Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 2952536 C 2019/09/24

(11)(21) **2 952 536**

(12) BREVET CANADIEN / CANADIAN PATENT

(13) **C**

(86) **Date de dépôt PCT/PCT Filing Date:** 2015/07/01
(87) **Date publication PCT/PCT Publication Date:** 2016/01/07
(45) **Date de délivrance/Issue Date:** 2019/09/24
(85) **Entrée phase nationale/National Entry:** 2016/12/15
(86) **N° demande PCT/PCT Application No.:** EP 2015/064972
(87) **N° publication PCT/PCT Publication No.:** 2016/001295
(30) **Priorités/Priorities:** 2014/07/02 (US62/019,911); 2014/12/22 (NO20141552)

(51) **Cl.Int./Int.Cl.** ***C12N 15/53*** (2006.01), ***C07H 21/04*** (2006.01), ***C12N 9/08*** (2006.01), ***C12Q 1/30*** (2006.01)

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(54) **Titre : METHODE DE DETECTION D'UNE RESISTANCE AU H_2O_2 CHEZ LE CRUSTACE**

(54) **Title: METHOD FOR DETECTING H_2O_2 RESISTANCE IN CRUSTACEANS**

ACTTTAGTTTACAATTCAAATCCA□ACCGTATAAGAACCTTCCTCGTACTACAACATCCG□ACTAGCTGA
□TCTAAGGAAAA□TCAAAGATAGAAAGGAGATATTT□ACTTGAATATT□TAAACAAAGAAAAAGGTAAGT
AGGATCATAGTT□ATTCAACAAAA□TAGTG□AAATA□CAAAA□GCAAAGACAGGCACTATGGAAAAATCA
ACACAA□CTCAAAGTTTAGTTTGT□AAAGTGTAATC□GGATACTACGTCTAAGAACAATAAATATGCCTC
□TCGTGATTGTGCCACAGAACAAC□TGCCAACTATTCAAAGTCGATAAAGAAAGAAGACC□TCTCACATC
CAGTCACGGAATCCCACTGGGTGA□AAAAA□AACATAGTTACCGTTGGCAGAAGAGGTCC□TCTTTGATT
CAAGATCTTGCA□TTATGGACGAAATCGCCCATTTTCTACCTCACCCTATTCCCCACACACTAGTTCATC
CAAAAGGAGCTGGAGCTT□TGGTTACTTGGAAATAACTCATGACATTACAAAGTACTGCAAGGCTGAAAT
ATTTGA□ACAAT□GGAAAGAGAACAGATCT□GCCGTAAGGTTCAGTACAGTTGGAGGTGAATCTGGATCT
GCTGATACTGAGAGAGATCCAAGAGGATTTGCCGTCAAATTT□ATACGAAGGAGGGTAAT□GGGAC□TGG
□TGGAAACAATACTCCTA□TTTTTT□TATTCGTGATCCCATTC□TTTTGAGAGTT□TATTCATTCACAAAA
AAGAAA□CCTGTAACACA□TTGAAGGATCCAGATATGTTTTGGGACTT□GTAAC□CTGAGGCCTGAAACA
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GATCTCATACCT□TAAAT□AGTTAATAAAAAGAACGAGGCTG□ATATTGTAAGT□TCACT□TAAAACTAA
CCAAGG□ATAAAGAATCT□TCAAG□CAAGAGGCTGCAAATATGGCCAGAGATGA□CCTGA□TACTC□ATT
CGCGAT□TATATAATGCTATTGACCAAGAAAACTTTCCTTCC□ATACCATGCACATTCAAGTGATGACTA
□GGATGAAGCAGACACCA□AGATT□TGATCCTTTTGATTTGACAAAGG□TTGGTCTCATAAAACCT□CCC
ACTCAT□GAAGTCGGACAACTAGTCCTCAA□AGAAA□CCATCCAATTACTTTGC□GAGGT□GAACAAATT
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□AACCTATAGTGATGCTCACCGCTATCGTA□TTCTGGTGAAG□GGGACGCCATGATTCCTCTGAGGAAGA
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AATATTATTTTT□TTCATGCATATGATAAAAAAAAA□ACTTGCTCACAAGACAAATGGTAATGAAACTTG
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AATCAC□CGGCA□TTATG□AGGGG□TTTTG□TTATA□CTGCT□GGCAACAGTCCGCTCAG□GGGGA□CTA
□TTTTG□TGCCAGAAGCAATTTCA□TCGTT□ATATGATTAAA□AAACA□ACAAC□CAAAA□CTATAGTCA
AATATTATTTCACTAGAT□ATATT□TGCTT□ATGCTGTAGTA□TAAG

(57) **Abrégé/Abstract:**
The present invention relates to a method for detection of elevated levels of catalase in crustaceans, such as Lepeophtheirus salmonis, related to resistance toward hydrogen peroxide (H_2O_2), and oligonucleotide sequences and kits useful in the method of the present invention. The present invention furthermore provides kits and reagents useful for the detection of elevated levels of catalase in Lepeophtheirus salmonis, related to resistance toward hydrogen peroxide (H_2O_2).

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 January 2016 (07.01.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/001295 A1

(51) **International Patent Classification**:
C12Q 1/68 (2006.01)

(21) **International Application Number**:
PCT/EP2015/064972

(22) **International Filing Date**:
1 July 2015 (01.07.2015)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
62/019,911 2 July 2014 (02.07.2014) US
20141552 22 December 2014 (22.12.2014) NO

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

[Continued on next page]

(54) Title: METHOD FOR DETECTING H_2O_2 RESISTANCE IN CRUSTACEANS

Figure 1

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ACTTTAGTTTACAATTCAAATCCATACCGTATAAGAACCTTCCTCGTACTACAACATCCGTACTAGCTGA
TTCTAAGGAAAATTCAAAGATAGAAAGGAGATATTTTACTTGAATATTTTAAACAAAGAAAAAGGTAAGT
AGGATCATAGTTTATTCAACAAAATTAGTGTAAATATCAAAATGCAAAGACAGGCACTATGGAAAAATCA
ACACAATCTCAAAGTTTAGTTTGTTAAAGTGTAATCTGGATACTACGTCTAAGAACAATAAATATGCCTC
TTCGTGATTGTGCCACAGAACAACTTGCCAACTATTCAAAGTCGATAAAGAAAGAAGACCTTCTCACATC
CAGTCACGGAATCCCACTGGGTGATAAAAATAACATAGTTACCGTTGGCAGAAGAGGTCCTTCTTTGATT
CAAGATGTTGCATTTATGGACGAAATGGCCCATTTTGTACGTGAGCGTATTCCCGAGAGAGTAGTTCATG
CAAAAGGAGCTGGAGCTTTTGGTTACTTGGAAATAACTCATGACATTACAAAGTACTGCAAGGCTGAAAT
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GCTGATACTGAGAGAGATCCAAGAGGATTTGCCGTCAAATTTTATACGAAGGAGGGTAATTGGGACTTGG
TTGGAAACAATACTCCTATTTTTTTTATTCGTGATCCCATTCTTTTTGAGAGTTTTATTCATTCACAAAA
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CCAAGGTATAAAGAATCTTTCAAGTCAAGAGGCTGCAAATATGGCCAGAGATGATCCTGATTACTCTATT
CGCGATTTATATAATGCTATTGACCAAGAAAACTTTCCTTCCTATACCATGCACATTCAAGTGATGACTA
TGGATGAAGCAGACACCATAGATTTTGATCCTTTTGATTTGACAAAGGTTTGGTCTCATAAAACCTTCCC
ACTCATTGAAGTCGGACAACTAGTCCTCAATAGAAATCCATCCAATTACTTTGCTGAGGTTGAACAAATT
GCTTTTAGTCCAGGGAACTTGATCCCTGGAATTGAAGCAAGCCCTGACAAAATGTTGCAAGGTCGGATAT
TAACCTATAGTGATGCTCACCGCTATCGTATTTCTGGTGAAGTGGGACGCCATGATTCCTCTGAGGAAGA
TAATTTTTATCAAGTCGGAGTGTTTTATAGGGATGTTTTAAACGAAGAACAAAAGAATAACTTGGTAGAC
AACATTGCAGTAAATTTAGTCCATGCACAAGAAAAATTCCAAGTGAAAGCCATCAAACAATTCGGGCAAT
GTGATCCTGATTATGGGAAAAGACTTGAGACTAGATTGGCTTATTATAAAAATAAATGATAAATTTTGTA
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AATATTATTTTTTTTCATGCATATGATAAAAAAAAATACTTGCTCACAAGACAAATGGTAATGAAACTTG
TGACAATTTCCATTAAATACCGTTGAGATATGAGCATCCACATCACTCTGACTATTTGCCAAACTGACAA
AATCACTCGGCATTTATGTAGGGGTTTTTGTTTATATCTGCTTGGCAACAGTCCGCTCAGTGGGGATCTA
TTTTTGTTGCCAGAAGCAATTTCATTCGTTTATATGATTAAATAAACATACAACTCAAAATCTATAGTCA
AATATTATTTCACTAGATTATATTTTGCTTTATGCTGTAGTATTAAG
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(57) **Abstract**: The present invention relates to a method for detection of elevated levels of catalase in crustaceans, such as *Lepeophtheirus salmonis*, related to resistance toward hydrogen peroxide (H_2O_2), and oligonucleotide sequences and kits useful in the method of the present invention. The present invention furthermore provides kits and reagents useful for the detection of elevated levels of catalase in *Lepeophtheirus salmonis*, related to resistance toward hydrogen peroxide (H_2O_2).

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— *with sequence listing part of description (Rule 5.2(a))*

Method for detecting H_2O_2 resistance in crustaceans

Field of the invention

The present invention relates to a method for detection of elevated levels of catalase in crustaceans, such as *Lepeophtheirus salmonis*, related to resistance toward hydrogen peroxide (H_2O_2), and oligonucleotide sequences and kits useful in the method of the present invention. The present invention furthermore provides kits and reagents useful for the detection of elevated levels of catalase in *Lepeophtheirus salmonis*, related to resistance toward hydrogen peroxide (H_2O_2).

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 has recently been estimated to €300 million (Costello M. J. (2009), The global economic cost of sea lice to the salmonid farming industry. Journal of Fish Diseases. 32. 115–118) and the aquaculture industry relays heavily on a few chemotherapeutants for 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., Copley L., Wallace C., Jackson D., Horsberg T.E. (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.

Although various chemotherapeutants have been in use in the aquaculture industry for more than 30 years, it is only during the last 15 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). So far, drug resistance in sea lice has been detected by various types of bioassays as a significant increase in EC50/LC50. Although the bioassays can detect any type of resistance to a given drug, such methods are not very accurate or sensitive.

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). Interox® 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-louse treatment with least environmental risk. Recent studies comparing the effects of Interox® 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.

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 308,000 Kg, in 2010, 3,071,000 kg and in 2011, 1,927,000 kg.

Treatments with hydrogen peroxide are rarely fully effective and from 85% to 100% of the mobile stages may be removed (Thomassen J.M. (1993) Hydrogen peroxide as a delousing agent for Atlantic salmon. In: Pathogens in Wild and Farmed Fish: Sea Lice (ed. by G.A. Boxshall & D. Defaye), pp. 290-295. Ellis Horwood, Chichester). Hydrogen peroxide may induce mechanical paralysis caused when bubbles form in the body (Thomassen J.M. (1993) Hydrogen peroxide as a delousing agent for Atlantic salmon. In: Pathogens nf Wild and Farmed Fish: Sea Lice (ed. by G.A. Boxshall & D. Defaye), pp. 290-295. Ellis Horwood, Chichester), and these gas bubbles in the haemolymph detach the lice and cause the lice to float to the water surface (Bruno D.W. & Raynard R. (1994) Studies on the use of peroxide as a method for the control of sea lice on Atlantic salmon. Aquaculture International 2, 10-18). Many sea lice subsequently recover from treatment (Johnson S.C., Constible J.M. & Richard (1993). Laboratory investigations of the efficacy of hydrogen peroxide against the salmon louse *Lepeophtheirus salmonis* and its toxicological effects on Atlantic salmon *Salmo salar* and chinook salmon *Oncorhynchus*

tshawytscha. Diseases of Aquatic Organisms 17, 197-204, Bruno D.W. & Raynard R. (1994) Studies on the use of peroxide as a method for the control of sea lice on Atlantic salmon. Aquaculture International 2, 10-18). It has been suggested that lice could therefore resettle on salmon (Hodneland K., Nylund A., Nisen F. & Midttun B. (1993). The effect of Nuvan, azamethiphos and hydrogen peroxide on salmon lice (*Lepeophtheirus salmonis*). Bulletin of the European Association of Fish Pathologists 13, 203-206.), but this was not observed in farm treatments (Treasurer J. W. & Grant A. (1997) The efficacy of hydrogen peroxide for the treatment of farmed Atlantic salmon, *Salmo salar L.*, infested with sea lice (Copepoda: Caligidae). Aquaculture 148, 265-275).

Hydrogen peroxide (H_2O_2) is the simplest peroxide (a compound with an oxygen-oxygen single bond). It is also a strong oxidizer. Hydrogen peroxide is a clear liquid, slightly more viscous than water. In dilute solution, it appears colorless. Due to its oxidizing properties, hydrogen peroxide is often used as a bleach or cleaning agent. The oxidizing capacity of hydrogen peroxide is so strong that it is considered a highly reactive oxygen species. Concentrated hydrogen peroxide, or 'high-test peroxide', is therefore used as a propellant in rocketry (Hill, C. N. (2001). A Vertical Empire: The History of the UK Rocket and Space Program, 1950–1971. Imperial College Press. ISBN 978-1-86094-268-6).Organisms also 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 peroxidases, which harmlessly and catalytically decompose low concentrations of hydrogen peroxide to water and oxygen.

Two theories 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 protistans and monogeneans, is the liberation of molecular oxygen as a result of catalase action (Schaperclaus W., Kulow H. & Screkenbach K., eds. (1979) Fishkrankheiten, 4th edn. Academie-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 (eds by R.T. Roush & B.E.Tabashnik), 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 concentrations of hydrogen peroxide (Barnes, Bowden, Horne & Ellis 1999 Barnes A.C., Balebona M.C., Horne M.T. & Ellis A.E. (1999a) Superoxide dismutase and catalase in *Photobacterium damselae subsp. piscicida* and their roles in resistance to reactive oxygen species. Microbiology 145, 483–494 &(Barnes A.C., Bowden T.J., Horne M.T. & Ellis A.E. (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.

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 P, Fita I, Loewen PC (January 2004). "Diversity of structures and properties among catalases". Cell. Mol. Life Sci. 61 (2): 192–208. doi:10.1007/s00018-003-3206-5. PMID 14745498). 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 of hydrogen peroxide to water and oxygen each second (Goodsell DS (2004-09-01). "Catalase". Molecule of the Month. RCSB Protein Data Bank. Retrieved 2007-02-11).

Orr et al., 1992, Archieves of biochemistry and biophysics 297:1, 35-41 reports of a study considering whether overexpression of catalase activity above the novel level prolongs the life span and provides enhanced protection against oxidative stress in *Drosophila melanogaster*. Similar findings are discussed by Mockett et al., 2003, Free radical and Biology and Medicine, 34:2, 207-2017.

Sanford et al. 1989, Journ. Of bacteriology, 171:3, 1492-1995 reports that *Dictyostelium discoideum* with catalase deficiency had an increased sensitivity to hydrogenperoxide.

In Vattanaviboon et al. 2001, FEMS microbiology Letters, 221:89-95 and Vattanabivoon and Mongkolsuk (2001),FEMS Microbiology Letters, 200: 111-116 the resistance against hydrogen peroxide in a bacteria (*Vibrio harveyi*) and is discussed.

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, particularly organophosphates and pyrethroids, is well established. However, resistance towards hydrogen peroxide (H_2O_2) has only been reported ones (J W Treasurer, S Wadsworth & A Grant. (2000) Resistance of sea lice, *Lepeophtheirus salmonis* (Kroyer), to hydrogen peroxide on farmed Atlantic salmon, *Salmo salar L.* Aquaculture Research, 31, 855-860). However, in the autumn of 2013, reduced treatment efficacy was reported from salmon farms in the north of Norway. The cause or mechanisms behind such resistance in salmon lice against hydrogen peroxide has not been known previously.

Up to date, resistance in sea lice towards hydrogen peroxide (H_2O_2) has not been acknowledged as a major concern in the aquaculture industry, except for the report by Orr et al. in 1992, *supra*. No documentation related to the mechanism of such resistance have existed. Also, no specific mechanism on the molecular level has been associated with resistance towards hydrogen peroxide (H_2O_2) in any parasites, including sea lice.

Efficient and sensitive methods for diagnosing resistance are crucial in order to manage and control drug resistance. Early detection of reduced sensitivity to a chemical can enable effective countermeasures to be enforced at a time point when these have a greater probability of being effective. Therefore, accurate and speedy identification of hydrogen peroxide (H_2O_2) resistant sea lice is crucial. Detection of hydrogen peroxide (H_2O_2) resistance prior to treatment, and the use of such analyses after treatment to evaluate treatment efficacy constitutes an important determinant for the 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 (H_2O_2) commonly used to combat sea lice infestation is linked to the expression level of the enzyme catalase. The present inventors have identified the gene encoding a catalase enzyme, and its relevance in the development of hydrogen peroxide resistance in sea lice. Furthermore, the present inventors have developed Real-Time PCR-assays to quantify the expression of this catalase encoding gene, and correlated this expression to the resistance toward hydrogen peroxide (H_2O_2) in sea lice. More particularly, the present invention is based on the quantification of the expression of the gene encoding the catalase of the sea lice (*Lepeophtheirus salmonis*) shown to be involved in the resistance towards hydrogen peroxide (H_2O_2) -based chemotherapy.

Thus, according to one embodiment, and in vitro method is provided for the detection of hydrogen peroxide resistance in one or more crustaceans comprising the steps of determining the catalase activity in the crustaceans to be analyzed.

According to one embodiment, an in vitro method is provided for detection of hydrogen peroxide (H_2O_2) resistance in one or more crustaceans comprising the steps of quantifying the expression of a catalase gene of the crustaceans to be analyzed. According to one embodiment, said catalase gene has a sequence as depicted in SEQ ID No. 1, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 1.

The crustacean may be one or more copepods, e.g. belonging to the family Caligidae. According to one embodiment, the copepod is selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei, Caligus elongatus*, and *Caligus rogercresseyi*.

According to one embodiment, said catalase gene origins from *Lepeophtheirus salmonis* and has a sequence as depicted in SEQ ID No. 1, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 1, such as 80% identical with SEQ ID No.1.

According to another embodiment, said catalase gene origins from *Lepeophtheirus salmonis* and has a sequence as depicted in SEQ ID No. 1, or variants or fragments thereof being at least 80 % identical with SEQ ID No. 1.

