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Characterization of Immune Aging in the Japanese Medaka (*Oryzias latipes*)

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Abstract: The prevalence of chronic inflammation increases with age and may be aggravated by environmental exposures. Similarly, during immune aging, inflammatory disease incidence increases as protective immunity decreases. To better understand disease and exposure risks, an immune aging model outlining key changes in immune function is crucial. Utilizing the lowest possible vertebrate class, we propose the Japanese medaka (*Oryzias latipes*) as a model to investigate sex-specific immune aging including changes in immune gene expression, leukocyte profiles, and organismal level immune response. Evaluating the expression of immune initiators (*CRP*, *TLR5-s*, *TLR5-m*, *TCRb*, and *MHCII*), immune mediators (*MYD88*, *Nf-kß*, *C3*, and *IL1b*), and immune effectors (*LYZ* and *C8*) in concomitance with alterations in leukocyte populations and host resistance to pathogens will inform about immune competence across ages. The data presented here demonstrate a critical decrease in the expression of immune initiators (*CRP*, *TLR5-soluble*, *TCRb*, and *MHCII*), mediators (*MYD88*, *Nf-kß*, *C3*, and *IL1b*), and effector (*LYZ*) in both females and males after 11 months post hatching (mph). Interestingly, both sexes displayed an upregulation for the immune effector, *C8*, during this older life stage (11–13 mph). Gene expression profiles for both sexes at the most elderly age (20 or 23 mph) appear to revert to a younger profile of expression indicating a second change in immune function during aging rather than a steady decline. Significant changes in leukocyte populations were observed in both male and female medaka after peaking sexual maturation at 3 mph. Organismal level immune competence data revealed male medaka at the elderly age to be more vulnerable than their female and younger male counterparts while no differences were observed in females based on age. Together, these data provide a holistic profile for immune aging in medaka, a useful tool for future immunological studies considering age as a factor influencing disease susceptibility.

Keywords: immune system; aging; medaka

Key Contribution: Japanese medaka present a common immune aging profile and are proposed as a useful model for future aging studies.

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1. Introduction

Aging is characterized by the declining function of the major homeostatic systems including the endocrine, nervous, and immune systems [1]. Being the major danger detection system in an organism, the aging of the innate and adaptive immune system directly impacts an organism's homeostasis and health. An aging immune system is manifested in an increased risk of infection and disease due to the reduced physical barrier efficiency and higher levels of self-antigens. Immunosenescence is a major factor for infectious disease occurrence and cancer entailing higher morbidity and mortality with age [2–5].

Immune pathologies such as chronic inflammation increase with age and affect 50 million Americans with annual direct costs averaging between USD 90 and 300 billion in the US [6]. There is significant research interest surrounding disease-specific mechanisms and vulnerability related to aging, but a suitable animal model for immune aging is currently unavailable.

The basic understanding of immune aging includes the idea that adaptive immunity declines with age, referred to as immunosenescence [7–9]. Immune aging has also been characterized by increased levels of cytokines, chemokines, and other factors leading to more chronic inflammation which is referred to as inflamm-aging [10]. During inflamm-aging, the innate immune system becomes more activated as opposed to immunosenescence in which adaptive immunity responses decline. High levels of proinflammatory markers leading to altered responses can increase the risk of autoimmunity and disease occurrence in aging organisms. Immune aging occurs on multiple biological levels from molecular changes in gene expression to cellular and tissue level changes which ultimately affect the organismal immune competence and the prevalence of disease. Alterations at different biological levels associated with immune aging include the following:

I Molecular changes such as the alteration of T-cell signaling, decreased level of Toll-like receptors (TLRs), and increased levels of complement protein 3 (C3) which are negatively correlated with longevity, and changes in the expression of key genes like *nuclear factor κβ* (*Nf-kß*) and *interleukin 1β* (*IL1b*) [9,11–15].

II Tissue and cellular level changes in immune system function like the involution of the thymus leading to changes in the number of helper and regulatory T-cell populations [16,17].

III Organismal level changes such as decreased immunity and increased infection and disease prevalence [1,10].

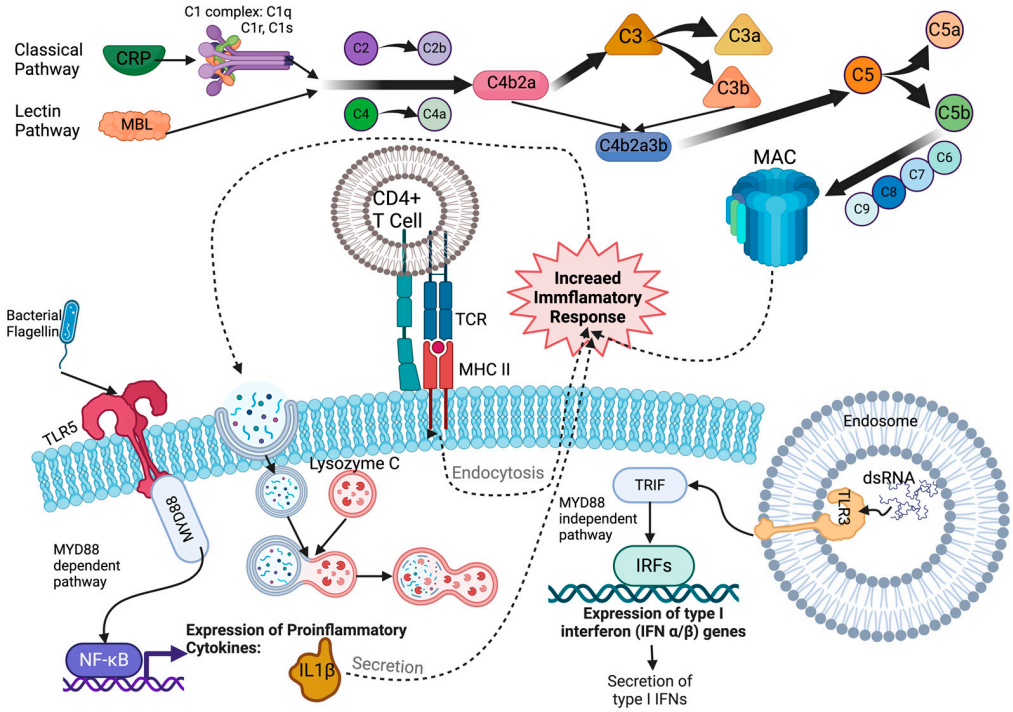
Age-related changes at the molecular level of an immune response consist of a modified expression of immune initiators like *C-reactive protein* (*CRP*), *Toll-like receptor 5 soluble* and *membrane-bound* (*TLR5-s* and *TLR5-m*), *T-cell receptor β* (*TCRb*), and *major histone compatibility complex II* (*MHCII*) (Figure 1).

