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Hydrogen peroxide treatment and its impacts on *Lepeophtheirus salmonis* originating from the Bay of Fundy, Canada

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Abstract

Hydrogen peroxide (H_2O_2) is used to treat sea lice infections of farmed salmonids in the Atlantic and Pacific Oceans and issues with resistance to this treatment, and others are a major threat to the sustainability of the industry. The objectives of this study were to determine how H_2O_2 exposure affects survival and antioxidant-related gene expression in salmon lice (*Lepeophtheirus salmonis*) collected from the Bay of Fundy, New Brunswick. The maximum recommended dose of H_2O_2 is 1,800 mg/L, while the EC_{50} values (with 95% CI) for the population tested were 1,486 (457, 2,515) mg/L for males and 2,126 (984, 3,268) mg/L for females. Neither temperature nor pre-treatment with emamectin benzoate (EMB) impacted survival after H_2O_2 exposure. RT-qPCR was performed on pre-adult sea lice exposed to H_2O_2 and showed that four genes classically involved in the response to oxidative stress were unchanged between treated and control groups. Seven genes were found to be significantly up-regulated in males and one in females. This is the first report on the efficacy and molecular responses of Atlantic Canada sea lice to H_2O_2 treatment.

KEYWORDS

bioassay, gene expression, hydrogen peroxide, sea lice

1 | INTRODUCTION

The salmon louse (*Lepeophtheirus salmonis*) is a parasitic copepod affecting salmonids in both the Atlantic and Pacific Oceans (ssp. *salmonis* and *oncorhynchi*, respectively; [Skern-Mauritzen et al., 2014]). The cost of sea lice control was calculated using production data from 2006 to be approximately $480 million globally (Mustafa et al., 2009), while more recent estimates suggest a large increase in this cost (Abolofia et al., 2017). Sea lice feed on the mucus, skin and blood of their host, causing lesions that disrupt homeostasis and increase susceptibility to secondary infections (Fast et al., 2006; Wagner et al., 2008). Chemical-based therapies are the primary method of controlling *L. salmonis* in aquaculture; however, due to few available treatments and poor management, resistant populations have arisen, making control increasingly difficult.

Hydrogen peroxide (H_2O_2) has been commonly used to treat sea lice infestations in salmon aquaculture over the past two decades in Europe due to its high efficacy and low environmental toxicity (Torrissen et al., 2013). The mechanism of action of H_2O_2 against sea lice is not well known; however, it has been observed to cause a gas bubble within the haemolymph of the louse, potentially leading to detachment from the host. This detachment may only be temporary as one study found that 89% of lice had recovered within 36 hr after exposure to H_2O_2, while another study showed the ability of treated lice to re-infect hosts (Johnson et al., 1993; Treasurer & Grant, 1997).

The efficacy of H_2O_2 against *L. salmonis* is dependent on both treatment time and water temperature (Treasurer & Grant, 1997). After a 20-min exposure to 1,500 mg/L H_2O_2, ~50%–80% of chalimi and 100% of pre-adults/adults were inactive, indicating a stage-dependent response. It has also been found that exposing egg strings to as little as 470 mg/L H_2O_2 for ~36 min can inhibit egg hatching and development of copepodids (Aaen et al., 2014). Nonetheless, this treatment has a narrow therapeutic index, especially at higher temperatures, causing gill irritation, respiratory distress and an increase in plasma cortisol levels of fish (Bowers et al., 2002; Johnson et al., 1993). In Canada, the maximum treatment concentration in the field is 1,800 mg/L H_2O_2 (Department of Fisheries and Oceans Canada, 2013).

Resistance to H_2O_2 was first reported in Scotland in 2000 wherein exposure to 2,500 mg/L H_2O_2 for 23 min resulted in an average reduction in lice numbers of only 67% (Treasurer et al., 2000). More recently, resistant strains of *L. salmonis* were observed in Norway, exhibiting EC_{50} values of ~1,700–2,100 mg/L H_2O_2, which is approximately 8- to 10-fold higher than susceptible strains (Helgesen et al., 2015). A possible mechanism for resistance against H_2O_2 was demonstrated by Helgesen et al. (2017), where resistant populations had higher baseline gene expression and enzyme activity of catalase (*CAT*) compared to susceptible strains. A marker based on this mechanism of resistance has not yet been published.

Stress responses in salmon lice have been characterized at the molecular level in the context of hyposalinity (Sutherland et al., 2012), drug exposure (Poley et al., 2016) and microsporidian infection (Poley et al., 2017). H_2O_2 is commonly used for studies aiming to characterize stress and antioxidant responses as exposure increases reactive oxygen intermediates (ROS). Cells possess a set of enzymes including *CAT*, superoxide dismutase (*SOD*) and glutathione peroxidase (*GPX*), among others, that detoxify ROS. Specifically, superoxide radicals ($\bullet O^{2-}$) are converted to H_2O_2 via *SOD* and then to water and oxygen (O_2) via *CAT* and *GPX*. Cellular responses to H_2O_2 have been examined in another species of sea louse, *Caligus rogercresseyi*, which showed an increase in the expression of glutathione reductase (*GR*) and a decrease in the expression of *SOD* after exposures (Chávez-Mardones et al., 2015; Seo et al., 2006). There is also evidence of a sex-dependent response in this species with males having a higher expression of *SOD*, *CAT* and phospholipid hydroperoxide glutathione peroxidase (*PGPXb)* after exposure compared to females (Chávez-Mardones et al., 2015).

In the current study, we examine the effects of H_2O_2 exposure on the survival and gene expression profiles of *L. salmonis*. The objectives of the current study were to determine (a) the effects of H_2O_2 on lice survival in vivo; (b) the sensitivity of an Atlantic Canada population of *L. salmonis* to H_2O_2; (c) the effect of water temperature on lice responses to H_2O_2 exposure; (d) how exposure to H_2O_2 affects the expression of genes related to stress and detoxification in sea lice; and (e) how pretreatment with another delousing agent, emamectin benzoate (EMB), affects EMB-resistant lice survival post-exposure to H_2O_2.

2 | MATERIALS AND METHODS

2.1 | Lice populations and culture

Atlantic *L. salmonis* F1 copepodids were obtained from the Huntsman Marine Science Centre in St. Andrew's, New Brunswick. The lice were transported in chilled coolers to the AVC where they were immediately stored at 12°C with air stones. After three hours, three 15-mL aliquots were removed for enumeration and assessment of survival, similar to Poley et al. (2016). Adult *L. salmonis* were maintained in similar conditions to the copepodids before use in a bioassay the following day.

2.2 | Salmon infection with lice and H_2O_2 treatment

All experimental protocols for the use of fish followed the Guidelines provided by the Canadian Council on Animal Care (2005; https://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf) and were submitted for review and approval to the UPEI Animal Care Committee (UPEI Animal Care Protocol #10-014).

