SARF SP 001

Literature review

To ascertain the state of knowledge on how hydrogen peroxide affects different life stages of organisms such as *Lepeophtheirus salmonis*: furthermore to review information on the likely target structures and physiological processes

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1. Introduction

Aim of this review

The purpose of this literature review, as set out by Scottish Aquaculture Research Forum (SARF), is to ascertain the state of knowledge on how hydrogen peroxide (H_2O_2) affects different life stages of *Lepeophtheirus salmonis* and similar organisms; also what are the physiological processes and/or structures targeted by this chemical as a chemotherapeutant for sea lice infestation. It is implicit that the remit is broader than just *L. salmonis* as there is little direct information currently available for sea lice and there may be benefit in drawing parallels with findings for other organisms. The overarching goal is to understand the effects of H_2O_2 on sea lice as a chemotherapeutant for their control so that treatment strategies can be refined and improved.

Key questions

(1) Can the literature explain the mode of action of H_2O_2 as a sea lice treatment, especially for *Lepeophtheirus* and *Caligus* genera, in terms of target physiological process and/or structures?
(2) Can the literature explain the mode of action of H_2O_2 on sea lice in relation to other organisms, especially other crustacea, and arthropods related in evolutionary terms to crustaceans?
(3) Can the literature on H_2O_2 occurrences in nature provide an indication of processes in sea lice which may involve H_2O_2 and thus provide the organism with mitigating enzymes or pathways which could reduce the efficacy of H_2O_2 as a chemotherapeutant?

General

The salmon louse *Lepeophtheirus salmonis* (Krøyer,1837) is a naturally occurring ectoparasite that has been found historically on wild and farmed salmonids. It has the potential to cause significant damage to the quality and condition of farmed fish due to the mobile stages feeding on fish mucus, skin tissue and blood. In 2012, the Scottish aquaculture industry produced 162,000 tonnes of farmed Atlantic salmon (*Salmo salar* L.) with an estimated market value of £600 million for the Scottish Economy (Marine Scotland, 2014), the first sale value having virtually doubled over the previous six years (Scottish Salmon Producers Organisation, 2013).

Economic losses due to *L. salmonis* in farmed Atlantic salmon in Scotland remain at a high level and were estimated by Costello (2009) at £30 million per year. Current estimates of sea lice treatment costs in Scotland are not available but models generated to explore the costs of different treatment strategies for sea lice in Norway

estimated the cost of treatments at about 2.28 NOK (Norwegian Kroner)/kg to 5.03 NOK/kg with an average cost of 2.42 NOK/kg (Liu & Bjelland, 2014). This predicted value was comparable with an estimate of 2.45 NOK/kg for some aquaculture companies in Norway (Jensen, 2013). Though not transferable to Scotland, it does indicate the potential for an impact on industry profit levels (and thus potential for sustainability/expansion) of controlling *L. salmonis* infestations.

L. salmonis can also parasitise wild salmonids, as well as farmed fish and there exist environmental concerns over the impact on wild salmonids of high sea lice numbers dispersed from salmon farms (Middlemas *et al*., 2012; Krkošek *et al*., 2013; Vollset *et al*., 2014).

Looking to the future, the Scottish Government is encouraging industry to increase production in a sustainable way (Marine Scotland, 2014). At a time of planned expansion in production a major concern of the industry is to reduce the sea lice burden and significant research has been undertaken in this area.

Sea lice treatments

Chemotherapeutic treatments for sea lice control were summarised by Pike & Wadsworth (2000) within their broad review of sea lice in salmonids; this covered the range of available chemical compounds with comments on their efficacy. All compounds are currently approved by the Scottish Environment Protection Agency (SEPA) for release to the environment and approved by the Veterinary Medicine Directorate (VMD) for marketing purposes.

Currently, H_2O_2 is one of four options to control sea lice infestation on farmed sites in Scotland (Table 1).

Table 1. Current treatments for sea lice

Trade Name	**Active Compound**
®Paramove™ 50	H_2O_2
Samaki Salartect	H_2O_2
Alpha max®	Deltamethrin
Salmosan®	Azamethiphos
SLICE®	Emamectin benzoate

®Paramove™50 (Solvay Interox) and Samaki Salartect (James A Mackie) are trade names of commercial products currently used across Scotland, containing approx. 50% w/v H_2O_2.

Use of H_2O_2 has some disadvantages, and negative factors include increased toxicity to the host at higher temperatures, inherent problems of bath treatment i.e. fish stress, labour, time and weather dependency, the cost of purchase and transport of chemical, growth inhibition (Speare *et al.,* 1999) and inappetance after repeated treatment (Treasurer *et al*., 2000a) (Table 2).

Table 2. Advantages and disadvantages of H_2O_2 treatment

Advantages	**Disadvantages**
Low environmental burden: breakdown products are water and oxygen.	Toxicity for fish increases with temperature – need to use a non-toxic dose at ambient temperature.
Effective in removal of attached adult sea lice (Treasurer & Grant 1997).	Problems of bath treatment: fish stress (Kiemer & Black, 1997), time, labour and weather-dependency.
Effective in reducing the hatching ability of egg strings of *L. salmonis* (McAndrew *et al*., 1998; Toovey & Lindon, 2000; Aaen *et al*., 2014).	Expensive in terms of chemical and transport logistics.
	Safety implications for personnel in industry.
	Growth inhibition with repeated use (Speare *et al*., 1999).
	Reduced efficacy after repeated use e.g. 41 previous treatments (Treasurer, et *al*., 2000b), and reports of 10 times variance in sensitivity to H_2O_2 between *L. salmonis* strains in Norway, with evidence of hereditary resistance (Helgesen *et al*., 2015).

Anti-parasite treatments for sea lice have been used in combination for maximum effect e.g. sequential treatment with SLICE® and H_2O_2 is reported by Merck Animal Health[1], www.aqua.merck-animal-health.com. http://www.merck-animal-health-usa.com/news/2014-9-25%20a.aspx accessed 11/5/2015

2. Hydrogen peroxide (H_2O_2)

Chemistry

Anhydrous H_2O_2 is a colourless liquid at room temperature with a high vapour pressure, a pungent odour, specific gravity of 1.45 g ml^{-1}, and which boils at 70 ^oC. Hydrogen peroxide, diluted in water or seawater, is a clear colourless liquid at room temperature. Hydrogen peroxide acts as an oxidising agent when used as a dilute solution in water, essentially as the reaction:

$$H_2O_2 = H_2O + \bar{O}$$

where an atom of oxygen dissociates from the H_2O_2 molecule giving a negative charge to the oxygen atom ($\bar{O}$). An oxidation reaction can be defined as any chemical change or process where there is an elemental electronegative increase or a net loss of electrons.

A benefit of H_2O_2 in the aqueous environment is that it is broken down to water and oxygen with a half-life of 7 days, though this can vary dependent on temperature, so there are no harmful waste products (Burridge *et al*., 2014). As shown by the reaction below, when H_2O_2 decomposes with the release of heat, two molecules of H_2O_2 combine to give two molecules of water and one of oxygen released as a gas.

$$2H_2O_2 = 2H_2O + O_2\uparrow$$

Mechanisms of biocidal action of H_2O_2 to cells and tissues

Hydrogen peroxide is used for treatment of ectoparasites in the salmon farming industry at the standard dose of 1500 ppm for 20 min dependent on water temperature. It is also used for effective control of numerous external pathogens of fish e.g. bacterial pathogens of the genera *Flavobacterium, Cytophaga* and *Tenacibaculum* (Avendano-Herrera *et al*., 2006).

Treasurer *et al.* (2000b) described the two main theories to explain the biocidal and cytotoxic action of H_2O_2. The first theory is based on the production of highly reactive hydroxyl radicals (·OH) by either of two mechanisms:
(a) from the interaction of the superoxide anion ($\cdot O_2^-$) with H_2O_2, a reaction first proposed by Haber & Weiss (1934)

$$\cdot O_2^- + H_2O_2 = O_2\uparrow + OH^- + \cdot OH$$

(b) from the production of extremely short-lived hydroxyl radicals within the cell catalysed *in vivo* by the presence of transition metal ions, especially Fe^{2+}, which act as electronic donors according to the Fenton reaction

$$Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + \cdot OH$$

In vitro it has been shown that hydroxyl radicals in the two electron reduction state and other oxygenated species can act as potent oxidising agents, reacting with nucleic acids, lipids and proteins, and denaturing them (Liochev,1999, in Linley *et al*., 2012). Thus all free radicals can denature essential genetic, metabolic and structural constitutive biomolecules and it is this that is the basis of cell and tissue toxicity.

A second plausible theory arises from the suggestion of Schaperclaus *et al*. (1979) (in Treasurer *et al*., 2000b) that toxicity to protists and monogeneans from H_2O_2 is due to the release of molecular oxygen as a result of catalase action.

$$2H_2O_2 = 2H_2O + O_2 \uparrow$$

The distribution of H_2O_2 in nature

Hydrogen peroxide is formed naturally as part of cell metabolism and is a potent source of further reactive oxygen species (ROS), taking a central position in cell metabolism of ROS in general.

Hydrogen peroxide is produced in the cytoplasm of cells firstly by the dismutation of $\cdot O_2^-$ by Cu/Zn-containing superoxide dismutase (SOD) and secondly in the mitochondrion by aerobic respiration i.e. oxidation of molecular oxygen to the $\cdot O_2^-$ and thence to H_2O_2 by a Mn-containing SOD (Veal *et al*., 2007).

Detoxification systems

Detoxification systems are the pathways and mechanisms by which reactive oxygen species such as the $\cdot O_2^-$ and H_2O_2 are inactivated by antioxidant enzymes. Antioxidants essential for the survival of living cells in organisms thus play a key role as regulators of H_2O_2 signalling. However, H_2O_2 is also generated constitutively, as a by-product of oxidative metabolism by the mitochondrial electron transport chain, and following activation of various oxidative enzyme complexes by chemical and physical stimuli including integrin ligands, growth factors, cytokines and neurotransmitters. These systems generate $\cdot O_2^-$ which is then converted into H_2O_2 and O_2 by SOD isoenzymes. The H_2O_2 is then reduced to H_2O by catalase and glutathione peroxidase enzymes (Goitre *et al*., 2012).

