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Isolated proteins from caligid copepods, polynucleotides encoding the same, and antigens and vaccines comprising the same, in particular for the treatment or prevention of caligid copepod infection in fish. Proteins are peroxiredoxin-2 (Prx-2), fructose bisphosphate aldolase (FBP); enolase, transitionally-controlled tumour protein homolog (TCTP) and triosephosphate isomerase (TIM).

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(57) **Abstract:** Isolated proteins from caligid copepods, polynucleotides encoding the same, and antigens and vaccines comprising the same, in particular for the treatment or prevention of caligid copepod infection in fish. Proteins are peroxiredoxin-2 (Prx-2), fructose biphosphate aldolase (FBP); enolase, transitionally-controlled tumour protein homolog (TCTP) and triosephosphate isomerase (TIM).

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Sea Lice Antigens and Vaccines

The present invention relates to isolated proteins from caligid copepods, and polynucleotides encoding the same, and antigens and vaccines comprising the same, in particular for the

5 treatment or prevention of caligid copepod infection in fish.

Parasitic copepods in the family Caligidae (caligid copepods) infect and cause disease in fish. Collectively, these species are referred to as sea lice. There are three major genera of sea lice: *Pseudocaligus*, *Caligus* and *Lepeophtheirus*. In the northern hemisphere, the salmon louse
10 (*Lepeophtheirus salmonis*), is responsible for most disease outbreaks on farmed salmonids. This parasite is responsible for substantial indirect and direct losses in aquaculture.

All developmental stages of sea lice, which are attached to the host, feed on host mucus, skin and blood. The attachment and feeding activities of sea lice result in lesions that vary in their
15 nature and severity depending upon: the species of sea lice, their abundance, the developmental stages present and the species of the host (Johnson et al., 2004). In the southern hemisphere, *Caligus rogercresseyi*, is the primary caligid affecting the salmon farming industry in Chile (Gonzalez and Carvajal, 2003).

Caligid copepods have direct life cycles consisting of two free-living planktonic Nauplius stages, one free-swimming infectious copepodid stage, four to six attached chalimus stages, one or two preadult stages, and one adult stage (Kabata, 1970). Each of these developmental stages is separated by a moult. Once the adult stage is reached, caligid copepods do not
20 undergo additional moults. In the case of *L. salmonis*, eggs hatch into the free-swimming first nauplius stage, which is followed by a second nauplius stage, and then the infectious
25 copepodid stage. Once the copepodid locates a suitable host fish, it continues its development through four chalimus stages, first and second preadult stages, and then a final adult stage (Schram, 1993). The moults are characterized by gradual changes as the animal grows and undertakes physical modifications that enable it to live as a free-roaming parasite, feeding
30 and breeding on the surface of the fish.

Feeding of caligid copepods on the mucus, skin and blood of their hosts leads to lesions that vary in severity based on the developmental stage(s) of the copepods present, the number of copepods present, their site(s) of attachment and the species of host. In situations of severe

disease, such as is seen in Atlantic salmon (*Salmo salar*) when infected by high numbers of *L. salmonis*, extensive areas of skin erosion and haemorrhaging on the head and back, and a distinct area of erosion and sub-epidermal haemorrhage in the perianal region can be seen (Grimnes et al., 1996). Sea lice can cause physiological changes in their hosts including the development of a stress response, reduced immune function, osmoregulatory failure and death if untreated.

There are several management strategies that have been used for reducing the intensity of caligid copepod (sea lice) infestations. These include: fallowing of sites prior to restocking, year class separation and selection of farm sites to avoid areas where there are high densities of wild hosts or other environmental conditions suitable for sea lice establishment (Pike et al., 1999). Although the use of these strategies can in some cases lessen sea lice infection rates, their use individually or in combination has not been effective in eliminating infection.

A variety of chemicals and drugs have been used to control sea lice. These chemicals were designed for the control of terrestrial pests and parasites of plants and domestic animals. They include compounds such as hydrogen peroxide, organophosphates (e.g., dichlorvos and azamethiphos), ivermectin (and related compounds such as emamectin benzoate), insect molting regulators and pyrethrins (MacKinnon, 1997; Stone et al., 1999). Chemicals used in treatments are not necessarily effective against all of the stages of sea lice found on fish, and can create environmental risk. As seen in terrestrial pest and parasites there is evidence for the development of resistance in *L. salmonis* to some chemical treatments, especially in frequently-treated populations (Denholm, 2002). To reduce the costs associated with sea lice treatments and to eliminate environmental risks associated with these treatments new methods of sea lice control such as vaccines are needed.

A characteristic feature of attachment and feeding sites of caligid copepods on many of their hosts is a lack of a host immune response (Johnson et al., 2004; Jones et al., 1990; Jónsdóttir et al., 1992). This lack of an immune response is similar to that reported for other arthropod parasites such as ticks on terrestrial animals. In those instances, suppression of the host immune response is due to the production of immunomodulatory substances by the parasite (Wikel et al., 1996). These substances are being investigated for use as vaccine antigens to control these parasites. Sea lice, such as *L. salmonis*, like other arthropod ectoparasites, produce biologically active substances at the site of attachment and feeding that limits the

host immune response. As these substances have potential for use in a vaccine against sea lice we have identified a number of these substances from *L. salmonis* and have examined their effects of host immune function *in vitro*.

- 5 Secretory proteins produced by the sea lice may act as immunomodulatory agents or assist in the feeding activities on the host (Fast et al., J Parasitol 89: 7-13, 2003, 2004). Neutralization of these activities by host-derived antibodies may impair sea lice growth and survival on salmon.
- 10 Vaccines are generally safer than chemical treatments, both to the fish and to the environment. Vaccine development has been hindered by a lack of knowledge of the host-pathogen interactions between sea lice and their hosts. There is therefore a need for further or improved commercial vaccines against sea lice.
- 15 WO 2006/010265 relates to recombinant vaccines against caligid copepods (sea lice) based on antigens isolated from sea lice.

The circum-oral glands are putative exocrine glands related to the mouth parts of sea lice. Isolated proteins from circum-oral glands may provide a source of potential antigens for use

20 in vaccines against caligid copepods.

The present invention aims to provide alternative or improved vaccines and/or antigens or the treatment or prevention of caligid copepod infection in fish.

- 25 Accordingly, the present invention provides one or more isolated circum-oral gland (COG) protein for use for use in the treatment or prevention of caligid copepod infection in fish.

In embodiments of the invention, the or each protein is selected from the group consisting of: fructose bisphosphate aldolase (FBP); triosephosphate isomerase (TIM); peroxiredoxin-2

30 (Prx-2); enolase; and transitionally-controlled tumour protein homolog.

Transitionally controlled tumor protein homolog (TCTP)

TCTP is a highly conserved protein, expressed in all eukaryotic organisms. The protein sequence places it close to a family of small chaperone proteins and is often designated as a stress-related protein because TCTP expression is up-regulated during stress (Bommer and Thiele, 2004; Gnanasekar et al., 2009). For instance, TCTP can prevent hydrogen peroxide induced cell death (Nagano-Ito et al., 2009; 2012). The protein also functions in several cellular processes, such as cell growth, cell cycle progression, malignant transformation, and apoptosis (Boomer and Thiele, 2004). TCTP is also believed to have an extracellular cytokine-like function whereby it modulates the secretion of cytokines from mast cells, basophils, eosinophils, and T and B-lymphocytes (Boomer and Thiele, 2004; Sun et al., 2008). Parasites actively secrete TCTP proteins during host infection as part of their immune evasion strategy (Meyvis et al., 2009; Gnanasekar et al., 2002). Parasitic TCTP proteins have been shown to cause infiltration of eosinophils and/or histamine release from basophils (Bommer and Thiele, 2004; Gnanasekar et al., 2002). When TCTPs from *Brugia malayi* (Brug, 1927), a human filarial parasite, were injected intra-peritoneally into mice, an influx of eosinophils into the peritoneal cavity was observed suggesting filarial TCTP may play a role in allergic inflammatory responses in the host (Gnanasekar et al., 2002). In addition, intracellular expression of TCTP was shown to protect *B. malayi* against oxidative stress (Gnanasekar and Ramaswamy, 2007). The TCTP homolog from the parasite *Schistosoma mansoni* (Sambon, 1907), a human blood fluke, was shown to bind a variety of denatured proteins and protected the parasite from the effects of thermal shock (Gnanasekar et al., 2009). Knockdown of TCTP in *Caenorhabditis elegans* (Maupas, 1900), a free living nematode, using RNA interference resulted in the reduction in the number of eggs laid in the F₀ and F₁ generations by 90% and 72%, respectively, indicating the important role TCTP plays in reproduction (Meyvis et al., 2009). Interestingly, a TCTP from *Plasmodium* was shown to protect the parasite from the anti-malarial drug, artemisinin. Increased expression of TCTP correlated with increased resistance to the drug (Walker et al., 2000). These results suggest that the parasitic form of TCTP may be involved in certain pathological processes in the host.

Peroxiredoxin-2 (Prx-2)

Peroxiredoxins are a family of peroxidase proteins that are highly conserved and ubiquitously found in all living organisms. Their main role is to protect organisms from oxidative damage that can result from the generation of reactive oxygen species. 2-Cys peroxiredoxin produced

in *Fasciola gigantica* (Cobbold, 1855), a parasite of livestock, was shown to reduce hydrogen peroxide levels and provide protection from oxidative damage (Sangpairoj et al., 2014).

Some other proposed cellular functions include differentiation, apoptosis, and proliferation.

Protein characterization studies in the hard tick have shown that Prx is expressed in all life

stages of the parasite (Tsuji, Kamio et al. 2001). Using immunohistochemistry, Tsuji et al.

(2001) was able to show strong Prx reactivity in the salivary glands of *Haemaphysalis*

longicornis (tick). A DNA nicking assay showed *H. longicornis* recombinant Prx inhibits

oxidative nicking of plasmid DNA (Tsuji et al., 2001). When the larval secretory-excretory

antigens glyceraldehyde 3-phosphate dehydrogenase (G3PDH), a glycolytic enzyme, and Prx

of the human trematode parasite *S. mansoni* were administered subcutaneously with papain, an allergen that induces T-helper 2 mediated responses, worm burdens and worm egg load in the liver and small intestine of mice were reduced 60-78% (El Ridi et al., 2013).

Peroxiredoxin-2 secreted by *F. hepatica* and *S. mansoni* has been found to activate

alternatively activated macrophages and induce a Th2 driven inflammatory response leading

to an increase in IL-4, IL-5, and IL-13 secretion from naïve T helper cells (Donnelly et al., 2008).

Enolase

Enolase is a key glycolytic enzyme found in the cytoplasm of prokaryotic and eukaryotic cells that catalyzes the conversion of D-2-phosphoglycerate to phosphoenolpyruvate (PEP)

and water. It is highly conserved and one of the most abundantly expressed cytosolic proteins of organisms and requires magnesium ions (Mg^{2+}) to be enzymatically active (Diaz-Ramos et al., 2012).

There are three different isoforms of α , β , and γ . Alpha enolase is found in almost

all human tissues whereas β and γ are found in muscle and neuron and/or neuroendocrine

tissues, respectively (Diaz-Ramos et al., 2012). During cellular growth α -enolase is

significantly upregulated. It has been identified in hematopoietic cells such as T and B cells,

neuronal cells, monocytes, and endothelial cells as a plasminogen receptor (Diaz-Ramos et al., 2012).

Studies have also shown that α -enolase can act as a heat-shock protein and a

hypoxic stress protein. It is often referred to as a “moonlighting protein” because it has multiple functions at different cellular sites (Diaz-Ramos et al., 2012; Pal-Bhowmick et al., 2007). Enolase has been shown to bind plasmin in other parasitic models and aid in the invasion and migration within host tissues through its fibronolytic activity.

5

Triose phosphate isomerase (TIM a.k.a TPI)

Triose phosphate isomerase is a glycolytic enzyme that catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Furthermore, the interaction of TIM on the surface of parasites (e.g. with lamin and fibronectin) suggests it might be an important virulence factor (Pereira et al., 2007). For example, in *S. aureus*, TIM is displayed at the cell surface and acts as an adhesion molecule (Furuya et al. 2011). Its location outside the cell suggests it might be important in the adherence and invasion of host tissues. The mechanism(s) of protection are not yet fully understood, however, vaccination studies with a TIM DNA vaccine has proven to be protective against *S. japonicum* in a mouse model. Mice vaccinated with the TIM DNA vaccine observed worm and egg reduction rates of 30.2% and 52.9% compared to the control (Zhu et al., 2004).

Fructose biphosphate aldolase (FBP)

20

Fructose biphosphate aldolase is a highly conserved enzyme in the glycolytic pathway that catalyzes the reversible cleavage of fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Its primary importance is energy metabolism for all living things, but it also has been shown to induce strong humoral and cell mediated immune responses in parasitic infection models (McCarthy, Wieseman et al. 2002; Saber, Diab et al. 2013). For example, mice vaccinated with *Schistosoma mansoni* FBP DNA vaccine observed a significant reduction in worm burden and intestinal egg counts (Saber et al., 2013). Immunolocalization studies have also shown FBP aldolase is most highly expressed in metabolically active tissues and at all developmental stages of the parasite, *Onchocerca volvulus* (McCarthy et al., 2002).

30

In embodiments of the invention, the protein comprises the amino acid sequence of one or more of the group consisting of: SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4; SEQ ID NO:5; SEQ ID NO:6; and homologues thereof.

In all aspects of the present invention, “homologues” are sequences having at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or at least 99% identity to the recited sequence.

5

SEQ ID NO:1:

MPIKHIHARQIYDSRGNPTVEVDLTTERGIFRAAVPSGASTGVHEALELRDKDSTWHGKS
 GLKAVKKNVNDVLGPPELVKKNLDPVKQEEIDDFMISLDGTDNKSKEFGANSILGISMAVCKA
 10 GAAHKGVPPLYRHIADLAGVKEVMMPVPAFNVINGGSHAGNKLAMQEFMILPTGAPSFTEA
 MRMGSEIYHHLKALIKKKYGLDATAVGDEGGFAPNFQANGEAIDLLVGAIEKAGYTGKIK
 IGMDVAASEFYKNGKYDLDFKNEESKEADWLTSEALGEMYKGFIKDAPVISIEDPYDQDD
 WEGWTALTSQTDIQIVGDDLTVTNPKRIQMAVDKKSCNCLLLKVNQIGSVTESIRAHNLA
 KSNWGTMTVSHRSGETEDCFIADLVVGLCTGQIKTGAPCRSERLSKYNQLLRIEEELGSN
 15 AKYVGDKFRMPF

SEQ ID NO:2:

MMPVPAFNVINGGSHAGNKLAMQEFMILPTGAPSFTEAMRMGSEIYHHLKALIKKKYGLDAT
 20 AVGDEGGFAPNFQANGEAIDLLVGAIEKAGYTGKIKIGMDVAASEFYKNGKYDLDFKNEESK
 EADWLTSEALGEMYKGFIKDAPVISIEDPYDQDDWEGRTALTSQTDIQIVGDDLTVTNPKRI
 QMAVDKKSCNCLLLKVNQIGSVTESIRAHNLA KSNWGTMTVSHRSGETEDCFIADLVVGLCT
 GQIKTGAPCRSERLSKYNQLLRIEEELGSNAKYVGDKFRMPF

25 SEQ ID NO:3:

MGLEGIVPPGVITGDNLIKLFYCRDHKVALPAFNCTSSSTINAVLQAARDIKSPVIVQF
 SNGGA AFMAGKGIKNDGQKASVLGAIAGA QHVRLMAKHYGVPVVLHSDHCAKKLLPWFDG
 MLEADEEYFKQNGEPLFSSHMLDLSEEFDEENISTCAKYFTRMTKMKMWLEMEIGITGGE
 30 EDGVDNTNVKAESLYTKPEQVYNVYKTLSEIGPMFSIAAAF GNVHGVYKAGNVVLSPHLL
 ADHQYIKEQINSPLDKPAFLVMHGGSGSTREEIAEAVSNGVIKMNIDTDTQWAYWDGLR
 KFYEEKKEYLQGQVGNPEGADKPNKKFYDPRVWVRAAEESMIKRANESFESLNAVNVLGD
 SWKH

SEQ ID NO:4:

MSLQPTNDAPQFKAMAVVNKEFKEVSLKDYTGKYVVLFFYP LDFTFVCPTEIIAFGDRAA
 DFRKIGCEVLACSTD SHF SHLHWINTPRKEGGLGDM DIPLIADKNMEISRAYGVLKEDDG
 5 VSFRGLFIIDGTQKLRQITINDLPVGRCD ETLRLVQAFQYTDVHG EVC PAGWKPGKKSM
 KPSKEGVSSYLADAEQSKK

SEQ ID NO:5:

10 MGGGRKFFVGGNWKMN GDKKSIDGIVDFLSKGD LDPNCEVVVGASPCYLDYSRSKLPANI
 GVAAQNCYKVAKGAF TGEISPQMIKDVGC EWAILGHSERRNVFGESDELIGEKVAFAL ES
 GLKIIPCIGEKLDERESGKTEEVCFKQLKAISDKVSDWDLVVLAYEPVWAIGTGKTATPA
 QAQETHLALRKWLKENVSEEVSQKVRILYGGSVSAGNCKELGTQPDIDGFLVGGASLKPD
 FVQIINATK

15

SEQ ID NO:6:

MKIFKDVFSGDELFS DTYKFLLDDCLYEVY GKYVTRTEGDVVL DGANASAE EAMDDCDS
 SSTSGVDVVLNHR L VETGFGSKKDYTVYL KDYMKKVVTYLEENGKQAEVDTFKTNINKVM
 20 KELLPRFKDLQFY TGETMDPEAMIIMLEYKEVDGKDIPVLYFFKHGLNEEKF

In embodiments of the invention, the protein is a recombinant protein.