According to another embodiment, said catalase gene origins from *Lepeophtheirus salmonis* and has a sequence as depicted in SEQ ID No. 1, or variants or fragments thereof being at least 90 % identical with SEQ ID No. 1

According to one embodiment, said catalase gene origins from *Caligus clemensei* and has a sequence as depicted in SEQ ID No. 13, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 13, such as 80% identical with SEQ ID No. 13.
According to another embodiment, said catalase gene origins from *Caligus clemensei* and has a sequence as depicted in SEQ ID No. 13, or variants or fragments thereof being at least 90 % identical with SEQ ID No. 13

According to yet another embodiment, said catalase gene origins from *Caligus rogercresseiy* has a sequence as depicted in SEQ ID No. 14, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 14, such as 80% identical with SEQ ID No. 14. According to yet another embodiment, said catalase gene origins from *Caligus rogercresseiy* has a sequence as depicted in SEQ ID No. 14, or variants or fragments thereof being at least 90 % identical with SEQ ID No. 14

According to another embodiment, the present method comprise the steps of detecting increased RNA-expression levels associated with hydrogen peroxide (H_2O_2) resistance in a crustacean to be analyzed, wherein said crustacean is resistant to hydrogen peroxide (H_2O_2) resistance if having elevated levels of catalase RNA-expression.

According to another aspect of the present invention, a method is provided comprising the steps of:

a) collecting sea lice from infested fish or water samples;

b) isolating genomic material from the any life stage of collected sea lice;

c) determining the expression level of a catalase gene.

According to one embodiment, the determination of expression level in c) is determined by measuring the catalase RNA-expression level, wherein said sea lice is resistant to hydrogen peroxide (H_2O_2) if the catalase RNA-expression level is elevated.

According to another embodiment of the present method, said step c) is performed using a primer selected from the group consisting of SEQ ID No.´s 2, 3, 4, 5, 7, 8, 10 and 11.

According to another embodiment of the present method, said step c) is performed using a primer selected from the group consisting of SEQ ID No.´s 15, 16, 18 and 19.

According to another embodiment of the present method, said step c) is performed using at least one probe selected from the group consisting of SEQ ID No.´s 6, 9 and 12.

According to another embodiment of the present method, said step c) is performed using at least one probe selected from the group consisting of SEQ ID No.´s 17 and 20.

According to another embodiment of the present method, said step c) comprises nucleic acid amplification.

According to another embodiment of the present method, the nucleic acid amplification is performed using polymerase chain reaction.

According to another embodiment of the present method, said step c) is performed by contacting the genomic material of the sea lice to be analyzed with a detection reagent, and determining the expression level of catalase mRNA.

According to the above embodiments, the determination of hydrogen peroxide (H_2O_2) resistance takes the advantage of determining the expression level of the gene encoding catalase in the crustaceans to be analyzed. Hydrogen peroxide (H_2O_2) resistance may also be determined by analyzing the level of catalase activity, as increased expression level of the catalase gene may be shown as an increased catalase activity level in the hydrogen peroxide resistant crustaceans.

According to another embodiment, the present method comprises the steps of detecting increased catalase activity in sea lice. Detection of catalase activity in sea lice may e.g. be performed using commercially available kits for measuring catalase enzyme activity, wherein said crustacean is resistant to hydrogen peroxide (H_2O_2) resistance if having elevated levels of catalase activity as a result of increased catalase gene expression.

According to another aspect of the present invention, a method is provided comprising the steps of:

a) collecting sea lice from infested fish or water samples;

b) homogenization of sea lice tissue samples from any life stage of collected sea lice;

c) determining the catalase activity, wherein said sea lice is resistant to hydrogen peroxide (H_2O_2) if the catalase activity is elevated.

According to one embodiment, the determining of catalase activity step c) is performed by collecting a sample isolated from the crustaceans to be analyzed with a pre-determined amount of hydrogen peroxide, and then with horseradish peroxidase and a chromophore, resulting in the formation of a chroma signal, and wherein the decomposition of hydrogen peroxide is proportional with the level of catalase in the sample. According to one embodiment, the formation of said chroma signal is measured colorometrically.

The present invention also provides for an isolated oligonucleotide sequence encoding a catalase of which the expression level is associated with hydrogen peroxide (H_2O_2) resistance. According to one embodiment, said nucleic acid sequence has the sequence as depicted in SEQ ID No. 1.

According to yet another embodiment, the oligonucleotide sequence of the present invention is identical or has at least 70% sequence identity with the SEQ ID No. 1 SEQ ID No. 13 or SEQ ID No. 14 and sequences having at least 70% sequence identity with the SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14 , respectively, or a fragment thereof, and complementary sequences of SEQ ID No 1 and fragments thereof, provided that said sequence are not identical with the sequence depicted in SEQ ID No. 21 or SEQ ID No. 22.

SEQ ID No. 21 correspond to a sequence published the 27th March 2009 assigned the Genbank accession number BT081149 (mRNA sequence encoding catalase from *Caligus clemensi*).

SEQ ID No. 22 correspond to a sequence published the 11th June 2013 assigned the Genbank accession number KF233999 (mRNA sequence encoding catalase from *Octopus vulgaris*).

The present invention furthermore provides for oligonucleotide sequences, such as primers and probes, useful in the detection of catalase gene expression in crustaceans according to the present invention. The said oligonucleotide sequences may be selected from the group consisting of SEQ ID No.´s 2-12, and fragments and variants thereof, having at least 70 % sequence identity with the said sequences SEQ ID No.´s 2-12, and complementary sequences thereof.

Furthermore, the present invention provides a kit for detection of hydrogen peroxide (H_2O_2) resistance in crustaceans comprising at least one oligonucleotide according to the present invention.

Finally, the present invention provides the use of one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 1 or a complementary oligonucleotide thereof for the determination of hydrogen peroxide in crustaceans.

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

Figures

Figure 1 shows the Catalase gene sequence from the *Lepeophtheirus salmonis* (SEQ ID No. 1).
Figure 2 shows the difference in catalase expression is significant between the H2O2-resistant strain (labelled H2O2), and the other two strains when using the calculated ratio value for catalase / elongation factor in each sample.

Figure 3 shows the distribution of normalized Ct-values in the whole dataset. A biphasic distribution with one peak at Ct ~16 and one at Ct ~20 can be observed.

Figure 4a shows the distribution of normalized Ct-values within treatments.
Figure 4b shows the distribution of normalized Ct-values within treatments and survival status.

Figure 5 shows the Wilcoxon test of rank sums for the difference in normalized Ct-values between immobilized and alive parasites for both treatment groups. For both groups, the difference is highly significant ($p<0.0001$).

Figure 6a shows the Wilcoxon test of rank sums for the difference in normalized Ct-values between immobilized ("immob") parasites in the two treatment groups, and a corresponding test for live ("lev") parasites.

Figure 6b. Estimated probability of immobilization (red line) with 95% confidence intervals (dotted lines) as a function of normalized Ct values in the experiments exposing parasites alone (Parasitt) and parasites on fish (Fisk) to H_2O_2. Vertical symbols along y = 0 and Y = 1 denote Ct values of the live and immobilized lice, respectively.

Figure 7 shows the output from the ANOVA analysis of catalase activity (U/ug protein) versus the strain of salmon lice.

Figure 8 shows the output from the ANOVA analysis of expression of the catalase gene (normalized Ct-values) versus the strain of salmon lice.

Figure 9 shows the output from the ANOVA analysis of normalized Ct-values versus the strain of salmon lice.

Figure 10 shows the output from the Tukey-Kramer HSD test of normalized Ct-values versus the strain of salmon lice.

Figure 11 shows the clustering of strains based on the normalized Ct-values.

Figure 12 shows the alignment between the catalase genome sequence from *Lepeophtheirus salmonis, Caligus rogercresseyi* and *Caligus clemensi.*

Detailed description of the invention

The present invention provides an in vitro method for determination of hydrogen peroxide (H_2O_2) resistance in crustaceans, including copepods, in particular *Lepeophtheirus salmonis*, based on the surprising findings that the expression level of catalase gene is linked with resistance against hydrogen peroxide.

The increased level of catalase gene expression results in increased catalase activity in the hydrogen peroxide resistant crustacean. The expression “catalase activity” as used in the present invention is thus to be read in the context of being a result of increased catalase gene expression, irrespective of the method used to determine said increased catalase gene expression and/or catalase activity.

Although the experimental data linking the catalase gene expression level and hydrogen peroxide resistance was identified in the sea lice species *Lepeophtheirus salmonis,* the skilled person will acknowledge, based on the teaching herein, that the present method and the present oligonucleotides may be used to determine hydrogen peroxide resistance in crustaceans, in particular copepods, in particular copepods belonging to the family Caligidae. In particular, it is to be understood that the present method and the present oligonucleotides may be used to determine hydrogen peroxide resistance in copepods affecting farmed fish, such as e.g. fish belonging to the family *Salmonidae.* According to one embodiment, the present method and present oligonucleotides are useful for detection of hydrogen peroxide resistance in copepod selected from the group consisting of *Lepeophteirus salmonis*, *Caligus clemensei, Caligus elongatus*, and *Caligus rogercresseyi.*

Throughout the application, 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 in the present application, ”sea lice” or “sea louse” refer to the species *Lepeophtheirus salmonis*. By establishing a bioassay to differentiate sensitive vs resistant populations of sea lice, characterizing several lice strains with respect to sensitivity, documenting that the resistance is inheritable, measuring catalase enzyme activity, sequencing the catalase gene and establishing Real-Time PCR-assays, the present inventors have found that the hydrogen peroxide resistance is linked with the level of expression of the catalase gene.

In particular, the present application provides a method wherein at least one hydrogen peroxide resistant sea louse is determined by determining the catalase expression level of the sea lice to be tested.

The sequences provided in table 3 below may according to one embodiment be used to determine hydrogen peroxide resistant sea lice of the species *Lepeophtheirus salmonis*.

The present inventors have furthermore provided sequences that may be used according to the present method to determine hydrogen peroxide resistant sea lice of the species *Caligus clemensei* and *Caligus rogercresseyi*, i.e. having the following sequences:

Species	Forward primer	Revers primer	Probe
Caligus rogercressеyi	CR-ST-F AAGAGGAATCCTGTGACACACTTG (SEQ ID No. 15)	CR-ST-R CTGGTCGGAGTGTGACAAAGTC (SEQ ID No. 16)	CR-ST-P ACCCCGACATGGTATG (SEQ ID No. 17)
Caligus clemensei	Cali-Cata-F GCGCACTTTGTCCGAGAAC (SEQ ID No. 18)	Cali-Cata-R CTGCACCCTTGGCATGAAC (SEQ ID No. 19)	Cali-Cata-P CATTCCCGAGCGCGT (SEQ ID No. 20)

According to one embodiment, the present invention provides a method for characterizing the hydrogen peroxidase sensitivity of one or more sea lice, comprising the steps of

a) determining in a biological sample comprising DNA or RNA of one or more sea lice the expression level of the catalase gene according to SEQ ID No. 1 or a variant or fragment thereof;

b) comparing the determined catalase expression level to a control standard or the expression of the catalase gene, or a variant or fragment thereof, in a control sample;

c) determining whether the difference in expression level in the sample as compared to the control standard or the expression of catalase in a control sample; and

d) characterizing the sea lice, wherein the sea lice to be tested has a higher expression level compared with the control standard or the control sample.

According to one embodiment, said method includes 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 of step a)

c) providing a pair of PCR primers specific for the catalase gene of SEQ ID No. 1;

d) performing PCR on the nucleic acid of step b) using the pair of PCR primers of step c);

e) determining the expression level of the catalase gene by comparing the level of catalase expression with the level of control standard or a catalase gene expression sample control.

According to one embodiment, the level of catalase gene expression control is the level of catalase expression in H_2O_2 sensitive sea lice.

According to another embodiment, a H_2O_2 resistant sea louse is a sea louse having a catalase expression level that are significantly higher compared with the catalase expression level in a H_2O_2 sensitive sea louse.

According to another embodiment, a method is provide wherein one or more H_2O_2 resistant sea lice is determined using a primer pair selected from the group of primer pair consisting of SEQ ID no. 2 and 3, SEQ ID No. 4 and SEQ ID No. 5, SEQ ID No. 7 and SEQ ID No. 8, and SEQ ID No. 10 and SEQ ID No. 11.

According to another aspect of the present invention, the catalase expression level is measured using a catalase activity assay, e.g. wherein the method of the invention comprises the steps of:

a) providing a sample to be tested;

b) add H_2O_2 to the sample of a) to the sample of a)

c) stop the reacting of b) by adding a catalase inactivator

d) determine the remaining amounts of H_2O_2

e) determine the amount of catalase present in the sample

f) compare the results of step e) with a control sample of a H_2O_2 sensitive sea louse and determine the catalase expression level of the test sample.

Furthermore, 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 of the catalase enzyme gene, e.g. the catalase enzyme gene depicted in SEQ ID No 1. An “oligonucleotide sequence” or “nucleic acid sequence” used to determine the expression level of catalase gene is capable of hybridize to a nucleic acid sequence with a complementary sequence, such as e.g. mRNA extracted from the copepod to be analyzed for hydrogen peroxide resistance.

The skilled person is well 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 the present invention may be designed to hybridize to either strand.

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.

Oligonucleotide probes and oligonucleotide primers

The present invention provides oligonucleotide probes and oligonucleotide primers that may be used for determining the level of expression of the catalase gene being linked with hydrogen peroxidase resistance in a crustacean to be tested in accordance with the present invention. 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.

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 crustacean to be tested.

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 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.

A 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 a crustacean 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 invention typically comprises a nucleotide sequence which under stringent conditions hybridize to at least 8, 10, 12, 16, 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 crustacean to be analyzed according to the present invention. According to one embodiment, the oligonucleotide probe according to the present invention comprises about 13 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 provided which are selected from the group consisting of SEQ ID No. 6, SEQ ID No. 9 and SEQ ID No. 12.

The present invention furthermore provides oligonucleotide primers useful for amplification of any given region of a nucleotide sequence. An oligonucleotide primer according to the present invention typically comprises a nucleotide sequence at least 8, 10, 12, 16, 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 18 - 25 consecutive nucleotides, more preferably about 20 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. It is to be understood that the oligonucleotide primer according to the present invention according to one embodiment may comprise SNP´s or the complement thereof. According to one embodiment, the primers useful in order to determine hydrogen peroxide resistant sea lice may be selected from the group consisting of the primer pairs SEQ ID No. 2 and SEQ ID No. 3, SEQ ID No. 4 and SEQ ID No. 5, SEQ ID No. 7 and SEQ ID No. 8, and SEQ ID NO. 10 and SEQ ID No. 11.

Oligonucleotide probes and oligonucleotide primers according to the present invention may be synthesizes according to methods well known to the skilled person.

The present invention furthermore relates to isolated nucleic acid sequences and variants or fragments thereof having at least 70% identity with the nucleic acid sequences depicted in SEQ ID NO.´s 1-12 or fragments thereof. 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, 80% identity, 85% 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.

According to one embodiment, the invention includes oligonucleotide sequence encoding a catalase of which the expression level is associated with hydrogen peroxide resistance in sea lice, wherein said nucleic acid sequence has the sequence as depicted in SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14 or fragments thereof, and sequences having at least 80% sequence identity with the SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14, respectively, and complementary sequences of SEQ ID No 1, SEQ ID No. 13, and SEQ ID No. 14, respectively.

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 identity program useful for determining the percent homology 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).

Methods for determination of the catalase activity by measuring the expression level of catalase gene.

Based on the teaching herein, i.e. that the expression level of catalase gene are linked with hydrogen peroxide resistance in crustacean, the skilled person will acknowledge that various well known methods are available for the determination of the expression level of genes, such as the expression of catalase gene within a population of crustacean, may be applicable in the present method. For example, methods based on genome sequencing, hybridization and enzyme assay based methods are applicable for determining whether a crustacean is hydrogen peroxidase resistant in accordance with the present inventions.

Various enzyme based methods are available for the skilled person for this purpose, of which a number of polymerase chain reaction (PCR) based methods are available. Oligonucleotide primers according to the present invention useful in such a method may be selected from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 8, SEQ ID NO. 10 and SEQ ID No. 11.

Several hybridization methods for detection of a nucleic acid sequence of interest, such as e.g. a gene sequence or a mRNA encoding the catalase enzyme (or one or more parts thereof) are available to the skilled person, and which may be utilized in accordance with the method of the present invention. For example, the oligonucleotides according to the present invention may be detected utilizing molecular beacon technology. According to this aspect of the present invention, oligonucleotide primers may be synthesized comprising complementary regions at each end allowing the formation of a hairpin loop, and wherein a fluorophore is attached at one end of the oligonucleotide primer, and a quenching agent is attached to the other end, and wherein fluorescence signal is produced upon binding to a target sequence.

Isolation of sea lice genomic material

The method according to the present invention may according to one embodiment involve the isolation of a biological sample from a crustacean and measuring the level of expression of catalase in order to determine whether the crustacean is hydrogen peroxide resistant.

In order to determine whether a crustacean, such as sea lice, is hydrogen peroxide resistant in accordance with the present invention, genomic material may be isolated. 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 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). 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. 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/~gcat-seek/protocols/DNeasy_Blood_&_Tissue_Handbook.pdf).

Catalase gene expression detection kits

Based on the teaching herein, the skilled person will acknowledge that , based on the identification of the link between hydrogen peroxide resistance and catalase activity in crustaceans, reagents applicable in determination of catalase activity and/or catalase gene expression level can be developed for the determination of hydrogen peroxidase resistance. The term “kit” as used herein in the context of catalase activity determination detection reagents is intended to cover reagents useful for determination of catalase activity, both in respect of determining the level of catalase gene expression or by determining the activity of catalase present in a sample to be analyzed.

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 ore more oligonucleotide probe(s), oligonucleotide 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 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 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.

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 catalase gene expression utilizing oligonucleotide primers or primer pair(s). According to one embodiment, the kit according to the present invention comprises a forward primer and a reverse primer for amplifying a region of the catalase gene. Said kit may furthermore optionally comprise further reagents (enzymes and nucleotide triphosphates) necessary for conducting PCR or real time PCR.

Detection of catalase activity

According to the present invention, the high expression level of catalase seen in hydrogen peroxide resistance sea lice may also be determined measuring the catalase activity in sea lice to be analyzed, as increased catalase gene expression results in increased catalase activity.

Various protocols useful for measuring catalase activity are known to the skilled person and are useful in measuring catalase activity in accordance with the present invention. Many of the well known catalase assays available to the skilled person measure the catalase activity by contacting the sample to be analyzed with a predetermined amount of hydrogen peroxide, and then with horseradish peroxidase and a chromophore. Catalase present in the sample to be analyzed decomposes hydrogen peroxide according to the following equation:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

The horseradish peroxidase reacts with the chromophore using the remaining hydrogen peroxide as an oxidizing agent, resulting in the formation of a chroma signal which may be measured using well known colorimetric methods. The mere catalase present in the sample, the less chroma signal is formed. Non-limiting examples of chromophores to be used according to this aspect of the present invention is 3,3',5,5'-tetramethylbenzidin, 3,3'-diaminobenzidine and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid).