Atlantic salmon smolts were obtained from the Cooke Aquaculture commercial production facility in Pennfield, New Brunswick, Canada. The fish were transported to the Atlantic Veterinary College aquatic holding facility at the University of Prince Edward Island (UPEI) in Charlottetown, Prince Edward Island, Canada. Fish were held in circular flow-through tanks containing 250 L of 11°C (10.6 – 11.1°C) freshwater for the first 21 days as an acclimatization. Each fish was individually tagged and identified with a passive integrated transponder (PIT) tag (AVID® Inc.) and allowed to recover for 14 days. The system was then switched to saltwater (Instant Ocean®) recirculation over the course of 7 days, resulting in a final salinity of 33 ppt. Fish were held for a further 14 days to acclimatize to the saltwater. During this period, fish were fed a commercial diet at 1% body weight per day, divided over two feeds. Fish were kept on a light: dark cycle of 14:10 hr. At the start of the study, fish weighed 189.5 ± 53.1 g (mean ± *SD*).

Two infection trials were carried out involving copepodid exposures using the same methodology. Inflows were shutoff to all tanks, and water level dropped approximately 5 cm, or just below the outflow level. Copepodids were added to the designated tanks (75 lice/fish) and water flow restored after approximately 2 hr to give sufficient time for attachment. Supplemental oxygen was added to maintain tanks at >8 mg/L. Prior to re-starting the recirculation system, 100 μm mesh bags were placed over inflows to prevent movement of copepodids between tanks. These mesh bags were replaced with 500 μm bags after 7 days to prevent movement of latter life stages between tanks.

2.2.1 | Infection trial 1

Triplicate tanks were randomly assigned 20 fish each (250.0 ± 59.27 g) and were exposed to copepodids as described

above, and lice were allowed to develop to the adult stage over a 65-day period post-infection (dpi). At this point, all fish were individually anaesthetized (100 mg/L MS-222) and lice enumerated on the body surface of each fish before being recovered and then returned to their home tank. Six days later, 10 fish from each tank were randomly selected to receive an H_2O_2 bath, and the remaining 10 to go through a sham bath. In short, fish were removed from their home tank, PIT tag read and placed into a treatment tote. When treatment totes reached five fish (i.e. 4 treatment totes per tank), treatment was administered (Interox Paramove 50®) at a concentration of 1,500 mg/L H_2O_2 for two totes; equal volume of SW for two sham totes; for a period of 20 min) and temperature monitored to ensure it never exceeded 13°C. After the 20-min treatment, fish were separated in two tanks (treatment and sham; $n = 3$ per condition) to prevent reassortment between treatment groups. Twenty-four hours after the treatment, all fish were killed (250 mg/L MS-222) and lice enumerated for individual fish.

2.2.2 | Infection trial 2

Triplicate tanks were set up as above; however in this case, fish underwent H_2O_2 or sham treatment 6 hr prior to infection with copepodids. Ten fish were randomly selected from each tank and given a H_2O_2 bath and the remaining 10 fish given a sham bath, as above. Following the 20-min bath, fish were replaced in their home tank and then exposed to *L. salmonis* copepodids (75 lice/fish) 6 hr later using the exposure method above. Two weeks after lice exposure (i.e., prior to moulting to mobile life stages), fish were killed (250 mg/L MS-222) and lice counted on all body surfaces of each fish.

2.3 | Bioassays

For all bioassays, H_2O_2 treatments were made by diluting 50% H_2O_2 (Interox Paramove 50®) in sea water, unless stated otherwise. Sea water was maintained at 11°C with a salinity of 32 ± 2 parts per thousand (ppt). Males and females were assessed for survival before being collected using criteria presented by Igboeli et al. (2014), with the exception of live and weak lice being considered healthy (i.e. mobile), while moribund and dead lice were considered dead (i.e. immobile). Triplicate Petri dishes were used for all adult treatments, whereas copepodid larvae exposures were done with six replicates per treatment.

Bioassays in plastic Petri dishes ($n = 6$) were used to expose *L. salmonis* to H_2O_2 (Poley et al., 2016). The first bioassay (Bioassay I-larval exposure) exposed copepodid larvae to 2,000 mg/L H_2O_2 in sea water for 20 min followed by a seawater rinse and 6-hr holding period in fresh sea water. A control group exposed only to sea water was run alongside the treatment group, including the rinse and holding phases. Each sample contained approximately 500 individuals. The same survival and enumeration assessments described above (in section 2.1) were completed 6 hr after H_2O_2 exposure, except three 5-mL aliquots were used for each sample ($n = 6$).

A second bioassay (Bioassay II-pre-adult exposure for molecular analysis) was completed with male and female pre-adult I/II *L. salmonis* and included 0 mg/L, 2,000 mg/L or 10,000 mg/L H_2O_2. This assay was carried out in triplicate dishes for each sex and treatment combination (for a total of 30–35 males and 32–38 females per treatment). Upon completion of this bioassay, lice were collected separately by treatment and sex and stored at −80°C until molecular analysis was performed.

Lice being used in a third bioassay (Bioassay III-hydrogen peroxide dilution series) were collected from fish once the female lice reached pre-adult II/ early adult stages (i.e. males were all adults). They were then removed from the fish and left in sea water overnight. The following day, a bioassay was performed using triplicate dishes for each sex in each of six treatment groups (0, 200, 1,000, 2,000, 5,000, 10,000 mg/L). Lice were exposed to one of six concentrations ($n = 14$–24 males and 10–25 females per group) of H_2O_2 for 20 min and then given a 6-hr recovery period in sea water. Half maximal effective concentrations were calculated for each sex separately as described by Pacheco et al. (2013).

A fourth bioassay (Bioassay IV-interaction of temperature and treatment) was used to determine the interaction of temperature and H_2O_2 treatment on lice survival. Lice were separated by sex into a control (0 mg/L) or treated (2,000 mg/L) group ($n = 12$–19 males and 24–31 females per treatment) and triplicate Petri dishes per treatment group maintained for a 20-min exposure followed by a rinse and a 24-hr recovery period in sea water. Throughout the entirety of the bioassay, lice were kept at either 5 ± 1°C or 10 ± 1°C. The lice were then assessed for survival, collected and stored at −80°C for further analysis.

Adult lice were collected and allowed to rest in sea water for 24 hr prior to being used in a 39-hr bioassay (Bioassay V-double drug treatment). On the first day, lice were separated into Petri dishes containing either sea water or emamectin benzoate (EMB) at 200 parts per billion (ppb). The Petri dishes were then kept in an incubator at 11°C for 24 hr, after which dead/immobile lice from both groups were removed. The surviving lice were then exposed to one of three treatments: sea water, 200 ppb EMB or 2,000 mg/L H_2O_2. Lice in the sea water and EMB groups were kept at 11°C for 15 hr, whereas the group exposed to H_2O_2 underwent a 20-min exposure to the treatment followed by a 14-hr 40-min recovery period in sea water at 11°C. At the end of the bioassay, survival was assessed. For the second part of this experiment, groups were named in an "x/y" format. In this case, "x" denotes the exposure in which the lice survived the initial 24 hr, while "y" denotes what treatment was used for the second exposure. For example, the "Control/EMB" group spent 24 hr exposed to sea water after which the survivors were exposed to EMB for 15 hr. Triplicate Petri dishes were used for all treatment groups. Between 47–64 males and 51–64 females per group were used in the first exposure, while 9–19 males and 11–20 females per group were used in the second exposure.