25 An aspect of the invention provides an antigen comprising one or more protein according to the invention.

30 An aspect of the invention provides a vaccine against caligid copepod infection in fish, the vaccine comprising an immunologically effective amount of one or more protein according to the invention, and a pharmaceutically-acceptable diluent or carrier, and optionally an adjuvant.

In embodiments of the invention, each of the one or more antigens is different from the other antigen or antigens in the vaccine.

In embodiments of the invention, the vaccine comprises five antigens, wherein one of the five antigens comprises FBP, one of the five antigens comprises TIM, one of the five antigens comprises Prx-2, one of the five antigens comprises enolase, and one of the five antigens comprises TCTP.

5

In embodiments of the invention, the vaccine comprises five antigens, wherein one of the five antigens comprises the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or homologues thereof, one of the five antigens comprises the amino acid sequence of SEQ ID NO:3 or homologues thereof, one of the five antigens comprises the amino acid sequence of
10 SEQ ID NO:4 or homologues thereof, one of the five antigens comprises the amino acid sequence of SEQ ID NO:5 or homologues thereof, and one of the five antigens comprises the amino acid sequence of SEQ ID NO:6 or homologues thereof.

15

In embodiments of the invention, the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.

In embodiments of the invention, the fish is a salmonid. In embodiments of the invention, the fish is a salmon or trout.

20

As aspect of the invention provides, the protein, antigen or vaccine according to the invention for use in the treatment or prevention of caligid copepod infection in fish.

In embodiments of the invention, the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.

25

In embodiments of the invention, the fish is a salmonid. In embodiments of the invention, the fish is a salmon or trout.

30

An aspect of the invention provides a polynucleotide comprising DNA encoding a protein isolated from the circum-oral gland (COG) or the frontal gland complex (FGC) of a caligid copepod.

In embodiments of the invention, the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.

In embodiments of the invention, the protein encoded by the polynucleotide is selected from the group consisting of: fructose bisphosphate aldolase (FBP); triosephosphate isomerase (TIM); peroxiredoxin-2 (Prx-2); enolase; and transitionally-controlled tumour protein

5 homolog (TCTP).

In embodiments of the invention, the polynucleotide according to the invention comprises DNA encoding the amino acid sequence of one or more of the group consisting of: SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4; SEQ ID NO:5; SEQ ID NO:6; and

10 homologues thereof.

In embodiments of the invention, the polynucleotide according to the invention comprises DNA comprising the nucleotide sequence of one or more of the group consisting of: SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ

15 ID NO:13; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; and homologues thereof.

SEQ ID NO:7:

ATGCCTATTAAACACATTCATGCACGTCAAATCTACGACTCTCGTGGTAACCCTACAGTGGA
20 GGTGGATCTCACCCTGAGCGAGGGATTTTCCGCGCTGCCGTCCCCAGTGGAGCTTCCACAG
GGGTTTCATGAGGCCCTGGAAGTGCAGCGACAAGGACTCTACCTGGCACGGGAAGAGTGGTCTC
AAGGCTGTGAAGAATGTGAACGACGTCCTTGGGCCCCGAGTTGGTGAAGAAGAACCCTTGACCC
CGTGAAGCAAGAGGAGATCGATGATTTTCATGATCAGCCTCGACGGGACGGATAACAAGAGCA
AATTTGGGGCTAATTCTATTTTGGGAATCTCGATGGCTGTGTGCAAGGCTGGTGCCGCCAC
25 AAGGGTGTTCCCCTCTACCGCCATATCGCTGACTTGGCGGGTGTGAAGGAAGTGATGATGCC
GGTGCCCGCATTTAATGTTCATTAACGGAGGTTCTCATGCTGGAAATAAGTTGGCGATGCAAG
AATTCATGATCCTTCCAAGTGGAGCTCCCTCCTTCACTGAAGCCATGAGGATGGGATCTGAA
ATCTATCACCATCTCAAGGCTCTTATCAAGAAGAAGTACGGGTTGGATGCTACAGCCGTTGG
AGATGAGGGTGGCTTTGCTCCCAACTTCCAAGCCAACGGCGAGGCTATCGACCTTCTTGTTG
30 GAGCCATTGAAAAGGCTGGATACACTGGAAAAATCAAGATCGGAATGGATGTTGCTGCTTCA
GAATTTTACAAAAATGGAAAGTACGATTTAGATTTCAAAAATGAAGAATCCAAGAGGCCGA
TTGGCTAACTTCCGAGGCTCTTGGTGAATGTACAAAGGATTCATCAAGGATGCACCTGTCA
TTTCCATTGAAGATCCCTACGATCAAGATGATTGGGAGGGATGGACTGCATTGACATCACAA
ACTGACATTGAGATTGTGCGAGATGATCTCACAGTCACAAACCCCAAGCGTATTCAAATG

GCTGTTGACAAGAAATCTTGCAACTGCCTCCTCTTGAAAGTAAATCAAATTGGTTCAGTAAC
TGAATCTATTTCGGGCCCCACAATCTTGCTAAGAGCAACGGCTGGGGTACCATGGTCTCTCATA
GATCTGGTGAGACAGAGGATTGTTTCATCGCTGATCTCGTCGTTGGTCTCTGCACTGGTCAA
ATCAAGACTGGAGCTCCTTGCAGATCCGAACGTTTGTCTAAATACAATCAATTGTTGCGTAT
5 TGAAGAGGAGTTGGGATCCAACGCTAAATATGTGCGGTGACAAGTTCAGAATGCCCTTTTAA

SEQ ID NO:8:

ATGCCGATTAAACACATCCATGCCCCGCCAAATCTATGACTCCCGTGGTAACCCGACCGTTGA
10 AGTTGACCTGACCACCGAACGTGGCATTTCCTGTCGCGCGGTGCCGAGCGGTGCATCTACGG
GTGTTTCATGAAGCTCTGGAAGTGCAGGATAAAGACTCAACCTGGCACGGCAAATCGGGTCTG
AAAGCGGTCAAAAACGTGAATGATGTTCTGGGCCCCGAACTGGTGAAGAAAAACCTGGACCC
GGTCAAAACAGGAAGAAATTGATGACTTTATGATCAGCCTGGATGGTACCGACAACAAATCTA
AATTCCGGCGCAAATAGTATTCTGGGTATCTCCATGGCAGTCTGTAAAGCTGGCGCAGCTCAT
15 AAAGGTGTGCCGCTGTATCGTCACATTGCGGATCTGGCCGGCGTCAAAGAAGTGATGATGCC
GGTTCCGGCCTTCAACGTCATTAATGGCGGTAGCCATGCAGGTAATAAACTGGCTATGCAGG
AATTTATGATTCTGCCGACCGGTGCCCCGTCATTACCGAAGCCATGCGCATGGGTTCGGAA
ATTTATCATCACCTGAAAGCGCTGATTAAGAAAAAATACGGCCTGGATGCAACGGCTGTTGG
TGACGAAGGCGGTTTTTGCCCCGAACTTCCAAGCGAATGGCGAAGCCATTGATCTGCTGGTTG
20 GTGCAATCGAAAAAGCTGGCTACACCGGTAAAATTAAAATCGGCATGGATGTCGCGGCCTCC
GAATTCTACAAAAACGGTAAATACGATCTGGACTTCAAAAATGAAGAAAGTAAAGAAGCGGA
TTGGCTGACCAGCGAAGCCCTGGGCGAAATGTACAAAGGTTTCATCAAAGATGCCCCGGTGA
TTAGCATCGAAGATCCGTACGACCAGGATGACTGGGAAGGCTGGACCGCACTGACGTCTCAG
ACCGATATTCAAATCGTGGGTGATGACCTGACCGTTACGAACCCGAAACGTATCCAGATGGC
25 GGTTGATAAAAAATCTTGCAACTGTCTGCTGCTGAAAGTCAATCAAATTGGCTCAGTGACCG
AATCGATCCGTGCGCATAACCTGGCCAAATCTAATGGCTGGGGTACGATGGTGTCTCACCGC
TCCGGCGAAACCGAAGATTGCTTCATTGCAGACCTGGTGGTTGGCCTGTGTACGGGTGAGAT
CAAAACCGGTGCTCCGTGCCGTAGCGAACGCCTGTCTAAATATAATCAACTGCTGCGCATCG
AAGAAGAACTGGGTAGCAATGCGAAATATGTGGGTGATAAATTCCGTATGCCGTTT

30

SEQ ID NO:9:

ATGGGTCTTGAAGGAATTGTTCCCCCTGGTGTCACTGGAGACAATCTTATTAAGTTGTT
CGAATACTGCAGAGACCATAAAGTTGCTCTCCCTGCTTTCAACTGCACGTCTTCTTCAACCA

TCAATGCAGTTTTGCAAGCAGCACGGGACATTAAATCCCCTGTGATTGTTCAATTTTCCAAT
GGTGGAGCTGCTTTTATGGCCGGCAAAGGCATCAAAAATGACGGTCAAAGGCTAGTGTCTC
TGGTGCAATTGCTGGGGCTCAACATGTTTCGTTTAAATGGCAAAGCACTATGGTGTTCCTGTAG
TTCTTCACTCTGATCACTGTGCTAAAAAACTCCTCCCATGGTTTGATGGAATGCTTGAAGCT
5 GATGAAGAGTATTTCAAACAAAATGGTGAACCTCTTTTCTCCAGTCACATGCTTGATCTCTC
GGAGGAGTTTGATGAAGAAAATATTTCCACTTGTGCAAAATATTTTACTCGCATGACTAAAA
TGAAAATGTGGTTAGAAATGGAAATTGGAATCACTGGGGGCGAAGAGGATGGTGTGACAAT
ACCAATGTGAAAGCGGAGTCTCTTTACACCAAACCCGAACAAGTTTACAACGTGTACAAAAC
ACTCAGCGAAATTGGACCAATGTTTTCCATTGCTGCCGCTTTTGGAACGTACATGGTGTAT
10 ACAAGGCAGGTAACGTTGTTCTTTCCCCACATTTGTTGGCTGATCATCAAAAATACATCAAG
GAGCAAATTAACCTCCCACTTGATAAACCCGCCTTCCTTGTCATGCACGGAGGCTCCGGCTC
CACCAGAGAAGAAATTGCTGAAGCAGTAAGCAACGGTGTGATCAAAATGAATATTGATACGG
ATACTCAATGGGCTTACTGGGATGGTCTCAGAAAGTTTTATGAAGAAAAGAAGGAGTATCTT
CAAGGACAGGTTGGAAATCCAGAAGGCGCTGACAAGCCAAACAAAAAGTTTTACGATCCACG
15 AGTTTGGGTTTCGTGCTGCTGAGGAGTCTATGATTAAGAGAGCCAATGAATCCTTTGAATCAT
TAAACGCTGTGAATGTCCTTGGTGACTCCTGGAAACACTAA

SEQ ID NO:10:

20 ATGGGTCTGGAAGGCATCGTTCCGCCGGGTGTCATTACGGGTGATAACCTGATTAACTGTT
CGAATACTGCCGCGACCACAAAGTGGCACTGCCGGCTTTTAACTGCACCAGCTCTAGTACGA
TTAATGCAGTGCTGCAGGCGGCCCCGTGATATTAAATCTCCGGTTATCGTCCAATTTAGTAAC
GGCGGTGCAGCTTTCATGGCGGGCAAAGGTATTAAAAATGATGGCCAGAAAGCCTCCGTTCT
GGGCGCCATCGCAGGTGCTCAACATGTTTCGCCTGATGGCCAAACACTATGGTGTCCCGGTGG
25 TTCTGCATTCTGATCACTGCGCGAAAAAACTGCTGCCGTGGTTCGATGGCATGCTGGAAGCC
GACGAAGAATACTTTAAACAGAACGGTGAACCGCTGTTCTCCTCACACATGCTGGATCTGTC
GGAAGAATTTGACGAAGAAAATATCAGCACCTGTGCGAAATATTTACCCGTATGACGAAAA
TGAAAATGTGGCTGGAAATGGAAATTGGCATCACGGGCGGTGAAGAAGATGGTGTGACAAC
ACCAATGTGAAAGCCGAAAGCCTGTATACGAAACCGGAACAGGTCTATAACGTGTACAAAAC
30 CCTGTCCGAAATTGGCCCGATGTTTTCAATCGCGGCCGCATTCCGCAACGTTTCATGGTGTCT
ATAAAGCCGGTAATGTCGTGCTGTCTCCGCATCTGCTGGCTGATCACCAGAAATACATCAAA
GAACAAATCAACAGTCCGCTGGACAAACCGGCGTTTCTGGTGTGATGCATGGCGGTTCCGGTAG
CACCCGTGAAGAAATTGCGGAAGCCGTGAGCAACGGTGTATTAAAAATGAATATCGATACCG
ACACGCAGTGGGCATATTGGGATGGCCTGCGCAAATTCTACGAAGAAAAGAAAGAATACCTG

CAGGGCCAAGTTGGTAACCCGGAAGGTGCTGATAAACCGAATAAAAAATTCTATGACCCGCG
 TGTGTGGGTTTCGTGCTGCCGAAGAAAGTATGATCAAACGCGCTAACGAATCCTTTGAATCCC
 TGAACGCAGTGAATGTGCTGGGTGACAGTTGGAAACAC

5 SEQ ID NO:11:

ATGAGTCTTCAACCAACGAATGATGCTCCTCAATTCAAGGCTATGGCCGTTGTGAACAAGGA
 ATTCAAGGAGGTGTCACTCAAGGACTATACCGGCAAATACGTGGTTCTCTTTTTCTACCCCT
 TGGACTTTACCTTTGTTTGCCCCACAGAAATCATTGCCTTTGGAGATCGGGCTGCAGATTTC
 10 CGTAAAATTGGATGTGAGGTCCTTGCCTGCTCCACTGACTCCCATTTTTCTCATCTCCACTG
 GATCAACACTCCTCGTAAGGAGGGAGGACTTGGGGACATGGACATTCCTTCATTGCGGATA
 AGAACATGGAAATTTCTAGAGCCTATGGCGTGCTCAAGGAAGACGATGGAGTGTCTTCAGA
 GGACTTTTCATCATTGACGGCACTCAGAACTCCGTCAAATCACCATCAATGATCTTCCTGT
 CGGAAGATGCGTAGACGAAACCTTAAGACTTGTACAAGCCTTCCAATACACGGACGTGCATG
 15 GCGAGGTTTGCCCTGCGGGATGGAAGCCAGGAAAGAAGTCTATGAAGCCCAGCAAGGAAGGT
 GTCTCATCTTACCTCGCAGATGCTGAACAATCAAAGAAATAA

SEQ ID NO:12:

ATGTCACTGCAACCGACGAACGACGCCCCGCAATTCAAAGCAATGGCAGTGGTTAACAAAGA
 ATTCAAAGAAGTTTTCGCTGAAAGATTACACCGGCAAATACGTCTGTGCTGTTTTTCTATCCGC
 TGGACTTTACCTTCGTCTGCCCCGACGGAATTATCGCATTGCGGATCGTGCGGCCGACTTC
 CGCAAAATTGGTTGCGAAGTGCTGGCTTGTAGCACCGATTCTCATTTTCAGTCATCTGCACTG
 GATCAACACGCCGCGTAAAGAAGGCGGTCTGGGCGATATGGACATTCGCTGATCGCAGATA
 25 AAAATATGGAAATTTCCCGCGCTTATGGTGTCTGAAAGAAGATGACGGCGTGTCTTTTCGT
 GGTCTGTTCATTATCGACGGCACCCAGAACTGCGCCAAATTACGATCAATGATCTGCCGGT
 TGGTCGTTGCGTCGACGAAACCCTGCGCCTGGTTTCAGGCGTTTCAATACACGGATGTGCACG
 GTGAAGTTTGTCCGGCCGGCTGGAAACCGGGTAAAAAATCTATGAAACCGTCAAAGAAGGC
 GTGTCGTCCTACCTGGCAGATGCTGAACAATCCAAAAA

30

SEQ ID NO:13:

ATGGGTGGAGGAAGAAAATTTTTCGTTGGTGGAACTGGAAAATGAATGGAGACAAGAAATC
 TATTGATGGAATCGTAGATTTTTTGTAGCAAGGGGGATTGGACCCAAATTGTGAGGTTGTTG

