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- (54) Title: METHOD FOR DETECTING HYDROGEN PEROXIDE RESISTANCE IN CRUSTACEANS

Figure 1

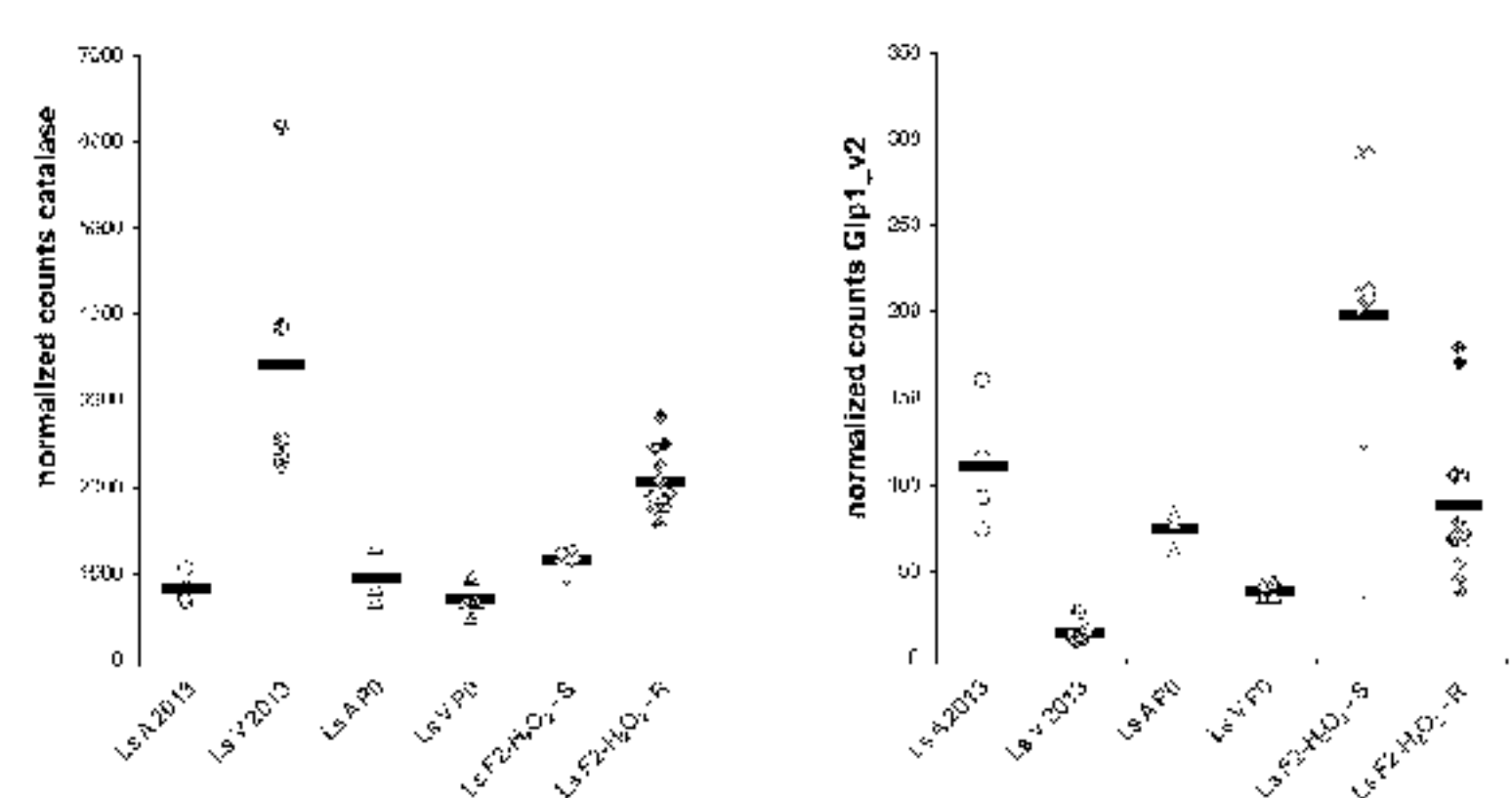
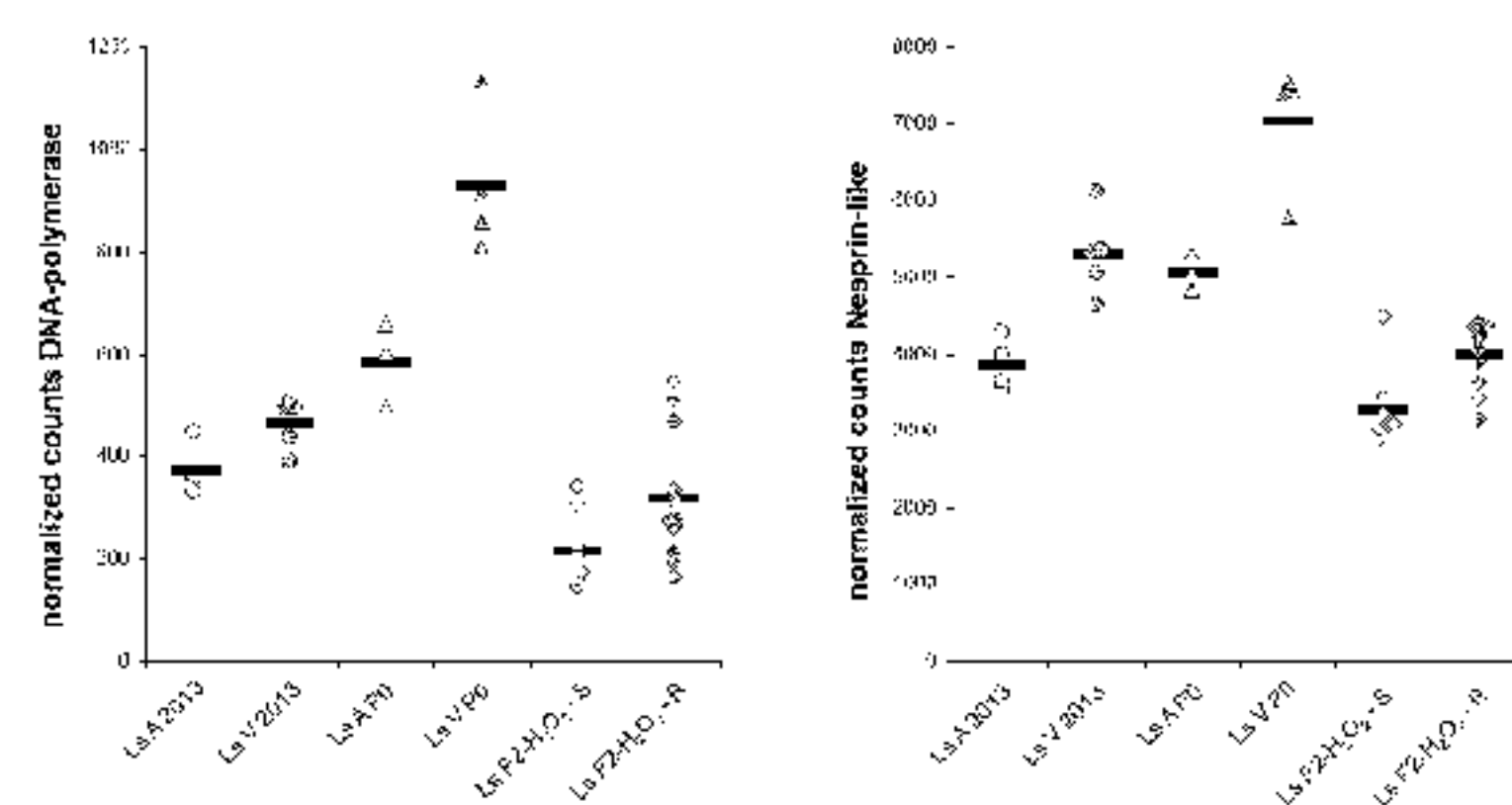


Figure 1 cont.



- (57) Abstract: The present invention relates to a method for detecting whether or not crustaceans, in particular sea lice, such as *Lepeophtheirus salmonis* and *Caligus rogercresseyi* is resistant towards hydrogen peroxide (H₂O₂). In particular, the method of the invention relates to the detection of the expression level of genes found to be either upregulated or downregulated in sea lice resistant towards H₂O₂. The present invention furthermore relates to oligonucleotide sequences and kits comprising oligonucleotide sequences useful in the method of the present invention.

TITLE: Method for detecting hydrogen peroxide resistance in crustaceans

Field of the invention

5 The present invention relates to a method for detecting whether or not crustaceans, in particular sea lice, such as *Lepeophtheirus salmonis* and *Caligus rogercresseyi* is resistant towards hydrogen peroxide (H₂O₂). In particular, the method of the invention relates to the detection of the expression level of genes found to be either upregulated or downregulated in sea lice resistant towards H₂O₂. The present invention furthermore relates to oligonucleotide sequences and kits useful in the method of the present invention.

10 Background of the invention

Sea lice are naturally occurring marine ectoparasites that attach to the skin and feed on the mucus, blood and surface tissues of salmon and other species of fish. Sea lice (*Lepeophtheirus salmonis* and *Caligus* spp.) are the major pathogens affecting global salmon farming industry and have a significant impact in many areas. The annual loss was for the global salmon farming industry were estimated to €300 million in 2009 (Costello M. J. (2009), The global economic cost of sea lice to the salmonid farming industry. Journal of Fish Diseases. 32. 115–118), and in 2013 it was estimated to USD 300 mill in Chile alone (Augusti C., Bravo S., Contreras G., Bakke M.J., Helgesen K.O., Winkler C., Silva M.T., Mendosa J., Horsberg T.E. (2016), Sensitivity assessment of *Caligus rogercresseyi* to anti-louse chemicals in relation to treatment efficacy in Chilean salmonid farms. Aquaculture 458, 195-205).

Thus, aquaculture industry relays heavily on a few chemotherapeutants for sea lice control. Emerging resistance development to these drugs increase the necessity to develop new treatment methods (biological, prophylactic and drugs) and tools to avoid increased loss due to sea lice and to ensure a sustainable salmon farming industry in the future. Control measures have relied upon a limited number of chemotherapeutants since the 1970s. Parasite resistance and reduced efficacy have now been reported for the majority of these compounds (Sevatdal S., et al. (2005), “Monitoring of the sensitivity of sea lice (*Lepeophtheirus salmonis*) to pyrethroids in Norway, Ireland and Scotland using bioassays and probit modeling”, Aquaculture 244, 19-27). A successful integrated louse-management strategy requires free access to a range of effective, chemically unrelated active ingredients deployed according to current best practice. Over-reliance on a limited number of products will lead, inevitably to resistance, which is difficult to counter.

35 Although various chemotherapeutants have been in use in the aquaculture industry for more than 35 years, it is only during the last 20 years that such use has been part of some kind of integrated pest management (IPM) system. Management practices include coordinated salmon production within a defined area, use of single year class of fish, limited production period, fallowing, coordinated restocking, use of wrasse, synchronized treatments during the winter and targeting female lice to reduce the impact of settlement during the spring (Pike A., Wadsworth S. L., (2000), “Sea Lice: A review. Advances in Parasitology”, Academic Press. 44. 232-337).

Further tools are required to progress towards a true IPM-system common to other forms of food production. One key to succeeding with an IPM is to develop tools for the management of resistance to the medicines in use (Brook K. (2009), “Considerations in developing an integrated pest management program for control of sea lice on farmed salmon in Pacific Canada”, Journal of Fish Diseases. 32, 59–73). Bioassays that require comprehensive numbers of parasites, equipment and labor were used to monitor sensitivity. However, these methods showed low sensitivity, and had limitations when comparing resistance in different regions, as well as predicting treatment efficacy. Resistance mechanisms can be identified in some cases, and molecular methods, with high precision, high-throughput potential and reduced total cost (Aaen et al., 2015, “Drug resistance in sea lice: a threat to salmonid aquaculture”, Trends in Parasitology, Vol 31, No. 2). Resistance towards organophosphates has been linked to Phe362Tyr mutation in the AChE coding gene. For pyrethroids several factors are likely to be involved in resistance, cytochrome P450 is involved in the detoxification of pyrethroids, as well as biomarkers in the mitochondrial DNA (Bakke et al., 2018, “Deltamethrin resistance in the salmon louse, *Lepeophtheirus salmonis* (Krøyer): Maternal inheritance and reduced apoptosis.” Scientific Reports, Vol. 8, 8450). Using these genes, it was demonstrated that medicinal treatments drive genetic selection towards a more resistant salmon lice population within a very short time span if there is no influx of sensitive genotypes (Jensen et al., 2017, “A selection study on laboratory-designed population of salmon lice (*Lepeophtheirus salmonis*) using organophosphate and pyrethroid pesticides”, PLOS one 12(5)e0178086).

PCR based methods for detection of resistance genes have been published for azamethiphos (EP 3 033 433 A1), pyrethroids (EP 3 030 673 A1, EP 3 030 674 A1) as well as the catalase gene for hydrogen peroxide resistance (EP 3 164 502 B1).

Hydrogen peroxide has been demonstrated to be the least harmful to non-target organisms, highly effective against certain stages of louse development and most environmentally responsible (Burridge, L. 2013, “A review of potential environmental risks associated with the use of pesticides to treat Atlantic salmon against infestations of sea lice in southwest New Brunswick, Canada”, DFO Can. Sci. Advis. Sec. Res. Doc. 2013/050. iv + 25 p). Interlox® Paramove® 50 is a commercially available hydrogen peroxide product for the treatment of sea lice. It contains 50% hydrogen peroxide.

Hydrogen peroxide is only efficacious on post-chalimus stages of sea lice; it must be used in conjunction with other pest management techniques to maximize treatment benefits. There are conflicting results regarding viability of sea lice post treatment as well as the ability of lice re-infection. Because there are no techniques currently developed to remove sea lice from a tarp treatment, timing of treatment to target post-chalimus stage lice is essential. Recent studies of egg viability and nauplii survival post-treatment do indicate that nauplii survival reaches 0 within days of hatch.

Hydrogen peroxide breaks down readily into water and is considered the anti-lice treatment with least environmental risk. Recent studies comparing the effects of Interlox® Paramove® 50, Salmosan®, and AlphaMax® on American lobster, mysid shrimp and sand shrimp showed that

Interox[®] Paramove[®] 50 had the least impact on these non-target organisms. Unlike other anti-louse chemotherapeutants, hydrogen peroxide does not have a withdrawal period.