2.4 | RNA isolation

Total RNA was isolated from pools of 500 copepodid lice in Bioassay I and pools of two lice in Bioassay II. For Bioassay IV, frozen lice were individually homogenized using 5 mm stainless-steel beads and a TissueLyser (Qiagen). RNA was isolated from lice using TRI-Reagent (Thermo Fisher Scientific; Chomczynski & Mackey, 1995; Chomczynski, 1993) following the manufacturer's instructions with modifications for lice from Bioassay IV. Specifically, following the organic phase extraction, the supernatant was removed, and RNA was then purified using RNeasy MinElute columns (Qiagen) with an on-column DNase I digestion to remove genomic DNA as per the manufacturers' instruction. RNA quantity and purity were analysed using spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific), and samples from Bioassays I and II were treated with Ambion® Turbo DNA-*free*™ DNase using a 1:1 ratio of buffer to DNase enzyme for copepodids and a 3:1 buffer to DNase ratio for pre-adults. Bio-Rad's RNA StdSens Analysis Kit was then used to assess RNA integrity of samples from Bioassays I and II using Experion automated gel electrophoresis as per the manufacturer's instructions. All samples were resuspended in nuclease-free water and stored at −80°C until further use.

2.5 | RT-qPCR

Synthesis of cDNA was performed with 0.5–1 μg of total RNA in 20 μl reactions using iScript™ Reverse Transcription Supermix kit (Bio-Rad) or a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per the manufacturer's instructions. The same cDNA synthesis kit was used for all samples within an experiment. Reactions without RT enzyme were included and confirmed no genomic contamination. To generate a standard curve, equimolar concentrations of RNA from H_2O_2 exposed and control lice were pooled, and PCR products generated using GoTaq Green PCR Mastermix (Promega) for Bioassays I and II. Transcript-specific standard curves (6-point, 5-fold series dilution) were designed to confirm primer efficiency (90%–110%). RT-qPCR amplification was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) in 11 μl reactions with 1 μl template and 0.1 μM of each primer using the following thermal regime: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 15 s. Melt curve analysis was then performed by increasing the temperature from 65°C to 95°C in 0.5°C increments every 5 s. Melt curves confirmed single product formation for all transcripts assayed. All RT-qPCRs were completed using the Bio-Rad CFX Connect™ for standard curves or Bio-Rad 384 Connect™ (Hercules) for individual samples. RT-qPCR data normalization was completed using qbase-PLUS (Biogazelle) with an output of $\log_2$ ratios relative to two reference genes, *elongation factor 1α* (*ef1α*) and *vinculin* for Bioassays I and II, and *ef1α* and *ribosomal protein s20* (*rps20*) for Bioassay IV. Different pairs of reference genes were selected based on their stability within an experiment using geNorm (Vandesompele et al., 2002) which showed collective M values of 1.630 and 0.211 with coefficients of variation (CV) of 0.586 and 0.073 for Bioassays II and IV, respectively. Due to insufficient amounts of RNA, gene expression analysis could not be performed on lice from Bioassay I.

2.6 | Statistical analysis

R Studio (v 1.0.153) was used to perform a principal component analysis (PCA) and general linear models to compare gene expression between treatment groups, temperatures, and sexes of *L. salmonis* with a p-value <.05 considered statistically significant. From the survival results of the EC_{50} bioassay, R Studio was also used to determine the concentration killing 50% of the individuals from this population. R scripts used for data analyses can be found in Appendix S1.

3 | RESULTS

3.1 | H_2O_2 bath treatment impact on lice

3.1.1 | Infection trial 1

At 65 dpi, the mean infection level was 2.32 ± 0.301 lice/fish, with 57% of the population consisting of adult males and 43% adult females. Prevalence of infected fish in the study population was 74.1%. Hydrogen peroxide baths were carried out on post-smolt salmon to mimic the time and concentrations used in field applications of Interox Paramove 50®. Post-treatment reductions were assessed by subtracting final counts from pretreatment counts on an individual basis. Six sham fish out of 30 exhibited increased counts (one louse in each case) at the post-treatment count, whereas only one H_2O_2 fish exhibited an increase. Under macroscopic/microscopic examination, some lice having undergone an H_2O_2 treatment were rendered immobile, while others were observed to be floating on the water surface with gas bubbles in the haemolymph (Figure 1). Lice counts were significantly lower in H_2O_2-treated fish compared to sham-treated fish ($p = .03$; Figure 2), resulting in a total of 74% less lice after H_2O_2 treatment (85% of fish had lower counts post-bath) compared to 31% less when undergoing the sham (45% of fish had lower counts post-bath). All lice remaining attached to H_2O_2-treated fish were male ($n = 16$), whereas 59% (23/39) of remaining lice on sham fish were male. In this case, H_2O_2 treatment was 100% effective against females and 67% effective against males.

3.1.2 | Infection trial 2

To determine whether H_2O_2 bath treatment impacted subsequent settlement of *L. salmonis* copepodids, a second infection trial was conducted similar to the first, but in this case, fish were randomly selected for administration of an H_2O_2 or sham bath, 6 hr prior to copepodid exposure. Infection intensity was assessed 14 days later demonstrating an average of 17.9 ± 1.11 and 18.2 ± 1.11 lice/fish

FIGURE 1 Adult female *Lepeophtheirus salmonis* following a 20-min exposure to 2,000 mg/L H_2O_2 and 6-hr recovery in sea water. Note presence of gas emboli in anterior portion of Cephalothorax and genital complex

