Fish Health

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7FP---KBBE---2013---603121
Annual Coordination Meeting
Bari 4-6 November 2014

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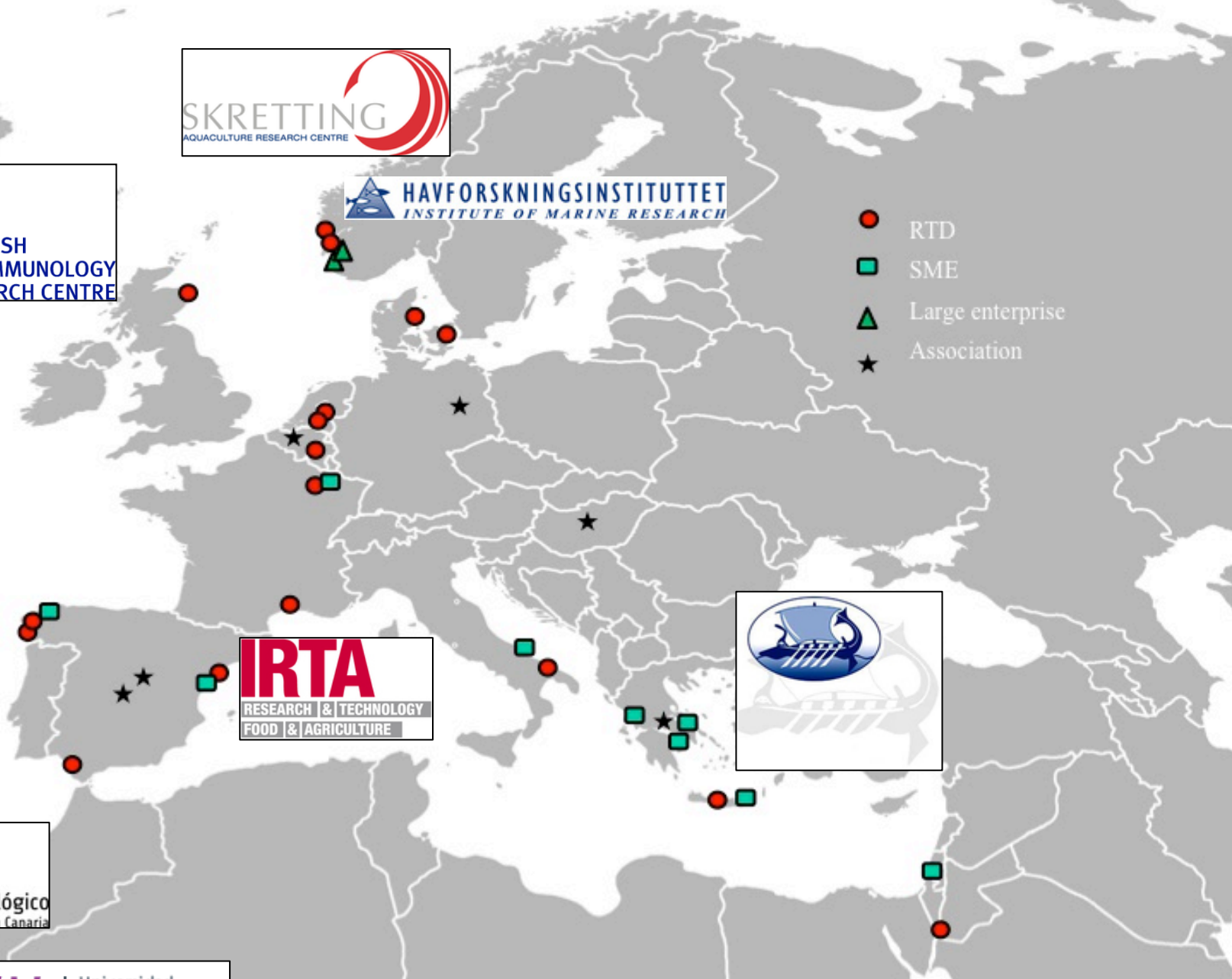
HCMR (P1)
FCPCT
IRTA
UNIABDN
SARC

WP 24 Fish health - meagre

HCMR
FCPCT
UNIABDN (P5)
IEO
ULL

WP 25 Fish health - greater amberjack

IMR (P7)

WP 26 Fish health - Atlantic halibut

WP24 Fish health: Meagre

This WP will address bottlenecks relating to meagre health. Tasks include:

(a) studies of key disease states.
(b) development of appropriate treatments.
(c) a first characterisation of the meagre immune system/ responses required for future immune intervention.

Milestones:

Month 12 (MS51) - Design of primers for amplification of meagre target gene DNA sequences.

Month 24 (MS52) - Grow-out of larvae and collection of samples from immune ontogeny time-line.

Month 30 (MS53) - Amplification and sequencing of target gene sequences from stimulated tissues.

Month 36 (MS54) - Completion of challenge and collection of samples for study of immune gene modulation.

Deliverables:

Month12 (BUT – asked to move to Month 20)
D24.1 - The effect of vitamin D inclusions in diets in the development of Systemic Granulomatosis in meagre.

Month 24
D24.2 - The effect of Ca/P ratio in the diet in the development of Systemic Granulomatosis in meagre.

Month 26
D24.3 - Cloning of key marker genes of innate and adaptive immune responses in meagre.

WP25 Fish Health: Greater amberjack

This WP will address bottlenecks relating to amberjack disease control. Tasks include:

(a) dietary regimes that improve larval and adult disease resistance.
(b) diagnostic tests for several major pathogens.
(c) immune markers to aid selection of resistance, with a focus on mucosal defences.

Milestones:

Month 18 (MS58) - Design of primers for amplification of amberjack target gene DNA sequences.

Month 30 (MS59) - Successful Chlamydia screening and sequencing.

Month 30 (MS60) - Samples collected from stimulated primary cultures/explants, ready for immune gene expression analysis.

Deliverables:

None before Month 39!!

WP26 Fish health: Atlantic halibut

Sonal Patel and Audun Nerland

Will liaise with the TargetFish programme (EU 7th FP).

This WP will address a key bottleneck relating to Atlantic halibut larval health, namely nodavirus (Viral Neural Necrosis, VNN) outbreaks in larval and juvenile stages. Tasks include:

Task 26.1 Production of VNN capsid protein.

Task 26.2 Monitor and assess immune response and protection – Activity in this Task planned in Yr 2-Yr 3.

Deliverables:

Month 24

D26.1 - Assess the use of two eukaryotic expression systems; microalgae and a protozoa Leishmania tarentolae) for production of nodavirus capsid protein

Month 36

D26.2 - Testing of the delivery of vaccine candidates via artemia to halibut larvae

Progress to date

Everything looks to be on target at this early stage.

Need to keep an eye on diets to be made and that the immune genes are cloned without difficulty over the coming year.

WP24 Fish Health

HCMR

Vitamin D experiments

- Started in July 2014
- Duration 3 months
- Ends in October 2014

Experimental design

- 3-month old fish (~5g)
- Twelve 500L tanks each containing 50 fish
- 4 treatments; D0, D1, D2, D3

Ingredient	D0	D1	D2	D3
Soyabean meal	10	10	10	10
Fish meal	50	50	50	50
Wheat	17,4	16,9	16,3	14,3
Corn gluten	14,6	14,6	14,6	14,6
Fish oil	7,5	7,5	7,5	7,5
Premix	0,5	0,5	0,5	0,5
Vitamin D		0,49	1,1	3,1
Vitamin D (IU/Kg)	4550	7000	10000	20000

Sampling

- 3 samplings performed to date
- Growth assessment
- Samples for granuloma assessment
- Histology
- Enzyme analysis
- Molecular analysis
- Last sampling at the end of experiment

Histology/pathology

- Several histochemical techniques are being tested for the best description of lesions.

- Both SEM and TEM of the granulomas has been conducted, as well as EDS analysis.

- This work is in progress and the description of the pathology is being prepared as a manuscript.

Nocardia work

- Fish farms in Greece working with meagre have been informed.

- Several attempts to isolate the pathogen have been made using different media, with no results so far.

- The type strain of *Nocardia seriolae* has been purchased from an international collection but there appears to be contamination in the strain sent!

WP24 Fish Health

FCPCT

Task 24.1: Systemic granulomatosis

1. Mass production of meagre juveniles for feeding trials (subtask 24.1.1).

2. Diets for feeding trial (subtask 24.1.1) will be finished and received at FCPCT by November 2014.

Task 24.7: Description, diagnosis and treatment of other bacterial/virus infection diseases occuring in meagre

1. Routine sampling for bacterial and viruses of natural occurence in meagre.

2. Challenge test nº1.

SYMPTOMS

None. Routine sampling.

MICROBIOLOGY

Several different strains of bacteria are isolated.