CRP expression can increase with age leading to an increased risk of altered immune response potentially manifesting as disease [18]. Decreases in TLR function have been correlated with aging in human cell lines, leading to a reduced immune response [19]. *TCRb* expression decreases with age; specifically, genes associated with the TCRb-Nf-kß pathway do not have sustained expression over age [20]. Adversely, *MHCII* expression from monocytes increases with aging and can lead to changes in monocyte production potentially implicating age-related disease manifestation [21]. Immune mediators like *myeloid differentiation factor 88* (*MYD88*), *nuclear factor κβ* (*Nf-kß*), *complement protein 3* (*C3*), and *interleukin 1β* (*IL1b*) have been previously evaluated for associations with aging. While *MYD88* expression levels are not directly impacted by age [11], *Nf-kß* expression increases with age in various tissues and can be associated with age-related inflammatory diseases [14]. The complement system and aging have commonly been linked; *C3* expression specifically increases with age and positively correlates with a decrease in longevity [15]. IL1b is a key mediator of immune responses and has been shown to be increased in some tissues and cell types with age like beta cells and decreased in other tissues like skin [12,13]. Finally, immune effector genes like *lysozyme C* (*LYZ*) and *complement protein 8* (*C8*) may also change with age. Increased LYZ induction is associated with increased age in mice [22]. C8 activation is dependent upon C3 activation, so it can be hypothesized that *C3* expression changes with age will directly affect *C8* expression with age. Therefore, the molecular deregulation of the immune function due to age is likely to entail changes in cellular and tissue function. Notwithstanding, the exact mechanisms behind the changes in cell populations, signaling, and response are still unclear [10]. However, modifications in leukocyte populations are known to be consistent and sensitive to age as demonstrated in mice [23]. Dendritic cells (DCs) which functionally recognize Toll-like receptors to stimulate an immune response are not thought to decrease in numbers in healthy aging; however, reduced populations of

both conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) as well as the decreased functionality of cDCs and pDCs is associated with increased age and can lead to altered immune responses found in chronic diseases [10,24]. The ability of dendritic cells to release proinflammatory cytokine IL12 declines with aging in mice. In combination with decreased dendritic cell numbers in the elderly, this could indicate an overall decline in immune responses related to dendritic cell function [25]. Neutrophils who are majorly recruited to an immune response site by IL1 and IL8 are key cells in an innate immune response to microbial infections. Age is not known to have a great impact on the number of neutrophils, but their cellular response functions like migration and adhesion may be diminished with aging [10,26]. A decline in chemokine circulation related to neutrophil activation has been observed in elderly compared to young mice, suggesting a potential impact of age on neutrophil function [27]. Macrophages are important for secreting cytokines and chemokines and presenting antigens to T-cells as well as the phagocytosis of antigens. In elderly mice, reduced proinflammatory cytokine release from macrophages is common and can lead to a decreased immune response [10,28]. Decreased phagocytosis activity by macrophages is also a characteristic of immune aging observed in mice [29]. Contrary to other immune cells, natural killer cells, which are important for recognizing MHC binding and subsequently secrete cytokines to perpetuate an immune response, greatly increase in number during aging [10,30]. Lymphocytes are also affected by aging. The naïve population of B-cells decreases with age and leads to a reduced response to new antigens, reducing overall antibody levels [10]. T-cells have also been commonly shown to be affected by aging. The shifting of T-cell populations from naïve T-cells to memory and effector T-cells is a classic example of normal aging in mammals [17]. Thymic involution is the main driver behind the decrease in naïve T-cells with aging. This decline in naïve T-cells can impact the population balance of different T-cell subtypes, ultimately impacting the effectiveness of an immune response [10]. The number of CD4 helper T-cells and CD8 cytotoxic T-cells decreased with increasing aging [16]. Changes in numbers, distribution, and function of immune cell populations can drastically impact immune function and are critical in understanding immune aging and potential increased vulnerabilities with age. Changes at both the molecular and cellular levels can manifest at the organismal level and affect immune response and function. It is understood that over time, organism homeostasis declines and there is an increased risk of death [31].

To better understand the aging process and the changes in the immune system function during aging, model organisms have been used to study specific pathways and genetic components. Classic model systems like yeast, worms, and flies have been used to identify some key genetic components and pathways involved in immune aging. However, these models lack significant similarities to human immunity such as a closed circulatory system, specific immune organs, and most notably, an adaptive immune system. For this reason, vertebrate models are better suited to mimic human immune aging. These vertebrate models traditionally include mice and fish. Current murine models have been used to outline changes in immune function related to age such as the dysregulation of immune cell populations like T-cells, dendritic cells, and natural killer cells [32].

Fish have been established as excellent models for vertebrate systems including in immunotoxicology, developmental biology, and behavioral studies [33]. The short life span, extrauterine development, and quick maturation of fish models like zebrafish and medaka make them useful experimental models [33,34]. Medaka is commonly used in ecotoxicological and biomedical studies to investigate cancer, behavior, and neurobiology [33,35–37]. A vast variety of molecular tools are available for the medaka model providing a great resource for application in aging studies [38]. The goal of the presented research is to outline the key characteristics of immune aging in medaka to aid in its use as a model organism for aging-related research specifically related to immune vulnerability in age.



CRP – c reactive protein	**MHCII** – class II major histocompatibility complex
MBL – mannose binding lectin	**TLR5** – toll-like receptor 5
C1q,r,s – complement protein 1 q, r, s	**MYD88** – myeloid differentiation primary response 88
C2 – complement protein 2	**NF-kß** – nuclear factor kappa beta
C4 – complement protein 4	**IL1ß** – interleukin 1 beta
C4b2a – complement C3 convertase	**Lysozyme C** – lysozyme C
C3 – complement protein 3	**TRIF** – toll receptor inducing interfeuron
C4b2a3b – complement C5 convertase	**IRFs** – interferon regulatory factors
C5 – complement protein 5	**IFN** – interfeurons (a/ß - alpha/beta)
C9,8,7,6 – complement proteins 9,8,7,6	**TLR3** – toll-like receptor 3
MAC – membrane attack complex	**dsRNA** – double stranded RNA
TCR – T-cell receptor	

Figure 1. Hypothetical molecular signaling pathways associated with immune aging in medaka. The humoral immune response is identified by the lectin and the classical complement pathway. T-cell responses are indicated through Toll-like receptor (TLR)- and T-cell receptor (TCR)-specific pathways.

2. Materials and Methods

2.1. Model Organism

Japanese medaka, *Oryzias latipes* (*OL*), is a commonly used model organism for research studies. This fish model is ideal for studying gene function and expression associated with immune aging as they have a relatively short life span and quick reproductive development, providing an alternative model for common fish and other organisms with long life spans [33]. The entire genome of *OL* has been drafted and described [39]. There are many strains of *OL* all of which have a robust breeding ability, adding to the features of this model for genetic study [36]. Transparent eggs and developing larvae make medaka fish ideal for developmental study [35,37]. Medaka has also been described to be a good model for ecotoxicological studies [37,40]. A medaka husbandry has been established at Texas A&M University-Corpus Christi (TAMUCC). All the animal research described in this study is in compliance with IACUC #03/19 and the City University of Hong Kong's Animal Ethics Commission. Japanese medaka aged 3 to 23 mph were used to evaluate immune aging with 80 being used for gene expression profile, 40 for cellular immune profiles, and 150 for host resistance assays. Medaka (>1 mph) were maintained in independent tank systems until the desired age for study. Water quality was evaluated weekly, and water changes (75%) were performed as necessary. Tanks were kept at a temperature range of

25 ± 1 °C with a 12 h photoperiod. Other water quality parameters were maintained as follows: dissolved oxygen at 6 mg/L with constant sponge filter aeration, nitrates below 20 mg/L, nitrites below 0.1 mg/L, ammonia below 0.001 mg/L, and pH ranging from 7.7 to 8.2. Embryo medaka were collected from breeding tanks and rinsed before being maintained in glass Petri dishes with 9 parts DI water and 1 part saltwater with methyl blue (0.01%) with water changes (100%) every other day until hatching. After hatching, the larvae were maintained in 2 L glass vases with 9 parts DI water and 1 part saltwater until 1 mph at a density of 50 larvae per 2 L with water changes (100%) weekly and water quality standards as mentioned above. Moribund embryos, larvae, and adult medaka were checked for daily and euthanized with MS222.

