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RESEARCH ARTICLE

Effects of vaccination against *Yersinia ruckeri* on oxidative stress biomarkers and liver and heart biochemistry in rainbow trout (*Oncorhynchus mykiss*)

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Abstract. To determine the effects of vaccination against *Yersinia ruckeri* on the health condition of rainbow trout, *Oncorhynchus mykiss* (Walbaum) in general, and oxidative stress biomarkers and metabolic parameters specifically, as well as to identify mechanisms that underpin the susceptibility of fish to vaccination, we compared the liver and heart function, and the oxidative mechanism underlying those effects, by detecting relevant lipid peroxidation and protein oxidation biomarkers, as well as aerobic-anaerobic metabolism in trout immunized against *Y. ruckeri* at 30 days post-vaccination and in healthy individuals. In our study, hepatic aminotransferase activities were positively associated with the oxidative stress biomarkers in the trout vaccinated against *Y. ruckeri*. Moreover, similar associations were observed in the cardiac tissue of the immunized trout. Decreased aldehydic and ketonic derivatives of oxidatively modified proteins and the reduction of aminotransferase and lactate dehydrogenase activities were sensitive to the vaccination of trout against *Y. ruckeri* and may potentially be used as biomarkers in evaluating vaccine effects in the liver of rainbow trout. Understanding the role of biochemical changes in the tissues of vaccinated trout has important implications for understanding of the complex physiological changes that occur in immunization, and also for improving aquaculture practices to maximize tissue growth and the health of vaccinated trout.

Keywords: rainbow trout *Oncorhynchus mykiss*, *Yersinia ruckeri,* immunization, oxidative stress, metabolic parameters, liver, heart

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Introduction

Yersinia ruckeri, a Gram-negative bacterium in the family *Enterobacteriaceae*, is the causative agent of Enteric Redmouth (ERM) disease, or yersiniosis, and causes acute or chronic infections in salmonids (Thompson and Adams 2004). Disease outbreaks appear related to conditions of stress or poor water quality, possibly because a carrier state exists with the bacterium lying dormant in fish until times of stress (Thompson and Adams 2004). Yersiniosis has been spreading around the world, and the classical symptoms of the disease are associated with serotype O1 of this bacterium. *Y. ruckeri* has been isolated from different fish species, but the most susceptible to infection are salmonids, especially rainbow trout,

Oncorhynchus mykiss (Walbaum) (Pękala and Antychowicz 2010). At least eight different serological groups are thought to exist based on whole cell reactions, O-antigens, and LPS profiles (Stevenson 1997). Two of the most predominant groups of *Y. ruckeri* belong to serovar type 1 (Hagerman), which is more commonly isolated from rainbow trout, and serovar type II (O'Leary), which was first isolated from coho salmon, *Oncorhynchus kisutch* (Walbaum) (Stevenson and Airdrie 1984, Daly et al. 1986). Serovar 1 was originally thought to be the most virulent serovar, but it has since been shown that serovar 2 can be as virulent as serovar 1 (Cipriano and Ruppenthal 1987). Infected fish and asymptomatic carriers are the main source of the infection, spreading bacteria with feces. Gills are regarded as the entry route of *Y. ruckeri* rods but the likelihood of the disease depends on the virulence of the given strain. Characteristic clinical signs of yersiniosis, such as hemorrhages around the oral cavity, are caused by extracellular products (ECPs) of *Y. ruckeri* (Pękala and Antychowicz 2010). Hemorrhages are found in internal organs as well. Post mortem examination proves the dysfunction of the swim bladder with bloody effusion fluid in its cavity. Reducing stress factors during the culture and transport of fish as well as the application of vaccines as an immune prophylactic mechanism effectively prevent yersiniosis (Pękala and Antychowicz 2010).

Vaccination, which is now a part of routine husbandry management in many aquaculture systems is used as a means of controlling bacterial disease outbreaks, and the use of vaccines is steadily increasing as the diversity of species being farmed expands, and new vaccines are developed for additional microbial agents (Thompson and Adams 2004). Both salmon and rainbow trout are vaccinated against three to five diseases during their production cycle, often with a multivalent vaccine, and productivity has increased as a result of vaccination (Gudding et al. 1999). Yersiniosis is successfully controlled with commercial vaccines, and, in fact, it was one of the first diseases to be controlled by vaccination (Thompson and Adams 2004). Most vaccines are bacterin preparations using whole cell preparations of serovar 1 (the Hagerman strain and the major cause of disease outbreaks). Bacteria are generally inactivated with formalin and sometimes pH lysed at pH 9.8 to expose internal cell components (Thompson and Adams 2004).

The results of Kozińska and Pękala (2012) indicate that vaccines prepared using *Y. ruckeri* strains originating from particular farms and applied only in these farms may result in the best prophylaxis against yersiniosis in rainbow trout. The study by Kozińska and Pękala (2012) compares the protective effect of two autologous vaccines against homologous and heterologous *Y. ruckeri* strains. Their studies were preceded by determining optimal vaccination conditions (antigen dose and fish exposition time to the vaccine suspension). Each of the vaccines contained antigens prepared from one of two strains of *Y. ruckeri*, either YR1 or Pt426, which originated from different farms and exhibited some biochemical differences. The fish were immersed in the vaccines containing 108 of antigens for 60 s (conditions determined as optimal). Six weeks after immunization, the fish that had been immunized with the different vaccines were divided into four groups. One of them was infected with the strain that is homologous to the vaccine. Each of the remaining groups was infected with one of the three heterologous strains. The relative percentage of survival (RPS) after infection with homologous strains was 90.5% for YR1 and 87% for Pt426, while for heterologous strains RPS it was markedly lower at a range of 33 to 67% (Kozińska and Pękala 2012). Grudniewska et al. (2010) evaluated the impact of vaccinations against furunculosis and yersiniosis (produced using field strains) administered by immersion on the growth and survival of rainbow trout fry. The control group of fish was subjected to the same immersion procedure in water without vaccines. The best rearing results (both survival and growth) with the vaccinated group were noted. The results also indicated that the vaccination resulted in decreased losses and reduced mortality, which has tangible benefits in the form of profit (Grudniewska et al. 2010).

