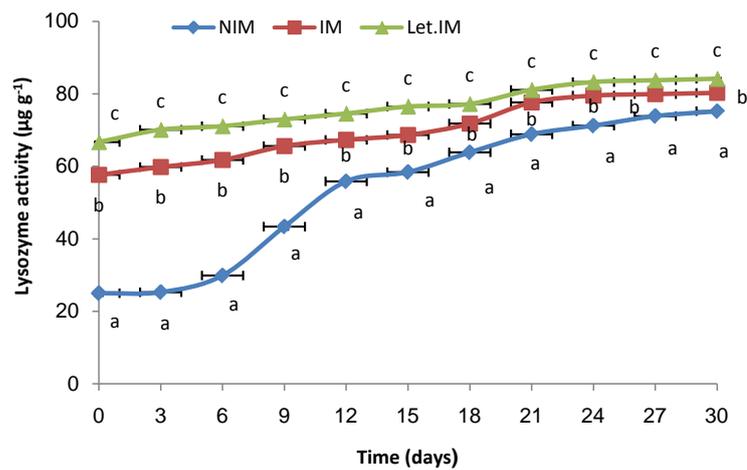


Figure 4. Total lysozyme level changes ($\mu\text{g/g}$ wet body weight) in larvae from only immunised (IM), injected weekly with letrozole and immunised and non-immunised (NIM) parents. Each data point represents the mean (\pm SE) of triplicates. Different letters indicate a significant difference ($P < 0.05$).

3.4. IgM Levels

Data on IgM in the larval extracts were shown in **Figure 5**. Significant difference ($P < 0.05$) was found among immunoglobulin levels between Let.IM, IM and NIM. No significant difference was found between IM and Let.IM ($P > 0.05$).

3.5. Susceptibility Test of Vaccinated Larvae from Injected Weekly with Letrozole and Immunised, Only Immunised and Non-Immunised Parents

Data on cumulative mortality rate in immunised (IM (L) and control non immunized (CNI) larvae of each female (IM, Let.IM and NIM females) after challenging with live *Lactococcus garvieae* (L) (1.2×10^8 cells/mL) were shown in **Figure 6**. The cumulative mortality rate was significantly lower in immunised larvae originating from Let.IM and IM females compared to immunised larvae from NIM females. Significant ($P < 0.05$) was

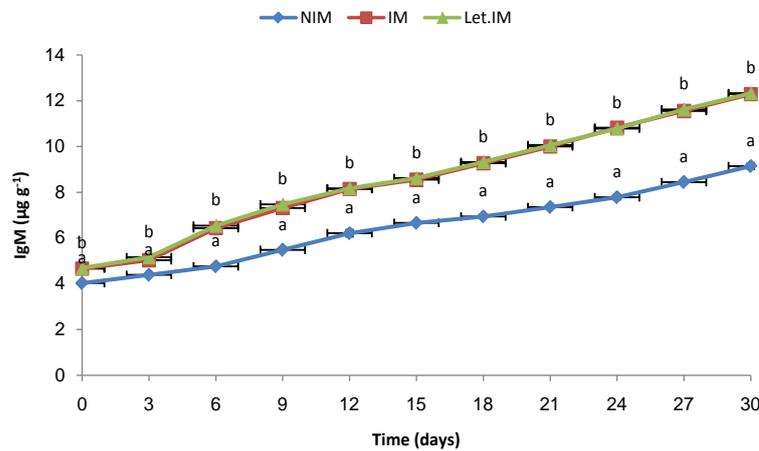


Figure 5. Total IgM level changes ($\mu\text{g/g}$ wet body weight) in larvae from immunised (IM), injected weekly with letrozole and immunised (Let.IM) and non-immunised (NIM) parents after immunised. Each data point represents the mean ($\pm\text{SE}$) of triplicates. Different letters indicate a significant difference ($P < 0.05$).

