AGD in Ireland: parasite culture and field studies

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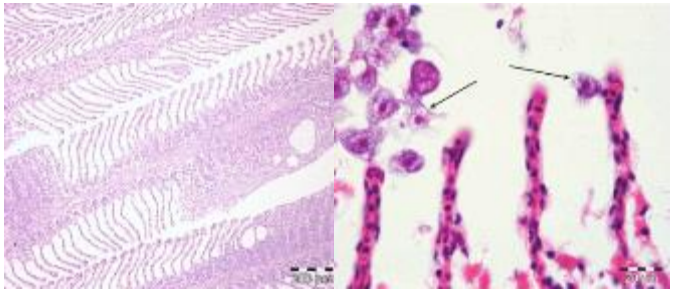
LÁRIONAD TAIGHDE MARA AGUS FIORUISCE

Vet Aqua INTERNATIONAL

"Gill disease in Finfish aquaculture with particular emphasis on AGD"

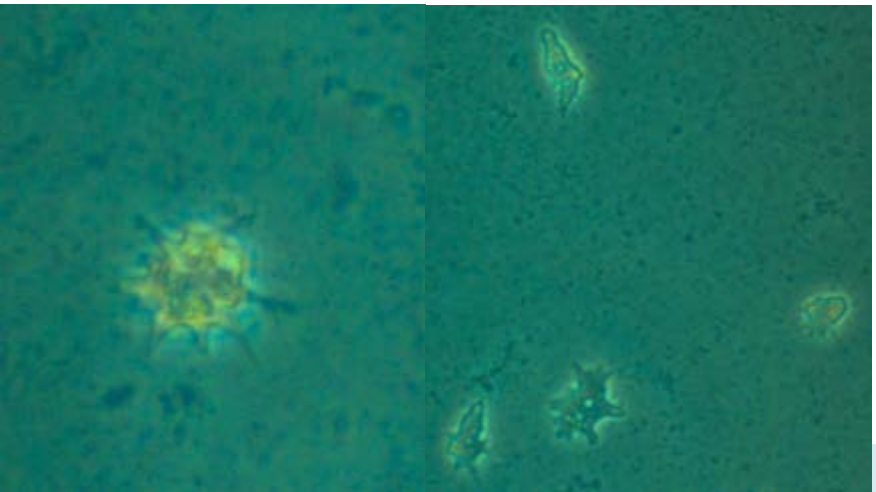
- Marine Institute PhD Fellowship
  - Marine and Freshwater Research Centre in Galway/Mayo Institute of Technology (GMIT)
  - Vet Aqua International
- 2013 – 2017
- Research Objectives
  - **Investigate molecular diagnostics**
  - Set up amoeba culture
  - Challenge trials
  - Field studies – longitudinal studies transmission pathways, reservoirs
  - Proteomics – host response



Severe hyperplasia and large vesicle formation (L) and amoeba present in histo (R) (courtesy: E. Collins)