2.2. Immune Gene Assessment across Ages and between Sexes

2.2.1. RNA Extraction and Reverse Transcription

Adult male and female *OL* at the ages of 3, 4, 5, 6, 7, 11, 13, and 20 or 23 mph (n = 5 per sex per age) were sacrificed using MS-222 immersion and ice water. Liver tissues were dissected out and stored at −80 °C. Tissues were homogenized using 500 µL of TRI-Reagent and a pestle in 1.5 mL sterile microcentrifuge tubes. The samples were maintained at room temperature (23 °C) for 5 min. Cell lysis was performed by adding 50 µL of chloroform to each sample tube followed by gentle mixing. After incubation for 10 min at room temperature, the samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The aqueous phase containing the RNA was transferred into a new sterile 1.5 mL microcentrifuge tube and washed by adding 250 µL isopropanol, vortexed for 10 s, and incubated at room temperature for 10 min. The samples were then centrifuged at 12,000 rpm for 8 min at 23 °C to spin down the RNA into a pellet. After centrifugation, the supernatant was discarded, and the pellet was resuspended using 500 µL of 75% molecular-grade ethanol. The samples were then centrifuged at 7500 rpm for 5 min at 23 °C. Then, the supernatant of ethanol was removed, and the tubes were left open to air-dry and allow for the evaporation of any remaining ethanol. Finally, each sample pellet was resuspended using 50 µL of RNA-/DNA-free sterile water and stored at −80 °C. RNA quality and concentration were determined using the BioSpectrometer (Eppendorf, Hamburg, Germany). Each sample was diluted at a 1:2 dilution with RNA-/DNA-free water and 2 µL was pipetted into the microcuvette. The RNA samples were measured using a 260 nm wavelength. The spectrometer measured the concentration of RNA in ng/µL. Sample concentrations below a threshold amount (130 ng/µL at dilution of 1:2) could not be used for further analysis due to the requirements for the reverse transcription protocol.

A reverse transcription was performed to obtain cDNA for RT-qPCR using the Promega Reverse Transcriptase kit following the manufacturer's instructions. Briefly, the RNA, oligo primers, random primers, and nuclease-free water were combined in a 0.2 mL PCR tube and incubated at 70 °C for 5 min. Then, a reaction mix containing the reaction buffer, $MgCl_2$, PCR nucleotide mix, reverse transcriptase, and water was prepared as a master reaction mix for all the samples. The preincubated samples were removed from 70 °C and placed on ice for 5 min. Next, 15 µL of the master reaction mix was added to each sample tube. Each sample tube was centrifuged briefly to collect any potential condensation. Then, the samples were placed in the thermocycler. The program consisted of 3 major steps: annealing at 25 °C for 5 min, extending at 42 °C for up to an hour, and then inactivating reverse transcriptase at 70 °C for 15 min. Once the reverse transcription protocol was completed, the cDNA samples were stored at −20 °C.

After reverse transcription, RT-qPCR was performed with *OL* immune gene primers (Table S1) to determine relative gene expression for both sex and age variations.

2.2.2. Real Time-qPCR Relative Gene Expression

Relative gene expression for key immune initiators including C-reactive protein (CRP), Toll-like receptor 5 soluble and membrane (TLR5-s and TLR5-m), T-cell receptor beta (TCRb), and major histone compatibility complex II (MHCII); immune mediators including

myeloid differentiation primary response 88 (MYD88), nuclear factor κβ (Nf-kß), complement protein 3 (C3), and interleukin 1β (IL1b); and immune effectors including lysozyme C (LYZ) and complement protein 8 (C8) was performed using a 1:2 dilution of cDNA template in a 96-well plate using a QuantStudio3 (ThermoFisher). Five replicates for each age and sex were assessed. Relative gene expression was determined by the standard ΔΔCT method [41] using 18S ribosomal RNA (18S), 60S ribosomal protein L7 (RPL7), and elongation factor 1 alpha (EF1a) as housekeeping genes. Due to the nature of these data lacking a standard control, ΔCt values were used for the statistical analysis to compare ages within sexes and sexes within ages. The statistical analysis included normality testing (QQ plots and Shapiro–Wilk), one-way ANOVA (parametric) or Kruskal–Wallis (non-parametric) test, post hoc testing (TukeyHSD or Dunn's test), and Cohen's d test for effect size determination.

2.3. Leukocyte Population Assessments

Adult male and female *OL* at the ages of 3, 7, 14, and 20 mph (n = 5 per sex per age) were used for whole-head kidney leukocyte population assessment via flow cytometry. *OL* was sacrificed using MS-222 immersion and ice water according to TAMUCC IACUC #03/19. Head kidney tissues were then extracted and placed in 1% Fetal Bovine Serum (FBS)/1X Phosphate-Buffered Saline (PBS). The tissues were dissociated by gentle pipetting. The samples were then centrifuged at 500× g for 5 min at 4 °C. After centrifugation, sterile Milli-Q water was added for the lysis of red blood cells. After 10–15 s, 10X PBS was added to restore the salt balance and preserve leukocytes. The samples were then sieved through a 50 µm cell strainer into a new sterile tube. The samples were then centrifuged (500× g for 5 m @ 4 °C) and the supernatant was discarded. The remaining leukocytes were resuspended in 1%FBS/PBS. The sample aliquots were stained with DAPI and counted for viability and concentration. The cells were then diluted to 1 million cells per µL. The samples were run on the BD Acuri CSampler Plus flow cytometer at medium rate for 15,000 events in Q1-UR which was predetermined to exclude most debris and include immune cell populations. Flow cytometry was used to assess leukocyte populations based on cell size (forward scatter) and complexity (side scatter). Distinct immune cell populations were then gated for lymphocytes and phagocytes (macrophages and granulocytes) and the percentage of each population per total event was calculated for each sample. Comparisons were made among the ages of the same sex and between the sexes of the same age. The statistical analysis included normality testing (QQ plots and Shapiro–Wilk), two-way ANOVA, and TukeyHSD post hoc test ($p < 0.05$).

2.4. Immune Competence Assessment—Host Resistance Assay

Adult male and female *OL* at the ages of 4, 8, 12, 16, and 20 mph (n= 15 per sex per age) were exposed to a known bacterial fish pathogen, *Edwardsiella tarda*, to test immune competence in a host resistance assay (HRA). The fish were obtained from the City University of Hong Kong and treated according to the guidelines from the City University of Hong Kong Ethics Committee. Intraperitoneal injection of 1 µL of *E. tarda* at 1 × 10^5 cfu was used to induce an immune response and fish were monitored for morbidity for 30 days post injection [42]. To evaluate sex-specific and age-specific vulnerabilities to bacterial infection, comparisons in survival rates based on sex and age were made. The statistical analysis included normality testing (QQ plots and Shapiro–Wilk) and log-rank survival test with Bonferroni adjustment for multiple comparisons ($p < 0.05$).

3. Results

3.1. Relative Immune Gene Expression

Key immune genes were measured for relative expression including immune initiators *(CRP*, *TLR5-s*, *TLR5-m*, *TCRb*, and *MHCII)*, immune mediators *(MYD88*, *Nf-kß*, *C3*, and *IL1b)*, and immune effectors (LYZ and C8). Sex-specific differences were measured in immune genes of unchallenged *OL* within specific age groups (Figure 2).