Immune defence mechanisms

Phagocytes can generate H_2O_2 as a defence against foreign matter or microbes (Veal *et al*., 2007). In the crayfish *Pacifastacus leniusculus*, amongst other Crustacea, a model has been proposed for the interaction of haemocytes, the analogue of vertebrate white blood cells, with invading microbes (Holmblad & Söderhäll, 1999). The interactions are proposed at two levels; firstly, invading microorganisms are opsonized by peroxinectin, a peroxidase, which triggers integrin-mediated binding by haemocytes. Secondly, peroxinectin also binds to extracellular SOD which can catalyse the production of H_2O_2 from superoxide anions; peroxinectin may then convert this H_2O_2 to either toxic hypochlorous acid (see below) or toxic peroxynitrite ($ONOO^-$). It is these toxic products which are active against microbes. Not only H_2O_2 but also superoxide production has been reported to be enhanced in invertebrate defence against pathogens (Holmblad & Söderhäll, 1999).

$$H_2O_2 + 2Cl^- = 2HOCl \text{ (hypochlorous acid)}$$

H_2O_2 in cell signalling

A signalling mechanism is a process whereby external stimuli (a molecule external to a cell or another cell/microorganism) activate a membrane-bound or cytoplasmic molecule, such as an enzyme. This activation cascades through intracellular signalling pathways. It is now known that ROS, including H_2O_2, act as short lived, signalling intermediates in these pathways. In the context of the production of H_2O_2 in the cell arising from signalling processes, growth factors, cytokines and integrins among others can activate the enzymes NADPH (nicotinamide adenine dinucleotide phosphate) oxidase and 5'-lipooxygenase, which reduce oxygen molecules to produce superoxide anions which are then converted to H_2O_2 by SOD. Hydrogen peroxide has itself been shown to act as a signalling molecule in the activation of cell proliferation, differentiation, migration or apoptosis pathways (references in Veal *et al*., 2007). The mechanism for regulating signalling activation by H_2O_2 is unclear but it may be mediated through the concentration of H_2O_2 dictating which pathways are activated, the localisation of antioxidants involved in H_2O_2 processing i.e. whether present in cytoplasm or in peroxisomes, and the potentially short transmission distances/short half-life of H_2O_2. Hydrogen peroxide exerts its signalling influence through oxidising susceptible proteins and modifying their activity. These can include transcriptional regulators or enzymes (Veal *et al*., 2007). An example of enzyme regulation by ROS including H_2O_2 is inactivation of tyrosine phosphatases, resulting in increased tyrosine kinase activity and resultant modulation of cellular signalling and effector pathways (Hordijk, 2006).

3. H_2O_2 and its effect on *L. salmonis*

Life cycle of L. salmonis

The life stages of *L. salmonis* comprise larval free swimming stages, the nauplii, a second free swimming stage, the copepodid which is infective, and attached development stages, the chalimus, preadult, and mature adult (Pike and Wadsworth, 2000). Thus there are eight developmental stages in total (Fig. 1) comprising two nauplii, a copepodid stage, two chalimii, two preadults and adult (Hamre *et al*., 2013). The nauplius stage consists of a head section without a trunk or abdomen whilst the copepodid stage develops a trunk and abdomen. Each stage is separated by a moult stage when the exoskeleton is shed. The adult male is approximately 6 mm in length and the mature female 20-25 mm (Pike & Wadsworth, 2000) (Fig. 2).

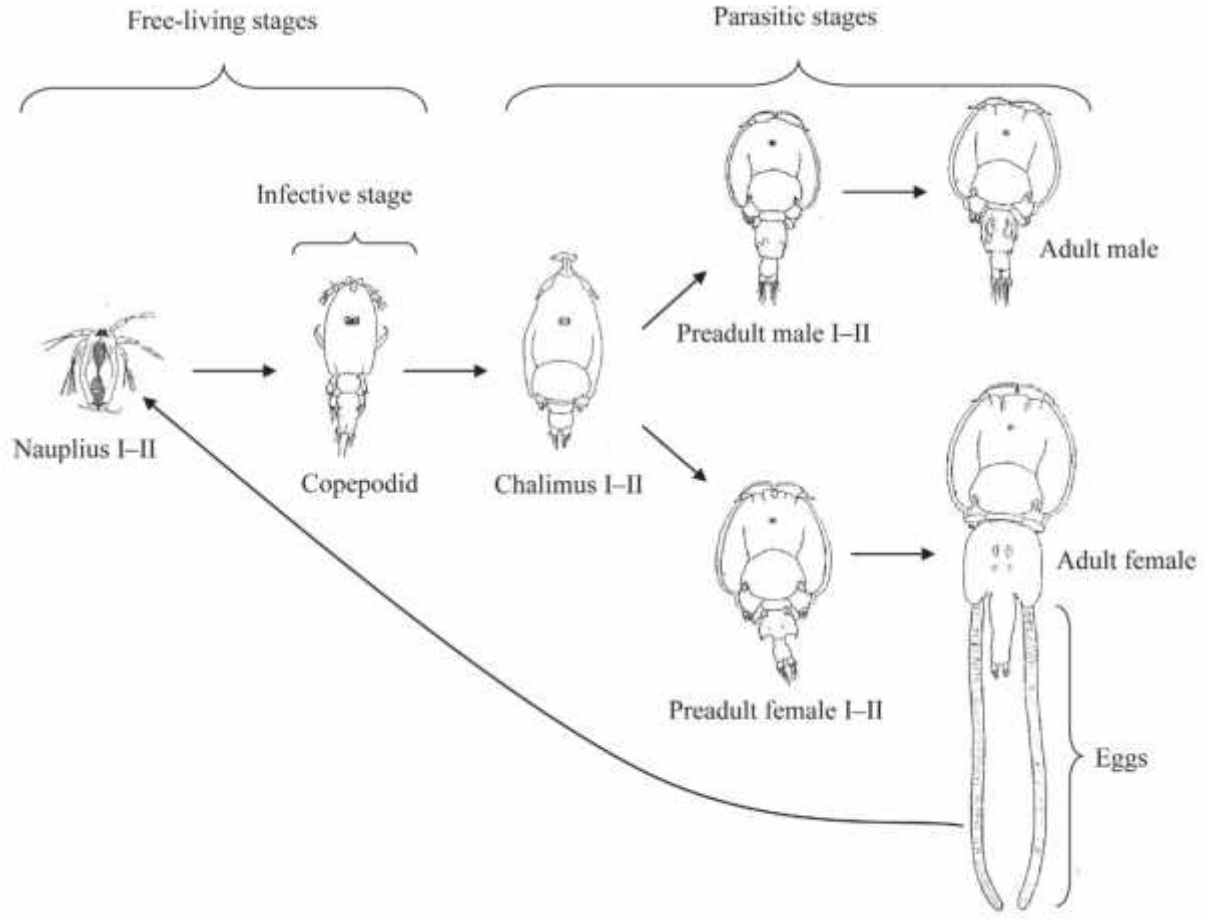

Fig. 1. Life cycle of *Lepeophtheirus salmonis* (Igboeli *et al*., 2014; reproduced with permission from authors).

Fig. 2. Adult gravid females with long tail egg strings amongst *L. salmonis* attached to the skin of a farmed Atlantic salmon grower. Crown copyright. (The gravid louse second from the left is approx. 25mm in length).

The effect of H_2O_2 treatment on L. salmonis life stages

Research groups in Scotland, Canada and Norway have described the efficacy of H_2O_2 in immobilizing, removing or killing the various *L. salmonis* life stages (Table 3a-c). Recovery following exposure is also a feature of these studies.

Whilst field use of H_2O_2 in the first instance seeks to treat and remove the attached feeding adult stages, in particular gravid females, past research has also investigated how the free-living stages as well as the attached chalimus and fertilised eggs are affected. McAndrew *et al*. (1998) used a laboratory bench top approach using Petri dishes and filtered seawater to study mobile stages. They reported all nauplii and copepodids were killed by 1 h after treatment with H_2O_2 at 1500 ppm for 20 min at 7.5 °C. Bruno & Raynard (1994) reported a delay in killing of copepodids, with 10% mortality after a 1250 ppm exposure for 20 min at 10 ^{o}C, rising to 100% mortality by 19 h. For chalimus I and II stages, McAndrew *et al*. *(*1998) and Johnson *et al*. (1993) both concluded there was no killing effect of 1500 ppm H_2O_2 at 7.5 °C, and at 1000 to 2000 ppm at 11 °C, respectively, for 20 min on either stage; 96% of chalimii recovered after 20 min exposure to 1000 - 2000 ppm at 11 ^{o}C (Johnson *et al*., 1993).

Studies have shown up to 100% dislodgement with up to 90% recovery by 24 h for preadults and adults (Treasurer & Grant, 1997; Johnson *et al.*, 1993). Treasurer & Grant (1997) showed in both laboratory experiments and on farms that although 100% of preadult and adults were inactive after 20 min exposure to 1500 ppm peroxide for 20 min, 35% had recovered by 1 h and 85% by 24 h. The question for sea lice control is therefore: can passively distributed clouds of removed sea lice act as a source of new infection? Treasurer & Grant (1997) suggested that tidal action might disperse adult sea lice removed by H_2O_2 that subsequently recover, also predation by juvenile saithe may reduce numbers of treated adults in the water. Johnson *et al.* (1993) found a similar result under laboratory conditions: approx. 80% reduction in the intensity of preadult and adults on Atlantic salmon after a 20 min exposure to 1500 ppm at 11 ^{o}C followed by 85-90% recovery of activity by 24 h. Recovery was commonly observed in these *in vitro* studies. Consequently, is it possible for the recovered adults to reattach? Johnson *et al.* (1993) held the negative view, as did Treasurer & Grant (1997) from three farm treatment studies. McAndrew *et al*. (1998) addressed this question with aquarium based reattachment studies (H_2O_2 at 1500 ppm for 20 min at 7.5 °C) and found that all mobile stages were capable of reattachment, though adult females significantly less so. Recent results from Marine Scotland Science (Pert *et al*., SARF SP 001 report) also demonstrated that adults treated with 1500 ppm at 10 ^{o}C off the host could attach to salmon in an aquarium setting.

Turning to egg susceptibility, three studies have provided positive evidence that the eggs of gravid females are sensitive to H_2O_2. McAndrew *et al.* (1998) found that eggs at an early stage of development (non-pigmented) failed to hatch after treatment, whereas those at a pigmented stage successfully hatched and produced viable copepodids, although with significantly reduced numbers. The authors inferred that a difference in egg permeability between early and later stages might explain this observed difference is sensitivity. Toovey & Lyndon (2000) reported that H_2O_2 at 1500 ppm for 20 min at 10 ^{o}C produced approx. 80% reduction in the number of sea lice hatching from treated sea lice eggstrings versus reference eggstrings. Hydrogen peroxide was more effective than 1 ppm dichlorvos or 5 ppb cypermethrin. Recently, Aaen *et al*. (2014) reported that no hatching was observed in field treatments when sea lice eggstrings were exposed to 1750 ppm for 32 min at 6 ^{o}C. Furthermore, in a laboratory study, treatment with 470 ppm for 36 min at 8 ^{o}C was almost completely effective at annulling hatching of eggstrings. This novel observation at a previously untested concentration was explained by a procedure using thorough stirring of H_2O_2 into the exposure water. These authors posed an interesting question for future studies i.e. can frequently treated peroxide-resistant ovigerous lice produce peroxide-resistant eggstrings?