Neoparamoeba perurans culture

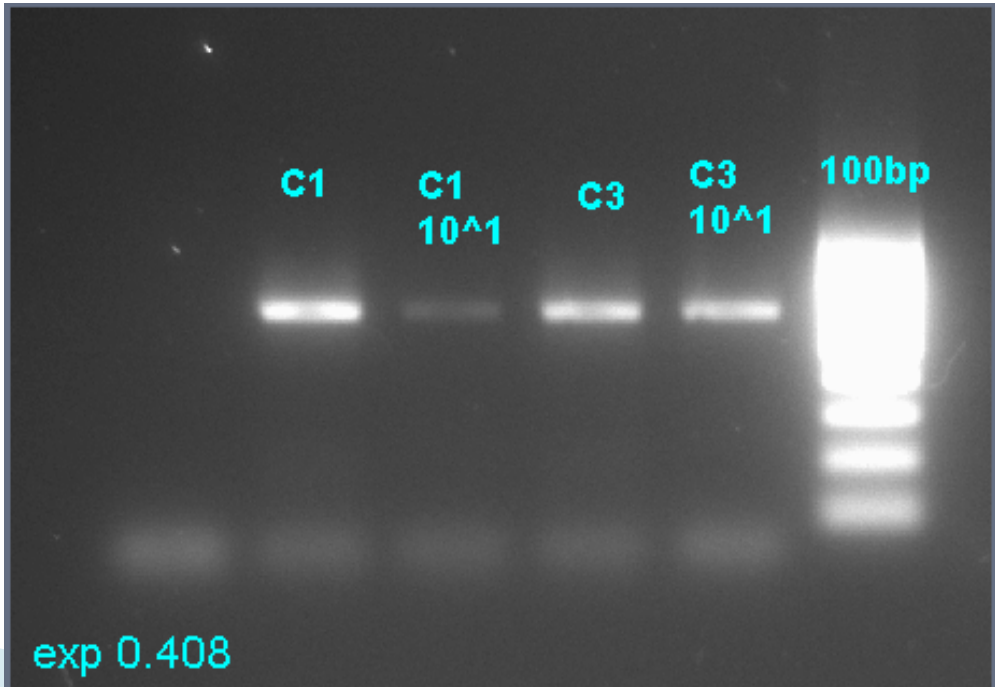
- Establishment of a *N. perurans* culture in July 2013
  - Gill acquired from infected sites with gill scores of 2
  - Placed in tissue culture flasks with sterile seawater and left refrigerated over night
  - Amoeba allowed to settle on flask and removed using trypsin and washing.
  - Cultured on MYA @ 18°C
- Culture tested positive using *N. perurans* real-time PCR
- Successfully utilised in infection trials
- Further infection trails planned in the near future.



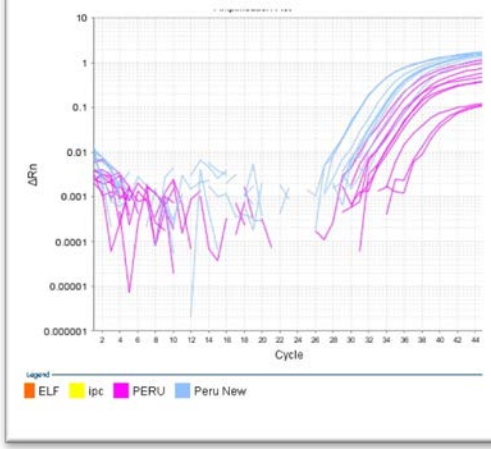
N. perurans free floating in culture (L) and attached to M/Y agar (R)

N. Perurans culture-PCR

- 636 bp sequence of the 18s rRNA gene (Young *et al*. 2008)
- Two extractions of the culture were tested
- Culture sequence shows 99% similarity with sequences from Norway (KF146713), Australia (GU574794) and Chile (GQ407108)