Various routes of administration (intraperitoneal injection, direct immersion, shower or spray, feeding and anal intubation) have been evaluated and provide good levels of protection, although commercial

vaccines for Yersiniosis tend to be administered by IP injection or by immersion (Thompson and Adams 2004). The success of the vaccine has been reported to be variable under field conditions, and often does not completely prevent disease outbreaks when the level of infection is high, as is seen when fish are stressed (Horne and Barnes 1999). Clearly, a greater understanding of the fish response against *Y. ruckeri* and during vaccination against yersiniosis would help improve this situation.

Therefore, exploring the effects of vaccination against *Y. ruckeri* on the health condition of trout in general, and oxidative stress biomarkers in different tissues specifically, would be valuable. The present study aims to clarify the effects of vaccination against *Y. ruckeri* on liver and heart function, and the oxidative mechanism underlying those effects, by detecting relevant lipid peroxidation and protein oxidation biomarkers, as well as metabolic parameters in rainbow trout immunized against *Y. ruckeri*.

Materials and Methods

Experimental animals

Thirty rainbow trout with a mean body mass of (107.9 ± 3.1) g were used in the experiments. The study was carried out at the Department of Salmonid Research, Inland Fisheries Institute near the town of Żukowo (Poland). Experiments were performed at a water temperature of 14.5 ± 0.5°C and the pH was 7.5. The dissolved oxygen level was about 12 ppm with additional oxygen supply with a water flow of 25 L/min, and a photoperiod of 7 hours per day. The fish were fed a commercial pelleted diet at an optimal level, using 12-hour belt feeders for fish. The daily dose of feed was calculated in accordance with the applicable feed table (From and Rasmussen 1984). All enzymatic assays were carried out at the Department of Zoology and Animal Physiology, Institute of Biology and Environmental Protection, Pomeranian University in Słupsk (Poland).

Experimental design

The fish were divided into two groups: I) control; II) immunized by vaccine against *Y. ruckeri*. The fish were held in 250-L square tanks (70-75 fish per tank) under the same conditions. The vaccine was produced at the Department of Fish Diseases, National Veterinary Research Institute in Puławy (Poland). The vaccine against yersiniosis was prepared using whole cells of *Y. ruckeri* inactivated with formalin. The vaccine contains three *Y. ruckeri* strains originated from rainbow trout cultured on the different farms where fish exhibited clinical signs of yersiniosis. All bacteria isolates belonged to O1 serotype biotype 2, which is pathogenic for fish, and showed some differences in their biochemical properties. The bacterial strains were inoculated onto trypticase soya broth (TSB, BioMerieux) and incubated for 48 h at 24 ± 1°C. After incubation the purity of the culture was checked. The density of the vaccine was estimated spectrophotometrically, and formalin was added to the final concentration of 0.4% in order to inactivate the bacterial strains. After 48 h incubation, the vaccine was monitored for sterility. The prepared vaccine at a concentration of 1 × 109 cells per mL was used for fish vaccination *per os*. The concentrated vaccine was mixed into the feed, and administered three times at intervals of one day.

One month after immunization, liver and heart samples from rainbow trout were collected. The fish were kept for 30 days after vaccination at a water temperature of 14.5 ± 0.5°C and pH 7.5. In our study, 15 rainbow trout from the unhandled control and 15 vaccinated trout were used one month after immunization.

Sampling

The animals were quickly caught and killed on day 31 post vaccination (n = 15 in each group). The liver and heart were removed *in situ*. Briefly, the liver and heart from each fish were excised, weighed, and washed in ice-cold Tris-HCl buffer. The organs were rinsed clear of blood with cold isolation buffer and

homogenized in a glass Potter-Elvehjem homogenizing vessel with a motor-driven Teflon pestle on ice in a proportion of 1:9 (weight/volume). The isolation buffer contained 100 mM Tris-HCl; pH of 7.2 was adjusted with HCl. Homogenates were centrifuged at 3,000 g for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at -20°C until analysis. One heart and one liver from each fish were used in the biochemical assay. There were 15 hearts and 15 livers in each group. Protein contents were determined using the method by Bradford (1976) with bovine serum albumin as the standard. Absorbance was recorded at 595 nm. All enzymatic assays were carried out at 22 ± 0.5°C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) in duplicate. The enzymatic reactions were started by the addition of the tissue supernatant. The specific assay conditions were as follows.

Oxidative stress biomarker assays

Assay of 2-thiobarbituric acid reactive substances (TBARS) level

An aliquot of the homogenate was used to determine the lipid peroxidation status of the sample by measuring the concentration of 2-thiobarbituric-acid-reacting substances (TBARS) according to the method by Kamyshnikov (2004). The reaction mixture contained sample homogenate (2.1 mL, 10% w/v) in Tris-HCl buffer (100 mM, pH 7.2), 2-thiobarbituric acid (TBA; 0.8%, 1.0 mL), and trichloracetic acid (TCA; 20%, 1.0 mL). The total volume was kept in a water bath at 100°C for 10 min. After cooling, the mixture was centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. TBARS values are expressed as nmoles malondialdehyde (MDA) per mg protein.

Assay of carbonyl groups of oxidatively modified protein levels

Carbonyl groups were measured as an indication of the oxidative damage to proteins according to the method of Levine et al. (1990) in modification of Dubinina et al. (1995). Samples were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) in 2M HCl. Blanks were run without DNTP. Afterward, proteins were precipitated with TCA and centrifuged for 20 min at 3,000 g. The protein pellet was washed three times with ethanol:ethylacetate (1:1) and incubated at 37°C until complete resuspension. The carbonyl content was measured spectrophotometrically at 370 nm (aldehydic derivatives, OMP_{370}) and at 430 nm (ketonic derivatives, OMP_{430}) (molar extinction coefficient 22,000 M^{-1} cm^{-1}) and expressed as nmol per mg protein.