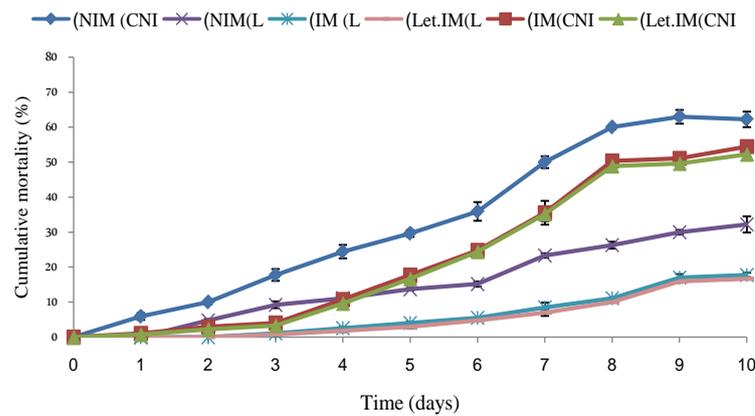


Figure 6. Cumulative mortality with LD_{50} infection of *Lactococcus garviea* (L) in larvae from immunised (IM), injected weekly with letrozole and immunised (Let.IM) and non-immunised (NIM) parents. Control non-immunised (CNI). Each data point represents the mean ($\pm\text{S.E.}$) of triplicates.

found among the cumulative mortality rate between IM (L), IM (CNI) and NIM (CNI), NIM (L) groups and Let.IM (L), Let.IM (CNI) and NIM (CNI), NIM (L) (Table 2).

The RPS values of larvae from immunised, injected weekly with letrozole and immunised and non-immunised parents vaccinated with *L. garvieae* was $67.36\% \pm 0.9\%$, $68.05\% \pm 0.66\%$ and $48.27\% \pm 2.79\%$ respectively (Table 3). Significant difference ($P < 0.05$) was found between IM and NIM, also Let.IM and NIM. No significant difference was found between IM and Let.IM ($P > 0.05$).

4. Discussion

The aquaculture industry is recently facing a serious setback due to infectious [23] diseases leading to severe economic loss [24]. Although mortalities have been reported in all stages of life, maximum mortalities in all the cultivated fish species have been reported at larval stages. Due to lack of immune competence at the early stages [25], an alternate method was evaluated for enhancing the resistance for more availability of healthy fish seed by immunisation.

Delamare-Deboutteville, Wood [26] used immunisation (*i.e.* by injection and immersion in formalin killed

Table 2. Difference tested by one-way ANOVA for Cumulative mortality (%) larvae* of experimental groups after immunized by bath exposure for 15 min with formalin (3%) treated *L. garvieae* 10⁹ cells.

Groups/days	1	2	3	4	5	6	7	8	9	10
NIM (CNI)	B	D	D	C	D	D	D	D	D	D
IM (CNI)	A	B	B	B	C	C	C	C	C	C
Let.IM (CNI)	A	B	AB	B	C	C	C	C	C	C
NIM (L)	A	C	C	B	B	B	B	B	B	B
IM (L)	A	A	A	A	A	A	A	A	A	A
Let.IM (L)	A	A	A	A	A	A	A	A	A	A

*Larva from immunized (IM), injected weekly with letrozole and immunized (Let.IM) and non-immunised (NIM) parents Control non-immunised (CNI). Different letters indicate a significant difference ($P < 0.05$).

Table 3. Difference tested by one-way ANOVA for RPS values (%) in larvae* of experimental groups after immunised by bath exposure for 15 min with formalin (3%) treated *L. garvieae* 10⁹ cells/mL.