Molecular Diagnostics

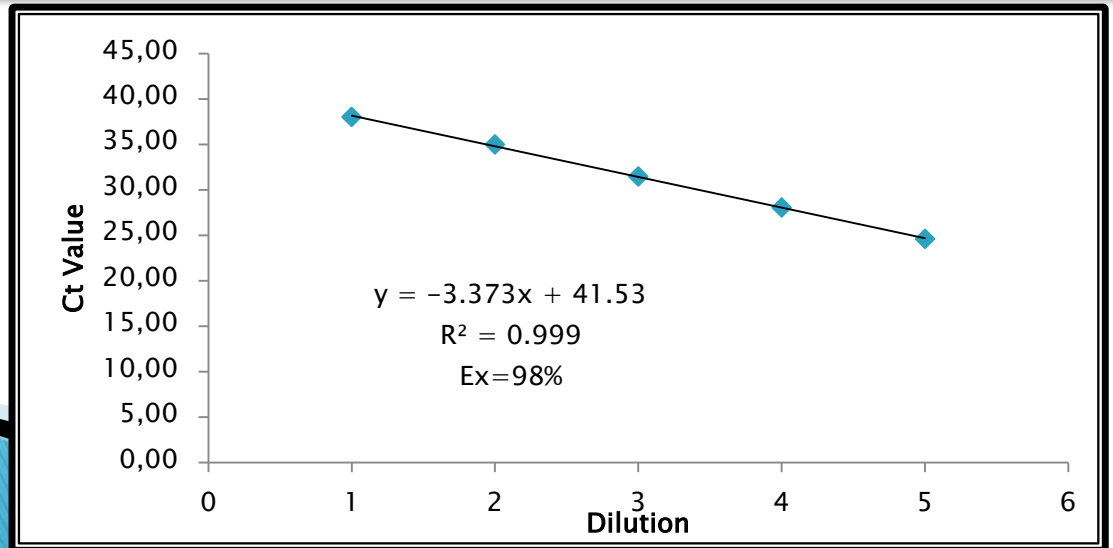


- Two published Real-Time PCR methods Bridle *et al*. 2010 (SYBR Green) and Fringuelli *et al* 2012 (TaqMan).
- Fringuelli method – based on FFPE samples and transition to testing field samples was not established.
- Alternative real-time assay developed, based on the 18S rRNA gene.

Amplification efficiency

- A key attribute of any real-time PCR reaction is the amplification efficiency of the PCR
  - There should be a doubling of PCR product in every cycle
- Accepted efficiency is 100 +/- 10% (TaqMan®)
- Amplification efficiency of the Real-Time PCR assay was established using the Ct slope method (Efficiency (Ex) = [10(-1/slope)]-1)
- New method has an amplification efficiency of 98%

	1st Run		2nd Run		3rd Run		Mean	Stdev
10^{-5}	37.64	38.02	38.17	38.08	38.04	38.07	38.00	0.19
10^{-4}	34.93	35	35.21	35.04	34.78	34.88	34.97	0.15
10^{-3}	31.55	31.53	31.60	31.58	31.27	31.27	31.47	0.15
10^{-2}	27.98	27.97	28.08	28.08	28.11	27.96	28.03	0.07
10^{-1}	24.49	24.59	24.55	24.66	24.73	24.63	24.61	0.08



Specificity

- Primers and probes were tested via theoretical means by Blast (Basic Local Alignment Search Tool) searches in order to identify similar sequences in the nucleotide database
- Did not cross react with *N. pemaquidensis* (ATCC®50172™)
- Plan to test other *Neoparamoeba* sp. if available

Sensitivity

- Cultured amoeba were tested in order to determine the LOD
  - Results from samples tested during analysis for the standard curve were used
  - The final dilution that produced all replicates positive prior to the signal drop off point was selected for further LOD identification
  - A C_t value of 40.72 was established as the working LOD for this assay.

Repeatability

- Naturally infected gills were used in order to examine the repeatability of the entire assay from extraction to PCR
- 10 samples of gill tissue from samples that have previously tested positive were pooled to ensure same amount of target analyte and matrix and taken through extraction process.
- The repeatability was carried out at two further concentrations by creating dilutions of 10^{-1} and 10^{-2}