Beware! Nocardia spp. sent for typing, discarded by others because they are frequently normal microbiota.

August 2014 Meagre (3.6 g)   Task 24.7

SYMPTOMS

Ulceration, erosion of the tail.

MICROBIOLOGY

Bacteriology and culture - Different strains of bacteria have been isolated. Species to be determined.

Probable cause: cannabalism.

September 2014. Meagre adults   Task 24.7

SYMPTOMS
Mass mortality in one culture tank.

MICROBIOLOGY
Bacteriology and culture – No important bacteria isolated.

Virus analysis in progress.

CHALLENGE TEST Nº 1.

Design: Sublethal dose of opportunistic bacteria.
INDIVIDUALS: Meagre fry (n=30).
BACTERIA/DOSE: *Photobacterium demselae* subsp. *piscicida*/10^3cfu/fish.

MORTALITY/MICROBIOLOGY

No fish died but recovered bacteria from 3 fish.

Sublethal dose did not produce losses and seems to be useful for immunological studies in this species.

WP24 Fish Health

IRTA + UNIABDN

Task 24.5: A First Description of the Immune Ontogeny of *Argyrosomus regius*

C.J. Secombes[2], A. Estevez[1], K.B. Andree[1], C. Campoverde[1], D. Milne [2], E. Gisbert[1], A. Roque[1], M.D. Furones[1]

[1]IRTA, St. Carles de la Ràpita, Spain
[2] University of Aberdeen, Scotland, UK

Location

Ebro River Delta

Location
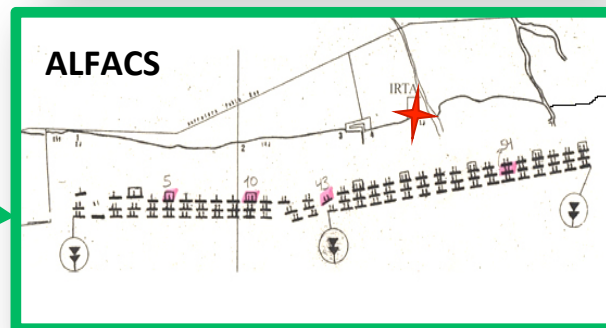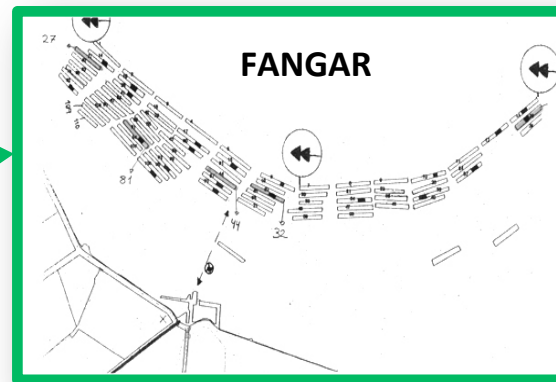
FANGAR

ALFACS

Ebro River Delta

Fangar Bay
Surface Area: 12 km^2
Temperature: 6-30ºC
Salinity: 21-38 psu
High renovation rate
Non-tidal.

Alfacs Bay
Surface Area: 50 km^2
Temperature: 7-31ºC
Salinity: 25-35 psu
Low renovation rate
Non-tidal.

Location
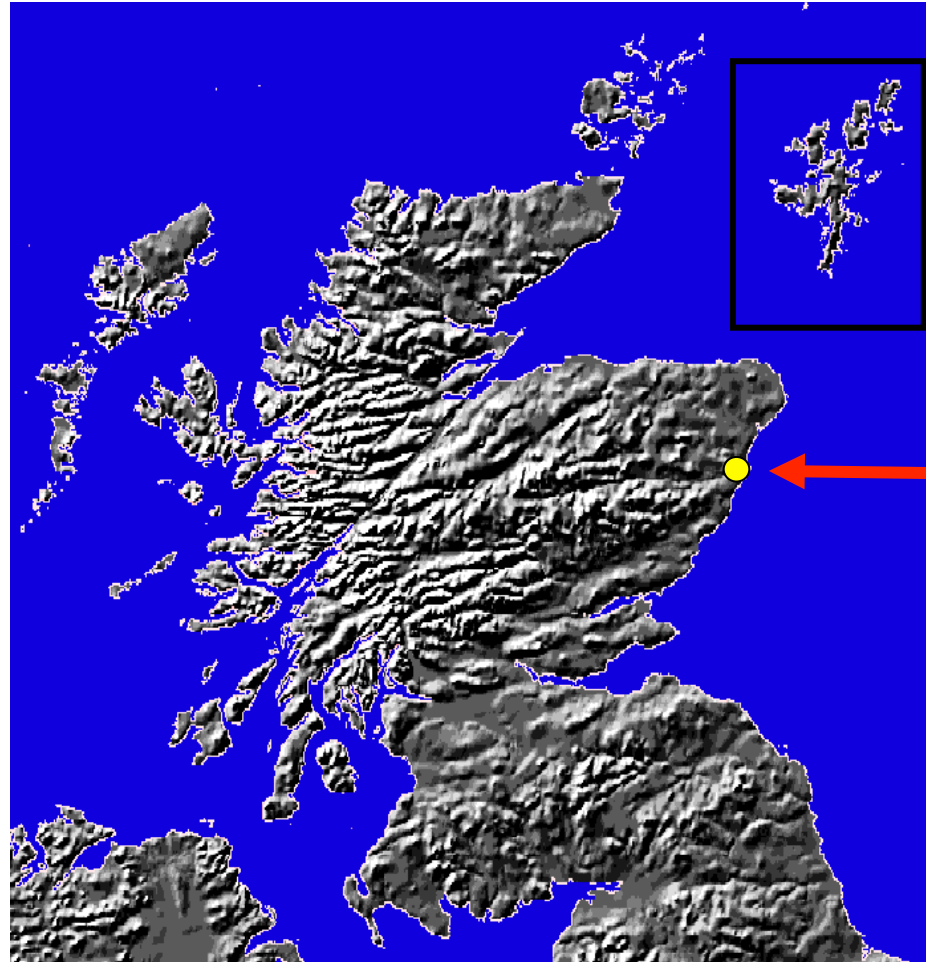
Aberdeen

Larval Culture Facilities
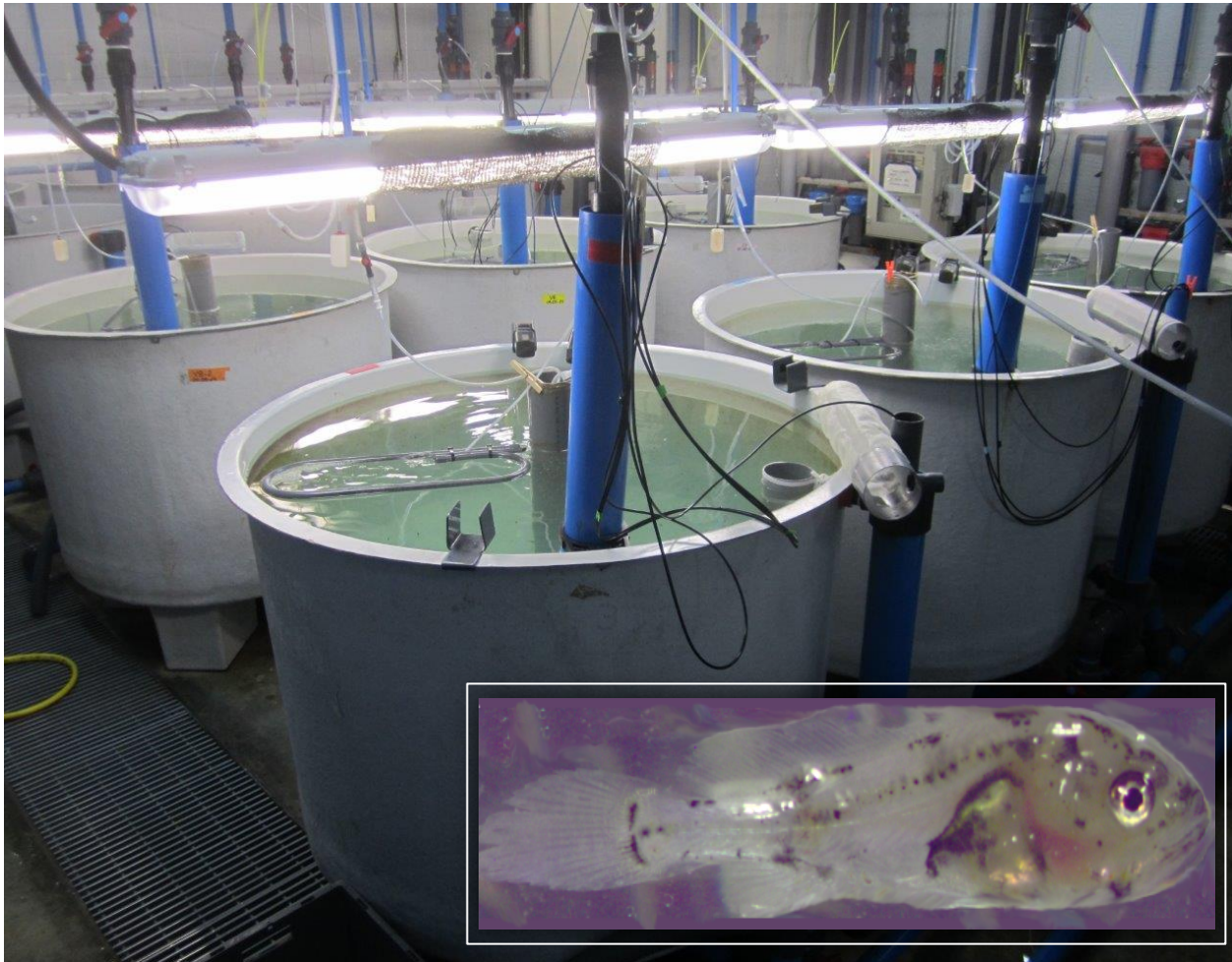
Larval grow-out 2000 L tanks with recirculation aquaculture system (RAS)