Days/Groups	NIM	IM	Let.IM
2	51.48 ± 4.55a	100 ± 0b	100 ± 0b
3	48.01 ± 1.01a	72.22 ± 2.77b	91.66 ± 8.33b
4	54.81 ± 1.70a	75.91 ± 2.14b	81.16 ± 2.38c
5	53.71 ± 1.03a	77.01 ± 2.30b	82.02 ± 2.76b
6	57.56 ± 1.26a	77.57 ± 0.69b	80.34 ± 1.07b
7	53.16 ± 2.60a	76.18 ± 2.10b	80.14 ± 1.83b
8	56.20 ± 1.18a	77.89 ± 0.70b	79.81 ± 0.62b
9	52.32 ± 0.50a	66.71 ± 1.24b	67.43 ± 0.43b
10	48.27 ± 2.79a	67.36 ± 0.9b	68.05 ± 0.66b

*Larvae from immunised (IM), injected weekly with letrozole and immunised (Let.IM) and non-immunised (NIM) parents. In a column different letters indicate a significant difference ($P < 0.05$).

bacteria) to look at the possibility of vaccination of *Lates calcarifer* against streptococcus (entrococcus). After one month, a positive effect vaccination was observed in immunised fish. Also the percent of mortality in control group was 75%. In our experiment, rainbow trout were vaccinated with *L. garvieae* bacterial cells. Larvae originating from IM, Let.IM and NIM parents were subjected to experimental infectivity trials with *L. garvieae* at day 48 post-hatch. The larvae originating from IM and Let.IM parents showed delayed onset and reduced mortality rates in comparison to the larvae originating from NIM parental group. The present results are comparable with other studies on transfer of maternal immunity in some fish species. In the sea bream, plaice, tilapia and red sea bream, transfer of specific protection against various pathogens has been reported [10] [14] [16]. However, experimental immunisation of Atlantic salmon brood females against yearsiniosis did not protect the offspring against the disease although specific antibodies were demonstrated in the eggs and fry [10]. Similarly, transfer of maternal antibody from red sea bream mother fish, immunised against *Vibrio* sp., and did not have protective value for the larvae. This is in accordance with the fact that the concentration of maternal IgM in the larval stages is generally greatly reduced from that observed in the ovarian stages [14]. The ability of larvae originating from Let.IM and IM parents to respond better to infectious was clearly seen in this study (RPS), Cumulative mortalities in larvae originating from Let.IM and IM parents in comparison to larvae originating from NIM group revealed a difference in favour about 10% (Figure 1). The mechanism underlying the transfer of maternal immunity and protection in offspring by injection of letrozole and immunisation of fish prior to spawning season needs further studies.

For assessing the ability and the levels of response from these larvae against *L. garvieae*, the larvae were vac-

cinated via immersion with *L. garviea* bacterial cells at day 58 (128 mg). The following 30 days larval extracts were evaluated for the presence of non-specific (lysozyme) and specific (IgM) humoral immune parameters and at day 88 they were subjected to experimental infectivity trials with *L. garviea* virulent bacterial cells in order to assess the effect of the above vaccinations on the protection of larvae.

The effectiveness of vaccination of cultured fish heavily depends on the degree of maturity of their immune system [14]. The first appearance of surface IgM in lymphocytes varies considerably in different fish species, this appearance being generally more dependent on size than age [27] [28], although this is not always the case [28]. Fresh water species like rainbow trout and channel cat fish show the first appearance of surface IgM at about 1 week after hatching [28]. Fish larval are very small in size and fragile at the early stages of their life cycle and vaccination by injection is unfeasible. Immersion and oral vaccination is an effective and practical method for mass vaccination of fish larvae.

The wet body weight of larvae was similar and no significant difference ($P > 0.05$) was found in all groups (Figure 3).