- 5 Hydrogen peroxide is believed to be available for use in all major salmon farming countries. It was a common louse treatment in the 1990's but was subsequently replaced by in-feed louse treatments and other bath treatments until a recent resurgence. In 2009, the use of H₂O₂ in aquaculture in Norway was 308 tons, in 2010 it was 3071 tons and in 2012 it was 2538 tons, further it was 8262 tons in 2013, 31577 tons in 2014, 43 246 tons in 2015, 26597 tons in 2016 and 9277 tons in 2017 (cf. Norwegian Institute of Public Health 2018 www.fhi.no).
- 10 Organisms naturally produce hydrogen peroxide as a by-product of oxidative metabolism. Consequently, nearly all living things (specifically, all obligate and facultative aerobes) possess enzymes known as catalase and peroxidases, which harmlessly and catalytically decompose low concentrations of hydrogen peroxide to water and oxygen. Two theories
- 15 have been proposed to explain the therapeutic effects of hydrogen peroxide. The first is that bactericidal action is through the formation of hydroxyl radicals and its effect on DNA (Imlay J. A. (1987), "The mechanisms of toxicity of hydrogen peroxide", PhD Thesis, University of California, Berkeley). The second, proposed to explain toxicity to protists and monogeneans, is the liberation of molecular oxygen as a result of catalase action
- 20 (Schaperclaus et al., eds. (1979), *Fishkrankheiten*, 4th edn. Akademie-Verlag. Berlin).
- Resistance of insects to pesticides develops through genetic selection of individuals (Soderlund D.M. & Bloomquist J.R. (1990), "Molecular mechanisms of insecticide resistance. In: *Pesticide Resistance in Arthropods*" (ed. by R.T. Roush & B.E. Tabashnik),
- 25 pp. 58-96. Chapman & Hall, London) and, in lice, this may be selection for individuals with cuticle that provides a barrier to penetration by hydrogen peroxide or the presence of detoxifying enzymes such as catalase, glutathione reductase, glutathione synthetase, superoxide dismutase, and glucose-6-phosphate dehydrogenase. An alternative explanation could be prior induction as reported for *Aeromonas salmonicida* pre-exposed to low
- 30 concentrations of hydrogen peroxide (Barnes et al., (1999a) "Superoxide dismutase and catalase in *Photobacterium damsela* subsp. *piscicida* and their roles in resistance to reactive oxygen species", *Microbiology* 145, 483–494 & Barnes et al. (1999b), "Peroxide-inducible catalase in *Aeromonas salmonicida* subsp. *salmonicida* protects against exogenous hydrogen peroxide and killing by activated rainbow trout, *Oncorhynchus mykiss*, *L. macrophages*", *Microbial Pathogenesis* 26, 149–158). These bacteria had catalase activity 20-fold higher when subsequently exposed to higher concentrations than in un-induced cultures.
- 35 Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen (Chelikani et al., January 2004, "Diversity of structures and properties among catalases", *Cell. Mol. Life Sci.* 61 (2): 192–208). It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules
- 40

of hydrogen peroxide to water and oxygen each second (Goodsell (2004-09-01), "Catalase. Molecule of the Month", RCSB Protein Data Bank. Retrieved 2007-02-11).

5 The development of resistance by sea lice to medicines and its management is one of the main concerns in sea lice control, particularly when the range of medicines is limited. Resistance of sea lice to pesticides, organophosphates, pyrethroids and H₂O₂ is well established. Reduced sensitivity towards hydrogen peroxide (H₂O₂) was first reported in Scotland (Treasurer et al. (2000), "Resistance of sea lice, *Lepeophtheirus salmonis* (Kroyer), to hydrogen peroxide on farmed Atlantic salmon, *Salmo salar L*", Aquaculture Research, 31, 855-860), and from Norway in 2013 (Helgesen et al., 2015, 10 "First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway", Aquaculture Reports 1, 37-42). It has been documented that increased catalase activity plays a role in H₂O₂ resistance in salmon lice. Increased catalase expression and subsequent enhanced catalase activity is a mechanism for 15 H₂O₂ resistance in salmon lice, and molecular screening methods could be developed on this basis. (Helgesen et al., 2015 "First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway", Aquaculture Reports 1, 37-42). The sequence expressing the catalase gene and the methods for using this gene as a resistance marker for H₂O₂ resistance in sea lice is disclosed in European 20 Patent application EP 3 164 502 B1.

Data shows that the catalase gene is probably induced by the exposure of lice to H₂O₂. It has however been found that if H₂O₂ is not applied as sea lice treatment for a period of time, the catalase expressions could return to its normal expression values, similar to the 25 sensitive louse levels (unpublished data). Thus, there is a need for improved methods for detection of H₂O₂ resistance in sea lice.

Efficient, sensitive, stable and reliable methods for diagnosing resistance are crucial in order to manage and control drug resistance. Early detection of reduced sensitivity to a 30 chemical can enable effective countermeasures to be enforced at a time point when these have a greater probability of being effective. Therefore, accurate, stable and speedy identification of hydrogen peroxide (H₂O₂) resistant sea lice is crucial. Detection of hydrogen peroxide resistance prior to treatment, and the use of such analyses after treatment to evaluate treatment efficacy constitutes an important determinant for the 35 integrated pest management (IPM) in the aquaculture industry.

Summary of invention

The present invention is based on the surprising finding that resistance towards hydrogen peroxide commonly used to combat sea lice infestation is linked to the expression of a set 40 of genes that are either downregulated or upregulated in hydrogen peroxide resistant sea lice.

In particular, it has been found that the expression of a gene encoding an aquaglyceroporin (Glp1_v2) is downregulated in H₂O₂ resistant adult female sea lice.

Also, the endoplasmic reticulum resident protein 29 (ERP29) is downregulated in H₂O₂ resistant sea lice.

5 Furthermore, it has been found that the genes encoding a DNA polymerase (delta subunit 3) is upregulated in sea lice resistant to H₂O₂.

It has furthermore been found that expression of the gene encoding Nesprin-like is upregulated in H₂O₂ resistant sea lice.

10 It has furthermore been found that a gene encoding a yet unknown protein (herein named NA, SEQ ID No. 10) is upregulated in H₂O₂ resistant sea lice.

The sea lice that may be analysed according to the present invention is one or more copepods, e.g. belonging to the family Caligidae.

15 According to one embodiment, the copepod is selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi*.

20 According to one embodiment, the copepod is selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei* and *Caligus elongates*.

According to one embodiment, the copepod is *Caligus rogercresseyi*.

25 According to one embodiment the copepod is *Lepeophtheirus salmonis*.

According to **a first aspect**, a method is provided for the detection of hydrogen peroxide resistance in one or more adult female sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi* comprising the steps of:

- 30
- a) collecting one or more adult female sea lice from infested fish or water samples;
 - b) isolating genomic material from the collected sea lice; and
 - c) determining the expression level of at least one of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2),
- 35 endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.

According to one embodiment of the first aspect, said method is provided for the detection of hydrogen peroxide resistance in one or more sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei* and *Caligus elongatus*.

40 According to one embodiment of the first aspect, said method is provided for the detection of hydrogen peroxide resistance in one or more adult female sea lice wherein the sea lice is *Lepeophtheirus salmonis*.

According to one embodiment of the first aspect, said method is provided for the detection of hydrogen peroxide resistance in one or more adult female sea lice wherein the sea lice is *Caligus rogercresseyi*.

5 According to one embodiment of the first aspect, said method is provided for determining the expression level of at least one of the genes encoding a protein having a sequence selected from the group consisting of:

SEQ ID No. 2 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 2;

10 SEQ ID No. 4 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 4;

15 SEQ ID No. 6 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 6;

SEQ ID No. 8 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 8;

20 SEQ ID No. 10 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 10.

According one embodiment of the first aspect, said method is provided for determining the expression level of at least one of the genes comprising a sequence selected from the group consisting of:

25 SEQ ID No. 1 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 1;

30 ,SEQ ID No. 3 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 3;

SEQ ID No. 5 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 5;

35 SEQ ID No. 7 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 7;

SEQ ID No. 9 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 9.

- According to one embodiment of the first aspect, the method according to the present invention involves determination of the expression levels of at least **two of the genes** encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10 is determined.
- According to one embodiment of the first aspect, the method according to the present invention involves determination the expression levels of a gene encoding catalase in addition to one or more of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10. Said catalase gene can be selected from the group consisting of SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, and SEQ ID No. 14 or variants or fragments thereof being at least 70 % identical with SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, and SEQ ID No. 14, respectively.
- According to one embodiment of the first aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding aquaglyceroporin (Glp1_v2) is downregulated compared with the expression level of aquaglyceroporin (Glp1_v2) in one or more H₂O₂ sensitive sea lice.
- According to one embodiment of the first aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding endoplasmic reticulum resident protein 29 (ERP29) is downregulated compared with the expression level of endoplasmic reticulum resident protein 29 (ERP29) in one or more H₂O₂ sensitive sea lice.
- According to one embodiment of the first aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding DNA polymerase (delta subunit 3) is upregulated compared with the expression level of DNA polymerase (delta subunit 3) in one or more H₂O₂ sensitive sea lice.
- According to one embodiment of the first aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding nesprin-like is upregulated compared with the expression level of nesprin-like in one or more H₂O₂ sensitive sea lice.
- According to one embodiment of the first aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding and the protein of SEQ ID No. 10 is upregulated compared with the expression level of and the protein of SEQ ID No. 10 in one or more H₂O₂ sensitive sea lice.
- According to one embodiment of the first aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more adult female sea lice according to the first aspect, wherein step (c) of the present invention comprise the steps of:

(c1) providing one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 9 or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 9, respectively; and

(c2) determining the expression level of at least one of the genes selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, or variants or fragments thereof being at least 70 % identical with the entire length of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, respectively, by measuring the RNA expression level of said genes in the collected sea lice, wherein said sea lice is resistant to H₂O₂ if having reduced levels of expression of the genes encoding aquaglyceroporin (Glp1_v2) and/or endoplasmic reticulum resident protein 29 (ERP29), and/or elevated levels of RNA-expression of the genes encoding DNA polymerase (delta subunit 3), and/or nesprin-like and/or the protein of SEQ ID No. 10 compared with one or more non-resistant sea lice.

According to one embodiment of the first aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more female adult sea lice according to the first aspect of the present invention, comprising the steps of

(a) collecting one or more sea lice from infested fish or water samples;
 (b) isolating DNA or RNA from the collected sea lice;
 (c) providing a pair of PCR primers capable of hybridising under stringent conditions to at least one of the genes selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, respectively;
 (d) performing PCR on the isolated DNA or RNA using the pair of PCR primers of (c);
 (e) determining the expression level of one or more of said genes comparing the level of the expression of said genes in the collected sea lice with the level of control standard or a gene expression sample control.

The expression of the one or more genes in the collected sea lice or water sample is according to one aspect of the invention compared with the expression level of said genes in one or more non-resistant sea lice.

According to **a second aspect**, a method is provided for the detection of hydrogen peroxide resistance in one or more sea lice wherein the sea lice is of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus* and *Caligus rogercresseyi* comprising the steps of:

a) collecting one or more sea lice from infested fish or water samples;
 b) isolating genomic material from any life stage of the collected sea lice; and
 c) determining the expression level of at least one of the genes encoding the proteins selected from the group consisting of endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.

According to one embodiment of the second aspect, said method is provided for determining the expression level of at least one of the genes encoding a protein having a sequence selected from the group consisting of:

5 SEQ ID No. 4 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 4;

SEQ ID No. 6 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 6;

10 SEQ ID No. 8 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 8;

15 SEQ ID No. 10 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 10.

According one embodiment of the second aspect, said method is provided for determining the expression level of at least one of the genes comprising a sequence selected from the group consisting of:

20 SEQ ID No. 3 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 3;

SEQ ID No. 5 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 5;

25 SEQ ID No. 7 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 7;

30 SEQ ID No. 9 or variants or fragments thereof being at least 70 % identical, such as 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with SEQ ID No. 9.

35 According to one embodiment of the second aspect, the method according to the present invention involves determination of the expression levels of at least **two of the genes** encoding the proteins selected from the group consisting of endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10 is determined.

40 According to one embodiment of the second aspect, the method according to the present invention involves determination the expression levels of a gene encoding catalase in addition to one or more of the genes encoding the proteins selected from the group consisting of endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10. Said catalase gene can be selected from the group consisting of SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, and

SEQ ID No. 14 or variants or fragments thereof being at least 70 % identical with SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, and SEQ ID No. 14, respectively.

5 According to one embodiment of the second aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding endoplasmic reticulum resident protein 29 (ERP29) is downregulated compared with the expression level of endoplasmic reticulum resident protein 29 (ERP29) in one or more H₂O₂ sensitive sea lice.

10 According to one embodiment of the second aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding DNA polymerase (delta subunit 3) is upregulated compared with the expression level of DNA polymerase (delta subunit 3) in one or more H₂O₂ sensitive sea lice.

15 According to one embodiment of the second aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding nesprin-like is upregulated compared with the expression level of nesprin-like in one or more H₂O₂ sensitive sea lice.

20 According to one embodiment of the second aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein the one or more sea lice is found to be resistant if the expression of the gene encoding and the protein of SEQ ID No. 10 is upregulated compared with the expression level of and the protein of SEQ ID No. 10 in one or more H₂O₂ sensitive sea lice.

25 According to one embodiment of the second aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more sea lice, wherein step (c) of the fourth aspect of the present invention comprise the steps of:

30 (c1) providing one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence of SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, SEQ ID No. 9 or a complementary oligonucleotide of SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, SEQ ID No. 9, respectively; and

35 (c2) determining the expression level of at least one of the genes selected from the group consisting of SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, or variants or fragments thereof being at least 70 % identical with the entire length of SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, respectively, by measuring the RNA expression level of said genes in the collected sea lice, wherein said sea lice is resistant to H₂O₂ if having reduced levels of expression of the genes encoding endoplasmic reticulum resident protein 29 (ERP29), and/or elevated levels of
40 RNA-expression of the genes encoding DNA polymerase (delta subunit 3), and/or nesprin-like and/or the protein of SEQ ID No. 10 compared with one or more non-resistant sea lice.

According to one embodiment of the second aspect, the present invention provides a method for determination of H₂O₂ resistance in one or more **sea lice** according to the second aspect of the present invention, comprising the steps of

- 5 (a) collecting one or more sea lice from infested fish or water samples;
- (b) isolating DNA or RNA from the collected sea lice;
- (c) providing a pair of PCR primers capable of hybridizing under stringent conditions to at least one of the genes selected from the group consisting of SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, respectively;
- 10 (d) performing PCR on the isolated DNA or RNA using the pair of PCR primers of (c);
- (e) determining the expression level of one or more of said genes comparing the level of the expression of said genes in the collected sea lice with the level of control standard or a gene expression sample control.

