PROJECT REPORT

HADDOCK AND COD IMMUNOLOGY AND VACCINE DEVELOPMENT

This TCS program is a project in collaboration between the University of Aberdeen and Sea Fish Aquaculture

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AIM OF TCS PROGRAMS

KTP (former **TCS**) as a government-funded scheme will enable Seafish Aquaculture to access the skills and resources of the University of Aberdeen for strategic advantage through the work developed by a graduate, known as an associate.

The Program 3593, "Haddock and Cod Immunology and Vaccine Development", started on the 21st of January of 2002 and will be completed after 3 years. The structure of this program is divided in milestones to be achieved on the research side of the project, as well as working on the associate's personal development by taking part in courses and conferences. Five of these courses are organised by the TTI itself and are compulsory.

The associate will also aim to gain a NVQ (Level 4) in Management as part of the personal development. Although this program is research based, during its duration the associate will satisfy the requirements to gain such qualification as most of these requirements are covered by the work undertaken in the program.

Throughout the program the associate work and the progress of the project is reviewed by the Local Management Committee (LMC) every four months, as well as by annual reports and a final report to be presented by the end of the program.

PROGRAM 3593

HADDOCK AND COD IMMUNOLOGY AND VACCINE DEVELOPMENT

1. Introduction

This progress report presents the work carried out in the first two years of a three-year project on Haddock and Cod Immune System and Vaccine Development. The aim of this project is to study the ontogeny of the immune system in Haddock and use this knowledge to develop a vaccine technology.

The progress on the following aspects is detailed in this report:

- Training
- Assessment of the immune system of the haddock
- Initial vaccine development trials

2. Training

As part of the Teaching Company Scheme, the Associate should carry out several tasks for personal development. These tasks include Modules organised by the TTI (Technology Transfer and Innovation), former TCD. During the first year of the project the associate has completed four of the five modules. The last of the modules will be undertaken in the next months (Task 8.0). It will explore the implication of globalisation of markets for business for a period of three days. The associate will carry out a structured review of the company of the impact of a factor still to be agreed.

The first module carried out was the Introductory Module, web based. This module served as an introduction of the role of the associate in both the project and the company.

Module 1 (Task 1.3) was completed in Bath in March 2002. A group of associates from all over the UK took part in this course. One of the main objectives was to learn how to manage the associate's projects. Subjects such as resource management were discussed, as well as handling negotiations and meetings. The associates also learnt how to develop their communication skills required for oral presentations.

During Module 2 (Task 1.3) the associate carried out the presentation of a Mini-Project on "Live food production in Aquaculture" agreed with both academic and industrial supervisors during the LMCII. During this course we also learnt about time and stress management, how to work in a team and the roles existing in it, and on conflict management. The course ended by giving and receiving feedback between all the associates. This helped all the associates to know and understand their own strengths and weaknesses.

Module 3 (Task 6.0) was the last module completed in Wolverhampton in October 2002. This course introduced the associates to the basics of business processes. The module covered different issues, from setting up a business to marketing, financial analysis and business performance. Having no background of such matters, this module showed to be extremely useful as well as interesting.

At the beginning of the program, training in both the University and the Company was completed (Tasks 1.0, 2.0 and 3.0). During the first two months the University provided training at their facilities. The associate was inducted on Health and Safety regulations by doing Risk Assessments for the procedures to be carried out during the project and was registered as part-time PhD student and as member of the University staff.

During this time the associate also received training on molecular techniques, which included RNA extraction from different tissues to obtain cDNA as well as PCR technique and primers design. Training was also given on different Immunoassays such as ELISA, Lysozyme assay, Antiprotease assay and Respiratory burst assay.

Once settled in the Company, induction on Health and Safety regulations were also given and further training on Husbandry techniques was received. This included tasks such as larval rearing, live feed production and fish husbandry.

As part of the training and personal development the associate attended a Fish Vaccination and Fish Immunology Workshop in Wageningen, The Netherlands in April of 2002 and 2003 respectively. Different areas were covered both academic and industrial. Subjects such as methods of fish vaccination (delivery and formulation), immunostimulation, secondary effects, economic aspects and how to plan vaccination trials were studied. A more academic side of the study of the immune responses in fish and shrimp was seen during the Fish Immunology workshop.

In September 2003 the associate attended to the European Association of Fish Pathologist 11th Conference at Malta where a poster of the most recent results was presented. During this conference a wide variety of subjects were studied, ranging from immunology of fish and shellfish to diagnostics and pathology in different fish species.

3. Initial assessment of the Immune System

Gene work. Fishing for immune genes

One of the aims of this project is to isolate genes that had been characterized in other fish species and play important roles in the immune system of the haddock (Task 4.0). Our work has concentrated on 4 of these genes: RECOMBINATION ACTIVATING GENE–1 (RAG-1), INTERLEUKIN-1β (IL-1β), INTERLEUKIN-8 (IL-8) and IMMNOGLOBULIN M (IgM). During the second year of the project partial sequences of these genes were obtained using the method outlined below.

In the first year of the project, RNA was extracted from haddock adult tissues, head kidney, spleen, and liver. cDNA was obtained from the RNA by RT-PCR (Reverse Transcriptase Polymerase Chain Reaction). As well as this genomic DNA (gDNA) was extracted from muscle tissue. Both were used to isolate the desired immune gene sequences using PCR (Polimerase Chain Reaction).

RAG-1

RAG-1 together with RAG-2, is responsible for the genetic recombination events leading to functional immunoglobulin and TcR genes being expressed in mature B and T lymphocytes, and therefore is an appropriate marker to follow the development of immune organs that contain these cells (Willet *et al.*, 1997).

Using gDNA and degenerated primers already previously used successfully to amplify the Cod RAG-1 sequence (Venkatesh *et al.*, 2001) the haddock RAG-1 gene was isolated. To amplify this fragment 2μl of gDNA from haddock was used along with 4μl of each of the degenerated primers, one forward and a reverse. Together with a master mix containing a reaction buffer, $MgCl_2$, dNTP's and Taq polymerase, the partial RAG-1 sequence was amplified by PCR. The PCR conditions are detailed in Table 1.

PCR cycling protocol					
Target gene	Denaturation	Annealing	Elongation	No. Cycles	Product size (bp)
RAG-1	94°C @ 5'	-	-	1	
	94°C @ 1'	56°C @ 1'	72°C @ 1'	38	
	-	-	72°C @10'	1	900

Table 1. Summary of conditions for the PCR used to amplify the RAG-1 gene sequence.