TTGGAGCCTCACCTGCTATTTGGACTATTCCCGTTCTAAACTTCCTGCCAATATCGGAGTG
GCTGCACAAAATTGTTATAAGGTGGCCAAAGGAGCATTTACCGGAGAAATCAGTCCTCAAAT
GATTAAAGATGTTGGTTGTGAATGGGCGATTCTTGGTCATTCAGAGCGTAGAAATGTCTTTG
GGGAATCTGATGAGCTCATTGGCGAAAAGGTTGCTTTTGCACCTTGAGTCTGGTCTCAAAATT
5 ATTCATGCATTGGAGAAAAATTAGACGAACGTGAATCTGGGAAGACTGAGGAGGTCTGCTT
TAAGCAACTTAAAGCCATTTCTGACAAAGTATCTGATTGGGATCTTGTCTCTTAGCTTATG
AACCAGTTTGGGCCATTGGAACCTGGCAAAACAGCTACACCTGCTCAGGCTCAAGAAACACAT
CTTGCTCTTCGTAAATGGCTAAAGGAGAACGTTTCTGAGGAAGTTTCACAAAAAGTGCGAAT
CCTCTATGGAGGTTCCGTGAGTGCTGGTAATTGCAAGGAACTTGGCACTCAGCCTGATATTG
10 ACGGCTTCCTTGTTGGAGGAGCCTCTCTCAAACCTGACTTTGTTCAAATCATCAACGCTACT
AAGTAA

SEQ ID NO:14:

15 ATGGGCGGCGGTGCGAAATTCTTTGTCGGCGGCAACTGGAAAATGAACGGCGATAAAAAATC
TATCGATGGTATCGTGGATTTTCTGAGCAAAGGCGATCTGGATCCGAATTGCGAAGTGGTTG
TGGGTGCGAGCCCGTGTTATCTGGATTACAGCCGTTCTAAACTGCCGGCAAACATTGGTGTG
GCCGCACAGAATTGCTATAAAGTTGCGAAAGGCGCCTTCACCGGTGAAATTAGCCCGCAGAT
GATCAAAGATGTTGGCTGTGAATGGGCAATTCTGGGTCAATTCTGAACGTCGCAACGTGTTTG
20 GCGAAAGTGATGAACTGATCGGTGAAAAAGTTGCATTTCGCGCTGGAAAGCGGCCTGAAAATT
ATCCCGTGTCATCGGTGAAAAACTGGATGAACGCGAATCTGGTAAAACGGAAGAAGTGTGTTT
TAAACAGCTGAAAGCCATTTCTGATAAAGTTAGTGATTGGGATCTGGTTGTGCTGGCGTATG
AACCGGTGTGGGCGATTGGTACCGGTAAAACCGCAACGCCGGCACAGGCACAGGAAACCCAC
CTGGCACTGCGTAAATGGCTGAAAGAAAACGTTAGCGAAGAAGTGTCTCAGAAAGTTTCGCAT
25 TCTGTACGGCGGTAGTGTTAGCGCGGGCAATTGCAAAGAACTGGGTACCCAGCCGGATATCG
ATGGCTTCCTGGTGGGTGGTGCTTCCCTGAAACCGGACTTTGTGCAGATTATCAACGCTACG
AAA

SEQ ID NO:15:

30 ATGAAGATCTTTAAGGACGTATTTTCTGGAGATGAATTATTTTCCGACACCTACAAGTTCAA
GTTGTTGGATGATTGCTTGTACGAGGTGTATGGAAAGTATGTCACACGGACTGAAGGAGATG
TGTTTCTTGATGGAGCCAACGCATCTGCTGAAGAGGCCATGGATGACTGTGATTCTCTTCC
ACCTCTGGTGTGATGTTGTCCTTAACCACCGTCTGGTCGAAACTGGGTTCGGTTCCAAGAA

GGACTACACCGTATACCTTAAGGACTACATGAAGAAGGTAGTGACATATTTAGAAGAAAATG
 GCAAACAAGCCGAAGTAGATACCTTCAAGACCAACATCAACAAGGTCATGAAGGAACTTTTA
 CCACGGTTTTAAGGATCTTCAATTCTATACTGGAGAAACGATGGACCCTGAGGCCATGATCAT
 CATGCTTGAATACAAGGAAGTTGATGGAAAGGATATTCCCGTCCTCTACTTTTTTAAACATG
 5 GATTAAATGAAGAAAAATTTTAA

SEQ ID NO:16:

ATGAAAATCTTCAAAGACGTGTTTAGCGGCGACGAACTGTTCTCGGATACCTACAAATTTAA
 10 ACTGCTGGATGATTGCCTGTATGAAGTGTACGGCAAATATGTTACCCGTACGGAAGGCGATG
 TGGTTCTGGATGGTGCGAACGCCAGCGCAGAAGAAGCGATGGATGATTGTGATAGCTCTAGT
 ACCTCTGGTGTGGATGTGGTTCTGAATCATCGCCTGGTTGAAACCGGCTTTGGTAGCAAGAA
 AGATTACACGGTGTATCTGAAAGATTACATGAAGAAAGTGGTTACGTATCTGGAAGAAAACG
 GCAAACAGGCGGAAGTGGATACCTTCAAAACGAACATCAACAAAGTTATGAAAGAAGTCTGCTG
 15 CCGCGTTTTAAAGATCTGCAGTTCTACACCGGTGAAACGATGGATCCGGAAGCCATGATTAT
 CATGCTGGAATATAAAGAAGTTGATGGCAAAGACATTCCGGTGCTGTACTTCTTCAAACACG
 GCCTGAACGAAGAAAAATTC

In embodiments of the invention, the DNA is cDNA.

20

An aspect of the invention provides an antigen comprising the polynucleotide according to the invention.

An aspect of the invention provides a vaccine against caligid copepod infection in fish, the
 25 vaccine comprising an immunologically effective amount of one or more polynucleotides according to the invention, or one or more antigen according to the invention, a pharmaceutically-acceptable diluent or carrier, and optionally an adjuvant.

In an embodiment of the invention, the vaccine comprises an immunologically effective
 30 amount of a combination of two or more antigens, wherein each of the one or more antigens independently comprises the DNA sequence selected from the group consisting of: SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; and homologues thereof.

In an embodiment of the invention, the one or more antigens is different from the other antigen or antigens in the vaccine.

5 In an embodiment of the invention, the vaccine comprises five antigens, wherein one of the five antigens comprises the DNA sequence of SEQ ID NO:7 or SEQ ID NO:8 or homologues thereof, one of the five antigens comprises the DNA sequence of SEQ ID NO:9 or SEQ ID NO:10 or homologues thereof, one of the five antigens comprises the DNA sequence of SEQ ID NO:11 or SEQ ID NO:12 or homologues thereof, one of the five antigens comprises the DNA sequence of SEQ ID NO:13 or SEQ ID NO:14 or homologues thereof, and one of the
10 five antigens comprises the DNA sequence of SEQ ID NO:15 or SEQ ID NO:16 or homologues thereof.

In an embodiment of the invention, the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.
15

In an embodiment of the invention, the fish is a salmonid. In an embodiment of the invention, the fish is a salmon or trout.

An aspect of the invention provides, the polynucleotide, antigen or vaccine according to the
20 invention for use in the treatment or prevention of caligid copepod infection in fish.

In an embodiment of the invention, the caligid copepod infection is a *Lepeophtheirus salmonis* or *Caligus rogercresseyi* infection.

25 In an embodiment of the invention, the fish is a salmonid. In an embodiment of the invention, the fish is a salmon or trout.

An aspect of the invention provides, a method of treatment or prevention of caligid copepod infection in fish, comprising administering a therapeutic amount of the protein,
30 polynucleotide, antigen, or vaccine of any one previous claim, optionally with the co-administration of an adjuvant.

In an embodiment of the invention, the caligid copepod infection is a *Lepeophtheirus salmonis* or *Caligus rogercresseyi* infection.

In an embodiment of the invention, the fish is a salmonid. In an embodiment of the invention, the fish is a salmon or trout.

- 5 The skilled person will appreciate that the claimed invention includes in its scope for the purposes of determining infringement variants of the claimed features that achieve substantially the same result in substantially the same way as the invention.

10 The invention will now be described by way of example with reference to the drawings in which:

Fig. 1 shows ELISA results for Atlantic salmon serum antibody response to TIM antigen with DNA antigen prime and protein boost;

- 15 Fig. 2 shows ELISA results for Atlantic salmon serum antibody response to TCTP antigen with DNA antigen prime and protein boost;

20 Fig. 3 shows ELISA results for Atlantic salmon serum antibody response to peroxiredoxin-2 antigen with DNA antigen prime and protein boost;

Fig. 4 shows ELISA results for Atlantic salmon serum antibody response to enolase antigen with DNA antigen prime and protein boost;

- 25 Fig. 5 shows ELISA results for Atlantic salmon serum antibody response to fructose biphosphate antigen with DNA antigen prime and protein boost;

Fig. 6 shows ELISA results for Atlantic salmon serum antibody response to TIM antigen with protein antigen prime and protein boost;

- 30 Fig. 7 shows ELISA results for Atlantic salmon serum antibody response to TCTP antigen with protein antigen prime and protein boost;

Fig. 8 shows ELISA results for Atlantic salmon serum antibody response to peroxiredoxin-2 antigen with protein antigen prime and protein boost;

Fig. 9 shows ELISA results for Atlantic salmon serum antibody response to enolase antigen with protein antigen prime and protein boost; and

- 5 Fig. 10 shows ELISA results for Atlantic salmon serum antibody response to fructose biphosphate antigen with protein antigen prime and protein boost.

For each of Figs 1 to 5, the data show the average absorbance at 450 nm. PBS is a vehicle control. DM1 (delivery method 1) is a vaccine prime using a cocktail of five DNA antigens
 10 (10 µg) with vaccine boost using cocktail of five recombinant proteins (50 µg). DM1 ctrl (delivery method 1 control) is a “prime” of DNA vaccine comprising empty pVAX1 vector (10 µg) with vaccine boost using mCherry recombinant protein (50 µg).

For each of Figs 6 to 10, the data show the blood serum IgM antibody response against the
 15 stated recombinant protein in individual Atlantic salmon parr vaccinated with the cocktail vaccine (DM2 cocktail), negative control fluorescent protein (DM2 ctrl) or no vaccination control (no vac ctrl) by delivery method 2 at 602 degree days post-vaccination at 14°C. Absorbance at 450nm shown for individual fish (circles, squares or triangles) with line
 indicating mean ± SEM (n = 12 fish per group). DM2 cocktail (delivery method 2 cocktail) is
 20 a vaccine prime using a cocktail of five recombinant antigens (50 µg) with vaccine boost using cocktail of five recombinant proteins (50 µg). DM2 ctrl (delivery method 2 control) is a “prime” using mCherry-His recombinant protein (250 µg) plus flagellin (50 ng) with vaccine boost using mCherry-His recombinant protein (250 µg).

25 Examples

Example 1 – Isolation of candidate antigen peptides from circum-oral glands

The circum-oral glands (COGs) were visualized in *L. salmonis* at chalimus stages using 3,3'-
 30 diaminobenzidine tetrahydrochloride (DAB). COGs were isolated by microdissection and transferred into microcentrifuge tubes containing protease inhibitor cocktail (AEBSF [4-(2-aminoethyl) benzenesulfonyl fluoride] at 2 mM, Aprotinin at 0.3 µM, Bestatin at 116 µM, E-64 at 14 µM, Leupeptin at 1 µM and EDTA at 1 mM in 100 ml stock solution; Sigma-Aldrich Cat. No. P2714) at a 1 to 10 dilution in cold, sterile crustacean Ringers saline. Ringers saline

was prepared by dissolving 0.58 M sodium chloride, 0.013 M potassium chloride, 0.013 M calcium chloride, 0.026 M magnesium chloride, 0.00054 M disodium hydrogen phosphate in 0.05M Tris-HCl, pH 7.5. Tissue was homogenised for two minutes at a frequency of 28 hertz using a TissueLyser II (Qiagen) by adding 100 µl 0.5 mm glass beads (BioSpec Products, catalog number 11079105) to 100 µl of sample. The supernatant was collected by centrifuging homogenate at 10,000 x g for 30 minutes at 4°C. Protein concentration was determined using a BCA protein assay kit (Pierce Cat. No. 23227). The COG supernatant yielded 610 µg of protein. Samples were stored at -80°C.

- Protein samples were concentrated with a 3K MWCO concentrator (Pierce) following manufacturer's instructions, and run on a SDS-PAGE gel. Gel slices containing proteins at 40 and 25 kDa were then analysed by nano-LC MS/MS.

Five proteins identified by the nano-LC MS/MS analysis were selected as candidate antigens: fructose biphosphate aldolase (FBP; Hu et al., 2015; Lorenzatto et al., 2012); triosephosphate isomerase (TIM; Furuya et al. 2011; Saramago et al., 2012); peroxiredoxin-2 (Prx-2; Knoops et al., 2016; Rhee et al., 2016; Wood et al., 2003); enolase (Diaz-Ramos et al, 2012; Wang et al., 2013); and transitionally-controlled tumour protein homolog (TCTP; Gnanasekar et al., 2009; Gnanasekar and Ramaswamy, 2007; Sun et al., 2008; Nagano-Ito et al., 2009 and 2012).

Example 2 – Production of recombinant vaccines from circum-oral glands peptides

The glycosylation of our protein targets was examined using NetNGlyc 1.0.

- The server identified one potential N-linked glycosylation site for both FBP and TIM.

NetOGlyc 4.0 software identified two potential O-linked glycosylation sites for Prx-2.

- Protein sequencing results from the nano-LC MS/MS analysis were used to blast NCBI database to obtain the complete mRNA coding sequence. As a quality control measure, the NCBI mRNA sequences of the targets were validated by performing RACE cDNA synthesis. To perform RACE cDNA synthesis, cDNA was prepared from RNA collected from 10 adult sea lice (RNeasyR Mini kit (Qiagen)). 5' and 3'-RACE-Ready cDNA was prepared using a SMARTer RACE 5'/3' cDNA synthesis kit (TaKaRa) for rapid amplification of cDNA ends.

Primers were specially designed for each protein to ensure amplification of the 5' end (5' RACE PCR) or 3' end (3' RACE PCR) of the mRNA (see Table 1 for list of primers used). PCR products were gel extracted using the NucleoSpin Gel and PCR clean up kit (Clontech).

5 *Table 1 – RACE primers*

Protein	5' RACE cDNA	3' RACE cDNA
FBP	GATTACGCCAAGCTTCAGCGCC TTCTGGATTTC CAACC (SEQ ID NO:36)	GATTACGCCAAGCTTGGCTCCAC CAGAGAAGAAATTGCTGAAG (SEQ ID NO:37)
Enolase	GATTACGCCAAGCTTAGTG CAG AGACCAACGACGAGATCAGCG (SEQ ID NO:34)	GATTACGCCAAGCTTGCCGTTGG AGATGAGGGTGGCTTTGCTC (SEQ ID NO:35)
TIM	GATTACGCCAAGCTTACGCTCT GAATGACCAAGAATCGCCCAT (SEQ ID NO:40)	GATTACGCCAAGCTTGAGGTTGT TGTTGGAGCCTCACCCCTGC (SEQ ID NO:41)
TCTP	GATTACGCCAAGCTTGGAACCG AACCCAGTTTCGACCAGACG (SEQ ID NO:42)	GATTACGCCAAGCTTCTGCTGAA GAGGCCATGGATGACTGTGA (SEQ ID NO:43)
Prx-2	GATTACGCCAAGCTTACACTCC ATCGTCTTCCTTGAGCAGGCC (SEQ ID NO:38)	GATTACGCCAAGCTTGGTCCTTG CCTGCTCCACTGACTCCCAT (SEQ ID NO:39)

- In-Fusion cloning of RACE products was then performed following the manufacturer's instructions. Single colonies (8-10) were isolated from culture plates and grown overnight in selective media (ampicillin) at 37°C with shaking (~180 rpm). Plasmid DNA was isolated from bacterial lysates using a QIAprep Spin Miniprep Kit following the manufacturer's instructions (Qiagen). To determine which clones contained our RACE insert, we analyzed the DNA by restriction digest using EcoRI and HindIII which flank the cloning site. Digested products were visualized on a 1% ethidium bromide gel.
- 15 Clones containing the largest gene specific inserts were sequenced. The mRNA sequencing results are shown in below (coding region underlined):

Enolase mRNA (SEQ ID NO:17):