Biochemical parameters assays

Assays of Alanine aminotransferase (AlAT, E.C. 2.6.1.2) and Aspartate aminotransferase (AsAT, E.C. 2.6.1.1) activities

AlAT and AsAT activity was analyzed spectrophotometrically with the standard enzymatic method (Reitman and Frankel 1957). The substrates in the reaction were α-ketoglutaric acid (α-KG) plus L-aspartate for AsAT and α-KG plus L-alanine for AlAT. The products formed by enzyme action in the presence of sample are glutamate and oxaloacetate for AsAT and glutamate and pyruvate for AlAT at pH 7.4. The addition of 2,4-dinitrophenyl hydrazine resulted in the formation of a hydrazone complex with ketoacids to form their respective hydrazone derivatives, which were measured colorimetrically at 530 nm. A red color was produced on the addition of 0.4M NaOH. The intensity of the color was related to enzymatic activity. In the measurement of both AlAT and AsAT, pyruvate was used as the standard for calibration graph composition. One unit of AsAT or AlAT is defined as the liberation of 1 μmol of pyruvate per hour at 37°C incubation per mg protein.

Assay of Lactate dehydrogenase (LDH, E.C. 1.1.1.27)

The colorimetric method by Sevela and Tovarek (1959) was used to determine LDH activity. Pyruvate

was formed by LDH action in the presence of sample and in the presence of NAD+. The reduction of NAD+ is coupled to the reduction of L-lactate. Briefly, 0.1 mL of tissue sample was mixed with 0.3 mL of NAD+ reagent (0.6 mg per sample), 0.8 mL of 0.03M tetrasodium pyrophosphate (pH 8.8), and 0.2 mL of 0.45M sodium lactate (pH 7.5) reagents. The samples were incubated at 37°C for 15 min. The addition of 2,4-dinitrophenyl hydrazine results in the formation of a hydrazone complex with ketoacids to form their respective hydrazone derivatives, which were measured colorimetrically at 530 nm. A red color is produced on the addition of 0.4M NaOH and is related to enzymic activity. In the measurement of LDH activity, pyruvate is used as the standard for calibration graph composition. One unit of LDH is defined as the formation of 1 μmol of pyruvate per hour at 37°C incubation per mg protein.

Assays of lactate and pyruvate concentration

Lactate and pyruvate concentrations were measured according to the procedure described by Herasimov and Plaksina (2000). One mL of sample was added to 6 mL of distilled water and 1 mL of 10% metaphosphoric acid. The mixture was centrifuged at 800 g for 5 min to separate the supernatant. One mL of 25% copper (II) sulfate and 500 mg of calcium hydroxide were added to the supernatant, which was then mixed for 30 min. The mixture was centrifuged at 1,000 g for 10 min. For the lactate concentration assay, the resulting supernatant was resuspended in 3 mL of *p*-dimethylamino benzaldehydic (0.5% in dimethyl sulfoxide), and 1 mL of 25% NaOH. The mixture was incubated at 37°C for 45 min, which was then centrifuged at 1,000 g for 10 min. The absorbance was measured at 420 nm. The mixture with 0.5% *p*-dimethylamino benzaldehydic and 25% NaOH was used as the blank. For the pyruvate concentration assay, the resulting supernatant was resuspended in 0.1 mL of 10% copper (II) sulfate, 4 mL of concentrated H2SO4, and 0.1 mL of 20% hydroquinone dissolved in alcohol, which was then heated in a water bath at 95°C for 15 min. Absorbance was measured at 430 nm. Calibration curves of lactate (0.1-5 mM) and pyruvate (0.1-5 mM) were used, and the results were expressed in μmol per mg protein.

Statistical analysis

Data are presented as the mean ± S.E.M. and were checked for assumptions of normality using the Kolmogorov–Smirnov one-sample test and Lilliefors test ($P > 0.05$). Homogeneity of variance was checked using the Levene test. In order to find significant differences (significance level, $P < 0.05$) between control and vaccinated groups, the Mann-Whitney U test was applied to the data (Zar 1999). Differences were considered significant at $P < 0.05$. In addition, the relationships between oxidative stress biomarkers and biochemical parameters of all individuals were evaluated using Spearman's correlation analysis. All statistical analysis was performed with STATISTICA 10.0 software (StatSoft, Poland).

Results

The level of lipid peroxidation in the liver and heart of trout treated with vaccine did not significantly differ from that in the controls (Fig. 1). The content of aldehydic derivatives of oxidatively modified proteins in the liver and heart was non-significantly lower in the group vaccinated against *Y. ruckeri* at the first month compared to the unhandled group (Fig. 2). Vaccination caused a significant decrease the ketonic derivatives in the liver by 35% ($P = 0.033$) compared to the control (Fig. 2). The ketonic derivatives of OMB content in cardiac tissue of fish vaccinated against *Y. ruckeri* at 30 days after immunization was non-significantly lower compared to the unhandled control (Fig. 2). AlAT, AsAT, and LDH activities were significantly decreased in the liver of the vaccinated group compared to those in the control (by 27.8%, $P = 0.009$; by 32%, $P = 0.006$; by 25.9%, $P= 0.008$, respectively) (Fig. 3). AlAT, AsAT, and LDH activities in the heart of trout treated with vaccine did not significantly differ from that in the controls (Fig. 3).

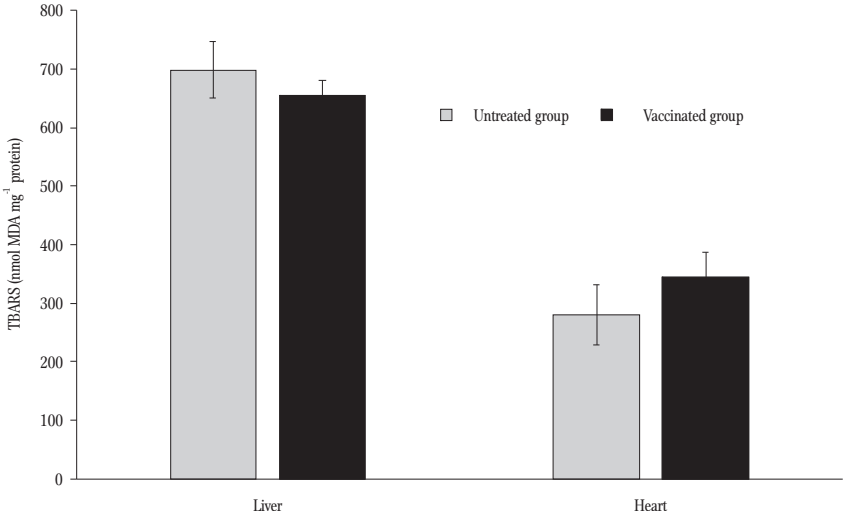

Figure 1. Level of lipid peroxidation (nmol MDA per mg protein) in the liver and heart of the trout vaccinated against *Y. ruckeri* 30 days after immunization (M ± m, n = 15).