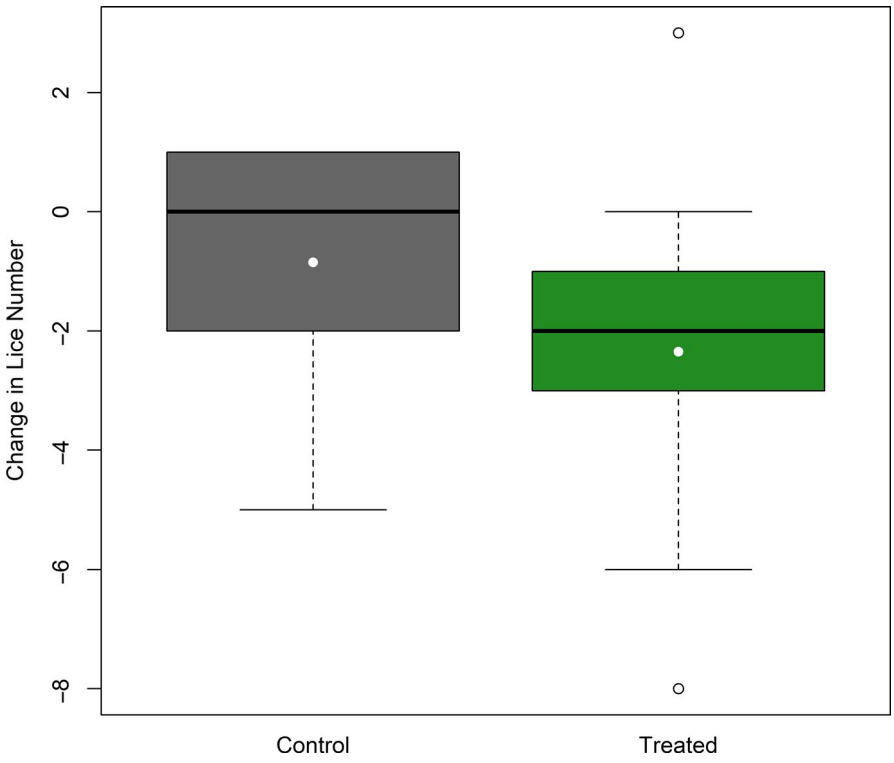

FIGURE 2 Change in post-treatment adult *Lepeophtheirus salmonis* numbers relative to pretreatment numbers in fish having been exposed to a sham bath (Control) or 2,000 mg/L H_2O_2 (Treated). Significant difference between groups following one-way ANOVA ($p = .03$). Data are presented in box-whisker plots where the bottom whisker is the lowest number in the data set and the top whisker is the highest number in the data set; the box denotes the Q1 and Q3 of the data, the median is the line within the box, and circles represent data point outliers

on sham- and H_2O_2-treated fish, respectively. While there were no differences between groups in overall lice infection ($p = .9$), the gills were impacted differently, where significantly more lice were found on the gills of H_2O_2-treated fish compared to sham-treated fish ($p = .004$; Figure 3).

3.2 | Lice tolerance to H_2O_2

Copepodid larvae from Bioassay I (larval exposure) exhibited high levels of survival post-exposure to the recommended treatment concentration of H_2O_2 (2,000 mg/L), with 40.4% surviving 6 hr post-exposure. Pre-adult males (Bioassay II-pre-adult exposure for molecular analysis) showed survival rates of 100% ± 0%, 97.1% ± 5.2% and 0% ± 0% after exposure to 0, 2,000 and 10,000 mg/L, respectively. When exposed to the same concentrations, pre-adult females showed survival rates of 100% ± 0%, 57.6% ± 6.9% and 0% ± 0%. Therefore, males showed an approximately 40% higher survival post-exposure to 2,000 mg/L H_2O_2 compared to females exposed to the same concentration.

Conversely, female lice from Bioassay III (hydrogen peroxide dilution series) showed greater tolerance to H_2O_2 than male lice with an

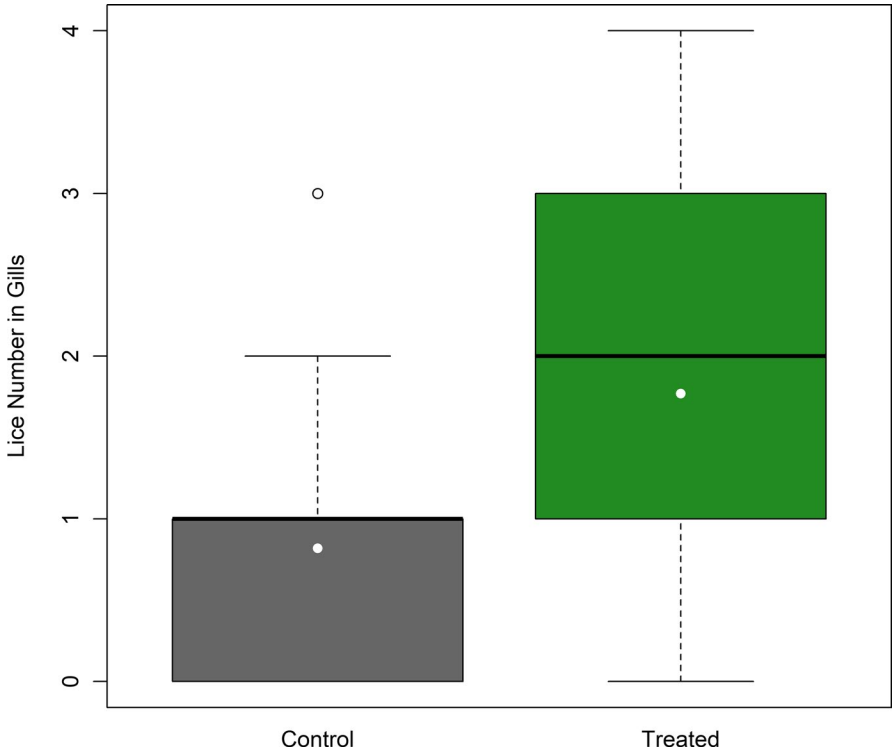

FIGURE 3 Number of *Lepeophtheirus salmonis* copepodids located in the gills of fish having been exposed to a sham bath (Control) or 2,000 mg/L H_2O_2 (Treatment) 6 hr before sea lice infection. Significant difference between groups following one-way ANOVA ($p = .004$). Data are presented in box-whisker plots where the bottom whisker is the lowest number in the data set and the top whisker is the highest number in the data set; the box denotes the Q1 and Q3 of the data, the median is the line within the box, and circles represent data point outliers

TABLE 1 Mean survival (%) ± *SEM* of *Lepeophtheirus salmonis* exposed to either a control (sea water) bath or 2,000 mg/L H_2O_2

Temperature	Treatment	Sex	Survival (%)	Sample size
5°C	Control	Male	76.9 ± 3.7	13
		Female	81.5 ± 1.6	27
	2,000 mg/L	Male	50 ± 3.5	12
		Female	84.6 ± 0.33	26
10°C	Control	Male	89.5 ± 3.6	19
		Female	93.5 ± 0.2	31
	2,000 mg/L	Male	37.5 ± 1.7	16
		Female	79.2 ± 1.7	24

EC_{50} value (95% confidence interval) of 2,126 mg/L (984, 3,268) H_2O_2 for females compared to 1,486 mg/L (457, 2,515) H_2O_2 for males.

There was no difference in survival between sexes (chi-square; $p > .05$) when exposed to H_2O_2 at different temperatures (Bioassay IV-interaction of temperature and treatment), so samples were grouped together. There was only a slight effect of temperature on treatment efficacy with survival at 5°C being 73% among lice treated with 2,000 mg/L H_2O_2, while survival at 10°C with the same concentration yielded a 67% survival (chi-square; $p = .4$). Control groups for both temperatures had survival rates of at least 80% (Table 1).