GCTCCGATTCACTTCTTATTTCTCAACGCTCATCGATACTTTATAAGGCTCAAATTCAAAAT
GCCTATTAAACACATTTCATGCACGTCAAATCTACGACTCTCGTGGTAAACCCTACAGTGGAGG
5 TGGATCTCACCCTGAGCGAGGGATTTTCCGCGCTGCCGTCCCCAGTGGAGCTTCCACAGGG
GTTTCATGAGGCCCTGGAAGTGC GCGACAAGGACTCTACCTGGCACGGGAAGAGTGGTCTCAA
GGCTGTGAAGAATGTGAACGACGTCCTTGGGCCCCGAGTTGGTGAAGAAGAACCCTTGACCCCG
TGAAGCAAGAGGAGATCGATGATTTTCATGATCAGCCTCGACGGGACGGATAACAAGAGCAAA
TTTGGGGCTAATTCTATTTTGGGAATCTCGATGGCTGTGTGCAAGGCTGGTGCCGCCACAA
10 GGGTGTTCCTCTACCGCCATATCGCTGACTTGGCGGGTGTGAAGGAAGTGATGATGCCGG
TGCCCGCATTTAATGTCATTAACGGAGGTTCTCATGCTGGAAATAAGTTGGCGATGCAAGAA
TTCATGATCCTTCCAAGTGGAGCTCCCTCCTTCACTGAAGCCATGAGGATGGGATCTGAAAT
CTATCACCATCTCAAGGCTCTTATCAAGAAGAAGTACGGGTTGGATGCTACAGCCGTTGGAG
ATGAGGGTGGCTTTGCTCCCAACTTCCAAGCCAACGGCGAGGCTATCGACCTTCTTGTTGGA
15 GCCATTGAAAAGGCTGGATACACTGGAAAAATCAAGATCGGAATGGATGTTGCTGCTTCAGA
ATTTTACAAAAATGGAAAGTACGATTTAGATTTCAAAAAATGAAGAATCCAAAGAGGCCGATT
GGCTAACTTCCGAGGCTCTTGGTGAATGTACAAAGGATTCATCAAGGATGCACCTGTCATT
TCCATTGAAGATCCCTACGATCAAGATGATTGGGAGGGATGGACTGCATTGACATCACAAAC
TGACATTCAGATTGTCGGAGATGATCTCACAGTCACAAACCCCAAGCGTATTCAAATGGCTG
20 TTGACAAGAAATCTTGCAACTGCCTCCTCTTGAAAGTAAATCAAATTGGTTCAGTAACTGAA
TCTATTTCGGGCCCAATCTTGCTAAGAGCAACGGCTGGGGTACCATGGTCTCTCATAGATC
TGGTGAGACAGAGGATTGTTTCATCGCTGATCTCGTCGTTGGTCTCTGCACTGGTCAAATCA
AGACTGGAGCTCCTTGCAGATCCGAACGTTTGTCTAAATACAATCAATTGTTGCGTATTGAA
GAGGAGTTGGGATCCAACGCTAAATATGTCGGTGACAAGTTCAGAATGCCCTTTTAATGATC
25 TAAAGGGTTGTTTCTTCATTGAAGAAAGTTCATTTCTATAGTCACAATAAATTATTTTCATGG
TTTTACAAGAAATTCACAGGACGAAAAACAAAAATCTTAATTTATTGAATTATTTCTATAT
GTATTACACGCGTACTCTAAGTAAAACCTTATAAAGGAATATAATTGTAATATAATTTATTG
TAATATTTTTTTTTTCATATTTAATTTATATTAAGGGTTGCCATTTAAATATATAAATTTCCC
CGTTGGTAAAAAAAAAAAA

30

FBP aldolase mRNA (SEQ ID NO:18):

GGGGGGAGTTAGTATAAGAGATCGACAGGCTCTGTTTCGCAACACTTGTTTCCTAAAGGCCAAAT
TATCTTAAATCTTAAAAATGGGTCTTGAAGGAATTGTTCCCCCTGGTGTGCATCACTGGAGAC
5 AATCTTATTAAGTTGTTTCGAATACTGTAGAGACCATAAAGTTGCTCTCCCTGCTTTCAACTG
CACGTCTTCTTCAACCATCAATGCAGTTTTTGCAAGCAGCACGGGACATTAAATCCCCTGTGA
TTGTTCAATTTTCCAATGGTGGAGCTGCTTTTATGGCCGGCAAAGGCATCAAAAATGACGGT
CAAAAGGCTAGTGTCTTGGTGCAATTGCTGGGGCTCAACATGTTCTTTAATGGCAAAGCA
CTATGGTGTTCCTGTAGTTCTTCACTCTGATCACTGTGCTAAAAAACTCCTCCCATGGTTTG
10 ATGGAATGCTTGAAGCTGATGAAGAGTATTTCAAACAAAATGGTGAACCTCTTTTCTCCAGT
CACATGCTTGATCTCTCGGAGGAGTTTGATGAAGAAAATATTTCCACTTGTCAGAAAATATTT
TACTCGCATGACTAAAATGAAAATGTGGTTAGAAATGGAAATTGGAATCACTGGGGGCGAAG
AGGATGGTGTGACAATACCAATGTGAAAGCGGAGTCTCTTTACACCAAACCCGAACAAGTT
TACAACGTGTACAAAACACTCAGCGAAATTGGACCAATGTTTTCCATTGCTGCCGCTTTTGG
15 AAACGTACATGGTGTATACAAGGCAGGTAACGTTGTTCTTTCCCCACATTTGTTGGCTGATC
ATCAAAAATACATCAAGGAGCAAATTAATCTCCCACTTGATAAACCCGCCTTCCTTGTCATG
CACGGAGGCTCCGGCTCCACCAGAGAAGAAATTGCTGAAGCAGTAAGCAACGGTGTGATCAA
AATGAATATTGATACGGATACTCAATGGGCTTACTGGGATGGTCTCAGAAAGTTTTATGAAG
AAAAGAAGGAGTATCTTCAAGGACAGGTTGGAAATCCAGAAGGCGCTGACAAGCCAAACAAA
20 AAGTTTTACGATCCACGAGTTTGGGTTCGTGCTGCTGAGGAGTCTATGATTAAGAGAGCCAA
TGAATCCTTTGAATCATTAACGCTGTGAATGTCCTTGGTGACTCCTGGAAACACTAAATAC
TTATTATTGGATATTCAGAATGTTTTAATTTCTATTTTGGAACTCCGAACTTACTAGTAATT
TATTTCTCTTTTAAAAAATGAATCAGTATATTTATTATTCTGTTTATAAAATTAAGTTATTG
TTAATTTCTTTAAATTTATTTATCAAAAATTAGAAATTGTTATACATGAAACATTGACATAA
25 ATCTAAAATTGAAACATTTTATGATTTTGTGTTTATAAATGCTAGATAAGAAGTCATAAAT
AAATGTATAATAAATTAACCTTCTTTCGTGATTAATTAACCTTGCTAATTAATGCATAATTTT
CATTTTTTTGAAGATATGCGCTAAAAAATTATTCAATAAAAAATTAAAATAG

PRX-2 mRNA (SEQ ID NO:19):

30 GGGGGAGTCTTATATCTGCTACCGGCAAGTGAACCTACCTCTGTCATCTCTCTTTGTAATATC
CGACTAAGTAACAAAATGAGTCTTCAACCAACGAATGATGCTCCTCAATTCAAGGCTATGGC
CGTTGTGAACAAGGAATTCAAGGAGGTGTCACTCAAGGACTATACCGGCAAATACGTGGTTCT
TCTTTTTCTACCCCTTGGACTTTACCTTTGTTTGCCCCACAGAAATCATTGCCTTTGGAGAT

CGGGCTGCAGATTTCCGTAAAATTGGATGTGAGGTCCTTGCCTGCTCCACTGACTCCCATT
 TTCTCATCTCCACTGGATCAACACTCCTCGTAAGGAGGGAGGACTTGGGGACATGGACATTC
 CCCTCATTGCGGATAAGAACATGGAAATTTCTAGAGCCTATGGCGTGCTCAAGGAAGACGAT
 GGAGTGTCTTCAGAGGACTTTTCATCATTGACGGCACTCAGAACTCCGTCAAATCACAAAT
 5 CAATGATCTTCCTGTCTGGAAGATGCGTAGACGAAACCTTAAGACTTGTACAAGCCTTCCAAT
 ACACAGACGTGCATGGCGAGGTTTGCCCTGCGGGATGGAAGCCAGGAAAGAAGTCTATGAAG
 CCCAGCAAGGAAGGTGTCTCATCTTACCTCGCAGATGCTGAACAATCAAAGAAATAATACAG
 AAGATCTCCCCTGTAGTTATTAGTTTCCATACCAATTCTCTCTTTTAATTCATTGATTGGA
 CACTGTTACCATGTTCCACTTTTTAATTGTACCTGGTCAGTCAGTGCCCAAGGTCATTGATT
 10 GATTAAAGTCTATCAAATATTTATGTATTCCCCGGTGTACTAATAGTTTTTAAGATATAAAAT
 ATACGACTTTTTAATATATT

TIM mRNA (SEQ ID NO:20):

15 GGGGGAGTTATAAAGCACTACTCGATTGCTAAGTACTTCGCGAGGTTCTACTAATTGTAAT
 ATAGTTGAAAAAATACATTCAAAAATGGGTGGAGGAAGAAAATTTTTCGTTGGTGGAACTG
 GAAAATGAATGGAGACAAGAAATCTATTGATGGAATCGTAGATTTTTTGAGCAAGGGGGATT
 TGGACCCAAATTGTGAGGTTGTTGTTGGAGCCTCACCTGCTATTTGGACTATTCCCGTTCT
 AAACCTTCCTGCCAATATCGGAGTGGCTGCACAAAATTGTTATAAGGTGGCCAAAGGAGCATT
 20 TACCGGAGAAATCAGTCCTCAAATGATTAAAGATGTTGGTTGTGAATGGGCGATTCTTGGTC
 ATTCAGAGCGTAGAAATGTCTTTGGGGAATCTGATGAGCTCATTGGCGAAAAGGTTGCTTTT
 GCACTTGAGTCTGGTCTCAAAATTATTCCATGCATTGGAGAAAAATTAGACGAACGTGAATC
 TGGGAAGACTGAGGAGGTCTGCTTTAAGCAACTTAAAGCCATTTCTGACAAAGTATCTGATT
 GGGATCTTGTCGTCTTAGCTTATGAACCAGTTTGGGCCATTGGAAGTGGCAAAACAGCTACA
 25 CCTGCTCAGGCTCAAGAAACACATCTTGCTCTTCGTAAATGGCTAAAGGAGAACGTTTCTGA
 GGAAGTTTCACAAAAGTGCGAATCCTCTATGGAGGTTCCGTGAGTGCTGGTAATTGCAAGG
 AACTTGGCACTCAGCCTGATATTGACGGCTTCCTTGTTGGAGGAGCCTCTCTCAAACCTGAC
 TTTGTTCAAATCATCAACGCTACTAAGTAAACAAAATACTGGATATTGACTCTTCTATAAT
 AGTCTTATCATCTCTTTAATGCTCTCACTCATTATTTGATAAATAACGAGGTTAAAATATTA
 30 TTTATTTGATTAAACGTAATCTAACGTAATACATATATATTAATTTTACGAATGCAGAAAA
 AAAATTATTGCATAAATACGTATTTTACA

TCTP mRNA (SEQ ID NO:21):

GAGGTTGTCGGCTTTCAAGGACCACTCAATTCCTCCCTAGTTCTAATTCACCTTTCACTCCGG
 ACTCTTCCCGTAAACACTCCTGCCTTATACAAAATGAAGATCTTTAAGGACGTATTTTCTGG
 5 AGATGAATTATTTTCCGACACCTACAAGTTCAAGTTGTTGGATGATTGCTTGTACGAGGTGT
 ATGGAAAGTATGTCACACGGACTGAAGGAGATGTGGTTCTTGATGGAGCCAACGCATCTGCT
 GAAGAGGCCATGGATGACTGTGATTCCTCTTCCACCTCTGGTGTGATGTTGTCCTTAACCA
 CCGTCTGGTTCGAAACTGGGTTTCGGTTCCAAGAAGGACTACACCGTATACCTTAAGGACTACA
 TGAAGAAGGTAGTGACATATTTAGAAGAAAATGGCAAACAAGCCGAAGTAGATACCTTCAAG
 10 ACCAACATCAACAAGGTCATGAAGGAACCTTTTACCACGGTTTAAGGATCTTCAATTCTATAC
 TGGAGAAACGATGGACCCTGAGGCCATGATCATCATGCTTGAATACAAGGAAGTTGATGGAA
 AGGATATTCCTGCTCTACTTTTTTTAAACATGGATTAAATGAAGAAAAATTTTAAACATTA
 GTGTCATCATTCTCAATTTCTTATAAATGTTTATATCTACAATATATTTTATATAGATA
 AAAAGAATTTCCGTTGACAATAATATGCGAACTACCTAATTAAATTATGTTGTATTTCATAT
 15 TTCTAATGCGATTTTTGGGAAATTTCTCGTTATAACTAAATTCATTTTTTAACGTACACGTC
 TGTATATGAATATATGTAAAGTGTTATTTACTTGTAAGAC

The mRNA sequencing data of the target proteins was aligned and compared with the
 corresponding NCBI mRNA sequence using the Clustal Omega multiple sequence alignment
 20 tool (EMBL-EBI).

In most cases, mRNA sequence data matched exactly or very closely (only single base pair
 differences) to the NCBI database, however, for one protein, enolase, an additional isoform
 was identified (new start codon identified upstream from the start site of the NCBI sequence).
 25 Using UniProt (Universal Protein Resource) the sequence matched with 99% identity to
Tribolium castaneum, the red flour beetle. Both the red flour beetle (hexapod) and sea louse
 (crustacean) belong to the clade Pancrustacea in the phylum arthropoda (www.uniprot.org).

The characteristics of sea lice antigens are provided in Table 2.

Table 2 – Characteristics of sea lice antigens

	Protein Size (kDa)	Amino acid length (residues)	mRNA length (bp)
FBP-aldolase	42.1	364	1539
Prx-2	24	199	888
Enolase	48.9	432	1630
Enolase (short)	31.8	290	1146
TCTP	21.6	172	846
TIM	28.7	249	1021

The edited sequences were used to produce the protein antigens by recombinant protein production in *E. coli*. The DNA sequence for each protein was codon optimized prior to gene synthesis and cloned into the pET-30a (+) expression vector with N-terminal His tag along with TEV cleavage site. Recombinant plasmids were then transformed into *E. coli BL21* (DE3) cells and grown overnight at 37°C. A single colony was selected and inoculated into 1 litre of LB media containing kanamycin and incubated at 200 rpm at 37°C.

The expression DNA sequences were as set out below:

Enolase (SEQ ID NO:22):

ATGCATCATCACCATCACCACGAAAACCTGTATTTTCAGGGCATGCCGATTAAACACATCCA
 15 TGCCCGCCAAATCTATGACTCCCGTGGTAACCCGACCGTTGAAGTTGACCTGACCACCGAAC
 GTGGCATTTCGTGCCGCGGTGCCGAGCGGTGCATCTACGGGTGTTTCATGAAGCTCTGGAA
 CTGCGCGATAAAGACTCAACCTGGCACGGCAAATCGGGTCTGAAAGCGGTCAAAAACGTGAA
 TGATGTTCTGGGCCCCGGAACCTGGTGAAGAAAAACCTGGACCCGGTCAAACAGGAAGAAATTG
 ATGACTTTATGATCAGCCTGGATGGTACCGACAACAAATCTAAATTCGGCGCAAATAGTATT
 20 CTGGGTATCTCCATGGCAGTCTGTAAAGCTGGCGCAGCTCATAAAGGTGTGCCGCTGTATCG
 TCACATTGCGGATCTGGCCGGCGTCAAAGAAGTGATGATGCCGGTTCCGGCCTTCAACGTCA
 TTAATGGCGGTAGCCATGCAGGTAATAAACTGGCTATGCAGGAATTTATGATTCTGCCGACC
 GGTGCCCCGTTCATTCACCGAAGCCATGCGCATGGGTTCGGAAATTTATCATCACCTGAAAGC
 GCTGATTAAGAAAAAATACGGCCTGGATGCAACGGCTGTTGGTGACGAAGGCGGTTTTGCCC
 25 CGAACTTCCAAGCGAATGGCGAAGCCATTGATCTGCTGGTTGGTGCAATCGAAAAAGCTGGC
 TACACCGGTAAAATTAAAATCGGCATGGATGTGCGGGCCTCCGAATTCTACAAAACGGTAA

ATACGATCTGGACTTCAAAAATGAAGAAAGTAAAGAAGCGGATTGGCTGACCAGCGAAGCCC
TGGGCGAAATGTACAAAGGTTTCATCAAAGATGCCCCGGTGATTAGCATCGAAGATCCGTAC
GACCAGGATGACTGGGAAGGCTGGACCGCACTGACGTCTCAGACCGATATTCAAATCGTGGG
TGATGACCTGACCGTTACGAACCCGAAACGTATCCAGATGGCGGTTGATAAAAAATCTTGCA
5 ACTGTCTGCTGCTGAAAGTCAATCAAATTGGCTCAGTGACCGAATCGATCCGTGCGCATAAC
CTGGCCAAATCTAATGGCTGGGGTACGATGGTGTCTCACCGCTCCGGCGAAACCGAAGATTG
CTTCATTGCAGACCTGGTGGTTGGCCTGTGTACGGGTCAGATCAAAACCGGTGCTCCGTGCC
GTAGCGAACGCCTGTCTAAATATAATCAACTGCTGCGCATCGAAGAAGAACTGGGTAGCAAT
GCGAAATATGTGGGTGATAAATTCCGTATGCCGTTT