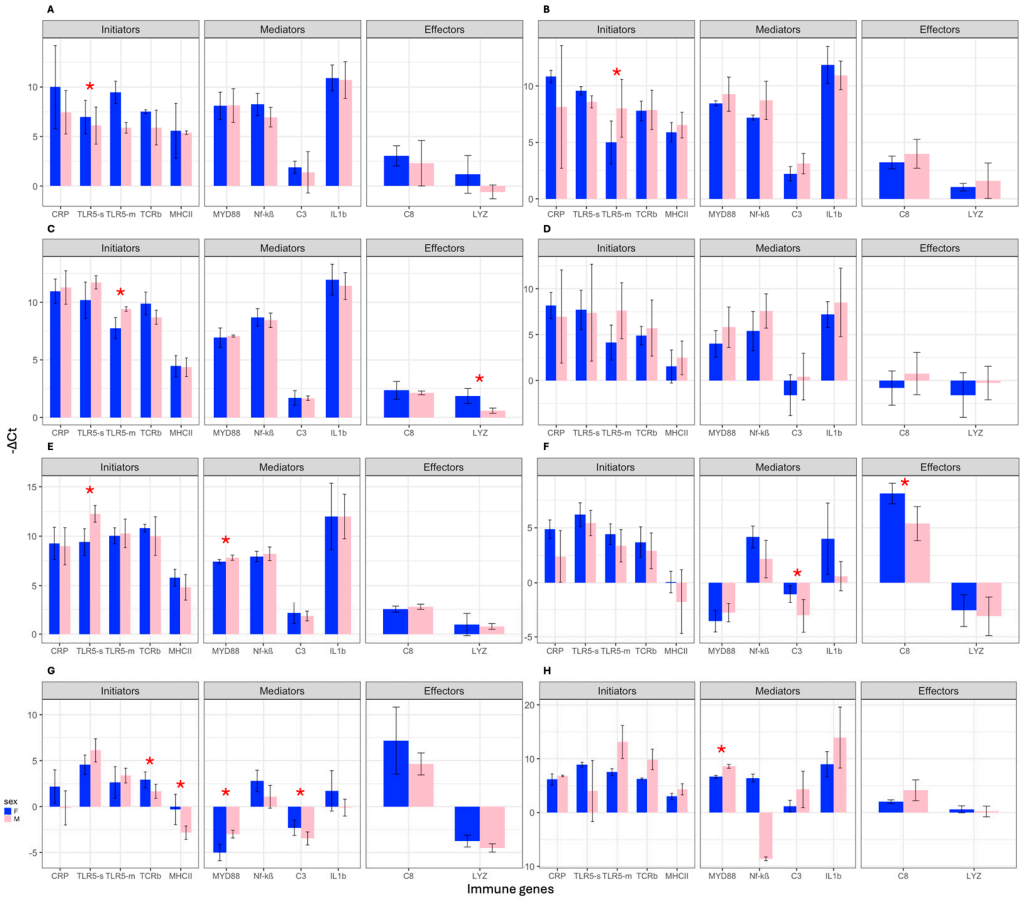


Figure 2. Relative gene expression of immune initiators (*CRP*, *TLR5-s*, *TLR5-m*, *TCRb*, and *MHCII*), mediators (*MYD88*, *Nf-kß*, *C3*, and *IL1b*), and effectors (*C8* and *LYZ*) in medaka at ages 3 (**A**), 4 (**B**), 5 (**C**), 6 (**D**), 7 (**E**), 11 (**F**), 13 (G), and 20–23 (**H**) mph. Significant differences between males (blue) and females (pink) are indicated by * above genes. Data are displayed as average ± standard error. ANOVA/TukeyHSD or Kruskal–Walis/Dunn ($p < 0.05$).

Differences were seen in the immune imitators *TLR5-s*, *TLR5-m*, *TCRb*, and *MHCII*. Specifically, at 3 mph, male *OL* had significantly higher expression of *TLR5-s.* However, at 7 mph, female *OL* had a significantly higher expression of *TLR5-s*. At 4 and 5 mph, female *OL* had higher *TLR5-m* expression. At 13 mph, OL displayed the highest number of immune genes (4) with sex-specific expression including immune initiators *TCRb* and *MHCII* and mediators *MYD88* and *C3*. Immune mediator *MYD88* was differently expressed among males and females with a higher expression in females at 7, 13, and 20/23 mph. *C3* was differently expressed in both 11 and 13 mph *OL* with males having higher expression levels. Immune effectors LYZ and C8 displayed sex-specific differences in only 5 and 11 mph *OL*, respectively, with higher levels in males.

A significant decrease in the expression of all the immune initiators and mediators was observed with an increase in age for females (Figure 3).

Specifically, the ages 11 and 13 mph tended to have decreased expression in immune genes compared to their younger counterparts. However, at 23 mph, the expression tended to resemble that of the younger ages and differed from the 11 and 13-mph age groups. Female OLs aged 11 and 13 mph generally represented a separate expression profile with a large effect difference from younger OLs aged 3–7 mph and the oldest group 23 mph. Specifically, the effect size difference from this 11 and 13 mph and younger 4 and 5 mph ranged from 2 to 6 pooled standard deviations (psd) different for immune initiators, 3 to 14 psd different for immune mediators, and 3 to 6 psd different for immune effectors (Cohen's d test).