The PCR product obtained was ligated into a cloning vector used to transect E.Coli cells, which are grown overnight at 37°C on agar plates. Bacterial clones were screened to check they contained the vector with the ligated PCR product and then sequenced. The partial sequence was aligned with those of other fish species to check it was the correct sequence. The isolated fragment shows high homology with other fish species with the highest identity found to be Cod, 84.2% at the amino acid level (See Fig.1).

```
Cod             ---------------------------------SQYHKMYRTVKPTSGRQIFQPLHTLRA
Flounder        ---------------------------------SQYHKMYRTVKATSGRQIFQPLHTLRA
Mullet          ---------------------------------SQSHKMYRTVKATSGRQIFQPLHALRA
Fugu            RQADELEAIMQGRGSGLHPAVCLAIRVNTFLSCSQYHKMYRTVKATTGRQIFQPLHSLRA
Trout           RQADELEAMMQGRGFGLHPAVCLAIRVNTFLSCSQYHKMYRTVKATSGRQIFQPLHTLRT
Haddock         ---------------------------------SQYHKMYRTVKATSGRQIFQPLHALRA
                                                 ** ********.*:*********:**:
Cod             AEKELLPGFHQFEWQPALKNVSTSCNVGIIN-GSGWASSVDESPADTITRRFRYDVALVS
Flounder        AEKELLPGFHEFEWQPALSNVSTSCNVGIIN-GSGWTSSVDDSPTETVTRRFRYDVSLVS
Mullet          AEKELLPGFHQFEWQPALKNVSTSCSVGIIN-GSGWASSVDDSPANTITRRFRYDVALVS
Fugu            VEKELLPGFHQFEWQPPLRNVSTSCSVGIINGLSGWTSSLDDSPAETITRRFRYDVAIVS
Trout           AEKELLPGYHPFEWQPALKSVSTSCHVGIIDGLSGWIASVDDSPADTVTRRFRYDVALVS
Haddock         AEKELLPGFHAFEWQPALASVSPSCHVGIIDGLSGWAASVDDAPADTITRRFRYDVALAS
                .********:* *****.* .**.** ****:  *** :*:*:::*::*:*********::.*

Cod             ALKDMEEDIMEGLRESGMEDSACTSGFSVMIKESCDGMGDVSEKHGGGPAVPEKVVRFSF
Flounder        ALKDLEEDIMEGLIEAGMEDSACTSSFNVMIKECCDGMGDVSEKHGGGPAVPEKVVRFSF
Mullet          ALKDLEEDIMDGLRESGMEDSACTSGFNVMIKECCDGMGDVSEKHGGGPAVPEKPVRFSF
Fugu            ALKDLEEDIMEGLRENEMEDSNCTLGFSVLIKESCDGMGDVSEKHGGGPLVPEKAVRFSF
Trout           ALKDLEEDIMEGLRERGLEDSACTSGFSVMIKESCDGMGDVSEKHGGGPPVPEKPVRFSF
Haddock         ALKDLEEDIMDGLRQQGLEDNACTEGFSVMIKESCDGMGDVSEKHGGGPAVPEKAVRFSF
                ****:*****:** :  :**. ** .*.*:***.*************** **** *****
Cod             TVMSVSVLADGED------REVTIFTEPKPNSELSCKPLCLMFVDESDHETLTAVLRPIV
Flounder        TVMSVSVLADGED------REVTVFTEPKPNSELSCKPLCLMFVDESDHETLTAVLRPIV
Mullet          TVMSVSVLADEEE------EEVTIFTEPKPNSELSCKPLCLMFVDESDHETLTAVMAPIV
Fugu            TIMSVSIKAEGE-------KDKVIFTEPKPNSELSCKPLSLMFVDESDHETLTAIMSPII
Trout           TIMSVSIQAEGED------EAITIFREPKPNSEMSCKPLSLMFVDESDHETLTGVLGPVV
Haddock         TVMSVSLRVADDGEEAGNAEEVIVFQEPKPNSELSCKPLCLMFVDESDHETLTAILGPVS
                *:****: .  :       .   :* *******:*****.*************.:: *:
Cod             AERNAMKESRLILSIGGLPRFFRFRFRGTGYDEKMVREMEGLEASGSTYICTLCDSSRAE
Flounder        AERNAMKESRLILSIGGLPRFFRFRFRGTGYDEKMVREMEGLEASGSTYICTLCDSSRAE
Mullet          AERNAMKESRLILSIGGLPRSFRFHFRGTGYDEKMVRELEGLEASGSTYVCTLCDSTRAE
Fugu            AERDAMKESRLIVSIGGLPRSFRIHFRGTGYDEKMVREMEGLEASGSMYICTLCDSSRAE
Trout           AERNAMKHSRLILSVGGLSRSFRFHFRGTGYDEKMVREMEGLEASGSTYICTLCDSTRAE
Haddock         AERSAIKRSRLILSIGGLSRLFSFRFRGSGYDEKMVRDVEGME----------------
                ***.*:*.****:*:***.* * ::***:********::**:*
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Figure 1. Multiple alignment of the predicted Haddock RAG-1 gene with known fish RAG-1's. Identical (*) and similar (: or .) residues identified using CLUSTAL W program are indicated.

IL 1-β AND IL-8

IL 1-β is a cytokine that initiates the expression of a pro-inflammatory cascade of proteins and cytokines by selective regulation of other molecules. It has stimulatory and regulatory effects in the growth and differentiation of many cell types regulating B and T cells and mediating inflammatory responses.

IL-8 is a chemokine, a member of the cytokine family that displays chemotactic activity for all known types of migratory immune cells, specifically activates neutrophil granulocytes and enhances the expression of adhesion molecules.

Both interleukins have the potential to be used as adjuvants to enhance the effect of various vaccines as well as being used to study the effect of immunostimulation.

Using gDNA from haddock the partial sequences of the IL-1β and IL-8 genes were obtained. The primers used were designed using a homology cloning approach. Fig. 2 shows the regions chosen to design the primers for isolation of IL-1β, which were biased towards the known Cod sequence.

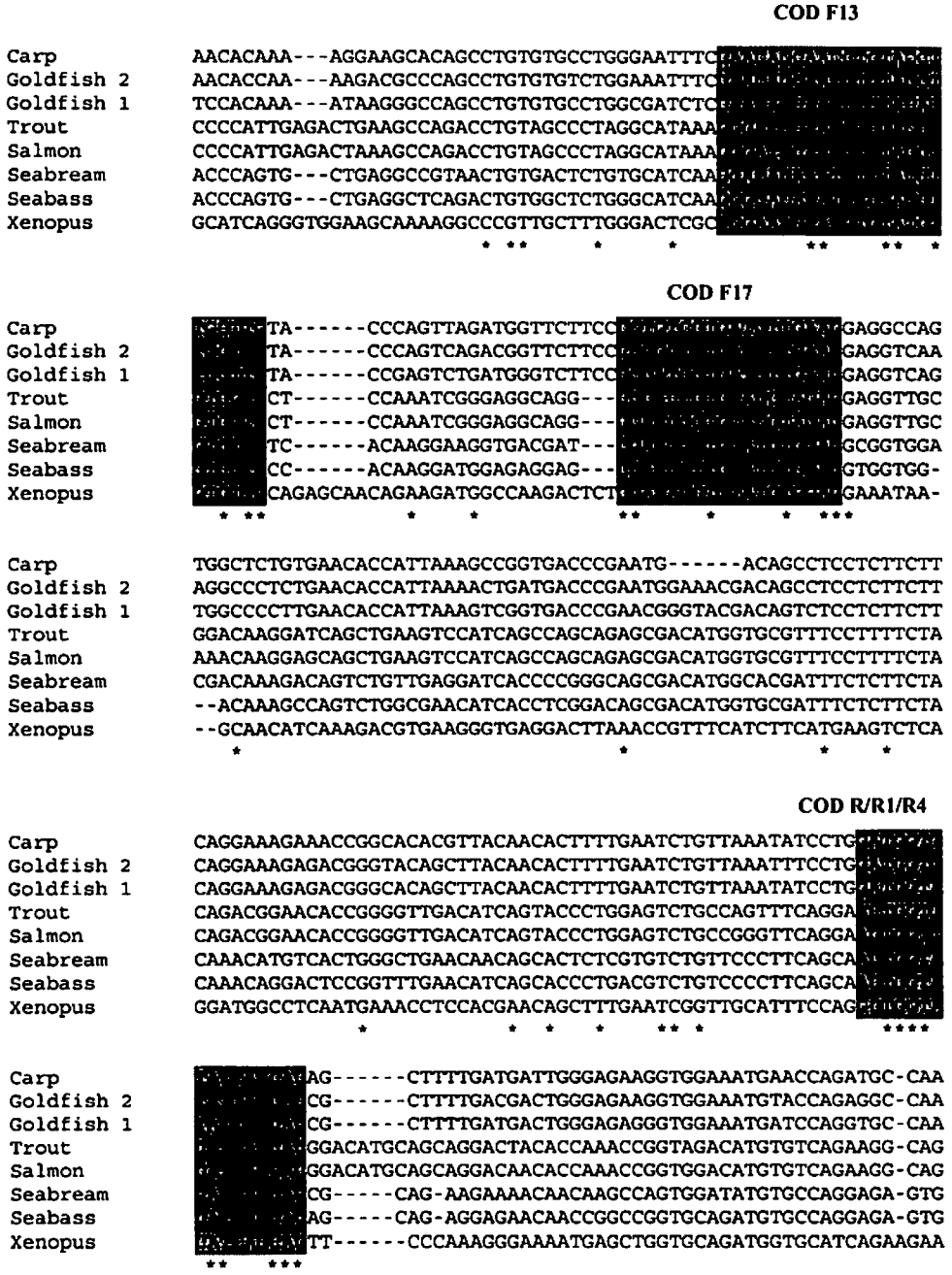

Figure 2. Primer design fr IL-1β. Fish sequences were aligned and areas of homology used to finally design the primers shown in the highlighted areas. Forward primers were named COD-F13 and COD-F17, Reverse primers were named COD-R-R1 and COD-R4. Cod sequence is omitted from alignment as it is confidential.

To amplify the haddock IL-1β fragment, 2μl of gDNA was used along with 4μl of the forward primer and 2μl of the reverse primer. Together with a master mix containing a reaction buffer, $MgCl_2$, dNTP's and Taq polymerase, the partial IL1-β sequence was amplified by PCR. The conditions are detailed in Table 2.

PCR cycling protocol					
Target gene	Denaturation	Annealing	Elongation	No. Cycles	Product size (bp)
IL-1β	94°C @ 5'	-	-	1	
	94°C @ 1'	60°C @ 1'	72°C @ 1'	5	
	94°C @ 1'	54°C @ 1'	72°C @ 1'	30	
	-	-	72°C @10'	1	631

Table 2. Summary of conditions for the PCR for the gene IL-1β gene.

Once the product was obtained it was ligated into a cloning vector. The procedure used to isolate the correct clone for the RAG-1 sequence was used for the IL-1β. Clones that were sequenced contained part if the haddock IL-1β gene which contained an intron (Fig 3.).

```
  1  CACACAGAAA GCAGACAGAC CTACCCTGAA TTTAGAGGTA AAACCATCCG
 51  CCAGCTTGCA TAGATCACTT CCAGTTCAAT TGAACTTCCG CGCCATCTTC
101  GTGCTTGTTC ACTATTCTTA TAACATTAAT AACGGTCAGT TATTCCAGAA
151  TGTTTTGATC AGTAAGAACG GAGATAGTGG TTTGAATTGA TTTTTGCGTG
201  ACTTTACATC ACGTGATTTA ATCCCTTCTA AAATCATAAT CTGACTACCA
251  GGGGAGGAGA TGTCTGTTGG GGACCCATCA CTTAATATAG GTCTTAAAAG
301  TACGATACAT CTATTTTTGG GACAAATACC TTTAATGTTA CGCTATTGGC
351  GAAATCAAAT GATAACAATA ATTTTCCGAA TTATTGCGTC CACCTGGCGG
401  GTATAGCCTA TATATTTTTT TGTATGACTG TGCTTCACAA TAAAACAACA
451  ATTCAAAACA CGTAACATTT TCAAAACAAC ACGCCATTAA TGTTGTCCTT
501  GTTCTTGTAG GAGGTGCAGA ACACGGACGA CCTGAAAAGC ATCAGCAAGA
551  ACAGTGACAT GGTGCGTTTC CTGTTCTACA GAACTGACAT CGGCGTCAGC
601  GCCAGCTCAC TCATGTCTGC CCGCTACTCC G
```

Figure 3. Partial nucleotide sequence for Haddock IL-1β obtained using the primers COD-F13 and COD-R4. The sequence contained an intron which is shown in italics. The intron begins classically with the nucleotides GT and ends with AG, which are boxed. The coding sequence is highlighted.

The coding region from this partial sequence was deduced and aligned with those of other fish species to check it was the correct sequence. The isolated fragment showed a good homology with other fish species. The highest identity was 88.4% at amino acid level (See Fig.4).