Neat	Ct 1	Ct 2	Ct Mean	Ct Stdev	10^{-1}	Ct 1	Ct 2	Ct Mean	Ct Stdev	10^{-2}	Ct 1	Ct 2	Ct Mean	Ct Stdev
1	29.93	29.92	29.93	0.01		33.04	33.3	33.17	0.18		35.94	35.97	35.96	0.02
2	29.97	30	29.99	0.02		32.11	32.3	32.21	0.13		36.2	36.29	36.25	0.06
3	30.06	30.09	30.08	0.02		32.93	32.85	32.89	0.06		36.32	36.35	36.34	0.02
4	30.45	30.55	30.5	0.07		32.79	32.73	32.76	0.04		36.07	36.53	36.3	0.33
5	30.66	30.66	30.66	0		32.57	32.75	32.66	0.13		35.77	35.51	35.64	0.18
6	29.94	29.94	29.94	0		32.25	32.17	32.21	0.06		36.94	36.37	36.66	0.4
7	29.94	29.91	29.93	0.02		33.41	33.63	33.52	0.16		36.93	36.82	36.88	0.08
8	30.04	30.07	30.06	0.02		32.69	32.82	32.76	0.09		35.87	36.5	36.19	0.45
9	30.11	30.07	30.09	0.03		32.05	32.19	32.12	0.1		35.48	35.16	35.32	0.23
10	29.91	29.94	29.93	0.02		32.3	32.34	32.32	0.03		35.88	35.9	35.89	0.01
			0.26	0.02				0.46	0.1				0.46	0.18

Molecular Results of Infection Trial

- Overall lower Ct values with new method and increased ability to detect low positive samples

Fish	Tank	Infected Y/N	Date	Days p.i	Tank gill score	AGD lesion Y/N	MI PCR	Fringueilli *et al* (2012)
1	1	Y	11.03.14	7	0	N	Neg	Neg
2	2	Y			0	N	37.56	Neg
3	3	Y			0	N	35.67	39.95
4	4	Y			0	N	Neg	Neg
5	5	Y			0	N	Neg	Neg
6	6	Y			0	N	Neg	Neg
7	7	N			0	N	Neg	Neg
8	1	Y	18.03.14	14	0	N	Neg	Neg
9	2	Y			0	N	32.72	37.54
10	3	Y			0	N	32.72	39.16
11	4	Y			0	N	Neg	Neg
12	5	Y			0	N	Neg	Neg
13	6	Y			0	N	32.88	37.24
14	7	N			0	N	Neg	Neg
15	1	Y	27.03.2014	23	0.6	Y	29.26	32.58
16	2	Y			0.9	Y	31.21	34.50
17	3	Y			0.6	Y	37.05	40.69
18	4	Y			0.4	Y	29.2	32.19
19	5	Y			0.75	N	36.61	Neg
20	6	Y			0.8	Y	33.30	37.52
21	7	N			0	N	Neg	Neg

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Longitudinal Study

- Site in the South West of Ireland was selected to be included in the Longitudinal study.
- Sampling commenced in late April 2013 shortly after smolts were transferred to sea.
- Samples (5 fish from 2 cages) were collected every 2-3 weeks
  - Gross assessment
  - Gill smears
  - Histology
  - PCR

Longitudinal Study

Date sampled	Mean gill score	Histological results	PCR results
07/05/2013	0.1	No amoeba, no gill pathology (or No evidence of AGD)	3/10 positive
23/05/2013	0	No amoeba, no gill pathology	Negative
05/06/2013	0	No amoeba, no gill pathology	Negative
11/06/2013	0.2	No amoeba, no gill pathology	Negative
28/06/2013	0	No amoeba, no gill pathology	1/10 positive
09/07/2013	0	No amoeba, no gill pathology	4/10 positive
31/07/2013	2.5	Pathology consistent AGD observed in all of the gills. Amoebae were observed	10/10 positive
16/08/2013	2	No amoeba observed, Gill pathology observed	1/10 positive
02/09/2013	0.35	No amoeba observed. Gill pathology is very slight	7/10 positive
25/09/2013	1.5	No amoeba observed. The gill changes suggest changes associated with AGD.	8/10 positive
18/10/2013	0	No amoeba observed, Low to moderate pathology	3/10 positive
04/11/2013	0.7	No amoeba observed, low level pathology	9/10 positive
29/11/2013	0.6	Amoeba observed, low to moderate of pathology	10/10 positive
17/12/2013	0.6	N/A	9/10 positive
17/01/2014	0.5	No amoeba observed. Gill pathology low	7/10 positive