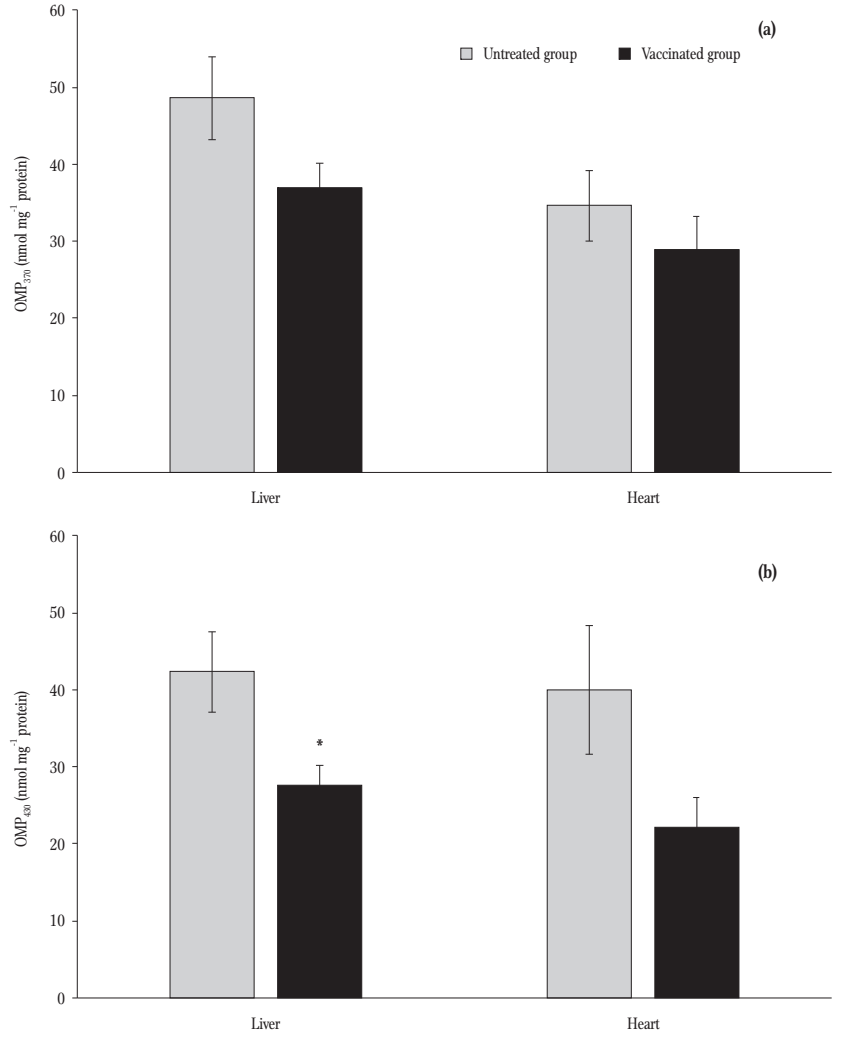

Figure 2. Aldehydic (a) and ketonic derivatives (b) of oxidatively modified proteins in the liver and heart of trout vaccinated against *Y. ruckeri* 30 days after immunization (M ± m, n = 15). *significant differences in values between unhandled and vaccinated groups 30 days after immunization are shown as $P < 0.05$