According to another embodiment of the present method, step c) of the above method is performed by detecting increased catalase activity in sea lice using commercially available kits for measuring catalase enzyme activity. Commercially available catalase assay kits are provided by e.g. Sigma-Aldrich ® (CAT100-1KT), cf. http://www.sigmaaldrich.com/catalog/product/sigma/cat100?lang=en&region=NO, Cayman Chemical Company ®, see https://www.caymanchem.com/pdfs/707002.pdf, Oxford Biochemical Research ®, see http://www.oxfordbiomed.com/catalase-assay-kit, and OxiSelectTM Catalase Activity Assay Kit from Cell Biolabs, Inc., see http://www.cellbiolabs.com/sites/default/files/STA-341-catalase-activity-assay.pdf.

EXAMPLES

Reduced efficacy of treatments with hydrogen peroxide (H_2O_2) has been observed in Nord-Trøndelag and Nordland counties in Norway. A salmon lice strain from this area (LS H_2O_2) has been cultivated in the wet-lab at the NIVA marine research station, Solbergstrand, Drøbak. The offspring of the original strain has been subjected to bioassays with hydrogen peroxide using the protocol of Sevatdal S and Horsberg TE (2003), "Determination of reduced sensitivity in sea lice (*Lepeophtheirus salmonis* Kroyer) against the pyrethroid deltamethrin using bioassays and probit modelling", Aquaculture 218, 21–31, adjusted for hydrogen peroxide using an exposure period of 30 minutes. The effect of the assay was determined immediately after exposure, as previous tests have demonstrated that around 80 % of the parasites will regain motility within 24 hours after exposure. The results were compared with a similar test conducted on a strain with no previous history of treatment failures, neither by H_2O_2, nor by any other chemical (LS Alta). The median immobilizing concentrations of H_2O_2 in mg/l (with 90 % CI) for these two strains were:

LS H_2O_2	1708 (1486-1962)
LS Alta	209 (157-277)

The mechanism behind the observed reduced sensitivity has not been determined previously. Studies at NMBU, School of Veterinary Science, have indicated overexpression of a metabolic enzyme catalase to be a factor. Based on these findings, catalase genes were sequenced using degenerated primers, and PatoGen has developed a TaqMan qPCR assay for the expression of this catalase gene. Several studies have been performed to confirm the correlation between H_2O_2-resistance and the overexpression of this catalase gene.

Example 1: H_2O_2-resistance is related to catalase gene expression

In order to determine if resistance towards H_2O_2 in sea lice is related to the expression level of the catalase gene, the genome sequence for the Lepeophtheirus salmonis catalase gene were sequenced using degenerated primers based on the known sequence for the catalase gene in *Caligus clemensi*. (http://www.ncbi.nlm.nih.gov/nucleotide/225719453?report=genbank&log$=nuclalign&blast_rank=1&RID=9VZC439Y01R). In order to quantify the expression level of the catalase gene in sea lice strains with known resistance status toward H_2O_2-treatments, a PCR-assay based upon the binding of the fluorescent dye SYBR-Green I into the PCR product (PE Applied Biosystems, Warrington, UK) was used. The primers used in this study are listed in table 1.

Table 1: Primers used in SYBR-Green I assay for study of expression level of the catalase gene in sea lice (*Lepeophtheirus salmonis*).

Name	Primer sequence	length	GC%	Tm	product length
CATALASE_F6	CCACAGAACAACTTGCCAAC SEQ ID No. 2	20	50	59.1	157
CATALASE_R6	GCCATTTCGTCCATAAATGC SEQ ID NO. 3	20	45	60.3	

Results

The catalase gene sequence from the *Lepeophtheirus salmonis* is presented in figure 1.

The difference in catalase expression is significant between the H_2O_2-resistant strain (labelled H_2O_2), and the other two strains when using the calculated ratio value for catalase / elongation factor in each sample (Figure 2). Thus, there is strong indication that the expression level of the catalase gene is suitable for differentiating between lice resistant and sensitive to H_2O_2 treatment.

Table 2: Expression level of the catalase gene in sea lice strains with known resistance status toward H_2O_2-treatments, using a PCR-assay based upon the binding of the fluorescent dye SYBR-Green I

			FIRST PARALEL		SECOND PARALEL		THIRD PARALEL		Ratio
Strain	Nr.	RNA quality (ng/µl)	Catalase	ELF1	Catalase	ELF1	Catalase	ELF1	Kat/ELF
H_2O_2	6	247	26,51	20,89	26,31	20,73	39,665	31,255	1,269
H_2O_2	7	87	27,96	21,49	27,85	21,23	41,885	32,105	1,305
H_2O_2	8	158	25,63	19,76	25,34	19,54	38,3	29,53	1,297
H_2O_2	9	168	27,43	20,46	27,28	20,63	41,07	30,775	1,335
H_2O_2	10	184	25,29	20,3	26,06	20,19	38,32	30,395	1,261
Strain A	21	173	27,69	19,4	27,16	19,62	41,27	29,21	1,413
Strain A	22	262	27,52	20,31	26,98	19,72	41,01	30,17	1,359
Strain A	23	154	28,2	20,86	27,28	20,62	41,84	31,17	1,342
Strain A	25	133	27,1	19,56	26,9	19,48	40,55	29,3	1,384
Strain A	26	338	26,75	20,1	26,13	19,19	39,815	29,695	1,341
Strain B	21	192	31,8	20,12	35,6	20,13	49,6	30,185	1,643
Strain B	22	161	31,3	21,02	31,09	20,72	46,845	31,38	1,493
Strain B	23	67	34,11	25,41	34,19	24,87	51,205	37,845	1,353
Strain B	24	125	33,11	22,02	32,99	21,51	49,605	32,775	1,514

Example 2: Lice mortality after experimental exposure correlate with the expression of a catalase gene, showing that H_2O_2 resistance is related to the expression of the catalase gene in sea lice

The aim of this study was to test if the expression level of a catalase gene using PatoGen's TaqMan qPCR-assay correlate with the ability of sea lice (*L. salmonis*) to withstand H_2O_2-

exposure. PCR-analyses were performed on dead and surviving parasites exposed to hydrogen peroxide in two experiments;

1. Sea lice infecting salmon were treated with 1500 mg hydrogen peroxide for 20 minutes.
2. Parasites in tanks without fish were treated with 2300 mg/l hydrogen peroxide for 20 minutes.

Based on the knowledge that the expression of the catalase gene is an important determinant for resistance towards H_2O_2-treatment in sea lice, PatoGen Analyse AS developed a sensitive Real-Time PCR (TaqMan) 5′-nuclease assay to quantify the expression level of the catalase gene. Real-time PCR has become a well-established procedure for quantifying levels of gene expression (c.f. e.g. de Kok JB, van Balken MR, Roelofs Rwhm, van Aarssen Yawg, Swinkels DW, Gunnewiek Jmtk: Quantification of hTERT mRNA and telomerase activity in bladder washings of patients with recurrent urothelial cell carcinomas. Clin. Chem. 2000, 46:2003-2007., Homey B, Dieu-Nosjean MC, Wiesenborn A, Massacrier C, Pin JJ, Oldham E, Catron D, Buchanan ME, Muller A, Malefyt RD, Deng G, Orozco R, Ruzicka T, Lehmann P, Lebecque S, Caux C, Zlotnik A: Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. J. Immunol. 2000, 164:6621-6632., Kubo A, Nishitani V, Minamino N, Kikumoto K, Kurioka H, Nishino T, Iwano M, Shiiki H, Kangawa K, Dohi K: Adrenomedullin gene transcription is decreased in peripheral blood mononuclear cells of patients with IgA nephropathy. Nephron 2000, 85:201-206.).The catalase assay were used together with an assay towards the Elongation factor 1 C (E1α), and results for the E1α-assay were used for normalization in order to standardize the results from different runs of the catalase-PCR-assay. The catalase-PCR-assay, in combination with the E1α-assay, was successfully applied to differentiate between H_2O_2-resistant and sensitive sea lice. The analyses were run in a commercially available thermocycling fluorimeter (Applied Biosystems 7500 Real-Time PCR System) at PatoGen Analyse AS laboratory in Ålesund. The assays were used qualitatively to determine the relative amount of the gene in individual samples, and samples from different life stages of sea lice were used. The Real-Time PCR-assay in combination with the E1α is ideal for the differentiating between H_2O_2-resistant and sensitive sea lice in the aquaculture industry.

TREATMENT TRIALS

Two treatment trials were performed where the present inventors analyzed lice surviving H_2O_2-treatment in the laboratory, and compared catalase gene expression levels in the population before treatment with the catalase gene expression level in surviving lice. In the first trial, H_2O_2-treatment were performed with sea lice attached to fish, while the second H_2O_2-reatment trial were performed using sea lice allowed to settle in a tank without fish. In both trials, lice affected by the treatment were collected in a system specially designed for these trials, for collecting lice in the outlet water from the tank.

Several strains were used in the study, which was conducted in the Licelab at the Sea lice Research Centre in Bergen. These included fully sensitive strains, a strain resistant to pyrethroids, a strain resistant to pyrethroids, organophosphates and emamectin benzoate, and a strain resistant to pyrethroids, organophosphates, emamectin benzoate and hydrogen peroxide. The strains were hatched at the same time, and the fish infested with a mixture of copepodites from all strains. The fish were divided into two groups kept in separate tanks.

Pre-test treatment

After development of pre-adult parasites, the fish were treated with cypermethrin, 15 µg/l for 30 minutes (Betamax) in another experiment. Two tanks were used for the original experiment. Remaining fish from this first experiment with surviving sea lice were used in the hydrogen peroxide treatment experiment. Though, sea lice included in this experiment had been pre-treated with cypermethrin.

Treatment trial 1: Fish experiment ("FISK")

Fish with a mixture of sensitive and resistant sea lice as described above, were treated with 1500 mg hydrogen peroxide for 20 minutes. Parasites that fell off the fish were collected in a filter on the outlet from the tank (immob). Parasites that were still attached to the fish were sampled from anesthetized fish 24 hours after the exposure (lev). In theory, the most resistant lice should be unaffected by the H_2O_2-treatment, and will remain attached to the fish, while the sensitive sea lice should be affected by treatment, loosen its grip of the fish, and is collected in the outlet water. We performed PCR-analyses for the expression level of the catalase gene on all lice from this trial.

Treatment trial 2: Parasite experiment ("PARASITT")

A mixed population of sea lice from sensitive and resistant populations as described above was allowed to settle in a tank without fish. The sea lice settled on the plastic surface of the tank walls. The lice in the tank were exposed to 2300 mg/l H_2O_2 for 20 minutes, and lice that lost its grip to the tank walls were collected in the water outlet of the tank with a filter unit. Surviving parasites attached to the wall of the incubator were sampled (lev). Immobilized parasites on the bottom of the incubator were also sampled (immob). Resistant lice would be unaffected by the exposure to H_2O_2, and still be attached to the tank walls, while the sensitive lice would lose its grip to the tank walls, and come out of the tank with the outlet water. We performed PCR-analyses for the expression level of the catalase gene on all lice from this trial.

Nucleic acid purification

In PatoGens laboratory, RNA and/or DNA were extracted from samples by methods well known to the skilled person. In short, tissue samples were transferred to Micro Collection Tubes and lysed and homogenized using QIAzol Lysis Reagent, steel beads and vigorous shaking using a TissueLyser system, followed by nucleic acid extraction using an RNAeasy

kit (Qiagen) or DNAeasy kit (Qiagen), all according to the manufacturer's instructions, and by methods well known to the skilled person. Chloroform were added to the samples and shaken vigorously. After resting and centrifuging, the relevant liquid phase were collected for further extraction of either RNA or DNA by vacuum technology using a Qiagen robot system, all according to the manufacturer's instructions, and by methods well known to the skilled person. Finally, nucleic acids were eluted in 25 ml of elution buffer and used for PCR by methods well known to the skilled person.

Real-Time PCR

Based on the newly sequenced *Lepeophtheirus salmonis* catalase gene, TaqMan® MGB Probe SNP Genotyping Assays using TaqMan® 5′ nuclease assay chemistry for detecting and quantifying the catalase gene in purified genomic DNA or RNA were established. Primers and probes are listed in table 3. The primers and probes used were ordered from Life Technologies Corporation. One-step amplification (45 cycles) was performed on an Applied Biosystems 7500 Real-Time PCR System performed at PatoGen Analyse AS laboratory in Ålesund, all according to the manufacturer's instructions, and by methods well known to the skilled person. The assays were used qualitatively to determine the expression level of the catalase gene in individual samples.

Table 3: Primers and probes used to quantify the expression of the catalase gene in sea lice.

Name	Forward primer	Revers primer	Probe
Cat1 Lepeophtheirus salmonis	Kata-ST-F TCACATCCAGTCACGGAATCC (SEQ ID No.4)	Kata-ST-R ACCTCTTCTGCCAACGGTAACTA (SEQ ID No. 5)	Kata-ST-P ACTGGGTGATAAAAAT (SEQ ID No. 6)
Cat2 Lepeophtheirus salmonis	Kata-St1-F GCCTGAAACAACACATCAAATGTC (SEQ ID No. 7)	Kata-ST1-R GCCGTTCATATGTCTATATCCATCTG (SEQ ID No. 8)	Kata-ST1-P TCTATTCTCAGACAGAGGAA (SEQ ID No. 9)
Cat3 Lepeophtheirus salmonis	Kata-ST2-F ACCGTTGAGATATGAGCATCCA (SEQ ID No. 10)	Kata-ST2-R TGCCGAGTGATTTTGTCAGTTT (SEQ ID No. 11)	Kata-ST2-P ATCACTCTGACTATTTGC (SEQ ID No. 12)

RESULTS

The result from Real-Time PCR-assay is interpreted by using the Ct-value for the catalase assay, normalizing the values toward the Ct-value for the E1α (Frost & Nilsen 2003, Validation of reference genes for transcription profiling in the salmon louse, *Lepeophtheirus salmonis*, by quantitative real-time PCR, Veterinary Parasitology 118 (2003) 169–174 Short) for the same individual lice by dividing the catalase Ct-value with the Ct-value for the E1α for the individual lice, and multiplying with the average Ct-value for the E1α for about 800 lice previously analyzed. The normalized Ct-value is used to establish the resistance level of individual lice and for lice populations. Sensitive sea lice have high Ct-values, indication a low expression of the catalase gene. Genetically resistant sea lice have lower Ct-values, indicating an elevated expression of the catalase gene, and a higher capacity to tolerate H_2O_2, and thus a higher resistance towards H_2O_2.

Statistical analyses

All normalized Ct values were first examined for normality using JMP 10.0.0. (SAS Institute). The distribution was clearly bi-phasic (Fig. 3) and could not be transformed to normality by log- or root transformation. Thus, the non-parametric Wilcoxon test of rank sums was used to examine the difference between the groups.

RESULTS

The distribution of normalized Ct-values for catalase in parasites from the groups "Fisk" (treatment of fish with lice) and "Parasitt" (treatment of lice only) are presented in Figure 3. The median value was almost identical for both groups (norm. Ct: 17.2 and 17.3, respectively).

Thereafter, the distribution of normalized Ct-values for catalase between alive (lev) and immobilized (immob) parasites from the two groups "Fisk" (treatment of fish with lice) and "Parasitt" (treatment of lice only) was examined (Figure 4). The results demonstrated similar normalized Ct-values for both live parasites (median: 16.7 and 15.5 for the "Fisk" and the "Parasitt" groups, respectively) and immobilized parasites (median: 20.1 and 19.4 for the "Fisk" and the "Parasitt" groups, respectively).

Then, the Wilcoxon test of rank sums was applied to test whether or not there was a statistically significant difference between surviving (lev) and immobilized (immob) parasites within each treatment group (parasites on the fish, "Fisk" or parasites alone, "Parasitt") (Figure 4). The analysis demonstrated a highly significant difference between normalized Ct-values for both treatment groups ($p<0.0001$ for both treatments).

Finally, a Wilcoxon test of rank sums was performed to see if there was a statistically significant difference in normalized Ct-values in surviving (lev) parasites between the treaments groups (parasites on fish, "Fisk" or parasites alone, "Parasitt"). Thereafter, the test was repeated for immobilized (immob) parasites (Figure 5). For the immobilized parasites, no significant difference was evident ($p=0.1495$). For the alive parasites, there was a highly significant difference ($p<0.0001$) with highest Ct-values (lowest expression) in the parasites on fish.

Finally we used logistic regression to test the effect of normalized Ct-values on the probability of lice being immobilized in the two experiments, according to the general linear model:

$\text{logit}(y) = b0 + b1x$,

where b0 is the intercept and b1 is the coefficient of the effect of normalized Ct-values (x). Statistics for these analyses from the two experiments is given in Table 4.

Table 4. Summary statistics for the logistic regression analyses of the experiments on H2O2 treatment of fish ("Fisk") and parasites alone ("Parasitt").

Experiment	Coefficients	Estimate	Std. Error	z value	P
"Parasitt"	Intercept	-11.73	1.829	-6.413	< 0.001
	Norm Ct-value	0.703	0.108	6.499	< 0.001
"Fisk"	Intercept	-9.15	2.340	-3.908	< 0.001
	Norm Ct-value	0.493	0.128	3.837	< 0.001

The results from the logistic regression analyses are presented in Figure 6b.

DISCUSSION

The analyses demonstrated highly significant differences between normalized Ct-values in surviving and immobilized parasites, both after H_2O_2 exposure of parasites on fish and H_2O_2 exposure of parasites alone. The highest Ct-values (lowest expression of the gene) were found in immobilized parasites in all cases. This observation demonstrates that a salmon louse with a higher expression of the enzyme catalase has a higher likelihood of surviving a treatment with hydrogen peroxide than parasites with a lower expression of the catalase enzyme.

In the study, significantly lower Ct-values (higher expression of the catalase gene) was found in live parasites exposed to hydrogen peroxide alone compared to live parasites exposed to hydrogen peroxide while still attached to the fish. Thus, an elevated expression of the catalase gene is related to resistance toward H_2O_2-treatment, and lice affected by H_2O_2-treatment have a lower expression of the catalase gene than resistant lice.

The difference in the mean normalized Ct-value was though only 1.2. The most likely reason is that the parasites exposed alone were exposed to a higher H_2O_2 concentration (2300 ppm) compared to the parasites exposed while still on the fish (1500 ppm). Thus, a higher proportion of parasites with intermediate sensitivities were immobilized in the group exposed alone compared to the group exposed on fish.

The Real-Time PCR -assay showed surprisingly good correlation with resistance status towards H_2O_2, and we believe that it will serve as a practical tool in the differentiation between H_2O_2 sensitive and resistant sea lice at individual and population level.

Thus, using the expression level of the catalase gene quantified by one of the Real-Time PCR-assays described here represent an ideal tool for differentiating between H_2O_2 resistant and sensitive sea lice in the aquaculture industry. Also, the prevalence of sensitive versus resistant sea lice in a population can be used to predict the best possible outcome of a treatment using H_2O_2 in the population. Also, the technique can become an important tool for optimizing sea lice treatments using H_2O_2, combination treatments using H_2O_2 in combination with other insecticides or other measures to reduce sea lice infection pressure, and to monitor the resistant status of populations of sea lice before treatment.