Higher lysozyme levels were found in immunised larvae originating from immunised IM and Let.IM females. Significant difference ($P < 0.05$) was found among lysozyme activity between IM and NIM (CNI) larvae and Let IM and NIM (Figure 4). The present result is comparable with investigation of Hanif, Bakopoulos [9] on seabream that larvae vaccinated with *Photobacterium damsela* subsp. *piscicida* and *liopo lysaccharides* showed significantly lysozyme activity compared to the controls [9]. Lysozyme is a bactericidal enzyme, involved in hydrolysis of the β -(1, 4) linked glycoside bonds of bacterial cell wall peptidoglycans. Lysozyme has also been detected in oocytes, fertilized eggs and larval stages of several species, for examples eggs of coho salmon and other salmonids [29]. Also there are several reports of lysozyme isolated from fish serum and ova, being bactericidal even for important fish pathogens like *Edwardsiella tarda* [25], *Escherichia coli* [30], *A. salmonicida* and *A. hydrophila* [16] and *Photobacterium damsela* [9].

Higher total immunoglobulin levels were found in immunised larvae originating from IM and Let.IM females. Significant difference was found among IgM levels between IM, and NIM (CNI) larvae and between Let IM and NIM (Figure 5). The present result is comparable with investigation of Hanif, Bakopoulos [9] on seabream that larvae vaccinated with *Photobacterium damsela* subsp. *piscicida* and *liopo lysaccharides* showed significantly total IgM levels compared to the controls [9].

Sex hormones are usually elevated during spawning season of fishes and the rise of sex hormones can severely affect the immunity of fishes [13]. It is now well established that estrogen receptors [31]. Mechanistically estrogens can influence immune responses by acting directly on lymphoid tissue or indirectly through multiple non lymphoid targets [31] [32]. For example, in *O. mykiss* and gilthead sea bream, shown that the rise of sex hormones can suppress the plasma IgM and IgM secreting cells during spawning period leading to immunosuppressive condition [32] [33]. Due to, elevation of sex steroids can make fish more vulnerable to several microbial infections during the spawning season and subsequent mortality following the spawning phase. We used the endocrine disrupter component (EDC) letrozole for rising lysozyme and IgM levels in immunised females. In the present study, effects of letrozole on sex steroid hormones have not been shown.

Eighty-eight days old immunised and non immunised larvae originating from IM, Let.IM and NIM females were subjected to bath challenges using the LD₅₀ dose (1.2×10^8 cells/MI). RPS values were calculated for larvae from three groups (Table 3). Significant difference was observed among the immunised groups (IM and Let.IM) and NIM parents. So it is evident that the immune system of rainbow trout larvae is efficient at this age (128mg) to respond against antigens. It is concluded that larvae can be vaccinated successfully as early as early as 58-days post hatch (128 mg) and that these larvae can be protected against *L. garviea*.

Rainbow trout is able for antibody production against *Aeromonas hydrophila* when exposed at 3 weeks post-hatch. The minimal size at which a response was detected in sockeye (*Oncorhynchus nerka*), pink (*O. gorbuscha*), chum, coho, Chinook salmon (*O. tshawytscha*) and rainbow trout (*O. mykiss*) was 1.0 - 2.5 g. Size was also shown to be a better indicator of immunological maturation than age in *Sebastiscus marmoratus* [29]. This is not comparable with our present study because immune response was detected in rainbow trout larvae when their body weight was 128 mg.

The production of fish larvae is often hampered by high mortality rates, and it is believed that financial losses due to infectious diseases are high [34]. This raises fundamental challenges with respect to the development of knowledge of early defense mechanisms in fish. Finally, the results of this study have shown that vaccine (*L. garvieae* bacterial cells) performed well as immunostimulants in rainbow trout larvae. Vaccinated larvae from

three groups (Let.IM, IM and NIM) showed less mortality, so immersion vaccination can be used in larvae at the age of 58 days (128 mg) when the immune system can respond well against antigen. Hence, strategies should be put in place to immunise brood stocks of rainbow trout prior to breeding for better health management and to overcome the challenges in vaccinating large number of larvae at a time.