In summary, McAndrew *et al*. (1998) commented: “the effect of peroxide on different life-cycle stages of lice is thus variable but the mechanisms which cause this are unknown”. At present, this statement still applies and leads onto a consideration of

the target structures in sea lice and related arthropods, as well as the possible physiological and biochemical mechanisms of action of H_2O_2.

Table 3a. Hydrogen peroxide effects on *L. salmonis* eggs from studies (1993-2015) using different doses, temperatures and times.

Life Stage	Treatment temperature °C	H_2O_2 dose (ppm)	Treatment time (min)	Treatment salinity	Experimental treatment	No. per treatment group	No. replicates/ cages	Hatching % (days post treatment pt)	Development (days post treatment pt)	Comments
[1]eggstring[a]	7.5 ± 1	0	20	33-34.5	in vitro	10 eggstrings	1	Immature eggs at time of treatment failed to hatch. Maturing eggs at time of treatment hatched.	Significant differences in nauplii numbers between treated and control egg strings. reduction).	[a]eggstrings taken from same ovigerous female lice for control and treatment groups- one string per group. Egg strings incubated at 10°C post exposure.
[1]eggstring[a]	7.5 ± 1	1500	20	33-34.5	in vitro	10 eggstrings	1			
[1]eggstring[a]	7.5 ± 1	0	20	33-34.5	in vitro	10 eggstrings	3		No signficant differnce in nauplii numbers until 7d pt.	
[1]eggstring[a]	7.5 ± 1	1500	20	33-34.5	in vitro	10 eggstrings	3		Nauplii surviving/maturing into copepodid stage were significantly reduced.	[b]eggstrings taken from different ovigerous female lice for control and treatment groups: similar total lengths of egg strings used for control and treatment groups. Egg strings incubated at 12°C post exposure. Control lice taken from sites not exposed to treatments for a minimum period of 9 weeks previously.
[2]eggstring[b]	10	1500	20	not specified	field	10 eggstrings	10	~20 (19d) compared to control [c]	Eggstrings from treated ovigerous female lice (19d pt) were more frequently pale and opaque and not pigmented- reflecting reduced capacity of eggstrings to produce viable larvae. ~30.8 % naup. (19d pt) compared to control, ~10 % cop. (19d pt) compared to control	
[3]eggstrings - low pigmentation[d]	6.4	0	31-6/9[e]	35	field	3 partial eggstrings	2	50 (18d)	2 copepodids[f] (18d)	[c]some of the adult ovigerous lice/eggstrings in treatment group were chalimus at time of treatment.
[3]eggstrings - low pigmentation[d]	6.4	1750	31-6/9[e]	35	field	3 partial eggstrings	3	no hatching (18d)		
[3]eggstrings - medium pigmentation[d]	6.4	0	31-6/9[e]	35	field	3 partial eggstrings	2	83 (18d)	12 viable copepodids[f] (18d)	
[3]eggstrings - medium pigmentation[d]	6.4	1750	31-6/9[e]	35	field	3 partial eggstrings	3	no hatching (18d)		[d]eggs held at 8 ± 0.4°C and 33.8 ± 0.1% in lab after field exposure. Eggs string cut into 10mm sections post exposure.
[3]eggstrings - high pigmentation[d]	6.4	0	31-6/9[e]	35	field	3 partial eggstrings	2	83 (18d)	135 viable copepodids[f] (18d)	
[3]eggstrings - high pigmentation[d]	6.4	1750	31-6/9[e]	35	field	3 partial eggstrings	3	22 (18d)	no viable nauplii	
[3]eggstrings - low pigmentation[g]	not specified	0	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	66 (15d)	9 viable copepodids[f] (15d)	[e]total time minus (-) infusion time to reach treatment dose.
[3]eggstrings - low pigmentation[g]	not specified	470	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	50 (15d)	viable larvae, all dead by day 15	
[3]eggstrings - low pigmentation[g]	not specified	1000	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	0 (15d)	0 viable larvae	[f]larvae considered to have reached copepodid stage if survived for more than 50 degree days post hatching.
[3]eggstrings - low pigmentation[g]	not specified	1500	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	25 (15d)	0 viable larvae	
[3]eggstrings - low pigmentation[g]	not specified	2000	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	33 (15d)	2 viable larvae, all dead at day 10	
[3]eggstrings - medium pigmentation[g]	not specified	0	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	100 (15d)	13 viable copepodids[f] (15d)	[g]ovigerous adult females collected 24hrs before treatment of eggs. Hydrogen peroxide not used on farmof origin but other treatments used - no information on prior treatment times on farm. Egg strings cut into 5mm sections, held at 12°C for 16-18 hrs post exposure and then at 7.8 ± 1.1 C and 34.4 ± 0.2% for remainder.
[3]eggstrings - medium pigmentation[g]	not specified	470	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	0 (15d)	0 viable larvae	
[3]eggstrings - medium pigmentation[g]	not specified	1000	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	0 (15d)	0 viable larvae	
[3]eggstrings - medium pigmentation[g]	not specified	1500	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	25 (15d)	0 viable larvae	
[3]eggstrings - medium pigmentation[g]	not specified	2000	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	0 (15d)	0 viable larvae	
[3]eggstrings - high pigmentation[g]	not specified	0	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	66 (15d)	40 viable copepodids[f] (15d)	
[3]eggstrings - high pigmentation[g]	not specified	470	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	50 (15d)	0 viable larvae	
[3]eggstrings - high pigmentation[g]	not specified	1000	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	50 (15d)	0 viable larvae	
[3]eggstrings - high pigmentation[g]	not specified	1500	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	50 (15d)	2 viable larvae, all dead on day 10	
[3]eggstrings - high pigmentation[g]	not specified	2000	36 ± 3 min	not specified	in vitro	4 partial eggstrings	1	50 (15d)	0 viable larvae	

[1]McAndrew *et al*. (1998); [2]Toovey & Lyndon (2000); [3]Aaen *et al*. (2014); [4]Pert *et al*. (2015); [5]Johnson *et al*. (1993).

Table 3a contd. Hydrogen peroxide effects on *L. salmonis* eggs from studies (1993-2015) using different doses, temperatures and times.

Life Stage	Treatment temperature °C	H_2O_2 dose (ppm)	Treatment time (min)	Treatment salinity	Experimental treatment	No. per treatment group	No. replicates/ cages	Hatching % (days post treatment pt)	Development (days post treatment pt)	Comments
[4]eggstrings[h]	10	0	20	34	in vitro	5	3		1268 copepodids (6-8d)	
[4]eggstrings[h]	10	600	20	34	in vitro	5	3		412 copepodids (6-8d)	
[4]eggstrings[h]	10	0	20	34	in vitro	5	3		911 copepodids (6-8d)	
[4]eggstrings[h]	10	1200	20	34	in vitro	5	3		354 copepodids (6-8d)	
[4]eggstrings[h]	10	0	20	34	in vitro	5	3		921 copepodids (6-8d)	
[4]eggstrings[h]	10	1500	20	34	in vitro	5	3		445 copepodids (6-8d)	
[4]eggstrings[h]	10	0	20	34	in vitro	5	3		901 copepodids (6-8d)	
[4]eggstrings[h]	10	1800	20	34	in vitro	5	3		237 copepodids (6-8d)	[h]eggstrings taken from lab lice strain, not exposed to treatments. Eggstrings detached from females 12 hrs pre-exposure. Pale egg strings displayed lower hatching compared to more pigmented egg strings.
[4]eggstrings[h]	13	0	20	34	in vitro	5	3		2024 copepodids (6-8d)	
[4]eggstrings[h]	13	600	20	34	in vitro	5	3		47 copepodids (6-8d)	
[4]eggstrings[h]	13	0	20	34	in vitro	5	3		1451 copepodids (6-8d)	
[4]eggstrings[h]	13	1200	20	34	in vitro	5	3		22 copepodids (6-8d)	
[4]eggstrings[h]	13	0	20	34	in vitro	5	3		1917 copepodids (6-8d)	
[4]eggstrings[h]	13	1500	20	34	in vitro	5	3		75 copepodids (6-8d)	
[4]eggstrings[h]	13	0	20	34	in vitro	5	3		1364 copepodids (6-8d)	
[4]eggstrings[h]	13	1800	20	34	in vitro	5	3		0 copepodids (6-8d)	
[5]eggstrings	not specified	1460	22	not specified	wellboat			hatch rates similar for controls and detached eggstrings of treated lice.		
[5]ovigerous females	not specified	1460	22	not specified	wellboat			hatch rates similar for controls and treated gravid females.		
[6]ovigerous females	assume 11°C	0	20,40	not specified	in vitro	7 ovigerous females	1	100% egg strings developed. 79-99% hatched.	0-47% had viable copepodids	
[6]ovigerous females	assume 11°C	1500	20, 40	not specified	in vitro	7 ovigerous females	1	43 % egg strings developed. 1.5 - 45.5% of these hatched.	Few active nauplii, no viable copepodid	
[1]females	7.5 ± 1	1500	20	33-34.5	in vitro[h]			female lice left on fish for 31d pt. No significant differences with controls in number lice producing egg strings at day 31.		

[1]McAndrew *et al*. (1998); [2]Toovey & Lyndon (2000); [3]Aaen *et al*. (2014); [4]Pert *et al*. (2015); [5]Beattie *et al*. (2012); [6]Johnson *et al*. (1993).

Table 3b. Hydrogen peroxide effects on *L. salmonis* nauplius/chalimus from studies (1993-2015) using different doses, temperatures and times.