Example 3: Enzymatic activity of the enzyme catalase and the expression of the gene coding for catalase in the salmon louse, correlate with resistance towards H2O2 in sea lice

The aim of this study was to test if the expression level of a catalase enzyme correlates with the expression of a catalase gene using PatoGens TaqMan qPCR-assay, and to establish if this expression correlates with the ability of sea lice (*L. salmonis*) to withstand H_2O_2-exposure. Enzyme quantification and PCR-analyses were performed on parasites collected in Norwegian aquaculture sites known to have varying ability to withstand exposure to hydrogen peroxide.

MATERIALS AND METHODS

SALMON LICE STRAINS

Ls Alta

This strain originated from a salmon farm located at the island Seiland in the Alta fjord in Finnmark county. It was collected in 2010 and has been kept in continuous culture at the NIVA marine research station Solbergstrand since then. It has been cultured for about 10 generations. There has been no history of insensitivity towards any chemicals at the farm or in the district from which these parasites originate.

Ls H_2O_2

This strain originated from a salmon farm in Vikna, Nord-Trøndelag. It was collected in 2013 and had been cultured for only one generation when the study was performed, on the offsprings of the original strain. At the site and in the district, there had been increasing problems with insensitivity of salmon lice towards available chemicals. The latest problem was with hydrogen peroxide, where the efficacy of several treatments was substantially lower than expected.

The parasites included were adult females. After sampling, each parasite was split along the mid-line with a scalpel. One half was immediately frozen at -80 degrees in a 0.5 ml Eppendorf vial and later used for the enzymatic assay. The other half was put on RNAlater, shipped to PatoGen Analyse AS, and used for the TaqMan PCR assay.

ASSAYS

Catalase activity assay

To determine if the catalase enzyme activity is important for resistance towards H_2O_2 in sea lice, an enzymatic assay using a standardized commercial kit for catalase activity was established. The catalase activity was measured in each split parasite. A commercial kit (Oxyselect catalase activity kit) was used according to the manufacturer's instructions in a 96 well microplate. The activity was read on an Epoch spectrophotometer, BioTek, USA, Gen5 verion 2.00 software. The activity was normalized against the protein content in the homogenate. Protein content was measured on a Take3 plate (BioTek) with a build-in protein calculation calibrated against bovine serum albumin on the same microtitre plate reader.

TaqMan assay

The expression of the catalase gene was measured using a TaqMan assay developed by PatoGen Analyse AS following procedures as described in example 2. The results were normalized against the expression of the elongation factor in the samples, also as described in example 2.

RESULTS

The correlation between normalized Ct-values and catalase activities (corrected for protein) were analysed using the statistical module incorporated in Excel (Microsoft). The correlation of all catalase activity values (U/µg protein) versus all Ct-values demonstrated a Pearson correlation factor of -0.3524. A regression analysis (Excel) demonstrated the association between enzyme activity and CT-values to be significant (p=0.016, Fig. 1).

Table 4: Output from the regression analyses of catalase activity versus gene expression (normalized Ct-values). Multiple R = Pearson's correlation coefficient.

SUMMARY OUTPUT								
Regression Statistics								
Multiple R	0,35240783							
R Square	0,12419128							
Adjusted R S	0,10428654							
Standard Err	1,56697035							
Observation	46							
ANOVA								
	df	*SS*	*MS*	*F*	*Significance F*			
Regression	1	15,319905	15,319905	6,23928055	0,01630628			
Residual	44	108,037427	2,45539608					
Total	45	123,357332						
	Coefficients	*tandard Erro*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95*
Intercept	20,3217353	0,41708976	48,7226901	6,3368E-40	19,4811461	21,1623244	19,4811461	21,1623
X Variable 1	-0,36200971	0,14492822	-2,49785519	0,01630628	-0,65409335	-0,06992607	-0,65409335	-0,06992

The data were grouped by strain, and the differences in mean catalase activity (U/µg protein) and mean normalized Ct-values were calculated using the statistical software JMP (SAS Institute). An analysis of variance was used for testing each parameter against strain.

For catalase activity, a higher enzyme activity was demonstrated in the H_2O_2 strain compared to the Alta strain. The difference was statistically significant (p= 0.0429, Fig. 7). For the normalized Ct-values, lower values were found in the H_2O_2 strain compared to the Alta strain. This difference was statistically highly significant (p<0.0001, Fig. 8).

DISCUSSION

The observed difference in sensitivity between these two strains, as demonstrated by bioassays, was associated with the expression of the gene coding for the catalase enzyme and the activity of this enzyme. The correlation between activity and expression was not

perfect, but still significant. The enzyme activity assay used showed a big variation between parallel samples, thus the estimated activities were somewhat inaccurate.

In conclusion, an association between bioassay results, enzymatic activity of the catalase enzyme and expression of the gene coding for catalase were demonstrated.

Example 4: Expression levels of the gene coding for the enzyme catalase in field population of sea lice.

The aim of this study was to test if the expression of the catalase gen in sea lice populations correlates with the known geographic distribution of resistance towards H_2O_2, or to areas with high treatment frequencies using H_2O_2. Several strains of salmon lice were sampled from different sites along the Norwegian coast.

MATERIALS AND METHODS

SALMON LICE STRAINS

Salmon lice (*Lepeophtheirus salmonis*), different developmental stages (pre-adult 1, pre-adult 2 and adult lice of both sexes) were sampled from fish farms along the west coast of Norway with unknown resistance status towards H_2O_2. Sea lice were collected using forceps, and 60 lice per site were conserved in 70% ethanol and kept at 4℃. Samples were sent refrigerated to PatoGen by express mail carrier.

The strains were labelled by county and location within the county (N, M or S for north, middle and south, 1, 2, ... for different sites).

TaqMan ASSAY

The expression of the catalase gene was measured using a TaqMan assay developed by PatoGen Analyse AS following procedures as described in example 2. The results were normalized against the expression of the elongation factor in the samples, also as described in example 2.

STATISTICAL ANALYSES

All normalized Ct values from each site were first examined separately for normality using JMP 10.0.0. (SAS Institute). The distribution within a site could for most sites be described by a normal distribution according to the Shapiro-Wiik W test. The strains not displaying a normal distribution were Nordland N2, Nordland M2, S-Trøndelag M and Rogaland N. As 9 of the 13 sample sets followed normal distribution, ANOVA was used to examine differences in mean values between the strains.

Furthermore, recursive partitioning was used (JMP 10.0.0.) to split the strains into three clusters:
1) High expression of the catalase gene (low Ct values)
2) Intermediate expression of the catalase gene (intermediate Ct values)
3) Low expression of the catalase gene (high Ct values)

RESULTS

The distribution within each group and the ANOVA analysis of normalized CT values versus strain is presented in Figure 9, and demonstrates that the confidence intervals for several strains are not overlapping, indicating a significant difference in the expression.

Thereafter, the expression values for all strains were compared using the Tukey-Kramer HSD test. The results are presented in Figure 10, and demonstrate in which strains the expression levels differed significantly (strains not sharing the same letter).

Finally, the clustering of results was examined using recursive partitioning. The results are presented in Figure 11, and demonstrated that the strains could be separated into

1) High expression: N-Trøndelag N, Nordland S, Nordland M1, Nordland M2,
2) Intermediate expression: Hordaland S, Sogn M, S-Trøndelag M, Finnmark N
3) Low expression: Rogaland N, S-Trøndelag N, Nordland N1, Nordland N2

DISCUSSION

The analyses demonstrated that there were significant differences between salmon lice strains in their expression of the catalase gene. The highest expressions were found in strains originating from the county N-Trøndelag and the south and middle part of the county Nordland. This is as expected, as these are areas where reduced treatment efficacy by hydrogen peroxide has been reported. The lowest expression of the catalase enzyme was found in samples from the northern part of the county Nordland and some scattered sites further south, all areas where there has been little or no use of hydrogen peroxide as a delousing agent. However, somewhat surprising, the strain from Finnmark, where hydrogen peroxide has never been used to treat for salmon lice, clustered in the intermediate group.

In conclusion, there seems to be a strain-specific variation in the expression of the enzyme which is fairly well correlated to the use of hydrogen peroxide as a delousing agent in Norway.

SEQUENCE LISTING

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<120> Method for detecting H2O2 resistance in sea lice

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<150> US62019911
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CLAIMS

1. 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 a catalase gene of collected sea lice.

2. Method according to claim 1, wherein said catalase gene has a sequence as depicted in SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 1 SEQ ID No. 13 and SEQ ID No. 14, respectively.

3. Method according to claim 1, wherein said catalase gene has a sequence as depicted in SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14, or variants or fragments thereof being at least 80 % identical with SEQ ID No. 1 SEQ ID No. 13 and SEQ ID No. 14, respectively.

4. Method according to any one of claims 1 to 3, comprising the steps of detecting increased RNA-expression levels associated with hydrogen peroxide (H_2O_2) resistance in a sea lice to be analyzed, wherein said sea lice is resistant to hydrogen peroxide (H_2O_2) resistance if having elevated levels of catalase RNA-expression.

5. Method according to any one of claims 1 to 4, comprising in step c:

- providing one or more isolated oligonucleotide sequence(s) comprising at least 8 contiguous nucleotides of the sequence SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14 or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14, respectively;

- determining the expression level of the catalase gene as depicted in SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14, or variants or fragments thereof being at least 70 % identical with SEQ ID No. 1 SEQ ID No. 13 and SEQ ID No. 14, respectively, in the collected sea lice by measuring the catalase RNA expression level in the collected sea lice, wherein said sea lice is resistant to hydrogen peroxide (H_2O_2) if having elevated levels of catalase RNA-expression compared with a non-resistant sea lice.

6. Method according to claim 5, wherein said determination is performed using a primer selected from the group consisting of SEQ ID No.'s 2, 3, 4, 5, 7, 8, 10 and 11, or by using at least one probe selected from the group consisting of SEQ ID No.'s 6, 9 and 12, or by

using a primer selected from the group consisting of SEQ ID No.'s 15, 16, 18 and 19, or by using at least one probe selected from the group consisting of SEQ ID No.'s 17 and 20.

7. 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 sea lice from infested fish or water samples;

b) homogenization of sea lice tissue samples from any life stage of collected sea lice;

c) determining the catalase activity, wherein said one or more sea lice is resistant to hydrogen peroxide (H_2O_2) if the catalase activity is elevated.

8. Method according to claim 7, wherein the determining of catalase activity step c) is performed by contacting a sample isolated from the crustaceans to be analyzed with a pre-determined amount of hydrogen peroxide, and then with horseradish peroxidase and a chromophore, resulting in the formation of a chroma signal, and wherein the decomposition of hydrogen peroxide is proportional with the level of catalase in the sample.

9. Method for characterizing the hydrogen peroxidase sensitivity of one or more sea lice, comprising the steps of

a) determining the expression level of the catalase gene according to SEQ ID No. 1 or a variant or fragment thereof in a biological sample comprising DNA or RNA of one or more sea lice;

b) comparing the determined catalase expression level to (i) a control standard or (ii) the expression of the catalase gene or a variant or fragment thereof in a control sample;

c) determining the difference in expression level in the sample as compared to the control standard or control sample; and

d) characterizing the hydrogen peroxidase sensitivity of the one or more sea lice, wherein the one or more sea lice to be tested is resistant if having a higher expression level compared with the control standard or the control sample.

10. Method according to claim 9, wherein step a) comprises

- collecting sea lice from infested fish or water samples;

- isolating DNA or RNA from the collected sea lice;

- providing a pair of PCR primers specific for the catalase gene of SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14, respectively; and

- performing PCR on the isolated DNA or RNA using the pair of PCR primers.

11. An oligonucleotide probe comprising a nucleotide sequence, wherein the sequence consists of that selected from the group of SEQ ID No.'s 6, 9, and 12 and fragments and variants thereof both having at least 70 % sequence identity over the entire length of SEQ ID No.'s 6, 9, and 12.

12. An oligonucleotide primer comprising a nucleotide sequence, wherein the sequence comprises 18 - 25 consecutive nucleotides and consist of that selected from the group of SEQ ID No.'s 2, 3, 4, 5, 7, 8, 10, and 11 and fragments and variants thereof both having at least 70 % sequence identity over the entire length of SEQ ID Nos. 2, 3, 4, 5, 7, 8, 10, and 11.

13. An oligonucleotide primer pair, wherein the sequences of said primer pair is selected from the groups consisting of SEQ ID Nos. 2 and 3, 4 and 5, 7 and 8, and, 10 and 11, and fragments and variants thereof both having at least 70 % sequence identity over the entire length of SEQ ID Nos. 2 and 3, 4 and 5, 7 and 8, and, 10 and 11.

14. Kit for detection of hydrogen peroxide (H_2O_2) resistance in crustaceans comprising at least one oligonucleotide probe, primer and/or primer pair according to any of the claims 11-13.

15. 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. 13 or SEQ ID No. 14 or a complementary oligonucleotide of SEQ ID No. 1, SEQ ID No. 13 or SEQ ID No. 14, respectively, for the determination of hydrogen peroxide resistance in sea lice selected from the group consisting of *Lepeophtheirus salmonis*, *Caligus clemensei, Caligus elongatus*, and *Caligus rogercresseyi*.

Figure 1

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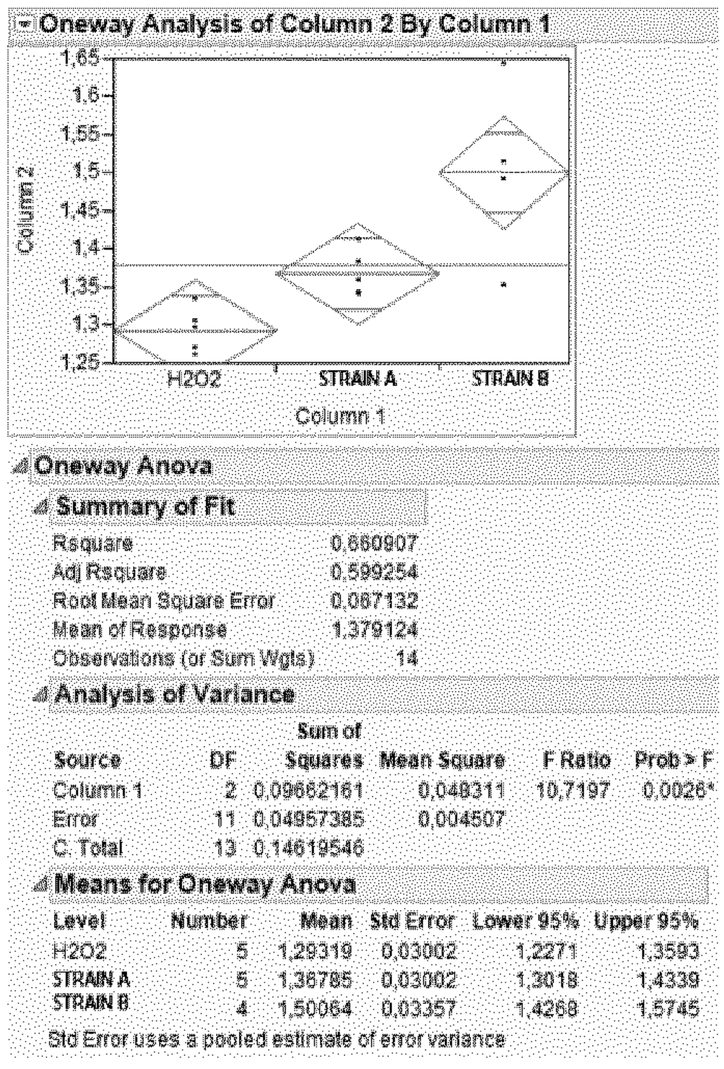
Figure 2



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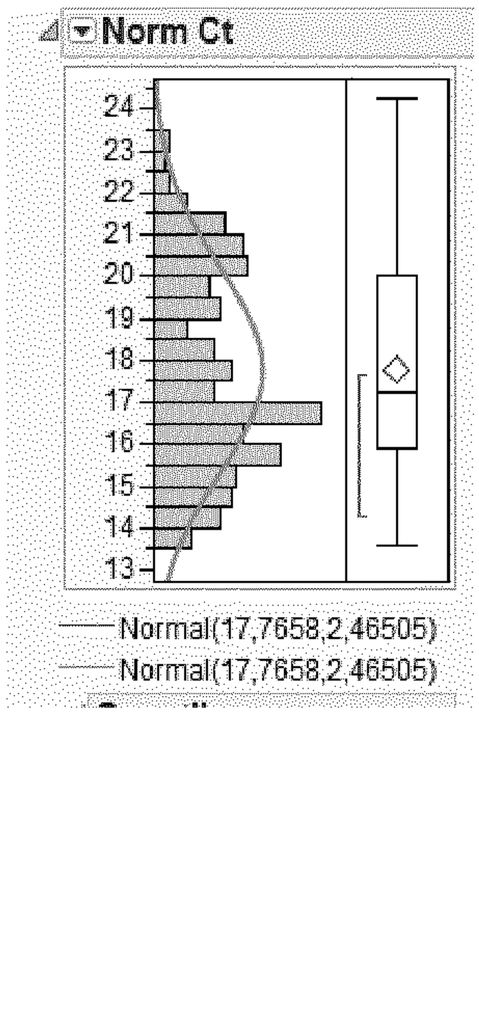