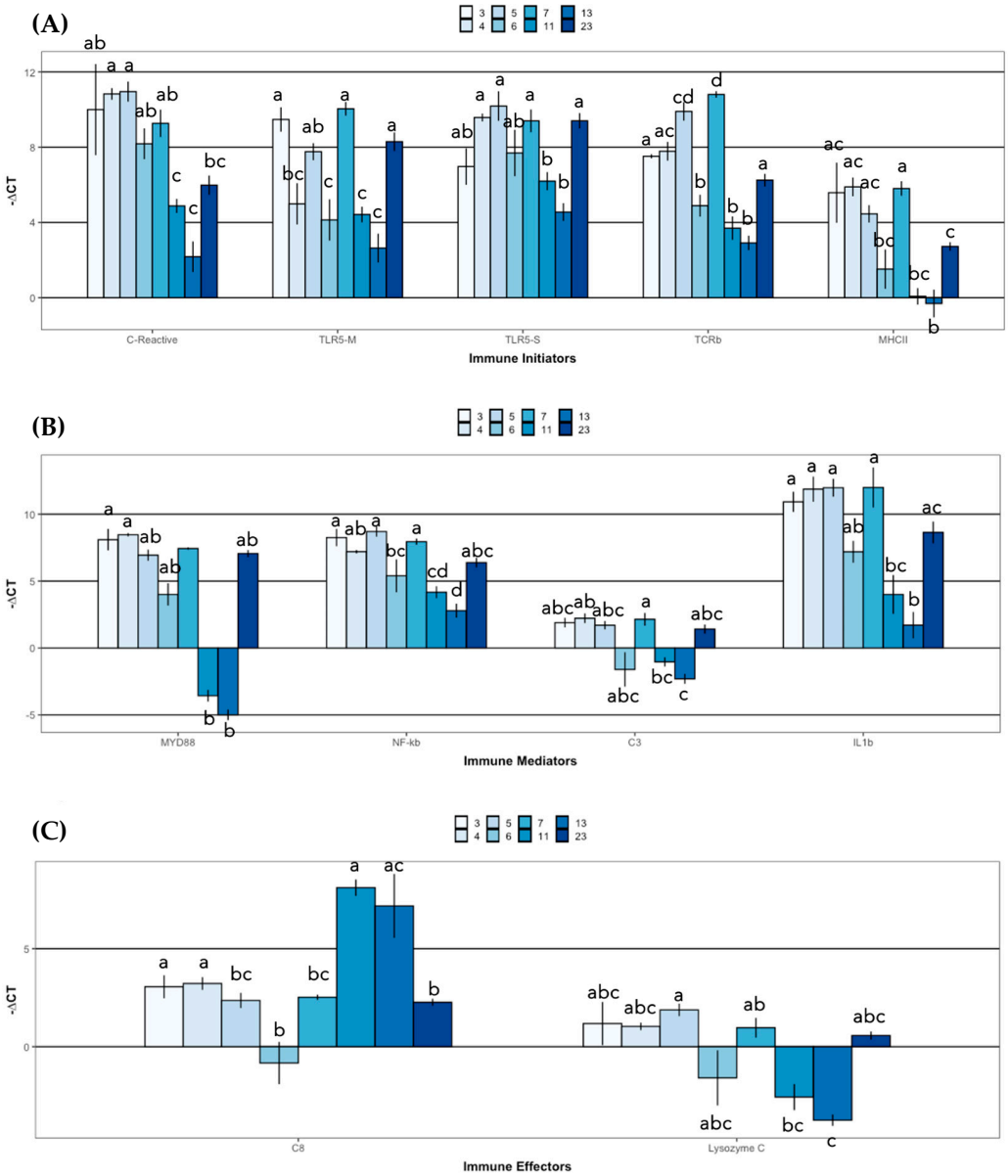


Figure 3. Relative gene expression of (**A**) immune initiators (*CRP(C-reactive)*, *TLR5-s*, *TLR5-m*, *TCRb*, and *MHCII*), (**B**) mediators (*MYD88*, *Nf-kß*, *C3*, and *IL1b*), and (**C**) effectors (*C8* and *LYZ(Lysozyme-C)*) in female medaka at ages 3, 4, 5, 6, 7, 11, 13, and 23 mph. Significant differences are indicated by different letters. Data are displayed as average ± standard error. ANOVA/TukeyHSD or Kruskal–Walis/Dunn ($p < 0.05$).

A similar trend was observed in males as previously described in females (Figure 4).

Male medaka of 11 and 13 mph also tended to have decreased expression in key immune genes when compared to their younger counterparts. For male *OL* at 11 and 13 mph, TLR5-*s*, *MYD88*, *IL1b*, *C8*, and *LYZ* were only different from one younger age (7 mph) compared to the female *OL* at 11 and 13 mph which were different from most if not all the younger ages (3–7 mph). Often, 11 mph was not significantly different from the younger *OL* while the expression was changed at 13 mph as seen for *CRP*, *TCRb*, and *LYZ*. At 20 mph, this gene expression resembled a profile closer to the younger groups than that of the 11 and 13 mph medaka, indicating some shift in immune function. There was a large effect size difference for the 13 mph compared to the 7 mph male *OL* which ranged from 4 to 6 psd for initiators, 6 to 23 psd for mediators, and 24 to 13 psd for effectors (Cohen's d test).

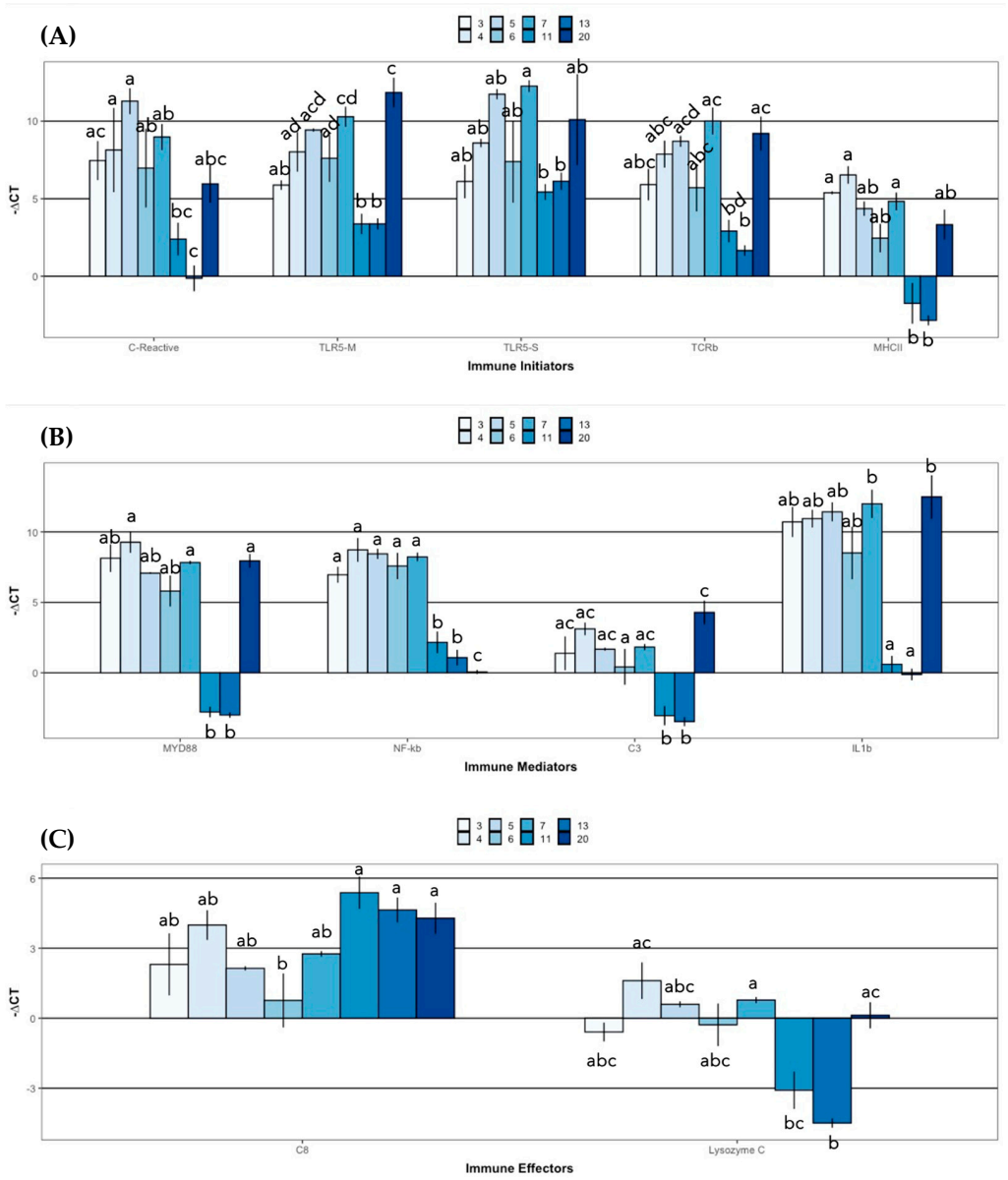
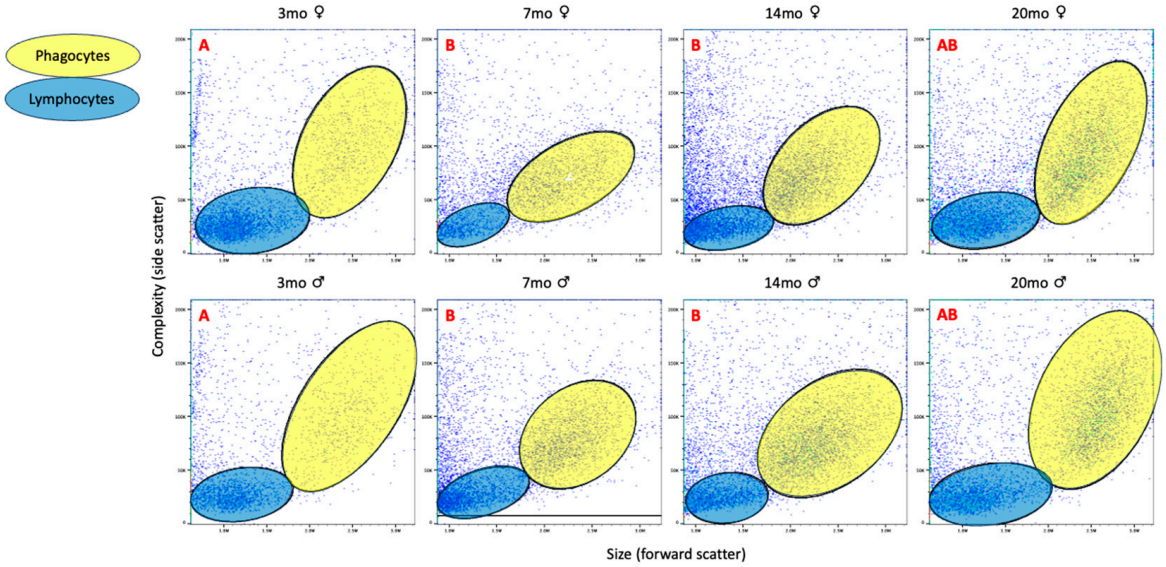