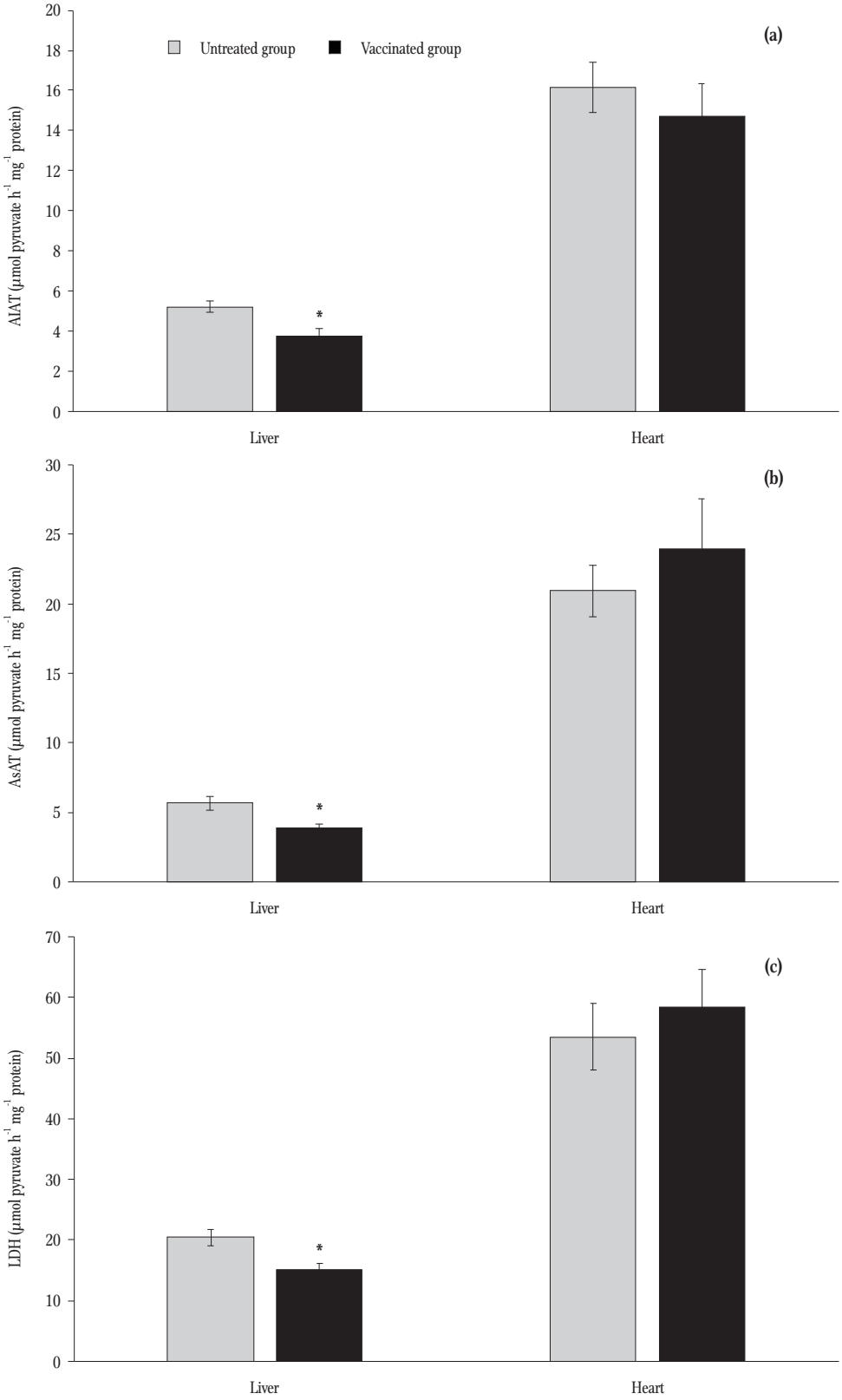

Figure 3. Alanine (AlAT), aspartate aminotransferase (AsAT), and lactate dehydrogenase (LDH) activity in the liver and heart of the trout vaccinated against *Y. ruckeri* 30 days after immunization (M ± m, n = 15). *significant differences in values between unhandled and vaccinated groups 30 days after immunization are shown as $P < 0.05$

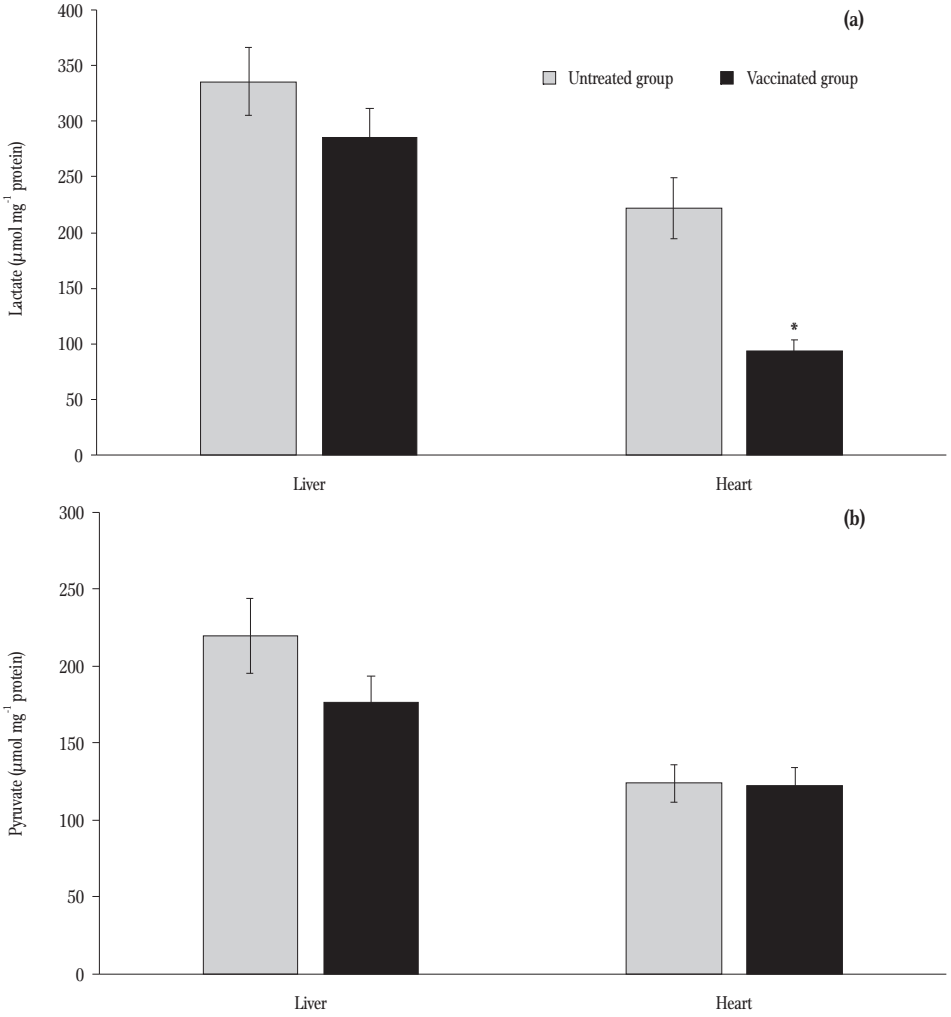

Figure 4. Lactate (a) and pyruvate (b) levels in the liver and heart of trout vaccinated against *Y. ruckeri* 30 days after immunization (M ± m, n = 15). *significant differences in values between unhandled and vaccinated groups 30 days after immunization are shown as $P < 0.05$

Vaccination caused a non-significant decrease in lactate and pyruvate levels in the liver compared to the control (Fig. 4). The lactate level in the cardiac tissue of fish treated with the vaccine against *Y. ruckeri* at 30 days after immunization was significantly lower compared to the unhandled control (Fig. 4).

Correlations between oxidative stress biomarkers and biochemical parameters in the liver and cardiac tissue of the unhandled control and in trout vaccinated against *Y. ruckeri* at 30 days after immunization are presented in Figs. 5-8. Our results indicate that the TBARS level was correlated inversely with AlAT and LDH activities ($r = -0.607$, $P = 0.000$ and $r = -0.626$, $P = 0.022$, respectively), while aldehydic derivatives of oxidatively modified proteins were correlated positively with LDH activities ($r = 0.752$, $P = 0.003$) and lactate level ($r = 0.715$, $P = 0.006$) in the liver of the unhandled control (Fig. 5).

In contrast to the unhandled control, in the liver of trout vaccinated against *Y. ruckeri* at 30 days after immunization, AlAT and AsAT activities were correlated positively with TBARS level ($r = 0.796$, $P = 0.001$ and $r = 0.733$, $P = 0.003$, respectively), as well as with aldehydic derivatives of oxidatively modified proteins ($r = 0.604$, $P = 0.022$ and $r = 0.776$, $P = 0.001$, respectively) (Fig. 6).