10

FBP aldolase (SEQ ID NO:23):

ATGCATCATCACCATCACCACGAAAACCTGTATTTTCAGGGCATGGGTCTGGAAGGCATCGT
TCCGCCGGGTGTCATTACGGGTGATAACCTGATTAACTGTTTGAATACTGCCGCGACCACA
15 AAGTGGCACTGCCGGCTTTTAACTGCACCAGCTCTAGTACGATTAATGCAGTGCTGCAGGCG
GCCCCGTGATATTAAATCTCCGTTATCGTCCAATTTAGTAACGGCGGTGCAGCTTTCATGGC
GGGCAAAGGTATTAAAAATGATGGCCAGAAAGCCTCCGTTCTGGGCGCCATCGCAGGTGCTC
AACATGTTCGCCTGATGGCCAAACACTATGGTGTCCCGGTGGTTCTGCATTCTGATCACTGC
GCGAAAAAACTGCTGCCGTGGTTCGATGGCATGCTGGAAGCCGACGAAGAATACTTTAAACA
20 GAACGGTGAACCGCTGTTCTCCTCACACATGCTGGATCTGTGGAAGAATTTGACGAAGAAA
ATATCAGCACCTGTGCGAAATATTTACCCGTATGACGAAAATGAAAATGTGGCTGGAAATG
GAAATTGGCATCACGGGCGGTGAAGAAGATGGTGTGACAACACCAATGTGAAAGCCGAAAG
CCTGTATACGAAACCGGAACAGGTCTATAACGTGTACAAAACCCTGTCCGAAATTGGCCCGA
TGTTTTCAATCGCGGCCGCATTCGGCAACGTTTCATGGTGTCTATAAAGCCGGTAATGTCGTG
25 CTGTCTCCGCATCTGCTGGCTGATCACCAGAAATACATCAAAGAACAATCAACAGTCCGCT
GGACAAACCGGCGTTTCTGGTGATGCATGGCGGTTCCGGGTAGCACCCGTGAAGAAATTGCGG
AAGCCGTGAGCAACGGTGTATTAAAAATGAATATCGATACCGACACGCAGTGGGCATATTGG
GATGGCCTGCGCAAATTCTACGAAGAAAAGAAAGAATACCTGCAGGGCCAAGTTGGTAACCC
GGAAGGTGCTGATAAACCGAATAAAAAATTCTATGACCCGCGTGTGTGGGTTTCGTGCTGCCG
30 AAGAAAGTATGATCAAACGCGCTAACGAATCCTTTGAATCCCTGAACGCAGTGAATGTGCTG
GGTGACAGTTGGAAACAC

Prx-2 (SEQ ID NO:24):

ATGCACCATCACCACCACCACGAAAATCTGTACTTCCAAGGCATGTCACTGCAACCGACGAA
CGACGCCCCGCAATTCAAAGCAATGGCAGTGGTTAACAAAGAATTCAAAGAAGTTTCGCTGA
5 AAGATTACACCGGCAAATACGTCGTGCTGTTTTTCTATCCGCTGGACTTTACCTTCGTCTGC
CCGACGGAAATTATCGCATTTGGCGATCGTGC GGCCGACTTCCGCAA AATTGGTTGCGAAGT
GCTGGCTTGTAGCACCATTCTCATTTTCAGTCATCTGCACTGGATCAACACGCCGCGTAAAG
AAGGCGGTCTGGGCGATATGGACATTCGCTGATCGCAGATAAAAATATGGAAATTTCCCGC
GCTTATGGTGTCTGAAAGAAGATGACGGCGTGTCAATTCGTGGTCTGTTTATTATCGACGG
10 CACCCAGAACTGCGCCAAATTACGATCAATGATCTGCCGGTTGGTCGTTGCGTCGACGAAA
CCCTGCGCCTGGTTTCAAGCGTTTCAATACACGGATGTGCACGGTGAAGTTTGTCCGGCCGGC
TGGAACCGGGTAAAAAATCTATGAAACCGTCAAAGAAGGCGTGTCTCTACCTGGCAGA
TGCTGAACAATCCAAAAA

15 TIM (SEQ ID NO:25):

ATGCATCATCATCATCATCACGAAAATCTGTACTTTCAAGGCATGGGCGGCGGTTCGCAAATT
CTTTGTGCGCGGCAACTGGAAAATGAACGGCGATAAAAAATCTATCGATGGTATCGTGGATT
TTCTGAGCAAAGGCGATCTGGATCCGAATTGCGAAGTGGTTGTGGGTGCGAGCCCGTGTTAT
20 CTGGATTACAGCCGTTCTAAACTGCCGGCAAACATTGGTGTGGCCGCACAGAATTGCTATAA
AGTTGCGAAAGGCGCCTTCACCGGTGAAATTAGCCCGCAGATGATCAAAGATGTTGGCTGTG
AATGGGCAATTCTGGGTCAATCTGAACGTCGCAACGTGTTTGGCGAAAGTGATGAACTGATC
GGTGAAAAAGTTGCATTCGCGCTGGAAAGCGGCCTGAAAATTATCCCGTGCATCGGTGAAAA
ACTGGATGAACGCGAATCTGGTAAAACGGAAGAAGTGTGTTTTAAACAGCTGAAAGCCATTT
25 CTGATAAAGTTAGTGATTGGGATCTGGTTGTGCTGGCGTATGACCGGTGTGGGCGATTGGTA
CCGGTAAAACCGCAACGCCGGCACAGGCACAGGAAACCCACCTGGCACTGCGTAAATGGCTG
AAAGAAAACGTTAGCGAAGAAGTGTCTCAGAAAGTTCGCATTCTGTACGGCGGTAGTGTTAG
CGCGGGCAATTGCAAAGAACTGGGTACCCAGCCGGATATCGATGGCTTCCTGGTGGGTGGTG
CTTCCCTGAAACCGGACTTTGTGCAGATTATCAACGCTACGAAA

30

TCTP (SEQ ID NO:26):

ATGCACCACCACCATCACCACGAAAATCTGTACTTCCAAGGCATGAAAATCTTCAAAGACGT
GTTTAGCGGCGACGAACTGTTCTCGGATACCTACAAATTTAAACTGCTGGATGATTGCCTGT

ATGAAGTGTACGGCAAATATGTTACCCGTACGGAAGGCGATGTGGTTCTGGATGGTGCGAAC
 GCCAGCGCAGAAGAAGCGATGGATGATTGTGATAGCTCTAGTACCTCTGGTGTGGATGTGGT
 TCTGAATCATCGCCTGGTTGAAACCGGCTTTGGTAGCAAGAAAGATTACACGGTGTATCTGA
 AAGATTACATGAAGAAAGTGGTTACGTATCTGGAAGAAAACGGCAAACAGGCGGAAGTGGAT
 5 ACCTTCAAAACGAACATCAACAAAGTTATGAAAGAACTGCTGCCGCGTTTTAAAGATCTGCA
 GTTCTACACCGGTGAAACGATGGATCCGGAAGCCATGATTATCATGCTGGAATATAAAGAAG
 TTGATGGCAAAGACATTCCGGTGCTGTACTTCTTCAAACACGGCCTGAACGAAGAAAAATTC

10 To evaluate the level of expression of our targets, small-scale cultures (4 ml) were grown to optimize the temperature, expression time, and Isopropyl β -D-1-thiogalactopyranoside (IPTG) concentration. SDS-PAGE and western blot were used to monitor expression over the different conditions. Once the optimum conditions were identified, culture volume was scaled up to 1L to ensure ≥ 10 mg of protein per target.

15 After IPTG induction, the 1L culture was spun down to collect cell pellets. Pellets were then lysed with lysis buffer and sonicated. Both supernatant and pellet fractions were collected and evaluated by SDS-PAGE to identify which fractions contained the target protein. For all proteins except for enolase, the proteins were located in the supernatant and therefore were soluble.

20 Soluble proteins were purified by adding the supernatant of the cell lysate to several millilitres of Ni-NTA (nickel-nitrilotriacetic acid) resin for high capacity, high performance nickel-IMAC (immobilized metal affinity chromatography), which is used for routine affinity purification of His-tagged proteins.

25 For insoluble proteins, pellets from the cell lysate were solubilized with urea, purified by N-column purification under denaturing conditions, and then refolded. Protein fractions were pooled and filter sterilized (0.22 μ m).

30 To ensure $\geq 90\%$ purity of the proteins, an additional two steps of purification by densitometric analysis of Coomassie blue stained SDS-PAGE gel was performed. Proteins were further analysed by western blot using primary mouse-anti-His mAb (GenScript, Cat. No. A00186). Protein concentration was determined using the Bradford protein assay with BSA standards (Pierce).

Aliquots were prepared in 1X PBS buffer with 10% glycerol (pH 7.4) and stored in -80°C.

The expression product of the Enolase expression DNA sequence (SEQ ID NO:22) is SEQ ID NO:27, which has the following sequence (TEV protease cleavage site is underlined, and the leading 6His tag is apparent):

MHHHHHHENLYFQGMPIKHIHARQIYDSRGNPTVEVDLTTERGIFRAAVPSGASTGVHEALE
LRDKDSTWHGKSGLKAVKNVNDVLGPELVKKNLDPVKQEEIDDFMISLDGTDNKSFKGANSI
10 LGISMAVCKAGAAHKGVPPLYRHIADLAGVKEVMMPVPAFNVINGGSHAGNKLAMQEFMILPT
GAPSFTEAMRMGSEIYHHLKALIKKKYGLDATAVGDEGGFAPNFQANGEAIDLLVGAIEKAG
YTGKIKIGMDVAASEFYKNGKYDLDFKNEESKEADWLTSEALGEMYKGF IKDAPVISIEDPY
DQDDWEGWTALTSQTDIQIVGDDLTVTNPKRIQMAVDKKSCNCLLLKVNQIGSVTESIRAHN
LAKSNGWGTMVSHRSGETEDCFIADLVVGLCTGQIKTGAPCRSERLSKYNQLLRIEEEELGSN
15 AKYVGDKFRMPF

The expression product of the FBP aldolase expression DNA sequence (SEQ ID NO:23) is SEQ ID NO:28, which has the following sequence (TEV protease cleavage site is underlined, and the leading 6His tag is apparent):

MHHHHHHENLYFQGMLEGIVPPGVITGDNLIKLFYCRDHKVALPAFNCTSSSTINAVLQA
ARDIKSPVIVQFSNGGAAFMAGKGIKNDGQKASVLGAIAGAQHVRMLMAKHYGVPVVLHSDHC
AKKLLPWFDGMLEADEEYFKQNGEPLFSSHMLDLSEEFDEENISTCAKYFTRMTKMKMWLEM
EIGITGGEEDGVDNTNVKAESLYTKPEQVYNVYKTLSEIGPMFSIAAAFGNVHGVYKAGNVV
25 LSPHLLADHQKYIKEQINSPLDKPAFLVMHGGSGSTREEIAEAVSNGVIKMNIDTDTQWAYW
DGLRKFYEEKKEYLQGQVGNPEGADKPNKKFYDPRVWVRAAEESMIKRANESFESLNAVNVL
GDSWKH

The expression product of the Prx-2 expression DNA sequence (SEQ ID NO:24) is SEQ ID NO:29, which has the following sequence (TEV protease cleavage site is underlined, and the leading 6His tag is apparent):

MHHHHHHENLYFQGMSLQPTNDAPQFKAMAVVNKEFKEVSLKDYTGKYVVLFFYP LDFTFVC
PTEIIAFGDRAADFRKIGCEVLACSTD SHF SHLHWINTPRKEGGLGDM DIPLIADKNMEISR

AYGVLKEDDGVSFRLFIIDGTQKLRQITINDLPVGRCDVETLRLVQAFQYTDVHGEVCPAG
WKPGKKSMKPSKEGVSSYLADAEQSKK

The expression product of the TIM expression DNA sequence (SEQ ID NO:25) is SEQ ID
5 NO:30, which has the following sequence (TEV protease cleavage site is underlined, and the
leading 6His tag is apparent):

MHHHHHHENLYFQGMGGGRKFFVGGNWKMNKGDKKSIDGIVDFLSKGDLDPNCEVVVGASPCY
LDYSRSKLPANIGVAAQNCYKVAKGAFTEGEISPQMIKDVGCWAILGHSERRNVFGESDELI
10 GEKVAFALESGLKIIPCIGEKLDERESGKTEEVCFKQLKAISDKVSDWDLVVLAYEPVWAIG
TGKTATPAQAQETHLALRKWLKENVSEEVSQKVRILYGGSVSAGNCKELGTQPDIDGFLVGG
ASLKPDFVQIINATK

The expression product of the TCTP expression DNA sequence (SEQ ID NO:26) is SEQ ID
15 NO:31, which has the following sequence (TEV protease cleavage site is underlined, and the
leading 6His tag is apparent):

MHHHHHHENLYFQGMKIFKDVFSGDELFSDTYKFKLLDDCLYEYVGKYVTRTEGDVVLDGAN
ASAEEMDDDCSSSTSGVDVVLNHRIVETGFGSKKDYTVYLKDYMKKVVTYLEENGKQAEVD
20 TFKTNINKVMKELLPRFKDLQFYTGETMDPEAMIIMLEYKEVDGKDIPVLYFFKHGLNEEKF

The expression products were typically applied as antigens. Antigens may also be applied
after 6His tag removal using TEV protease. Thus, the antigens may have a leading G residue.
The variants of SEQ ID NOs:27 to 31 produced by TEV protease cleavage or as defined by
25 SEQ ID NOs:1-6 are considered to achieve substantially the same result in substantially the
same way as SEQ ID NOs:27 to 31 and as defined by SEQ ID NOs:1-6 with a leading G
residue. Polynucleotide antigens encoding the same proteins are also considered to achieve
substantially the same result in substantially the same way as their polynucleotide variants.

30 Thus, the presence or absence of a His tag or an equivalent standard tag and the present or
absence of a TEV cleavage site, an equivalent cleavage site or the post-cleavage remnants
thereof, are not considered to affect the antigenic properties of the protein or polynucleotide
antigens.

For DNA vaccine production, each of the five antigens were cloned into the pVAX1™ plasmid vector (Invitrogen). A 3 kb vector was designed to promote high-copy number replication in *E. coli* and high level expression in most mammalian cell lines.

- 5 TIM was additionally cloned into the pVAC1 vector (InvivoGen). pVAC1 is a DNA vector vaccine plasmid designed to stimulate a humoral immune response via intramuscular injection. Antigenic proteins are targeted and anchored to the cell surface by cloning the gene of interest in frame upstream of the C-terminal transmembrane anchoring domain of placental alkaline phosphatase (InvivoGen). The antigenic peptide produced on the surface of muscle
10 cells is believed to be taken up by antigen presenting cells and processed through the major histocompatibility complex class II pathway (InvivoGen).

- The pVAC1-mcs backbone was selected over pVAC2-mcs for cloning because 1) the gene of interest does not contain a signal peptide even though it is secreted *in vivo* and 2) the vector
15 induces a humoral immune response. The signal sequence IL-2 and the 3' glycosyl-phosphatidylinositol (GPI) anchoring domain of human placental alkaline phosphatase directs cell surface expression of the antigenic protein (InvivoGen). The 3737 bp vector contains a Zeocin™ resistance gene and was designed for high-copy number replication in *E. coli*. The EF1- α gene of the pVAC1 vector ensures high levels of expression in skeletal muscle cells
20 and antigen presenting cells. Furthermore, the SV40 enhancer gene heightens the ability of the plasmid to be transported into the nucleus, especially in non-dividing cells (InvivoGen).

- The vectors, pVAX1 and pVAC1, are non-fusion vectors, therefore, the inserts needed to include a Kozak translation initiation sequence (e.g. **ANNATGG**) containing the initiation
25 codon and a stop codon for proper translation and termination of the gene. Primers were designed using SnapGene software to amplify a region that included the restriction enzyme site, the start codon, and the stop codon of the mRNA sequence of our target proteins. The primers are as set out in Table 3. The primers were used to amplify gene products from *L. salmonis* cDNA via PCR. PCR products of the expected size were PCR or gel purified,
30 digested with the appropriate restriction enzymes, and then PCR purified again.