OBJECTIVE

Detailed study of the ontogeny of the immune system from larval development through to mature juvenile. Using molecular biology tools we will describe the timing of onset of expression of specific immune genes and this will be compared to microscopy data from the main immune tissues to understand what developmental tissue markers correspond to a fully mature adaptive immune response.
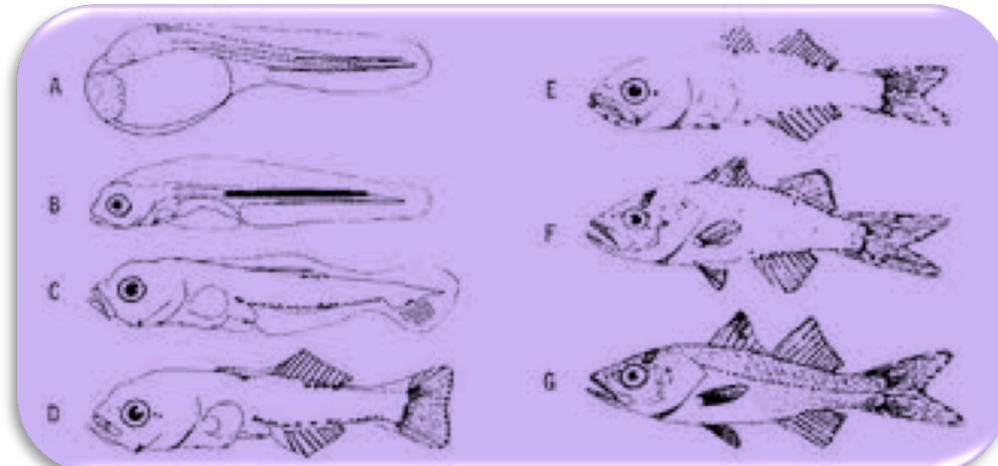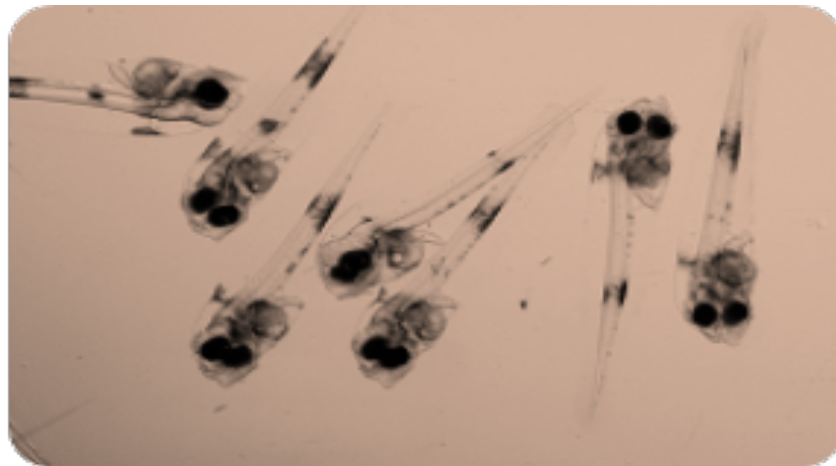
Rationale for Immune Study

The study is more than a zootechnological project as it strives to provide knowledge useful for prevention and management of diseases from pathogens, yet to be described, that may affect this species during captive rearing.

The focus of this study is to gain knowledge on the development of the immune system from the perspective of its development and the basics of its regulation to facilitate development of future vaccine protocols or other biotechnological approaches to advance this field for this species.

With this basic knowledge future areas of study can be approached such as immune modulation and the adoption of protocols for probiotics that can help to mitigate the impact of infectious diseases of this species during intensive rearing.
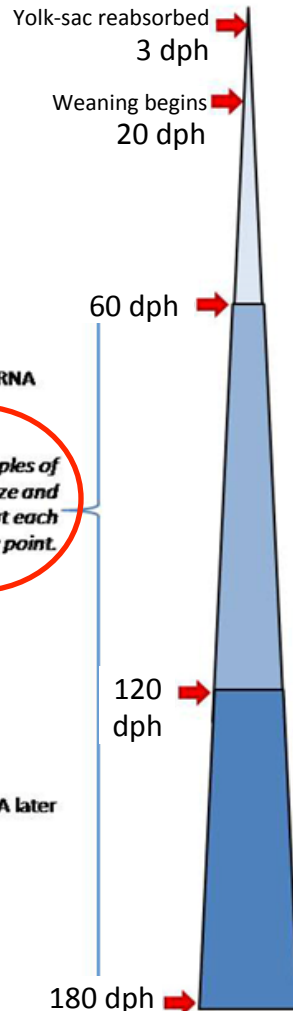
Methodology
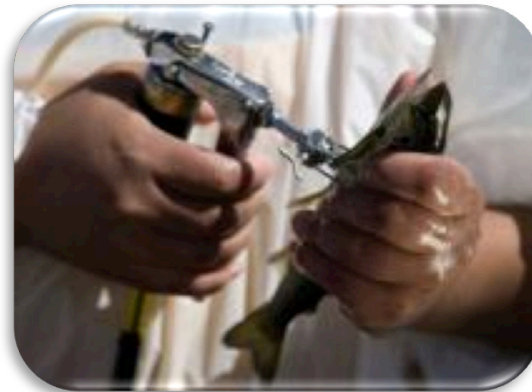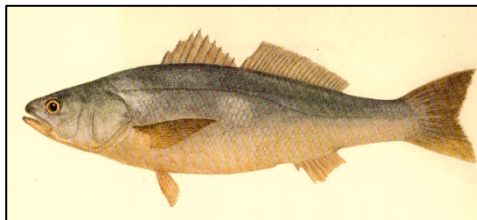
Sampling Schedule

Larval and post-larval stage ▢ :
Twice weekly sampling during the first 60 days.
Each sample:
30 larvae collected in RNA later
5 larvae collected in formol
(Total n = 16)

Weaned Juvenile stage ▢ :
Weekly sampling after weaning.
Each sample:
10-20 fish* Spleen, head kidney, peripheral blood in RNA later
5 fish collected in formol
(n = 8 x 2 = 16)

*Until fish are large enough for organ dissection collect all organ tissue as a unit as shown.

Collect samples of median size and large size at each sampling point.