After 24 hr, lice exposed to EMB (Bioassay V-double drug exposure) showed significantly higher survival (χ^2; $p = .047$) than their control (sea water) counterparts (78.6% and 65.6%, respectively). After the second (15 hr) exposure, the group exposed to EMB again showed higher survival than the control group (86.2% and 70%, respectively), but this was not significant (χ^2; $p = .3$). After exposure to H_2O_2, lice from the control group showed only slightly higher survival than those from the EMB-treated group (14.3% and 10.3%, respectively). Again, this difference was not significant (χ^2; $p = .9$).

3.3 | Gene expression responses to H_2O_2

Pre-adult *L. salmonis* exposed to 2,000 mg/L H_2O_2 (Bioassay II-pre-adult exposure for molecular analysis) showed significantly higher expression of *LsPGPXb* than those exposed to 10,000 mg/L H_2O_2 (two-way ANOVA; $p < .05$; Figure 4), although no difference in expression was observed between treated and control lice (two-way ANOVA; $p > .05$). Males showed significantly higher expression of *LsGR* than females (two-way ANOVA; $p < .001$; Figure 4), while no significant differences in the expression of *LsMnSOD* or *LsCat* were observed (Figure 4).

PCA separated male lice from female lice on a combination of the first and second principal components (PC), contributing 60.2% (*x*-axis) and 12.1% (*y*-axis) of the variation, respectively (Figure 5). The expression of *LsCat* depended on both temperature and sex (interaction effect; $p = .03$), where exposure to H_2O_2 at 10°C resulted in significantly higher expression in males compared to females (Bioassay IV-interaction of temperature and treatment). Temperature had a significant effect on the expression of *LsCathL* ($p = .02$) and *LsPGPXb* ($p = .02$), with both being more highly expressed at 5°C compared to lice maintained at 10°C, regardless of sex or treatment (i.e. main effect). Finally, eight genes including *LsGST-1D*, *LsMnSOD*, *LsCathL*, *LsHSP-ß1*, *LsPGPXb*, *LsGPRX* and *LsGR* were all more highly expressed in males compared to females (all $p < .01$; Figure 6a–g), while *LsPXD4* was more highly expressed in females compared to males ($p < .01$; Figure 6h).

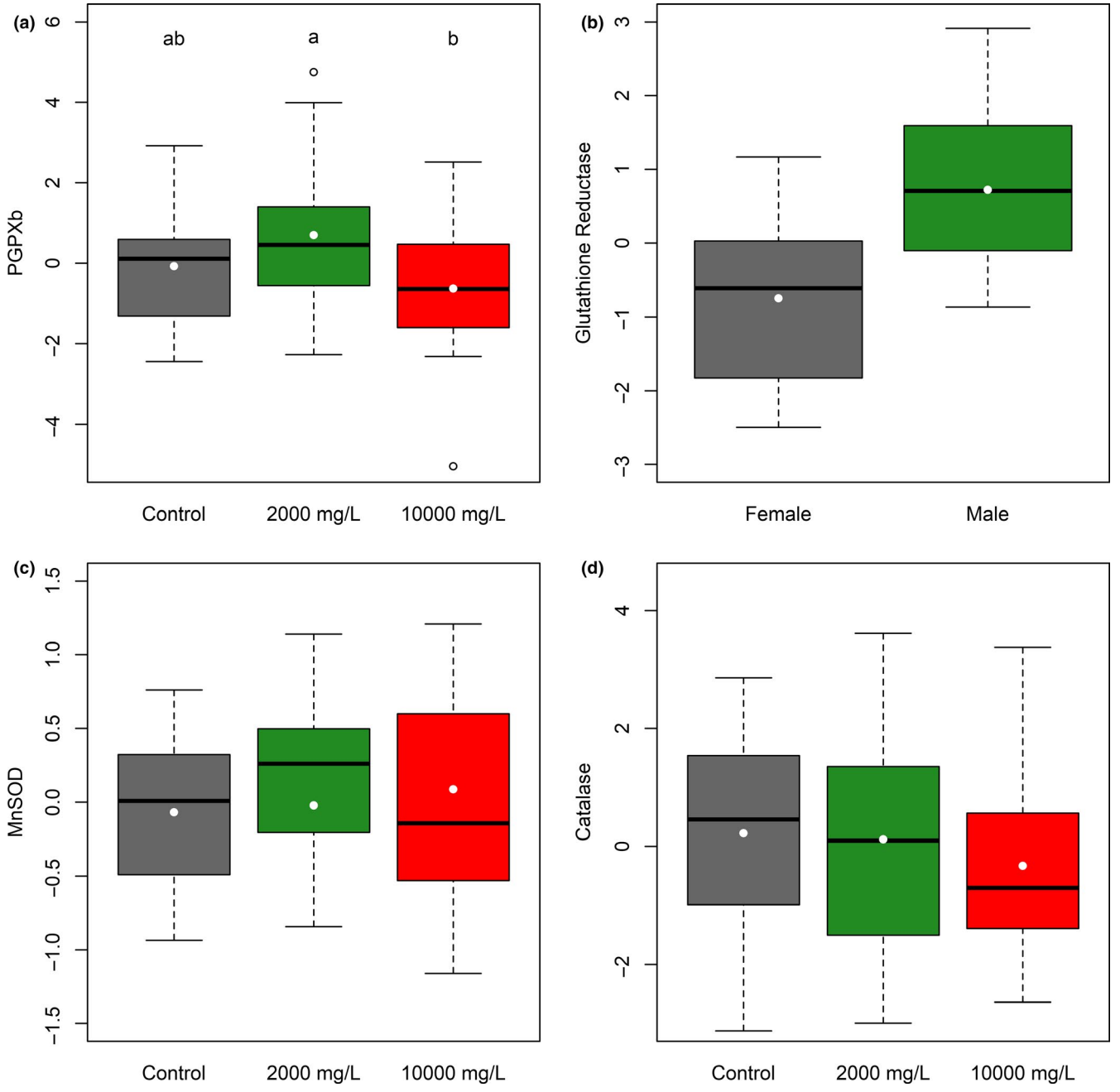

FIGURE 4 Antioxidant gene expression of pre-adult *Lepeophtheirus salmonis* following a 20-min exposure to 0, 2,000 or 10,000 mg/L H_2O_2 and 6-hr recovery in sea water. Data are represented as $\log_2$ calibrated normalized relative quantities (CNRQ) using *ef1α* and *vinculin* as reference genes. Primer sequences can be found in Appendix S2. Significant difference between groups following two-way ANOVA ($p < .05$) denoted by different letters. Data are presented in box-whisker plots where the bottom whisker is the lowest number in the data set and the top whisker is the highest number in the data set; the box denotes the Q1 and Q3 of the data, the median is the line within the box, and circles represent data point outliers

4 | DISCUSSION

Hydrogen peroxide is a chemotherapeutant commonly used to treat sea lice infections of farmed fish, with resistance to the treatment becoming an increasingly common occurrence (Bruno & Raynard, 1994; Helgesen et al., 2015). In this study, we show that *L. salmonis* tolerance to H_2O_2, from the Bay of Fundy, Canada, is similar to that of resistant strains in Norway (Helgesen et al., 2015) and that tolerance may be dependent on both life stage and sex.