```
Carp 1-2          SSDP-QNDGQ---PVCLGISNSNLYIACTQSGGS--PPVLLLKEVSGPLNTITAGDPNGY
Carp 2-2          SSAQ-QNNGQ---PVCLGISNSNLYIACTQSGGS--PPVLLLKEVSGPLNTITADDPNGY
Goldfish 1        SSAP-QNKGQ---PVCLAISNSNLYIACTESDGS--SPILILKEVSGPLNTIKVGDQNGY
Goldfish 2        SSAT-PKDAQ---PVCLEISNSNLYLACTQSDGS--SPALILKEVKGPLNTIKTDDPNGN
Carp 1            SSAT-QKEAQ---PVCLGISNSNLYLACTQLDGS--SPVLILKEASGSVNTIKAGDPN--
Red bream         PPTP-SAEAV---TVTLCIKDTNLYLSCHK-EGD--EPTLHLEAVDDKDSLLSITPGSDM
Seabream          PPTP-SAEAV---TVTLCIKDTNLYLSCHK-EGD--DPSLHLEAVDDKDSLLRITPGSDM
Seabass           DRTP-SAEAQ---TVALGIKGTNYYLSCHK-DGE--EPTLHLEVVD-KASLANITSDSDM
Tetraodon         ELQS-NREAQ---TVTLAIRNTNFYLSCHK-DGD--EPTLHLEAVEDKEQLKSISSDSDM
Haddock           ----------------------------TQ-KAD--RPTLNLEEVQNTDDLKSISKNSDM
Trout             TPVPIETEAR---PVALGIKGSNLYLSCSK-SGG--RPTLHLEEVADKDQLKSISQQSDM
Halibut           HPSP-TIEAR---PVVLGIKDTDFFLSCQK-NGA--EPTLHLERVENKCDLEAFSRDSEM
Turbot            HPSP-STEAR---PVALGIRGTNLYLSCQQ-EDG--VPTLHLEEVEDKSSLLAISGESDM
Xenopus           TQPHQGGS---KRPVALGLAGKNLYLSCRATEDGQDSPKLYLEEISNIKDVKGE----DL
Human             VQGEESND---KIPVALGLKEKNLYLSCVLKDD---KPTLQLESVDPKNYPKKK----ME
Chicken           PRGPRGSAGTGQMPVALGIKGYKLYMSCVMSGT---EPTLQLEEADVMRDIDSV----EL
Catshark          TTPTTEDDLP----VVMAISNQNLFLSCVGTQDS---PRVQLEKWD--RKLQNISSTTDL
                                                      * : *:

Carp 2-1          DSLLFFR--KETG-TDYNTFESVKYPGWFISTAFDDW--KRVEMSQVPTD-RTTDFTLQD
Carp 2-2          DSLLFFR--KETG-TAYNTFESVKYPGWFISTAFDDW--KRVEMSQVPTD-RTTDFTLQD
Goldfish 1        DSLLFFR--KETG-TAYNTFESVKYPGWYISTAFDDW--ERVEMIQVPTD-RTTNFTLED
Goldfish 2        DSLLFFR--KETG-TAYNTFESVKFPGWFITTAFDDW--EKVEMYQRPTE-RIPDFTLED
Carp 1            DSLLFFR--KETG-TRYNTFESVKYPGWFISTAFDDW--EKVEMNQMPTT-RTTNFTLED
Redbream          VRFLFYK--HVTG-LNNSTLVSVPFSSWYISTAEENN--KPVEMCQETTR-RHRIFKF--
Seabream          ARFLFYK--HVTG-LNNSTLVSVPFSNWYISTAEENN--KPVDMCQESAR-RHRIFKF--
Seabass           VRFLFYK--QDSG-LNISTLTSVPFSNWYISTAEENN--RPVQMCQESAR-RHRAFNIDN
Tetraodon         VRFLFYK--HDSG-VNLCTLVSVPYSDWYISTAVEDN--KPVEMCLESAL-RYTSFTIQP
Haddock           VRFLFYR--TDIG-VSASSLMSARYS--------------------------------
Trout             VRFLFYR--RNTG-VDISTLESASFRNWFISTDMQQDYTKPVDMCQKAAPNRLTTFTIQR
Halibut           VRFLFYK--QDSGGVSISTLMSARFPNWYISTSEQDN--RPVMVGQKNAR-CYQTFNIQH
Turbot            VRFLFYK--RDSG-VNISTLMSARFPNWYISTSEQDN--KPVEMCQESAQ-RYQTFSIQR
Xenopus           NRFIFMKSQDGLNETSTNSFESVAFPGWYISTSQREN--ELVQMVHQKNQEAIKDFNLFS
Human             KRFVFNK----IEINNKLEFESAQFPNWYISTSQAEN--MPVFLGGTKGGQDITDFTMQF
Chicken           TRFIFYR--LDSPTEGTTRFESAAFPGWFICTSLQPR--QPVGITNQPDQVNIATYKLSG
Catshark          LRFVFFKKVSSSG--LHFELESAMYRGWYVSTSRRNR--QPIELDEKKNHKRITIFTAD-
                    ::* :            : *. :
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Figure 4. Multiple alignment of the predicted Haddock IL-1β gene with selected known vertebrate IL-1β's. Identical (*) and similar (: or .) residues identified using CLUSTAL W program are indicated.

Fig.5 shows the regions chosen to design the primers for isolation of IL-8 which were biased towards the known Cod sequence. As we did with the IL-1β we used gDNA to obtain the sequence of the IL-8.

```
Flounder IL-8     ---------ATGAGCAGCAGAGTCATCGTTGTTGCTGTGATGGTGCTCCTGGCCTCTCTG
Halibut IL-8      ---------ATGAGCAGCAGAGTCATCGTTGTTGCTGTGATGGTGCTCCTGGCCTCTCTG
Trout IL-8        ---------ATGAGCATCAGAATGTCAGCCAGCCTTGTCGTTGTGCTCCTGGCCCTCCTG
                           *  **   ** * *              *  * ** ******* *   ***

Flounder IL-8     GCCATCAG-GAAGCGGTGAGCCTGAGAAGCCTAGGAGTGT
Halibut IL-8      GCCATCAGTGAAGCGGTGAGCCTGAGAAGCCTAGGAGTGT
Trout IL-8        ACCATTAC-GAGGGGATGAGTCTGAGAGGCATGGGGGCTG
                   **** *  ** *     ** *****  *  * **      ****  ** ********
                                                        CODIL8-F1
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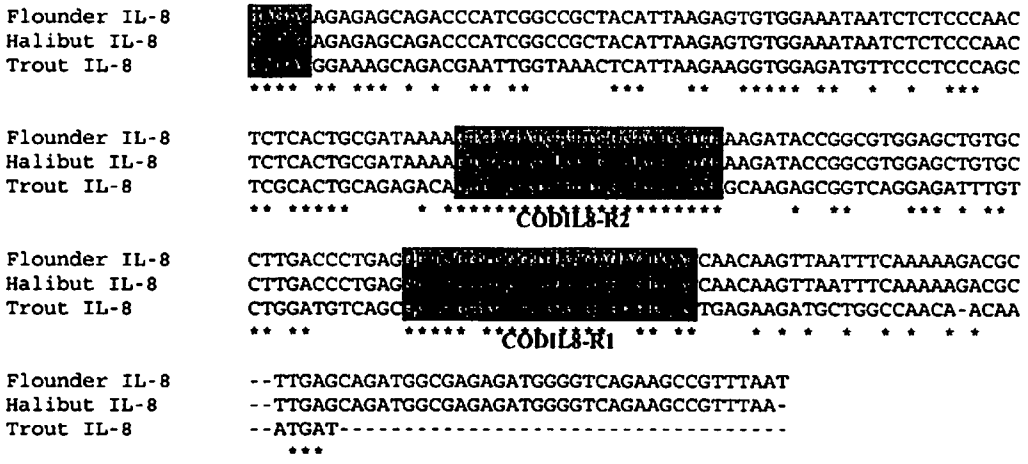

Figure 5. Primers design for known IL-8 fish sequences were aligned and areas of homology used to finally design primers in the highlighted areas biased towards Cod sequence. Forward primer was named CODIL8-F1 and reverse primers were named CODIL8-R1 and CODIL8-R2.

To amplify the haddock IL-8 fragment, 2µl of gDNA from haddock was used along with 4µl of each of the degenerated primers, one forward and a reverse. Together with a master mix containing a reaction buffer, $MgCl_2$, dNTP's and Taq polymerase, the partial IL-8 sequence was amplified by PCR. The PCR conditions are detailed in Table 3.

PCR cycling protocol					
Target gene	Denaturation	Annealing	Elongation	No. Cycles	Product size (bp)
IL-1β	94°C @ 5'	-	-	1	
	94°C @ 1'	60°C @ 1'	72°C @ 1'	5	
	94°C @ 1'	54°C @ 1'	72°C @ 1'	30	
	-	-	72°C @10'	1	114

Table 3. Summary of conditions for the Touchdown Polymerase Chain Reaction for the gene IL-8

The PCR product obtained was ligated into a cloning vector. The procedure used to isolate the correct clone for the RAG-1 was used for IL-8. Clones that were sequenced were aligned with those of other fish species to check it was the correct sequence. The isolated fragment showed good homology with other fish species. The highest identity was 86.8% at amino acid level (See Fig.6).