Mature Juveniles ▢ :
Continue sampling every two weeks until 180 dph.
Each sample:
10-20 fish Spleen, head kidney, peripheral blood in RNA later
5 fish collected in formol
(n = 4 x 2 = 8)

Yolk-sac reabsorbed → 3 dph

Weaning begins → 20 dph

60 dph →

120 dph →

180 dph →
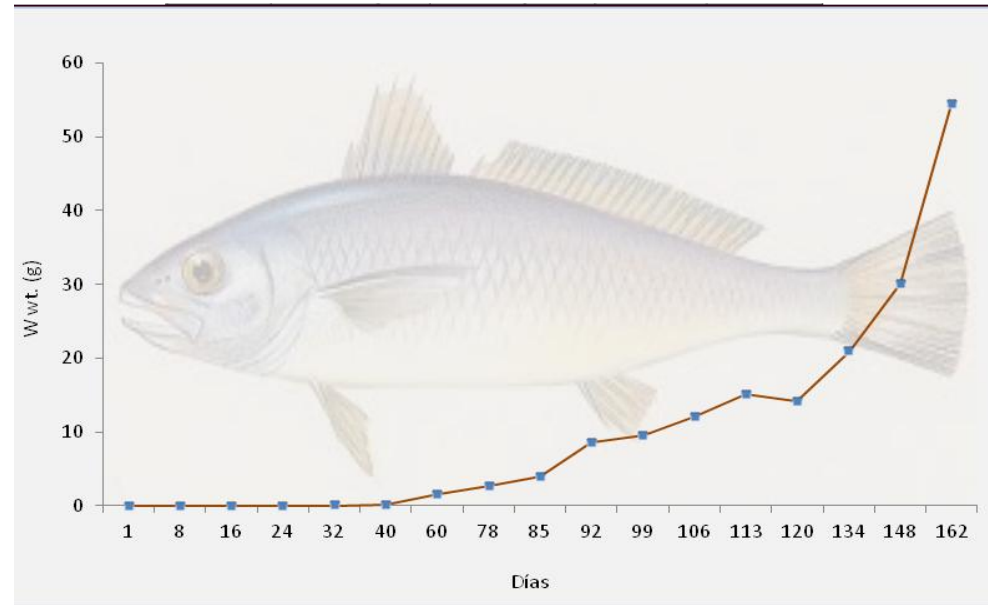
| JUVENILES						
Corvina	Wm1	64	30-jun	√		
Corvina	Wm2	71	07-jul	√	6400	2693,5
Corvina	Wm3	78	14-jul	√	7050	3940
Corvina	Wm4	82	18-jul	√	7900	8600
Corvina	Wm5	92	28-jul	√	8500	9560
Corvina	Wm6	99	04-ago	√	9200	12104
Corvina	Wm7	107	12-ago	√	9350	15080
Corvina	Wm8	113	19-ago	√	17255	14185
Corvina	Mm1	127	02-sep	√	11200	20915
Corvina	Mm2	141	16-sep	√	12600	30035
Corvina	Mm3	155	29-sep	√	15500	54470
Corvina	Mm4	162	06-oct			
Corvina	Mm5	169	13-oct			

Methodology
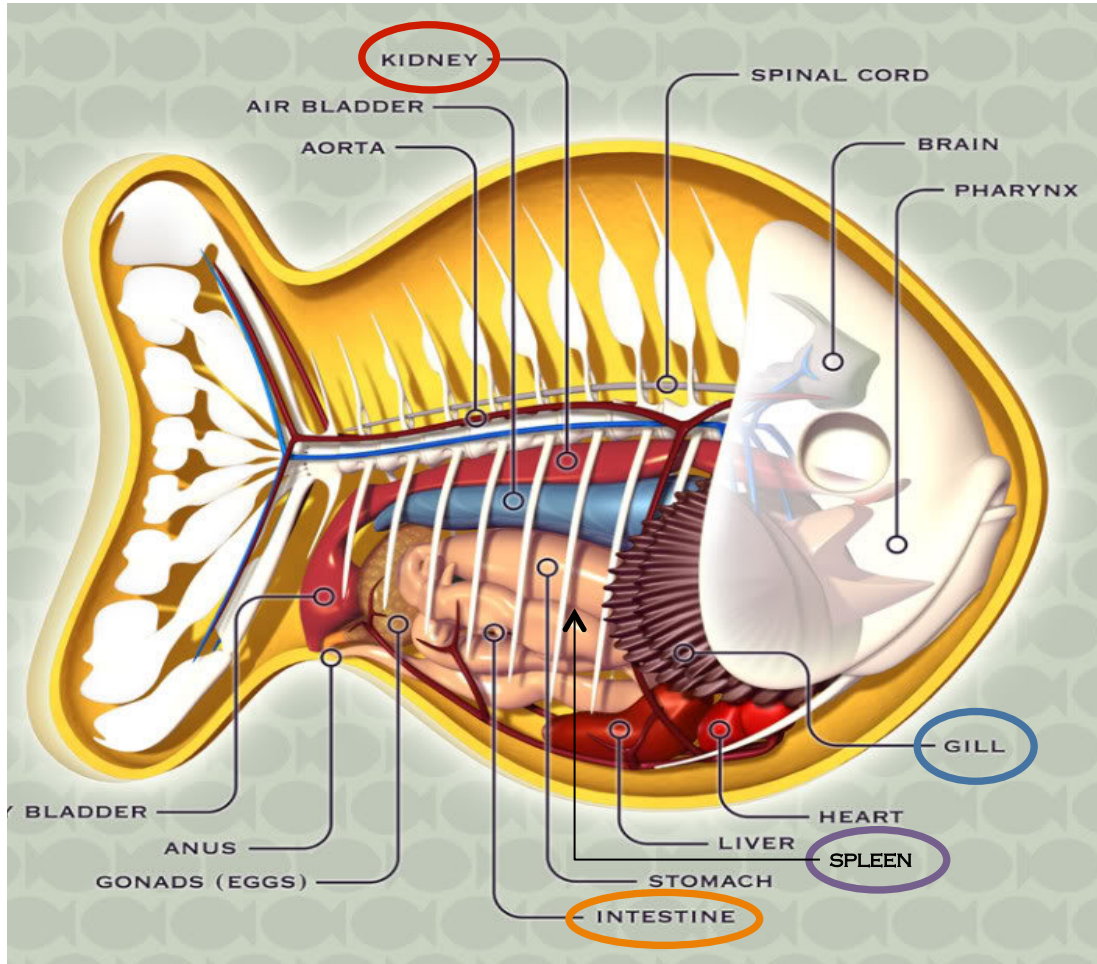
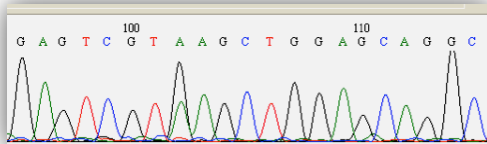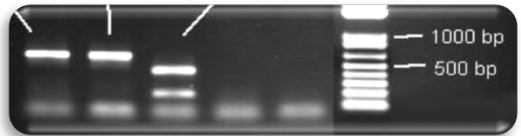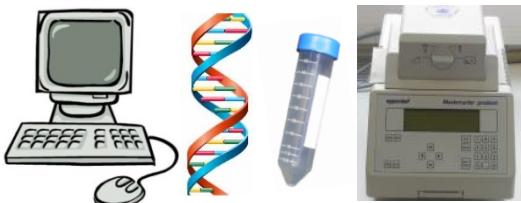## Tissues collected:
(stored in RNAlater @ -80ºC)

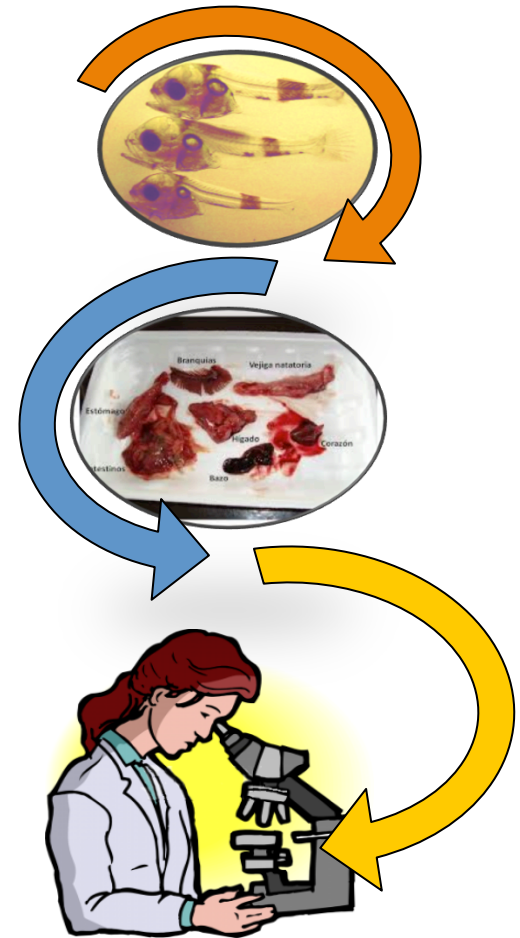Gill (epithelial cells + circulating blood)

Kidney

Intestine (GALT)

Spleen

Methodology
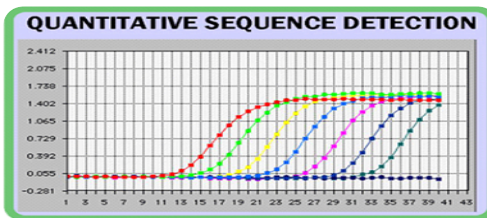
Histological analysis of immune tissues from developmental stages will be compared to ontogeny of gene expression within those tissues.
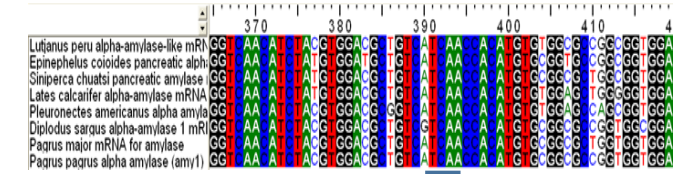
Methodology

Locate genes from phylogenetically similar species
in GenBank (NCBI), and align the gene sequences from all species.
From this alignment choose conserved areas to design consensus
or degenerate primers.