4.1 | Lice tolerance to H_2O_2

A common measure to determine sensitivity of a population to a toxicant is through the generation of EC_{50} values. In this case, we

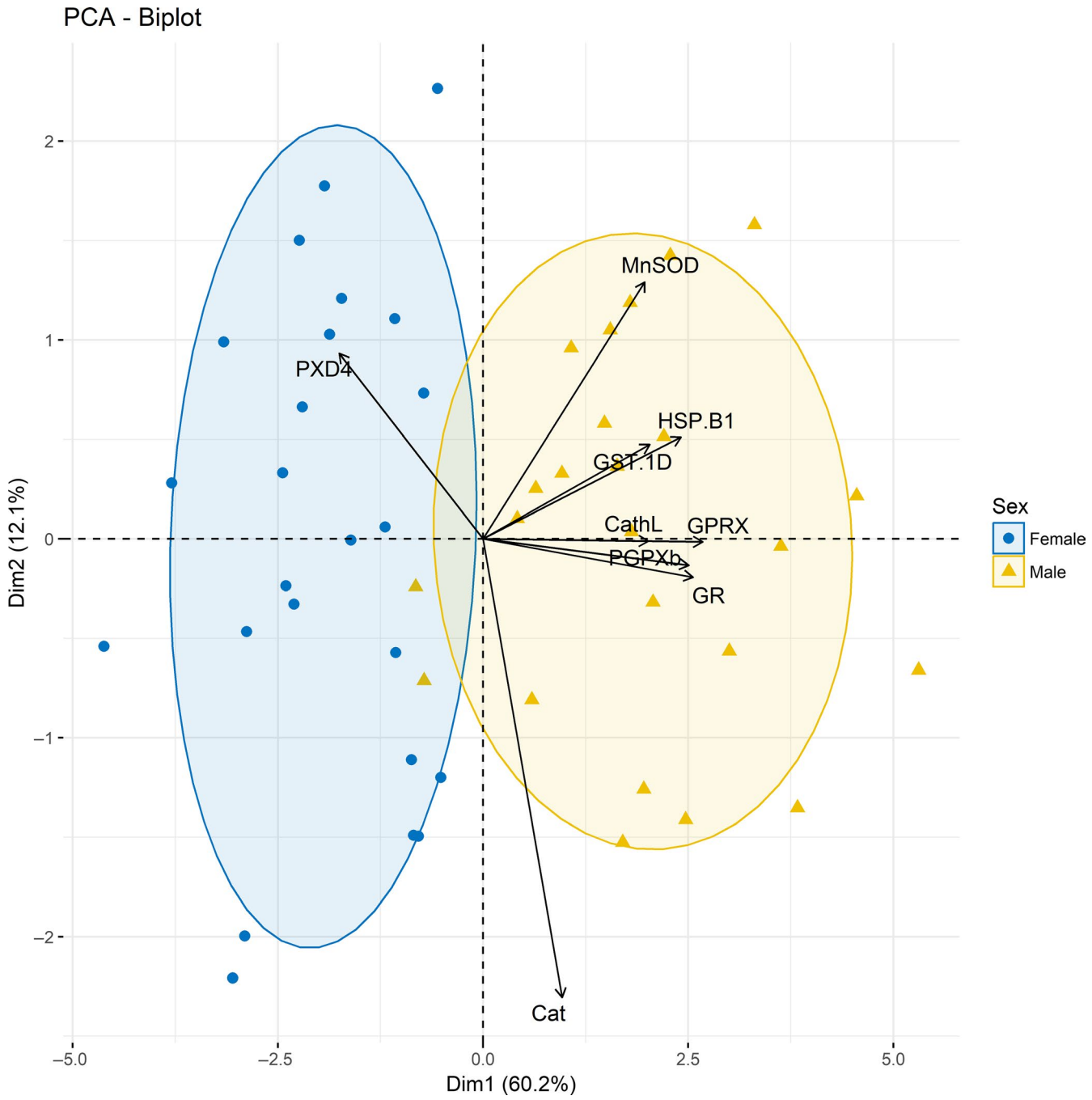

FIGURE 5 Principal component analysis (PCA) of male (triangles) and female (squares) *Lepeophtheirus salmonis* samples ($n = 48$). These samples are separated from each other on the *x*-axis (PC1; 60.2%) and the *y*-axis (PC2; 12.1%). The remaining principal components represent unexplained variation in the data (i.e. biological replicates from both groups span this axis, water temperature or H_2O_2 exposure)

wanted to determine the concentration of H_2O_2 required to immobilize or kill 50% of individuals in a sample of *L. salmonis* from the Bay of Fundy in New Brunswick. In this study, the EC_{50} value for males was found to be 1,486 mg/L H_2O_2, which was lower than that for females, which had an EC_{50} value of 2,126 mg/L H_2O_2. It has been shown previously that drug tolerance differs between sexes of lice, where EC_{50} values are higher in females having been exposed to H_2O_2 or deltamethrin, while males have higher EC_{50} values post-EMB exposure (Igboeli et al., 2014; Jones et al., 2013; Treasurer et al., 2000; Whyte et al., 2014). According to Fisheries and Oceans Canada (2013), the prescribed dose of H_2O_2 is up to 1,800 mg/L, or 0.18%. This means that in the sample of sea lice tested from the Bay of Fundy, just over half of the males exposed to this concentration, and just below half of the females exposed would be killed. These data contrast with two other unpublished bioassay results of bioassays showing the opposite effect (Pers. Obs.). In these assays, males showed higher EC_{50} values (1,890 and 3,362 mg/L) than females (1,251 and 1,093 mg/L). Despite the