15 The expression of the one or more genes in the collected sea lice or water sample is according to one aspect of the invention compared with the expression level of said genes in one or more non-resistant sea lice.

20 In a **third aspect** the present invention furthermore provides the use of one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, respectively, for *in vitro* determination of hydrogen peroxide resistance in sea lice according to the method of the first aspect.

25 In one embodiment according to the third aspect, the one or more isolated oligonucleotide sequence is used for determination of hydrogen peroxide resistance in adult female sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*.

30 According to a **fourth aspect** the present invention furthermore provides the use of one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, or a complementary oligonucleotide of SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, respectively, for *in vitro* determination of hydrogen peroxide resistance in sea lice according to the second aspect wherein the sea lice is selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus* and *Caligus rogercresseyi*,

40 According to yet another aspect, one or more mentioned isolated oligonucleotide sequence according to the third or fourth aspect may be used together with isolated oligonucleotide sequence for determining catalase expression wherein the isolated oligonucleotide sequence used in determining hydroxy peroxide resistance according to the present use is selected from the group consisting of SEQ ID No. 18, SEQ ID No. 19, SEQ ID NO. 20 and
45 SEQ ID No. 21.

5 The present invention furthermore provides according to yet another aspect a kit for detection of hydrogen peroxide resistance in sea lice comprising one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9, respectively, and wherein the one or more isolated oligonucleotide sequence is not SEQ ID No. 23.

10 The present invention provides according to yet a further aspect a DNA molecule encoding a protein comprising an amino acid sequence selected from the group consisting of SEQ ID No. 4, SEQ ID No. 6. SEQ ID No. 8, and SEQ ID No. 10.

15 According to yet another aspect, a DNA molecule is provided comprising a sequence selected from the group consisting of
SEQ ID No. 1 or variants thereof being at least 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with the entire length of SEQ ID No. 1,
SEQ ID No. 3 or variants thereof being at least 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with the entire length of SEQ ID No. 3,
20 SEQ ID No. 5 or variants thereof being at least 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with the entire length of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5
SEQ ID No. 7 or variants thereof being at least 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with the entire length of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5.
25 SEQ ID No. 7, and
SEQ ID No. 9, or variants thereof being at least 80 % identical, such as 85% identical, such as 90% identical, such as 92% identical, such as 95% identical, such as 98% identical, such as 99% identical with the entire length of SEQ ID No. 9.
30

35 According to yet another aspect, a DNA molecule is provided comprising a sequence selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9.

According to yet another aspect, a DNA molecule is provided comprising a sequence selected from the group consisting of SEQ ID No. 3, SEQ ID No. 5. SEQ ID No. 7, and SEQ ID No. 9.

40 The present invention and its various embodiments will be described in more detail in the following.

Figures

Figure 1 show gene expression data (normalized counts) of catalase and the five genes significantly differentially expressed in the 2013, P0 and F2-H₂O₂ groups (DNA-polymerase delta subunit 3, Nesprin-like, NA, ERP29 and Glp1_v2): Ls A-2013 (white circles), Ls V-2013 (grey circles), Ls A-P0 (white triangles), Ls V-P0 (grey triangles), Ls F2- H₂O₂-S (sensitive lice, white diamonds), Ls F2- H₂O₂-R (resistant lice, grey diamonds). Solid lines represent the mean in each group. Dark grey and black diamonds in the Ls F2-H₂O₂-R group represent the same individual lice in both catalase and Glp1_v2 graphs.

Figure 2 illustrates the number of genes differentially expressed in the H₂O₂ resistant lice (2013, P0 and F2- H₂O₂ groups), separately for up- and down-regulated genes. Numbers in the circles represent the unique genes differentially expressed in each group. Numbers in the intersection of the circles represent the differentially expressed genes shared between two or three groups.

Figure 3. qPCR validation for catalase and Glp1_v2 genes in the RNAseq louse groups: Ls A-2013 (white circles), Ls V-2013 (grey circles), Ls A-P0 (white triangles) and Ls V-P0 (grey triangles) lice. Solid lines represent the mean in each group. Data shown as fold change ($\log_2^{-(\Delta\Delta Ct)}$) referred to Ls A 2013 and Ls A P0 lice (calibrator sample). Letters above the data points (a, b and c) represent the statistically significant differences between the louse groups, groups sharing the same letter are not statistically different ($\alpha = 0.05$).

Detailed description of the invention

The present invention provides an *in vitro* method and means for determination of hydrogen peroxide resistance in crustaceans, in particular hydroxy peroxide resistance in sea lice. The method is based on the findings that determination of the expression levels of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10 with an unknown function can be used to reveal whether sea lice is sensitive towards hydroxy peroxide treatment or not.

The present invention thus provides method for the detection of hydrogen peroxide resistance in one or more adult female sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi* comprising the steps of:

- a) collecting one or more sea lice from infested fish or water samples;
- b) isolating genomic material from adult female of the collected sea lice; and
- c) determining the expression level of at least one of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.

Optionally, in addition to the determination of the at least one mentioned genes of step (c), said method may also involve the determination of the expression level of the gene encoding catalase.

According to one aspect, the sea lice to be analysed is one or more adult female sea lice.

According to one aspect adult female sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*.

5 In particular, it has been found that in hydroxy peroxide resistant adult female sea lice the expression of the gene encoding aquaglyceroporin (Glp1_v2) is significantly downregulated compared with the expression levels of said genes in sea lice being sensitive to hydroxy peroxide.

10 The present invention further provides method for the detection of hydrogen peroxide resistance in one or more sea lice wherein the sea lice is selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus* and *Caligus rogercresseyi* comprising the steps of:

- a) collecting one or more sea lice from infested fish or water samples;
- b) isolating genomic material from any life stage of the collected sea lice; and
- 15 c) determining the expression level of at least one of the genes encoding the proteins selected from the group consisting of endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.

20 Optionally, in addition to the determination of the at least one mentioned genes of step (c), said method may also involve the determination of the expression level of the gene encoding catalase.

Further, it has been found that in hydroxy peroxide resistant, the expression of the gene encoding endoplasmic reticulum resident protein 29 (ERP29) is significantly downregulated compared with the expression levels of said genes in sea lice being sensitive to hydroxy peroxide.

25 Furthermore, it has been found that expression of genes encoding DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10, respectively, in resistant sea lice is significantly upregulated compared with the expression level of said genes in hydroxy peroxide sensitive sea lice.

30 Glp1_v2 is one of the aquaglyceroporins identified by Stavang et al. (2015) in *L. salmonis* (Stavang et al, 2015, Phylogenomic and functional analyses of salmon lice aquaporins uncover the molecular diversity of the superfamily in Arthropoda. BMC Genomics 16:618. Stavang et al. (2015) identified a total of seven aquaporins in the salmon louse: two classical aquaporins (Bib and Prip-like or “PripL”), three aquaglyceroporins (Glp1_v1, Glp1_v2, Glp2, Glp3_v1 and Glp3_v2) and two unorthodox aquaporins (Aqp12-like1 or
35 “Aqp12L1” and Aqp12-like 2 or “Aqp12L2”).

Aquaporins are protein channels that facilitate transport of water, other small solutes such as H₂O₂ and gasses (Bienert et al., 2007, Specific Aquaporins Facilitate the Diffusion of Hydrogen Peroxide across Membranes. The Journal of biological chemistry. 282. 1183-92; Herrera and Garvin, 2011, Aquaporins as gas channels. Pflugers Arch - Eur J Physiol 462: 623 – 630; Miller et al., 2010, Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signalling, Proceedings of the National Academy of
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Sciences, 107 (36) 15681-15686; Stavang et al., 2015, *supra*; Thiagarajah et al., 2017, Aquaporin-3-mediated colonic epithelial responses, Proceedings of the National Academy of Sciences, 114 (3) 568-573; Zwiazek et al., 2017, Significance of oxygen transport through aquaporins, Scientific Reports, 7:40411).

- 5 Stavang et al. (2015), *supra*, identified a total of seven aquaporins in the salmon louse: two classical aquaporins (Bib and Prip-like or “PripL”), three aquaglyceroporins (Glp1_v1, Glp1_v2, Glp2, Glp3_v1 and Glp3_v2) and two unorthodox aquaporins (Aqp12-like1 or “Aqp12L1” and Aqp12-like 2 or “Aqp12L2”). Glp3_v2 was found to be expressed mostly in Nauplius II stage.
- 10 It has been demonstrated that certain aquaglyceroporins and unorthodox aquaporins are able to transport H₂O₂ through cell membranes in several organisms (Miller et al., 2010, *supra*; Thiagarajah et al., 2017, *supra*). Glps have an open pore configuration in *L. salmonis* (Stavang et al., 2015, *supra*), also allowing bigger molecules than water, like urea and glycerol, to pass through the channel. Miller et al. (2010), *supra*, found that one
- 15 aquaglyceroporin (AQP3) and one unorthodox aquaporin (AQP8) transported H₂O₂ through mammalian cell membranes. However, the classical aquaporin AQP1, did not transport H₂O₂.

- Several authors have reported the role of aquaporins as drug transporters in other parasites, and the link between aquaporins and drug resistance (reviewed in Song et al., 2014,
- 20 Parasite aquaporins: Current developments in drug facilitation and resistance, Biochimica et biophysica acta. 1840: 1566-1573). In Faghiri and Skelly (2009), The role of tegumental aquaporin from the human parasitic worm, *Schistosoma mansoni*, in osmoregulation and drug uptake, FASEB J. 23(8): 2780–2789, it was shown that the presence of a putative aquaglyceroporin (SmAQP) in the tegument of the parasitic worm *Schistosoma mansoni*. It
 - 25 was proven that SmAQP can transport water and an anti-parasitic compound (potassium antimonyl tartrate) across the parasite tegument. In addition, parasites with reduced levels of SmAQP exhibited a greater resistance to the anti-parasitic agent. In Trypanosomatid parasites, like *Leishmania* or *Trypanosoma* spp., certain aquaporins transport trivalent metalloids (SbIII and AsIII) through the parasite membranes (reviewed in Mandal et al.,
 - 30 2014, Trypanosomatid Aquaporins: Roles in Physiology and Drug Response. Diseases). The aquaglyceroporin LmAQP1 transports SbIII in *Leishmania* spp (Gourbal et al., 2004, Drug Uptake and Modulation of Drug Resistance in *Leishmania* by an Aquaglyceroporin. The Journal of biological chemistry. 279. 31010-7). Drug resistant parasites showed down-regulation of LmAQP1 (Marquis et al., 2005, Modulation in aquaglyceroporin AQP1 gene
 - 35 transcript levels in drug-resistant *Leishmania*, Mol Microbiol. Sep;57(6):1690-9), and the RNA levels correlated with the drug concentration. Resistance to melarsoprol and pentamidine is common among African trypanosomes (Baker et al., 2012, Aquaglyceroporin 2 controls susceptibility to melarsoprol and pentamidine in African trypanosomes, Proceedings of the National Academy of Sciences of the United States of
 - 40 America, 109(27), 10996-11001).

The authors found that the loss of function of an aquaglyceroporin, AQP2, was linked to drug resistance. Interestingly, a mitogen activated protein kinase 2 (MPK2) stabilizes LmAQP1 protein by phosphorylation in *Leishmania major* (Mandal et al., 2012), and dephosphorylation made LmAQP1 more susceptible to degradation. Altered AQP1 and

- MPK2 (by site-directed mutagenesis) reduced the drug uptake and drug sensitivity. Catalase activity can also be regulated by reversible phosphorylation via kinase enzymes by increasing the affinity of the enzyme for H₂O₂ (Dawson and Storey, 2016, The reversible phosphorylation of catalase from the freeze-tolerant North American wood frog, *Rana sylvatica*, *Biochim Biophys Acta*. 1860(3):476-85). These studies suggest that drug sensitivity can be linked to regulation of gene expression, but also to post-translational modifications of proteins. In our *L. salmonis* RNAseq data (cf. examples below), we found four putative mitogen activated protein kinases differentially expressed in H₂O₂ sensitive and resistant lice (data not shown).
- Based on the finding of the inventors, determination of the expression level of one of the genes mentioned above each in particular is suitable to indicate whether one or more sea lice are resistant or sensitive towards hydrogen peroxide resistance. However, without being limited by theory, and based on the inventors findings regarding determination of hydroxy peroxide resistance based on catalase gene expression level in sea lice, it is according to one aspect provided a method for the detection of hydrogen peroxide resistance in one or more sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi* comprising the steps of:
- a) collecting one or more sea lice from infested fish or water samples;
 - b) isolating genomic material from any life stage of the collected sea lice; and
 - c) determining the expression level of at **least two** of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.
- According to one aspect the sea lice is an adult female sea lice.
- According to one aspect the sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*,
- According to one aspect the sea lice is *Caligus rogercresseyi*
- According to yet another aspect, the above method may optionally also involve the determination of the expression level of catalase in the analysed one or more sea lice.
- According to another aspect, step (c) involves determining the expression level of at least three, such as at least four of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10. According to yet another aspect, step (c) involved determining the expression level of the genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.
- According to yet another aspect, the method of the invention involves the determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2) and endoplasmic reticulum resident protein 29 (ERP29), and optionally also determination of the expression level of a gene encoding catalase.

According to yet another aspect, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (GlpI_v2) and DNA polymerase (delta subunit 3), and optionally also determination of the expression level of a gene encoding catalase.

- 5 According to yet another aspect, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (GlpI_v2) and nesprin-like, and optionally also determination of the expression level of a gene encoding catalase.

- 10 According to yet another aspect, the method of the invention involves determination of the expression level of aquaglyceroporin (GlpI_v2) and the protein of SEQ ID No. 10, and optionally also determination of the expression level of a gene encoding catalase.

According to yet another aspect, the method of the invention involves determination of the expression level of the genes encoding endoplasmic reticulum resident protein 29 (ERP29) and DNA polymerase (delta subunit 3), and optionally also determination of the expression level of a gene encoding catalase.

- 15 According to yet another aspect, the method of the invention involves determination of the expression level of the genes encoding endoplasmic reticulum resident protein 29 (ERP29) and nesprin-like, and optionally also determination of the expression level of a gene encoding catalase.

- 20 According to yet another aspect, the method of the invention involves determination of the expression level of the genes encoding endoplasmic reticulum resident protein 29 (ERP29) and the protein of SEQ ID No. 10, and optionally also determination of the expression level of a gene encoding catalase.