In the cardiac tissue of the unhandled trout (Fig. 7), aldehydic derivatives of oxidatively modified proteins were correlated positively ($r = 0.628$, $P = 0.022$ and $r = 0.804$, $P = 0.001$, respectively), and AsAT

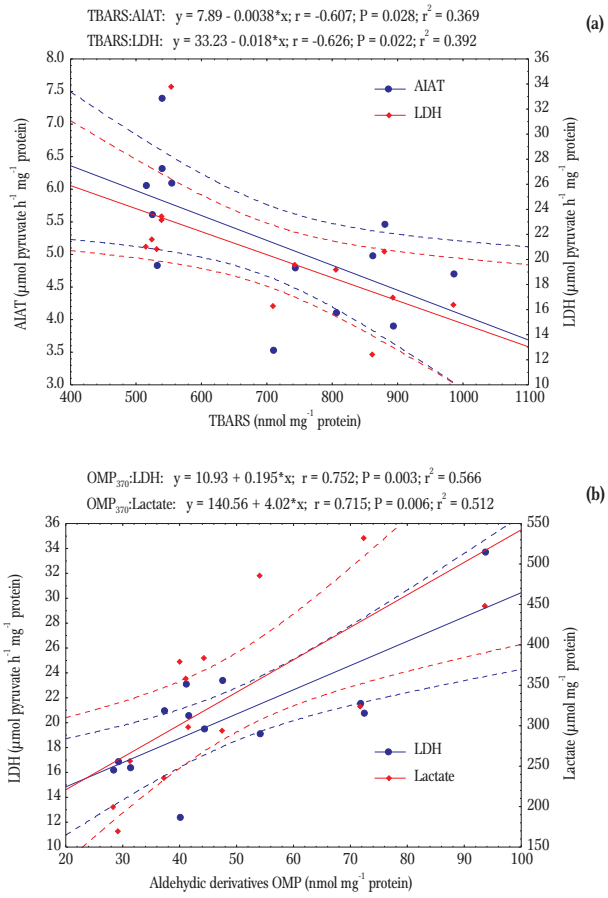

Figure 5. Correlations between oxidative stress biomarkers and biochemical parameters in the liver of unhandled controls (n = 15).

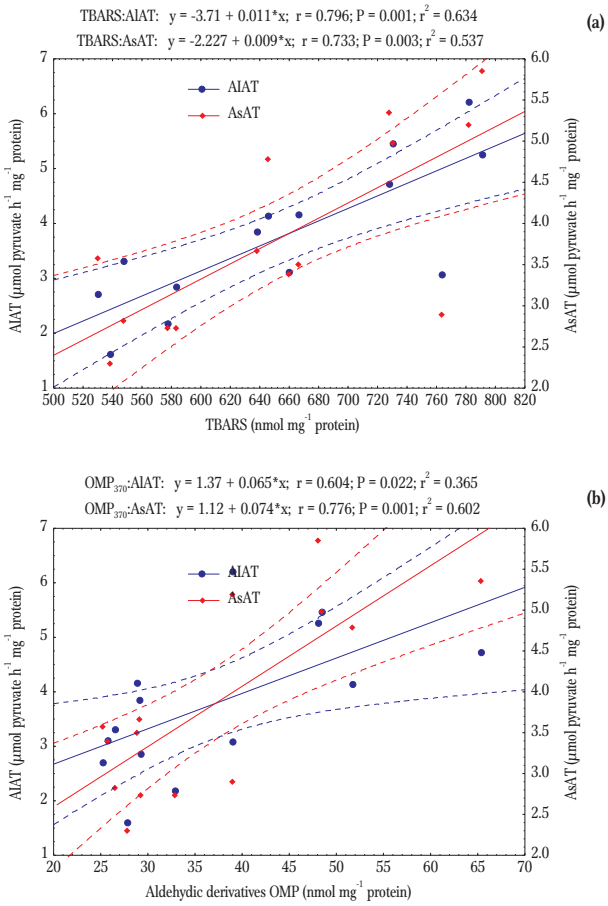

Figure 6. Correlations between oxidative stress markers and biochemical parameters in the liver of trout vaccinated against *Y. ruckeri* 30 days after immunization (n = 15).

activity was dependent on the TBARS level (r = 0.690, P = 0.009) and pyruvate level (r = 0.765, P = 0.002).

In the cardiac tissue of fish vaccinated against *Y. ruckeri* at 30 days after immunization, the TBARS level was correlated positively with aldehydic derivatives of oxidatively modified proteins (r = 0.536, P = 0.048) and AsAT activity (r = 0.599, P = 0.024). Ketonic derivatives of oxidatively modified proteins were correlated positively with AlAT and LDH activities (r= 0.610, P = 0.021 and r = 0.580, P = 0.030, respectively) (Fig. 8).

Discussion

We studied the effects of oral vaccination against *Y. ruckeri* on the oxidative stress biomarkers of the liver and heart in rainbow trout and compared these to the unhandled control in an attempt to elucidate the individual effects of vaccination on the metabolic alterations in the liver and heart that occur over 30 days following immunization. There are several similarities and differences in the responses. Many studies have been carried out to examine the efficacy of oral vaccines in fish (Quentel and Vigneulle 1997). These studies have looked at the types of immune responses stimulated by oral vaccination and the levels of protection obtained, but varying degrees of success have been reported in the literature. These variations are believed to be due to differences in experimental design among studies, including antigen preparation, the age and species of fish, the water temperature at the time of vaccination, the duration of feeding the vaccine, and antigen integrity when it reaches the