Life Stage	Treatment temperature °C	H_2O_2 dose (ppm)	Treatment time (min)	Treatment salinity	Experimental treatment	No. per treatment group	No. replicates/ cages	Killing % (hrs post treatment pt)	Immobilisation % (hours/days post treatment pt)	Recovery % (hrs post treatment pt)	Development (days post treatment pt)
[1]nauplius I/II	7.5 ± 1	0	20	33-34.5	in vitro	10	3x2	2.8 (1hr), 17.9 (24hr), 38.2 (48hr)			
[1]nauplius I/II	7.5 ± 1	1500	20	33-34.5	in vitro	10	3x2	100 (1hr)			
[1]copepodids	7.5 ± 1	0	20	33-34.5	in vitro	10	3x3	1 (1hr), 18.6 (24hr), 33.3 (48hr)			
[1]copepodids	7.5 ± 1	1500	20	33-34.5	in vitro	10	3x3	2 copepodids viable at 24hr			
[2]copepodite[a]	10	0	20	not specified	in vitro	11, 16	2		0, 12.5 (0hr) 0, 0 (2hr) 9, 6.25 (19hr)		
[2]copepodite[a]	10	1250	20	not specified	in vitro	10	1		10 (0hr), 20 (2hr), 100 (19 hr)		
[2]copepodite[a]	10	3000	20	not specified	in vitro	7, 12	2		100, 100 (0) , 100, 100 (2hr), 100, 100 (19hr)		
[3]chalimus	12	1000	not specified		in vivo				5 (% removed) (assume 0hr)		
[3]chalimus	8	2000	not specified		in vivo				60 (% removed) (assume 0hr)		
[4]chalimus[b]	11	0	20	not specified	in vitro	31, 33	2	6.1, 12.9 (24hr)			
[4]chalimus[b]	11	1000	20		in vitro	31	1	0 (24hr pt)			
[4]chalimus[b]	11	1500	20		in vitro	30	1	3.3 (24hr pt)			
[4]chalimus[b]	11	1500	40		in vitro	31	1	3.2 (24hr pt)			
[4]chalimus[b]	11	2000	20		in vitro	33	1	3 (24hr pt)			
[4]chalimus[b]	11	3000	20		in vitro	42	1	19 (24hr pt)			
[4]chalimus[b]	11	4000	20		in vitro	34	1	41.2 (24hr pt)			
[5]chalimus[c]	not specified	1500	20	32	in vitro	10	2		50-80 (0hr)	90-100 (1hr)	
[1]chalimus I/Iid	7.5 ± 1	1500	20	33-34.5	in vivo	6 fish	1				chalimus I: chalimus II ratio: 1:2.42 vs 1: 2.13, chalimus III: chalimus IV ratio: 1: 0.02 vs 1: 0.48 in treated vs controls 11d pt.
[6]chalimus[e]	10	1500, 1800	20	34	in vivo	30 fish control, 30 x 2 treated					Significantly fewer male and female chalimii moulted through to preadults at both concentrations.
[6]chalimus[e]	13	1500, 1800	20	34	in vivo	30 fish control, 30 x 2 treated					Significantly fewer male and female chalimii moulted through to preadults at 1500 concentrations. No significant difference for female chalimii/preadult ratio at 1800.
[7]non-motile lice	6.4	1750	31-6/9[f]	35	field	65-67 fish	1		77.8 (% removed) (6 d)[g]		
[4]chalimus/early preadults	11	1500	20	not specified	in vivo	20 fish	2[h]		no significant difference in treated vs controls(7d)		

[1]McAndrew *et al*. (1998); [2]Bruno & Raynard (1994); [3]Thomassen (1993); [4]Johnson *et al*. (1993); [5]Treasurer & Grant (1997); [6]Pert *et al*. (2015); [7]Aaen *et al*. (2014).
[a]immobilised lice described as not showing respiratory and appendage movement and no swimming response when touched with glass rod; [b]attached to excised fins. Held at 9.9 to 10.1°C for 24 h post exposure; [c]held at 10 ± 1°C post treatment; [d]chalimus stages based on old 10 stage life cycle; [e]infection generated from eggs taken from lab lice strain, not exposed to treatments; [f]total time minus (-) infusion time to reach treatment dose; [g]non-motile stages may have developed to motile stages; [h]one replicate each of Chinook and Atlantic salmon.

Table 3c. Hydrogen peroxide effects on. *L. salmonis* preadult/adult from studies (1993-2015) using different doses, temperatures and times.

Life Stage	Treatment Temperature °C	H_2O_2 dose	Treatment time (min)	Treatment salinity	Experimental treatment	No. lice per treatment group	No. replicates/ cages	Killing	Immobilisation/removal % (hours/days post treatment pt)	Recovery % (hrs post treatment pt)	Reattachment %
[1]preadults	12	1000	not specified	not specified	on fish[a]				~ 88		
[1]preadults	8	2000	not specified	not specified	on fish[a]				~ 97		
[2]preadult	10	0	20	not specified	in vitro	10	2		0		
[2]preadult	10	500	20	not specified	in vitro	10	2		20, 10 (0hr), 0, 0 (2hr), 0, 0 (19hr)		
[2]preadult	10	1250	20	not specified	in vitro	10	2		70, 70 (0hr) 40, 0 (2hr). 40, 0 (19 hr)		
[2]preadult	10	2000	20	not specified	in vitro	10	2		100, 90 (0hr) 50 (2hr), 40, 50 (19 hr)		
[2]preadult	10	3000	20	not specified	in vitro	10	2		100 (0hr) 30, 50 (2hr), 30, 50 (19hr)		
[3]preadult	7.5 ± 1	0	20	33-34.5	in vitro[b]	60	3				43.3 - 60 (24hr)
[3]preadult	7.5 ± 1	1500	20	33-34.5	in vitro[b]	60	3				30 - 55 (24hr): reinfection not significantly different to control.
[4]preadult/adult	11	1500	20	not specified	in vivo		2		80 (0hr)	89 (36hr)	
[4]preadult/adult[c]	11	0	20	not specified	in vitro	13	1	0 (24hr)			
[4]preadult/adult[c]	11	1000	20	not specified	in vitro	12	1	0 (24hr)			
[4]preadult/adult[c]	11	1500	20	not specified	in vitro	13,20	2	15.4, 10 (24hr)			
[4]preadult/adult[c]	11	1500	40	not specified	in vitro	14, 20	2	21.4, 55 (24hr)			
[4]preadult/adult[c]	11	2000	20	not specified	in vitro	12,20	1	0, 0 (24hr)			
[4]preadult/adult[c]	11	3000	20	not specified	in vitro	19	1	68.4 (24hr)			
[4]preadult/adult[c]	11	4000	20	not specified	in vitro	18	1	77.8 (24hr)			
[5]preadult/adult[d]	not specified	0	20	not specified	in vitro	5	2		0	0 (24hr)	
[5]preadult/adult[d]	not specified	1500	20	not specified	in vitro	5	4		100 (0hr)	35 (1hr), 50 (3hr), 80 (6hr), 85 (24hr)	
[5]preadult/adult	9	400	60	not specified	in vitro	5	2		50 (0hr)	100 (24hr)	
[5]preadult/adult	9	600	60	not specified	in vitro	5	2		70 (0hr)	90 (24hr)	
[5]preadult/adult	9	800	60	not specified	in vitro	5	2		90 (0hr)	80 (24hr)	
[5]preadult/adult	9	1000	60	not specified	in vitro	5	2		100 (0hr)	50 (24hr)	
[5]preadult/adult	12	400	60	not specified	in vitro	5	2		60 (0hr)	90 (24hr)	
[5]preadult/adult	12	600	60	not specified	in vitro	5	2		80 (0hr)	~ 98 (24hr)	
[5]preadult/adult	12	800	60	not specified	in vitro	5	2		100 (0hr)	30 (24hr)	
[5]preadult/adult	12	1000	60	not specified	in vitro	5	2		100 (0hr)	20 (24hr)	
[5]preadult/adult	15	400	60	not specified	in vitro	5	2		80 (0hr)	40 (24hr)	
[5]preadult/adult	15	600	60	not specified	in vitro	5	2		90 (0hr)	20 (24hr)	
[5]preadult/adult	15	800	60	not specified	in vitro	5	2		100 (0hr)	50 (24hr)	
[5]preadult/adult	15	1000	60	not specified	in vitro	5	2		100 (0hr)	10 (24hr)	
[5]preadult/adult	6.5	1565	20	32	field	7	1		87.7 (0hr)	85.7 (12hr)	
[5]preadult/adult	6.5	1403-2153	14-20	32	field	14	1		100 (0hr)	57.1 (12hr)	
[5]preadult/adult	11.5	1500-1650	15-16	32	field	19-79	1		100 (0hr)	1.3 - 11.1 (0.5hr), 33.3-97.5 (1hr), 51.9-66.7 (1.5hr), 90.9 (2.5hr), 90.3-97.5 (12hr)	

[1]Thomassen (1993); [2]Bruno & Raynard (1994); [3]McAndrew *et al*. (1998); [4]Johnson *et al*. (1993); [5]Treasurer & Grant (1997); [6]Thomassen & Lekang (1994); [7]Helgesen *et al*. (2015); [8]Hodneland *et al*. (1993); [9]Treasurer *et al*. (2000b); [10]Aaen *et al*. (2014) ; [11]Beattie et al. (2012); [12]Pert *et al*. (2015).

[a]not specified if in field or in aquarium; [b]treated *in vitro* then added to fish to study reattachment. Lice collected ~ 24 h before treated; transferred to 9.9-10.1°C after exposure; [d]held at 10°C after treatment;

Table 3c contd. Hydrogen peroxide effects on. *L. salmonis* preadult/adult from studies (1993-2015) using different doses, temperatures and times

Life Stage	Treatment Temperature °C	H_2O_2 dose	Treatment time (min)	Treatment salinity	Experimental treatment	No. lice per treatment group	No. replicates/ cages	Killing	Immobilisation/removal % (hours/days post treatment pt)	Recovery % (hrs post treatment pt)	Reattachment %
[6]preadult/adult[e]	6-14	3500-5000	20	not specified	field	5-10 fish	1		100 (0hr)		
[6]preadult/adult[e]	6-14	1500-2000	20	not specified	field	5-10 fish	1		100 (0hr)		
[6]preadult/adult[e]	6-14	2700	20	not specified	field	5-10 fish	1		94 (0hr)		
[6]preadult/adult[e]	6-14	1000	20	not specified	field	5-10 fish	1		83 (0hr)		
[6]preadult/adult[e]	6-14	420	120	not specified	field	5-10 fish	1		10 (0hr)		
[6]preadult/adult[e]	6-14	700	60	not specified	field	5-10 fish	1		38 (0hr)		
[6]preadult/adult[e]	6-14	1340	20	not specified	field	5-10 fish	1		85 (0hr)		
[6]preadult/adult[e]	6-14	600	20	not specified	field	6-12 fish	1		48 (0hr)		
[6]preadult/adult[e]	6-14	1400	20	not specified	field	6-12 fish	1		95 (0hr)		
[6]preadult/adult[e]	6-14	800	20	not specified	field	6-12 fish	1		60 (0hr)		
[6]preadult/adult[e]	6-14	950	20	not specified	field	6-12 fish	1		75 (0hr)		
[7]preadult/adult males[f g]	10-12	EC_{50} 216	30	not specified	in vitro	6-13			50 (0hr)		
[7]preadult/adult males [f h]	10-12	EC_{50} 1767	30	not specified	in vitro	6-13			50 (0hr)		
[7]preadult/adult males [f h]	10-12	EC_{50} 2127	30	not specified	in vitro	6-13			50 (0hr)		
[7]preadult/adult males [f i]	10-12	EC_{50} 539	30	not specified	in vitro	6-13			50 (0hr)		
[7]preadult/adult males [f i]	10-12	EC_{50} 693	30	not specified	in vitro	6-13			50 (0hr)		
[7]preadult/adult males [f i]	10-12	EC_{50} 563	30	not specified	in vitro	6-13			50 (0hr)		
[7]preadult/adult males [f i]	10-12	EC_{50} 541	30	not specified	in vitro	6-13			50 (0hr)		
[7]preadult/adult males [f i]	10-12	EC_{50} 538	30	not specified	in vitro	6-13			50 (0hr)		
[7]preadult/adult males[f g]	12	EC_{50} 45.9	24hr	not specified	in vitro	8-17			50 (0hr)		
[7]preadult/adult males[f g]	12	EC_{50} 64.7	24hr	not specified	in vitro	8-17			50 (0hr)		
[7]preadult/adult males [f h]	12	EC_{50} 138	24hr	not specified	in vitro	30-61			50 (0hr)		
[5]mobile	6	assume 1500	assume 20	32	field	10 fish	2		64 - 87 (24-48hr)		no observed reattachment
[5]mobile	9	assume 1500	assume 20	not specified	field	10 fish	2		84 - 92 (24-48hr)		no observed reattachment
[5]mobile	9.5	assume 1500	assume 20	not specified	field	10 fish	2		71-88 (24-48hr)		no observed reattachment
[8]mobile[j]	8	1500	25	34	in vitro	100	2	cumulative mortality slope: control/ H_2O_2 (over 136hr pe): 0.54/0.57). Females more susceptible than males, females with egg strings more susceptible than females without eggstrings.			