- 25 According to yet another aspect, the method of the invention involves determination of the expression level of the genes encoding DNA polymerase (delta subunit 3) and nesprin-like, and optionally also determination of the expression level of a gene encoding catalase.

According to yet another aspect, the method of the invention involves determination of the expression level of the genes encoding DNA polymerase (delta subunit 3) and the protein of SEQ ID No. 10, and optionally also determination of the expression level of a gene encoding catalase.

- 30 According to another aspect, the method of the invention involves determination of the expression level of the genes encoding nesprin-like and the protein of SEQ ID No. 10, and optionally also determination of the expression level of a gene encoding catalase.

- 35 According to yet another aspect, a method is provided for detection of hydrogen peroxide resistance in one or more sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi* comprising the steps of

- 40 a) collecting one or more sea lice from infested fish or water samples;
b) isolating genomic material from any life stage of the collected sea lice; and
c) determining the expression level of at least **three of the genes** encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2),

endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.

According to one aspect the sea lice is an adult female sea lice.

5 According to one aspect the sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*.

According to one aspect the sea lice is *Caligus rogercresseyi*

10 According to yet another aspect, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), and optionally also determination of the expression level of a gene encoding catalase.

According to yet another, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), and the protein of SEQ ID No. 10, and optionally also determination of the expression level of a gene encoding catalase.

15 According to yet another, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), and nesprin-like, and optionally also determination of the expression level of a gene encoding catalase.

20 According to yet another, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), DNA polymerase (delta subunit 3), and nesprin-like, and optionally also determination of the expression level of a gene encoding catalase.

25 According to yet another, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), DNA polymerase (delta subunit 3), and the protein of SEQ ID No. 10, and optionally also determination of the expression level of a gene encoding catalase.

30 According to yet another, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), nesprin-like, and the protein of SEQ ID No. 10, and optionally also determination of the expression level of a gene encoding catalase.

According to yet another, the method of the invention involves determination of the expression level of the genes encoding endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), and nesprin-like, and optionally also determination of the expression level of a gene encoding catalase.

35 According to yet another, the method of the invention involves determination of the expression level of the genes encoding endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), and the protein of SEQ ID No. 10; and optionally also determination of the expression level of a gene encoding catalase.

40 According to yet another, the method of the invention involves determination of the expression level of the genes encoding endoplasmic reticulum resident protein 29 (ERP29),

nesprin-like, and the protein of SEQ ID No. 10; and optionally also determination of the expression level of a gene encoding catalase.

5 According to yet another, the method of the invention involves determination of the expression level of the genes encoding DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10; and optionally also determination of the expression level of a gene encoding catalase.

10 According to yet another aspect, a method is provided for detection of hydrogen peroxide resistance in one or more sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi* comprising the steps of

- a) collecting one or more sea lice from infested fish or water samples;
- b) isolating genomic material from any life stage of the collected sea lice; and
- 15 c) determining the expression level of at least **four of the genes** encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.

According to one aspect the sea lice is an adult female sea lice.

According to one aspect the sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*.

20 According to one aspect the sea lice is *Caligus rogercresseyi*

According to one embodiment, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), and nesprin-like; and optionally also determination of the expression level of a gene encoding catalase.

25 According to one embodiment, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), and the protein of SEQ ID No. 10; and optionally also determination of the expression level of a gene encoding catalase.

30 According to one embodiment, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), nesprin-like and the protein of SEQ ID No. 10; and optionally also determination of the expression level of a gene encoding catalase.

35 According to one embodiment, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10; and optionally also determination of the expression level of a gene encoding catalase.

According to one embodiment, the method of the invention involves determination of the expression level of the genes encoding endoplasmic reticulum resident protein 29 (ERP29),

DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10; and optionally also determination of the expression level of a gene encoding catalase.

According to one embodiment, the method of the invention involves determination of the expression level of the genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10; and optionally also determination of the expression level of a gene encoding catalase.

Based on the teaching herein, i.e. that the expression level of genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10 are linked with hydrogen peroxide resistance in sea lice, the skilled person will acknowledge that various well known methods are available for the determination of the expression level of genes, and to determine whether the expression of a certain gene are downregulated or upregulated.

When referring to that the expression of a gene is “upregulated” it is to be understood to mean that the expression of the gene in question is increased compared with the expression level of said gene in a sea louse being sensitive towards hydrogen peroxide.

When referring to that the expression of a gene is “downregulated” it is to be understood to mean that the expression of the gene in question is decreased compared with the expression level of said gene in a sea louse being sensitive towards hydrogen peroxide.

The expression level may be measured by quantifying the levels of the gene product in question and may thus be determined by e.g. quantifying the amount of protein or quantifying the amount of mRNA.

The skilled person is aware of a number of methods that may be used to determine the mRNA level of an organism, such as a sea louse. For example, the skilled person would know the major methods of quantitatively detect mRNA levels including electrophoretic methods (e.g. Northern blotting), DNA microarray-based methods and quantitative PCT (Real-time PCR). Various protocols for determining expression levels are available, cf. e.g. the step by step guide to Northern blot analysis by ThermoFisher Scientific (<https://www.thermofisher.com/no/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/northern-blotting.html>). Other available analysis is the QuantiGene RNA Assay for gene expression profiling of Invitrogen® provided ThermoFisher Scientific (<https://www.thermofisher.com/no/en/home/life-science/gene-expression-analysis-genotyping/quantigene-rna-assays.html>).

Real-time PCR based methods (also called quantitative PCR or qPCR) allows monitoring DNA amplification during the PCR run in real time via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCT product molecules (amplicons) that are generated. Real time PCT methods is an efficient and by many considered as the preferred method for detection and quantification of DNA or RNA. An overview of the Real Time PCR technology is found in Methods in Molecular Biology, “Quantitative Real-Time PCR, Methods and Protocols, edited by Roberto Biassoni and Alessandro Raso, Springer Science+Business Media, New York, 2014 (<https://www.gene-quantification.de/biassoni-raso-quantitative-real-time-pcr-ebook-2014.pdf>).

It is to be understood that the methods used in EP 3 164 502 B1 to quantify the expression level of catalase gene in sea lice by quantification of genomic material isolated from a sea lice suspected to be hydrogen peroxidase resistant may be used to quantify the expression level of the genes encoding the proteins selected from the group consisting of
5 aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.

Although the experimental data linking hydrogen peroxide resistance with the expression level of genes encoding aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of
10 SEQ ID No. 10, respectively, were identified in the sea lice species *Lepeophtheirus salmonis*, the skilled person will acknowledge, based on the teaching herein, that the present method and may be used to determine hydrogen peroxide resistance in copepods belonging to the family *Caligidae*.

In particular, it is to be understood that the present method may be used to determine
15 hydrogen peroxide resistance in copepods affecting farmed fish, such as e.g. fish belonging to the family Salmonidae.

According to one embodiment disclosed herein, the present method is applicable for detection of hydrogen peroxide resistance in copepod selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi*.

20 According to one aspect the sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*.

According to one aspect the sea lice is *Caligus rogercresseyi*

Throughout the disclosure, the term "sea louse" or "sea lice" is to be understood to mean one or more copepod belonging to the family *Caligidae*. In the experimental data provided
25 in the present disclosure, "sea lice" or "sea louse" refer to the species *Lepeophtheirus salmonis*.

Based upon the information provided herein in respect of the linkage between hydrogen peroxidase resistance and the expression levels of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident
30 protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10, the skilled person would, based on his common general knowledge of the well-known analysis and methods for determining expression levels be able to provide the means necessary to perform such analysis. For example, the skilled person will, based on the teaching herein, identify and construct applicable oligonucleotides, such as probes or
35 primers, useful in the various methods mentioned above.

As used herein, an "oligonucleotide sequence" or "nucleic acid sequence" is to be understood to mean an oligonucleotide sequence or a nucleic acid sequence useful in determining the expression level of genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29
40 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10, respectively.

5 An "oligonucleotide sequence" or "nucleic acid sequence" used to determine the expression level of one or more of the above listed genes is capable of hybridize to a nucleic acid sequence with a complementary sequence, such as e.g. genomic material, extracted from the one or more sea lice to be analyzed for hydrogen peroxide resistance. The skilled person will understand that the genomic material may be e.g. mRNA or DNA.

10 The skilled person is also aware of the fact that nucleic acid molecules may be double stranded or single-stranded, and that reference to a particular site of one strand refers, as well, to the corresponding site on a complementary strand. Thus, reference to an adenine (A), a thymine (T) (uridine (U)), a cytosine (C) or a guanine (G) at a particular site on one strand of a nucleic acid is also to be understood to define a thymine (uridine), adenine, guanine, or cytosine, respectively, at the corresponding site on a complementary strand of the nucleic acid molecule. Thus, reference may be made to either strand in order to refer to a particular position. The oligonucleotide probes and oligonucleotide primers according to 15 the present invention may be designed to hybridize to either strand.

20 An "isolated nucleic acid" useful in the detection method of the present invention, i.e. such as primers and probes, as used herein is generally one that contains at least 8 nucleotides and which is capable of hybridizing a nucleic acid with a complementary sequence, and is separated from most other nucleic acids present in the natural source of the nucleic acid, and is thus substantially free of other cellular material.

25 The present invention provides the use of oligonucleotide probes and oligonucleotide primers being useful in determining the expression level of any one of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10. The determination of the expression level of a gene is widely applied in both human and veterinary diagnosis, wherein nucleic acids from e.g. pathogens present in biological samples are isolated and hybridized to one or more hybridizing probes or primers are used in order to amplify a target sequence.

30 One or more oligonucleotide probes may be constructed based on the teaching herein and used in hybridization-based detection methods where upon the binding of the oligonucleotides to the target sequence enables determination of the level of expression of a gene present in the sea lice to be tested.

35 The skilled person will acknowledge that an oligonucleotide probe according to the present invention may be a fragment of DNA or RNA of variable length used herein in order to hybridize to the target sequence, e.g. single-stranded DNA or RNA. The oligonucleotide probe according to the present invention may furthermore be labeled with a molecular 40 marker in order to easily visualize that hybridization have been achieved. Molecular markers commonly known to the skilled person may be used, e.g. a radiolabel, and more preferably, a luminescent molecule or a fluorescent molecule enabling the visualisation of the binding of the probe(s) to a target sequence.

45 An oligonucleotide probe according to the present invention is able to hybridize to another

nucleic acid molecule, such as the single strand of DNA or RNA originating from one or more sea lice to be analysed, under appropriate conditions of temperature and solution ionic strength, cf. e.g. Sambrook et al., *Molecular Cloning: A laboratory Manual* (third edition), 2001, CSHL Press, (ISBN 978-087969577-4). The condition of temperature and ionic strength determine what the skilled person will recognise as the "stringency" of the hybridization. The suitable stringency for hybridisation of a probe to target nucleic acids depends on inter alia the length of the probe and the degree of complementation, variables well known to the skilled person. A oligonucleotide probe according to the present disclosure typically comprises a nucleotide sequence which under stringent conditions hybridize to at least 8, 10, 12, 14, 16, 18, 20, 22, 25, 30, 40, 50 (or any other number in-between) or more consecutive nucleotides in a target nucleic acid molecule, e.g. single-stranded DNA or RNA isolated from the sea lice to be analyzed according to the present invention. According to one embodiment, the oligonucleotide probe according to the present invention comprises about 10 to 25 consecutive nucleotides. New technology like specific Locked Nucleic Acid (LNA) hybridization probes allows for the use of extremely short oligonucleotide probes (You Y.; Moreira B.G.; Behlke M.A. and Owczarzy R. (2006). "Design of LNA probes that improve mismatch discrimination". *Nucleic Acids Res.* 34 (8): e60. doi:10.1093/nar/gkl175. PMC 1456327. PMID 16670427) According to one embodiment, probes are used in the present method or according to the present use, which hybridize under stringent conditions to a gene encoding a protein selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.

Also oligonucleotide primers may be used in methods according to the present method for determination of the expression level of a gene encoding a protein selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10, wherein the oligonucleotide primers are used for amplification of any given region of said genes. An oligonucleotide primer according to the present disclosure typically comprises a nucleotide sequence at least 8, 10, 12, 14, 16, 18, 20, 22, 25, 30, 40, 50 (or any other number in-between) or more consecutive nucleotides. According to one embodiment, the oligonucleotide primer according to the present invention comprises about 14 - 25 consecutive nucleotides, more preferably about 15 nucleotides.

As used herein, the term "oligonucleotide primer" is to be understood to refer to a nucleic acid sequence suitable for directing an activity to a region of a nucleic acid, e.g. for amplification of a target nucleic acid sequence by polymerase chain reaction (PCR).

The skilled person will acknowledge that an oligonucleotide primer according to the present invention may be a fragment of DNA or RNA of variable length used herein in order to determine the expression level of the target sequence, e.g. single-stranded DNA or RNA, upon alignment of the oligonucleotide probe to complementary sequence(s) of the said target sequence to be analyzed. An oligonucleotide primer according to the present invention may furthermore be labeled with a molecular marker in order to enable visualization of the results obtained. Various molecular markers or labels are available. An

oligonucleotide primer according to the present invention typically comprises the appropriate number of nucleotides allowing that said primer align with the target sequence to be analyzed.

- 5 Oligonucleotide probes and oligonucleotide primers may be manufactured according to methods well known to the skilled person.

According to one aspect, oligonucleotides (primers or probes) may be used determine the expression level of a gene encoding a protein selected from the group consisting of
10 aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10. According to yet another aspect, oligonucleotides (primers or probes) may be used to determine the expression level of nucleic acid molecule comprising a sequence selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID NO. 5, SEQ ID No. 7 and SEQ ID No.
15 9 or variants or fragments thereof having at least 70% identity with SEQ ID No. 1, SEQ ID No. 3, SEQ ID NO. 5, SEQ ID No. 7 and SEQ ID No. 9, respectively.

The term "% identity" is to be understood to refer to the percentage of nucleotides that two or more sequences or fragments thereof contains, that are the same. A specified percentage of nucleotides can be referred to as e.g. 70% identity, 75% identity, 80% identity, 85%
20 identity, 90% identity, 95% identity, 99% identity or more (or any number in between) over a specified region when compared and aligned for maximum correspondence.

The skilled person will acknowledge that various means for comparing sequences are available. For example, one non-limiting example of a useful computer homology or
25 identity program useful for determining the percent sequence identity between sequences includes the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990, J. of Molec. Biol., 215:403-410 , "The BLAST Algorithm; Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402 , , Karlin and Altschul 1990, Proc. Nat'l Acad. Sci. USA, 87:2264-68 ; 1993, Proc. Nat'l Acad. Sci. USA 90:5873-77).