GenBank

Immune Ontogeny Gene Expression		
Endogeneous Controls	**Innate Immunity**	**Inflammatory Response**
EF1 (Elongation Factor)	Piscidin1 ("Defensin")	COX2 (cyclooxygenase 2)
GAPDH (Glyceraldehyde Phosphate Dehydrogenase)	Piscidin2 ("Defensin")	MyD88 (myeloid differentiating factor)
18S	Piscidin3 ("Defensin")	
	Lysozyme	
	Metallothionein	
	MX protein	
	NOD2 (Toll Like Receptor - TLR)	
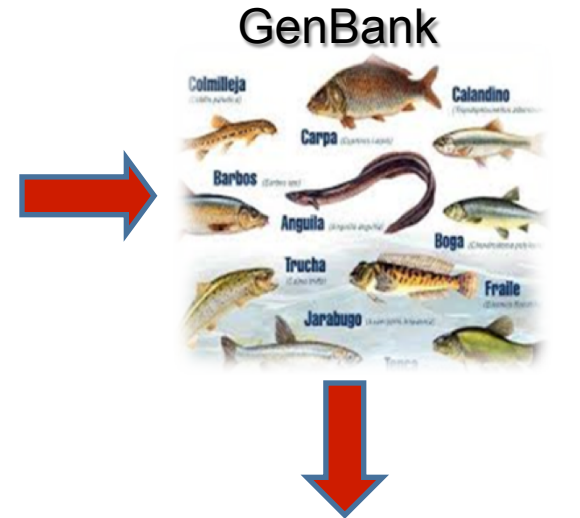
Innate and Inflammatory Response Degenerate Primers

Species - Target	Name	Sequence	bp	G/C	TmºC	Size (bp)
Argyrosoma regius Piscicidin 1 degenerate (sense)	dgPisc1F	GRATGAGGCTGYRTCRTTTCCC	22	10	64	110
Argyrosoma regius Piscicidin 1 degenerate (antisense)	dgPisc1R	ACWRGAATCCCTTKCCACAGCC	22	11	66	110
Argyrosoma regius C3 degenerate (sense)	dgC3F	ACTGGAGGCCACAGCTTAYGCTC	23	13	72	1201
Argyrosoma regius C3 degenerate (antisense)	dgC3R	GCCAGTACTCYATCCAGGTTCTC	23	12	70	1201
Argyrosoma regius COX2 degenerate (sense)	dgCOX2F	ATATTTGGACAAAACCAACCAGTGG	25	10	70	1500
Argyrosoma regius COX2 degenerate (antisense)	dgCOX2R	CARTTTGTACACAAAATCTATTTTGCTG	28	8	72	1500
Argyrosoma regius MX Protein degenerate (sense)	dgMXPF	GACATAGCAACCACAGAGGCYYTGA	25	12	74	570
Argyrosoma regius MX protein degenerate (antisense)	dgMXPR	GTCTTGTAGTTGARGAABCCDGGKAG	26	11	74	570
Argyrosoma regius NOD2 degenerate (sense)	dgNOD2F	CCTGTWTACACACCYTCACAAMAGG	25	11	72	1390
Argyrosoma regius NOD2 degenerate (antisense)	dgNOD2R	CAGGASAYAAKCCCTTBASYAGSACTTC	28	9	74	1390
Argyrosoma regius Lysozyme degenerate (sense)	dgLysoF	CTGGTGTTTCTGCTYCTGGTGGC	23	13	72	220
Argyrosoma regius Lysozyme degenerate (antisense)	dgLysoR	CCAKRAGCGYCTYTTYATCTGYAAYATG	28	9	74	220
Argyrosoma regius EF1 degenerate (sense)	dgEF1F	GACTTCATCAAGAACATGATCACTG	25	10	70	230
Argyrosoma regius EF1 degenerate (antisense)	dgEF1R	GATCTTCTTGATGTAGGTGCTCAC	24	11	70	230
Argyrosoma regius GAPDH degenerate (sense)	dgGAPDHF	GGASTACATGGTCTACATGTTCAAGTA	27	10	74	239
Argyrosoma regius GAPDH degenerate (antisense)	dgGAPDHFR	TGGTTGACYCCCATGACYAACATG	24	12	72	239
Argyrosoma regius MET degenerate (sense)	dgMETF	AARASTGGRACCTGCAACTGCGGWG	25	12	74	70
Argyrosoma regius MET degenerate (antisense)	dgMETR	GCAGCCAGAGGCGCARTTGSTGC	23	14	74	70
Argyrosoma regius MYD88 degenerate (sense)	dgMYD88F	CCYGARCTSTTTGATGCCTTCATCT	25	10	70	130
Argyrosoma regius MYD88 degenerate (antisense)	dgMYD88R	CACCTCRCTCRTCAATGAGTTCYC	24	11	70	130
Argyrosoma regius TNFa degenerate (sense)	dgTNFaF	GGCGTTYGCTCAGGGCGGCTTC	22	15	74	250
Argyrosoma regius TNFa degenerate (antisense)	dg TNFaR	GCTGAAACACVGCYCCCAGATAYATG	26	12	76	250

UNIABDN:

Lead for Tasks 24.5 and 24.6

Douglas Milne
Started 1st Sept 2014
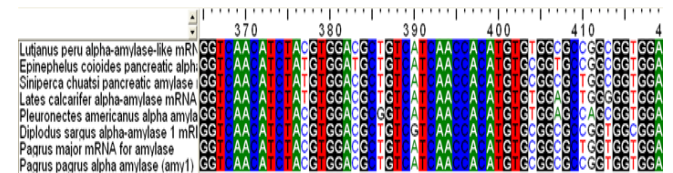
Methodology

Locate genes from phylogenetically similar studied species in GenBank (NCBI), prepare alignment of gene sequences from all species. From this alignment choose conserved area to design consensus or degenerate primers.

GenBank

Immune Ontogeny Gene Expression	
Adaptive Immunity	
RAG 1	IFN type I
IgM	IFN gamma
IgT	IL-1
TcR	IL-4
C3	IL-10
TNFa	IL-17
	IL-22