Table 3 – Primers for amplification

Protein	5' forward	3' reverse
FBP	GCTATCAAGCTTAAAATGGGTC TTGAAGGAATTGTTC (SEQ ID NO:46)	TCAGATGGATCCTTAGTGTTTC CAGGAGTCACCA (SEQ ID NO:47)
Enolase	TATCGCCTGCAGAAAATGCCTA TTAAACACATTCATGCACGTC (SEQ ID NO:44)	ATCGTAGCGGCCGCTTAAAAGG GCATTCTGAACTTGTC (SEQ ID NO:45)
TIM	TAGCTGGGTACCTTACTTAGTA GCGTTGATGATTTG (SEQ ID NO:50)	CGTATCAAGCTTAAAATGGGTG GAGGAAGAAAATTTTTC (SEQ ID NO:51)
TCTP	GTCATTCTGCAGAAAATGAAGA TCTTTAAGGACGTAT (SEQ ID NO:52)	TCAGTAGCGGCCGCTTAAAATT TTTCTTCATTTAATCCATG (SEQ ID NO:53)
Prx-2	TCGACGAAGCTTAAAATGAGTC TTCAACCAACGAATG (SEQ ID NO:48)	TCGACTGGTACCTTATTTCTTT GATTGTTTCAGCATCTGCGAG (SEQ ID NO:49)

Vectors were linearized with the appropriate restriction enzymes for each insert. Linearized
 5 vector and insert were ligated with T4 DNA ligase (Invitrogen) and transformed into *E. coli*
 Stellar competent cells (Clontech). Transformants were cultured on LB plates containing
 50 µg/ml kanamycin overnight at 37°C.

Single colonies were isolated and cultured overnight in 5 ml LB media + kanamycin
 10 (50 µg/ml) at 37°C with shaking. Glycerol stocks were prepared and stored at -80°C for each
 clone. Plasmid DNA was isolated from bacterial lysates using a QIAprep Spin Miniprep Kit
 (Qiagen) and then digested with the appropriate restriction enzymes and ran on a 1%
 ethidium bromide gel. Digested clones showing two bands corresponding to the size of the
 vector and insert were submitted for sequencing using T7 forward and BGH reverse primers
 15 (pVAX1 vector) or pVAC1 forward and pVAC1 reverse primers (pVAC1 vector) – see Table
 4 for primer sequences.

Table 4 – Primers for sequencing

Vector	5' forward	3' reverse
pVAX1	TAATACGACTCACTATAGGG ("T7"; SEQ ID NO:54)	TAGAAGGCACAGTCGAGG ("BGH"; SEQ ID NO:55)
pVAC1	ACTTGGTGGGTGGAGACTGAAGAGT (SEQ ID NO:56)	AGGCACCACAGACCTTCCAGGAT (SEQ ID NO:57)

Clones containing inserts that shared high sequence similarity with the target sequence and in the correct orientation were selected for large-scale plasmid isolation. Two different kits were used for large-scale DNA vaccine preparation: Invitrogen's PureLink™ HiPure Expi Megaprep kit and Qiagen's QIAfilter plasmid giga kit. Due to the low plasmid yields obtained from the Invitrogen kit, the Qiagen giga kit was the preferred method of isolation.

A 500 ml (PureLink™ kit) or 2.5 L culture (Qiagen giga kit) was prepared following the manufacturer's instructions. Briefly, glycerol stocks of positive clones were used to streak a LB + kanamycin plate. A single colony was selected to inoculate 5 ml LB media + kanamycin and grown for 8h at 37°C with shaking (~180 rpm). One milliliter was then transferred to 5-500ml aliquots of LB media + kanamycin and grown overnight (12-14h) for large-scale plasmid isolation the following day. All steps were performed following the manufacturer's instructions. Plasmid DNA was resuspended in nanopure water and the total amount (mg) of plasmid DNA was quantified using the NanoDrop 8000 Spectrophotometer (Thermo Scientific). Aliquots were prepared and stored at -20°C. As a quality control measure all plasmids were ran on a 1% ethidium bromide gel to check for bacterial contamination and insert. All DNA vaccines were re-sequenced before use in vaccine trial.

Example 3 – Immunological response to circum-oral glands peptide recombinant antigens

To evaluate the ability of the five candidate sea lice antigens identified in Example 1 to produce an immunological response in Atlantic salmon, the fish were vaccinated with five antigens simultaneously and the systemic antibody titer at 600 degree days after vaccination.

Treatment Groups

In more detail, Atlantic salmon of around 40 g in weight were divided into five treatment groups, each group consisting of two duplicate tanks of six salmon. The treatment groups were as follows:

1. pVAX1 vector DNA delivering all five antigens prime (i.m.; 10 µg per antigen) with subsequent i.p. boost of recombinant protein cocktail of all five antigens plus Montanide ISA 763A VG (50 µg per antigen; Delivery Method 1; “DM1”);
2. Recombinant protein cocktail of all five antigens prime plus (i.d.; 50 µg per antigen) plus flagellin (50 ng) with subsequent i.p. boost of recombinant protein cocktail of all five antigens plus Montanide ISA 763A VG (50 µg per antigen; Delivery Method 2; “DM2”);
3. Empty pVAX1 vector (i.m.) with subsequent i.p. administration of mCherry-His recombinant protein plus Montanide ISA 763A VG (“DM1 ctrl”);
4. mCherry-His prime (i.d.; 250 µg antigen) plus flagellin (50 ng) with subsequent boost of mCherry-His (i.p.; 250 µg) and
5. No vaccine control (“PBS”).

Thus, treatment groups 3 and 4 received sham treatments that contained none of the five antigens, and treatment group 5 served as a control for any non-specific immune responses to injury at vaccination of naïve fish.