[1]Thomassen (1993); [2]Bruno & Raynard (1994); [3]McAndrew *et al*. (1998); [4]Johnson *et al*. (1993); [5]Treasurer & Grant (1997); [6]Thomassen & Lekang (1994); [7]Helgesen *et al*. (2015); [8]Hodneland *et al*. (1993); [9]Treasurer *et al*. (2000b); [10]Aaen *et al*. (2014); [11]Beattie et al. (2012); [12]Pert *et al*. (2015).

[e]reference refers to all lice - it is assumed that this implies mobile lice since removed from fish by treatment. However other studies have recorded removal of chalimii stages; [f]bioassays initiated within 8 h post lice collection. Lice attached to walls or swimming in straight line considered alive. All others considered immobilised or dead. 0% mortality in 30 min control groups; 0-8.3% mortality in 24 h control groups. Preadults/adult males not distributed evenly between experiments; [g]lice from lab reared strain - not exposed to H_2O_2; [h]lice from farms reporting H_2O_2 resistance; [i]lice from farms where H_2O_2 used but resistance not reported; [j]lice exposed to treatment 24 h after collection. Lice classified as active (adhere to walls of tank); non-active (not able to swim or adhere but with movement at extremities; dead (no movement).

Table 3c contd. Hydrogen peroxide effects on. *L. salmonis* preadult/adult from studies (1993-2015) using different doses, temperatures and times.

Life Stage	Treatment Temperature °C	H_2O_2 dose	Treatment time (min)	Treatment salinity	Experimental treatment	No. lice per treatment group	No. replicates/ cages	Killing	Immobilisation/removal % (hours/days post treatment pt)	Recovery % (hrs post treatment pt)	Reattachment %
[9]mobile[k]	12-14	1500-1700	20	32	field	10 fish	2cages/3 farms		76 - 89 (24hr)		
[9]mobile[k]	not specified	2000	20		field	10 fish			7.5 (0hr)[k]		
[9]mobile[k]	not specified	2350	23		field	10 fish			70 (0hr)[k]		
[9]mobile[k]	not specified	2350	27		field	10 fish			90 (0hr)[k]		
[9]mobile[k]	not specified	2500	23		field	10 fish			63 (0hr)[k]		
[9]mobile[k]	6-6.5	2000	20	21	in vivo	10 fish	2		25 (24-48hr)[k]		
[9]total mobile[l]	6-6.5	2000	20	31	in vivo	10 fish	2		97 - 99 (24-48hr)		
[9]ovigerous females[k]	6.0-6.5	2000	20	21	in vivo	10 fish	2		15-16 (24-48hr)[k]		
[9]ovigerous females[l]	6.0-6.5	2000	20	31	in vivo	10 fish	2		87-90 (24-48hr)[m]		
[10]motile lice minus adult female	6.4	1750	31-6/9[n]	35	field	65-67 fish	1		85.7 (6d)		
[2]adult	10	0	20		in vitro	10	2	0	0		
[2]adult	10	500	20		in vitro	10	2		30, 70 (0hr), 10, 0 (2hr), 10, 0 (19hr)		
[2]adult	10	1250	20		in vitro	10	2		100, 90 (0hr), 30, 10 (2hr), 20, 10 (19hr)		
[2]adult	10	2000	20		in vitro	10	2		100, 100 (0hr), 40, 50 (2hr), 40, 30 (19hr)		
[2]adult	10	3000	20		in vitro	10	2		100, 100 (0hr), 50, 40 (2hr), 20, 50 (19hr)		
[11]adult	not specified	1460	22	not specified	wellboat						30 (treated) vs 43 (control)
[3]adult males	7.5 ± 1	0	20	33-34.5	in vitro[b]	19, 9	2				57.9 - 77.8 (24hr)
[3]adult males	7.5 ± 1	1500	20	33-34.5	in vitro[b]	19, 9	2				57.9 - 66.6 (24hr): attachment not significantly different to control.
[3]adult females	7.5 ± 1	0	20	33-34.5	in vitro[b]	30	3				50 - 83.3 (24hr)
[3]adult females	7.5 ± 1	1500	20	33-34.5	in vitro[b]	30	3				26.7 - 33.3 (24hr):attachment significantly lower than control.
[10]adult female	6.4	1750	31-6/9[n]	35	field	65-67 fish	1		100 (6d)		
[11]adult female	not specified	1460	22	not specified	wellboat					80 (7days), 20 (19 days)[o]	

[1]Thomassen (1993); [2]Bruno & Raynard (1994); [3]McAndrew *et al*. (1998); [4]Johnson *et al*. (1993); [5]Treasurer & Grant (1997); [6]Thomassen & Lekang (1994); [7]Helgesen *et al*. (2015); [8]Hodneland *et al*. (1993); [9]Treasurer *et al*. (2000b); [10]Aaen *et al*. (2014); [11]Beattie et al. (2012); [12]Pert et al. (2015).

[b]treated *in vitro* then added to fish to study reattachment. Lice collected ~ 24 h before treated; [k]H_2O_2 used 41 times in 6 years at farm: potential evidence of resistance; [l]H_2O_2 not used previously on farms; [m]ovigerous females less susceptible than other mobile stages; [n]total time minus (-) infusion time to reach treatment dose.

Table 3c contd. Hydrogen peroxide effects on. *L. salmonis* preadult/adult from studies (1993-2015) using different doses, temperatures and times.

Life Stage	Treatment Temperature °C	H_2O_2 dose	Treatment time (min)	Treatment salinity	Experimental treatment	No. lice per treatment group	No. replicates/ cages	Killing	Immobilisation/removal % (hours/days post treatment pt)	Recovery % (hrs post treatment pt)	Reattachment %
[12]adults	10	0	20	34	in vitro	30 fish	1		0		
[12]adults	10	600	20	34	in vitro	30 fish	2		23.8 (0hr), 4.8 (1hr), 0 (3hr-96hr)		
[12]adults	10	1000	20	34	in vitro	30 fish	2		42.9 (0hr), 23.8 (1hr), 4.8 (3hr) 4.8 (6hr), 0 (12-96hr)		
[12]adults	10	1500	20	34	in vitro	30 fish	2		81 (0hr), 61.9 (1hr), 14.3 (3hr), 9.5 (6hr), 0 (12-96hr)		
[12]adults	10	1800	20	34	in vitro	30 fish	2		100 (0hr), 85.7 (1hr), 19.0 (3hr), 9.5 (6hr), 0 (12-96hr)		
[12]adults	10	2300	20	34	in vitro	30 fish	2		100 (0hr), 100 (1hr), 33.33 (3hr), 23.8 (6hr), 9.5 (12hr), 4.8 (24hr), 4.8 (48hr), 0 (72-96hr)		
[12]adults	10	3000	20	34	in vitro	30 fish	2		100 (0hr), 85.7 (1hr), 23.8 (3hr), 14.3 (6hr), 4.8 (12hr), 4.8 (24hr), 0 (48-96hr)		
[12]adults	13	0	20	34	in vitro	30 fish	1		9.5 (0hr), 0 (1-48hr), 4.8 (72hr), 4.8 (96hr)		
[12]adults	13	300	20	34	in vitro	30 fish	2		9.5 (0hr), 0 (1-72hr), 14.3 (96hr)		
[12]adults	13	600	20	34	in vitro	30 fish	2		14.3 (0hr), 14.3 (1hr), 9.5 (3hr), 0 (6-72hr), 4.8 (96hr)		
[12]adults	13	1000	20	34	in vitro	30 fish	2		52.4 (0hr), 0 (1-72hr), 4.8 (96hr)		
[12]adults	13	1500	20	34	in vitro	30 fish	2		81 (0hr), 95.2 (1hr), 0 (3-96hr)		
[12]adults	13	1800	20	34	in vitro	30 fish	2		90.5 (0hr), 33.3 (1hr), 4.8(3hr), 0 (6hr), 4.8 (12hr), 4.8 (24hr), 4.8 (48hr), 4.8 (72hr), 4.8 (96hr)		
[12]adults	13	3000	20	34	in vitro	30 fish	2		100 (0hr), 66.7 (1hr), 33.3 (3hr), 19 (6hr), 28.6 (12hr), 28.6 (24hr), 23.8 (48hr), 23.8 (72hr), 33.3 (96hr)		
[12]adults	10	0	20	34	in vitro[b]	30	1				63 (96hr)
[12]adults	10	1500	20	34	in vitro[b]	30	2				51.6 (96hr)
[12]adults	10	1800	20	34	in vitro[b]	30	2				33.3 (96hr)

[1]Thomassen (1993); [2]Bruno & Raynard (1994); [3]McAndrew *et al*. (1998); [4]Johnson *et al*. (1993); [5]Treasurer & Grant (1997); [6]Thomassen & Lekang (1994); [7]Helgesen *et al*. (2015); [8]Hodneland *et al*. (1993); [9]Treasurer *et al*. (2000b); [10]Aaen *et al*. (2014); [11]Beattie et al. (2012); [12]Pert et al. (2015).
[b]treated *in vitro* then added to fish to study reattachment. Lice collected ~ 24 h before treated.

4. Target physiological processes and organs/structures in relation to H_2O_2 treatment

Observed or hypothesised effects of H_2O_2 in relation to L. salmonis

The literature reports that bubbles form quickly within the sea louse haemolymph and gut on treatment with H_2O_2 compounds such as Paramove® (Thomassen *et al*. 1993, Bruno and Raynard, 1994, McAndrew *et al*., 1998), causing muscle paralysis (Burridge *et al*., 2014) and resulting in sea lice floating to the water surface. A reasonable inference is that the formation of gaseous bubbles will cause a lifting force for attached sea lice and the concurrent muscle paralysis will cause the mouthparts to unlock their grip on the skin tissue. Whilst this observation has been corroborated by more than one author, there is no evidence in the literature that there has been experimental confirmation of which gas is contained in the bubbles. Two possibilities are, that it is gaseous H_2O_2 released out of solution, or that it is oxygen formed by the action of endogenous catalase or peroxidase in the body fluids of sea lice. The existence of such enzymes has been suggested by the staining of *L. salmonis* glands by 3 3'-diaminobenzidine tetrahydrochloride (DAB) (Bell *et al*., 2000), and the isolation of gene sequences from the *L. salmonis* genome representative of putative peroxidases and catalases.