Adaptive Response Degenerate Primers

Species - Target	Name	Sequence	bp	G/C	Tm°C
Argyrosomus regius IL -1B (sense)	IL-1BF	GCTCCACGCGGTGATG	16	68.8	56.9
Argyrosomus regius IL-1B (anti-sense)	IL-1BR	AGGTAGAGGTTTGTGCCC	18	55.6	54.4
Argyrosomus regius IL-2 (sense)	IL-2F	AGATTCGAGGTTCTTGCTCC	20	50	54.5
Argyrosomus regius IL-2(anti-sense)	IL-2R	TCGCACTCCTCCTTGAC	17	58.8	54.2
Argyrosomus regius IL-4/13 (sense)	IL-4F	GTGAATGGGATCCTGAATGG	20	50	60.1
Argyrosomus regius IL-4/13 (anti-sense)	IL-4R	TTCCAGTCCCGGTATATGCT	20	50	59.4
Argyrosomus regius IL-17A/F (sense)	IL-17F	TGATGATGATGGTGGCGGCA	20	55	59.6
Argyrosomus regius IL-17A/F (anti-sense)	IL-17R	CAGCAGCAGCACCTGGC	17	65	61.8
Argyrosomus regius IL-22 (sense)	IL-22F	CTGCATGCTAACATCCT	17	47.1	49.2
Argyrosomus regius IL-22 (anti-sense)	IL-22R	GATCTCTCCAATGGCTTTCT	20	45	52
Argyrosomus regius TNFa (sense)	TNFaF	ACAGTCAAGCGTCCTTC	17	59	52
Argyrosomus regius TNFa (anti-sense)	TNFaR	GGTTGAACACAGCTCCCAT	19	52.6	55.3
Argyrosomus regius IFN type 1 (sense)	IFN1F	TTGAGGAGGATCACAGC	17	52.9	51.2
Argyrosomus regius IFN type 1 (anti-sense)	IFN1R	CAGCTCCCAGGATTCAG	17	58.8	52.3
Argyrosomus regius IFN type 2 (sense)	IFN2F	ACCATCCATAGGCTGTTGC	19	52.6	55.2
Argyrosomus regius IFN type 2 (anti-sense)	IFN2R	GCTCGCCTCTTCGTACAG	18	61.1	55.4
Argyrosomus regius IgM (sense)	IgMF	AAGAGACAGGACTGGGA	17	52.9	51.9
Argyrosomus regius IgM (anti-sense)	IgMR	TTTCACAAAGCAAGTCAGGG	20	45	53.4
Argyrosomus regius IgT (sense)	IgTF	GGTCACTCTGTTGTGTCTG	19	52.6	52.9
Argyrosomus regius IgT (anti-sense)	IgTR	GTGGTGTAAAGACTCGTAAC	20	45	50.5
Argyrosomus regius beta defensin (sense)	DefF	GTGTGGGTACGGAGGAC	17	64.7	54.9
Argyrosomus regius beta defensin (anti-sense)	DefR	CGCACAGCACAGCATCT	17	58.8	56.1
Argyrosomus regius Piscadin (sense)	PisF	GATGGTCGTCCTCATGGCTG	20	45	52.9
Argyrosomus regius Piscadin (anti-sense)	PisR	CTTTCAGATGAACCGCCATAGAT	23	43.5	54.9
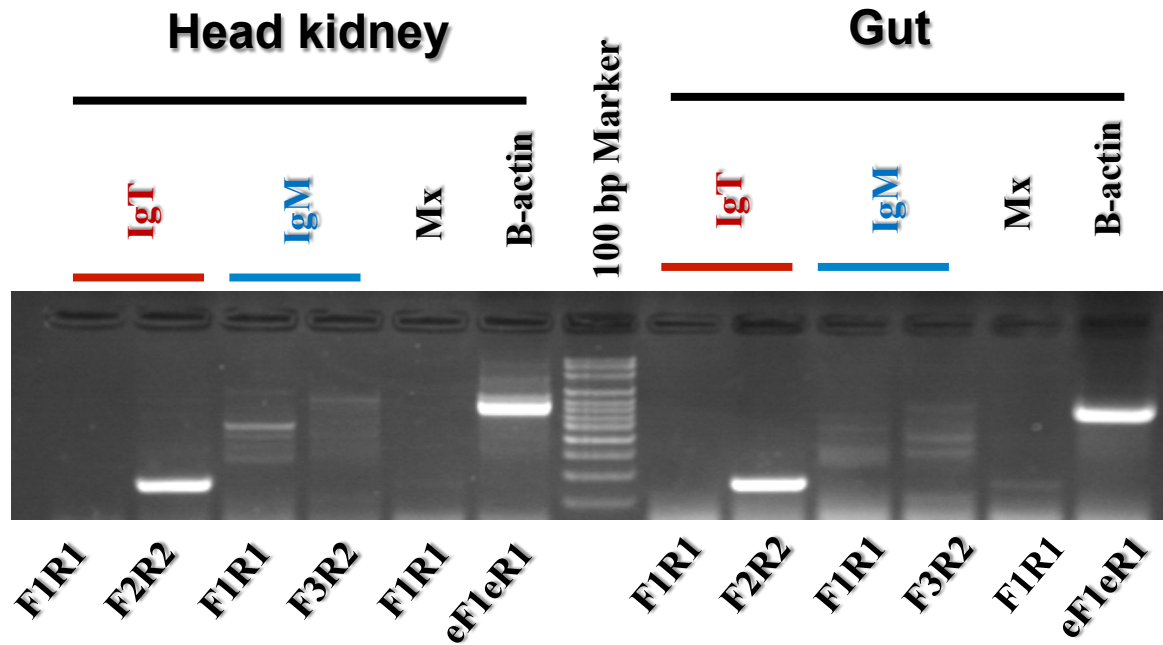
PCR was performed at 55°C for 38 cycles

Business Strategy

Polyclonal & monoclonal antibodies
Production utilising
Peptide Technology

The main advantage of applying **Peptide Technology** compared to cell based and recombinant approach is the **high degree of specificity** that can be achieved especially to distinguish closely related family members

VERTEBRATE ANTIBODIES
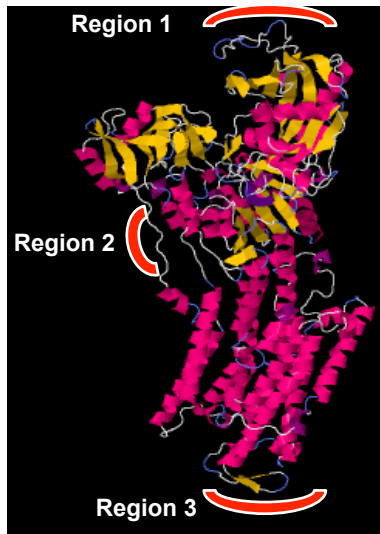
Regions Selection → **Peptide Design** → **Carrier Conjugation** → **Immunisation**

Region 1

Region 2

Region 3

N- Target of interest -C

Peptide 1   Peptide 2   Peptide 3

Carrier protein

Peptides selection & synthesis:
- One peptide per selected region
- Short peptides (10-12 amino acids)
- High antigenicity & solubility
- Unique blast

Region selection:
- Surface exposed regions
- Hydrophilic regions
- Flexible & low complexity regions
- beta-turns
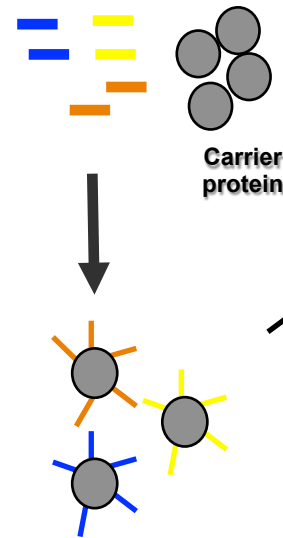
Carrier Conjugation:
- Peptides conjugated to immunogenic carrier protein

Immunisation:
- 5 to 10 different antigens (peptide) per single injection
- Optimal antigen concentrations
- Optimal number of immunisations, boosts and injection routes

Serum ELISA screening to check immune response

Hybridoma Generation

→

Hybridoma Cloning & Screening

→

Hybridoma Expansion

Spleenocytes   **Myeloma cells**
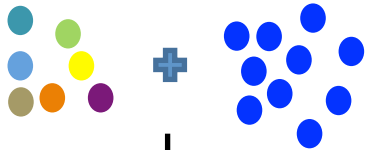
Cells fusion hybrids plated in multiple wells

Test supernatants for anti-peptide1, anti-peptide2 and anti-peptide 3 antibodies by ELISA followed by the evaluation test of interest (e.g. FACS)
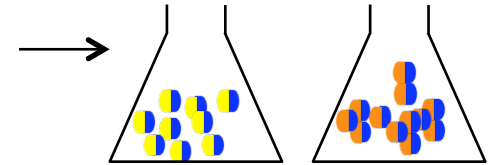
Hybridoma cells grown on the selective medium

Secreted anti-**peptide 1** antibody

Secreted anti-**peptide 2** antibody
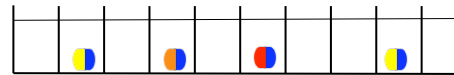
Allow cells to grow and re-test supernatants for anti-peptide 1, anti-peptide 2 and anti-peptide3 antibodies

Positive clones provide continues source of anti-peptide 1 & anti-peptide 2

N- **IL22 (salmonids)** -C

L7   L8

Immunodetection of rIL22 with anti-IL22 mAbs #L7 and #L8.
MW = Molecular weight marker
1 = Test sample
2 = Negative control

Spleenocytes fusion:

- **High throughput**; simultaneous screening to antibodies with specificity to different antigens
- **Fast** screening compared to conventional method (day 5 screening)

Can also be used for immunohistochemistry

Immunohistochemical detection of CD4 and FoxP3 in zebrafish infected for 28 days with ESX-5 deficient *M. marinum*.

A: CD4+ cells surround granulomas in the liver, spleen and pancreas.