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References

- [1] Kim, J.H., Go, J., Cho, C.R., Kim, J.I., Lee, M.S. and Parka, S.C. (2013) First Report of Human Acute Acalculous Cholecystitis Caused by the Fish Pathogen *Lactococcus garvieae*. *Journal of Clinical Microbiology*, **51**, 712-714. <http://dx.doi.org/10.1128/JCM.02369-12>
- [2] Harikrishnan, R., Balasundaram, C. and Heo, M.S. (2010) *Lactobacillus sakei* BK19 Enriched Diet Enhances the Immunity Status and Disease Resistance to Streptococcosis Infection in Kelp Grouper, *Epinephelus bruneus*. *Fish and Shellfish Immunology*, **29**, 1037-1043. <http://dx.doi.org/10.1016/j.fsi.2010.08.017>
- [3] Hosseini, M.H., Akhlaghi, M. and Moazzeni Jula, G. (2011) Experimental Vaccine against Lactococcosis in Cultured Rainbow Trout (*Oncorhynchus mykiss*). *Archives of Razi Institute*, **66**, 51-57. http://www.archrazi.com/browse.php?a_code=A-10-1-1
- [4] Haghighi Karsidani, S., Soltani, M., Nikbakhat-Brojeni, G., Ghasemi, M. and Skall, H.F. (2010) Molecular Epidemiology of Zoonotic *Streptococcus/Lactococcus* in Rainbow Trout (*Oncorhynchus mykiss*) Aquaculture in Iran. *Iran Journal of Microbiology*, **2**, 198-209. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3279792/>
- [5] Erfanmanesh, M.S., Pirali, E., Mohammadian, S. and Taherimirghaed, A. (2012) Genetic Characterization of *Streptococcus iniae* in Diseased Farmed Rainbow Trout (*Oncorhynchus mykiss*) in Iran. *Scientific World Journal*, 594073-594079.
- [6] Ricci, G., Ferrario, C., Borgo, F., Rollando, A. and Fortina, M.G. (2012) Genome Sequences of *Lactococcus garvieae* TB25, Isolated from Italian Cheese, and *Lactococcus garvieae* LG9, Isolated from Italian Rainbow Trout. *Journal of Bacteriology*, **194**, 1249-1250. <http://dx.doi.org/10.1128/JB.06655-11>
- [7] Ravelo, C., Magariños, B., Herrero, M.C., Costa, L., Toranzo, A.E. and Romalde, J.L. (2006) Use of Adjuvanted Vaccines to Lengthen the Protection against Lactococcosis in Rainbow Trout (*Oncorhynchus mykiss*). *Aquaculture*, **251**, 153-158. <http://dx.doi.org/10.1016/j.aquaculture.2005.05.027>
- [8] Petrie-Hanson, L. and Ainsworth, A.J. (2001) Ontogeny of Channel Catfish Lymphoid Organs. *Veterinary Immunology and Immunopathology*, **81**, 113-127. [http://dx.doi.org/10.1016/S0165-2427\(01\)00331-2](http://dx.doi.org/10.1016/S0165-2427(01)00331-2)
- [9] Hanif, A., Bakopoulos, V., Leonardos, I. and Dimitriadis, G.J. (2005) The Effect of Sea Bream (*Sparus aurata*) Broodstock and Larval Vaccination on the Susceptibility by *Photobacterium damsela* subsp. Piscicida and on the Humoral Immune Parameters. *Fish and Shellfish Immunology*, **19**, 345-361. <http://dx.doi.org/10.1016/j.fsi.2004.12.009>
- [10] Zapata, A., Diez, B., Cejalvo, T., Frías, C.G. and Cortés, A. (2006) Ontogeny of the Immune System of Fish. *Fish and Shellfish Immunology*, **20**, 126-136. <http://dx.doi.org/10.1016/j.fsi.2004.09.005>
- [11] Pavone, M.E. and Bulun, S.E. (2012) Aromatase Inhibitors for the Treatment of Endometriosis: A Review. *Fertility and Sterility*, **98**, 1370-1379. <http://dx.doi.org/10.1016/j.fertnstert.2012.08.053>
- [12] Chumsri, S., Howes, T., Bao, T., Sabnis, G. and Brodie, A. (2011) Aromatase, Aromatase Inhibitors, and Breast Cancer. *The Journal of Steroid Biochemistry and Molecular Biology*, **125**, 13-22. <http://dx.doi.org/10.1016/j.jsbmb.2011.02.001>
- [13] Milla, S., Depiereux, S. and Kestemont, P. (2011) The Effects of Estrogenic and Androgenic Endocrine Disruptors on the Immune System of Fish: A Review. *Ecotoxicology*, **20**, 305-319. <http://dx.doi.org/10.1007/s10646-010-0588-7>
- [14] Zhang, S.C., Wang, Z.P. and Wang, H.M. (2013) Maternal Immunity in Fish. *Developmental & Comparative Immunology*, **39**, 72-78. <http://dx.doi.org/10.1016/j.dci.2012.02.009>
- [15] Li, L., Zhang, S.C., Tong, Z. and Liu, J. (2010) *In Vivo* Effect of 17- β -Estradiol on Plasma Immunoglobulin Levels and Leukocyte Density in Zebrafish *Danio rerio*. *Chinese Journal of Oceanology and Limnology*, **28**, 527-532. <http://dx.doi.org/10.1007/s00343-009-9048-8>
- [16] Swain, P. and Nayak, S.K. (2009) Role of Maternally Derived Immunity in Fish. *Fish & Shellfish Immunology*, **27**, 89-99. <http://dx.doi.org/10.1016/j.fsi.2009.04.008>
- [17] Sharifiyazdi, H., Akhlaghi, M., Tabatabaei, M. and Mostafavi Zadeh, S.M. (2010) Isolation and Characterization of