```
Halibut     MSSRVIVVAVMVLLASLAISEAVSLRSLGVSLHCRCIETESRPIGRYIKSVEIISPNSHD
Trout       MSIRMSASLVVVLLALLTITEGMSLRGMGADLRCRCIETESRRIGKLIKKVEMFPPSSHR
Haddock     ------------------------------LRCRCIQTESRRIGHHIRKVEIIPANSHC
Chicken     -MNGKLGAVLALLLVSAALSQGRTLVKMGNELRCQCISTHSKFIHPKSIQDVKLTPSGPH
Human       MTSKLAVALLAAFLISAALCEGAVLPRSAKELRCQCIKTYSKPFHPKFIKELRVIESGPC
Shark       MNSKVILAVLALFILYLASTQAASLRHAGVSLRCQCIKTNSKFIHPRRMENIEIFPSGPC
                                          *:*:**.* *: :     .  .  ..
```

```
Halibut         -KTEIIATLKDTGVELCLDPEAPWVKRVINKLISKRRLSRWREMGSEAVC
Trout           -DTEIIATLSKSGQEICLDVSAPWVKRVIEKMLANNKC------------
Haddock         EESEIIATL-----------------------------------------
Chicken         KNVEIIATLKD-GREVCLDPTAPWVQLIVKALMAKAQLNSDAPLC-----
Human           ANTEIIVKLSD-GRELCLDPKENWVQRVVEKFLKRAENSH----------
Shark           SNVEIIATLKN-GTPVCLNPEAAWVKKIIDMIIKNSEKTES---------
                 . ***..*
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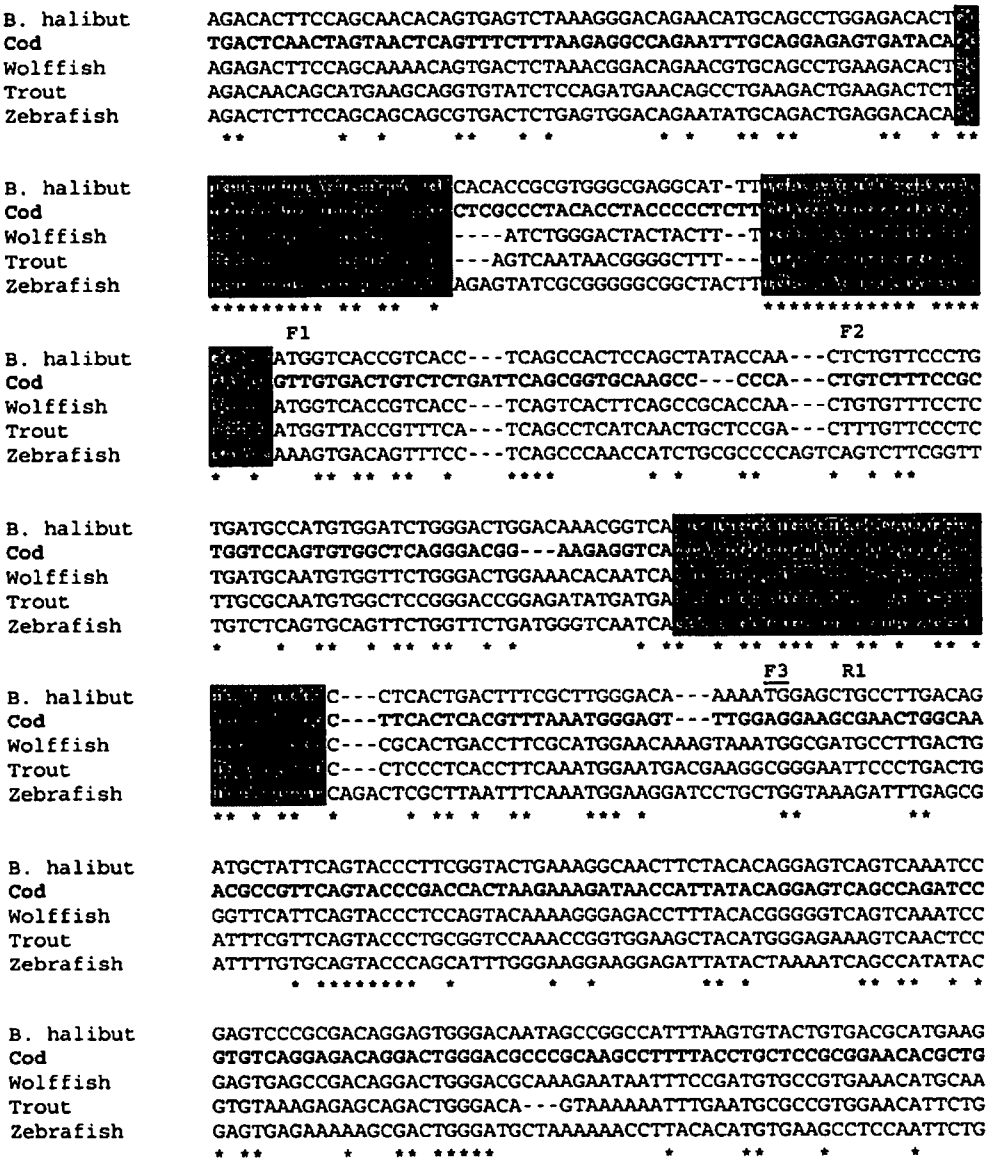
Figure 6. Multiple alignment of the predicted Haddock IL-8 gene with selected known vertebrate IL-8's. Identical (*) and similar (: or .) residues identified using CLUSTAL W program are indicated.

IgM

To obtain the sequence of the haddock IgM, seven primers (four forward and three reverse) were designed using a homology cloning approach. Fig. 2 shows the regions chosen to design the primers for the isolation of IgM, which were biased towards the known Cod sequence.