B: CD4 and FoxP3 antibodies stain the same areas of both necrotic and solid granulomas.
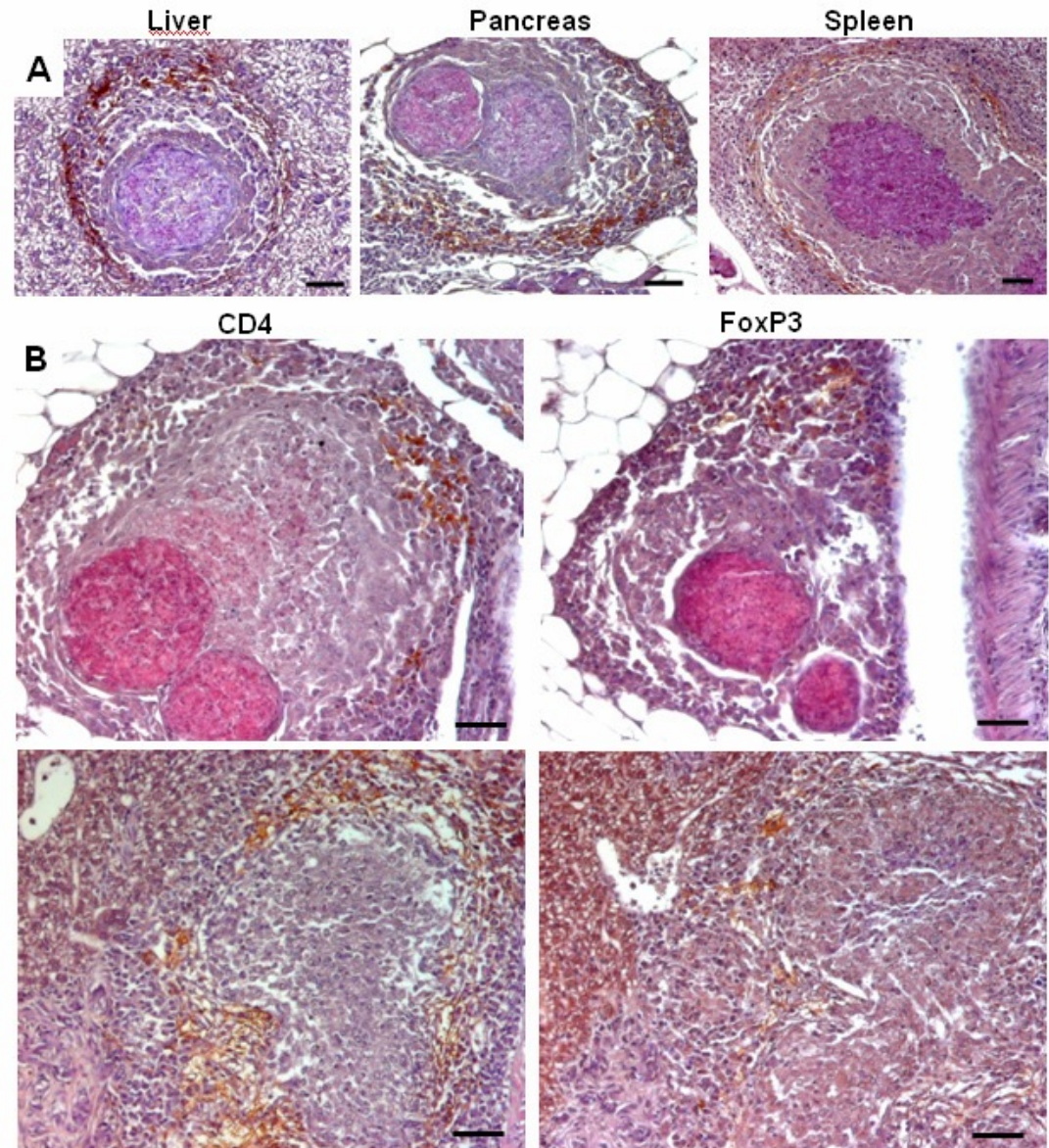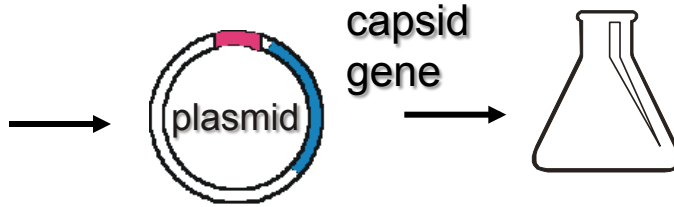
WP26 Fish Health
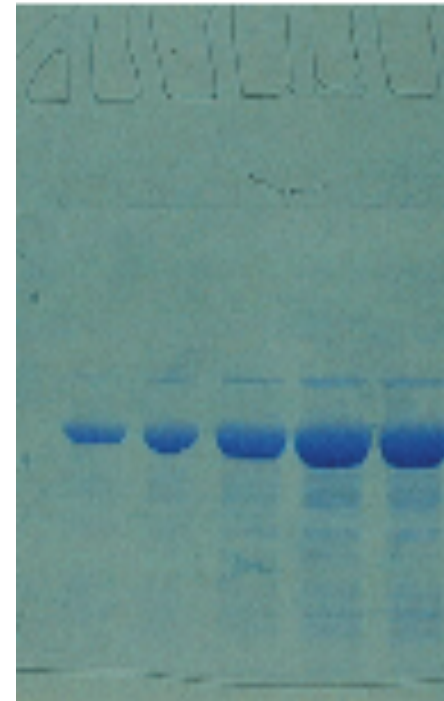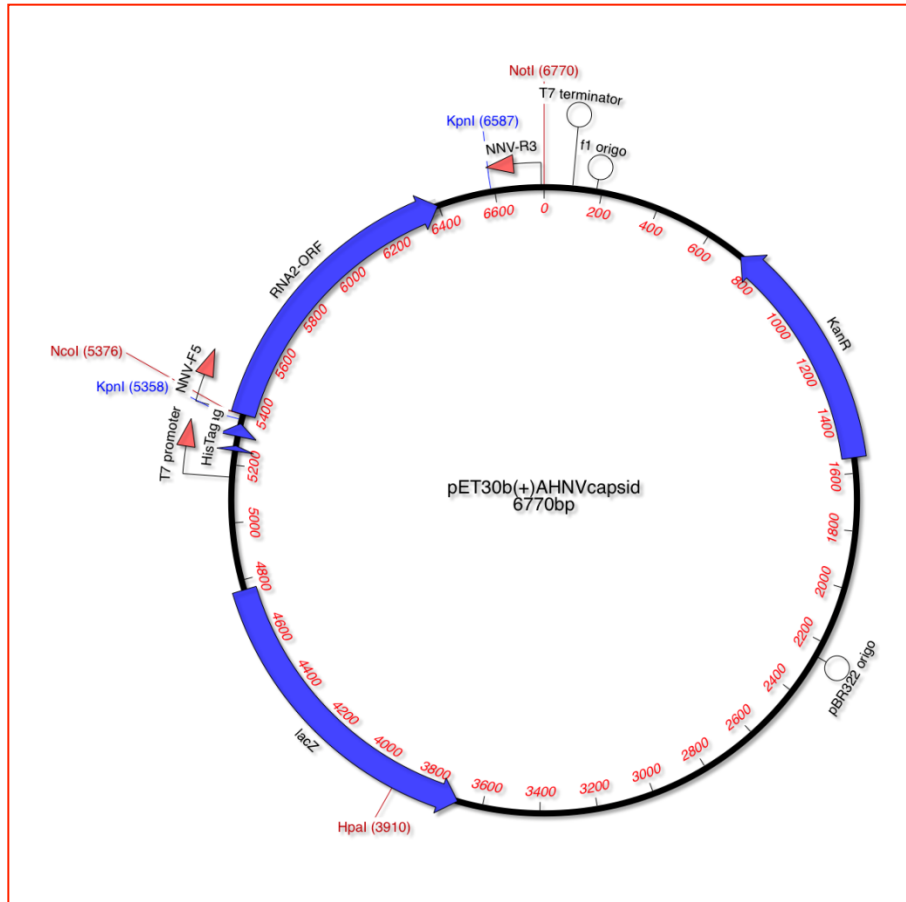
IMR