- Lactococcus garvieae* from Diseased Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) Cultured in Iran. *Iranian Journal of Veterinary Research*, **11**, 342-350.
- [18] Romalde, J.L., Alvarez, A.L., Ravelo, C., Toranzo, A.E. and Mendez, J.B. (2004) Oral Immunization Using Alginate Microparticles as a Useful Strategy for Booster Vaccination against Fish Lactococcosis. *Aquaculture*, **236**, 119-129. <http://dx.doi.org/10.1016/j.aquaculture.2004.02.028>
- [19] Shilling, A.D., Carlson, D.B. and Williams, D.E. (1999) Rainbow Trout, *Oncorhynchus mykiss*, as a Model for Aromatase Inhibition. *The Journal of Steroid Biochemistry and Molecular Biology*, **70**, 89-95. [http://dx.doi.org/10.1016/S0960-0760\(99\)00090-4](http://dx.doi.org/10.1016/S0960-0760(99)00090-4)
- [20] Bridle, A.R., Koop, B.F. and Nowak, B.F. (2012) Identification of Surrogates of Protection against Yersiniosis in Immersion Vaccinated Atlantic Salmon. *PLoS ONE*, **7**, 40841-40850. <http://dx.doi.org/10.1371/journal.pone.0040841>
- [21] Ellis, A.E. (1990) Lysozyme Assays. In: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Roberson, B.S. and Van Muiswinkel, W.B., Eds., *Techniques in Fish Immunology*, SOS Publications, Fair Haven, 101-103.
- [22] Bakopoulos, V., Peric, Z., Rodger, H., Adamms, A. and Richards, R. (1997) First Report of Fish Pasteurellosis from Malta. *Journal of Aquatic Animal Health*, **9**, 26-33. [http://dx.doi.org/10.1577/1548-8667\(1997\)009<0026:FROFPF>2.3.CO;2](http://dx.doi.org/10.1577/1548-8667(1997)009<0026:FROFPF>2.3.CO;2)
- [23] Buschmann, A.H., Tomova, A., López, A., Maldonado, M.A., Henríquez, L.A., Ivanova, L., *et al.* (2012) Salmon Aquaculture and Antimicrobial Resistance in the Marine Environment. *PLoS ONE*, **7**, 42724-42735. <http://dx.doi.org/10.1371/journal.pone.0042724>
- [24] Peeler, E.J. and Taylor, N.G.H. (2011) The Application of Epidemiology in Aquatic Animal Health—Opportunities and Challenges. *Veterinary Research*, **42**, 94-109. <http://www.veterinaryresearch.org/content/42/1/94> <http://dx.doi.org/10.1186/1297-9716-42-94>
- [25] Wang, Z., Zhang, S., Tong, Z., Li, L. and Wang, G. (2009) Maternal Transfer and Protective Role of the Alternative Complement Components in Zebrafish *Danio rerio*. *PLoS ONE*, **4**, 4498-4505. <http://dx.doi.org/10.1371/journal.pone.0004498>