Other than the descriptive observational account by Bruno & Raynard (1994) showing photographs of treated sea lice with gas bubbles, observations of emboli in the haemocoel mentioned by McAndrew *et al*. (1998) and mention of gas bubbles in haemolymph and gut of treated sea lice by Thomassen *et al*. (1993), there is no other information in the literature on other precise target organs for H_2O_2 in *L. salmonis.* This is an area for further research.

A fundamental property of H_2O_2 is that, acting as an oxidising agent on cells and tissues, its breakdown will produce oxygen, and any native enzymes that mitigate the effects of H_2O_2 as a treatment chemical will produce oxygen. In this context, the suggestion that it is the formation of molecular oxygen (Schaperclaus *et al*., 1979 in Treasurer *et al*., 2000b) which produces the observed bubbles is entirely plausible.

A variety of *L. salmonis* organs could be affected by H_2O_2. Potential target organs and structures are reviewed below as well as evidence for enzymes involved in H_2O_2 breakdown and mitigation of damaging intermediates.

General anatomy of copepods

The Copepoda are an order within the class Maxillopoda (a first pair of thoracic appendages modified as feeding maxillipeds), within the sub-phylum Crustacea and phylum Arthropoda. There are some 10,000 species of copepods widespread in both freshwater, and marine habitats and they constitute a significant part of the planktonic biomass. Approximately 5000 species are found in freshwater.

The copepod body comprises a head, trunk and tail sections (Kabata, 1979; Boxshall, 2005). The head carries five pairs of appendages: in order, the anntenules, anntenae, mandibles and maxillules which are involved in handling food. The trunk carries one pair of maxillipeds to aid feeding, and six swimming legs. The tail is comprised of four or five body segments or somites and bears caudal rami at its tip. The sex products are carried on the genital somite. The excretory system consists of two maxillary glands in the first part of the thorax.

Lepeophtheirus salmonis is an exclusively ectoparasitic species and belongs to the family *Caligidae* within the suborder Siphonostomatoida of the order Copepoda. The suborder Siphonostomatoida contains families of copepodids which possess siphon-like mandibles used in feeding, and frontal filaments used for larval stage attachment to hosts (Kabata 2003).

Anatomy of L. salmonis

This section discusses key tissues or structures in *L. salmonis* which might be affected by the free radicals or other products generated by H_2O_2 treatment and discusses evidence for the presence of endogenous enzymes in the tissues that may break down H_2O_2 e.g. peroxidase and catalase in particular.

Alimentary canal

The anatomy and histology of the alimentary canal has been described for a range of copepods and reviewed by Nylund *et al*. (1992). The alimentary canal of *L. salmonis* comprises an oesophagus, midgut and hindgut; whereas Scott (1901) divided the midgut of *Lepeophtheirus pectoralis* into three regions, a caecum, stomach and intestine. The midgut of *L. salmonis* is divided according to the cell types that make up the lining, different enterocytes being found along the length of the lining. These were suggested to be involved in secretion/excretion, food absorption and intracellular digestion (Nylund *et al*., 1992).

Bell *et al*. (2000) reported DAB staining of the midgut epithelial cells, indicating the presence of H_2O_2 degrading enzymes in the gut wall. Enzymes suggested, though not confirmed, by Bell *et al*. (2000) to generate a reaction with the DAB stain included

endogenous peroxidases, catalase and cytochrome oxidase. *Lepeophtheirus salmonis* adults frequently take blood meals from their salmon hosts and these enzymes may form an important part of dealing with the generation of reactive oxygen species by haem (Graça-Souza *et al*., 2006). Catalases and peroxidases have been shown to generate oxygen during the breakdown of H_2O_2 (George, 1947; Hernandez-Ruiz *et al*., 2001). This may result in the formation of oxygen bubbles in the gut, contributing to the observation of H_2O_2 treatment generating gas bubbles in *L. salmonis* resulting in potential temporary muscle paralysis and detachment of the parasite from the host (Bruno & Raynard, 1994). It is possible that in addition to a role in counteracting the damaging byproducts of haem breakdown, *L. salmonis* also generates H_2O_2 as a byproduct of breakdown of other food sources such as fish mucus. In mammals enzymes involved in the breakdown of certain amino-acids and fatty acids produce H_2O_2 as a byproduct. Due to the damaging nature of H_2O_2 to tissues these reactions are often contained within organelles called peroxisomes which also contain H_2O_2 degrading enzymes (Alberts *et al*., 2002; Smith & Aitchison, 2013). Peroxisomes have been recorded in crustacea (Lobodacunha, 1995, Orbea *et al*., 2000). The presence of these organelles in caligid copepods including *L. salmonis* is not known though expression of putative ABCD genes (a subfamily of ATP–binding cassette (ABC) proteins) which are associated with peroxisomes in other organisms, has been found in chalimus stages of *Caligus rogercresseyi* exposed to chemotherapeutants (Valenzuela-Muñoz *et al*., 2015). Antibodies against P-glycoprotein, an ATP-binding cassette protein, were found to bind to the epithelial lining of the *L. salmonis* gut (Tribble *et al*., 2007). A role for P-glycoprotein in response to H_2O_2 is not clear, with variable induction levels in response to H_2O_2 exposure being observed in higher organisms (Gray, 2005; Ziemann *et al*., 1999).

An interesting observation by transmission electron microscopy (TEM) in occasional feeding *L. salmonis* was the presence of bacteria both between the microvilli of midgut cells lining the lumen and inside enterocytes. In addition to these internal bacteria, bacteria are associated with external surfaces of the lice (Nylund 1992; Barker *et al.*, 2009). This was taken to indicate that *L. salmonis* might be passive carriers of bacteria surviving in the environment they inhabit or alternatively be acting as active or passive carriers of fish pathogenic bacteria, such as *Vibrio anguillarum* and *V.* (now *Aliivibrio*) *salmonicida* (Hoff *et al.*, 1989). Bacterial species can produce catalase enzymes as part of their defence against ROS (Ma *et al*., 1992; Chelikani *et al*., 2004). These enzymes catalyse the production of water and oxygen from H_2O_2. It is possible therefore, that bacteria associated with lice could act to reduce the concentration of H_2O_2 in the immediate environment.

Mouth/mode of feeding

The mouth and mouth-tube of caligid copepods and *Caligus curtis* in particular, a parasite of gadoids, has been described by Kabata (1974). The mouth tube or cone

comprises a forward facing labrum and hindmost labium, the forward facing labrum being supplied with internal musculature to lever the mouth tube forward over the fish skin surface. Mounted at the lowest forward edge of the labium there are up to 100 fine teeth on a divided bar, the strigil, which is set in front of a row of coarser teeth on the mandible. A rhythmic fluctuation of pressure effecting application of the labium into the fish skin allows the divided strigil bar to move apart so that the teeth of the two bars bring about fine sideways sawing movements into the fish skin. Movements of the upper and lower mandibles of the mouth tube then bring about the movement of skin debris up the mouth tube to the mouth.

Again, Bell *et al*. (2000) described the presence of DAB stained cells in glands associated with the mouth tube, indicating the presence of H_2O_2 degrading enzymes. As with the enzymes observed in cells of the *L. salmonis* midgut, these may be involved in breakdown of H_2O_2 generated during catalytic reactions of amino acids and fatty acids (Bell *et al.*, 2000), or haem breakdown during feeding. This would indicate that while excessive H_2O_2 might have detrimental effects, H_2O_2 uptake through feeding during treatments might be counteracted to a certain extent within the *L. salmonis* gut and mouth-tube.

Cuticle

The cuticle of arthropods is secreted by a basal layer of actively dividing epidermal cells which constitute the epidermis. It consists of an outer epicuticle, with surface cement and wax acting as an aqueous barrier, overlying an outer exocuticle and endocuticle made of chitin and either hardened or unhardened proteins[2]. The fine structure of the cuticle of free-living and parasitic copepods was reviewed by Bresciani (1986) which the reader should consult for information on the ultrastructure of the many diverse families of copepods parasitic on fishes (Boxshall, 2005 Fig. 2).

The cuticle of the chalimus II stage larva of *L. salmonis* is of the standard four layers and 'likely to constitute an important barrier to the action of externally applied pesticides' as reported by Bron *et al.* (2000a). These authors reported that there was no evidence of subcuticular secretory cells or glands within the epidermis, nor were pore canals observed in the cuticle. The absence of pore canals had also been reported previously for larval stages of brown shrimp and the authors made the point that the cuticle structure of larval *L. salmonis* stages may not reflect that of adult stages and differences in structure may account for life stage differences in susceptibility to chemotherapeutants. Bron *et al*. (2000a) describe a thin layer of mucoid appearance on the surface of the *L. salmonis* chalimus epicuticle. The epicuticle itself consisted

[2]Biology of the Arthropod Cuticle (2012) A.C. Neville https://books.google.co.uk/books?id=VQHtCAAAQBAJ&pg=PA408&lpg=PA408&dq=filshie+arthropod&source=bl&ots=soSPnwDqPw&sig=ODUKKUZWuO4uVCMZMZlIPPpXT58&hl=en&sa=X&ei=9BhSVYXFPIPq7AaF4IGACA&ved=0CCEQ6AEwAA#v=onepage&q=filshie%20arthropod&f=false accessed 12/05/2015

of four distinct layers and this is in line with general findings for arthropods, where the epiculticle is divided into layers of “cement”, wax, external epicuticle, internal epicuticle, going from the external surface to internal surface respectively. This suggests that the epicuticle, providing an outer hydrophobic external layer, would form a natural barrier to the ingression of water-soluble H_2O_2. However, certain areas of the epicuticle in arthropods lack the waxy layer to allow for gaseous exchange (Ruppert *et al*., 2004). Bron *et al*. (2000a) also noted near absence of a procuticle layer around foregut, hindgut and joint articulations and setae which may increase permeability to dissolved solutes in these regions. Additional TEM and biochemical analysis of the *L. salmonis* cuticle in different life stages would be beneficial to understand likely susceptibility to chemotherapeutants of different formulations.