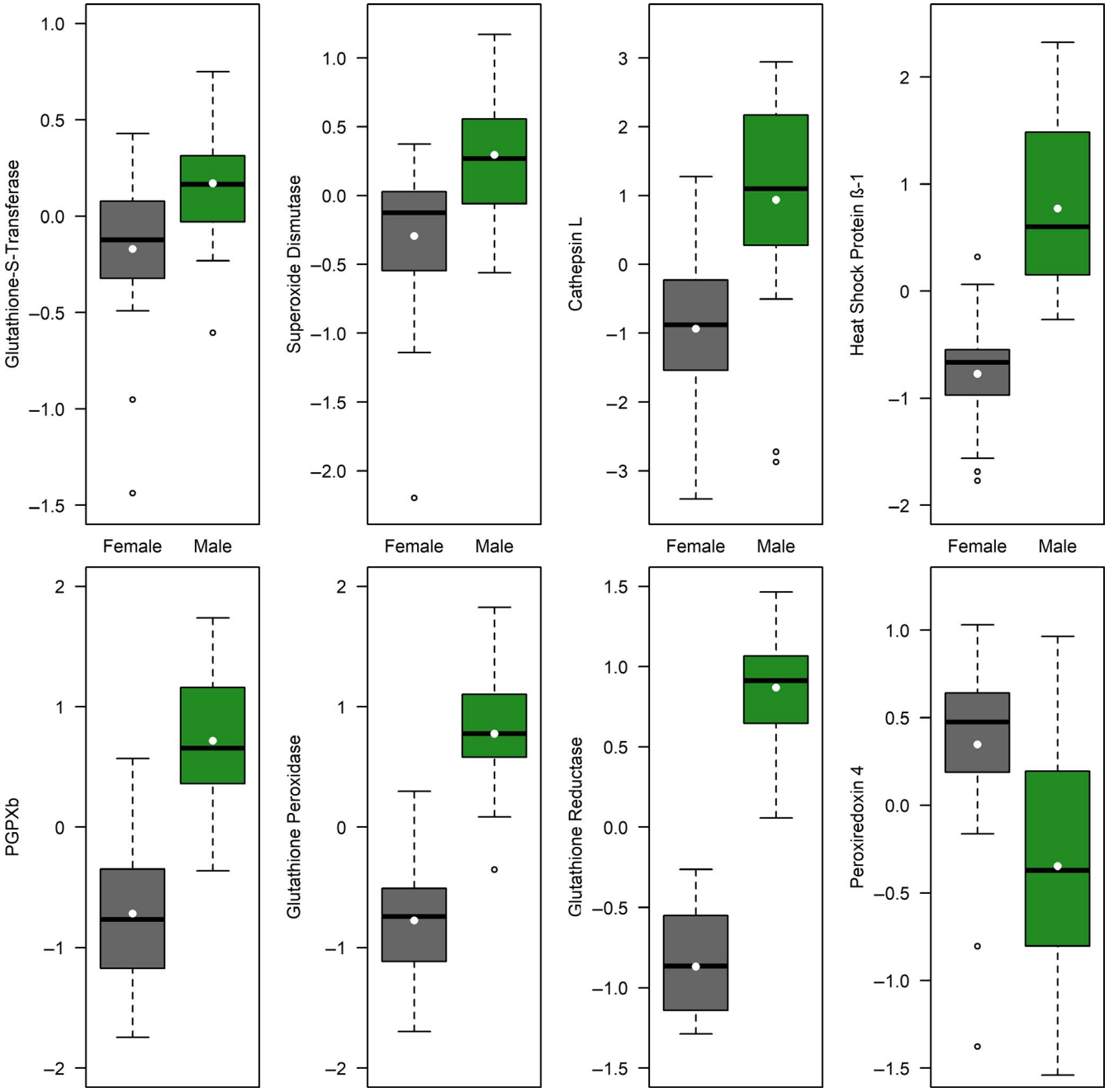

FIGURE 6 Antioxidant/stress gene expression of adult male and female *Lepeophtheirus salmonis* demonstrating increased expression of most genes in males and one in females. Data are represented as $\log_2$ calibrated normalized relative quantities (CNRQ) using *ef1α* and *RPS20* as reference genes. Primer sequences can be found in Appendix S1. Significant difference between sex was observed and represented in each graph following two-way ANOVA ($p < .01$). Data are presented in box-whisker plots where the bottom whisker is the lowest number in the data set and the top whisker is the highest number in the data set; the box denotes the Q1 and Q3 of the data, the median is the line within the box, and circles represent data point outliers

potential for contrasting differences in H_2O_2 sensitivity between sexes, bioassay results using lice from Atlantic Canada indicate a reduced sensitivity to the drug compared to what would be expected in naïve populations. EC_{50} calculations were performed in Norway by Helgesen et al. (2015), who found that resistant populations had EC_{50} values of 1,767–2,127 mg/L while populations having no prior exposure to the drug had an EC_{50} value of 216 mg/L. It should also be noted that these values are for a 30-min bioassay and that a 24-hr bioassay resulted in much lower EC_{50} values. In Scotland, Treasurer et al. (2000) found that when exposed to 2,000 mg/L H_2O_2, lice on farms having used the anti-parasitic many times in the past had only a 25% mortality while those having no prior exposure showed 97%–99% mortalities. Using the results of these studies for indirect comparisons to the data presented here, it is likely that H_2O_2 resistance will continue to become an increasingly severe problem in the Bay of Fundy.

Field data obtained by Gautam et al. (2017) suggest that treatment with H_2O_2 is more efficacious against adult females compared to pre-adult/adult males. Under laboratory conditions used in our study, treatment with 1,500 mg/L H_2O_2 was 100% and 67% efficacious against female and male lice, respectively. For the sham bath, lice removal was 39% and 26% for females and males, respectively. This means that in both groups, removal of male lice was 0.67 that of females, suggesting that while H_2O_2 significantly reduced lice numbers on the fish, it had no bias towards males or females. This may be similar to conditions on the farm where crowding of the fish within the pen/tank or holding of the fish during pretreatment may contribute to efficacy differences observed both in the field and in the laboratory using pre- and post-count methods; especially for bath administered pesticides. Finally, another reason behind this discrepancy may have to do with the ability of male lice to reattach after being dislodged, as males were observed to show up on previously uninfected fish and/or occur at higher numbers during the post-treatment counts compared to pretreatment counts.

While it was not the case in the current study, H_2O_2 efficacy has been shown to be temperature-dependent when attached to a host (Thomassen, 1993). There are also several other chemotherapeutic drugs that are temperature-dependent, such as the organophosphate trichlorfon, which increases in efficacy with increased temperature (Howe et al., 1994). This study only examined survival at two temperatures; however, a wider range of temperatures may be needed to determine any trends in H_2O_2 efficacy.