30 *Isolation of genomic material of sea lice*

The method according to the present invention may according to one embodiment involve the isolation of a biological sample from one or more sea lice and measuring the level of expression of the genes of interest in order to determine whether the sea lice is hydrogen
35 peroxide resistant.

Various methods for obtaining genomic material well known to the skilled person are available. The skilled person will acknowledge that any tissue (i.e. any part of the sea lice) may be used in order to extract genomic material. Furthermore, the genomic material to be
40 analyzed according to the present invention may be obtained from sea lice of any life stages, e.g. the free-swimming stages (nauplius stage I and II), the copepod stage, the pre-adult (chalimus stages 1-4), or the adult stage (adult male or adult female).

In one embodiment, the sea lice are adult female.

According to one embodiment, tissue removed from sea lice to be tested is maintained in 70% ethanol or other conservation liquid prior to further isolation of genomic material.

5 DNA may be extracted from the obtained tissue using commonly available DNA extraction/isolation methods, such as e.g. DNeasy DNA Tissue Kit according to the protocol of the manufacturer (http://lycofs01.lycoming.edu/~gcatseek/protocols/DNeasy_Blood_&_Tissue_Handbook.pdf).

Hydrogen peroxidase resistance kits

10 Based on the teaching herein, the skilled person will acknowledge that the identification of the link between hydrogen peroxide resistance and expression levels of genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10, in sea lice, reagents applicable in determination of the expression level of said genes can be developed for the determination of hydrogen
15 peroxidase resistance.

For example, according to the present invention, a kit may comprise oligonucleotide probe(s) or oligonucleotide primer(s) or primer sets, arrays/microarrays of nucleic acid molecules, and beads that contain one more oligonucleotide probe(s), oligonucleotide
20 primer(s) or other detection reagents useful in the method of the present invention. It is furthermore to be understood that the detection reagents in a kit according to the present invention may furthermore include other components commonly included in such kits, e.g. such as various types of biochemical reagents (buffers, DNA polymerase, ligase, deoxynucleotide triphosphates for chain extension/amplification, etc.), containers, packages, substrates to which detection reagents are attached., etc. necessary to carry the
25 method according to the present invention.

According to one embodiment of the present invention, a kit is provided which comprises the necessary reagents to carry out one or more assays in order to determine the catalase gene expression level according to the method of the present invention. A kit according to the present invention may preferably comprise one or more oligonucleotide probes that
30 hybridize to a nucleic acid target molecule (i.e. genetic material) enabling determination of the catalase gene expression level in the material analyzed. Multiple pairs of probes may be included in the kit to simultaneously analyze for determination of catalase gene expression at the same time. The probes contained in the kit according to the present invention may according to one embodiment be immobilized on a carrier, such as e.g. an array or a bead.

35 According to one embodiment, a kit according to the present invention comprises oligonucleotide primer(s) and optionally further reagents useful in methods for the determination of the expression level of one or more of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the
40 protein of SEQ ID No. 10. According to one embodiment, the kit according to the present invention comprises a forward primer and a reverse primer for amplifying a region of one or more of said genes. Said kit may furthermore optionally comprise further reagents (enzymes and nucleotide triphosphates) necessary for conducting PCR or real time PCR.

Examples

Salmon louse strains

Two well-characterized laboratory *L. salmonis* strains were used in this study: Ls A, sensitive to all anti-salmon lice chemicals used in Norway (tested by bioassays), and Ls V, resistant to azamethiphos, deltamethrin, emamectin benzoate and hydrogen peroxide (field reports and bioassays). Ls A was a strain originally collected on a fish farm in the Northern part of Norway in 2011. Ls V was collected from a fish farm in Mid-Norway in October 2013 with high anti-lice chemical treatment pressure and reported diminished H₂O₂ treatment efficacy. A total of 14 anti-lice chemical treatments were performed from August 2012 to September 2013 in that farm: six H₂O₂ treatments (up until one month before the lice collection), six combined treatments with deltamethrin and azamethiphos, one treatment with diflubenzuron and one with emamectin benzoate. The Ls V-2013 samples referred to in the current study were the original field samples of this strain. Ls A and Ls V strains were reared in continuous cultures at the research facilities of Solbergstrand (The Norwegian Institute for Water Research, NIVA, Drøbak, Norway), as described by Hamre et al. (2009), in “Establishment and characterization of salmon louse (*Lepeophtheirus salmonis* (Kroyer 1837)) laboratory strains”. *Parasitol. Int.* 58, 451-460

Example 1 Crossing experiment and bioassays

In order to obtain a lice sample from the same generation and a range of hydroxy peroxide sensitivities, a batch crossing experiment was designed. The experiment was performed as described by Bakke et al. (2018) in 2015 (Bakke et al., (2018), “Deltamethrin resistance in the salmon louse, *Lepeophtheirus salmonis* (Krøyer): Maternal inheritance and reduced apoptosis”, *Scientific Reports* 8, Article number: 8450).

In short, two Atlantic salmon (one fish per aquarium) were infested with approximately 50 Ls A copepodites each and another two fish (one fish per aquarium) with the same number of Ls V copepodites to produce the parental generation (P0). All salmon lice were collected from all fish when the lice were in the pre-adult II stage, before the mating occurred. Then 10 pre-adults II Ls A females and 10 pre-adult II Ls V males from the P0 generation were put back on 2 individual fish (5 on each fish) kept in individual tanks to produce the F1 generation of the family group 1. Two other fish in separate tanks were infested with the same number of Ls A males and Ls V females to produce the F1 generation of family group 2. All P0 lice from both family groups were preserved in RNAlater (Sigma) after removal of the egg strings which were set aside to hatch. After ~24h at room-temperature, the preserved samples were stored at -80 °C. Four fish were infested with copepodites from the F1 generation, two fish with family group 1 and two fish with family group 2. The lice developed to the adult stage, mated, and egg strings for the F2 generation were collected. Approximately 500 copepodites from each of the family groups 1 and 2 (F2) were used for infestation of eight Atlantic salmon for each family group, with the two-family groups separated in different tanks.

F2 parasites were allowed to develop to adults. and were selected for sensitivity towards hydrogen peroxide (Interox Paramove 50, H₂O₂ 50%, w/w, Solvay Chemicals, Belgium). The selection was performed *in vitro* using two-dose bioassays at Faculty of Veterinary

Medicine, NMBU (University of Life Sciences, Oslo, Norway) within 6 hours after sampling. All exposures were done in 1 L glass bottles held at 10-12 °C with constant aeration. Three bioassays were performed where the females were exposed to 600 and 1800 ppm H₂O₂ for 30 min (two bioassays with lice from the family group 1 and one from family group 2) and recording of the results immediately following the exposure time (Helgesen et al., (2015), *supra*).

Control groups not exposed to H₂O₂ were included to check the general performance of the parasites. Parasites affected/immobilized at the lowest H₂O₂ concentrations were considered sensitive, whereas parasites that were not visibly affected at the highest concentrations were considered resistant. Lice were classified as affected when they were unable to attach to the container wall (lice could show weak swimming pattern, being partially or completely immobilized at the bottom of the container or floating at the surface). Immediately after exposure and the immobilization rate recorded, lice were fixed in RNAlater and kept at -80 °C following ~24h at room-temperature. H₂O₂ sensitive and resistant F2 adult females were used in the RNAseq analysis.

In order to obtain H₂O₂ sensitive and resistant lice for the RNAseq study, F2 adult females were selected with two-dose H₂O₂ bioassays. Lice affected at the lowest dose were considered sensitive and lice unaffected at the highest were considered resistant. Table 1 shows the number of lice affected at the different H₂O₂ doses. For adult females, there were no significant differences between family groups, indicating that inheritance of resistance was not gender-specific.

Table 1. Number of F2 adult female lice affected in two-dose H₂O₂ bioassays (30 min exposure). Results indicated as fractions (number of affected lice out of total lice per dose) and percentages (in brackets)

Crossing and bioassays			
	Family group 1	Family group 1	Family group 2
0 ppm (Control)	1/18 (6 %)	0/5 (0 %)	1/18 (6 %)
600 ppm	2/16 (13 %)	1/18 (6 %)	8/32 (25 %)
1800 ppm	13/15 (87 %)	12/18 (67 %)	16/25 (64 %)

Family group 1: females from the sensitive Ls A strain were crossed with males from the H₂O₂-resistant Ls V strain in the P0 generation. Family group 2: males from the sensitive Ls A strain were crossed with females from the H₂O₂-resistant Ls V strain in the P0 generation.

30

Example 2 Transcriptome analysis

In total, 36 adult female parasites were enrolled. From the original strains (2013), four laboratory reared Ls A and five field collected Ls V were used. From the P0 generation for the batch crossing experiment (2015), three Ls A and four Ls V parasites were included. None of these parasites were selected for sensitivity towards any agent prior to sampling.

35

From the F2 generation after the crossing experiment, 20 parasites were included. These were selected for sensitivity towards H₂O₂, prior to sampling. Additional details are given in Table 2.

5 **Table 2.** Information about the 36 samples (all adult female lice) enrolled in the RNAseq study. N, sample size.

Group	N	Description
Ls A-2013	4	Laboratory strain, sensitive to all delousing chemicals. Collected in Northern Norway in 2011. Sixth generation.
Ls V-2013	5	Field strain, resistant to azamethiphos, deltamethrin, emamectin benzoate and hydrogen peroxide. Collected in Mid-Norway in 2013.
Ls A-P0	3	Laboratory strain. 12th generation of Ls A (sensitive). Not exposed to delousing chemicals during cultivation of any generation.
Ls V-P0	4	Laboratory strain. Sixth generation of Ls V (resistant). Not exposed to delousing chemicals during cultivation of any generation.
Ls F2-H ₂ O ₂ -S	8	Second generation after crossing of Ls A-P0 and Ls V-P0, affected by 600 ppm H ₂ O ₂ for 30 min (sensitive). Three lice from family group 1 and five lice from family group 2.
Ls F2-H ₂ O ₂ -R	12	Second generation after crossing of Ls A-P0 and Ls V-P0, unaffected by 1800 ppm H ₂ O ₂ for 30 min (resistant). Seven lice from family group 1 and five lice from family group 2.

RNA extraction

10 Total RNA was extracted from the 36 individual adult females using a Trizol protocol combined with RNeasy Mini kit for animal tissues (Qiagen, CA, USA) (one individual per extraction). Lice tissues were disrupted and homogenized in 1 ml Trizol using TissueLyser MM 301 (Qiagen Retsch) and one stainless steel bead of 5 mm diameter (Qiagen). After mixing with 0.2 ml of chloroform and a centrifugation step, the aqueous phase was

15 transferred to a new vial and mixed with one volume of 70% ethanol. Total RNA was then isolated with RNeasy spin columns following manufacturer's protocol. Genomic DNA was removed from the extracted RNA (10 µg) with Turbo DNA-free TM kit (TURBO™ DNase Treatment and Removal Reagents, Ambion, Life Technologies Thermo Fisher Scientific, USA). Subsequently, the RNA was cleaned and concentrated with RNA Clean &

20 ConcentratorTM-5 (Zymo Research). The RNA was quantified with ND-100 Spectrophotometer (Thermo Fisher Scientific, DE, USA) and the quality was checked with a 2100 Bioanalyzer instrument (Agilent Technologies) and the Agilent RNA 6000 Nano kit.

RNAseq

Total RNA samples were used for library preparation and Illumina sequencing at the Norwegian Sequencing Centre (Oslo, Norway). Thirty-six RNA-seq libraries (one per individual lice), each with unique index barcodes, were prepared using the TruSeq
 5 Stranded total RNA library preparation Kit v2 (Illumina, USA) by following manufacturer's protocol including the polyA enrichment step. Libraries were pooled together and sequenced on NextSeq500 platform (Illumina, USA) using 150 bp paired end High output reagents. Raw bcl files were generated using RTA v2.4.11 and were later demultiplexed (using the sample specific index) and converted to fastq format using
 10 bcl2fastq v2.17.1.14.

RNAseq gene expression analysis (DESeq2) showed that the groups Ls V-2013 and Ls F2-H₂O₂-R each had more than 2000 genes differentially regulated compared to the corresponding, presumably sensitive groups, Ls A-2013 and Ls-F2- H₂O₂-S. The Ls V-P0 lice had less than 150 genes differentially regulated compared to Ls A-P0 (Fig. 2).

15

Example 3: Gene expression analysis

Demultiplexed raw reads were cleaned using Trimmomatic v0.33 (Bolger et al., 2014, "Trimmomatic: a flexible trimmer for Illumina sequence data", *Bioinformatics* 30, 2114-2120) to remove/trim low quality reads and sequencing adapters as well as using BBDMap
 20 v34.56 (<https://sourceforge.net/projects/bbmap/>) to remove reads mapping to PhiX genome (Illumina spike-in). Cleaned fastq reads for each parasite were aligned to the *L. salmonis* transcriptome (coding sequences) using Hisat2 v2.1.0 (Kim et al., 2015, "HISAT: a fast-spliced aligner with low memory requirements", *Nature Methods* 12, 357-360. <https://www.nature.com/articles/nmeth.3317>). The transcriptome file
 25 (ftp://ftp.ensemblgenomes.org/pub/metazoa/release-44/fasta/lepeophtheirus_salmonis) contained the predicted transcriptome from genomic data. It was modified for the aquaporin genes by substituting the predicted cds sequences in the original transcriptome with experimentally determined cds sequences from Stavang et al. (2015), *supra*. Unmapped reads were filtered out using SAMtools version 1.4 (Li et al., 2009, The
 30 Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078-2079). Gene annotation files in GTF format were generated for each parasite and then merged using Cufflinks version 2.2.1. (Trapnell et al., 2010, "Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell
 35 differentiation", *Nat. Biotechnol.* 28, 511-515). Counts of fragments aligning to each transcript were calculated using FeatureCounts version 1.5.2. (Liao et al., 2014, "FeatureCounts: an efficient general-purpose program for assigning sequence reads to
 40 genomic features", *Bioinformatics* 30, 923-930). Analysis of the differential expression within each group (Ls A-2013 versus Ls V-2013; Ls A-P0 versus Ls V-P0; Ls F2-H₂O₂-S versus Ls H₂O₂-R) were done using DESeq2 (Love et al., "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2", *Genome Biol.* 15, 550) (default settings for the count normalization method). The significance level was set to $\alpha = 0.05$.