Plausibly, there are also endogenous peroxidases, catalase or cytochrome oxidase associated with the cuticle of *L. salmonis* and *C. elongatus* based on DAB staining, (Bell *et al*., 2000). Bell *et al*. described discrete DAB staining regions closely associated with the dorsal cuticle of *L. salmonis*, referred to as ‘dorsal surface regions (DSR)’. These increased in number from chalimus to adult stages, with sexual dimorphism evident with respect to distribution in adult lice. If DSR do produce enzymes capable of degrading H_2O_2 then this observation may reflect reasons for differences in male and female susceptibility to H_2O_2 treatment observed in some studies (McAndrew *et al*., 1998). In relation to the cuticle and protection against H_2O_2, positive granular DAB staining material (potentially containing enzymes) was also observed on the surface of the cuticle, thought to have originated, via ducts, from exocrine glands. Bell *et al*. (2000) suggested that these excretions may have a role in sea lice survival in keeping the surface cuticle free of contaminating organisms. Gresty & Warren (1993) demonstrated the presence of ciliate epibionts on the surface of *L. salmonis* using scanning electron microscopy (SEM). If the excretions contain H_2O_2 reducing enzymes, based on positive DAB staining, then this again may help to reduce damaging effects of external H_2O_2 anti-sea lice treatment.

Cuticle and moulting

A key question centering on moulting is whether at the time of shedding of the exoskeleton the cuticle is more or less sensitive to H_2O_2. There is no precise information in the literature on testing between-instar life stages of *L. salmonis* for H_2O_2 susceptibility but the finding of H_2O_2 degrading enzymes would suggest that these help protect the developing new cuticle.

Bron *et al*. (2000b) described the moult cycle of the *L. salmonis* chalimus phase in order to provide a baseline for future comparative studies examining changes in cuticle structure/moulting following exposure to exogenous compounds or from the use of vaccines. An experimental approach was taken to allow copepodids to infect Atlantic salmon, then later chalimus II stages were fixed for transmission electron microscopy

(TEM) to study the dynamics of the moulting cycle. Skinner (1962) described moulting of the chalimus larva as: shedding of the old cuticle (ecdysis); followed by post-moult (metecdysis), during which time the new endocuticle is formed; then intermoult (anecdysis), a resting phase that can be quite long; then lastly premoult (proecdysis), when the old cuticle separates from the epidermis beneath. From the TEM observations of *L. salmonis* chalimus stage, it was clear that the new endocuticle, with an electron-dense epicuticle, was formed completely and early in the post-moult stage. One of the main conclusions was that there was no resting phase in *L. salmonis* exoskeleton formation.

Appendages

Using the stain, DAB, Bron (1993) first reported that thoracic appendages stained strongly for H_2O_2-degrading enzymes. Bell *et al.* (2000) also found DAB staining of cells in the glands in the swimming legs in the mobile stages. These authors suggested that the selective advantage of having glandular enzymes is that the enzymes reduce epibiotic surface fouling by microorganisms on the surface of the cuticle particularly in the mobile stage where constancy of swimming is important. Again, either in relation to degrading H_2O_2 which has managed to enter the tissues, or in degrading external H_2O_2 at the surface (DAB staining material having been seen on preadult caudal rami and on the caudal rami, thoracic legs and marginal membranes of the cephalothorax of adults) (Bell *et al*., 2000), these enzymes may reduce effects of H_2O_2 treatment.

Frontal filament

The frontal filament attaches the chalimus stages of *L. salmonis* to the sea louse's host. It is also thought to occur in a modified form in the preadult stage, and internally in late stage copepodids (González-Alanis, 2000). It has been described in *L. salmonis* as a "short and stumpy stem" with a basal plate embedded in the host epidermis, adhering to the host skin basement membrane. The frontal filament is produced by the frontal organ, located centrally at the anterior margin of the dorsal shield. The frontal organ consists of a number of different cell groups or glands, and with a collecting and axial duct (Bron *et al*., 1991). The cells secrete "glue like" substances with polysaccharide and proteinaceous characteristics which form the plate and filament (Bron *et al*., 1991). The stem part of the filament contains an axial duct through its center and is covered externally by a lamina which is continuous with the cuticle of the chalimus larvae (Bron *et al*., 1991; Johnson & Albright, 1992). Bell *et al*. (2000) observed DAB staining in the "frontal gland complex" of *L. salmonis* which they suggested was similar to the filament producing glands described by Bron *et al*. (1991), with particularly strong staining in the regions analagous to the "A" glands in Bron *et al*. (1991).

Eggs

L. salmonis adult females produce two egg strings containing 500-1000 eggs and can produce up to 11 paired egg strings following a single mating (Heuch, 2000). A number of studies, summarised in section 3 above, have examined the effect of H_2O_2 treatment on egg viability, hatching success and subsequent viability of larval stages. A positive relationship between the degree of egg pigmentation (darker eggs indicating more mature stages) and subsequent successful hatching following H_2O_2 treatment was observed in a number of cases (McAndrew *et al*., 1998; Toovey & Lyndon, 2000) though unsuccessful hatching following treatment, irrespective of pigmentation, has also been reported (Aaen *et al*., 2014). Differences in membrane permeability between early and late stage eggs were suggested as a potential reason for poorer hatching success in early stage eggs (McAndrew *et al*., 1998). However, differences may also arise due to pigmentation. Sea lice eggs have been shown to contain canthaxanthin-like and astaxanthin carotenoid pigments (Noack *et al*., 1997). These pigments have been demonstrated to possess antioxidant properties and to reduce detrimental effects of H_2O_2 in mammalian cells (Nakajima *et al*., 2008). They are considered to play a role as antioxidants, among other functions, in copepodids, protecting against ultraviolet radiation (UVR) and lipid peroxidation (Andersson *et al*., 2003; Rhodes, 2007; Caramujo *et al*., 2012). Therefore, if pigmented eggs are indeed less susceptible to H_2O_2 treatments then antioxidant activity of pigment may also be responsible. Pigmented eggs also indicate greater maturity of eggs, which in turn may reflect more advanced development of cells/tissues in the sea lice embryos capable of H_2O_2 breakdown.

5. Reactive oxygen species (ROS): action and mitigation

General

The concept of oxidative stress is an important one to discuss for all living organisms, of relevance to the metabolism of invertebrates just as much as to vertebrates including man. Oxidative stress amounts to an excess in the production of oxidising agents and reactive oxygen species (ROS) in cells and tissues above the organism's ability to detoxify the ROS or reactive intermediates and repair the resulting damage. Imbalances to the normal redox state of cells, the dynamic exchange of electron donor and electron acceptor molecules in viable metabolising cells will naturally generate peroxides and free radicals that damage key cell constituents such as proteins, lipids and DNA. In essence, severe oxidative stress can cause cell death and even moderate oxidation can precipitate apoptosis, programmed cell death (Dröge 2002; Poljsak *et al*., 2013).

Setting the ROS in perspective for Crustacea, Holmblad & Söderhäll (1999) make the point that oxidative metabolism is a fundamental part of the metabolism of every living

cell but the cell must protect itself from the deleterious accumulation of reactive oxygen species, of which the superoxide anion $\cdot O_2^-$, H_2O_2 and the hydroxyl radical, •OH are the most damaging.

Pathways of mitigation of H_2O_2 including resistance/tolerance: ROS generated by H_2O_2.

Oxidative stress modulating enzymes

Antioxidant enzymes play an important role in protection of cells against free radical damage. In insects the key enzymes are: thioredoxin reductase (TrxR), reducing disulphide $TrxS_2$ to dithiol $Trx(SH)_2$, SODs and catalase (Graça-Souza *et al*., 2006). SODs are enzymes that alternately catalyze the dismutation or separation of $\cdot O_2^-$ into either molecular oxygen O_2 or H_2O_2. The mechanism of action is that the enzyme alternately removes or adds an electron to $\cdot O_2^-$ so converting the radical to less damaging species. SODs are widespread in cells and tissues and represent a very important antioxidant enzymatic defense for all cells exposed to oxygen. H_2O_2 generated during SOD dismutation of $\cdot O_2^-$ is in turn converted to H_2O and O_2 by catalase. However, to mitigate/minimize the formation of •OH the organism needs sufficient of the antioxidant enzymes.

L. salmonis: possible resistance mechanisms to H_2O_2.

How then might resistance to H_2O_2 be developed in *L. salmonis*? Treasurer *et al*. (2000b) outlined two probable causes of resistance from field trials and experimental treatments:

(1) selection for sea lice with a thicker cuticle that provides a barrier to penetration by H_2O_2.

(2) the presence of detoxifying enzymes such as catalase, glutathione reductase, SOD and glucose-6-phosphatase dehydrogenase.

Both causes are plausible but the need for further research in sea lice was recommended. This is the only reference found to suggest theories of resistance to H_2O_2 in *L. salmonis*. This is an area needing further biochemical and morphological research.

Gene sequences have been identified in the *L. salmonis* genome putatively considered to code for oxidative stress modulating enzymes e.g. SOD 1, phospholipid-hydroperoxide glutathione peroxidase (phgp) and peroxiredoxin -4 and -6**.** Table 4 lists details of gene sequences from *L. salmonis* considered to represent enzymes that are involved in negating the effects of ROS. Given the ubiquity of similarly functioning

genes across the different kingdoms, it is likely that they have similar functions in *L. salmonis*. The ability to counteract the effects of H_2O_2 by producing these enzymes and antioxidants is significant for understanding the mechanism of resistance of *L. salmonis* to H_2O_2 and planning for new strategies to mitigate sea lice infestation on farmed Atlantic salmon.

Table 4. Crustacean and arthropod examples of oxidant and antioxidant: enzymes and genes reported

Gene or Enzyme	**Oxidant/Substrate**	**Antioxidant**	**Species/ Reference**
Gene	Superoxide anion	Superoxide dismutase 1	*L. salmonis* Yasuike. *et al. Genbank* BT077716
Gene	Superoxide anion	Superoxide dismutase (extracellular)	*L.salmonis* Yasuike *et al*. Genbank BT077648
Gene	Superoxide anion	Superoxide dismutase	*Drosophila* Graça-Souza *et al.* (2006)
Enzyme	Superoxide anion	Superoxide dismutase	*Rhodnius prolixus* Graça-Souza *et al.* (2006)
Enzyme	Hydrogen peroxide	Catalase	*Rhodnius prolixus* Graça-Souza *et al.* (2006)
Gene	Hydrogen peroxide	Catalase	*Drosophila* In Graça-Souza *et al.* (2006)
Gene	Hydrogen peroxide	Peroxiredoxin-4 & 6	*L. salmonis* Yasuike *et al*. *Genbank* BT121969, BT121155
Enzyme	Haem	Haem-peroxidase	*Rhodnius prolixus Ribeiro* (1998)
Gene	Thioredoxin	Thioredoxin reductase	*Drosophila* Graça-Souza *et al.* (2006)
Gene	Thioredoxin	Thioredoxin reductase	*Anopheles gambiae* Graça-Souza *et al.* (2006)
Gene	Phospholipid-hydroperoxide glutathione peroxide	Phospholipid-hydroperoxide glutathione peroxidase	*L. salmonis* Eichner *et al*. (2008) *Genbank* EF490927

Recently the first report of reduced sensitivity or resistance towards H_2O_2 in *L. salmonis* in Norway showed conclusively, using a bioassay protocol, that reduced sensitivity was strongly associated with genetic strains of *L. salmonis* (Helgesen *et al*., 2015). Moreover, reduced sensitivity in progeny of the strains was observed showing that the resistance trait was hereditary. This reinforces the need to understand

resistance mechanisms to H_2O_2 in *L. salmonis* to improve predictions on resistance emergence and strategies to reduce such emergence.