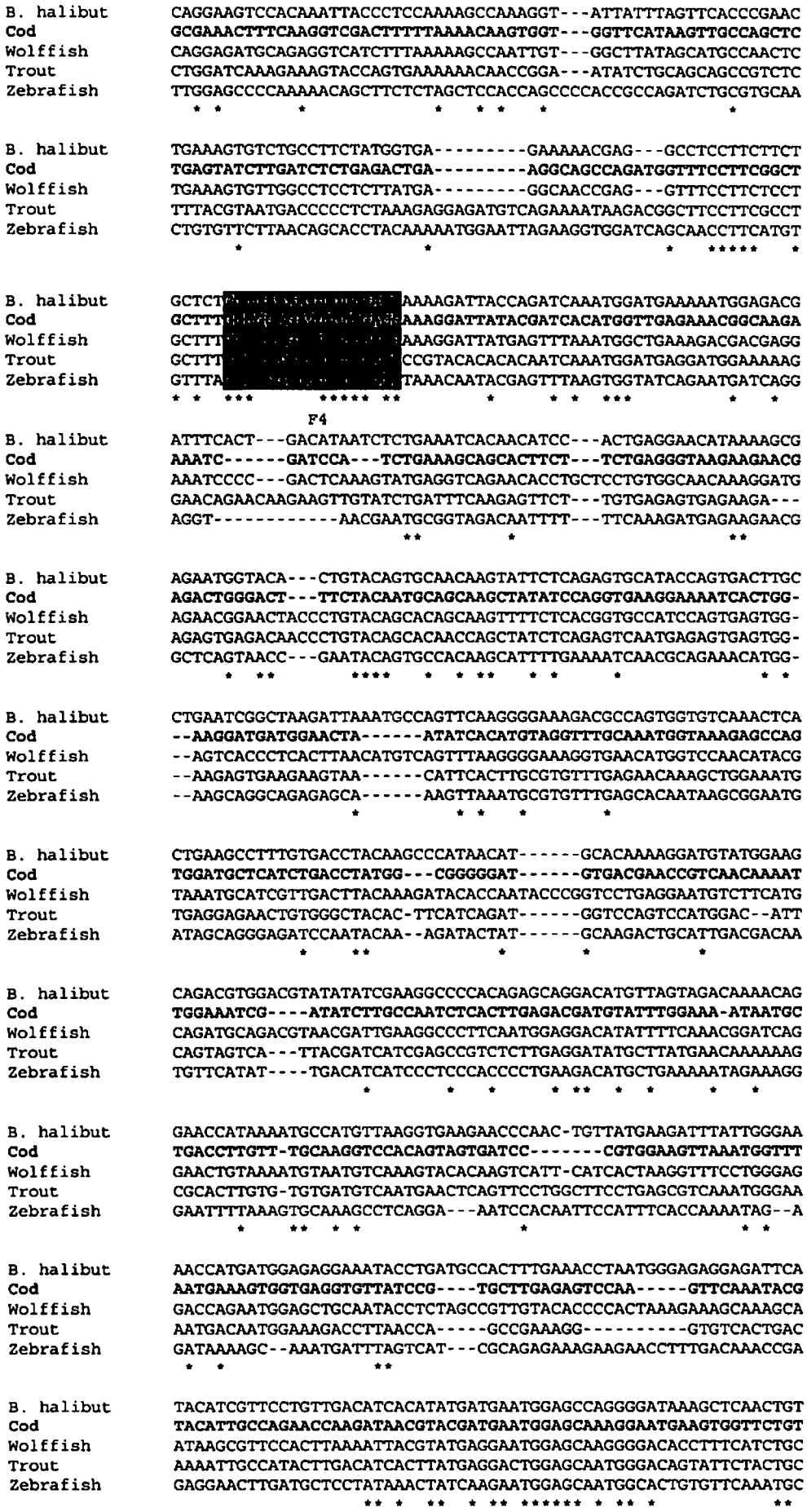

B. halibut CAGGAAGTCCACAAATTACCCTCCAAAAGCCAAAGGT---ATTATTTAGTTCACCCGAAC
Cod GCGAAACTTTCAAGGTCGACTTTTTAAAACAAGTGGT---GGTTCATAAGTTGCCAGCTC
Wolffish CAGGAGATGCAGAGGTCATCTTTAAAAAGCCAATTGT---GGCTTATAGCATGCCAACTC
Trout CTGGATCAAAGAAAGTACCAGTGAAAAAACAACCGGA---ATATCTGCAGCAGCCGTCTC
Zebrafish TTGGAGCCCCAAAAACAGCTTCTCTAGCTCCACCAGCCCCACCGCCAGATCTGCGTGCAA
B. halibut TGAAAGTGTCTGCCTTCTATGGTGA---------GAAAAACGAG---GCCTCCTTCTTCT
Cod TGAGTATCTTGATCTCTGAGACTGA---------AGGCAGCCAGATGGTTTCCTTCGGCT
Wolffish TGAAAGTGTTGGCCTCCTCTTATGA---------GGCAACCGAG---GTTTCCTTCTCCT
Trout TTTACGTAATGACCCCCTCTAAAGAGGAGATGTCAGAAAATAAGACGGCTTCCTTCGCCT
Zebrafish CTGTGTTCTTAACAGCACCTACAAAAATGGAATTAGAAGGTGGATCAGCAACCTTCATGT
B. halibut GCTCT[redacted]AAAAGATTACCAGATCAAATGGATGAAAAATGGAGACG
Cod GCTTT[redacted]AAAGGATTATACGATCACATGGTTGAGAAACGGCAAGA
Wolffish GCTTT[redacted]AAAGGATTATGAGTTTAAATGGCTGAAAGACGACGAGG
Trout GCTTT[redacted]CCGTACACACACAATCAAATGGATGAGGATGGAAAAAG
Zebrafish GTTTA[redacted]TAAACAATACGAGTTTAAGTGGTATCAGAATGATCAGG
F4
B. halibut ATTTCACT---GACATAATCTCTGAAATCACAACATCC---ACTGAGGAACATAAAAGCG
Cod AAATC------GATCCA---TCTGAAAGCAGCACTTCT---TCTGAGGGTAAGAAGAACG
Wolffish AAATCCCC---GACTCAAAGTATGAGGTCAGAACACCTGCTCCTGTGGCAACAAAGGATG
Trout GAACAGAACAAGAAGTTGTATCTGATTTCAAGAGTTCT---TGTGAGAGTGAGAAGA---
Zebrafish AGGT------------AACGAATGCGGTAGACAATTTT---TTCAAAGATGAGAAGAACG
B. halibut AGAATGGTACA---CTGTACAGTGCAACAAGTATTCTCAGAGTGCATACCAGTGACTTGC
Cod AGACTGGGACT---TTCTACAATGCAGCAAGCTATATCCAGGTGAAGGAAAATCACTGG-
Wolffish AGAACGGAACTACCCTGTACAGCACAGCAAGTTTTCTCACGGTGCCATCCAGTGAGTGG-
Trout AGAGTGAGACAACCCTGTACAGCACAACCAGCTATCTCAGAGTCAATGAGAGTGAGTGG-
Zebrafish GCTCAGTAACC---GAATACAGTGCCACAAGCATTTTGAAAATCAACGCAGAAACATGG-
B. halibut CTGAATCGGCTAAGATTAAATGCCAGTTCAAGGGGAAAGACGCCAGTGGTGTCAAACTCA
Cod --AAGGATGATGGAACTA------ATATCACATGTAGGTTTGCAAATGGTAAAGAGCCAG
Wolffish --AGTCACCCTCACTTAACATGTCAGTTTAAGGGGAAAGGTGAACATGGTCCAACATACG
Trout --AAGAGTGAAGAAGTAA------CATTCACTTGCGTGTTTGAGAACAAAGCTGGAAATG
Zebrafish --AAGCAGGCAGAGAGCA------AAGTTAAATGCGTGTTTGAGCACAATAAGCGGAATG
B. halibut CTGAAGCCTTTGTGACCTACAAGCCCATAACAT------GCACAAAAGGATGTATGGAAG
Cod TGGATGCTCATCTGACCTATGG---CGGGGGAT------GTGACGAACCGTCAACAAAAT
Wolffish TAAATGCATCGTTGACTTACAAAGATACACCAATACCCGGTCCTGAGGAATGTCTTCATG
Trout TGAGGAGAACTGTGGGCTACAC-TTCATCAGAT------GGTCCAGTCCATGGAC--ATT
Zebrafish ATAGCAGGGAGATCCAATACAA--AGATACTAT------GCAAGACTGCATTGACGACAA
B. halibut CAGACGTGGACGTATATATCGAAGGCCCCACAGAGCAGGACATGTTAGTAGACAAAACAG
Cod TGGAAATCG----ATATCTTGCCAATCTCACTTGAGACGATGTATTTGGAAA-ATAATGC
Wolffish CAGATGCAGACGTAACGATTGAAGGCCCTTCAATGGAGGACATATTTTCAAACGGATCAG
Trout CAGTAGTCA---TTACGATCATCGAGCCGTCTCTTGAGGATATGCTTATGAACAAAAAAG
Zebrafish TGTTCATAT----TGACATCATCCCTCCCACCCCTGAAGACATGCTGAAAAATAGAAAGG
B. halibut GAACCATAAAATGCCATGTTAAGGTGAAGAACCCAAC-TGTTATGAAGATTTATTGGGAA
Cod TGACCTTGTT-TGCAAGGTCCACAGTAGTGATCC-------CGTGGAAGTTAAATGGTTT
Wolffish GAACTGTAAAATGTAATGTCAAAGTACACAAGTCATT-CATCACTAAGGTTTCCTGGGAG
Trout CGCACTTGTG-TGTGATGTCAATGAACTCAGTTCCTGGCTTCCTGAGCGTCAAATGGGAA
Zebrafish GAATTTTAAAGTGCAAAGCCTCAGGA---AATCCACAATTCCATTTCACCAAAATAG--A
B. halibut AACCATGATGGAGAGGAAATACCTGATGCCACTTTGAAACCTAATGGGAGAGGAGATTCA
Cod AATGAAAGTGGTGAGGTGTTATCCG----TGCTTGAGAGTCCAA-----GTTCAAATACG
Wolffish GACCAGAATGGAGCTGCAATACCTCTAGCCGTTGTACACCCCACTAAAGAAAGCAAAGCA
Trout AATGACAATGGAAAGACCTTAACCA-----GCCGAAAGG----------GTGTCACTGAC
Zebrafish GATAAAAGC--AAATGATTTAGTCAT---CGCAGAGAAAGAAGAACCTTTGACAAACCGA
B. halibut TACATCGTTCCTGTTGACATCACATATGATGAATGGAGCCAGGGGATAAAGCTCAACTGT
Cod TACATTGCCAGAACCAAGATAACGTACGATGAATGGAGCAAAGGAATGAAGTGGTTCTGT
Wolffish ATAAGCGTTCCACTTAAAATTACGTATGAGGAATGGAGCAAGGGGACACCTTTCATCTGC
Trout AAAATTGCCATACTTGACATCACTTATGAGGACTGGAGCAATGGGACAGTATTCTACTGC
Zebrafish GAGGAACTTGATGCTCCTATAAACTATCAAGAATGGAGCAATGGCACTGTGTTCAAATGC

Figure 6. Primers design for known IgM fish sequences were aligned and areas of homology used to finally design primers in the highlighted areas biased towards Cod sequence. Forward primers were named F1, F2, F3 and F4 and reverse primers were named R1, R2 and R3.

To amplify fragments from the IgM gene 2µl of each combination of the designed primers were used along with 2µl of cDNA from haddock spleen together with a master mix containing a reaction buffer, $MgCl_2$, dNTP's and Taq polymerase. Three partial IgM sequences were amplified by PCR as detailed in Table 4. The three fragments obtained were between the primers F2 and R1, F1 and R1 and the largest with 636 bp between F4 and R3.

PCR cycling protocol					
Target gene	Denaturation	Annealing	Elongation	No. Cycles	Product size (bp)
IgM	94°C @ 5'	-	-	1	
	94°C @ 1'	48°C @ 1'	72°C @ 1'	35	
	-	-	72°C @10'	1	145-636

Table 4. Summary of conditions for the Polymerase chain reaction for the gene IgM.

The PCR products obtained were ligated into a cloning vector. The procedure used to isolate the correct clone for the previous genes was used for IgM. Clones that were sequenced were aligned with those of other fish species to check it was the correct sequence. The isolated fragments showed good homology with other fish species reaching a 90% identity at the nucleotide level and 85% at amino acid level in the largest fragment when aligned with the Cod sequence (see Fig 7).

```
Cod             AADFSPKDYTITWLRNGKKIDPSESSTSSEGKKNETGTFYNAASYIQVKENHWKDDGTNI
Haddock         AADFSPKDYTITWLRNGKKIDPSESRTSSEGKKNETGLFYTAASYIQVEENRWKDDGTNI
                ************************** *********** **.*******:**:*********

Cod             TCRFANGKEPVDAHLTYGGG-CDEPSTKLEIDILPISLETMYLENNADLVCKVHSSDPVE
Haddock         TCRFANSEAHVDASLTYGSGPGCEASTKLEIDILPISLETMYLENNAELVCKVHSSDPVE
                ******.:  *** ****.*   *.**********************:************

Cod             VKWFNESGEVLSVLESPSSN--TYIARTKITYDEWSKGMKWFCEASIKDSIEVPTRKYFV
Haddock         VKWFNESGEVLSKLEKSSSNRPTYIATAKITYDEWSKGMNWYCRAGIKDSIEEPTRKYFS
                ************ **..*** **** :************:*:*.*.****** ******

Cod             KNNGRNRVPPSVYLLPPVDDLSCTNMTLTCFVKD
Haddock         KNNGPNRVPPSVYLLPPVDDLSCTNMTLTCFV--
                **** ***************************
```

Figure 7. Alignment of the predicted Haddock IgM gene with Cod known IgM gene. Identical (*) and similar (: or .) residues identified using CLUSTAL W program are indicated.

FUTURE WORK

1) cDNA has been made to obtain the full coding sequence of RAG-1, IL-1β, IL-8 and IgM, using 3'RACE and 5'RACE.

2) A Gene Walking library has been constructed which will allow the promotor of these genes and their genomic organisation to be determined.

3) Tissues will be isolated from immune stimulated and non-stimulated fish, cDNA will be prepared from mRNA of these tissues and the expression of RAG-1, IL-1β, IL-8 and IgM investigated.

4. Trials

Trial 1. Ontogeny of the immune system

A trial to study the ontogeny of the immune system started in March 2002 (Task 10.0). The aim of this experiment was the collection of egg and larval material for RNA extraction and production of cDNA from samples. The cDNA will be used in RT-PCR to determine when the immune system starts to develop using the RAG-1 gene as a marker.

Samples were collected at intervals of 2 days during the egg phase and 5 days during larval stage, with a more intense sampling around hatching (110°days), when it was on a daily basis. Every sample consisted of three replicates, from two populations: Wild Broodstock and Hatchery reared Broodstock (second generation). The reason for sampling these two was to find out if there is any difference on the immune system development between wild and hatchery-bred fish. Sampling started in March collecting eggs (500 in each sample per replicate), and following larval development until weaning. Larval samples consisted of 100 larvae per sample in duplicate for the first six days post-hatching and from then on 50 larvae per sample in duplicate. The replication of the sampling was done in case during RNA extraction any sample was lost. Sampling was completed by the end of May.

In July 2002 RNA extraction of all the samples was carried out at the facilities of the University of Aberdeen. Together with the egg and larval material, tissues extracted from adult haddock were taken for more RNA extraction. This RNA was then reverse transcribed into cDNA and then as a positive control of the RT-PCR the housekeeping gene βactin was amplified by a PCR. All samples of cDNA were then stored at -20°C for later investigation.