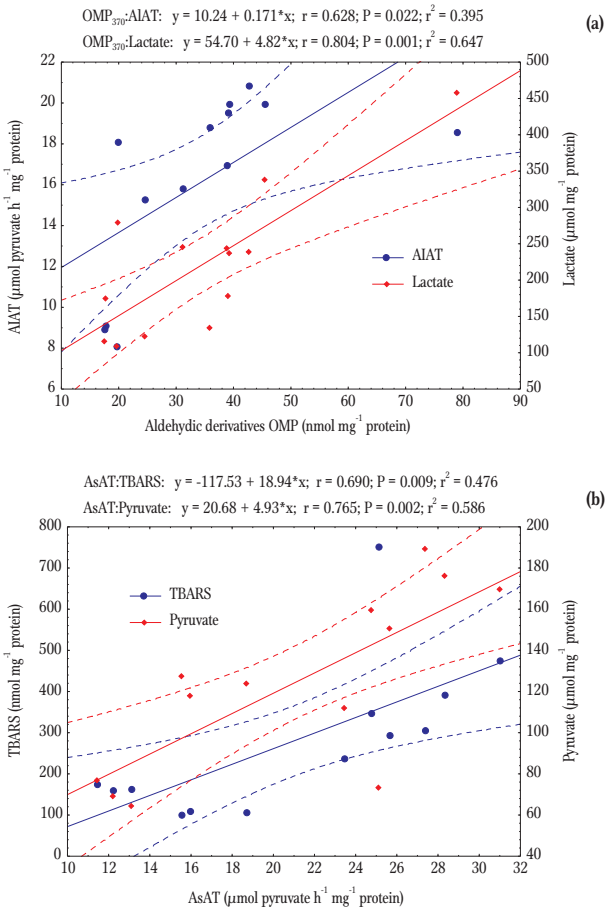

Figure 7. Correlations between oxidative stress markers and biochemical parameters in the cardiac tissue of unhandled controls (n = 15).

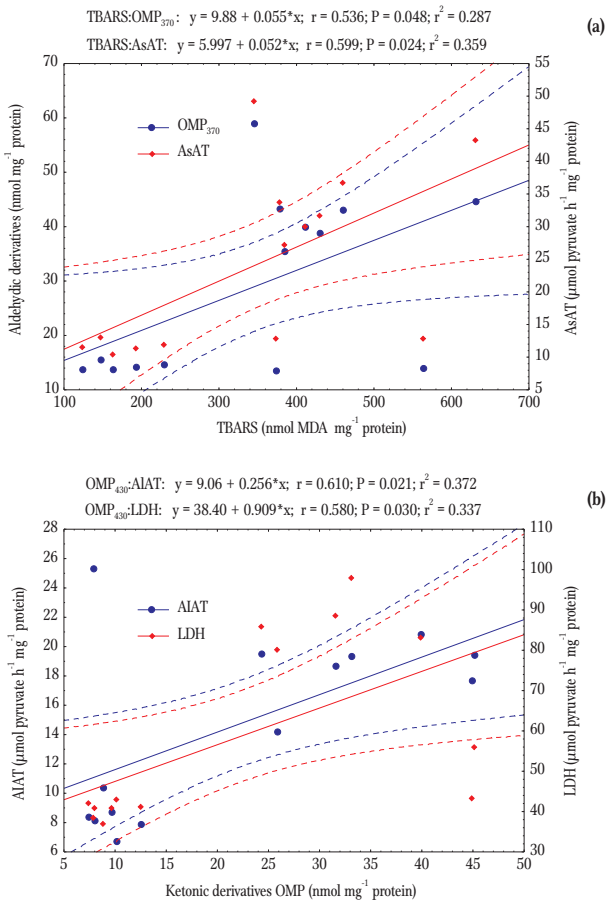

Figure 8. Correlations between oxidative stress markers and biochemical parameters in the cardiac tissue of trout vaccinated against Y. ruckeri 30 days after immunization (n = 15).

hind gut (Thompson and Adams 2004). Our data shed some light on the individual effect of the long-term effect of vaccination against *Y. ruckeri* in rainbow trout that may be contributing to metabolic changes and the health condition of vaccinated trout.

In the present study, vaccination against *Y. ruckeri* showed significant association with decreased carbonyl derivatives of oxidatively modified proteins as biomarkers of protein damage and liver enzyme activities (Fig. 6). Transaminases like AlAT and AsAT play significant roles in amino acid and protein metabolism, and they might be released into the plasma following tissue damage and dysfunction. In the present study, the levels of both AsAT and AlAT and LDH decreased over a 30-day period after immunization (Fig. 3). The non-significantly decreased levels of lactate and pyruvate noted 30 days after immunization (Fig. 4) might be a result of the lower activities of aminotransferase and LDH (Fig. 3). Lactate is considered to be a central player in cellular, regional, and whole body metabolism (Gladden 2004). It now appears that lactate is an anaerobic metabolite in the presence of anoxia, a hypoxic metabolite in the presence of dysoxia, and an aerobic metabolite in the presence of an adequate oxygen supply (Gladden 2004). When tissue hypoxia is present, pyruvate oxidation in the Krebs cycle is decreased. Lactate production is increased and ATP formation continues via glycolysis. Because lactic acid is a product of anaerobic metabolism, elevated lactate levels represent inadequate tissue oxygen delivery (Shinde et al. 2005).

In this study, we demonstrated that hepatic AlAT and AsAT activities were positively associated with

oxidative stress biomarkers (TBARS as biomarker of lipid peroxidation, aldehydic, and ketonic derivatives of oxidatively modified proteins as biomarkers of protein damage) in the trout vaccinated against *Y. ruckeri* (Fig. 6). Moreover, similar associations were observed in the cardiac tissue of immunized trout (Fig. 8). The TBARS level was correlated with AsAT activity, while aldehydic derivatives of oxidatively modified protein levels were associated with AlAT and LDH activities. The TBARS and aldehydic derivatives of OMP levels were not significant in the liver or heart of vaccinated trout (Figs. 1 and 2). This reinforces our conclusion that decreased aldehydic and ketonic derivatives of oxidatively modified protein levels and lower aminotransferase and LDH activities were sensitive to the vaccination of trout against *Y. ruckeri* and can potentially be used as biomarkers in evaluating vaccine toxicity in the liver of rainbow trout.