Figure 4. Relative gene expression of (**A**) immune initiators (*CRP(C-reactive)*, *TLR5-s*, *TLR5-m*, *TCRb*, and *MHCII*), (**B**) mediators (*MYD88*, *Nf-kß*, *C3*, and *IL1b*), and (**C**) effectors (*C8* and *LYZ(Lysozyme-C)*) in male medaka at ages 3, 4, 5, 6, 7, 11, 13, and 20 mph. Significant differences are indicated by different letters. Data are displayed as average ± standard error. ANOVA/TukeyHSD or Kruskal–Walis/Dunn ($p < 0.05$).

3.2. Leukocyte Population Assessment

FCM data presented two distinct populations of leukocytes in medaka, lymphocytes and phagocytes, including both macrophages and granulocyte populations. No differences between the leukocyte populations of male and female medaka of the same age were observed (Figure 5).

However, for both males and females, the phagocyte numbers were significantly increased in 7, 14, and 20 mph medaka. No differences were observed in lymphocyte populations for either sex or age.



Sex	Average % Phagocytes ± SD	ANOVA based on age - Phagocytes	Average % Lymphocytes ± SD	ANOVA based on age - Lymphocytes
Female	1.24±1.29	A	4.30±3.33	A
Female	6.45±4.17	B	16.92±5.91	A
Female	7.76±2.10	B	19.42±8.70	A
Female	10.77±3.55	B	10.58±3.32	A
Male	1.55±1.31	a	15.82±12.96	a
Male	6.14±2.63	ab	18.82±17.89	a
Male	9.20±1.68	b	14.65±6.30	a
Male	13.96±6.29	b	12.42±4.48	a

Figure 5. FCM leukocyte populations of 3, 7, 14, and 20 mph female (A1–A4) and male (B1–B4) medaka. Phagocyte populations are shown gated in yellow and include the presence of macrophages and granulocytes. Lymphocyte populations are shown gated in blue. The table displays the percentages of phagocytes and lymphocytes based on FCM. Differences in the phagocyte populations based on age are indicated in letters for females (uppercase) and males (lowercase). No differences based on sex were observed. No differences in lymphocyte populations were observed.

3.3. Immune Competence Assay

Host immune challenge by *E. tarda* revealed significant differences in survival between young males (4 and 8 mph) and old males (20 mph) (Figure 6).

There were no variations in survival among females based on age. Similarly, differences in survival based on sex at younger ages were not observed to be different; however, at 20 mph, males had significantly decreased survival compared to females. Overall, males at 20 mph were observed to have significantly lowered survival rates compared to both females of the same age and younger males, indicating this age is a particularly sensitive window.

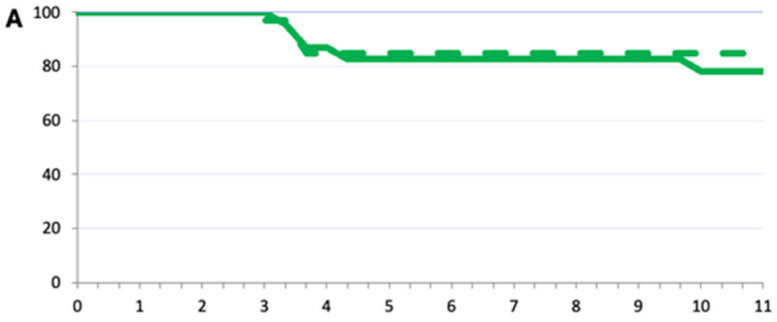


Figure 6. *Cont.*

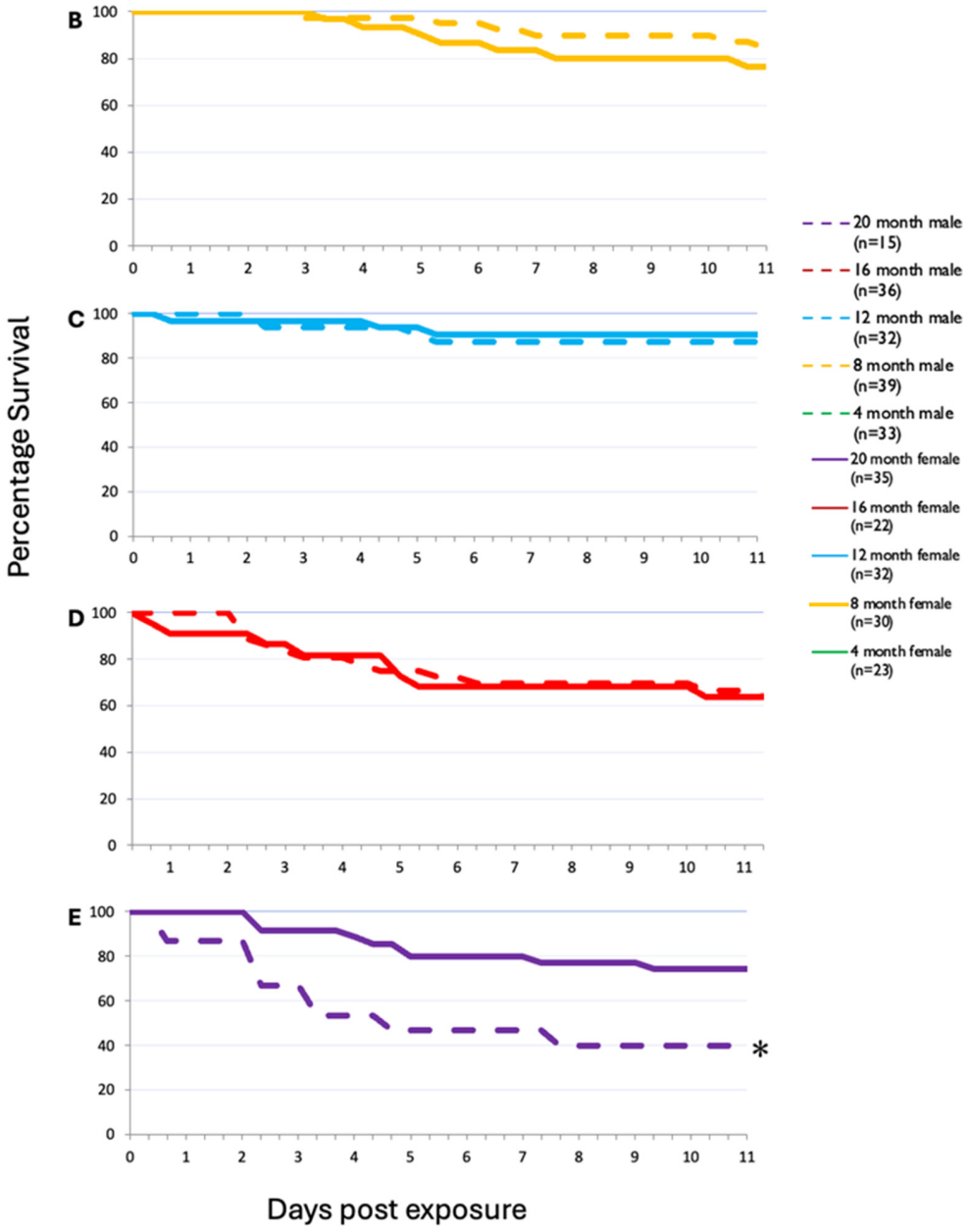


Figure 6. Host resistance assay survival of adult medaka 4 (**A**), 8 (**B**), 12 (**C**), 16 (**D**), and 20 (**E**) mph challenged by *E. tarda*. Pairwise log-rank comparison ($p < 0.05$) indicated by *.