Other organisms: resistance mechanisms to H_2O_2

(i) Oxidative stress genes and their modulation in a marine copepod Tigriopus japonicus

The intertidal marine copepod *T. japonicus* has been recognised as a relevant model species for marine pollution and xenobiotics testing. Lee *et al*. (2008) showed that when expression of glutathione S-transferase (*GST*) genes was studied by quantitative real-time PCR, expression of *GST*-Sigma was highly upregulated when *T. japonicus* was exposed to 1-4 mM H_2O_2 for 96 h. GSTs are a family of phase II metabolic isozymes known for their ability to catalyze the conjugation of the reduced form of glutathione, via a sulhydryl group, to xenobiotic substances for the purpose of detoxification (Sheehan *et al*., 2001).

(ii) Oxidative stress of haem in blood-sucking arthropods and how it is mitigated

Because of the fast-acting toxicity of haem, blood-feeding arthropods have evolved a variety of biochemical strategies to mitigate and avoid the effects of haem toxicity: haem aggregation, haem degradation, antioxidant enzymes, haem-binding proteins and low molecular weight antioxidants (Graça-Sousa *et al*., 2006).

Haem toxicity is partly due to the generation of the free-radicals alkoxyl and peroxyl as an outcome of lipid peroxidation (Graça-Sousa *et al*., 2006) but is also due to haem serving as a cofactor for the hydroperoxidases: catalases, which liberate molecular oxygen from H_2O_2 (Vlasits *et al*., 2007):

$$PorFE^{III} + H_2O_2 \rightarrow Por^{\cdot+}Fe^{IV}=O + H_2O \quad \text{(reaction 1)}$$
$$Por^{\cdot+}Fe^{IV}=O + H_2O_2 \rightarrow Por\ Fe^{III} + O_2 + H_2O \quad \text{(reaction 2)}$$

Therefore, available haem may promote the toxic effect of H_2O_2 treatment through increasing the production of molecular oxygen from H_2O_2. This will also apply to endogenous H_2O_2 generated by *L. salmonis* during various cellular functions, with potential examples described in section 2. As such, it is to the advantage of adult *L. salmonis* to have adequate methods to remove haem. The reactions above promote free radical damage due to haem and it is to the advantage of the haematophagous organism to sequester or degrade haem immediately after its release by proteolysis of haemoglobin, Hb. Haem aggregation in *Plasmodium*, the causative organism of malaria, is centred on the digestive vacuole and there is a comprehensive set of data characterising a special kind of haem aggregate, the ‘malaria pigment’ or Hz. In Hz, haem molecules form dimers stabilised by iron-carboxylate bonds and the dimers interact through hydrogen bonds between the propionate side chains of the porphyrin

ring. Hz is not unique to *Plasmodium* but it has also been demonstrated in other blood-feeding organisms such as the triatomine insect *Rhodnius prolixus*, (where 70% of the haem in the mid-gut is in the form of Hz), the helminth *Schistosoma mansoni*, the causative organism of human Schistosomiasis and the parasitic protozoan *Haemoproteus columbae.* So a variety of blood feeders have evolved this biochemical adaptation quite independently to avoid haem toxicity (Graça-Souza *et al*., 2006).

Haem degradation is an important route of haem removal and this is evident from the kissing bug *R. prolixus* and the mosquito *Aedes aegypti*. (Paiva-Silva *et al*., 2006; Pereira *et al*., 2007). Another route for haem mitigation and sequestering is the role for haem-binding proteins in blood-feeding arthropods. This is evident from the *Rhodnius* haem-binding protein (RHDP) and by the haem lipoprotein (HeLp) from the haemolymph of the cattle tick, *Boophilus microplus* (Maya-Monteiro *et al*., 2000)

Low molecular mass antioxidants also protect against oxidative damage in insects. Herbivorous arthropods easily acquire tocopherol, carotene and flavonoids from the diet, all potent antioxidants. Urate levels were also found to be high in the haemocoel of *Rhodnius* (ten times that of human plasma) and as a very effective antioxidant this strongly mitigates endogenous and haem-induced accumulation of lipid peroxidation products (Souza *et al*., 1997). Sea lice contain canthaxanthin-like and astaxanthin carotenoid pigments (Noack *et al*., 1997) which display antioxidant properties in mammalian systems. It is likely that the sea lice acquire these pigments from their salmon hosts.

These well studied strategies discussed above in haematophagous organisms have potential relevance to Crustacea and the blood feeder *L. salmonis*. Mitigating the effects of interaction between haem and H_2O_2, through reducing the level of their interaction, might offer another area where sea lice may adapt and develop increased resistance to H_2O_2 treatment. To speculate further, higher numbers of female lice containing blood meals were observed compared to males by Brandal *et al*. (1976) (in Flik and Wiegertjes, 2004), though reported evidence for this as a general finding is limited. However, if combining the above observations regarding haem's promoting the efficacy of H_2O_2 treatment through acting as a catalase cofactor in the breakdown of H_2O_2 and generation of oxygen, and female sea lice more commonly having blood feeds, then this might offer another reason why in some studies (McAndrew *et al*., 1998) H_2O_2 treatment seemed to have a greater impact on adult female sea lice compared to males. Fewer females reattached to hosts within a 24 hour period following treatment. McAndrew *et al.* 1998 also reported that emboli formation was more common in adult female lice but did not say if these lice contained blood meals. None of the above strategies have been reported in the literature on *L. salmonis* so this represents a knowledge gap.

(iii) Oxidative stress from ultraviolet radiation (UVR) in a freshwater copepod and its mitigation

The field of freshwater limnology research has shown how fast enzyme responses to UVR are adaptive for the ecological fitness and survival of freshwater copepods. Souza *et al*. (2012) reported that short-duration exposure to UVR causes the freshwater calanoid copepod *Eudiaptomus gracilis* to rapidly activate production of enzymes that either prevent widespread collateral peroxidation, i.e. GST, or regulate apoptotic cell death i.e. caspase 3 or facilitate neuronal transmission i.e. cholinesterase. Souza *et al*. (2012) emphasised that these enzymes must act together to reduce the stress level of the organism but in doing so they markedly reduce UVR-induced trauma within a short time scale, i.e. 6 hours, within a long day of brilliant sunshine. Through this fast enzyme activation the negative effects of oxidative stress from UVR can be quickly mitigated to benefit adaptive survival.

6. Summary

Effect of H_2O_2 on sea lice

In relation to those sea lice stages found on farmed salmon, the effect of hydrogen peroxide has been tested on attached copepodid to adult stages including eggs. Hydrogen peroxide results in egg mortality or greatly reduced hatching rates. It does not appear to affect development of larval stages through to adults overall, though rate of maturation is delayed. Hydrogen peroxide causes mobile stages to detach, but they can reattach. As such, confidence that detached stages do not re-infect or infect other farms is required. Some appropriate field based studies may be useful to confirm. Continued efforts to develop collection systems for detached mobile sea lice stages after treatment should be encouraged. Knowledge of the self-infection and connectivity probabilities for farms, based on local hydrodynamics and knowledge of sea lice dispersal, may help inform on risk from detached, but still viable, sea lice. This may influence H_2O_2 treatment strategies for certain sites.

Can the literature explain the mode of action of H_2O_2 as a sea lice treatment, especially for Lepeophtheirus and Caligus genera, in terms of target physiological process and/or structures?

Very little is reported about the actual effects of H_2O_2 on sea lice, apart from observed formation of bubbles in the haemolymph which is suggested to give rise to detachment due to temporary muscle paralysis and positive buoyancy from the generated gas forcing sea lice to the water surface. Though not confirmed it is likely that these bubbles are oxygen bubbles, formed during breakdown of the H_2O_2, though a second possibility is gaseous H_2O_2 released out of solution.

To advance knowledge on effects on *L. salmonis*, it would be beneficial to look in more detail at the biochemistry of different organs, to complement previous ultrastructural investigations. This could help improve e.g. treatment formulations. It may also be beneficial to look at histopathological changes (light and electron microscopy) in different sea lice stages following H_2O_2 treatment using parameters (dose, time, temperature) currently used on aquaculture sites. This could be extended to looking at the effects of longer treatments at lower doses than currently used, the success of which was illustrated by Aaen *et al*. (2014) for treatment of eggs.

Can the literature explain the mode of action of H_2O_2 on sea lice in relation to other organisms, especially other crustacea, and arthropods related in evolutionary terms to crustaceans?

The mode of action of reactive oxygen species is known, and because of the conserved nature of pathways involved in both generating ROS and mitigating their effects, findings from other organisms can be used to give an informed opinion on potential effects on *L. salmonis*. Apart from the role of (potentially) molecular oxygen mentioned above which has also been suggested as responsible for death in protists

and monogeneans following H_2O_2 exposure, reactive oxygen species result in damage to membranes, cellular proteins and DNA which over time can reduce the viability of exposed organisms and their reproductive output. While hatching and developmental effects have been shown on eggs and larval stages of *L. salmonis*, there is lack of knowledge on how H_2O_2 treatment affects subsequent egg production and larvae viability over the life span of exposed juvenile/adult lice. The extent of this effect and consequences for population numbers and infection dynamics in the marine environment is unknown. The effect of light (UV) levels at time of treatment could also be investigated; additional ROS generation from UV radiation may have a synergistic effect on the treatment, although increases in antioxidant enzymes to counteract increased UVR are known to occur in copepods. Canthaxanthin-like and astaxanthin carotenoid pigments have been reported in sea lice, likely acquired from salmon hosts which in turn acquire the pigments through their diet. These enzymes display antioxidant properties in mammalian systems. It may be worthwhile considering whether current salmon diet composition could contribute to response of sea lice to H_2O_2 treatments.

Can the literature on H_2O_2 occurrences in nature provide an indication of processes in sea lice which may involve H_2O_2 and thus provide the organism with mitigating enzymes or pathways which could reduce the efficacy of H_2O_2 as a chemotherapeutant?

There are numerous potential ways in which sea lice may reduce impact of H_2O_2 treatment, including development of thicker cuticles, expression of proteins involved in degradation and repair, improvement of haem sequestering. These could represent candidates for monitoring in relation to H_2O_2 resistance so that basis for and potential rate of resistance development can be better monitored and understood. Genes encoding for putative antioxidant enzymes have been identified in the *L. salmonis* genome, and there is some evidence of *L. salmonis* ability to develop resistance against H_2O_2, the resistance trait in one study demonstrated to be hereditary.

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