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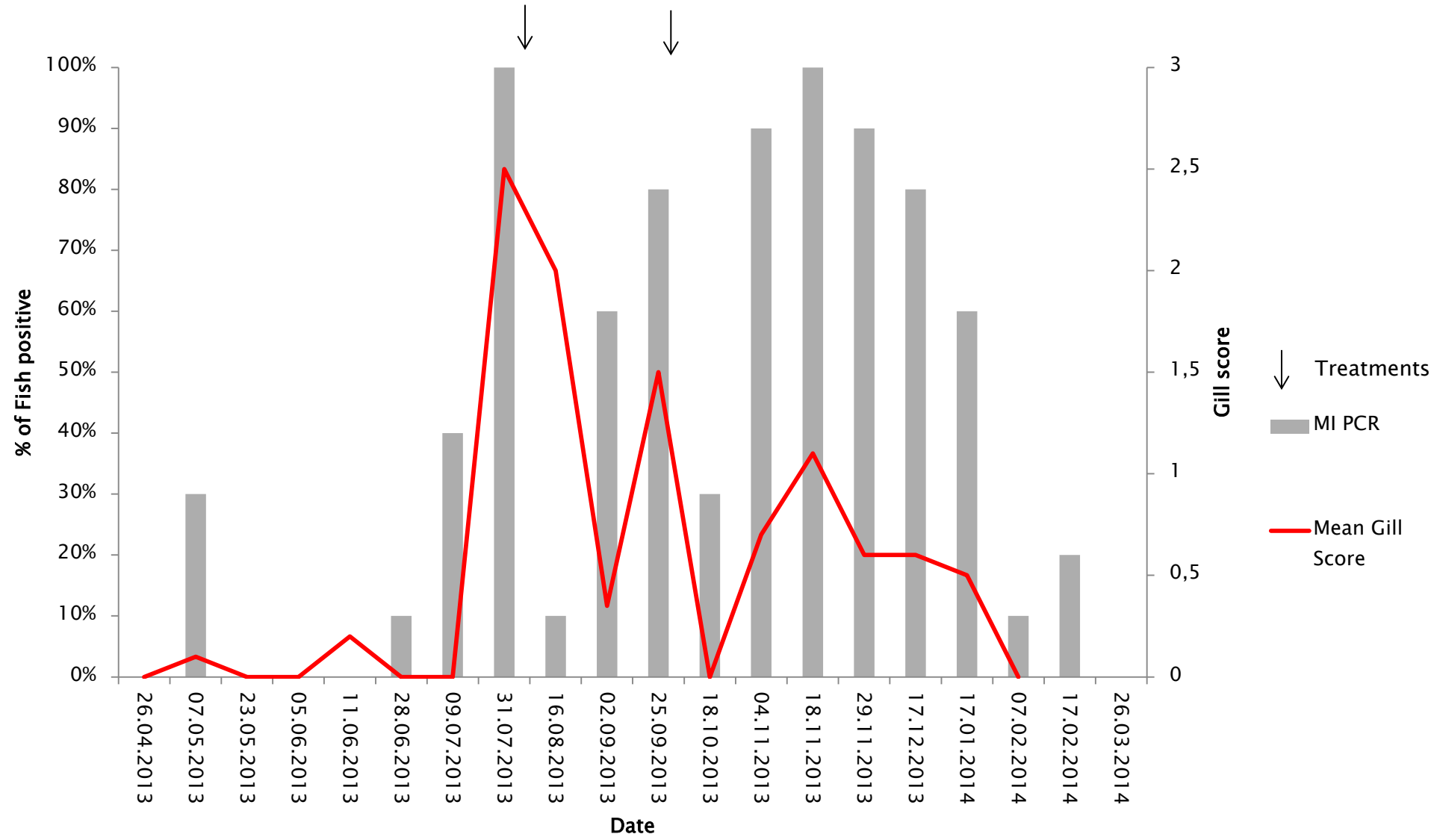