The control mCherry recombinant protein was produced using the following mRNA (SEQ ID NO:32):

```
ATGCATCATCACCATCACCACGAAAACCTGTATTTTCAGGGCATGGTTTCCAAAGGCGAAGA
AGACAATATGGCAATCATCAAAGAATTTATGCGTTTCAAAGTCCACATGGAAGGTTTCAGTCA
ATGGCCATGAATTTGAAATTGAAGGCGAAGGTGAAGGCCGTCCGTATGAAGGTACCCAGACG
GCAAAACTGAAAGTCACCAAAGGCGGTCCGCTGCCGTTTGCTTGGGATATTCTGTCACCGCA
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ATTCATGTATGGTTTCGAAAGCGTACGTTAAACACCCGGCCGATATCCCGGACTACCTGAAAC
 TGAGCTTTCCGGAAGGCTTCAAATGGGAACGTGTTATGAACTTCGAAGATGGCGGTGTGGTT
 ACCGTCACGCAGGATAGCTCTCTGCAAGACGGTGAATTCATCTACAAAGTGAACTGCGCGG
 TACCAATTTCCCGTCTGATGGCCCGGTTATGCAGAAGAAAACCATGGGCTGGGAAGCGAGTT
 5 CCGAACGTATGTACCCGGAAGACGGTGCCCTGAAAGGCGAAATCAAACAGCGCCTGAACTG
 AAAGATGGCGGTCATTATGACGCAGAAGTGAAAACCACGTACAAAGCTAAAAAACCGGTCCA
 ACTGCCGGGCGCATACAACGTGAACATCAAACCTGGATATCACCAGCCACAACGAAGACTACA
 CGATCGTTGAACAATATGAACGTGCGGAAGGTCGTCACTCTACGGGCGGTATGGATGAACTG
 TACAAATAATGA

10

The recombinant mCherry protein had the following sequence (SEQ ID NO:33):

MHHHHHHENLYFQGMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQT
 AKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVV
 15 TVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKL
 KDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDEL
 YK

Thus, mCherry may have the sequence recited above, which has a His tag (HHHHHH; SEQ ID
 20 NO:58) and a TEV cleavage site (ENLYFQG; SEQ ID NO:59), a TEV cleaved variant
 sequence, or another tagged or untagged variant sequence.

A further 12 Atlantic salmon were held in duplicate tanks of 6 fish each. These fish were
 acclimatized for 25 days in the system prior to sampling for basal level immune responses of
 25 the population prior to vaccination. This group served as a control for basal specific antibody
 responses to the antigens.

All the immune sampling (i.e. blood and mucus sampling post-vaccination) occurred at
 602 degree days post-priming vaccination, which the period after which you can begin to
 30 detect specific antibody titers to the vaccine antigens. Degree day was calculated by
 multiplying the average temperature by the number of days ($DD = ((T_0 + T_1 + \dots)/\text{no. of days}) \times \text{no. of days}$).

Experimental Methods

Atlantic salmon parr approximately 40 g in weight were obtained from the USDA, Franklin, ME facility. Fish were maintained in a recirculating fresh water flow through system in 100-gallon tanks at a stocking density of 25 kg/m³ and were fed at a rate of 1.5% body weight per day. Water quality and fish condition were monitored daily.

After a 25-day acclimation period, Atlantic salmon parr were vaccinated. Atlantic salmon were anaesthetized prior to tagging and vaccination by netting fish into 100 mg/L of MS222 supplemented with 200 mg/L sodium bicarbonate as a buffer to sustain neutral pH. The fish were tagged with elastomer along the jaw line for ease of identification.

The fish were primed by intramuscular injection of the vaccine at a dose of 10 µg per antigen per fish (DM1), a cocktail recombinant protein vaccine at a dose of 50 µg per antigen per fish in a total volume of 30 µl in sterile phosphate buffered saline with 50 ng ultrapure flagellin from *Pseudomonas aeruginosa* (InvivoGen; “DM2”; n = 48 fish per treatment group; duplicate tanks of 24 fish per group), or with the control formulation as appropriate. Post-tagging and vaccination the fish were returned to their respective housing tanks and monitored continuously until full recovery.

Two weeks after prime vaccination, the fish were anaesthetized and received a boost vaccination of recombinant proteins intraperitoneally at a dose of 50 µg per protein per fish, adjuvanted with Montanide™ ISA 763 A VG in a total volume of 100 µl (DM1 and DM2).

To measure the specific antibody response to louse antigens post-vaccination the blood and mucus of 12 Atlantic salmon per treatment group were sampled at 602-degree days for ELISA and dot blot analysis, respectively. Fish were euthanized with a lethal dose of 250 mg/L MS-222 buffered with 100 mg/L sodium bicarbonate. Blood was collected by bleeding the fish via the caudal vein. Blood samples were incubated at 4°C overnight and serum was isolated by centrifugation at 3716 x g for 10 min at 4°C. Serum was isolated and stored at -80°C until further use. Skin mucus samples were collected by placing the fish in a bag containing 10 ml phosphate buffered saline and massaging the fish for 2 minute each to wash off mucus. Mucus was centrifuged at 3716 x g for 10 minutes at 4°C and the supernatant transferred into sterile tubes and stored at -80°C.

The efficacies of the vaccines in eliciting a systemic immune response were evaluated for each vaccine candidate. All ELISA's were optimized prior to running serum samples from each fish. Optimal protein concentration, primary, and secondary antibody concentrations were determined for each antigen by running a checkerboard assay (Table 5).

Table 5 – Checkerboard assay results for antibody detection of sea louse antigens.

Protein	Stock $\mu\text{g/ml}$	Coating	Primary	Secondary
TIM	12600	2 $\mu\text{g/ml}$	1/500	1/500
FBP	12500	2 $\mu\text{g/ml}$	1/1000	1/1000
Prx-2	11800	2 $\mu\text{g/ml}$	1/1000	1/2000
TCTP	17900	2 $\mu\text{g/ml}$	1/500	1/1000
Enolase	620	2 $\mu\text{g/ml}$	1/500	1/2000

One hundred microliters of antigen (2 μg per well in carbonate:bicarbonate coating buffer; Sigma) was coated onto the wells of a 96-well polystyrene microtiter plate (Thermo Scientific). Plates were washed with low salt wash buffer (3x) and then blocked overnight at 4°C with 3% (w/v) casein in deionized water. After three more washes with low salt wash buffer, serum dilutions (1/100) in PBS were added to each well and allowed to incubate overnight at 4°C (100 μl per well). Plates were washed 5x with high salt wash buffer to remove residual serum and unbound antibodies. Primary antibody, mouse anti salmonid Ig monoclonal (Biorad; cat # MCA2182), was diluted to the appropriate concentration in PBS (Table 5) and added to each well (100 μl /well) and incubated at room temperature for 1h. Plates were washed with high salt wash buffer (5X) to remove unbound antibody. The secondary antibody, goat anti-mouse IgG peroxidase (Sigma; cat # A4416), was diluted to the appropriate concentration with conjugate buffer (1 % (w/v) bovine serum albumin diluted in low salt wash buffer) and added to the wells. After a 1 hr incubation at room temperature followed by 5X wash with high salt wash buffer, 100 μl of the chromogen (TMB) was added to each well and incubated for 10 min at room temperature. The reaction was stopped by adding 50 μl 2 M sulfuric acid to each well. Plates were mixed and the absorbance was recorded at 450 nm using a spectrophotometer. Each plate contained relevant controls: 1) pooled positive serum, 2) pooled negative serum, and 3) no serum controls (PBS). The

coefficient of variation of the A450nm of sample replicates within a plate, and the pooled positive serum between plates was always $\leq 20\%$.

Results

5

At 602 degree days after vaccination, Atlantic salmon serum antibody levels were measured to the five sea louse antigens included in the vaccine. ELISA analysis data showed Atlantic salmon responded to all five antigens delivered in the cocktail vaccine with a DNA prime (Figs 1-5), or a recombinant protein prime (Figs 6-10).

10

An immunological response was also induced by prime vaccination with 10 μ g TIM DNA antigen either in a pVAX1 vector or a pVAC1 vector, following by a boost using 50 μ g of TIM recombinant protein.

15 Thus, TIM, FBP, Prx-2, TCTP and Enolase each provides an antigen that elicits an immunogenic response in fish.

Example 4 – Efficacy of sea lice vaccine candidates

20 The efficacy of sea lice vaccine candidates against *Lepeophtheirus salmonis* (salmon louse) infection in Atlantic salmon (*Salmo salar*) was evaluated.

The specific antibody response was measured across 6 treatments (n =15 fish per treatment). Controls included a control for the His-tag as well as a no injection control (phosphate buffered saline [PBS]). The His-tag control served as a control for the His tag on the bacterially expressed sea louse antigens. PBS served as a control for any non-specific immune responses to injury at vaccination and to allow for the evaluation of sea lice settlement of non-vaccinated fish. An additional 42 fish per treatment were vaccinated and sampled to measure vaccine efficacy post sea lice challenge.

30

Treatments:

Vaccine 1: enolase (SEQ ID NO:1)

Vaccine 2: Prx-2 (SEQ ID NO:4)

Vaccine 3: TIM (SEQ ID NO:5)

5 Vaccine 4: FBP (SEQ ID NO:3)

Vaccine 5: TCTP (SEQ ID NO:6)

Vaccine 6: vehicle control (phosphate buffered saline - PBS)

10 For the prime vaccination, each recombinant protein vaccine contained 100 ng of purified flagellin from *Pseudomonas aeruginosa* (FLA-PA Ultrapure, InvivoGen) and was adjuvanted (Montanide™ ISA 763 A VG; Seppic™). For the boost vaccination, each vaccine formulation was adjuvanted (Montanide™ ISA 763 A VG; Seppic™).

Vaccine production

15

Recombinant protein vaccines were prepared by inoculating lysogenic broth (LB)-kanamycin (50 µg) agar plates with glycerol stocks of *E. coli* BL21 (DE3) cells, which contain the pET-30a (+) expression plasmid (Novagen) with gene insert, and growing each vaccine candidate overnight at 37°C. Single colonies were isolated and used to inoculate 2-50 ml flasks of LB
20 with kanamycin (50 µg). Cultures were allowed to grow at 37°C with shaking for 2-4 hours or until the optical density at 600nm was reached (0.6 to 0.8). Approximately 16.6 ml of culture media was added to 500 ml of LB with kanamycin (50 µg) in a flask for overnight growth at 200 rpm and 37°C. Once target optical densities were reached (i.e. 0.6 to 0.8), IPTG was added at 1 mM dose to each 500 ml flask and temperature was reduced to 18°C
25 with shaking at 200 rpm. After 15-18 hr of induction, the optical density was measured (target optical densities of 1-7) and cultures were centrifuged at 10,000 x g for 10 min at 4°C. The weight of each pellet was measured in each centrifugation bottle. Based on that weight, the amount of lysis buffer was calculated (2 ml of lysis buffer per 100 mg of cell pellet), and pellets were resuspended with vortexing. DNase was added (2 U per ml of lysis buffer) to
30 each bottle and mixed gently. Pellets were sonicated on ice in 20 second bursts for a total of 4 min and then incubated on ice for 15 min with intermittent mixing followed by centrifugation for 20 min at 10,000 x g at 4°C. The supernatant was decanted and added to a nickel-iminodiacetic acid-based protein purification resin (His60 Ni Superflow Resin; Takara), and allowed to incubate for 2 to 24 hours with gently stirring at 4°C.

Some proteins (e.g. Prx-2) were shown to have a high affinity for the resin and therefore lower incubation times were preferred (~2 h). Lower affinity proteins (e.g. FBP and TCTP) were allowed to mix with the resin for at least 24 h. Resin and supernatant (~250-300 ml) was added to 4-10 ml polypropylene gravity flow purification columns (Thermo Scientific, catalog # 29924). Once the resin settled to the bottom of the column, 10 ml of equilibration buffer was added (x 2). This was followed by 10 ml of wash buffer (x 2). The protein was eluted from the column by adding multiple 10 ml aliquots of elution buffer until protein detection by 280 nm light absorbance was negligible. For high affinity proteins, elution buffer containing 400 mM imidazole was added. For lower affinity proteins, 300 mM imidazole elution buffer was used. The eluate for each protein was combined and concentrated using 20 ml, 5 kDa, MWCO concentrators (GE Healthcare catalog # 28-9329-59). Excess imidazole was removed by adding concentrates to PD-10 desalting columns (GE Healthcare). Protein was concentrated briefly again and then filter sterilized with 0.22 µM, 13 mm diameter, PVDF syringe filters (Celltreat® catalog # 229742). A sterile 80% glycerol solution was added to each protein aliquot to give 8-10% glycerol per tube prior to storage at -80°C (Acros Organics CAS 56-81-5). Protein concentration was determined using a Pierce® BCA Protein Assay Kit (Thermo Scientific catalog # 23227). Proteins that were difficult to express at the quantities required (e.g. enolase) were produced by enhanced methods known to the skilled person (GenScript® protein expression service).

Atlantic salmon

Atlantic salmon post smolts (n = 342) approximately 70 to 100 grams in weight were maintained in a recirculating artificial salt water system on a 12:12 hr light:dark cycle in 100-gallon tanks at a stocking density of 25 kg/m³. Water quality, ammonia, nitrite, and fish condition were monitored daily. Salmon were fed a daily ration of BioTrout 3 mm pellets (Bio-Oregon®) at 1.5% body weight per day and maintained at temperatures of 13 ± 1°C, 32 ± 1‰ salinity, and 8 ± 1 mg/L dissolved oxygen (means ± standard deviations).

Fish vaccination

Fish size ranged from 98 to 295 grams at prime vaccination (average size 180 g). There were two vaccine treatments per tank (n = 19 fish per antigen) in replicates of three tanks (n = 38

fish per tank). During the vaccination phase, fish stocking density was $\leq 18.1 \text{ kg/m}^3$. Prior to vaccination, 20 fish were euthanized for mucus and blood collection with a lethal dose of MS-222 (250 mg/L). These fish served as a measure of the basal level of immunity of the fish. Fish to be vaccinated were anaesthetized with 100 mg/L MS-222 and then primed intradermally using a sterile 25-gauge needle and syringe. A 200 μg dose was prepared for the following recombinant proteins: enolase, Prx-2, TIM, FBP and TCTP (n = 57 fish per treatment). The number of injections per antigen ranged between two to three 10- μl injections per fish to achieve the target dose. For the PBS control, a single 10 μl dose was injected into each fish (n = 57). To distinguish fish between vaccine groups, an elastomer tag (Northwest Marine Technology, Inc.) was injected under the skin along the jawline following the intradermal injection of antigen. Each recombinant protein vaccine contained 100 ng of FLA-PA Ultrapure flagellin from *P. aeruginosa* (InvivoGen cat# tlrl-pafla). Each vaccine formulation was adjuvanted with Montanide™ ISA 763 A VG (Seppic™). Once primed, fish were returned to their respective treatment tanks to recover.