Figure 4a

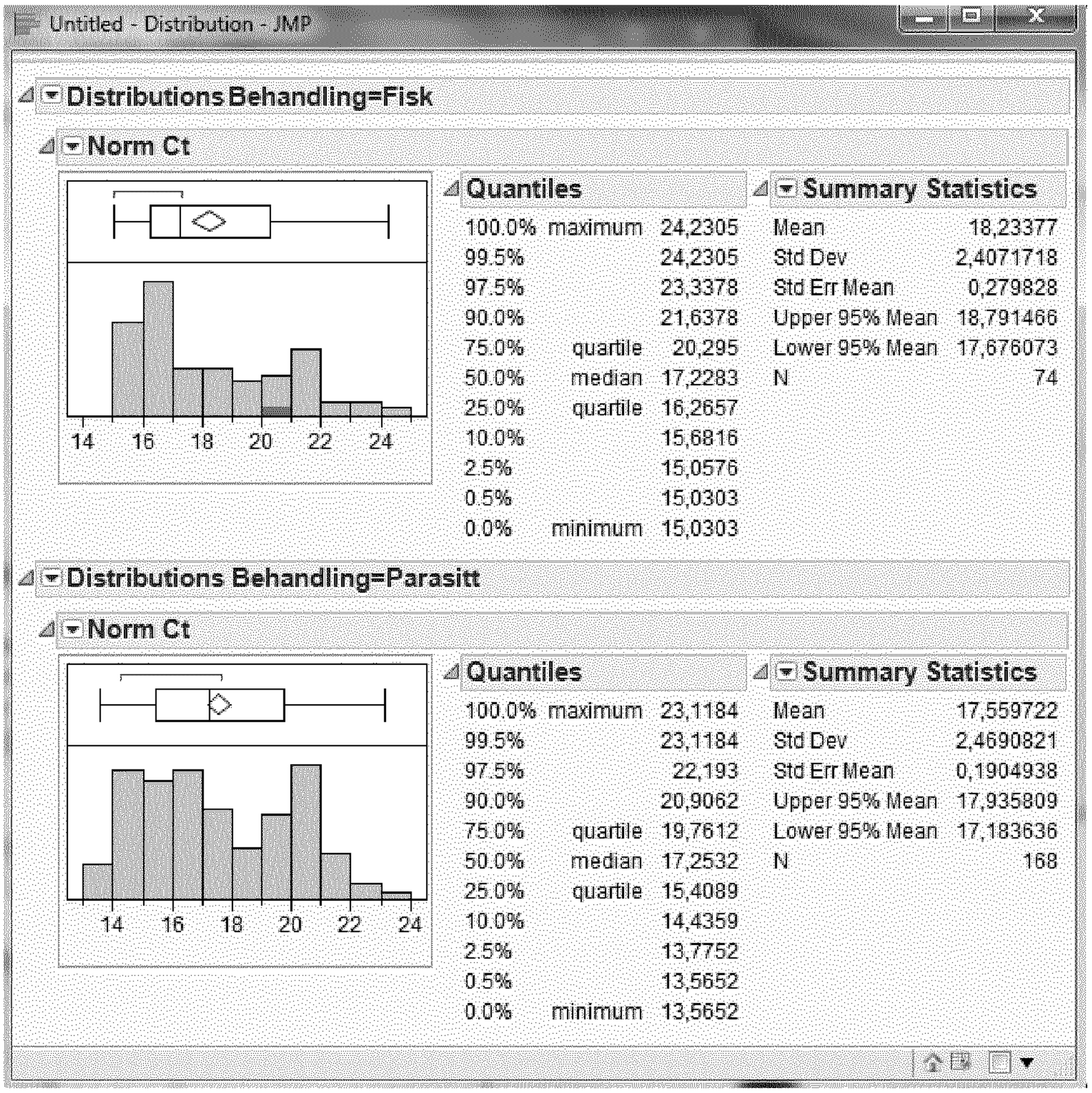

Untitled - Distribution - JMP
Distributions Behandling=Fisk
Norm Ct
14 16 18 20 22 24
Quantiles
100.0% maximum 24,2305
99.5% 24,2305
97.5% 23,3378
90.0% 21,6378
75.0% quartile 20,295
50.0% median 17,2283
25.0% quartile 16,2657
10.0% 15,6816
2.5% 15,0576
0.5% 15,0303
0.0% minimum 15,0303
Summary Statistics
Mean 18,23377
Std Dev 2,4071718
Std Err Mean 0,279828
Upper 95% Mean 18,791466
Lower 95% Mean 17,676073
N 74
Distributions Behandling=Parasitt
Norm Ct
14 16 18 20 22 24
Quantiles
100.0% maximum 23,1184
99.5% 23,1184
97.5% 22,193
90.0% 20,9062
75.0% quartile 19,7612
50.0% median 17,2532
25.0% quartile 15,4089
10.0% 14,4359
2.5% 13,7752
0.5% 13,5652
0.0% minimum 13,5652
Summary Statistics
Mean 17,559722
Std Dev 2,4690821
Std Err Mean 0,1904938
Upper 95% Mean 17,935809
Lower 95% Mean 17,183636
N 168

Figure 4b

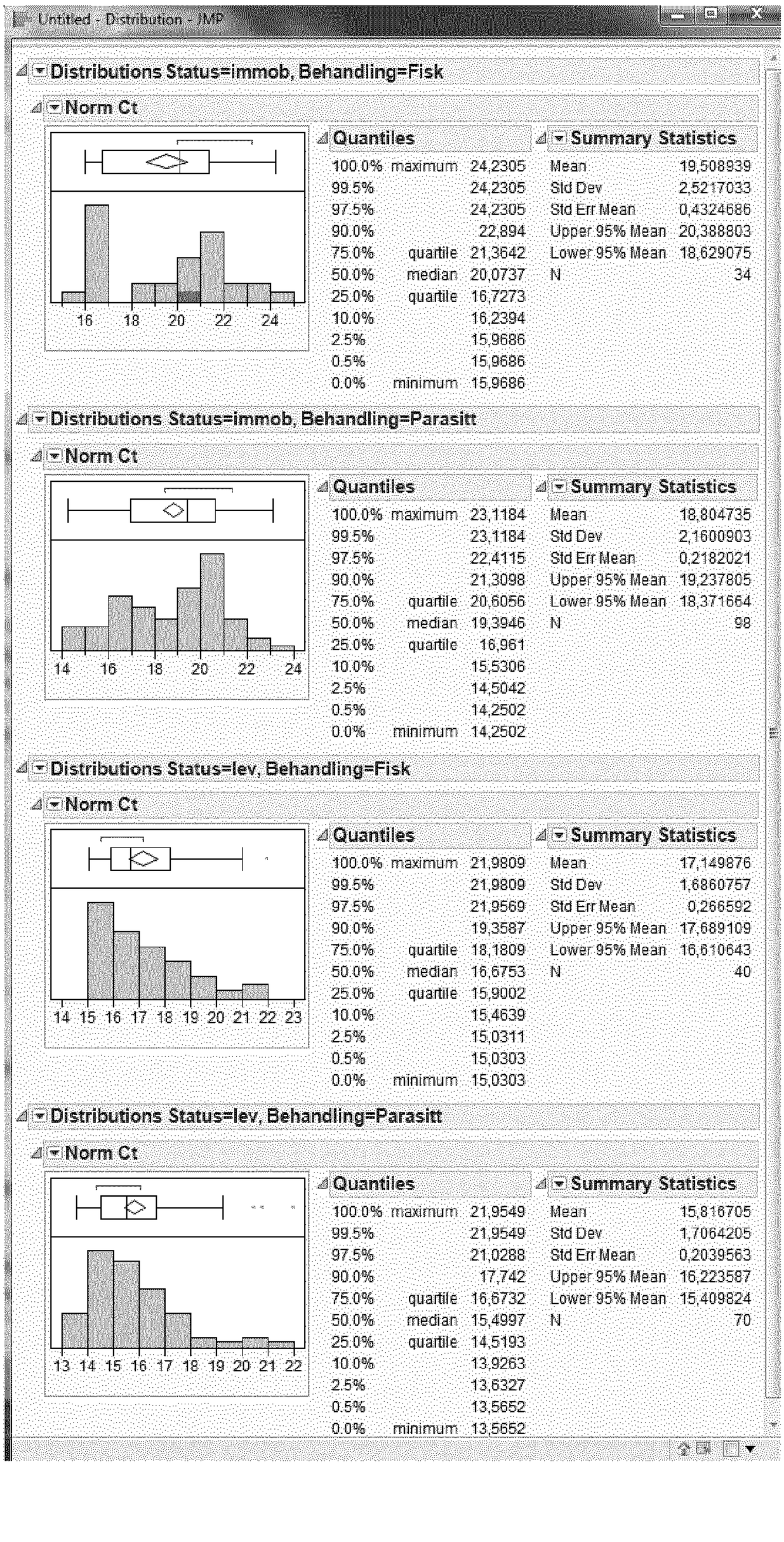

Untitled - Distribution - JMP
Distributions Status=immob, Behandling=Fisk
Norm Ct
16 18 20 22 24
Quantiles
100.0% maximum 24,2305
99.5% 24,2305
97.5% 24,2305
90.0% 22,894
75.0% quartile 21,3642
50.0% median 20,0737
25.0% quartile 16,7273
10.0% 16,2394
2.5% 15,9686
0.5% 15,9686
0.0% minimum 15,9686
Summary Statistics
Mean 19,508939
Std Dev 2,5217033
Std Err Mean 0,4324686
Upper 95% Mean 20,388803
Lower 95% Mean 18,629075
N 34
Distributions Status=immob, Behandling=Parasitt
Norm Ct
14 16 18 20 22 24
Quantiles
100.0% maximum 23,1184
99.5% 23,1184
97.5% 22,4115
90.0% 21,3098
75.0% quartile 20,6056
50.0% median 19,3946
25.0% quartile 16,961
10.0% 15,5306
2.5% 14,5042
0.5% 14,2502
0.0% minimum 14,2502
Summary Statistics
Mean 18,804735
Std Dev 2,1600903
Std Err Mean 0,2182021
Upper 95% Mean 19,237805
Lower 95% Mean 18,371664
N 98
Distributions Status=lev, Behandling=Fisk
Norm Ct
14 15 16 17 18 19 20 21 22 23
Quantiles
100.0% maximum 21,9809
99.5% 21,9809
97.5% 21,9569
90.0% 19,3587
75.0% quartile 18,1809
50.0% median 16,6753
25.0% quartile 15,9002
10.0% 15,4639
2.5% 15,0311
0.5% 15,0303
0.0% minimum 15,0303
Summary Statistics
Mean 17,149876
Std Dev 1,6860757
Std Err Mean 0,266592
Upper 95% Mean 17,689109
Lower 95% Mean 16,610643
N 40
Distributions Status=lev, Behandling=Parasitt
Norm Ct
13 14 15 16 17 18 19 20 21 22
Quantiles
100.0% maximum 21,9549
99.5% 21,9549
97.5% 21,0288
90.0% 17,742
75.0% quartile 16,6732
50.0% median 15,4997
25.0% quartile 14,5193
10.0% 13,9263
2.5% 13,6327
0.5% 13,5652
0.0% minimum 13,5652
Summary Statistics
Mean 15,816705
Std Dev 1,7064205
Std Err Mean 0,2039563
Upper 95% Mean 16,223587
Lower 95% Mean 15,409824
N 70

Figure 5

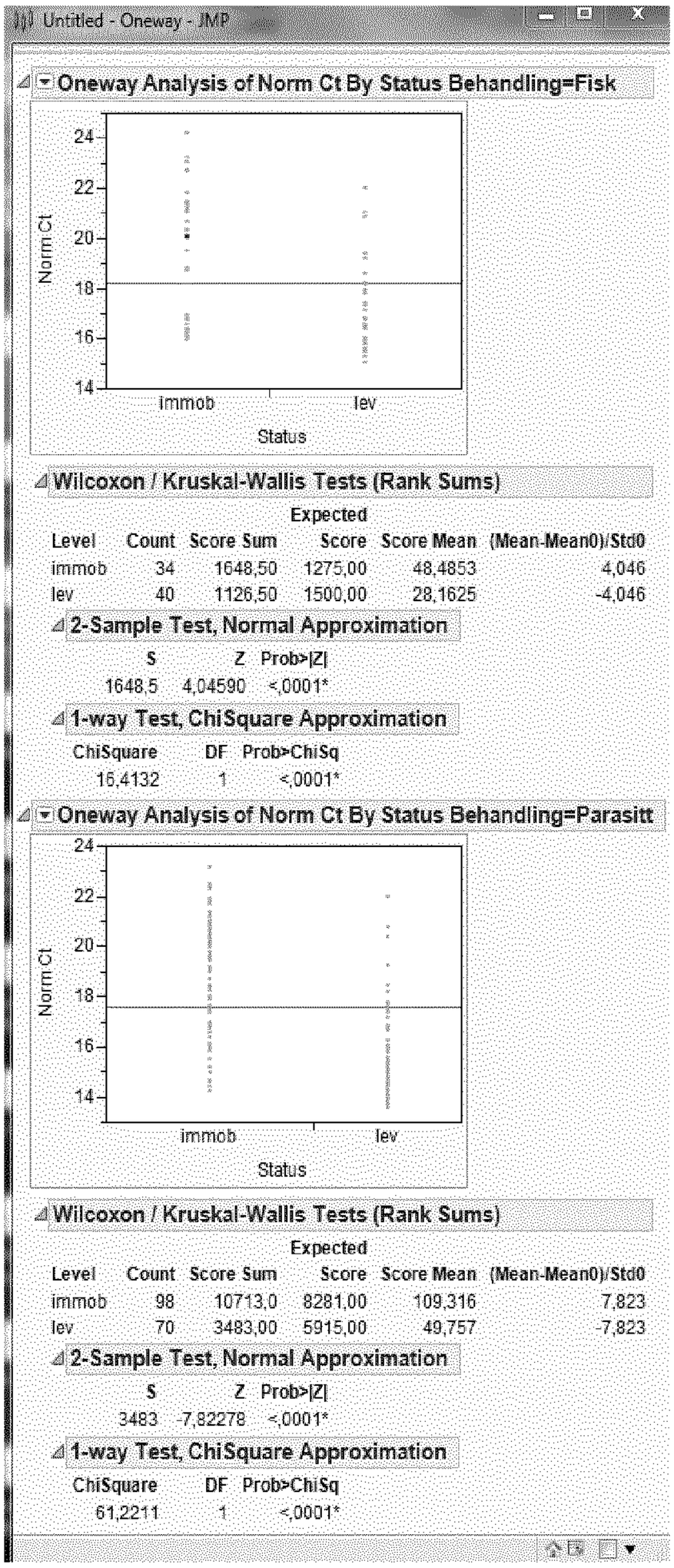

Untitled - Oneway - JMP
Oneway Analysis of Norm Ct By Status Behandling=Fisk
Norm Ct
24
22
20
18
16
14
immob
lev
Status
Wilcoxon / Kruskal-Wallis Tests (Rank Sums)
Level Count Score Sum Expected Score Score Mean (Mean-Mean0)/Std0
immob 34 1648,50 1275,00 48,4853 4,046
lev 40 1126,50 1500,00 28,1625 -4,046
2-Sample Test, Normal Approximation
S Z Prob>|Z|
1648,5 4,04590 <,0001*
1-way Test, ChiSquare Approximation
ChiSquare DF Prob>ChiSq
16,4132 1 <,0001*
Oneway Analysis of Norm Ct By Status Behandling=Parasitt
Norm Ct
24
22
20
18
16
14
immob
lev
Status
Wilcoxon / Kruskal-Wallis Tests (Rank Sums)
Level Count Score Sum Expected Score Score Mean (Mean-Mean0)/Std0
immob 98 10713,0 8281,00 109,316 7,823
lev 70 3483,00 5915,00 49,757 -7,823
2-Sample Test, Normal Approximation
S Z Prob>|Z|
3483 -7,82278 <,0001*
1-way Test, ChiSquare Approximation
ChiSquare DF Prob>ChiSq
61,2211 1 <,0001*

Figure 6a

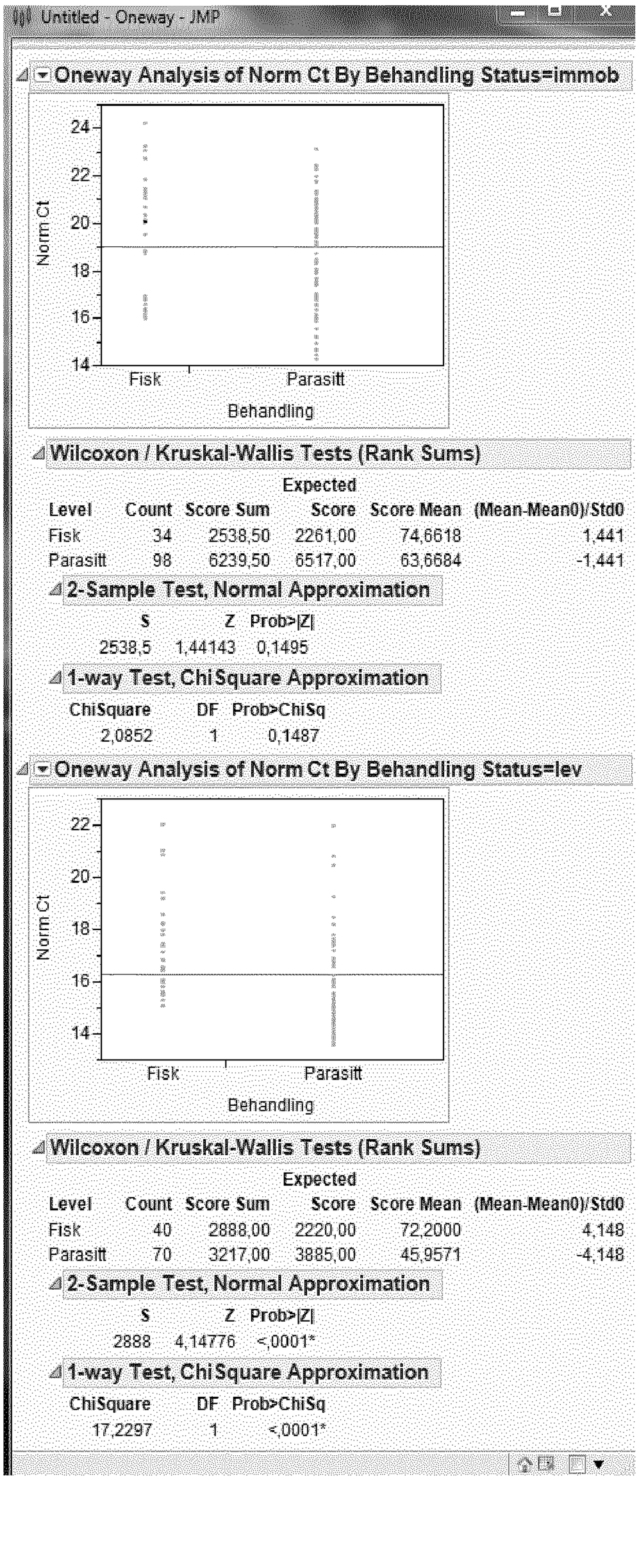

Untitled - Oneway - JMP
Oneway Analysis of Norm Ct By Behandling Status=immob
Norm Ct
24
22
20
18
16
14
Fisk
Parasitt
Behandling
Wilcoxon / Kruskal-Wallis Tests (Rank Sums)
Level Count Score Sum Expected Score Score Mean (Mean-Mean0)/Std0
Fisk 34 2538,50 2261,00 74,6618 1,441
Parasitt 98 6239,50 6517,00 63,6684 -1,441
2-Sample Test, Normal Approximation
S Z Prob>|Z|
2538,5 1,44143 0,1495
1-way Test, ChiSquare Approximation
ChiSquare DF Prob>ChiSq
2,0852 1 0,1487
Oneway Analysis of Norm Ct By Behandling Status=lev
Norm Ct
22
20
18
16
14
Fisk
Parasitt
Behandling
Wilcoxon / Kruskal-Wallis Tests (Rank Sums)
Level Count Score Sum Expected Score Score Mean (Mean-Mean0)/Std0
Fisk 40 2888,00 2220,00 72,2000 4,148
Parasitt 70 3217,00 3885,00 45,9571 -4,148
2-Sample Test, Normal Approximation
S Z Prob>|Z|
2888 4,14776 <,0001*
1-way Test, ChiSquare Approximation
ChiSquare DF Prob>ChiSq
17,2297 1 <,0001*