4. Discussion

Understanding how changes in immune system regulation and function at the molecular, cellular, and organismal levels is crucial for evaluating immune vulnerabilities related to disease manifestation and infections. This study puts forth Japanese medaka fish (*Oryzias latipes*) as a model for immune aging, providing evidence that there are changes in immune competence during the aging of medaka, which is relevant for understanding both fish and human immune vulnerabilities related to age. The molecular components of the immune system are highly conserved among vertebrate animals including fish and humans, allowing the use of lower taxonomic models for understanding relevant human health concerns such as the increased prevalence of disease with age. The decline in the function of major homeostatic systems, including the immune system, endocrine system, and nervous system, is a characteristic feature of aging. Immune aging can implicate an increased risk of infection and disease due to reduced physical barrier efficiency and

higher levels of self-antigens. Understanding these processes is essential to comprehend age-related immune diseases.

The data presented indicate that Japanese medaka undergo similar aging processes of the immune system as other vertebrates, namely turquoise killifish and humans [43]. As sex hormones play a pivotal role in the orchestration of the immune response, it is expected to observe sex differences in immune competence and that immune aging differs between sexes [44]. Only a few studies are available to characterize the sex differences in the immune response of fish, indicating a possible stronger male immunocompetence [45–47], but which is also more susceptible to environmental stressors and internal cortisol levels [45,47–49]. This sex-specific immune aging is also noted in the murine model [50]. The 20 mph male medaka displayed a decline in host-resistant immune competence with increased age, highlighting their increased vulnerability with age. The flow cytometry data illustrated an increase in phagocyte populations with an increase in age in both sexes. Phagocytes, including macrophages, neutrophils, eosinophils, and basophils, play crucial roles in innate immunity by combating infections and regulating inflammatory responses [9]. The observed increase in phagocyte populations with age in both male and female fish suggests a potential compensatory response to age-related changes in immune competence or increased inflammatory status. Understanding the functional implications of altered phagocyte dynamics in the context of immune aging could provide valuable insights into the mechanisms underlying age-related immune dysfunction. In many vertebrates, including humans, neutrophils are typically the most abundant granulocytes, playing a crucial role in an effective immune response, such as antiviral functions, interactions with B- and T-cell, and involvement in many autoimmune pathologies [51]. The age-related increase in phagocyte numbers may be associated with changes in neutrophil functions as described in humans, including less accurate cell migration, increased neutrophil kinase activity, increased degranulation, reduced phagocytosis, and reduced bactericidal function [52–54]. However, in fish, the composition of granulocyte populations is diverse and can differ significantly from that in mammals. For example, in several teleost species, granulocytes such as acidophilic granulocytes, which perform similar functions to mammalian neutrophils, can be predominant [55–57]. Additionally, in cartilaginous fish, granulocyte populations may be dominated by heterophils, which also participate in innate immune responses [58]. This variability in granulocyte types suggests that the age-related increase in phagocyte numbers observed in medaka could involve shifts in specific granulocyte populations, potentially reflecting species-specific adaptations in immune function. These findings underscore the importance of understanding species-specific immune aging processes, which may reveal broader insights into the mechanisms of immune dysfunction across vertebrates. The reduced function and accuracy of neutrophils could lead to increased inflammation and reduced immune competence specifically in response to a bacterial pathogen.

In addition to organismal and cellular levels, sex-specific differences in immune gene expression profiles were observed at 3, 4, 5, 7, 11, 13, and 20/23 mph. At 3, 4, 5, and 13 mph, immune initiator differences were observed with higher expression levels in the males at the oldest and youngest ages (3 and 13 mph) and higher expression in the females at 4, 5, and 7 mph. Immune mediator expression sex-specific differences were seen at 7, 11, 13, and 20/23 mph. Specifically, female *OL* had higher levels of *MYD88* and males had higher levels of *C3* when differences were seen. At 5 mph, male medaka had higher levels of immune effector *LYZ* and at 11 mph, males also had higher levels of immune effector *C8* when compared to females. Sex-specific variation in immune gene expression has been reported in humans [59]. In general, females tend to have more humoral and cell-mediated immune responses while males have stronger inflammatory responses [60]. Women have been reported to rely more heavily on TLR-medicated pDC activation than men after viral infection indicating a sex-specific difference in cell-mediated immune responses [61]. Females are also known to have higher expression levels of genes along TLR pathways including *MYD88* [62]. As presented here, male medaka demonstrate a reduced immune competence at the organismal level when presented with pathogen challenge, no changes

at the cellular level in phagocyte numbers after 3 mph, and significant changes at the molecular level depending on age with significantly less *MYD88* expression in the elderly age of 20 mph. This manifestation of reduced immune competence after pathogen challenge when compared to females at the elderly age could potentially be explained by neutrophilic immune dysfunction or a potential compensation by the female *OL* maybe through *MYD88* mediated mechanisms. Thus, medaka may present a good model for further investigating age-specific immune responses or vulnerabilities for both mammals and fish. Additionally, this finding hints at potential sex-specific vulnerabilities of immune competence associated with aging for which medaka would be ideal to model. Previous research has shown that mammals display a similar sex-specific immunosenescence with a faster decline of male immune parameters during aging compared to females [7].

Several molecular-level changes associated with immune aging in medaka fish have been noted and are considered characteristics of typical immune aging within specific sexes. The trend for immune initiators (*CRP*, *TLR5-s*, *TCRb*, and *MHCII*) and mediators (*MYD88*, *Nf-kß*, *C3*, and *IL1b*) to decline after 11–13 mph in both male and female *OL* can be equated to a similar decline in the expression of related genes in the murine model at around 20 months [63]. This decline of immune initiators and mediators could imply a reduced or delayed response to immune stimuli, potentially leading to increased susceptibility and reduced immune competence. Alterations in the expression of immune initiators, mediators, and effectors are observed with increasing age. For example, immune initiators, such as *CRP*, *TLR5-s*, *TCRb*, and *MHCII*, show decreased expression with age, leading to potential alterations in immune response and disease manifestation. Specifically, CRP, a known proinflammatory marker, can modulate granulocyte activity and has been noted in humans to inhibit chemotaxis [64]. TLR5 is associated with the recognition of bacterial flagellum and the activation of immune mediator *Nf-kß*, so decreased expression with increasing age may indicate a decreased immune response to bacteria stimuli which can lead to reduced immune competence [65]. While there is some evidence that *TLR5* expression can increase with age in humans [66], there are contradictory data that *TLR5* decreases in perimenopausal women [67]. Data here demonstrate decreased TLR5 expression in our elderly medaka; this variation could be indicative of differences in TLR function between mammals and teleost, and distinguishment between membrane-bound or soluble Toll-like receptors could account for the differences in expression levels. It is well documented that aging in vertebrates results in reduced thymus size as well as reduced T-cell numbers [1]. The changes in T-cell receptor signaling and expression are associated with normal immunoescence in both humans and mice [68]. Here, a reduction in *TCRb* expression with age in medaka aligns with the expectation of reduced T-cells and related signaling found in vertebrates [16,17]. Similarly, it would be expected that a reduction in *TCR* expression would be accompanied by reduced *MHCII* expression due to the joint immune signaling pathways [69]. *MHCII* expression is characteristic of macrophages and is documented to decline with normal aging in vertebrates which was also found in our medaka aging model [69]. Reduced immune initiator expression is characteristic of a normal aging immune system in vertebrates.