Two weeks post-prime vaccination, fish were anesthetized and boosted with an intraperitoneal (i.p.) injection of the recombinant protein vaccines, except for Prx-2 proteins, which was boosted 3 weeks and 4 days post-prime vaccination (n = 11). One hundred microliters of a 200 μg dose was prepared for the following recombinant proteins: enolase, Prx-2, TIM, FBP and TCTP (n = 57 fish per treatment). Each vaccine formulation was adjuvanted with Montanide™ ISA 763 A VG (Seppic™ Lot #36017Z). One hundred microliters of antigen at the described doses (above) plus Montanide was i.p. injected into each fish. For the PBS control, 100 μl PBS was added with adjuvant. Once boosted, fish were returned to their respective treatment tanks to recover.

At least three weeks prior to sea lice challenge, Atlantic salmon approximately 240 g in size were cohabitated into eight replicate tanks. Around 5 fish per treatment were transferred into each tank giving a total of 65 fish per tank or a stocking density of 41.3 kg/m^3 .

Serum and mucus collection

At 602 degree days, 43 days after boost vaccination and 588 degree days (42 days after boost), 15 fish per treatment were euthanized by exposing fish to an overdose of M-S222 (250 mg/L) to measure specific antibody responses after vaccination (n = 90 fish). Serum was

collected by bleeding the fish via the caudal vein using a sterile 23-gauge needle with a 3 ml syringe. Samples were processed by incubating samples at 4°C overnight and then centrifuging the blood at 3000 x g for 10 min at 4°C. The supernatant containing the plasma was collected and transferred into 2-1.5ml microcentrifuge tubes and stored at -80°C for ELISA analysis. Skin mucus samples were collected by placing each fish into a bag containing 10 ml phosphate buffered saline and massaging the fish for 2 minute each to wash off mucus. Samples were centrifuged for 15 minutes at 1500 x g at 4°C. Mucus was transferred into two 1.5 ml microcentrifuge tubes and stored at -80°C for dot blot analysis.

10 *L. salmonis* challenge

Two thousand *L. salmonis* egg strings were collected from gravid females and transferred to a sea lice hatchery. *L. salmonis* copepodids of similar age (3-4 days old) were pooled and the number of copepodids were calculated by counting ten 1-ml aliquots of lice using a dissecting scope to give the mean number of copepodids per ml of seawater. Infections were performed by reducing the volume of the tank holding the fish to a third of the original volume and copepodids were added to each of the replicate tanks to give an infection density of 80 copepodids per fish. The dissolved oxygen was monitored continuously throughout the 1-hour bath infection to maintain dissolved oxygen at 8.5 ± 1.0 mg/L (mean $\pm$ standard deviation). After one hour, the tank water level was restored. Dissolved oxygen was monitored for another 1.5 hours before turning the flow back on to each tank. Fish were monitored for an additional hour to ensure dissolved oxygen and flow rate were maintained in each tank at the appropriate levels.

25 To evaluate vaccine efficacy against salmon louse attachment, Atlantic salmon (n = 42 fish per treatment; n = 252) were challenged with *L. salmonis* copepodids 980 or 994 degree days after boost vaccination). Eight to eleven days after sea lice challenge, the salmon were exposed to an overdose of MS-222 to perform sea lice counts. Blind counts of the chalimus stages were recorded from the skin and gills of each fish for each treatment using a dissecting microscope and forceps. To reduce count variation, the same four individuals manually counted the number of lice on each fish. After counts were completed, the length (mm) and weight (g) of each fish was recorded.

Data analysis

The relative intensity (RI), which is the total number of lice per gram body weight [RI = total number lice / total weight (g)], was calculated for each individual fish subject to a vaccine treatment (Myksvoll et al., 2018). The RI values between vaccine treatments were compared. The average relative intensity (ARI = average number of lice / average weight [g]) was calculated to determine the percent change in lice intensity between vaccinated treatments and the PBS control (Myksvoll et al., 2018). Using these values, the % change was calculated (ARI PBS control – ARI vaccine antigen) / (ARI PBS control) x 100).

Results

The data from the sea lice vaccine trial showed that vaccination with recombinant protein antigens identified from the circum-oral glands of the chalimus stages reduced the number of chalimus per fish caused by the sea lice challenge.

Prx-2 and FBP were shown to be the most protective of the tested antigens, as shown in the RI values reported in Table 6.

Table 6 – Mean relative intensity of sea lice post vaccination and challenge with L. salmonis.

Antigen	RI (mean ± SEM)
PBS control	0.164 ± 0.016
Prx-2	0.114 ± 0.012
FBP	0.117 ± 0.014
TCTP	0.125 ± 0.010
Enolase	0.132 ± 0.013
TIM	0.135 ± 0.010

The percent reductions in chalimus counts for Prx-2, FBP, enolase, TCTP, and TIM were 28.9% to 13.1% (Table 7).

Table 7 – Percent reduction of *L. salmonis* chalimus stages after vaccination.

Antigen	Reduction (%)
Prx-2	28.9
FBP	25.1
Enolase	19.1
TCTP	17.9
TIM	13.1

Atlantic salmon were vaccinated with 5 different *L. salmonis* candidate antigens and challenged with the infective stage of the parasite. Using the average relative intensity, the percent change between the PBS control and candidate vaccine was calculated.

The antigens (FBP, TCTP, TIM, Prx-2, and enolase) had no negative effect on the growth of the vaccinated fish. Thus, vaccination with the *L. salmonis* antigens identified from the circum-oral glands of the chalimus stages reduced the relative intensity of chalimus infestation on Atlantic salmon.

The immunogenicity of the candidate antigens was assessed by western blot. Data showed that the pooled serum samples from vaccinated and sea lice challenged fish contained antibodies to the sea lice vaccine antigens. Protein bands of the correct sizes were detected on the nitrocellulose membrane after development (FBP, 42.1 kDa; TCTP, 21.6 kDa; enolase, 48.9 kDa; TIM, 28.7 kDa; and Prx-2, 24.0 kDa). These results suggest that the monovalent recombinant protein vaccines, Prx-2, FBP, Enolase, TIM, and, TCTP induced an antibody response in the host. Furthermore, the results show that the antigenic response to the vaccines by the host was protective upon secondary challenge with sea lice.

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Claims

1. An isolated protein, which is isolated from the circum-oral gland (COG) or the frontal gland complex (FGC) of a caligid copepod.

5

2. The protein according to claim 1, wherein the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.

3. The protein according to claim 1 or claim 2, wherein the protein is selected from the group consisting of: peroxiredoxin-2 (Prx-2); fructose biphosphate aldolase (FBP); enolase; transitionally-controlled tumour protein homolog (TCTP); and triosephosphate isomerase (TIM).

10

4. The protein according to any one of claims 1 to 3, comprising the amino acid sequence of one or more of the group consisting of: SEQ ID NO:4; SEQ ID NO:3; SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:6; SEQ ID NO:5; and homologues thereof.

15

5. The protein according to any one of claims 1 to 4, wherein the protein is a recombinant protein.

20

6. An antigen comprising one or more protein according to any one of claims 1 to 5.

7. A vaccine against caligid copepod infection in fish, the vaccine comprising an immunologically effective amount of one or more protein according to any one of claims 1 to 5 or one or more antigen according to claim 6, and a pharmaceutically-acceptable diluent or carrier, and optionally an adjuvant.

25

8. A vaccine according to claim 7, wherein each of the one or more antigens is different from the other antigen or antigens in the vaccine.

30

9. A vaccine according claim 8, wherein the vaccine comprises five antigens, wherein one of the five antigens comprises FBP, one of the five antigens comprises TIM, one of the five antigens comprises Prx-2, one of the five antigens comprises enolase, and one of the five antigens comprises TCTP.

10. A vaccine according claim 8, wherein the vaccine comprises five antigens, wherein one of the five antigens comprises the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 or homologues thereof, one of the five antigens comprises the amino acid sequence of
5 SEQ ID NO:3 or homologues thereof, one of the five antigens comprises the amino acid sequence of SEQ ID NO:4 or homologues thereof, one of the five antigens comprises the amino acid sequence of SEQ ID NO:5 or homologues thereof, and one of the five antigens comprises the amino acid sequence of SEQ ID NO:6 or homologues thereof.
- 10 11. The vaccine according to any one of claims 7 to 10, wherein the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.
12. The vaccine according to any one of claims 7 to 11, wherein the fish is a salmonid.
- 15 13. The vaccine according to claim 12, wherein the fish is a salmon or trout.
14. The protein, antigen or vaccine according to any one of claims 1 to 13 for use in the treatment or prevention of caligid copepod infection in fish.
- 20 15. The protein, antigen or vaccine for use according to claim 14, wherein the caligid copepod infection is a *Lepeophtheirus salmonis* or *Caligus rogercresseyi* infection.
16. The protein, antigen or vaccine for use according to claim 14 or claim 15, wherein the fish is a salmonid.
- 25 17. The protein, antigen or vaccine for use according to claim 16, wherein the fish is a salmon or trout.
18. A polynucleotide comprising DNA encoding a protein isolated from the circum-oral gland (COG) or the frontal gland complex (FGC) of a caligid copepod.
- 30 19. The polynucleotide according to claim 18, wherein the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.

20. The polynucleotide according to claim 18 or claim 19, wherein the encoded protein is selected from the group consisting of: peroxiredoxin-2 (Prx-2); fructose biphosphate aldolase (FBP); enolase; transitionally-controlled tumour protein homolog (TCTP); and triosephosphate isomerase (TIM).

5

21. The polynucleotide according to any of claims 18 to 20, comprising DNA encoding the amino acid sequence of one or more of the group consisting of: SEQ ID NO:4; SEQ ID NO:3; SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:6; SEQ ID NO:5; and homologues thereof.

10 22. The polynucleotide according to any of claims 18 to 21, wherein the DNA comprises the nucleotide sequence of one or more of the group consisting of: SEQ ID NO:12; SEQ ID NO:11; SEQ ID NO:10; SEQ ID NO:9; SEQ ID NO:8; SEQ ID NO:7; SEQ ID NO:16; SEQ ID NO:15; SEQ ID NO:14; SEQ ID NO:13; and homologues thereof.

15 23. An antigen comprising the polynucleotide according to any of claims 18 to 22.

24. A vaccine against caligid copepod infection in fish, the vaccine comprising an immunologically effective amount of one or more polynucleotide according to claim any of claims 18 to 22, or one or more antigen according to claim 23, a pharmaceutically-acceptable
20 diluent or carrier, and optionally an adjuvant.

25. The vaccine against caligid copepod infection in fish according to claim 24, wherein the vaccine comprises an immunologically effective amount of a combination of two or more antigens, wherein each of the one or more antigens independently comprises the DNA
25 sequence selected from the group consisting of: SEQ ID NO:12; SEQ ID NO:11; SEQ ID NO:10; SEQ ID NO:9; SEQ ID NO:8; SEQ ID NO:7; SEQ ID NO:16; SEQ ID NO:15; SEQ ID NO:14; SEQ ID NO:13;; and homologues thereof.

26. The vaccine according claim 24 or claim 25, wherein each of the one or more antigens
30 is different from the other antigen or antigens in the vaccine.

27. The vaccine according claim 26, wherein the vaccine comprises five antigens, wherein one of the five antigens comprises the DNA sequence of SEQ ID NO:7 or SEQ ID NO:8 or homologues thereof, one of the five antigens comprises the DNA sequence of SEQ

ID NO:9 or SEQ ID NO:10 or homologues thereof, one of the five antigens comprises the DNA sequence of SEQ ID NO:11 or SEQ ID NO:12 or homologues thereof, one of the five antigens comprises the DNA sequence of SEQ ID NO:13 or SEQ ID NO:14 or homologues thereof, and one of the five antigens comprises the DNA sequence of SEQ ID NO:15 or SEQ ID NO:16 or homologues thereof.

28. The vaccine according to any one of claims 24 to 27, wherein the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.

29. The vaccine according to any one of claims 24 to 28, wherein the fish is a salmonid.

30. The vaccine according to claim 29, wherein the fish is a salmon or trout.

31. The polynucleotide, antigen or vaccine according to any one of claims 18 to 30 for use in the treatment or prevention of caligid copepod infection in fish.

32. The polynucleotide, antigen or vaccine for use according to claim 31, wherein the caligid copepod infection is a *Lepeophtheirus salmonis* or *Caligus rogercresseyi* infection.

33. The polynucleotide, antigen or vaccine for use according to claim 31 or claim 32, wherein the fish is a salmonid.

34. The polynucleotide, antigen or vaccine for use according to claim 33, wherein the fish is a salmon or trout.

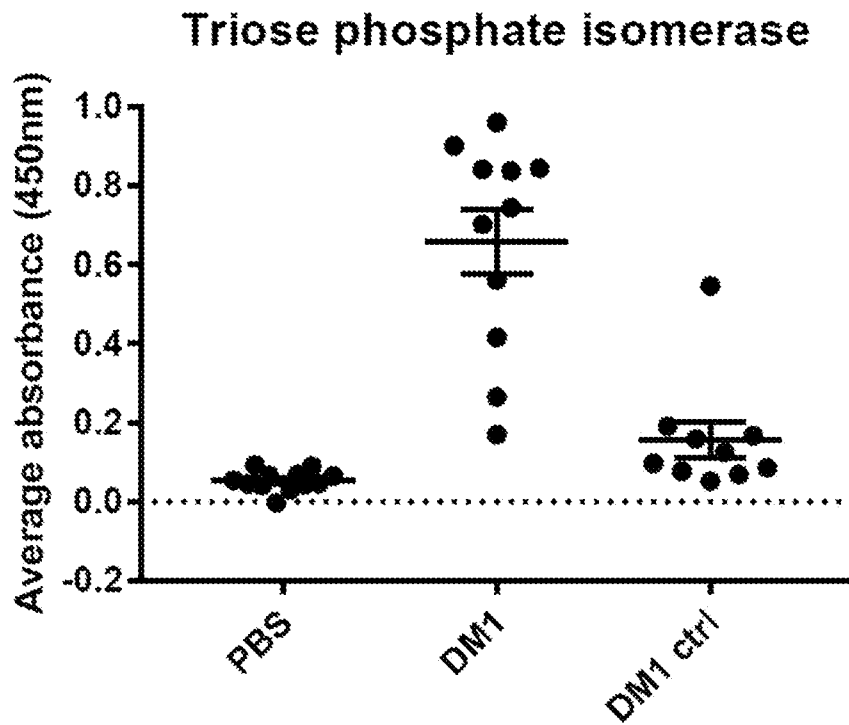
35. A method of treatment or prevention of caligid copepod infection in fish, comprising administering a therapeutic amount of the protein, polynucleotide, antigen, or vaccine of any one previous claim, optionally with the co-administration of an adjuvant.

36. The method according to claim 35, wherein the caligid copepod is *Lepeophtheirus salmonis* or *Caligus rogercresseyi*.

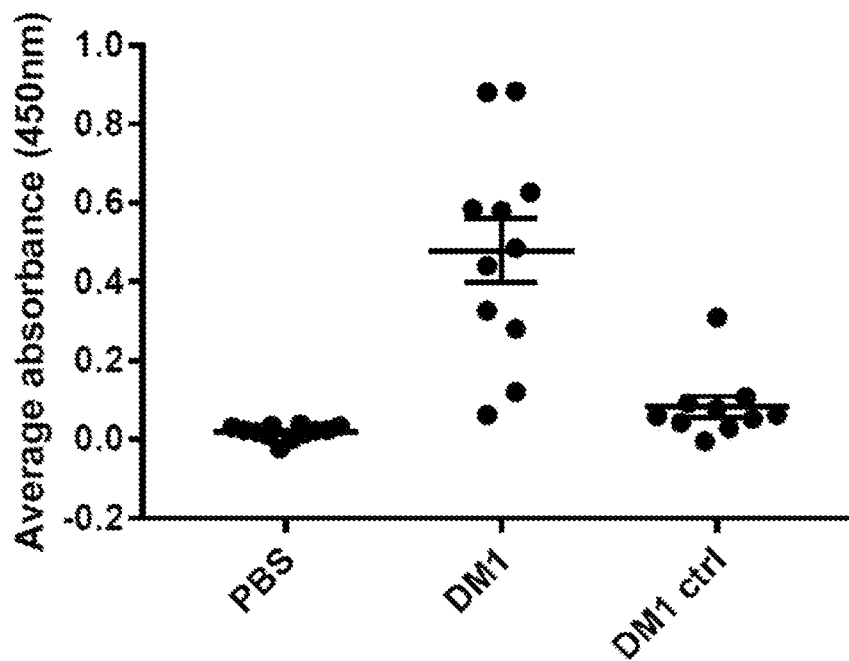
37. The method according to claim 36, wherein the fish is a salmonid.

38. The method according to claim 37, wherein the fish is a salmon or trout.

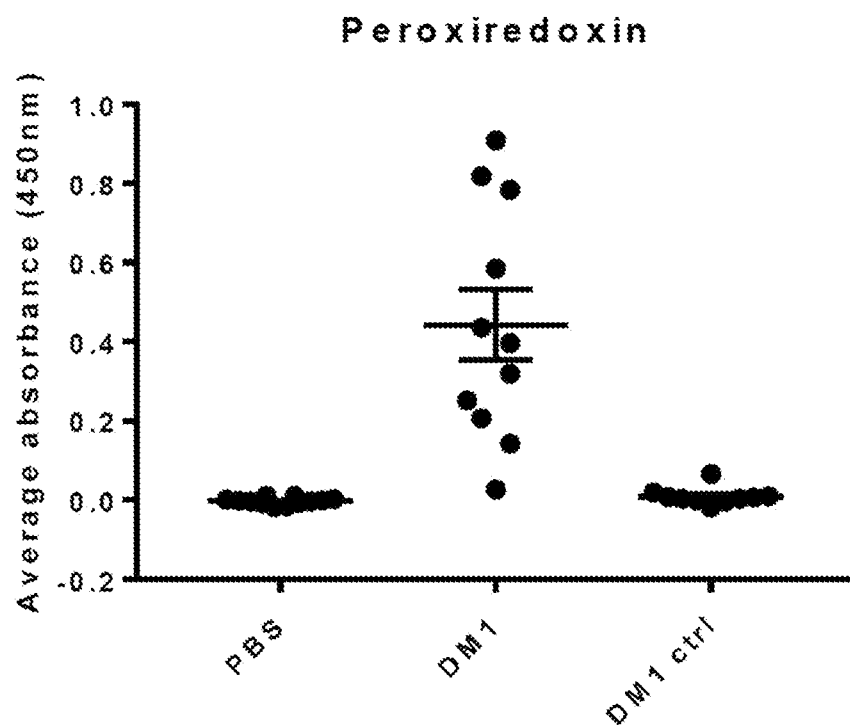
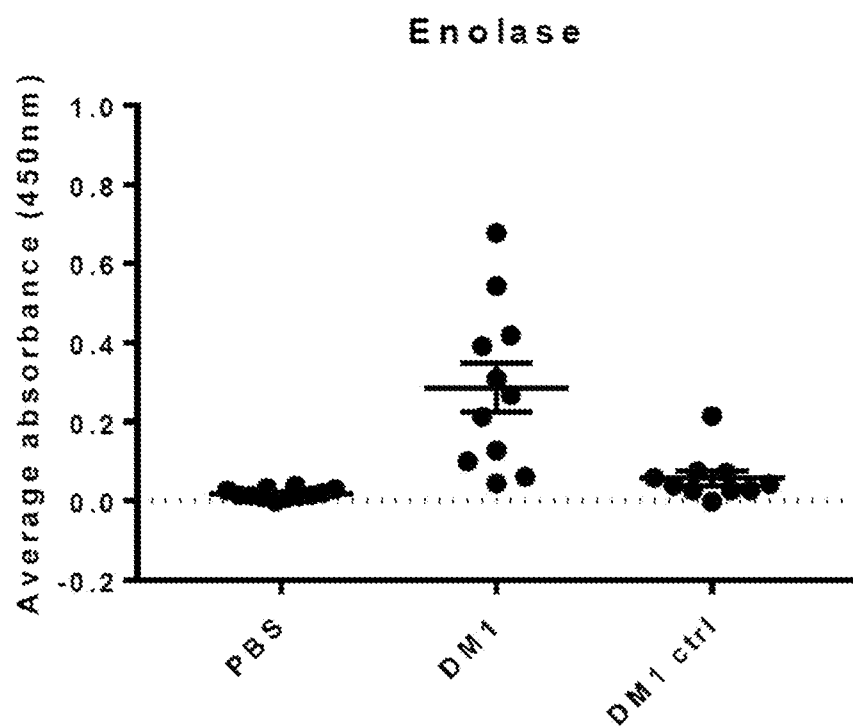
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*Fig. 1*

Transitionally controlled tumor protein homolog

*Fig. 2*

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*Fig. 3**Fig. 4*

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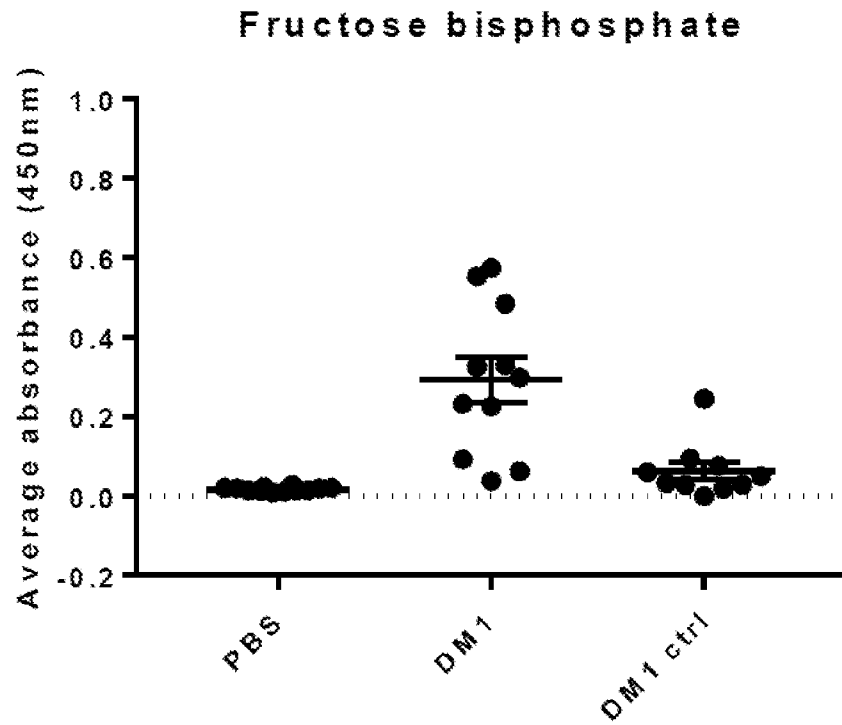


Fig. 5

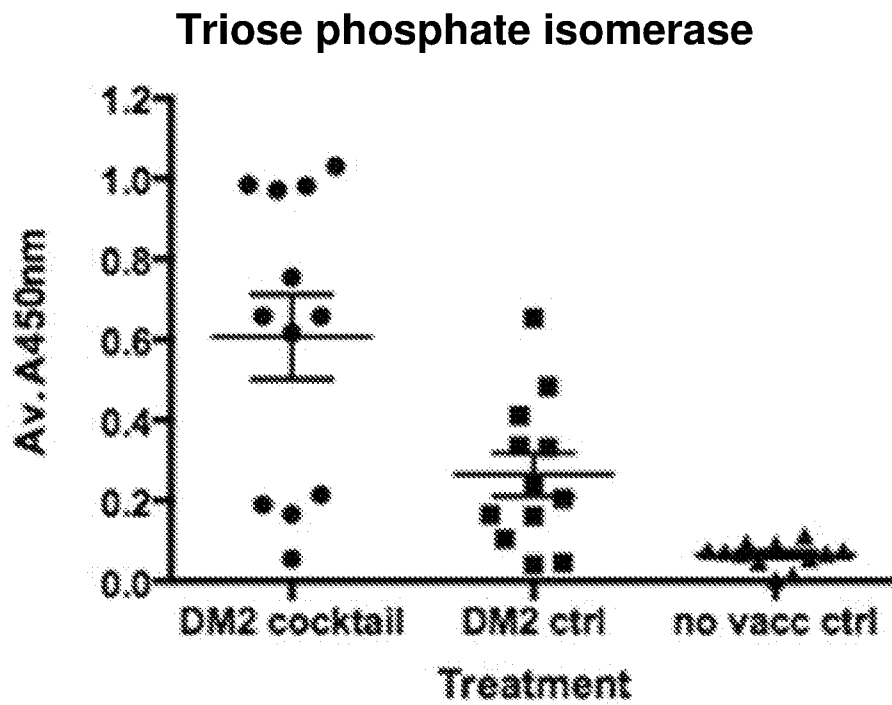


Fig. 6

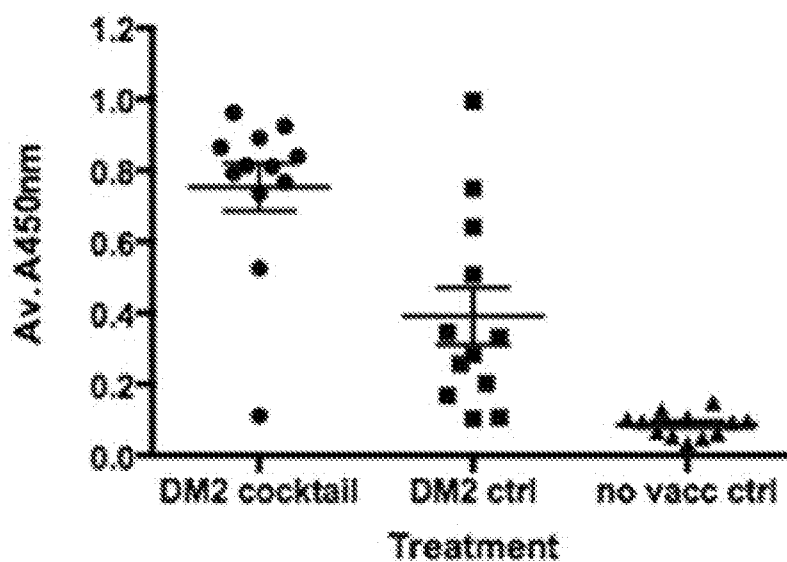


Fig. 7

Peroxioredoxin

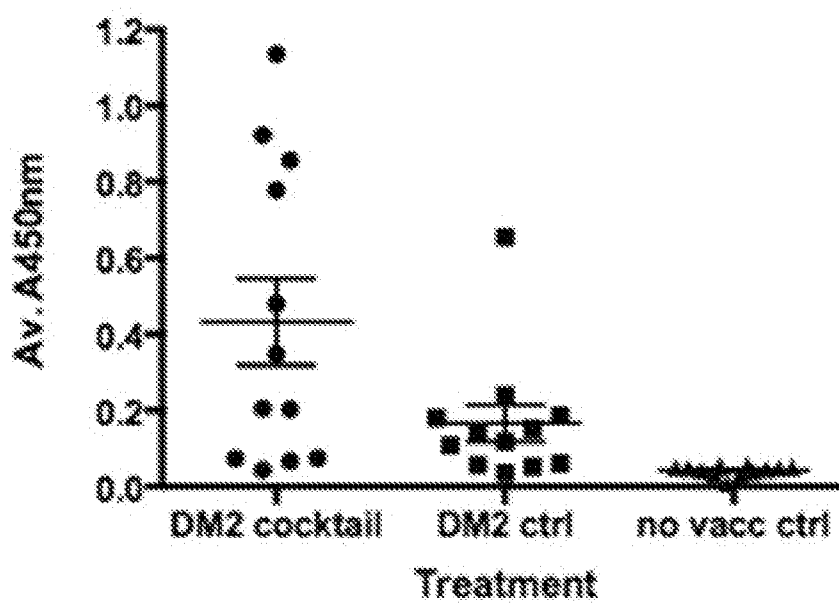
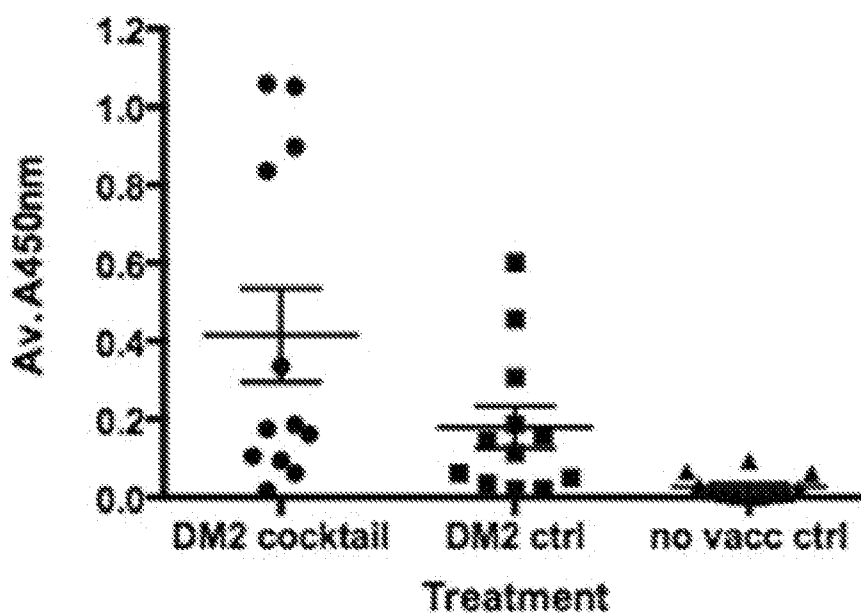
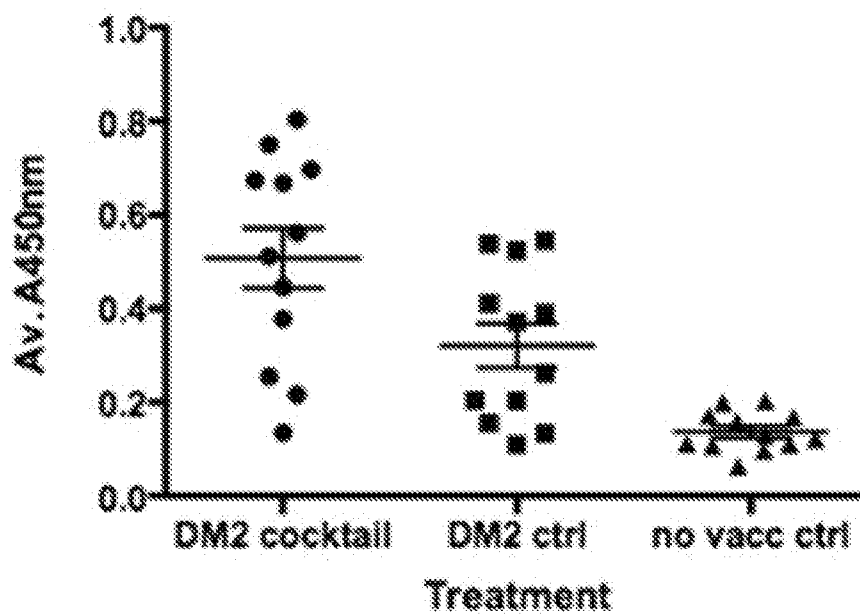


Fig. 8

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Enolase*Fig. 9***Fructose biphosphate***Fig. 10*