Figure 6b

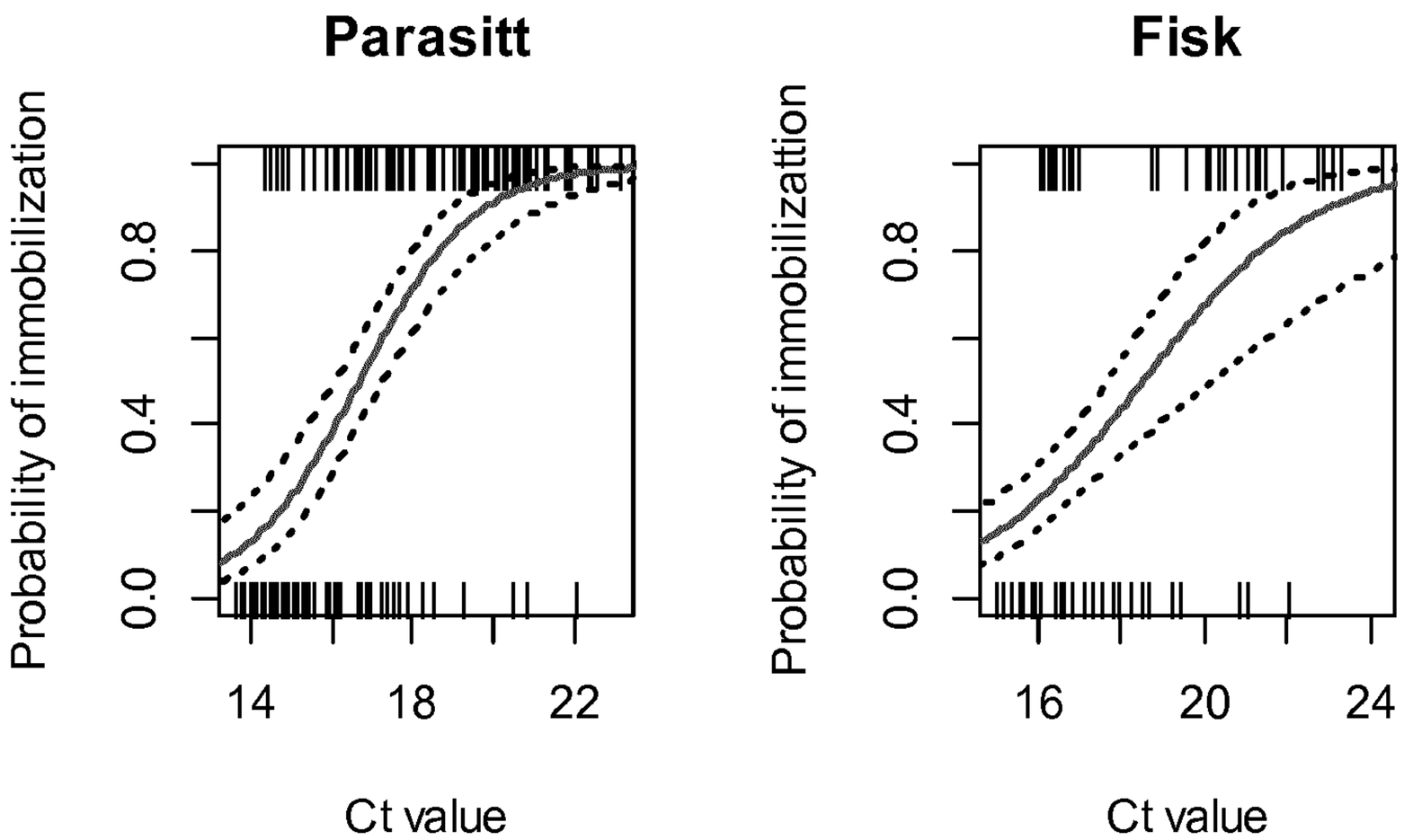

Parasitt
Probability of immobilization
0.0
0.4
0.8
14
18
22
Ct value
Fisk
Probabilitty of immobilization
0.0
0.4
0.8
16
20
24
Ct value

Figure 7

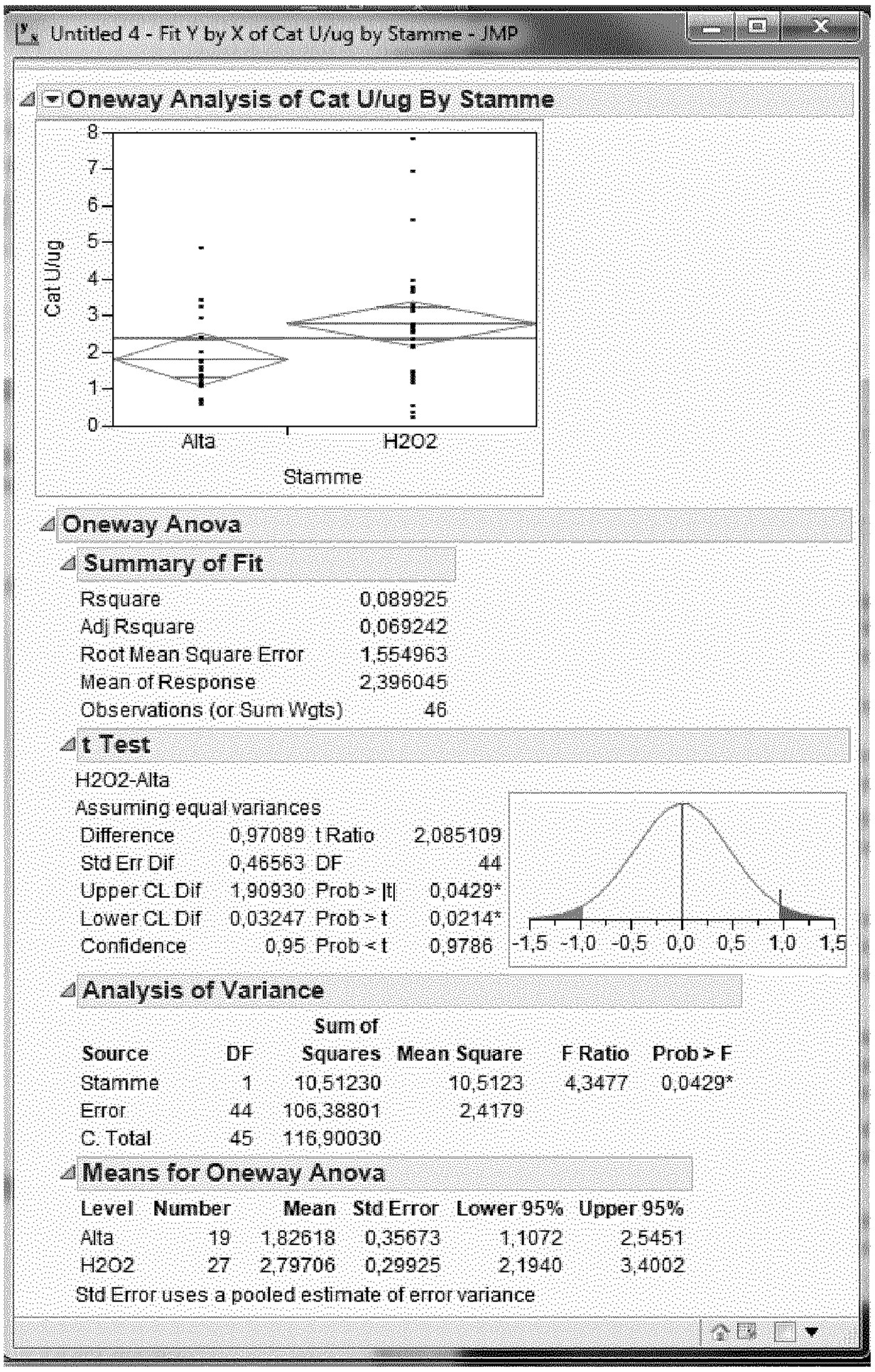

Untitled 4 - Fit Y by X of Cat U/ug by Stamme - JMP
Oneway Analysis of Cat U/ug By Stamme
Cat U/ug
8
7
6
5
4
3
2
1
0
Alta
H2O2
Stamme
Oneway Anova
Summary of Fit
Rsquare 0,089925
Adj Rsquare 0,069242
Root Mean Square Error 1,554963
Mean of Response 2,396045
Observations (or Sum Wgts) 46
t Test
H2O2-Alta
Assuming equal variances
Difference 0,97089 t Ratio 2,085109
Std Err Dif 0,46563 DF 44
Upper CL Dif 1,90930 Prob > |t| 0,0429*
Lower CL Dif 0,03247 Prob > t 0,0214*
Confidence 0,95 Prob < t 0,9786
-1,5 -1,0 -0,5 0,0 0,5 1,0 1,5
Analysis of Variance
Source DF Sum of Squares Mean Square F Ratio Prob > F
Stamme 1 10,51230 10,5123 4,3477 0,0429*
Error 44 106,38801 2,4179
C. Total 45 116,90030
Means for Oneway Anova
Level Number Mean Std Error Lower 95% Upper 95%
Alta 19 1,82618 0,35673 1,1072 2,5451
H2O2 27 2,79706 0,29925 2,1940 3,4002
Std Error uses a pooled estimate of error variance

Figure 8

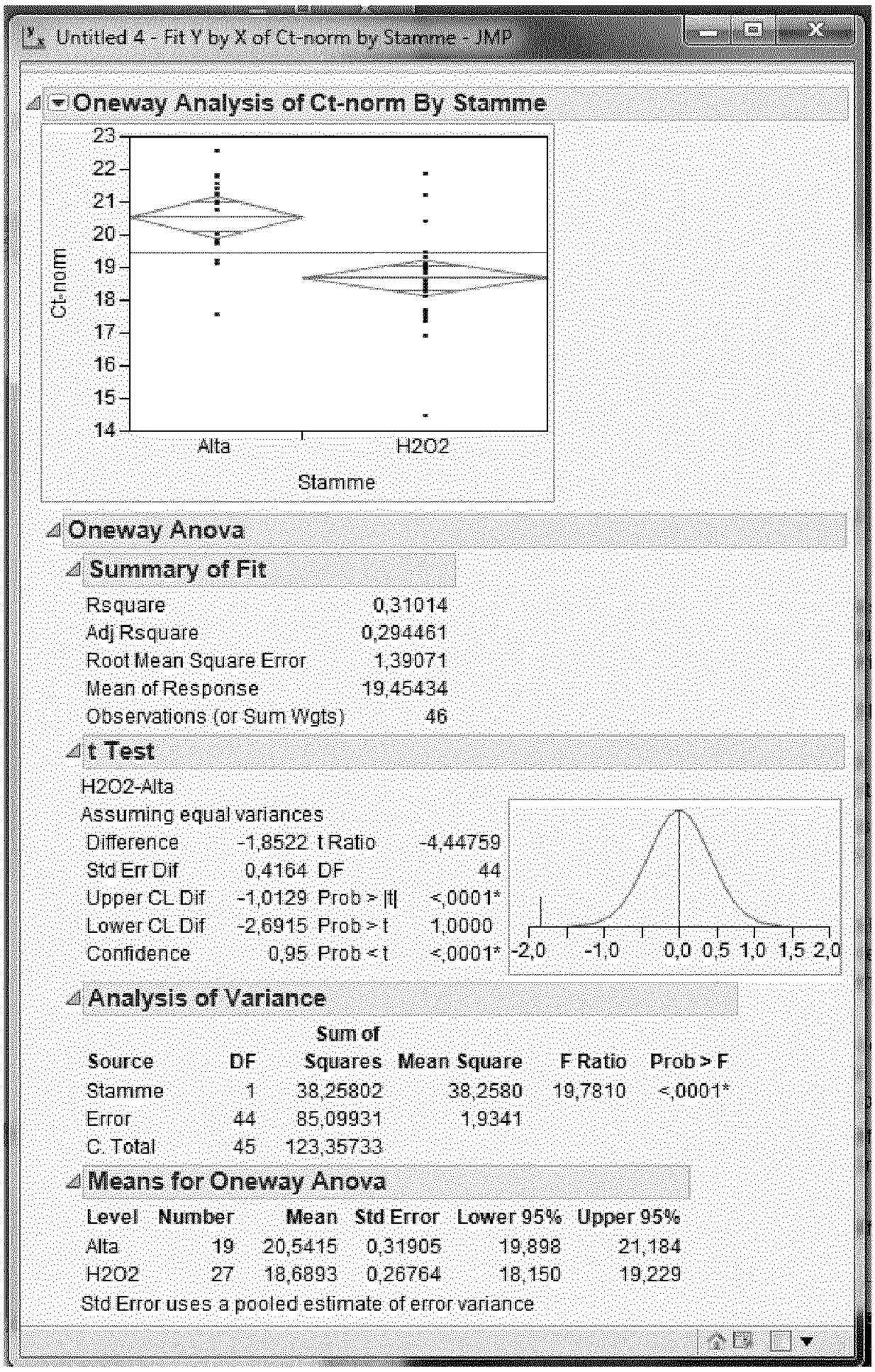
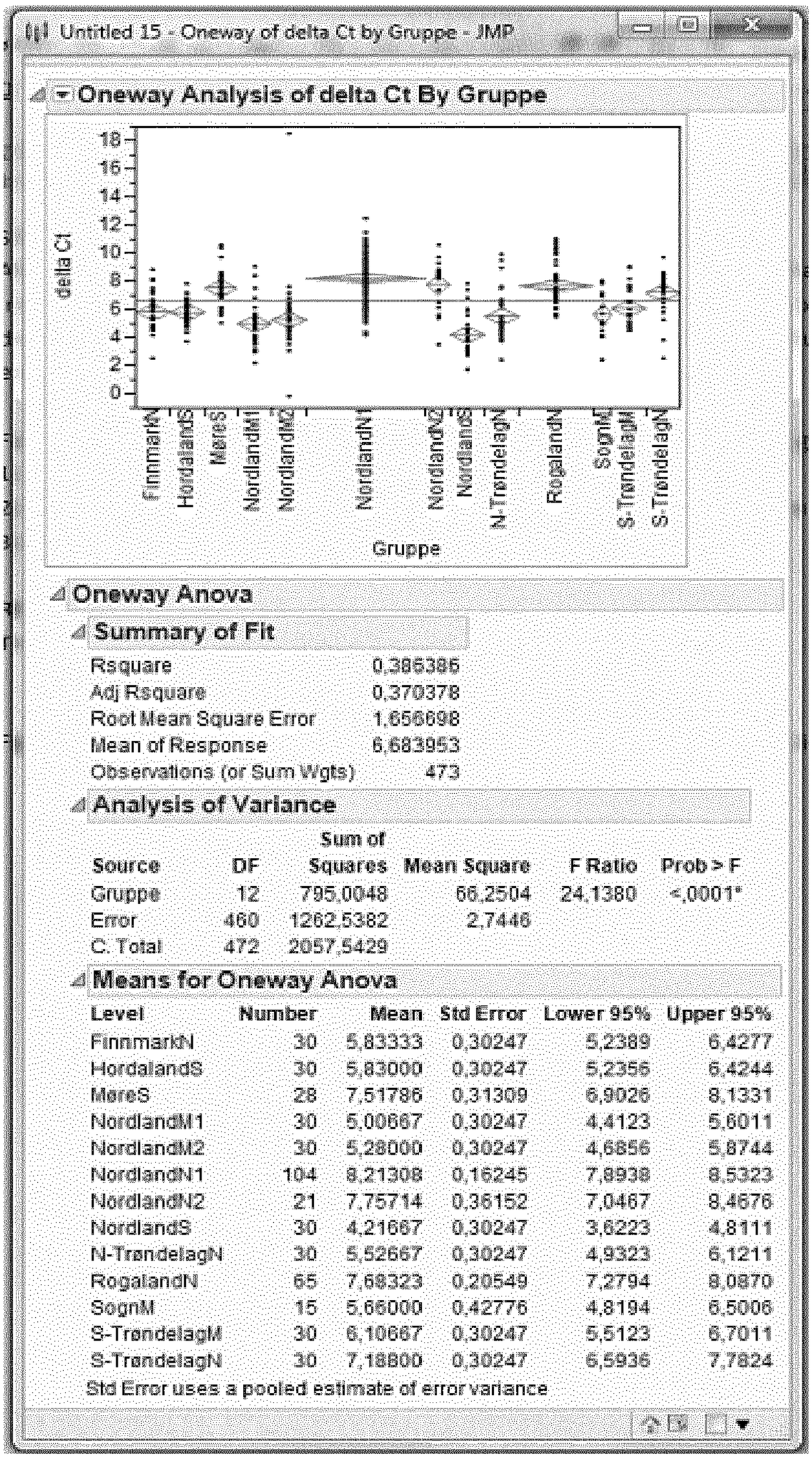

Untitled 4 - Fit Y by X of Ct-norm by Stamme - JMP
Oneway Analysis of Ct-norm By Stamme
Ct-norm
23
22
21
20
19
18
17
16
15
14
Alta
H2O2
Stamme
Oneway Anova
Summary of Fit
Rsquare 0,31014
Adj Rsquare 0,294461
Root Mean Square Error 1,39071
Mean of Response 19,45434
Observations (or Sum Wgts) 46
t Test
H2O2-Alta
Assuming equal variances
Difference -1,8522 t Ratio -4,44759
Std Err Dif 0,4164 DF 44
Upper CL Dif -1,0129 Prob > |t| <,0001*
Lower CL Dif -2,6915 Prob > t 1,0000
Confidence 0,95 Prob < t <,0001*
-2,0 -1,0 0,0 0,5 1,0 1,5 2,0
Analysis of Variance
Source DF Sum of Squares Mean Square F Ratio Prob > F
Stamme 1 38,25802 38,2580 19,7810 <,0001*
Error 44 85,09931 1,9341
C. Total 45 123,35733
Means for Oneway Anova
Level Number Mean Std Error Lower 95% Upper 95%
Alta 19 20,5415 0,31905 19,898 21,184
H2O2 27 18,6893 0,26764 18,150 19,229
Std Error uses a pooled estimate of error variance

Figure 9



Oneway Anova

Summary of Fit

Rsquare	0,386386
Adj Rsquare	0,370378
Root Mean Square Error	1,656698
Mean of Response	6,683953
Observations (or Sum Wgts)	473

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Gruppe	12	795,0048	66,2504	24,1380	<,0001*
Error	460	1262,5382	2,7446		
C. Total	472	2057,5429			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
FinnmarkN	30	5,83333	0,30247	5,2389	6,4277
HordalandS	30	5,83000	0,30247	5,2356	6,4244
MøreS	28	7,51786	0,31309	6,9026	8,1331
NordlandM1	30	5,00667	0,30247	4,4123	5,6011
NordlandM2	30	5,28000	0,30247	4,6856	5,8744
NordlandN1	104	8,21308	0,16245	7,8938	8,5323
NordlandN2	21	7,75714	0,36152	7,0467	8,4676
NordlandS	30	4,21667	0,30247	3,6223	4,8111
N-TrøndelagN	30	5,52667	0,30247	4,9323	6,1211
RogalandN	65	7,68323	0,20549	7,2794	8,0870
SognM	15	5,66000	0,42776	4,8194	6,5006
S-TrøndelagM	30	6,10667	0,30247	5,5123	6,7011
S-TrøndelagN	30	7,18800	0,30247	6,5936	7,7824

Std Error uses a pooled estimate of error variance

Figure 10

Connecting Letters Report

Level		Mean
NordlandN1	A	22,280769
NordlandN2	A	22,052381
S-TrøndelagN	A B	21,723333
RogalandN	A	21,667692
MøreS	A B C	21,542857
S-TrøndelagM	B C D	19,913333
HordalandS	C D	19,680000
FinnmarkN	D	19,476667
SognM	D E	18,973333
N-TrøndelagN	D E	18,460000
NordlandM2	D E	18,150000
NordlandM1	D E	18,056667
NordlandS	E	17,406667

Levels not connected by same letter are significantly different.