Immune mediators, such as *MYD88*, *Nf-kß*, *C3*, and *IL1b* exhibit decreased expression with age. MYD88 is a key immune mediator responsible for regulating an immune response; the reduction or absence of MYD88 can result in a dysregulated immune response including increased inflammation related to CD4+ T-cell functions [70]. While there is some evidence to support the finding of reduced *MYD88* expression with increasing age, there is also evidence which shows no change in the expression of this mediator with increasing age [11,71]. Nf-kß is a crucial player in inflammatory response and aging results in the reduction of Toll-like receptor expression and the subsequent MYD88 activity; reduced *Nf-kß* expression is also expected to be a characteristic of immune aging. This reduced Nf-kß activity can lead to changes in the regulation of inflammatory responses and the subsequent immune competence [72]. *C3* expression is indicative of the activity of the complement system; thus, reduced C3 activity with increasing age could lead to reduced

innate immune response via complement and ultimately impact immune competence. Aging is associated with reduced TLR activation, notably in monocytes/macrophages and neutrophils, and subsequently reduced MYD88 activity which can lead to reduced *IL1b* expression and activity [70]. This MYD88-IL1b axis is a crucial inflammatory response and IL1b is also associated with key neutrophil activation and activity; therefore, reduced *IL1b* expression could be responsible for the dysfunction of neutrophil-related immune functions with age [73,74]. As previously described, medaka demonstrate an increase in phagocytes with increasing age and key changes in immune gene expression which could indicate altered inflammatory responses; specifically, neutrophils present an interesting player in this immune aging characteristic as they are the typical major components of phagocyte populations and the dysfunction of neutrophils can lead to a dysregulated immune response. Conversely to immune initiator and mediator decline with age, immune effectors like *C8* are shown to increase in expression while effector *LYZ* decrease with age. Increased levels of C8 with increasing age are documented in humans [75]. Lysozyme activity is crucial in microbial immune response for vertebrates. Lysozyme in mammals is secreted by monocytes and then plays a role in inflammatory response and is also described to affect the activity and function of neutrophils [76,77]. Thus, here we describe reduced *LYZ* expression indicating changes to the inflammatory response and potential impacts on neutrophil function associated with increased age. The reverting of the immune molecular profile to a more similar profile of younger (3–7 mph) *OL* at the elderly stage (20/23 mph) could be explained by an increase in chronic inflammation later in life. The RT-qPCR data presented here represent the expression levels of unchallenged *OL*; therefore, while the molecular profile resembles that of a more effective immune response, chronic inflammation may be overshadowing the underlying reduced immune competence seen after immune activation and challenge on the organismal level, specifically in male *OL*. To best evaluate this discrepancy, immune gene expression levels should be measured both before and after the immune challenge which would allow for the visualization of this immune compromise at the molecular level and bring to light the potential mechanisms that female *OL* may be utilizing in order to maintain competence compared to their male counterparts at the elderly age.

Here, medaka males and females seem to age similarly at the cellular level; however, there are notable sex-specific differences in the organismal and molecular levels of immune aging with reduced male immune competence during the HRA. It is noted that assessments at the cellular and molecular level were of unchallenged immune responses, thus the significant impact seen at the organismal level after the challenge indicates an important difference in the functionality of the immune response mechanisms for elderly male *OL*. This discrepancy in male immune competence could be explained by hormonal differences between the males and females, sex-specific immune responses like differences in T-cell subpopulations, or variations in the functionality of immune response mechanisms. Male and female medaka differ in sex-specific hormonal responses, and estrogen and testosterone also differently impact immune function; therefore, persistent hormonal differences could result in differing immune signaling and function. It has been noted in the murine model that high levels of estradiol can protect females from harmful inflammatory responses increasing their survival when compared with both males and females with lower estradiol [78]. In addition, female and male medaka tend to have differing immune cell subpopulations including T-cells. It could be hypothesized that a discrepancy in T-cell subpopulations or activation of these cells could explain the differences between male and female survival after pathogen exposure. While an increase in phagocytes was observed for both aging males and females, it is argued that the functionality of these phagocytes which could be neutrophils may have declined functions including signally. Sex- and age-specific differences in immune cell populations and subsequent responses have been observed in humans; specifically, males tend to have a higher natural killer cell-mediated and proinflammatory response while females have higher levels of T-cell and B-cell signaling [50].

Medaka illustrates a senescent immune phenotype during typical aging characterized by reduced host resistance in elderly males, changes in phagocyte populations after sexual maturation at 3 mph, sex-specific molecular immune profiles at specific ages, and reduced immune initiator, mediator, and effector expression levels after 11–13 mph in both males and females. While here we present the underlying sex-specific differences in *OL* at each age and compare the similarities with other mammalian models, it is noted that we do see a more significant impact of age on the differences in immune response than sex which has been previously described in humans [50]. The comparative analyses of aging in teleost models such as zebrafish (*Danio rerio*) and sea bream (*Sparus aurata*) reveal similar trends in the immune aging process, with changes that mirror those observed here in medaka. In zebrafish, aging is associated with a shift towards a proinflammatory profile, marked by an increase in certain immune cell populations and the elevated expression of proinflammatory cytokines like TNF-a and IL6 [79,80]. These cytokines are known to contribute to chronic inflammation, a hallmark of aging across vertebrates. Similarly, sea bream exhibits age-related increases in proinflammatory markers along with changes in gene expression that suggest a decline in immune competence with age [81]. These findings support the idea that aging in teleosts including medaka is accompanied by a shift towards a more proinflammatory immune state, which may contribute to the observed decline in organismal survival and increased disease susceptibility. The medaka model, with its observed changes in phagocyte populations and immune gene expression profiles indicating a proinflammatory shift, aligns well with other teleost model species. This similarity between teleost species highlights the evolutionary conservation of immune aging mechanisms and the relevance of the medaka model for studying the effects of aging on immune function. The reduced survival observed in elderly male medaka, along with sex-specific differences in immune genes, further emphasizes the complexity of immune aging and its implications for organismal health. The organismal, cellular, and molecular changes with age and sex can impact the overall immune response of the organisms and contribute to age-related disease susceptibility; thus, based on data presented here, Japanese medaka are a good model for such future studies.

5. Conclusions

Japanese medaka may be a useful tool to model immune aging as these changes are similar to those seen in mice and human serum levels. The medaka fish offers advantages over other model systems like yeast, worms, and flies, including its vertebrate nature, relatively short lifespan, and quick reproductive development. Its utility in various research fields, such as immunotoxicology, developmental biology, and behavioral studies, makes it an attractive choice for investigating immune aging and its relationship with environmental toxicants. Medaka presents similar sex-specific and age-associated variations in immune profiles at the organismal, cellular, and molecular levels. The research presented lays the groundwork for using medaka fish as a model organism to further investigate immune vulnerability and age-related diseases, contributing to broader knowledge in the field of immunology and aging research. Future research outlining more detailed characteristics of immune function and competence considering both a challenged and unchallenged system at a variety of ages with sex-specific considerations would further expand the usefulness of this model for use in aging-related immunotoxicity studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9090333/s1, Table S1: *OL* immune gene primers.

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