Differentially expressed genes shared between presumed hydrogen peroxide resistant lice

The DESeq2 analysis generated two lists for each group, one list of genes up-regulated in resistant lice and another list for genes down-regulated in resistant lice, both compared to sensitive lice within the same group. We searched for genes that were differentially
 5 expressed in the same direction in at least two of the three groups. An R-script was developed to identify the shared genes across all the groups (Ls 2013, Ls P0 and Ls F2-H₂O₂) or between two of the groups (Ls 2013 vs Ls P0, Ls 2013 vs Ls F2-H₂O₂ and Ls P0 vs Ls F2-H₂O₂). The Uniprot database (www.uniprot.org), NCBI-Non-redundant protein sequences (nr) database (ncbi.nlm.nih.gov) and “ENSEMBL Metazoa (transcript)”
 10 “protein information” section (http://metazoa.ensembl.org/Lepeophtheirus_salmonis/Info/Index), were used to annotate the genes.

qPCR study

15 Quantitative polymerase chain reaction (qPCR) was used to validate the RNAseq results for two genes, catalase and Glp1_v2. An elevated expression of catalase has already been associated with resistance towards hydrogen peroxide (Helgesen et al. (2017), “Increased catalase activity — A possible resistance mechanism in hydrogen peroxide resistant salmon lice (*Lepeophtheirus salmonis*)”, Aquaculture, 468 (1), 135-140), and the expression of
 20 Glp1_v2 was significantly downregulated in the three groups of presumed H₂O₂-resistant parasites in the RNAseq study (Ls V-2013, Ls V-P0, Ls F2-H₂O₂-R). The samples enrolled in the validation study were not exposed adult females from the RNAseq study: Ls A-2013, Ls V-2013, Ls A-P0 and Ls V-P0.

25 RNA extraction, DNase treatment and RNA cleaning were performed for every sample the same way as samples prepared for RNAseq. First strand cDNA was produced from 1 µg of cleaned RNA using the qScriptTM cDNA synthesis (reverse transcriptase) kit (Quanta Biosciences, MD, USA). The cDNA was cleaned with the DNA Clean & ConcentratorTM-5 kit (Zymo Research) and diluted 1:10 before used as PCR template for qPCR using gene specific primers and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, CA, USA),
 30 following manufacturer’s protocol. Each qPCR reaction was optimized for 11 µl total reaction volume, 150/150 or 300/300 nM primer concentration and 2 µl of template, corresponding to 0.2 µg cDNA/RNA. Reactions were run in duplicate or triplicate and two negative controls were added, a non-template control and a no reverse transcriptase control. The range of efficiencies for qPCR reactions were 96-98% for reference and gene specific
 35 primers. The qPCR was run on a Bio-Rad CFX96 real-time system (Bio-Rad, CA, USA) under the following conditions: 95°C for 30 sec followed by 40 cycles of amplification at 95°C for 10 sec and 60°C for 50 sec. After qPCR, the homogeneity and specificity of the PCR products was confirmed by melting curve analysis, agarose gel electrophoresis and Sanger sequencing. Relative gene expression was determined by the ΔC_t method ($\Delta C_t = C_{t_{\text{target}}} - C_{t_{\text{reference}}}$), where $C_{t_{\text{target}}}$ is the C_t values for catalase or Glp1_v2, and $C_{t_{\text{reference}}}$ the
 40 average of the elongation factor 1-alpha (EF) and prohibitin-2 (Proh2) genes. (see table 3 below for primer details). Fold change in gene expression was calculated according to the $2^{-(\Delta\Delta C_t)}$ method, using the average of the C_t values for Ls A-2013 and Ls A-P0 lice as calibrator sample. Expression levels (ΔC_t) of catalase and Glp1_v2 were separately

subjected to analysis of variance (ANOVA) with Tukey HSD post hoc test for multiple comparisons. Group (Ls A-2013+P0, Ls V-2013 and Ls V-P0) was used as the model effect and the significance level was set to $p<0.05$.

5 **Table 3:** Primers used for the qPCR validation experiment. Genes: Cat, catalase; Glp1, aquaglyceroporin type 1; EF, elongation factor 1- α ; Proh2, prohibitin-2.

Gene	Primer name	Primer sequence	Primer concentration	Product size (bp)
Cat	Ls_CAT_6 F Ls_CAT_6 R	CCACAGAACAACCTTGCCAAC GCCATTTTCGTCCATAAATGC	150/150 nM	157
Glp1	Ls_Glp1_2 F Ls_Glp1_2 R	TCGGCTCCAGGAATTGTTCT GGTCCTAAATCTCTCGCTGGG	300/300 nM	200
EF	Ls_gEF_2 F Ls_gEF_2 R	ATGGCACGGAGACAACATGT CGGGCACTGTTCCAATACCT	150/150 nM	206
Proh2	Ls_gProhib2_2 F Ls_gProhib2_2 R	GCTCATCACACAGCGTCAAC CAGCTCTTTGGGCCTCTTGT	300/300 nM	176

Gene expression

10 The catalase gene was previously found differentially expressed in H₂O₂-sensitive and -
resistant lice (Helgesen et al., 2017, *supra*) and its expression level has recently been
introduced as H₂O₂ resistance marker in the salmon industry (Helgesen et al., 2017, *supra*,
EP 3 164 502 B1). The present RNAseq study was performed in order to validate the use of
the catalase gene expression as a resistance marker in adult females, as this developmental
15 stage was not included in the previous study (Helgesen et al., 2017, *supra*). There were
significantly higher numbers of catalase transcripts in resistant lice selected by H₂O₂ (Ls
V-2013 and Ls F2- H₂O₂-R) than in sensitive lice (Table 5 and Fig. 1). However, the
number of catalase transcripts in Ls V-P0 parasites, which were not exposed to H₂O₂ for
two years, did not differ significantly from Ls A-P0. The qPCR validation data showed a
20 similar gene expression pattern compared to the DESeq2 analysis for 2013 and P0 RNAseq
samples (Figs. 1 and 3). Ls V-2013 had statistically significantly higher catalase expression
than Ls A-2013 and Ls A-P0 (Fig. 3). Ls V P0 had a statistically similar catalase
expression as Ls A-2013 and Ls A-P0. The sensitivity of the unexposed Ls V strain was
tested after completion of the RNAseq study. The EC₅₀ value for pre-adult II females of the
25 Ls V lab-strain was 1635 ppm, eight times higher than the Ls A strain (216 ppm) (Table 4),
suggesting that Ls V-P0 lice were still resistant to H₂O₂ when enrolled in the RNAseq
study. In addition, more than 70% of the Ls V-P0 descendants (Ls F2- H₂O₂) were
unaffected at 600 ppm H₂O₂, and more than 10% at 1800 ppm (Table 1). DESeq2 analysis
for Ls F2- H₂O₂-R showed that the catalase transcript had the lowest p(adj) value among
30 the genes differentially expressed in that group of lice, and they had on average close to

three times higher numbers of catalase transcripts than their grandparents, Ls V-P0 (Table 5.

5 **Table 4.** Bioassay results for pre-adult II (males and females) and young adult males exposed to H₂O₂ for 30 min. N: total number of lice used in the bioassays (all chemical concentrations together). ppm: mg L⁻¹. EC₅₀: concentration immobilizing 50% of the lice. CI: confidence interval. Ls A: sensitive strain. Ls V: H₂O₂-resistant strain.

Louse strain	H ₂ O ₂ exposure data:	
	Year; water temperature; N; doses (ppm)	EC ₅₀ (ppm) (90% CI)
Ls A - Lab strain		
Helgesen et al. 2015, <i>supra</i> *	2013; 10-12°C	216 (153 - 305)
Ls V		
Helgesen et al. 2015, <i>supra</i> *	2013; 10-12°C	2127 (1253 - 3610)
Ls V- Lab strain	2017; 10-11°C	Females: 1635 (734 - 3643)
	25 females and 22 males	
	0, 600, 1400, 2200, 3000, 4200	Males: 1795 (1095 - 2943)

*Data for males and females together. 95% CI.

Table 5. Gene expression data of several genes differentially expressed in the louse groups Ls 2013, P0 and F2-H₂O₂. The ENSEMBL *L. salmonis* transcriptome was used in the analysis, but the sequences of genes coding for aquaporins were replaced by GenBank entries (Stavang et al., 2015). log2FC = log2 fold change (up-regulation is indicated as positive values, down-regulation as negatives); p(adj) = p-value for normalized counts ($\alpha = 0.05$). Statistical significance is indicated in bold. Asterisk (*) indicates no overlap in the range of normalized counts between the groups.

Average normalized counts (range)					
Gene	Lice group	Ls A / F2-H ₂ O ₂ -S	Ls V / F2-H ₂ O ₂ -R	log2FC	p(adj)
Catalase	2013	818 (675 – 1055)	3429 (2236 – 6165)	2.066	< 0.001*
	P0	954 (696 – 1326)	706 (491 – 963)	-0.433	0.784
	F2-H ₂ O ₂	1161 (891 – 1386)	2072 (1580 – 2821)	0.836	< 0.001*
DNA-polymerase	2013	374 (331 – 447)	464 (390 – 505)	0.318	0.044
	P0	585 (495 – 658)	930 (812 – 1134)	0.669	0.024*
	F2-H ₂ O ₂	217 (144 – 344)	320 (165 – 548)	0.561	0.045
Nesprin-like	2013	3864 (3522 – 4290)	5297 (4644 – 6116)	0.455	< 0.001*
	P0	5066 (4837 – 5304)	7036 (5803 – 7547)	0.474	0.034*
	F2-H ₂ O ₂	3271 (2887 – 4498)	4021 (3158 – 4403)	0.298	0.005
NA	2013	14 (8 – 17)	33 (19 – 52)	1.186	0.018*
	P0	21 (11 – 41)	94 (57 – 164)	2.162	0.026*
	F2-H ₂ O ₂	10 (4 – 20)	21 (5 – 38)	1.034	0.015
ERP29	2013	90 (77 – 102)	56 (40 – 74)	-0.692	0.015*
	P0	114 (96 – 128)	50 (45 – 55)	-1.203	< 0.001*
	F2-H ₂ O ₂	110 (76 – 140)	81 (44 – 118)	-0.443	0.019
Glp1_v2	2013	112 (74 – 164)	15 (10 – 26)	-2.894	< 0.001*
	P0	77 (64 – 86)	40 (35 – 44)	-0.934	0.025*
	F2-H ₂ O ₂	197 (39 – 292)	88 (40 – 181)	-1.161	0.002
Aqp12L1	2013	158 (140 – 173)	99 (73 – 152)	-0.679	0.013
	P0	162 (144 – 192)	148 (130 – 181)	-0.127	0.957
	F2-H ₂ O ₂	182 (130 – 219)	141 (104 – 185)	-0.370	0.010

Aqp12L2	2013	56 (42 – 75)	15 (13 – 20)	-1.905	< 0.001*
	P0	29 (11 – 46)	24 (19 – 30)	-0.289	0.960
	F2-H ₂ O ₂	98 (69 – 124)	66 (31 – 103)	-0.571	0.012
Glp2	2013	20 (15 – 31)	5 (0 – 14)	-1.980	0.045*
	P0	17 (7 – 26)	20 (16 – 30)	0.222	0.976
	F2-H ₂ O ₂	24 (6 – 42)	11 (1 – 17)	-1.119	0.008
Glp3_v1	2013	149 (110 – 182)	297 (183 – 365)	0,990	< 0.001*
	P0	222 (185 – 253)	174 (120 – 253)	-0.351	0.855
	F2-H ₂ O ₂	134 (91 – 203)	121 (82 – 148)	-0.160	0.378

ENSEMBL/GenBank gene names for the genes: Catalase, EMLSAT00000007315; DNA - polymerase, EMLSAT00000002584; Nesprin-like, EMLSAT00000005972; NA (not annotated), EMLSAT00000005947; ERP29, EMLSAT00000009549; Glp1_v2, KR005661.1; Aqp12L1, KR005665.1; Aqp12L2, KR005666.1; Glp2, KR005662.1; Glp3_v1, KR005663.1.

These results may indicate that the catalase gene is induced by H₂O₂ exposure. The induction of the catalase gene after H₂O₂ exposure was demonstrated in a penaeid shrimp (Wang et al., 2012, *supra*). The gene was significantly up-regulated at 2 h after injecting 0.1% H₂O₂ in the shrimp body. In addition, H₂O₂ resistance is hereditary, as demonstrated by Helgesen et al (2015, 2017, *supra*). The heritable factor may thus be the ability to quickly induce catalase expression. This also indicates that induction of the catalase gene can be problematic when using the expression of this gene as a H₂O₂ resistance marker, since resistant lice not exposed to H₂O₂ may have a normal expression of this gene and could erroneously be classified as sensitive. On the other hand, after a short exposure to H₂O₂, sensitive and resistant lice seem to be readily separable by expression of the catalase gene.

New putative molecular markers

To identify further genes associated with H₂O₂ resistance, differentially expressed genes from the Ls 2013, Ls P0 and Ls F2-H₂O₂ generations were compared. The resistant adult female lice that had been exposed to H₂O₂ (Ls V-2013 and Ls F2-H₂O₂-R) shared 790 differentially expressed genes (Fig. 2). This support the hypothesis that H₂O₂ exposure could induce the expression of several genes, even within a timespan of 30 minutes. Only five genes (three up-regulated and two down-regulated in resistant lice) were differentially expressed in all three groups (Ls V-2013, Ls V-P0 and Ls F2-H₂O₂-R) (Fig. 2), irrespective of H₂O₂ exposure. Table 5 shows the gene expression and annotation data for these genes. The log2 fold change ranged from ~|0.2| to ~|3|. The three genes consistently up-regulated in presumed resistant lice were a DNA polymerase (delta subunit 3), the Nesprin-like protein and a not annotated protein (NA). Nesprin-like, also known as enaptin or synaptic nuclear envelope protein 1, is an actin-binding protein involved in the maintenance of nuclear organization and structural integrity. The two genes downregulated in resistant adult female lice were the endoplasmic reticulum resident protein 29 (ERP29)

and an aquaporin protein (Glp1_v2). ERP29 plays an important role in the processing of secretory proteins within the endoplasmic reticulum.