Figure 11

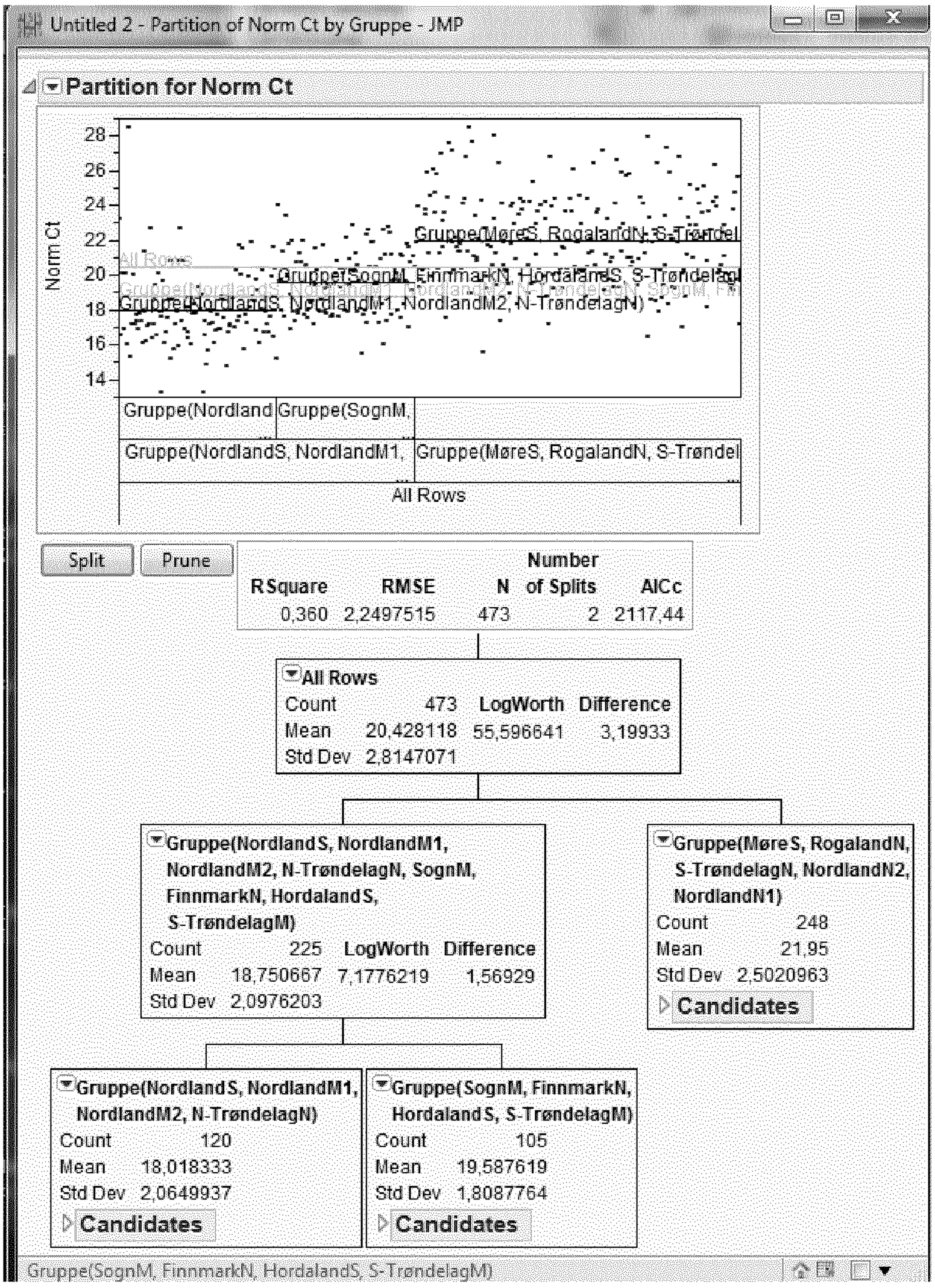

Untitled 2 - Partition of Norm Ct by Gruppe - JMP
Partition for Norm Ct
Norm Ct
28
26
24
22
20
18
16
14
Gruppe(Nordland
Gruppe(SognM,
Gruppe(NordlandS, NordlandM1,
Gruppe(MøreS, RogalandN, S-Trøndel
All Rows
Split
Prune
RSquare RMSE N Number of Splits AICc
0,360 2,2497515 473 2 2117,44
All Rows
Count 473 LogWorth Difference
Mean 20,428118 55,596641 3,19933
Std Dev 2,8147071
Gruppe(NordlandS, NordlandM1, NordlandM2, N-TrøndelagN, SognM, FinnmarkN, HordalandS, S-TrøndelagM)
Count 225 LogWorth Difference
Mean 18,750667 7,1776219 1,56929
Std Dev 2,0976203
Gruppe(MøreS, RogalandN, S-TrøndelagN, NordlandN2, NordlandN1)
Count 248
Mean 21,95
Std Dev 2,5020963
Candidates
Gruppe(NordlandS, NordlandM1, NordlandM2, N-TrøndelagN)
Count 120
Mean 18,018333
Std Dev 2,0649937
Candidates
Gruppe(SognM, FinnmarkN, HordalandS, S-TrøndelagM)
Count 105
Mean 19,587619
Std Dev 1,8087764
Candidates
Gruppe(SognM, FinnmarkN, HordalandS, S-TrøndelagM)