- [26] Delamare-Deboutteville, J., Wood, D. and Barnes, A.C. (2006) Response and Function of Cutaneous Mucosal and Serum Antibodies in Barramundi (*Lates calcarifer*) Acclimated in Seawater and Freshwater. *Fish & Shellfish Immunology*, **21**, 92-101. <http://dx.doi.org/10.1016/j.fsi.2005.10.005>
- [27] Rombouta, J.H.W.M., Abellie, L., Picchietti, S., Scapigliati, G. and Kiron, V. (2011) Teleost Intestinal Immunology. *Fish & Shellfish Immunology*, **31**, 616-626. <http://dx.doi.org/10.1016/j.fsi.2010.09.001>
- [28] Salinas, I., Zhang, Y.A. and Sunyer, J.O. (2011) Mucosal Immunoglobulins and B Cells of Teleost Fish. *Developmental & Comparative Immunology*, **35**, 1346-1365. <http://dx.doi.org/10.1016/j.dci.2011.11.009>
- [29] Uribe, C., Folch, H., Enriquez, R. and Moran, G. (2011) Innate and Adaptive Immunity in Teleost Fish: A Review. *Veterinarni Medicina*, **56**, 486-503.
- [30] Van Herreweghe, J.M., Vanderkelen, L., Callewaert, L., Aertsen, A., Compernelle, G., Declerck, P.J., *et al.* (2010) Lysozyme Inhibitor Conferring Bacterial Tolerance to Invertebrate Type Lysozyme. *Cellular and Molecular Life Sciences*, **67**, 1177-1188. <http://dx.doi.org/10.1007/s00018-009-0241-x>
- [31] Thilagam, H., Gopalakrishnan, S., Bo, J. and Wang, K.J. (2009) Effect of 17- β -Estradiol on the Immunocompetence of Japanese Sea Bass (*Lateolabrax japonicus*). *Environmental Toxicology and Chemistry*, **28**, 1722-1731. <http://dx.doi.org/10.1897/08-642.1>
- [32] Akbary, P., Mirvaghefi, A.R., Akhlaghi, M., Amiri, B.M. and Fereidouni, M.S. (2013) Effects of the Aromatase Inhibitor Letrozole on Serum Immunoglobulin and Lysozyme Levels in Immunized Rainbow Trout (*Oncorhynchus mykiss* Walbaum) Females. *International Journal of Aquatic Biology*, **1**, 273-280. <http://www.npajournals.com/ijab/index.php/ijab/article/view/115>
- [33] Sun, L., Shao, X., Wu, Y., Li, J., Zhou, Q., Lin, B., *et al.* (2011) Ontogenetic Expression and 17 β -Estradiol Regulation of Immune-Related Genes in Early Life Stages of Japanese Medaka (*Oryzias latipes*). *Fish & Shellfish Immunology*, **30**, 1131-1137. <http://dx.doi.org/10.1016/j.fsi.2011.02.020>
- [34] Ángeles Esteban, M., Cuesta, A., Chaves-Pozo, E. and Meseguer, J. (2013) Influence of Melatonin on the Immune System of Fish: A Review. *International Journal of Molecular Sciences*, **14**, 7979-7999. <http://dx.doi.org/10.3390/ijms14047979>