Despite the presence of tolerance differences between sexes, we show that both copepodids and more mature mobile stages of *L. salmonis* are more tolerant to the drug compared to naïve populations (Helgesen et al., 2015). This poses a greater challenge for salmon farms in the Bay of Fundy since resistance to other drugs, such as EMB, has already been established (Chang et al., 2011). One aspect of sea lice control involves the use of multiple drug treatments to avoid the development of resistance to a single treatment. In the field, it is not uncommon for lice to be treated with H_2O_2 following the use of another chemotherapeutant, which is why one of the goals of this study was to determine the efficacy of an H_2O_2 treatment following exposure to EMB. The results of this experiment showed that the survival rate after exposure to EMB was 78.6%. This high rate of survival is likely because lice in the Bay of Fundy are largely resistant to the effects of EMB (Chang et al., 2011). A concentration of 200 ppb EMB was chosen because it is well below the most recent EC_{50} calculations for lice in the Bay of Fundy (Igboeli et al., 2014). This way, it would provide a challenge to this population of lice, while being a lethal concentration in lice previously unexposed to the drug (Westcott et al., 2008). The development of resistance is one of the reasons that integrated pest management programmes need to be implemented in areas where sea lice are an issue. Upon exposure to H_2O_2, lice that were not exposed to EMB first showed only 14% survival while those that were first exposed to EMB had a survival rate of 10%. It should also be noted that the control group had only 65% survival after the first 24 hr in sea water and 70% survival after the second 15 hr in sea water. Prior work in bioassay development suggests a cut-off at 80% survival in controls (Whyte et al., 2014), which means that this assay failed. However, this standard is set for single drug assays, and while there has been some work done in two drug assays, it is a new method of assessing interactions between multiple drugs. Single drug assays are also done over a period of 24 hr, whereas two drug assays take much longer. Because of this, it may be unreasonable to always have the same expectations for survival in a double drug assay compared to their single drug counterparts. Assuming an 80% survival per day, a cut-off of 65%–70% survival in the controls may be more reasonable in cases where a bioassay takes multiple days.

As it has been previously mentioned, lice may be able to recover from H_2O_2 treatment (Johnson et al., 1993; Treasurer & Grant, 1997). Because of this, it is difficult to determine the optimal recovery period before assessing lice survival. For example, some studies have examined survival immediately after exposure, while others waited upwards of 24 hr (Bravo et al., 2010; Johnson et al., 1993). In this study, we chose to assess survival after a six-hour recovery period, since it is unlikely that lice being detached from a host for this amount of time would be able to re-infect a fish. Another factor making bioassay results difficult to interpret is that the length of drug exposure may differ. H_2O_2 bioassays have been completed using exposure times upward of 24 hr, while others, such as this one, used only a 20-min exposure (Helgesen et al., 2015). In terms of applying this to field treatments, both of these factors may play a role; however, it is also possible that, due to fish crowding or other factors, lice may be removed regardless of whether a chemotherapeutant is being used or not as is shown in our in vivo trial. It is also important to note that while increasing the exposure time means that a lower dose of the drug is required to achieve a greater efficacy, it is also a major source of stress for the fish (Bowers et al., 2002; Helgesen et al., 2015). It is for these reasons that bioassay protocols (both laboratory and field) should be more standardized to mimic what is, and should be, used in field treatments.

4.2 | Gene expression

One objective of the current study was to determine the gene expression responses to H_2O_2 exposure in the salmon louse *L. salmonis*. With H_2O_2 being a reactive oxygen species, the targets for this study were directed at antioxidant enzymes, as they are primarily responsible for the detoxification of reactive oxygen species (Ježek & Hlavatá, 2005). Glutathione reductase (*LsGR*), a mitochondrial enzyme responsible for the conversion of glutathione disulphide to glutathione, has been used as an indicator of oxidative stress in cells in vivo (Lüersen et al., 2013). It should be noted that in Bioassay II, higher expression of *LsGR* in males was associated with an approximately 40% increase in survival compared to females when lice were exposed to 2,000 mg/L H_2O_2.

Exposure to 10,000 mg/L H_2O_2 resulted in significant down-regulation of *LsPGPXb* compared to exposure to 2,000 mg/L H_2O_2, though neither treatment group showed differential expression

against controls. There are a limited number of studies examining the transcriptomic expression of this gene; however, a study by Chávez-Mardones et al. (2015) showed that the expression of two *PGPXb* variants in adult *Caligus rogercressyi* was increased following exposure to H_2O_2. It is possible that since exposure to 10,000 mg/L H_2O_2 resulted in 100% mortality of *L. salmonis*, that expression was compromised by factors involved in an inability to maintain homeostasis and eventual cell death.

A recent study done by Helgesen et al. (2017) elucidated a possible mechanism for H_2O_2 resistance in *L. salmonis*. The study examined the expression and enzymatic activity of catalase between two different populations of lice from Norway: one sensitive to the drug and one resistant to it. The results showed that in all stages tested (pre-adult I, pre-adult II and adult), the resistant population showed significantly higher expression and enzyme activity of catalase. Because of this, it is expected that exposure to the drug should result in increased expression of the enzyme; this, however, was not the case. In fact, there were no changes in catalase expression between treatment groups or sexes. This may indicate that while baseline overexpression of the enzyme is associated with resistance, exposure to H_2O_2 does not induce further expression of this gene. Due to the historic use of hydrogen peroxide in the Bay of Fundy (ACFFA, 2019), and the panmictic population structure of the *L. salmonis* in the North Atlantic (Besnier et al., 2014), it is expected that this population would have been sufficiently exposed to the drug for selection pressure to favour the development of an increase in baseline catalase to have occured.

The final result of this study was that sex had an impact on the expression of all nine genes examined in sea lice from Biossay IV (interaction of temperature and treatment). There was no interaction effect of temperature and treatment on any of the genes tested; however, *LsCat* was found to be significantly higher in males than in females when held at 10°C but not when held at 5°C. Differences in lice tolerance to H_2O_2 have been observed in other species, while among *L. salmonis*, the expression of catalase has been shown to be dependent on both sex and life stage (Chávez-Mardones et al., 2015; Farlora et al., 2017; Helgesen et al., 2017). Although sex differences were observed in the present study, they were inconsistent, suggesting that stage, population, intramoult variation, population or other potentially confounding variables may play a role in sea lice tolerance to H_2O_2. Nonetheless, higher tolerance in males could be linked to higher expression of *LsGST-1D*, *LsMnSOD*, *LsCathL*, *LsHSP-ß1*, *LsPGPXb*, *LsGPRX* and *LsGR*.

5 | CONCLUSIONS

The results of this study tell us a number of things: (a) H_2O_2 treatment causes a significant decrease in lice abundance on fish in vivo; (b) due to the high EC_{50} values, and survivorship under in vivo exposure, *L. salmonis* in the Bay of Fundy has already experienced significant selection pressure towards tolerance of H_2O_2 treatments; however, more bioassays on this population will be needed to determine the variability throughout the population and whether seasonality has a role in survival during exposure; (c) the lack of an effect of temperature on survival means temperature is either not a factor in the survival of lice exposed to H_2O_2 or that temperature only becomes a factor above 10°C; (d) H_2O_2 does not induce a change in gene expression of these four genes (*LsCat*, *LsMnSOD*, *LsGR and LsPGPXb*) compared to the controls 6 hr after exposure; (e) it can be concluded that due to widespread resistance in the Bay of Fundy, pretreatment with EMB did not increase mortalities compared to exposure to H_2O_2 alone; and (f) there are inherent differences present in the expression of some genes related to stress and antioxidation between males and females.

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CONFLICT OF INTEREST

The authors report no conflict of interest in production of this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.s

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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