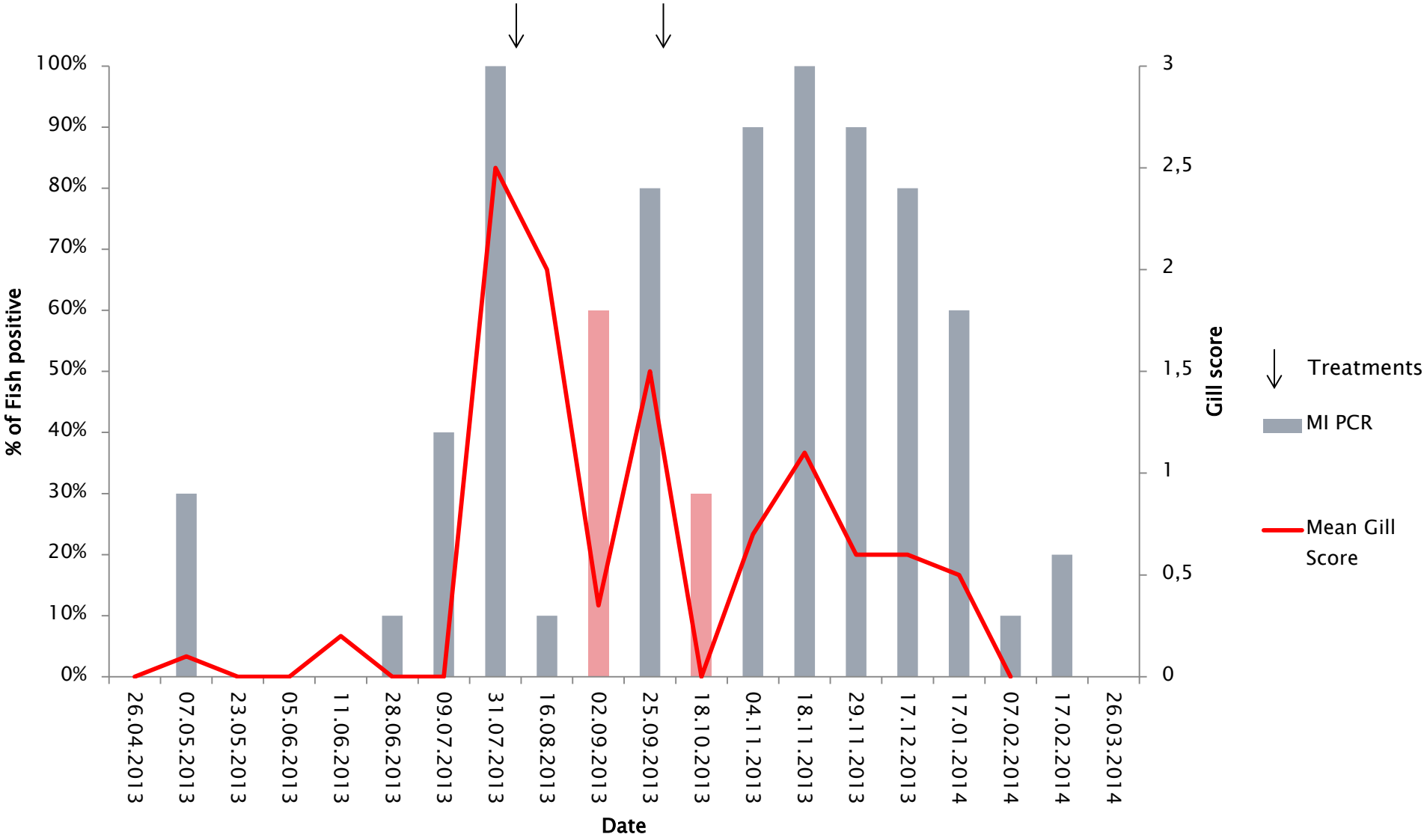
Molecular results