Now that the partial sequence of the RAG-1 has been obtained, probes for this gene have been designed to use with all the stored samples to determine when this gene starts to be expressed in young haddock.

Trial 2. Initial Immune Response Assessment

In order to study the immune response of haddock to immunisation, two populations of 10 fish each have been vaccinated. The vaccine used was an immersion vaccine from AVL (AquaVac Vibrio), which contains formalin killed *Vibrio anguillarum* (Biotype I and II)). The delivery method was injection of 100µl in the peritoneum. The aim of this immunisation is to follow the antibody synthesis throughout a period of 12 weeks.

The experiment started on the 27th of September of 2002 and finished on the 19th of December. Two populations were then vaccinated, one of 34 adult haddock (approx. 18 months-old) 10 of which were sampled. From the juvenile population, year class 2002 (140-176dd) with 57 fish, 10 were vaccinated and consequently sampled.

Juveniles had been already dip-vaccinated for *Vibrio anguillarum* with the same vaccine used for the immunisation trial. Blood sampling (0.5-1ml, depending on fish size) occurred every 4 weeks to obtain serum.

The serum obtained was used in agglutination assays to study the effect of the vaccination in the antibody production in both fish populations. The assays were carried out as follows. One 96-well plate (U bottom) was used for each sampling point, where 50µl of PBS was added to all the wells of each plate. Subsequent to this, 50µl of the serum from 8 of the fish sampled were inoculated in the first column and mixed by pippetting. Finally samples were serially diluted and 50µl of $5x10^8$cfu/ml of *Vibrio anguillarum* was added and mixed. Plates were covered and left for 48h at 4°C.

Positive agglutination was noted down in each of the plates and then expressed as the $-\text{Log}_2$ of the serum dilution which gave the positive agglutination (Fig. 8 and 9).

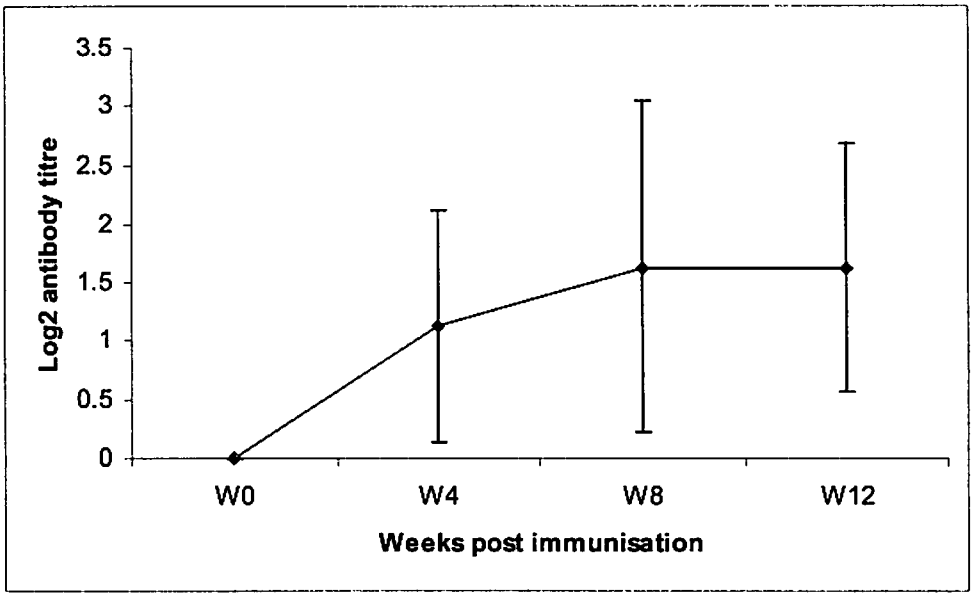


Figure 8. Antibody titres post immunisation in Adult Haddock population. Titre is indicated as the -Log2 of the dilution were agglutination takes place. Bars indicate the standard deviation of the eight replicates.

Both in adults and in juveniles there is an increase in the antibody presence in the serum, although at a very low level, having also great variability between individuals, as the standard deviation bars indicate.

Further analysis of these results are being done.

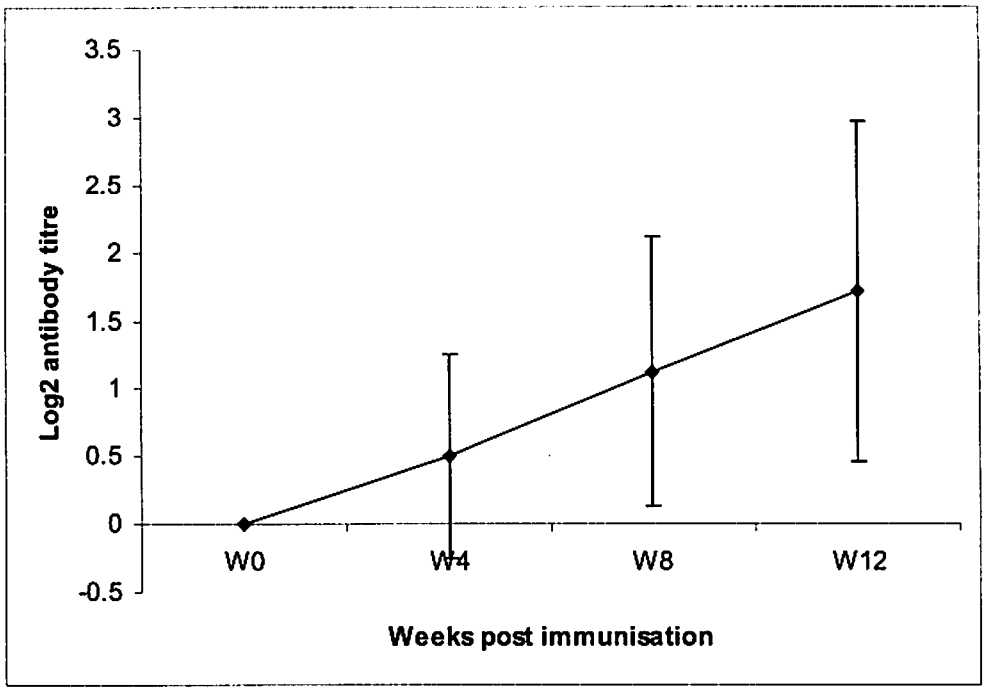


Figure 9. Antibody titres post immunisation in Juvenile Haddock population. Titre is indicated as the -Log2 of the dilution were agglutination takes place. Bars indicate the standard deviation of the eight replicates.

Trial 3. Assessment of Immune parameters in Haddock

This study started on the 28th of November 2002 and will continue for 12 months, sampling every three months. Ten adult fish (season 1999) are being held in a 3.6 m diameter, 16 m^3 tank and under normal husbandry procedures. Fish are pit-tagged to allow individual identification throughout the experiment.

At each sampling 1-2ml blood is collected and fish length and weight is recorded. Blood is collected by two different methods, with or without heparin. The first is used to obtain the hematocrit, a count of total numbers of blood cells and to produce blood smears to make differential cell counts. Serum is extracted from the blood that has not been heparinised and will be used to carry out Lysozyme and Antiprotease assays.

Overall this analysis will give a wide study of the behaviour of the immune system of haddock throughout the seasons. This will be helpful to assess the best time for vaccination.

Trial 4. Ontogeny of the immune organs

During the spawning season of 2003, samples were collected from a population of recently hatched larvae up to weaning. The aim of this sampling was to collect material to study the development of the immune organs in Haddock by histology.

Samples were taken in a daily basis for the first 15 days after hatching, after which it was decreased to every two days until 40 days post hatch (dph). Finally the last 25 days samples were collected every 5 days.

All samples are now stored in 70% Ethanol after being fixed overnight in 4% phosphate-buffered formaldehyde (pH 7.0), awaiting analysis.

Trial 5. Whole Mount *in situ* Hybridisation

The second sampling undertaken during the 2003 spawning season was made to collect material to be used for a technique which will allow us to locate the organs where a gene is being expressed. The procedure carried out for the collection of the samples was the following: Larvae were fixed overnight in Paraformaldehyde in the fridge at 4°C. After this, samples were transferred to 100% Ethanol and stored in groups of 10 eggs/larvae in the freezer at -20°C until further treatment. Samples were taken until 40 dph in a daily basis until 15 dph after which sampling was every two days.

Whole larvae will be hybridised using the RAG-1 probe previously designed for this purpose.

Trial 6. Cod larvae immunostimulation via live food

This trial is part of a project carried out at the Ardtoe facilities for EWOS during 2003, where a novel nucleotide enrichment for live food was delivered to cod larvae during first feeding. The purpose of this part of the experiment is to study the impact of this diet on the immune system of the larvae.

Each treatment consisted of 4 replicates in 80-litre tanks containing 50 larvae per litre. Once larvae hatched they were transferred to the tanks where they were fed rotifers for the first 30 days, after which they were then fed with artemia until they were weaned at 50 dph. Treatments were as follows:

- ✓ CONTROL: Larvae fed with rotifers and artemia enriched with *in house* enrichment.
- ✓ NUC-ROT: Larvae fed with rotifers and artemia enriched with nucleotides diet.
- ✓ NUC-ART: Larvae fed with rotifers enriched with *in house* enrichment and artemia enriched with nucleotide diet.

Larvae were sampled four times throughout the experiment, at 17 dph and 31 dph during the rotifer phase, and in the artemia phase at 40 dph and 50 dph. Fifty larvae from three tanks of each treatment were fixed in TRIzol (1ml per 50-100mg sample) and stored at -80°C.

RNA was extracted from the larval material, reverse transcribed into cDNA and as a positive control, the housekeeping gene β-actin was amplified by a PCR. Finally the cDNA was stored at -20°C.

Probes were then designed using a homology cloning approach for the following genes involved in immune system: IL1-β, TCRa, TCRb, IgM and RAG-1. Using these probes, the samples are currently being analysed.

3. Initial Vaccine Development

Trial 1. Study of Challenge Methods

An experiment to assess the most efficient method of challenge (Tasks 7.7-7.13) started on the 5th of November 2002 at the challenge facilities of Marine Harvest (MH), Lochailort. This study will allow the most effective method for the final assessment of the vaccination trial to be determined.