Figure 12

```
CLUSTAL 2.1 multiple sequence alignment


gi|225719453|gb|BT081149.1|      Caligus clemensi
gi|555555555|gb|BT555555.1|      Lepeophtheirus salmonis


gi|225719453|gb|BT081149.1|      ----------------------GGGGGGGCTTACTTCACTTG--TGTCT- 25
gi|555555555|gb|BT555555.1|      TTCTAAGGAAAATTCAAAGATAGAAAGGAGATATTTTACTTGAATATTTT 50
                                           *   **   ** ** *****  * * *


gi|225719453|gb|BT081149.1|      ----------------------GACC----------------------TG 31
gi|555555555|gb|BT555555.1|      AAACAAAGAAAAAGGTAAGTAGGATCATAGTTTATTCAACAAAATTAGTG 100
                                           ** *                        **


gi|225719453|gb|BT081149.1|      TGAAT-TTAAGA-----AGA-----------GGAAAAT--ATATA----- 57
gi|555555555|gb|BT555555.1|      TAAATATCAAAATGCAAAGACAGGCACTATGGAAAAATCAACACAATCTC 150
                                 * *** * ** *     ***           * *****  * * *


gi|225719453|gb|BT081149.1|      ------TAATTCAT-----------------CTCCATC----------- 72
gi|555555555|gb|BT555555.1|      AAAGTTTAGTTTGTTAAAGTGTAATCTGGATACTACGTCTAAGAACAATA 200
                                       ** **  *                  ** * **


gi|225719453|gb|BT081149.1|      ---ATGTCCGTTCGTGATCGAGCCTCCGAGCAACTGGCAGATTATGTTAA 119
gi|555555555|gb|BT555555.1|      AATATGCCTCTTCGTGATTGTGCCACAGAACAACTTGCCAACTATTCAAA 250
                                    *** *  ******** * *** * ** ***** **  * ***   **


gi|225719453|gb|BT081149.1|      AACGATTAAGGGCGCTGATCGCCTAACATCAAGTCATGGGATCCCTCTTG 169
gi|555555555|gb|BT555555.1|      GTCGATAAAGAAAGAAGACCTTCTCACATCCAGTCACGGAATCCCACTGG 300
                                   **** ***   *  ** *  ** ***** ***** ** ***** ** *


gi|225719453|gb|BT081149.1|      GCGACAAGACCAACATTGTTACCGTAGGTAAGAGAGGCCCTGCTTTGCTT 219
gi|555555555|gb|BT555555.1|      GTGATAAAAATAACATAGTTACCGTTGGCAGAAGAGGTCCTTCTTTGATT 350
                                 * ** ** *  ***** ******** ** *  ***** *** ***** **


gi|225719453|gb|BT081149.1|      CAAGACGTGGCTTTCATCGATGAAATGGCGCACTTTGTCCGAGAACGCAT 269
gi|555555555|gb|BT555555.1|      CAAGATGTTGCATTTATGGACGAAATGGCCCATTTTGTACGTGAGCGTAT 400
```

```
                                ***** ** ** ** ** ** ******** ** ***** ** ** ** **

gi|225719453|gb|BT081149.1|     TCCCGAGCGCGTGGTTCATGCCAAGGGTGCAGGGGCCTTCGGGTACTTTG 319
gi|555555555|gb|BT555555.1|     TCCCGAGAGAGTAGTTCATGCAAAAGGAGCTGGAGCTTTTGGTTACTTGG 450
                                ******* * ** ******** ** ** ** ** ** ** ** ***** *

gi|225719453|gb|BT081149.1|     AGGTGACCCACGACATCACGAAATACTCCAAAGCAGGTATCTTCTCTGAA 369
gi|555555555|gb|BT555555.1|     AAATAACTCATGACATTACAAAGTACTGCAAGGCTGAAATATTTGATACA 500
                                *  * ** ** ***** ** ** **** *** ** *  ** **   *  *

gi|225719453|gb|BT081149.1|     ATCGGAAAGAGAACCCCTTTGGCTGTAAGGTTCAGTACTGTTGGGGGCGA 419
gi|555555555|gb|BT555555.1|     ATTGGAAAGAGAACAGATCTTGCCGTAAGGTTCAGTACAGTTGGAGGTGA 550
                                ** ***********   * * ** ************** ***** ** **

gi|225719453|gb|BT081149.1|     GTCCGGATCTGCGGATAGTGCCCGGGATCCTAGGGGCTTTGCGGTTAAGT 469
gi|555555555|gb|BT555555.1|     ATCTGGATCTGCTGATACTGAGAGAGATCCAAGAGGATTTGCCGTCAAAT 600
                                 ** ******** **** **   * ***** ** ** ***** ** ** *

gi|225719453|gb|BT081149.1|     TCTACACCAAGGATGGAAACTGGGATTTGGTTGGGAACAACACGCCCATT 519
gi|555555555|gb|BT555555.1|     TTTATACGAAGGAGGGTAATTGGGACTTGGTTGGAAACAATACTCCTATT 650
                                * ** ** ***** ** ** ***** ******** ***** ** ** ***

gi|225719453|gb|BT081149.1|     TTCTTTATCCGAGATCCCAGTCTCTTTAGTAACTTCATTCACACCCAAAA 569
gi|555555555|gb|BT555555.1|     TTTTTTATTCGTGATCCCATTCTTTTTGAGAGTTTTATTCATTCACAAAA 700
                                ** ***** ** ******* *** ***   *  ** *****  * *****

gi|225719453|gb|BT081149.1|     AAGGAATCCAGTCACTCATTTGAGGGACCCTGATATGGTCTGGGACTCTG 619
gi|555555555|gb|BT555555.1|     AAGAAATCCTGTAACACATTTGAAGGATCCAGATATGTTTTGGGACTTTG 750
                                *** ***** ** ** ******* *** ** ****** * ******* **

gi|225719453|gb|BT081149.1|     TGACCCTTCGGCCAGAGACCAGCCATCAAATATCCTTCCTCTACTCTGAC 669
gi|555555555|gb|BT555555.1|     TAACTCTGAGGCCTGAAACAACACATCAAATGTCTATTCTATTCTCAGAC 800
                                * ** **  **** ** ** *  ******** **  * ** * *** ***

gi|225719453|gb|BT081149.1|     AGGGGAACCCCTGATGGATATCGACACATGAATGGCTACGGCTCACATAC 719
gi|555555555|gb|BT555555.1|     AGAGGAACACCAGATGGATATAGACATATGAACGGCTATGGATCTCATAC 850
                                ** ***** ** ********* **** ***** ***** ** ** *****
```

```
gi|225719453|gb|BT081149.1|     ATTTAAACTCGTGAATCAAAAGGACGAGGCTGTATATTGCAAGTTCCATA 769
gi|555555555|gb|BT555555.1|     CTTTAAATTAGTTAATAAAAAGAACGAGGCTGTATATTGTAAGTTTCACT 900
                                 ****** * ** *** ***** ********************* ***** **

gi|225719453|gb|BT081149.1|     TGAAAACCAACCAAGGAATCAAGAATCTCTCCAGCAAGGAGGCTGGAGAC 819
gi|555555555|gb|BT555555.1|     TTAAAACTAACCAAGGTATAAAGAATCTTTCAAGTCAAGAGGCTGCAAAT 950
                                * ***** ******** ** ******** ** **  * ******* * *

gi|225719453|gb|BT081149.1|     TTATCCCGTGATGATCCTGACTATGCCATCCGTGACTTATACAACTCTAT 869
gi|555555555|gb|BT555555.1|     ATGGCCAGAGATGATCCTGATTACTCTATTCGCGATTTATATAATGCTAT 1000
                                 *  ** * ********** **  * ** ** ** ***** **  ****

gi|225719453|gb|BT081149.1|     CGCCGGGGAAAATTATCCCTCGTACACCATGCACATCCAGGTCATGACTG 919
gi|555555555|gb|BT555555.1|     TGACCAAGAAAACTTTCCTTCCTATACCATGCACATTCAAGTGATGACTA 1050
                                 * *   ***** * *** ** ** ********** ** ** ******

gi|225719453|gb|BT081149.1|     AGGAACAAGCTGATAACTTGGAGTCTGATCCCTTTGACCTGACTAAGGTT 969
gi|555555555|gb|BT555555.1|     TGGATGAAGCAGACACCATAGATTTTGATCCTTTTGATTTGACAAAGGTT 1100
                                 ***  **** ** * * * ** * ****** *****  **** ******

gi|225719453|gb|BT081149.1|     TGGTCTCATAAAGCCTTCCCTCTCATCGAAGTGGGACAATTGGTCCTTAA 1019
gi|555555555|gb|BT555555.1|     TGGTCTCATAAAACCTTCCCACTCATTGAAGTCGGACAACTAGTCCTCAA 1150
                                ************ ******* ***** ***** ****** * ***** **

gi|225719453|gb|BT081149.1|     CAAGAACCCAGACAACTACTTCTCAGAAGTGGAGCAGATTGCCTTTAGCC 1069
gi|555555555|gb|BT555555.1|     TAGAAATCCATCCAATTACTTTGCTGAGGTTGAACAAATTGCTTTTAGTC 1200
                                 *  ** ***  *** *****  * ** ** ** ** ***** ***** *

gi|225719453|gb|BT081149.1|     CTGGGAACCTTGTACCTGGAATAGAACCCAGCCCTGACAAAATGCTTCAA 1119
gi|555555555|gb|BT555555.1|     CAGGGAACTTGATCCCTGGAATTGAAGCAAGCCCTGACAAAATGTTGCAA 1250
                                * ****** *  * ******** *** * *************** * ***

gi|225719453|gb|BT081149.1|     GGTCGCATTCTGTCTTATAGTGATGCTCATCGTTATCGCCTT------GG 1163
gi|555555555|gb|BT555555.1|     GGTCGGATATTAACCTATAGTGATGCTCACCGCTATCGTATTTCTGGTGA 1300
                                ***** **  *  * ************** ** *****  **      *
```

```
gi|225719453|gb|BT081149.1|      AGCGAATTAC----ACTCATCTTA--ACGTTAATTC--ACCA-------- 1197

gi|555555555|gb|BT555555.1|      AGTGGGACGCCATGATTCCTCTGAGGAAGATAATTTTTATCAAGTCGGAG 1350

                                 ** *     *    * ** *** *  * * *****   * **


gi|225719453|gb|BT081149.1|      -----TACAATAAGGCCTGCAATTACCAACGAGATGGTCCCATG------ 1236

gi|555555555|gb|BT555555.1|      TGTTTTATAGGGATGTTTTAAACGAAGAACAAAAGAATAACTTGGTAGAC 1400

                                      ** *   * *  *  **  *  *** * *   *  * **


gi|225719453|gb|BT081149.1|      -----TGCA-TGAGT--AATCAA------GGGAAGGGCCCAAATTACTAT 1272

gi|555555555|gb|BT555555.1|      AACATTGCAGTAAATTTAGTCCATGCACAAGAAAAATTCCAAGTGAAAGC 1450

                                      **** * * *  * ** *       * **    **** * *


gi|225719453|gb|BT081149.1|      CCCAACTCCTTTTGGGGGCCCAGTG-GATCCCAGGATAAATACGGAGAGC 1321

gi|555555555|gb|BT555555.1|      CATCAAACAATTCGGG----CAATGTGATCCT--GATTA-TGGGAAAAGA 1493

                                 *   *  *  ** ***    ** ** *****   *** * *  * * **


gi|225719453|gb|BT081149.1|      ----ACACTTTCTTTGCCTCGGGCGAAGCTAAAAGACACGAATCCGCT-C 1366

gi|555555555|gb|BT555555.1|      CTTGAGACTAGATTGGCTTATTATAAAAATAAATGATA-AATTTTGTAAC 1542

                                     * ***   ** ** *      **  **** ** *  * *  *   *


gi|225719453|gb|BT081149.1|      ATGAGGAC----------------AACTTTTCTCA-----AGTTGGAGAG 1395

gi|555555555|gb|BT555555.1|      ATAAGCATGTATTACACCCCAAAAAATTGTTCCCACCCTTAATTAAAAAA 1592

                                 ** ** *                 ** * *** **     * **  * *


gi|225719453|gb|BT081149.1|      TTT---------TACCGAGATATC------TTGAATGAA---GAGCAAAA 1427

gi|555555555|gb|BT555555.1|      TATATATATATATACCAAAATATTATTTTTTTTCATGCATATGATAAAAA 1642

                                 * *         **** * ****       **  *** *   **  ****


gi|225719453|gb|BT081149.1|      GAGCAACTTGGTGGACAACATTGCTAGTGATTTAATTCATGCACAAGAGC 1477

gi|555555555|gb|BT555555.1|      AAAATACTTGCTC-ACAAGACAAATGGTAATGAAACTTGTG-ACAA---- 1686

                                   *   ***** *  **** *    * ** **  ** *  ** ****


gi|225719453|gb|BT081149.1|      ACTTCCAGGAGAAGGCCATTAAGCAGTTT-TGTCAATGCGATCCCGACTA 1526

gi|555555555|gb|BT555555.1|      -TTTCCATTA-AATACCGTTGAGATATGAGCATCCACATCACTCTGACTA 1734

                                   *****  * **  ** ** **   *     ** *    *  * *****


gi|225719453|gb|BT081149.1|      C----------GGCAAAAG-ACTGG------AAGCAAGGCTGGCTCTCTA 1559
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gi|555555555|gb|BT555555.1|      TTTGCCAAACTGACAAAATCACTCGGCATTTATGTAGGGGTTTTTGTTTA 1784
                                           * *****  *** *      * * * ** *   * * **

gi|225719453|gb|BT081149.1|      CAAAAAT---AAAAAAGTCTA---AGGGGAG------TTCCGCTG--GAA 1595
gi|555555555|gb|BT555555.1|      TATCTGCTTGGCAACAGTCCGCTCAGTGGGGATCTATTTTTGTTGCCAGA 1834
                                  *          ** ****     ** ** *      **  * **    *

gi|225719453|gb|BT081149.1|      AATAATATTCAT--ATTTATAT--CTATGATGTATGTACA---------- 1631
gi|555555555|gb|BT555555.1|      AGCAAT-TTCATTCGTTTATATGATTA-AATAAACATACAACTCAAAATC 1882
                                  *  *** *****   *******   **  **  *  ****

gi|225719453|gb|BT081149.1|      TAT---------TTACTTAAATAAAAAA----------GCAATGAAATA 1661
gi|555555555|gb|BT555555.1|      TATAGTCAAATATTATTTCACTAGATTATATTTTGCTTTATGCTGTAGTA 1932
                                  ***         *** ** * ** *  *              ** * **

gi|225719453|gb|BT081149.1|      TC--- 1663
gi|555555555|gb|BT555555.1|      TTAAG 1937
                                  *
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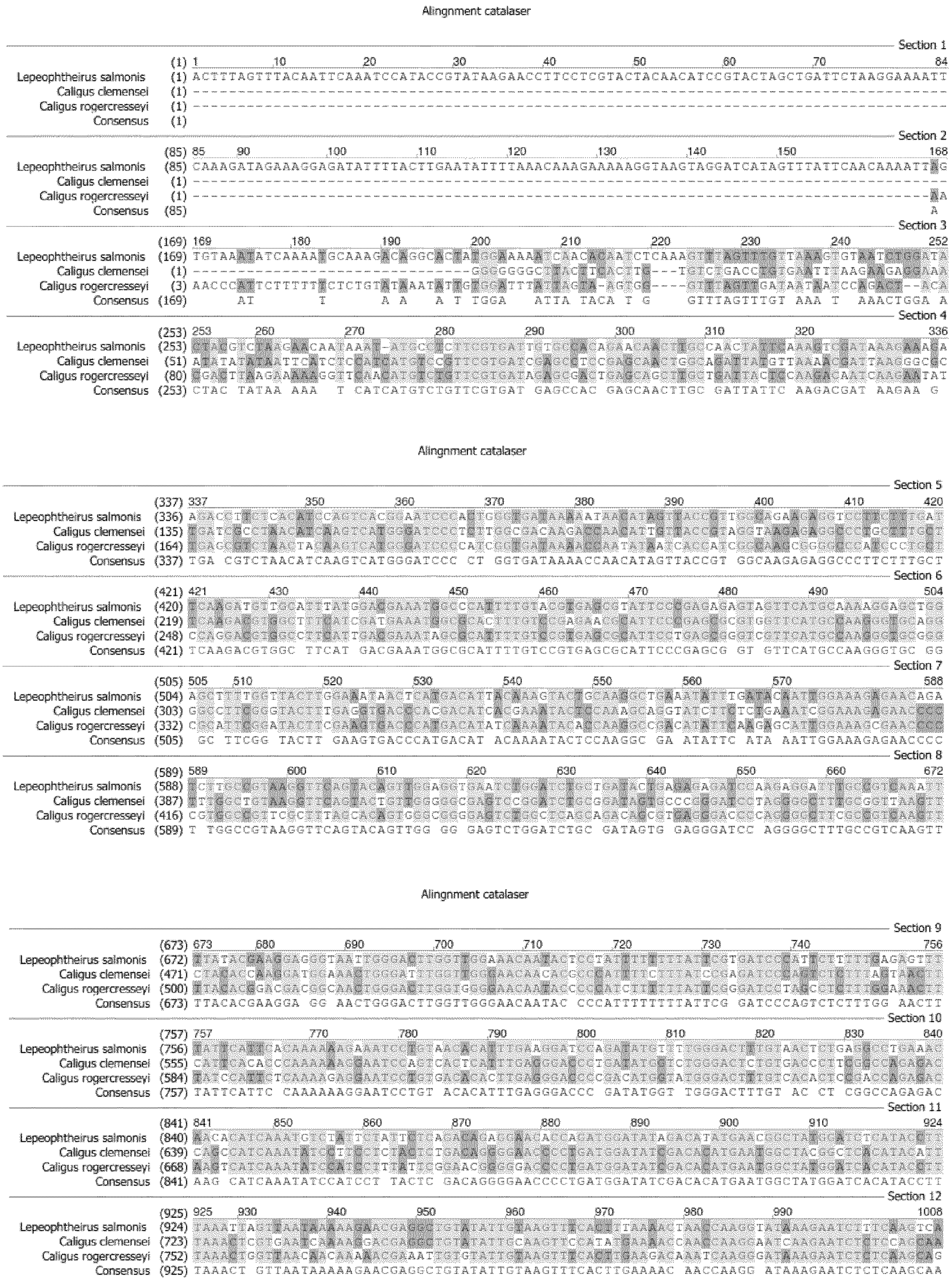
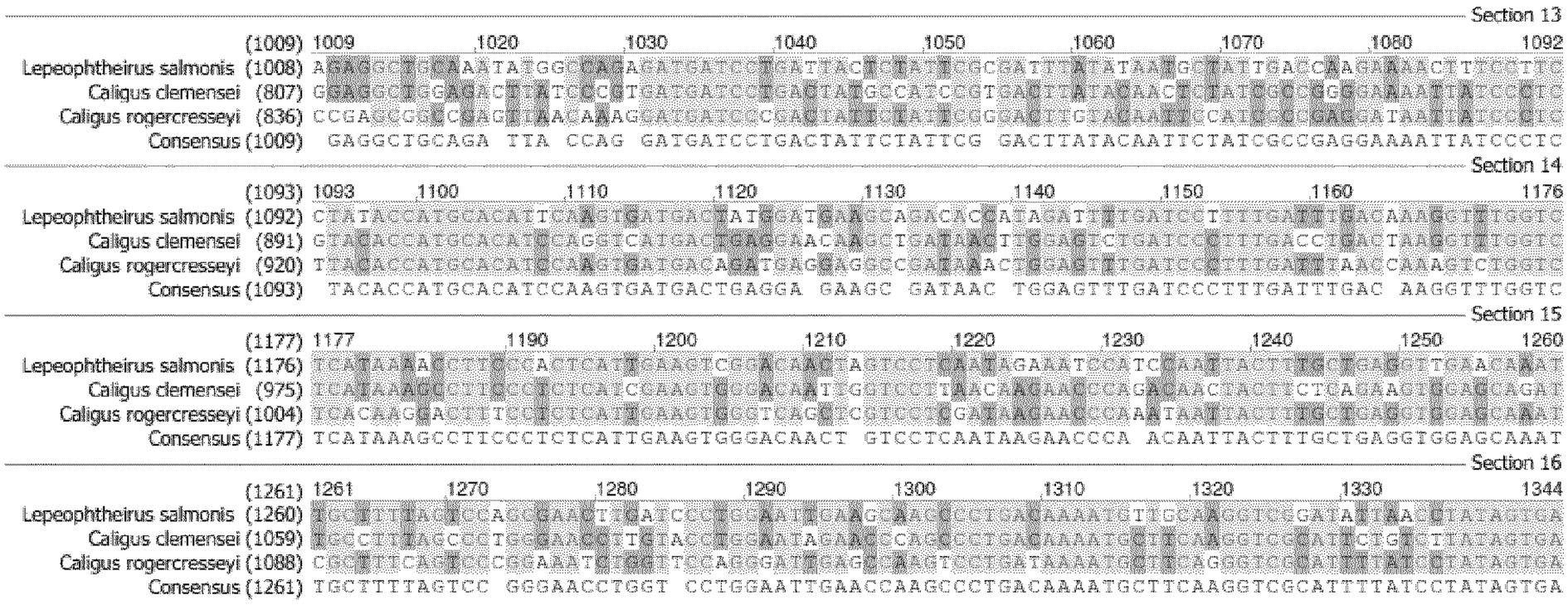
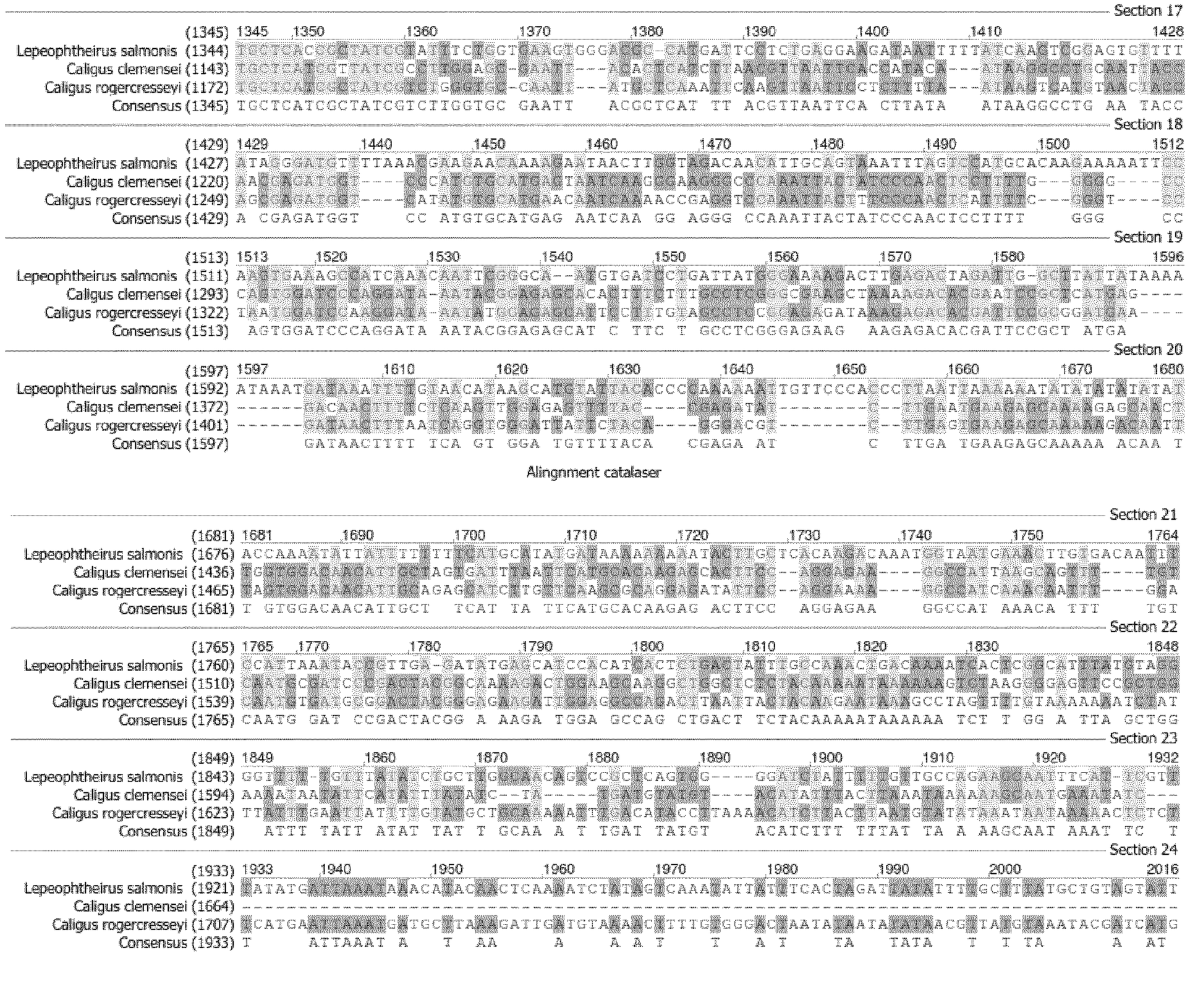
Figure 13


Alingnment catalaser
Section 1
Lepeophtheirus salmonis
Caligus clemensei
Caligus rogercresseyi
Consensus
Section 2
Section 3
Section 4
Alingnment catalaser
Section 5
Section 6
Section 7
Section 8
Alingnment catalaser
Section 9
Section 10
Section 11
Section 12

Alingnment catalaser



Alingnment catalaser



Alingnment catalaser

Alingnment catalaser

Section 25

(2017) 2017 2030 2040 2050 2060 2070 2080 2090 2100
Lepeophtheirus salmonis (2005) A G
Caligus clemensei (1664)
Caligus rogercresseyi (1791) T AAAAGAAAATAAATTGAAATATCATAATATATTAAAATATATATTTTTTGAAGTTGCGATAATACAAACGATGATTTTGTGC
Consensus (2017) A

Section 26

(2101) 2101 2110 2120 2130 2140 2150 2160 2170 2184
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (1875) AAGACTACTTCAGTCTCTTAGGAAGCGGGATTGGAATTCATTTAGTTATTTGTCTCTAGGGTTGGAGGGTGCAATTCATTTTTG
Consensus (2101)

Section 27

(2185) 2185 2190 2200 2210 2220 2230 2240 2250 2268
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (1959) ATATTATATTTCTTGATATATATATATAGACTATTTTTTAAAGAAAATAAATGAATTGAAAACTGCGAATATACCGTTTTACTA
Consensus (2185)

Section 28

(2269) 2269 2280 2290 2300 2310 2320 2330 2340 2352
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2043) TCCCAGATTTGCGCTTATCGTTGTTGTTGAACCCGGAACCATTAAAAATCATTGTATTTAATGTGTTAAATTGGTTAATAATAT
Consensus (2269)

Alingnment catalaser

Section 29

(2353) 2353 2360 2370 2380 2390 2400 2410 2420 2436
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2127) GGTCCGACTTGAGGAACTAAAAGAATAAAAAAATGTAAAATATTAATTGACTTCATTACTTAATTTTGTACTTTTTTCTCTTTT
Consensus (2353)

Section 30

(2437) 2437 2450 2460 2470 2480 2490 2500 2510 2520
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2211) TCTATCCTTTATAGTAATTGGATATGTAATGAAAAAATGGCCACTGTTTTCCGCATATATATGTTATGTTCAATACATTTATGT
Consensus (2437)

Section 31

(2521) 2521 2530 2540 2550 2560 2570 2580 2590 2604
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2295) AACAAACAATAAAATATCAATTTAGTTTATTAATAACATACATTTGTATTTAAGAATGTTTAGTATCAGGTTATTCAGTCAGCC
Consensus (2521)

Section 32

(2605) 2605 2610 2620 2630 2640 2650 2660 2670 2688
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2379) TAAGAATATAAACAAAATAAAAAGTTTAATAAAACGCTCTATAATAAAAAAGAAAGAATCACTAGAATGATGGGCATCACATGG
Consensus (2605)

Alingnment catalaser

Section 33

(2689) 2689 2700 2710 2720 2730 2740 2750 2760 2772
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2463) TTTTTTGTTTCAAATCCTTTCGTTTTAAAAGTTTACTTTTAACACTTCCTTCGTGAGGAAACTTAGGTGGCTGAAAATGTTGTG
Consensus (2689)

Section 34

(2773) 2773 2780 2790 2800 2810 2820 2830 2840 2856
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2547) TTTTATCTTTCTTTCCTATATTTGTTTTAATTAAGTTATTTAAGGTGAAATGTTTATTTGTACTCTTTTATATATAAACTGGGA
Consensus (2773)

Section 35

(2857) 2857 2870 2880 2890 2900 2910 2920 2930 2940
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2631) ATTTTATAAAAACTTAATCATTCGATTTCAATTTATTTCTCTTTCACACGCTGCTTCATGATTTGTAGTTTAATCACAAATCTC
Consensus (2857)

Section 36

(2941) 2941 2950 2960 2970 2980 2990 3000 3010 3024
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2715) AGGATCCTCCAATGTACAGTTTTGGTGTAAAGCACTCAAGCTATATCTACGGCGCCCTTCCCCTTCCAGGAGAGGAGACTCGTC
Consensus (2941)

Alingnment catalaser

Section 37

(3025) 3025 3030 3040 3050 3060 3070 3080 3090 3108
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2799) CTCTTCCTTACTAACATCATTCTCCCAACGTCCCACACAAACCTACTCAATGCCTCTAACAAGTTTTTCCTTTGCCTCAGGTGG
Consensus (3025)

Section 38

(3109) 3109 3120 3130 3140 3150 3160 3170 3180 3192
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2883) ACGCACTTCTTCATTCATCACCAAAAATCTCCTTCGGTATCCGACATTCTCCTTACATTACCATGCTTAAAGGCGACAACTATG
Consensus (3109)

Section 39

(3193) 3193 3200 3210 3220 3230 3240 3250 3260 3276
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (2967) TCACTACCTACACAGACAGTGTTGAGGAGGAACCCTACAGGTCTTCTTCTACTTCATTTGACGGAGCCTCTAATAAGGTCACAA
Consensus (3193)

Section 40

(3277) 3277 3290 3300 3310 3320 3330 3340 3350 3360
Lepeophtheirus salmonis (2008)
Caligus clemensei (1664)
Caligus rogercresseyi (3051) CTACCTACAGTTATAACATTGTGAAAAATGGATCCAACAATGTTAATAGTAATGAGTCCAGCTATATCGTTCAAGAGCCTTCCC
Consensus (3277)

ACTTTAGTTTACAATTCAAATCCATACCGTATAAGAACCTTCCTCGTACTACAACATCCGTACTAGCTGA
TTCTAAGGAAAATTCAAAGATAGAAAGGAGATATTTTACTTGAATATTTTAAACAAAGAAAAAGGTAAGT
AGGATCATAGTTTATTCAACAAAATTAGTGTAAATATCAAAATGCAAAGACAGGCACTATGGAAAAATCA
ACACAATCTCAAAGTTTAGTTTGTTAAAGTGTAATCTGGATACTACGTCTAAGAACAATAAATATGCCTC
TTCGTGATTGTGCCACAGAACAACTTGCCAACTATTCAAAGTCGATAAAGAAAGAAGACCTTCTCACATC
CAGTCACGGAATCCCACTGGGTGATAAAAATAACATAGTTACCGTTGGCAGAAGAGGTCCTTCTTTGATT
CAAGATGTTGCATTTATGGACGAAATGGCCCATTTTGTACGTGAGCGTATTCCCGAGAGAGTAGTTCATG
CAAAAGGAGCTGGAGCTTTTGGTTACTTGGAAATAACTCATGACATTACAAAGTACTGCAAGGCTGAAAT
ATTTGATACAATTGGAAAGAGAACAGATCTTGCCGTAAGGTTCAGTACAGTTGGAGGTGAATCTGGATCT
GCTGATACTGAGAGAGATCCAAGAGGATTTGCCGTCAAATTTTATACGAAGGAGGGTAATTGGGACTTGG
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