Several studies have reported a relationship between *Y. ruckeri* O1 infection and immune system activation (the expression of the IL-1β, IL-8, and IL-10 genes) in rainbow trout (Raida and Buchmann 2008). Raida and Buchmann (2008) investigated the development of adaptive immunity in rainbow trout surviving a primary infection with 5x105 CFU *Y. ruckeri* O1 (LD50 dose) with transcriptome analysis of spleen tissue. These fish that survived a primary infection also showed significantly increased survival following a secondary infection (same dose) when compared to naïve trout. The cytokines and chemokines comprised IL-1β, IL-1 receptor antagonist (Ra), IL-6, IL-8, IL-10, IL-11, and IFN-γ, IL-1 receptor I and II (IL-RI and IL-RII). Transcript levels of genes encoding cytokines and receptors were increased during the primary infection but not during the secondary infection. Changes in T cell occurrence or activity in the spleen during the infections were inferred from the transcript level of T cell receptor (TCR), CD4, and CD8α genes. No alteration in the expression of MHC class II and immunoglobulins IgM and IgT was detected. The amount of *Y. ruckeri* O1 in the spleen was correlated to the expression of IL-1β, IL-8, and IL-10 genes with a peak expression (first infection). The low transcript levels of the bacterial gene and the host's immune genes during re-infection can be interpreted as a result of the development of adaptive immunity. This would explain the relatively fast elimination of the bacteria during the secondary infection, whereby the activation of cytokines becomes less pronounced (Raida and Buchmann 2008).

Raida et al. (2011a) also show that antibody titers and the bactericidal effect of plasma increase in ERM immersion vaccinated rainbow trout. This is associated with a reduced bacteremia and increased protective immunity against *Y. ruckeri* infection. The study by Raida et al. (2011a) indicates that specific plasma antibodies with other humoral and cellular elements may take part in immunity against *Y. ruckeri* and induce protection of rainbow trout against ERM. Raida et al. (2011b) also show that the transcripts of cytokine genes in blood cells sampled three days after infection by bath challenge in a bacterial suspension (LD60 dose, 1.8x109 CFU/ml *Y. ruckeri* for 1 h) was significantly higher in fish which obtained a high bacteriemia and died at later time points when compared to both non-infected control fish and infected fish that survived the infection. They also demonstrate that highly susceptible trout contract early, heavy septicemia infections, which elicit high up-regulation of the transcript of pro-inflammatory cytokines. The transcript levels of a number of central cytokines, chemokines, and cytokine receptors (IL-1β, IL-6, IL-8, IL-10, TNF-α, IL-receptor II) were significantly increased in infected fish (Raida et al. 2011b).

The interactions with rainbow trout head kidney macrophages were investigated by Ryckaert et al. (2010). *In vitro* experiments were performed to measure uptake, intracellular survival, respiratory burst response, and macrophage viability after exposure to *Y. ruckeri*. Despite these toxic substances, *Y. ruckeri* is able to survive *in vitro* inside trout macrophages for at least 24 h. Ryckaert et al. (2010) showed that *Y. ruckeri* bacteria are sequestered in autophagocytic compartments without fusion with primary lysosomes. Bacteria were capable of replicating inside these compartments. Immersion infection of juvenile rainbow trout resulted in steadily increasing

numbers of bacteria in the head kidney over time. As the infection progressed, *Y. ruckeri* shifted from a predominantly extracellular phase during the first week after infection to an intracellular phase inside the host macrophages from day 7 onward (Ryckaert et al. 2010).

Reactive oxygen species (ROS) mediated reactions lead to the formation of protein carbonyl derivatives, which serve as a marker of ROS-mediated protein damage (Stadtman and Berlett 1998). Highly reactive oxygen species that are formed during normal metabolism and under conditions of oxidative stress are able to oxidize proteins or convert lipid and carbohydrate derivatives to compounds that react with functional groups on proteins (Stadtman and Berlett 1998). The response of oxidative stress biomarkers in different tissues of fish is dependent on immune system activation and reactive oxygen species (ROS) generation due to respiratory burst in response to microbe recognition induced by vaccination. Paiva and Bozza (2014) described the mechanisms by which ROS directly kill microbes or interfere with the immune response, and the role of ROS in pathogenic viral, bacterial, and protozoan infections. Phagocytes recognize microbes through many molecular patterns displayed by them and then try to engulf them. Once a microbe is phagocytosed, the nature of the molecules recognized on its surface dictates the treatment enacted within the phagosome. Respiratory burst, a process by which NADPH oxidase generates ROS in response to microbe recognition, is a possible outcome of this process, and it helps to get rid of many microbes (Paiva and Bozza 2014). Once a pathogen is phagocytosed, it must subvert the respiratory burst, withstand its oxidative power, or escape the phagosome to survive (Paiva and Bozza 2014). Microbe recognition sets the immune system in motion, and ROS are produced not only in the phagocyte respiratory burst, but also in other cell compartments, such as the mitochondria, as intermediaries in many signal transduction pathways, such as leukocyte pattern recognition receptor (PRR) signaling. The generation of ROS is a prerequisite to the formation of neutrophil extracellular traps (NETs) and is actively involved in the following: phagolysosomal formation and enzymatic degradation; autophagy; chemoattraction and inflammation; cell death of infection reservoirs; antigenic presentation, T-helper polarization, and lymphocyte proliferation; iron redistribution among tissues; cell compartment availability of iron (Paiva and Bozza 2014). ROS are used by cells of the adaptive immune system as regulators of signal transduction by cell surface receptors (Williams and Kwon 2004). In our study, immunization against *Y. ruckeri* did not induce increases in the oxidative stress biomarkers in trout liver or heart one month after immunization (Figs. 1 and 2).

Conclusion

Vaccination against *Y. ruckeri* results in metabolic plasticity, predominantly in the liver with little effect on oxidative stress or metabolic enzyme activity in the heart. We noted decreases of ketonic derivatives of oxidatively modified protein levels and aminotransferase and LDH activities in the liver induced during the first month after immunization against *Y. ruckeri*, while non-significant changes occurred in the cardiac tissue for oxidative stress biomarkers and aerobic-anaerobic metabolism. Changes in markers of protein oxidation and correlations between carbonyl contents and markers of aerobic-anaerobic metabolism suggest that proteins may contribute to the intermediate supply for the Kreb's cycle in the liver and heart. We did not find any changes in cardiac tissue 4 weeks after immunization unlike in the trout liver following immunization in which there is an decrease in glycolytic enzyme and protein oxidation capacity. This is likely a result of long-term adaptation to immunization. Understanding the role of biochemical changes in the tissues of vaccinated trout has important implications for understanding of the complex physiological changes that occur in immunization but also for improving aquaculture practices to maximize the tissue growth and health of vaccinated trout.

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