Three different delivery methods were studied; **immersion** in a bath containing 10^7cfu/ml, **injection of 10^5cfu/ml** in 100µl Phosphate Buffered Saline (PBS) and **cohabitation** of 30 fish with 10 others **injected with 10^7cfu/ml** in PBS. For the first two methods 10 fish were used per treatment. Fish were marked with a panjet accordingly to each treatment and allocated in three tanks for the duration of the experiment.

To carry out this trial, *V. anguillarum* from the lab at MH was used. Bacteria used for injection were grown by platting into petri dishes containing blood seawater agar with 1.5% salt. After colonies had grown they were suspended in PBS. Bacterial concentration was measured by Optical density. At 540 nm an OD of 0.95-1.05 is approximately equivalent to 10^9cfu/ml. Serial dilutions to obtain the required concentrations were then made in PBS. Bacteria for Immersion exposure were grown in liquid media, Trypton soy broth with 1.5% salt. They were then washed and resuspended in saline.

The experiment was to be finished after one month or earlier if mortality was faster, but some problems with the cultured bacteria used for the challenge resulted in the experiment being terminated early. It seems that the bacteria were insufficient or not virulent enough, as none of the fish had died after two weeks.

On the 10th of December of the same year the experiment was repeated with fish of 86g average weight. The experiment resulted, as before, with no mortalities after 4 weeks. It seems likely that this is due to a low virulence of the strain provided by MH.

Once more the challenge experiment was repeated in the 24th of March 2003. This time the strain used for the trial was obtained from the Fisheries Research Services, Marine Laboratory, Aberdeen. This *Vibrio* named MT 2582, was used successfully in previous trials carried out at the Marine Lab. Fish had an averaged weight of 177.55g and were challenged as described previously.

Five days after fish were challenged, the first mortalities started to occur and continued steadily for 20 days. After four weeks and 3 days after the last mortality was registered, the experiment was terminated.

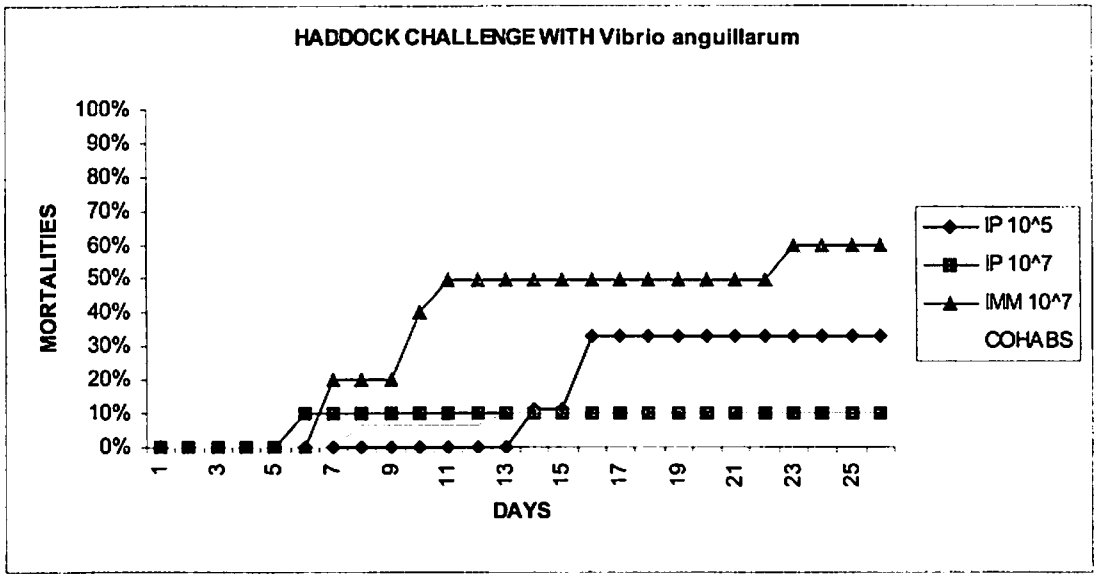


Figure 10. Cumulative mortalities in haddock post challenge. IP=Intraperitoneal injection of 100µl of 105 or 107 cfu/ml, IMM=Immersion in bath containing 10^7cfu/ml for a period of 30 min, COHABS=Cohabitation where a group of 30 fish was exposed to the group of fish infected with a 10^7cfu/ml IP injection

The group that proved to be more susceptible to the infection were those immersed in a bath containing 10^7cfu/ml of *Vibrio anguillarum* reaching 60% mortality (See Fig.10). This is indeed an ideal method to challenge, as requires little effort; and also less stressful for the fish, therefore the mortalities are more indicative of the infection and not due to stress. Also, injection can cause side infections due to the wound produced by the needle. These reasons together with the positive result of the challenge allowed us to make the decision to use this method as a delivery of *Vibrio* infection in haddock in following experiments.

Trial 2. Assessment of Vaccine Delivery.

An experiment to assess the most efficient delivery method of a formalin-killed *V. anguillarum* vaccine on fish of 86g average weight started on the 17th of December (Task 7.1-7.6, 4.10). It will have a duration of 4 months, when fish will be transported to MH, Lochailort to assess the vaccination by challenge with a virulent strain of *V. anguillarum*.

A number of delivery methods were chosen, which included:

- ✓ HANDLING CONTROL: Fish are only handled.
- ✓ PLACEBO CONTROL: Injected intraperitonally with 100µl PBS as placebo vaccination.
- ✓ IMMERSION: dipped for 60sec in vaccine.
- ✓ INTRA-PERITONEAL INJECTION: Injected with 100µl of vaccine into the peritoneum
- ✓ INTRA-MUSCULAR INJECTION: Injected with 100µl of vaccine into the dorsal muscle.
- ✓ ORAL: Administrated 100µl of vaccine orally by intubation.

A total of 45 fish were allocated to each treatment and marked with a panjet to recognize treatments (Fig. 11). Individual lengths and weights were recorded. Fish were then allocated to two tanks with all the treatments in equal number in each of the tanks to avoid tank effects. The vaccine used, formalin-killed *V. anguillarum*, was made with bacteria grown at the Lochailort, MH facilities adjusted to a concentration of 10^9cells/ml.

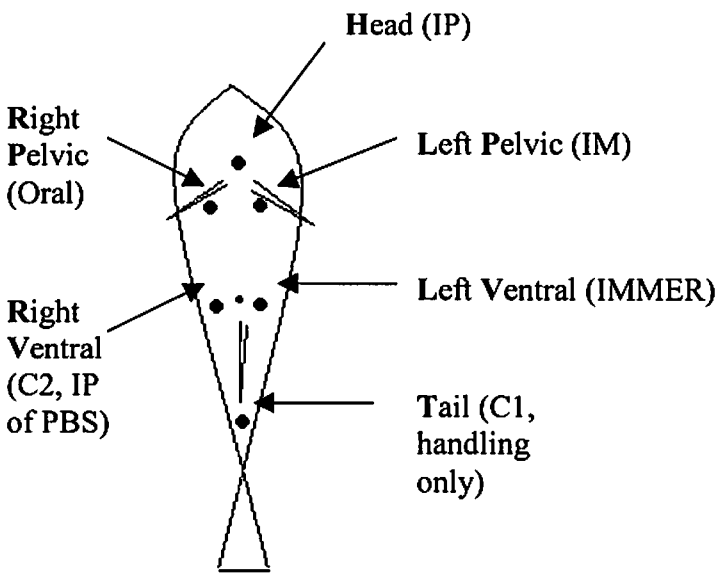


Figure 11. Panjet marks for each vaccination treatment. IP= Intraperitoneal, IM= Intramuscular, IMMER= Immersion, C1= Control 1, handling only, C2= Control 2, Injection intraperitoneal of PBS, Oral= Oral intubation

Vaccination efficacy was finally assessed by challenging the fish in a bath containing 10^7cfu/ml. Three days post-challenge mortalities started to occur in all treatments, reaching the pick of mortalities in only 4 days (See Fig.12). Two weeks after challenge the trial was terminated.

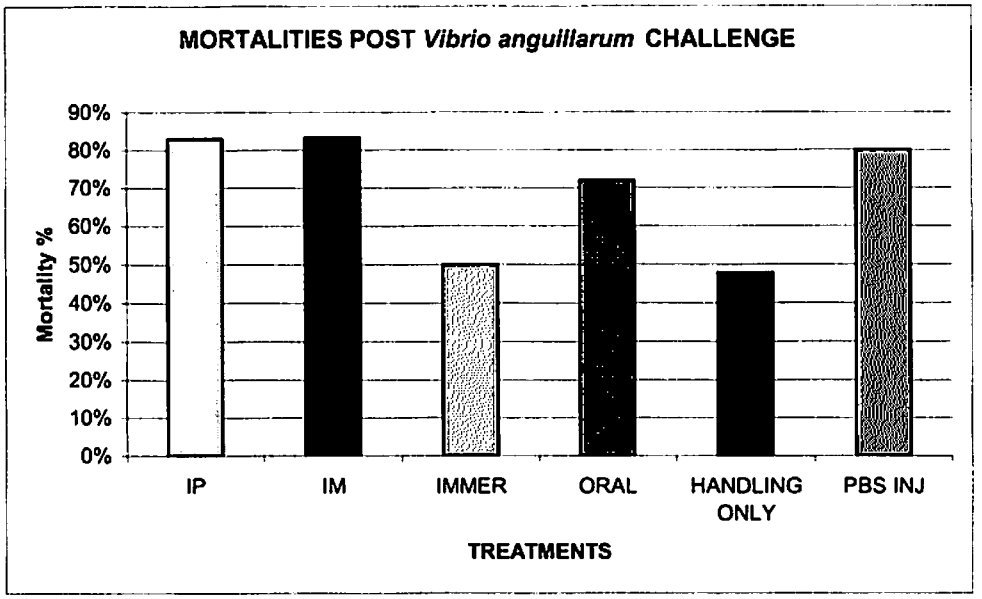


Figure 12. Cumulative mortalities after challenge with *Vibrio anguillarum* by immersion. IP= Intraperitoneal, IM= Intramuscular, IMMER= Immersion, C1= Control 1, handling only, C2= Control 2, Injection intraperitoneal of PBS, Oral= Oral intubation

The results obtained from the challenge showed that none of the treatments had protective effect on the fish. The treatments with a higher index of mortality were seen among the fish that were injected by the following methods: IP, IM or PBS (control group 2) and Orally intubated, reaching more than 80% mortality. This result is difficult to interpret, as mortalities do not follow the expected pattern of protection. Stress may have had an effect on the increased mortalities seen in those groups with a more traumatic delivery of the vaccine when compared to Immersion and Handling only Control which are typically less stressful. Even though challenge was made six months after vaccination, stress could still have a negative effect on the survival of the fish. This trial will be compared at a later stage with the next vaccination trial, currently in progress, to elucidate these results.