Task 26.1 Production of VNN capsid protein

- Expression in
  - *E. coli*
  - *Leishmania tarentolae*
  - *Tobacco plant*
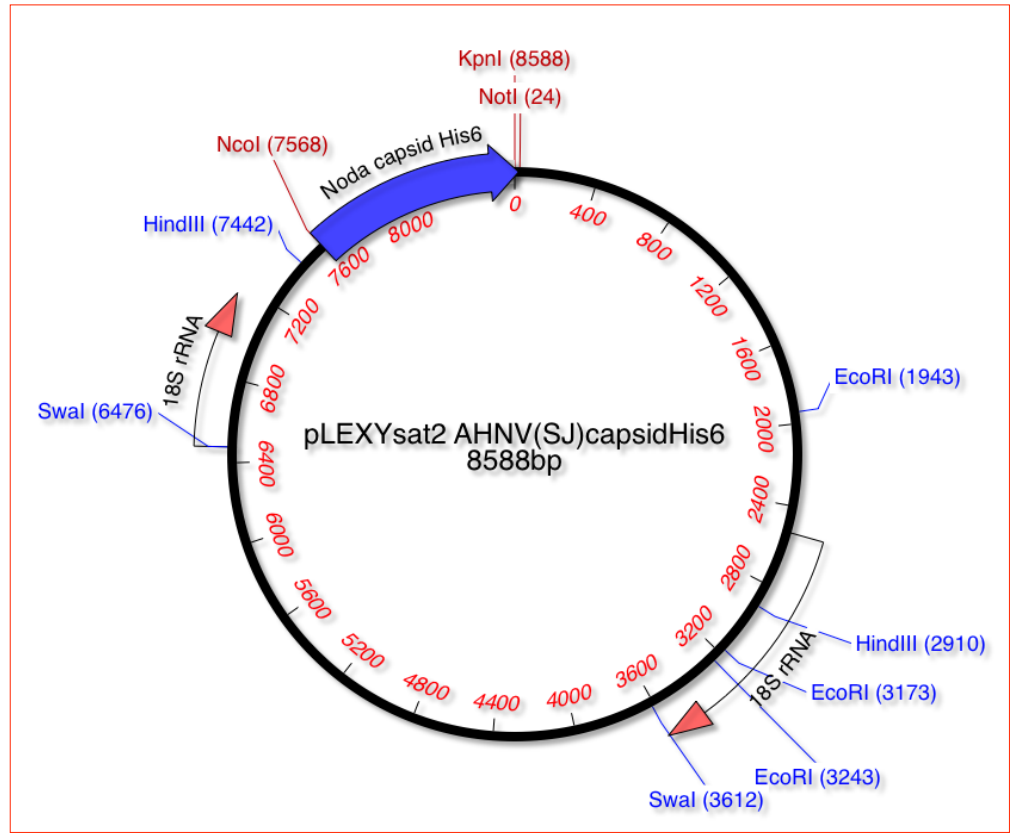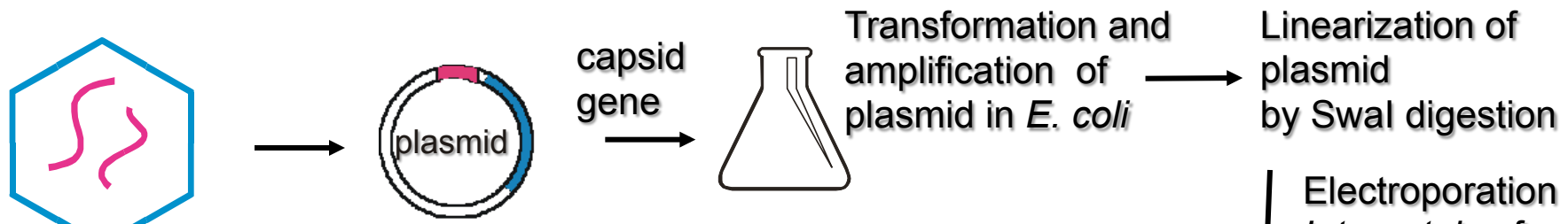
Expression in *E. coli*

capsid gene

Transformation and expression in *E. coli* as inclusion bodies

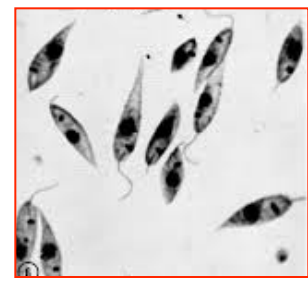Serial dilutions of purified recombinant protein

Expression in *Leishmania tarentolae*

capsid gene
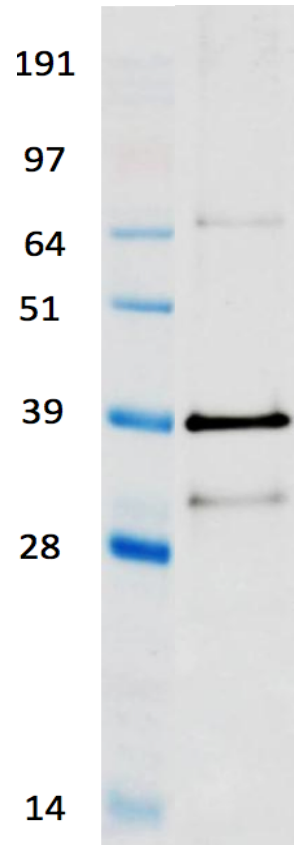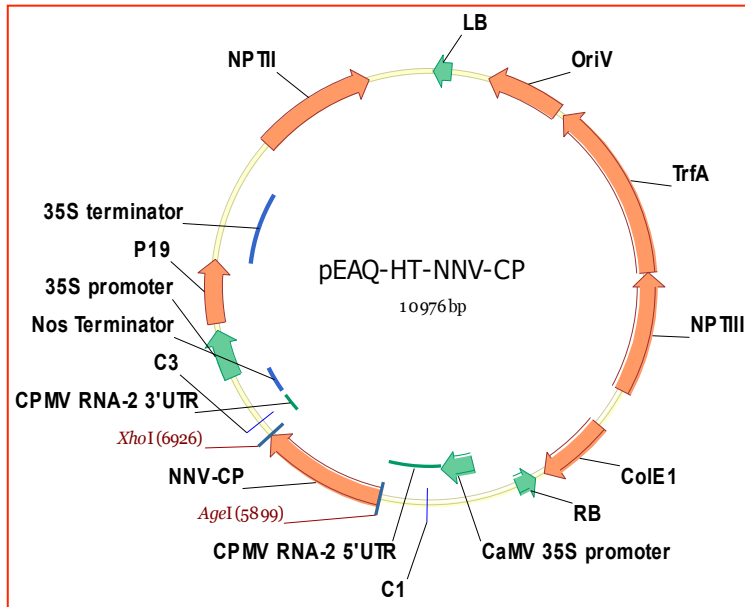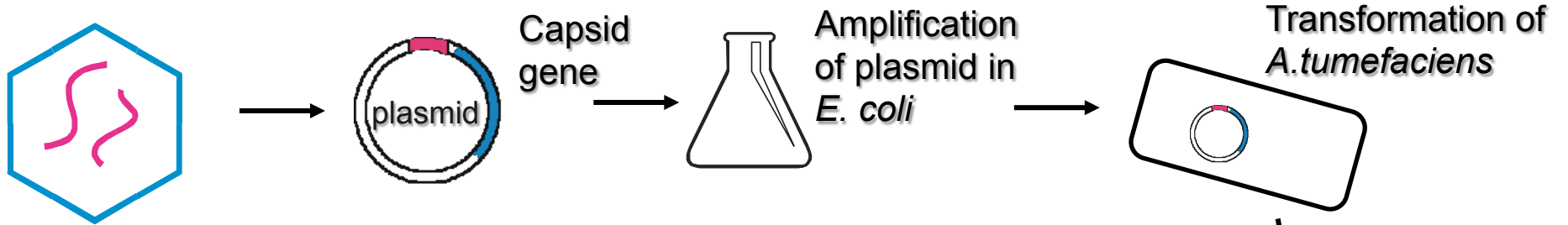
Transformation and amplification of plasmid in *E. coli*

Linearization of plasmid by SwaI digestion

Electroporation into *L.tarentolae* for integration in the 18S rRNA gene and expression of rekombinant capsid protein

KpnI (8588)
NotI (24)
NcoI (7568)
Noda capsid His6
HindIII (7442)
18S rRNA
SwaI (6476)
pLEXYsat2 AHNV(SJ)capsidHis6
8588bp
EcoRI (1943)
18S rRNA
HindIII (2910)
EcoRI (3173)
EcoRI (3243)
SwaI (3612)

0
400
800
1200
1600
2000
2400
2800
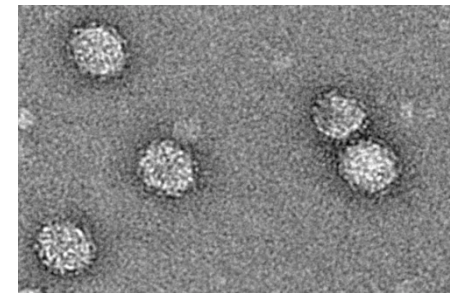3200
3600
4000
4400
4800
5200
5600
6000
6400
6800
7200
7600
8000

Western blot using rabbit anti-AHNVcapsid antibodies

Transient expression in *N.benthamiana* (performed at the John Innes Centre, UK)

Capsid gene

Amplification of plasmid in *E. coli*

Transformation of *A.tumefaciens*

Agroinfiltration

pEAQ-HT-NNV-CP
10976 bp

- LB
- OriV
- NPTII
- TrfA
- 35S terminator
- P19
- 35S promoter
- Nos Terminator
- NPTIII
- C3
- CPMV RNA-2 3'UTR
- *Xho*I (6926)
- NNV-CP
- ColE1
- *Age*I (5899)
- RB
- CPMV RNA-2 5'UTR
- CaMV 35S promoter
- C1

191
97
64
51
39
28
14

Western blot using anti-NNV antibodies

TEM of virus like particles formed by recombinant proteins

Thank you for your attention