All but Glp1_v1 and Glp3_v2 were detected in our RNAseq data. Table 5 shows the gene expression data for several aquaporins in our study. There were no statistically significant differences in the expression of Bib or PripL within any of the Ls 2013, Ls P0 or Ls F2-H₂O₂ groups (data not shown). However, Glp1_v2 was statistically significantly down-regulated in all presumed H₂O₂ resistant groups (Ls V-2013, Ls V-P0 and Ls F2-H₂O₂-R). The qPCR validation data showed a similar gene expression pattern compared to the DESeq2 analysis for 2013 and P0 RNAseq samples (Figs. 1 and 3). Ls V-2013 and Ls V P0 had statistically significantly lower Glp1_v2 expression than Ls A-2013 and Ls A-P0 (Fig. 3). Glp 2 was significantly down-regulated in two groups, Ls V-2013 and Ls F2-H₂O₂-R, but the expression of this gene was low. Glp3_v1 was up-regulated in Ls V-2013. The unorthodox aquaporins, Aqp12L1 and Aqp12L2, were statistically significantly down-regulated in Ls V-2013 and Ls F2-H₂O₂-R groups.

Glp1_v2 was down-regulated in all three groups of presumed H₂O₂ resistant adult female lice, indicating a possible involvement in H₂O₂ transport: The lower the number of Glp1_v2 channels is, the less amount the exogenous H₂O₂ would enter the louse body and cause toxic effects. The downregulation of Aqp12L1 and Aqp12L2 in presumably resistant lice exposed to H₂O₂ may also indicate a role of these proteins as H₂O₂ channels. This goes especially for Aqp12L2, with a $\sim |2|$ log₂ fold change and no overlap in the normalized count range in the Ls 2013 groups (Table 5). As in the case of Glps, Stavang et al. (2015), *supra*, also found an open pore configuration in the 3D modelling of Aqp12L2.

There was an overlap in the normalized count range for DNA-polymerase, Nesprin-like, NA, ERP29, Glp1_v2, Aqp12L1 and Aqp12L2 genes between sensitive and resistant F2-H₂O₂ lice (Table 5), although the differences were statistically significant. F2 lice is a louse population arisen from the mix of sensitive and resistant lice, with a wide range of H₂O₂ sensitivities. This gene expression overlap suggests that H₂O₂ resistance in F2 lice comes from a number of up- and down-regulated genes combined in slightly different ways depending on each individual louse, giving to all of them the capability to survive 1800 ppm H₂O₂. Only the expression of catalase was able to clearly separate sensitive from resistant F2 lice. As an example, the two F2-H₂O₂ resistant lice with high Glp1_v2 reads, are the ones with higher catalase expression, possibly suggesting a compensatory effect: high Glp1_v2 reads could mean that more exogenous H₂O₂ would enter the louse body, needing the louse more catalase for breaking down the H₂O₂ and survive the exposure (Fig. 1, dark grey and black triangles).

Table 6: Overview of sequence numbering

Sequence number	Description
SEQ ID No. 1	DNA sequence encoding aquaglyceroporin (Glp1_v2) identified in <i>Lepeophtheirus salmonis</i>
SEQ ID No. 2	Amino acid sequence of aquaglyceroporin (Glp1_v2) encoded by SEQ ID No. 1
SEQ ID No. 3	DNA sequence encoding endoplasmic reticulum resident protein 29 (ERP29) identified in <i>Lepeophtheirus salmonis</i>
SEQ ID No. 4	Amino acid sequence of endoplasmic reticulum resident protein 29 (ERP29) encoded by SEQ ID No. 3
SEQ ID No. 5	DNA sequence encoding DNA polymerase (delta subunit 3) identified in <i>Lepeophtheirus salmonis</i>
SEQ ID No. 6	Amino acid sequence of DNA polymerase (delta subunit 3) encoded by SEQ ID No. 5.
SEQ ID No. 7	DNA sequence encoding nesprin-like identified in <i>Lepeophtheirus salmonis</i>
SEQ ID No. 8	Amino acid sequence of nesprin-like encoded by SEQ ID No.7
SEQ ID No. 9	DNA sequence encoding a hitherto unknown protein identified in <i>Lepeophtheirus salmonis</i>
SEQ ID No. 10	Amino acid sequence of the protein encoded by SEQ ID No. 9
SEQ ID No. 11	DNA sequence encoding catalase of <i>L. salmonis</i>
SEQ ID No. 12	DNA sequence encoding catalase of <i>C. clemensei</i>
SEQ ID No. 13	DNA sequence encoding catalase of <i>C. rogercresseyi</i>
SEQ ID No. 14	DNA sequence encoding catalase of <i>C. elongatus</i>
SEQ ID No. 15	Primer used to detect expression level of catalase (Ls_CAT_6 F)
SEQ ID No. 16	Primer used to detect expression level of catalase (Ls_CAT_6 R)
SEQ ID No. 17	Primer used to detect expression level of aquaglyceroporin type 1 (Ls_Glp1_2 F)
SEQ ID No. 18	Primer used to detect expression level of aquaglyceroporin type 1 (Ls_Glp1_2 R)
SEQ ID No. 19	Primer used to detect expression level of elongation factor 1-alpha (Ls_gEF_2 F)
SEQ ID No. 20	Primer used to detect expression level of elongation factor 1-alpha (Ls_gEF_2 R)
SEQ ID No. 21	Primer used to detect expression level of prohibitin-2 (Ls_gProhib2_2 F)
SEQ ID No. 22	Primer used to detect expression level of prohibitin-2 (Ls_gProhib2_2 R)
SEQ ID No. 23	A revers primer of 24 nucleotides comprising the sequence CATACAAGTATAGGAACTGGCTCA wherein the primer is used for detection of a SNP in the zinc finger gene PLAGL1 from sheep (SEQ ID 12 of CN108866160 A)

CLAIMS

- 5 1. Method for the detection of hydrogen peroxide resistance in one or more adult female sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi* comprising the steps of:
 - a) collecting one or more adult female sea lice from infested fish or water samples;
 - b) isolating genomic material from the collected sea lice; and
 - 10 c) determining the expression level of at least one of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.
- 15 2. Method according to claim 1, wherein the adult female sea lice is selected from *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*.
3. Method according to claim, wherein the sea lice is *Lepeophtheirus salmonis*.
- 20 4. Method according to any one of the above claims, wherein said genes encodes a protein having a sequence selected from the group consisting of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 10, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6, SEQ ID No. 8, and SEQ ID no.10, respectively.
- 25 5. Method according to any one of the above claims, wherein said genes has a sequence selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 9, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ
30 ID no.9, respectively.
6. Method according to any of the above claims, wherein the expression levels of at least two of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29),
35 DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10 i determined.
7. Method according to any of the above claims, wherein in addition the expression levels of a gene encoding catalase is determined.

8. Method according to claim 7, wherein the catalase gene is selected from the group consisting of SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, and SEQ ID No. 14 or variants or fragments thereof being at least 70 % identical with SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, and SEQ ID No. 14, respectively.
- 5
9. Method according to any of the above claims, wherein the expression of the one or more genes in the collected sea lice or water sample is compared with the expression level of said genes in one or more non-resistant sea lice.
- 10
10. Use of one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, respectively, for *in vitro* determination of hydrogen peroxide resistance in sea lice.
- 15
11. Use according to claim 10, wherein the one or more oligonucleotide sequence is used for determination of hydrogen peroxide resistance in sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi*, in particular *Lepeophtheirus salmonis*, *Caligus clemensei* and *Caligus elongatus* and more particular *Lepeophtheirus salmonis*.
- 20
12. Use according to claims 10 or claim 11, wherein the isolated oligonucleotide sequence is selected from the group consisting of SEQ ID No. 18, SEQ ID No. 19, SEQ ID NO. 20 and SEQ ID No. 21.
- 25
13. Kit for detection of hydrogen peroxide resistance in sea lice comprising one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, respectively.
- 30
14. A DNA molecule encoding a protein comprising an amino acid sequence selected from the group consisting of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6, SEQ ID No. 8, and SEQ ID No. 10.
- 35
15. The DNA molecule according to claim 14, wherein the DNA molecule comprise a sequenced selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, or variants or fragments thereof being at least 70 % identical with the entire length of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9.
- 40

AMENDED CLAIMS

received by the International Bureau on 7 May 2021 (07.05.2021)

1. Method for the detection of hydrogen peroxide resistance in one or more adult female sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi* comprising the steps of:
 - a) collecting one or more adult female sea lice from infested fish or water samples;
 - b) isolating genomic material from the collected sea lice; and
 - c) determining the expression level of at least one of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10.
2. Method according to claim 1, wherein the adult female sea lice is selected from *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*.
3. Method according to claim 1, wherein the sea lice is *Lepeophtheirus salmonis*.
4. Method according to any one of the above claims, wherein said genes encodes a protein having a sequence selected from the group consisting of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6, SEQ ID No. 8, SEQ ID No. 10, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6, SEQ ID No. 8, and SEQ ID no.10, respectively.
5. Method according to any one of the above claims, wherein said genes has a sequence selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 9, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID no.9, respectively.
6. Method according to any of the above claims, wherein the expression levels of at least two of the genes encoding the proteins selected from the group consisting of aquaglyceroporin (Glp1_v2), endoplasmic reticulum resident protein 29 (ERP29), DNA polymerase (delta subunit 3), nesprin-like and the protein of SEQ ID No. 10 is determined.

7. Method according to any of the above claims, wherein in addition the expression levels of a gene encoding catalase is determined.
8. Method according to claim 7, wherein the catalase gene is selected from the group consisting of SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, and SEQ ID No. 14 or variants or fragments thereof being at least 70 % identical with SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, and SEQ ID No. 14, respectively.
9. Method according to any of the above claims, wherein the expression of the one or more genes in the collected sea lice or water sample is compared with the expression level of said genes in one or more non-resistant sea lice.
10. Use of one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, respectively, for *in vitro* determination of hydrogen peroxide resistance in sea lice.
11. Use according to claim 10, wherein the one or more oligonucleotide sequence is used for determination of hydrogen peroxide resistance in sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei*, *Caligus elongatus*, and *Caligus rogercresseyi*, in particular *Lepeophtheirus salmonis*, *Caligus clemensei* and *Caligus elongatus* and more particular *Lepeophtheirus salmonis*.
12. Use according to claims 10 or claim 11, wherein the isolated oligonucleotide sequence is selected from the group consisting of SEQ ID No. 18, SEQ ID No. 19, SEQ ID NO. 20 and SEQ ID No. 21.
13. Kit for detection of hydrogen peroxide resistance in sea lice comprising one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, respectively.
14. A DNA molecule encoding a protein comprising an amino acid sequence selected from the group consisting of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6, SEQ ID No. 8, and SEQ ID No. 10.
15. The DNA molecule according to claim 14, wherein the DNA molecule comprise a sequenced selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9, or variants or fragments thereof being at least 70 % identical with the entire length of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, and SEQ ID No. 9.

Figure 1

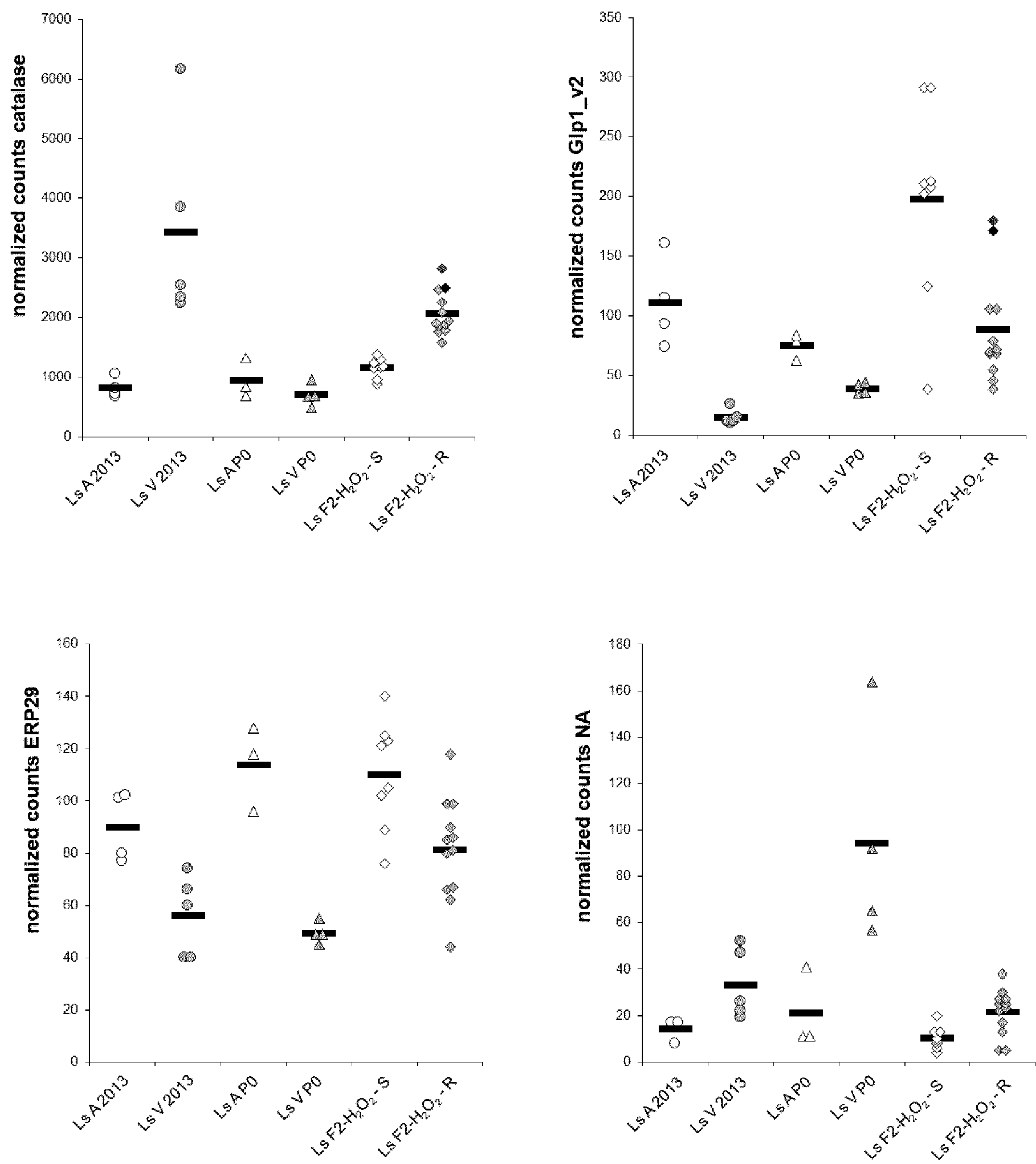


Figure 1 cont.