Blood samples were taken from each treatment before vaccination and a week before challenge. The serum obtained will be used for Agglutination, Lysozyme and Antiprotease assays.

Samples for histology were also taken for antibody analysis of the *Vibrio* bacteria in different tissues every six weeks. An assessment of secondary effects produced by the vaccination was made by studying growth loss, intra-abdominal adhesions and damage on gill structure. There was no indication of any secondary effects due to the vaccination.

Trial 3. Juvenile vaccination regimes

With this trial we aim to obtain a result that allow us to give the industry of haddock farming an indication of when and how to vaccinate juvenile haddock to obtain the most efficient protection. Using 900 juveniles of 3.4g average we separate them in 9 groups following different regimes of vaccination.

A: Immersion vaccination @ 3.4g, IP Boost @ 35g
B: Immersion vaccination @ 3.4g, Immersion Boost @ 15g, IP Boost @ 35g
C: Immersion vaccination @ 3.4g, Immersion Boost @ 15g
D: Immersion vaccination @ 3.4g
E: **E.1**: IP vaccination @ 35g
E.2: IP vaccination @ 35g with AVL vaccine
E.3: IP vaccination @ 35g with Lochailort strain (MH)
E.4: IP vaccination @ 35g with Lochailort strain plus adjuvant (MH+adjvt)
F: Control (no vaccination)

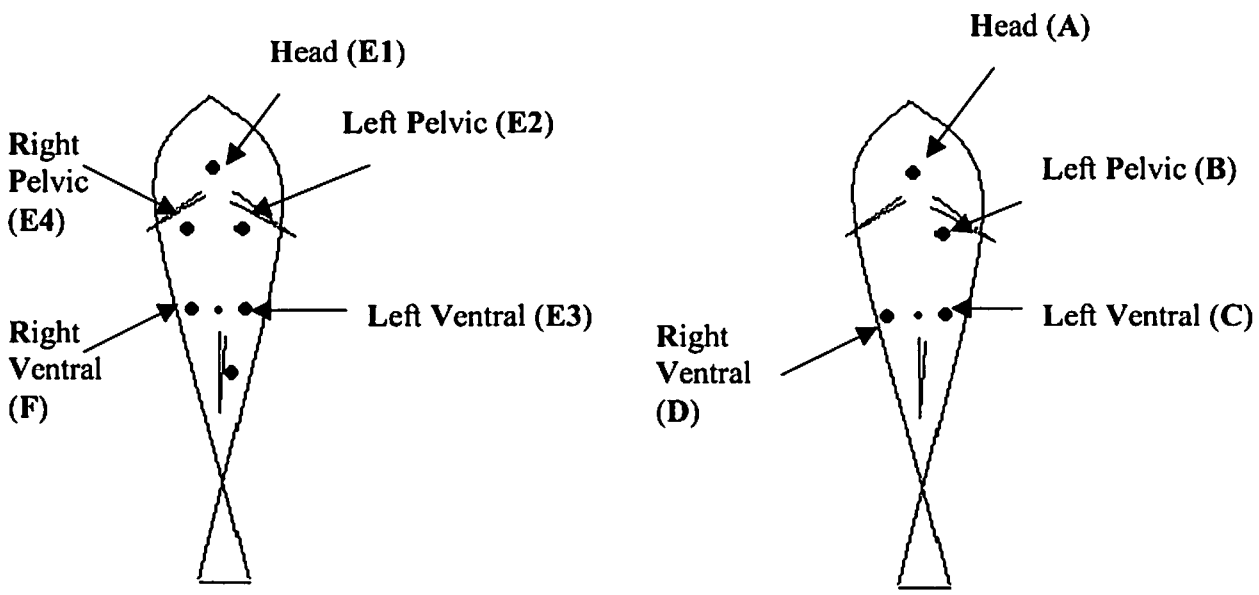


Figure 13. Panjet marks in different treatments. Panjet marks with a combination of low Left ventral plus other mark in group F: Control fish (not vaccinated) and fish vaccinated with IntraPeritoneal injection at 35g: E1: Aberdeen vaccine, E2: AVL vaccine, E3: Lochailort vaccine, E4: Lochailort vaccine plus adjuvant. Single marks in other treatments. A: Immersion at 3.5g

and IP injection Boost at 35g; B: Immersion at 3.5g, Immersion Boost at 15g, IP injection Boost at 35g; C: Immersion at 3.5g and Immersion boost at 15g; D: Immersion at 3.5g.

The fifth group (E) was divided in 4 subgroups where we will be testing the efficacy of different vaccines. This will allow us as well to determine the efficiency of each of these vaccines. We are using a commercial vaccine provided by AVL-Schering Plough Aquaculture which will be compared to two vaccines that consist in formalin-killed *Vibrio anguillarum*. The two vaccines that we manufactured are the strain MT2582, which gave a successful challenge results, and the same vaccine that was used for the previous vaccination trial, obtained from Marine Harvest, Lochailort. We have two groups of this last vaccine, one with the normal formalin-killed vaccine and another one that uses an adjuvant provided by AVL-Schering Plough Aquaculture. The vaccine used in all the other treatments is MT2582.

This trial was started in the 24th of July and vaccinations were made in a month interval, being the last batch vaccinated in mid September. On the 8th of December, 5 months after the beginning of the trial, a total of 50 fish per treatment were challenged with MT2582 at the Marine Harvest facilities. Fish were allocated in 5 tanks with 10 fish of each group in each of the tanks two weeks before challenge to allow the acclimatisation of the fish.

The method of challenge was as follows; 50 l tanks were filled with 40.5 l of water and mixed with a solution of 500ml containing 10^9cfu/ml of *Vibrio anguillarum* (MT2582) to obtain a bath of 10^7cfu/ml. A fresh bath was made for each of the 5 tanks. Fish were netted out of their tanks and immersed in the bath for a period of 30min, after which were quickly returned to their original tanks. Oxygen was provided for the duration of the challenge.

Mortalities started after day 7-post challenge and the trial was finished on day 30 post-challenge, 7 days after the last mortality. Even though mortalities only reached a 28%, it does show an interesting trend. As expected, the highest mortality corresponds to the control group, the rest of the other groups that were vaccinated mortalities were between 0 and 6% of mortality (see Fig 14.). All vaccinated groups are significantly different when compared to the control group.

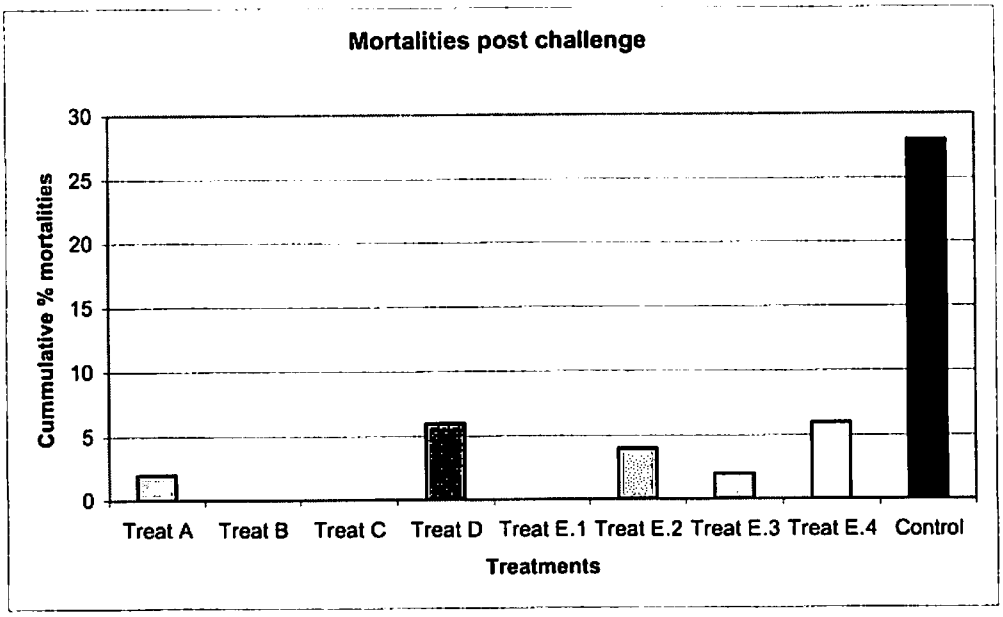


Figure 14. Cummulative mortalities post challenge. Treatments are as follows. A: Immersion at 2g and IP injection Boost at 20g; B: Immersion at 2g, Immersion Boost at 5g, IP injection Boost at 20g; C: Immersion at 2g and Immersion boost at 5g; D: Immersion at 2g; F: Control fish (not vaccinated) and fish vaccinated with IntraPeritoneal injection at 20g: E1: Aberdeen vaccine, E2: AVL vaccine, E3: Lochailort vaccine, E4: Lochailort vaccine plus adjuvant.

The highest mortalities among the vaccinated groups were recorded in the groups where the Lochailort vaccine with adjuvant and the fish vaccinated with an immersion at 2g. There is also a 2% mortality in the group IP vaccinated with the Lochailort vaccine confirming that this vaccine does not give as good protection as the Aberdeen strain, although it does protect compared to the control.

The only secondary effects registered in the trial were seen in the group E.4, were an adjuvant was used. All fish showed adhesions from severe to very severe.

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