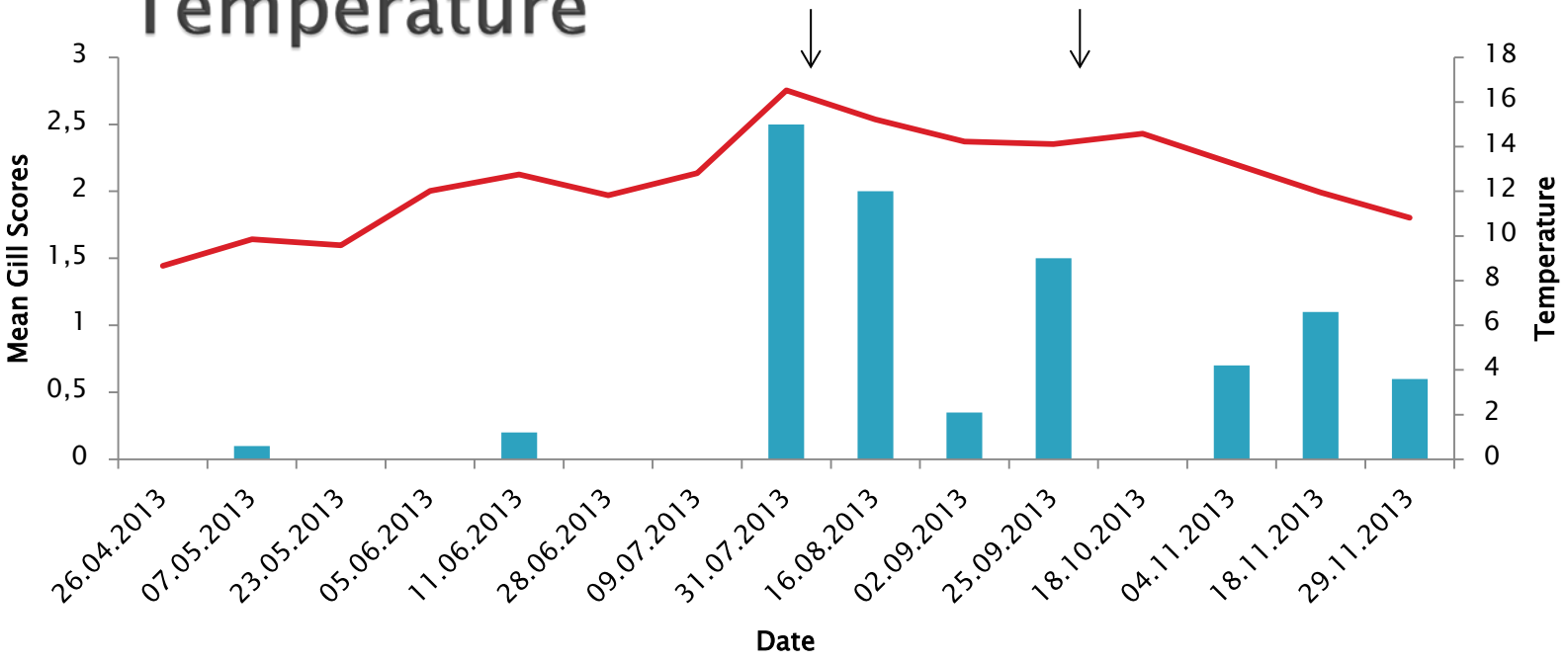
Molecular results

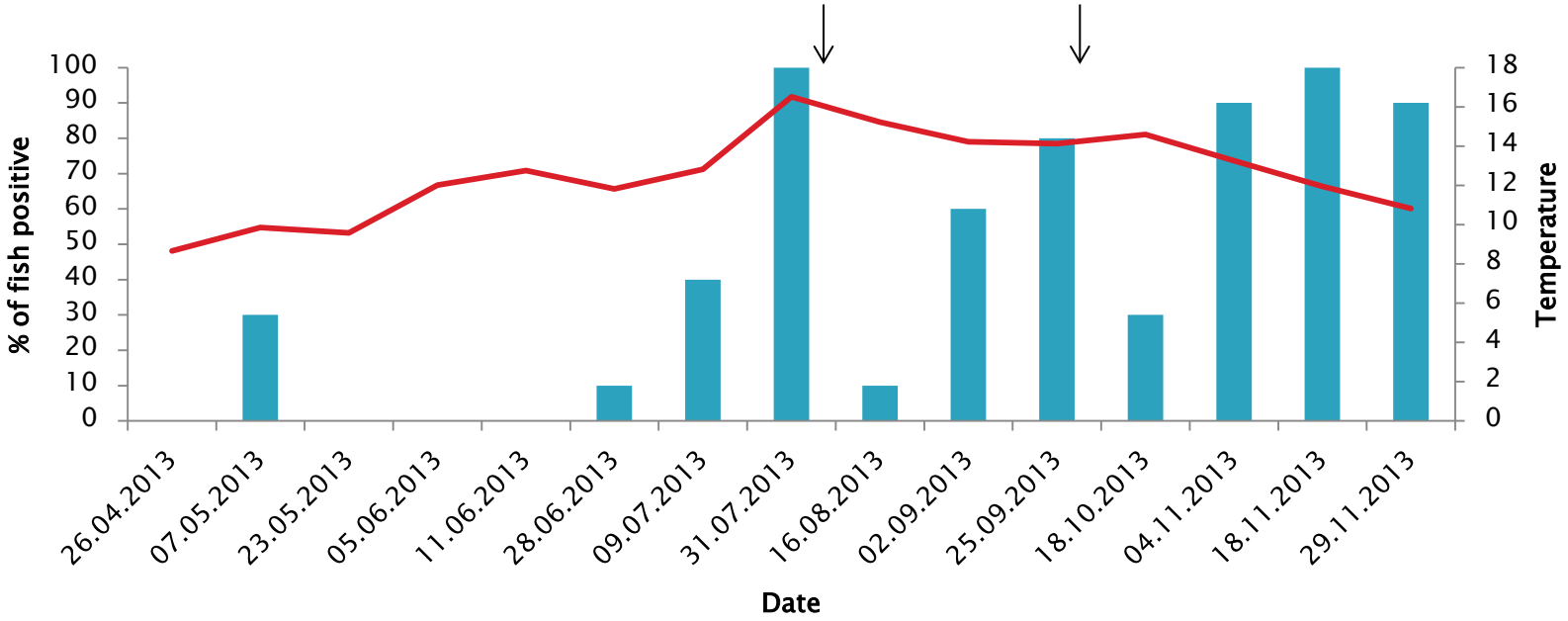


Temperature

- Water temperature had significant influence during AGD outbreak
  - Large increase in temp in July at onset of outbreak
  - Problematic for treatments
- Average temperature peaked at 19°C

Temperature





Longitudinal Study

- *N. perurans* first detected by PCR on June 28th before outbreak.
- Gill scores were 0 until July 31st.
- Treatments reduced % PCR positive fish and gill scores
- Amoeba detected by PCR until March 23rd.
- Although no samples positive in March for Cages 1 & 5, other cages on site are positive.
- New PCR methods appears to be sensitive enough to pick up infection 2/3 weeks prior to gross assessment

Future plans

- Longitudinal Study
  - Environmental data, O2, salinity
  - Growth, mortalities, husbandry
  - Other pathogens, Desmozoon lepeophtherii
- Molecular
  - Further sequencing
  - Cloning of culture DNA-allow for quantification
  - Archive samples
- Morphology
- Proteomics
  - Collect samples from infection trial or farm

Thank you for your attention.

Molecular results