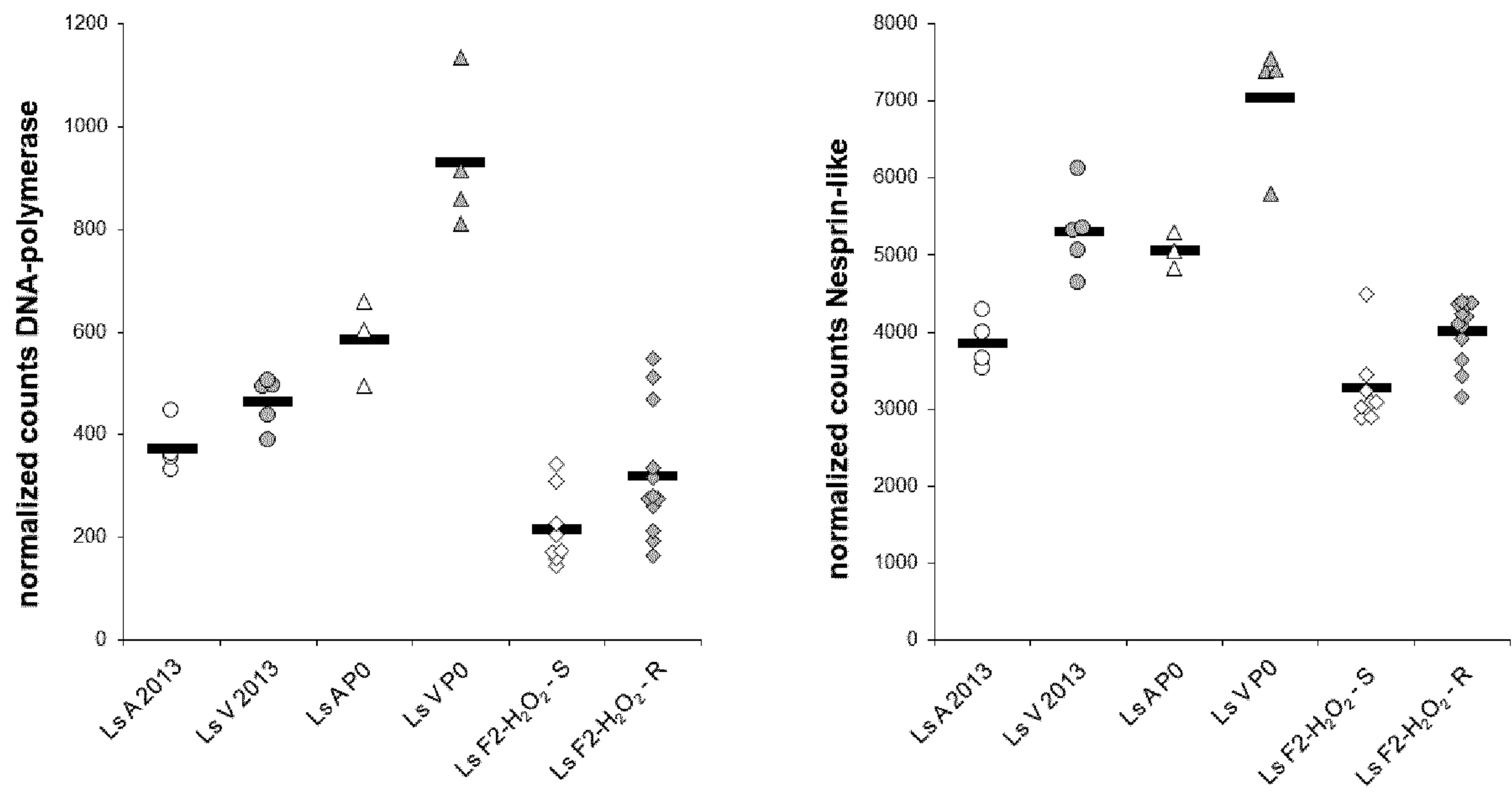


Figure 2

No. GENES **UP**-REGULATED IN **RESISTANT** LICE

No. GENES **DOWN**-REGULATED IN **RESISTANT** LICE

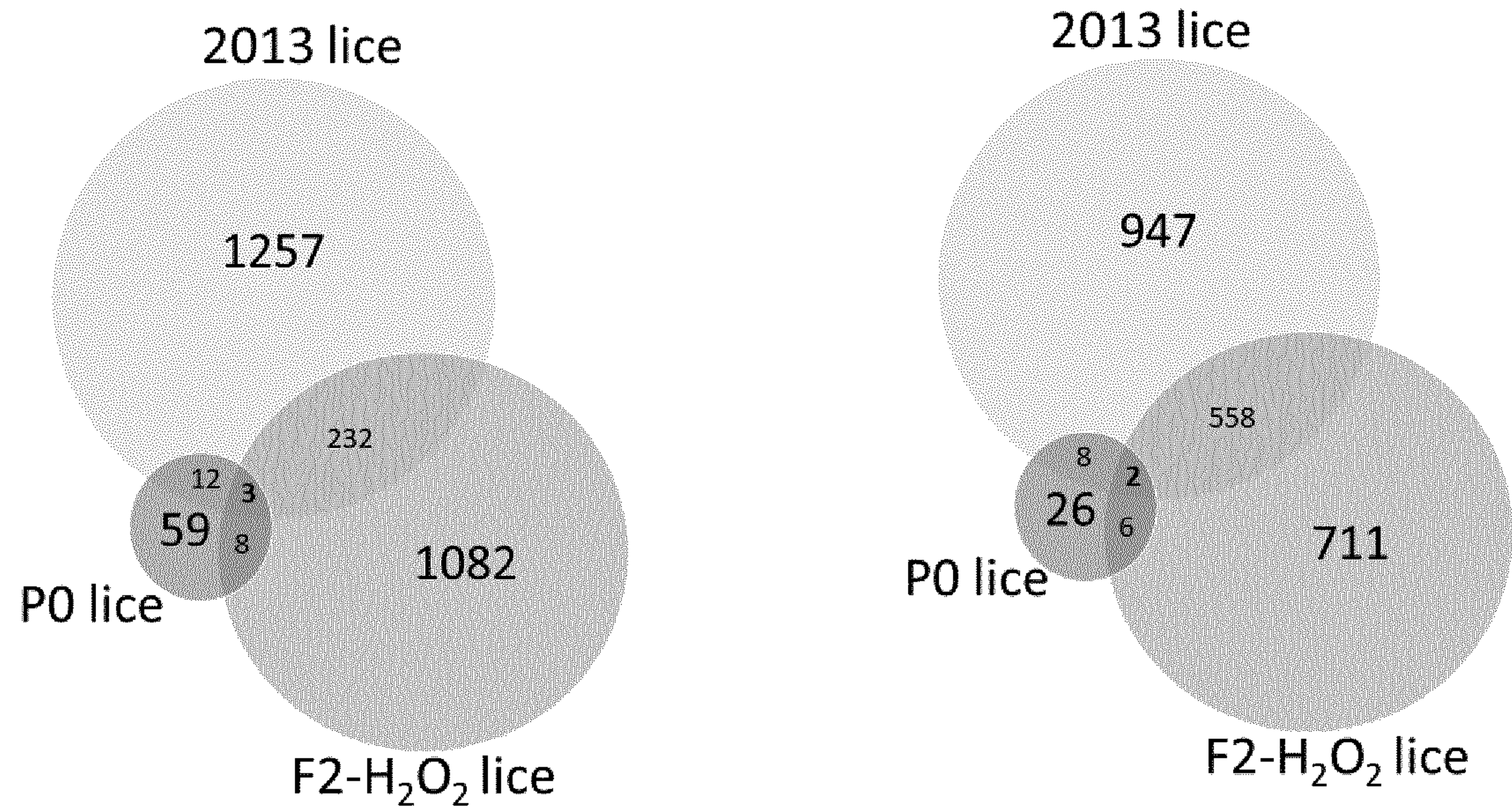
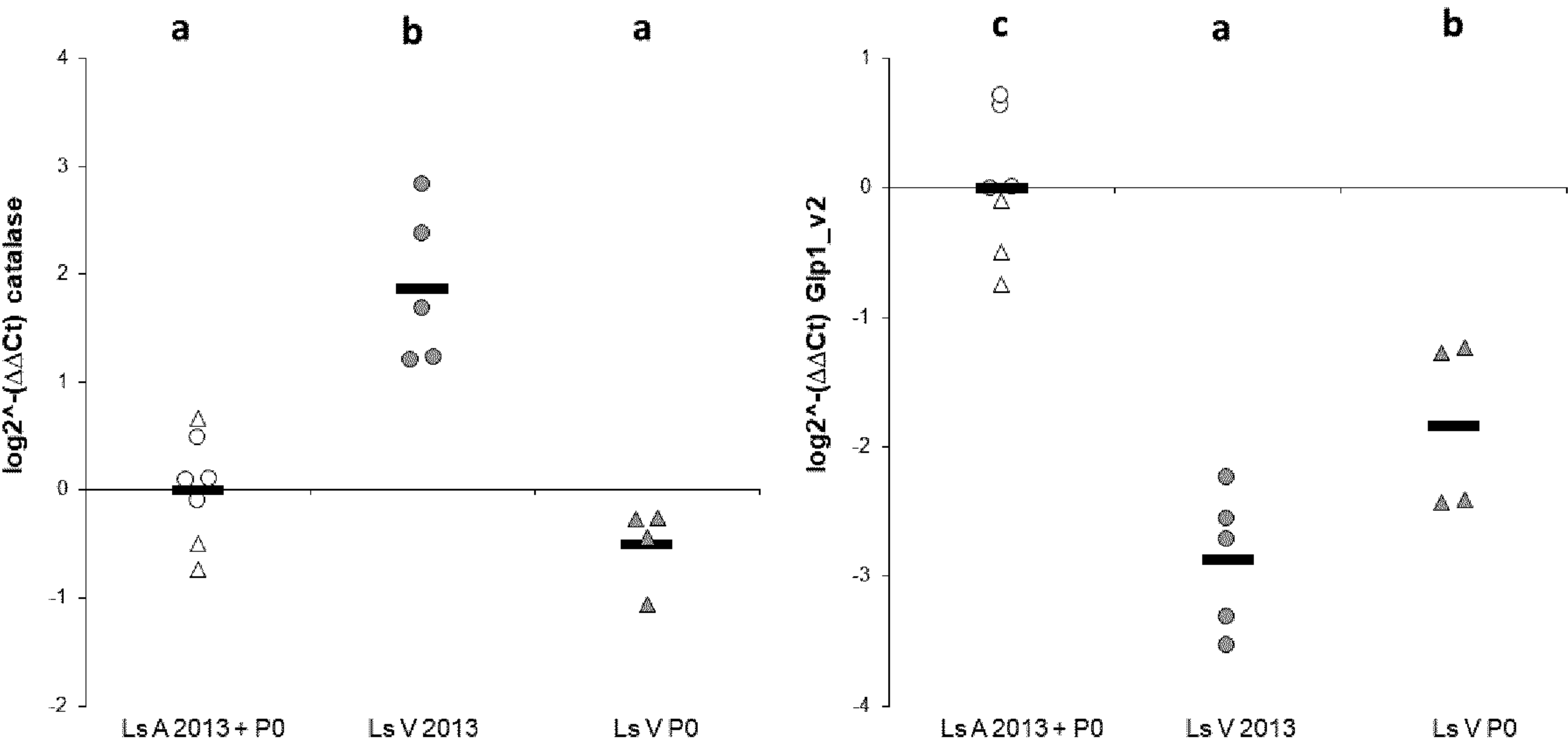


Figure 3



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2020/087625

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/6888

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 3 164 502 B1 (PATOGEN AS [NO]) 5 December 2018 (2018-12-05) cited in the application claims 1-16	1-12
X	----- DATABASE Geneseq [Online] 21 February 2019 (2019-02-21), "Sheep PLAGL1 gene INDEL locus-specific reverse PCR primer, SEQ ID 12.", XP055700267, retrieved from EBI accession no. GSN:BFX13486 Database accession no. BFX13486 sequence & CN 108 866 160 A (UNIV NORTHWEST A&F) 23 November 2018 (2018-11-23) sequence 12 ----- -/-	13



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 February 2021

Date of mailing of the international search report

26/02/2021

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Eveleigh, Anna

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2020/087625

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL [Online] 5 October 2007 (2007-10-05), "EST_lsal_evj_795477 lsalevj mixed_tissue_mixed_stage Lepeophtheirus salmonis (Pacific)_cDNA Lepeophtheirus salmonis cDNA clone lsal_evj_514_117_fwd 5', mRNA sequence.", XP055700660, retrieved from EBI accession no. EM_EST:EX485057 Database accession no. EX485057 sequence & RYOSUKE YAZAWA ET AL: "EST and Mitochondrial DNA Sequences Support a Distinct Pacific Form of Salmon Louse, Lepeophtheirus salmonis", MARINE BIOTECHNOLOGY, SPRINGER-VERLAG, NE, vol. 10, no. 6, 24 June 2008 (2008-06-24), pages 741-749, XP019636706, ISSN: 1436-2236, DOI: 10.1007/S10126-008-9112-Y the whole document -----	14,15
A	FARLORA RODOLFO ET AL: "Aquaporin family genes exhibit developmentally-regulated and host-dependent transcription patterns in the sea louse Caligus rogercresseyi", GENE, ELSEVIER, AMSTERDAM, NL, vol. 585, no. 1, 23 March 2016 (2016-03-23), pages 119-127, XP029512465, ISSN: 0378-1119, DOI: 10.1016/J.GENE.2016.03.035 the whole document -----	1-13
A	JON ANDERS STAVANG ET AL: "Phylogenomic and functional analyses of salmon lice aquaporins uncover the molecular diversity of the superfamily in Arthropoda", BMC GENOMICS, BIOMED CENTRAL, vol. 16, no. 1, 19 August 2015 (2015-08-19), page 618, XP021227385, ISSN: 1471-2164, DOI: 10.1186/S12864-015-1814-8 the whole document ----- -/--	1-13

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/087625

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	Celia Agusti-Ridaura ET AL: "Markers for monitoring hydrogen peroxide resistance in the salmon louse, Lepeophtheirus salmonis", 10 April 2020 (2020-04-10), XP055700521, DOI: 10.21203/rs.3.rs-22366/v1 Retrieved from the Internet: URL:https://www.researchgate.net/publication/340635661_Markers_for_monitoring_hydrogen_peroxide_resistance_in_the_salmon_ouse_Lepeophtheirus_salmonis/fulltext/5e966d66a6fdcca789189a85/Markers-for-monitoring-hydrogen-peroxide-resistance-in-the-salmon-ouse-Lepeophtheirus-salmonis.pdf [retrieved on 2020-06-03] the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2020/087625

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