Gene expression profile analysis of Manila clam (*Ruditapes philippinarum*) hemocytes after a *Vibrio alginolyticus* or *Perkinsus olseni* challenge using an immune-enriched oligo-microarray

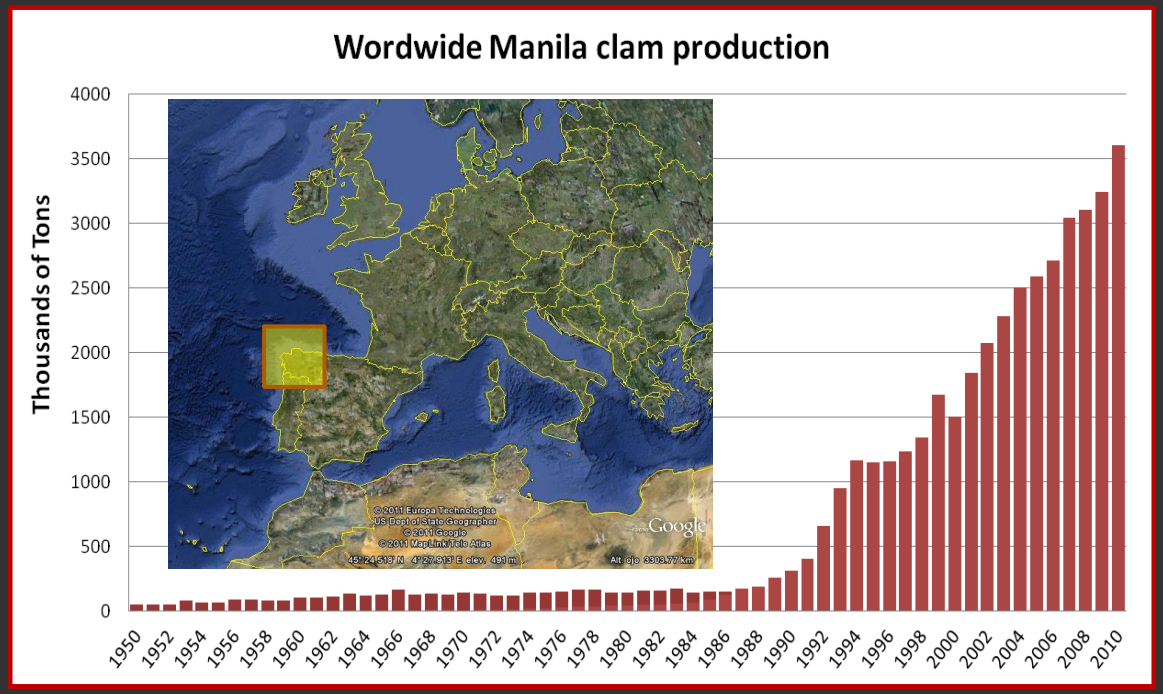
Moreira R, Romero A, Milan M, Bargelloni L, Novoa B, Figueras A

IIM Instituto de Investigaciones Marinas de Vigo
CSIC Consejo Superior de Investigaciones Científicas

International Symposium on Genetics in Aquaculture XII
SANTIAGO DE COMPOSTELA 2015

Economic importance

- Wordwide Manila clam production (FAO):

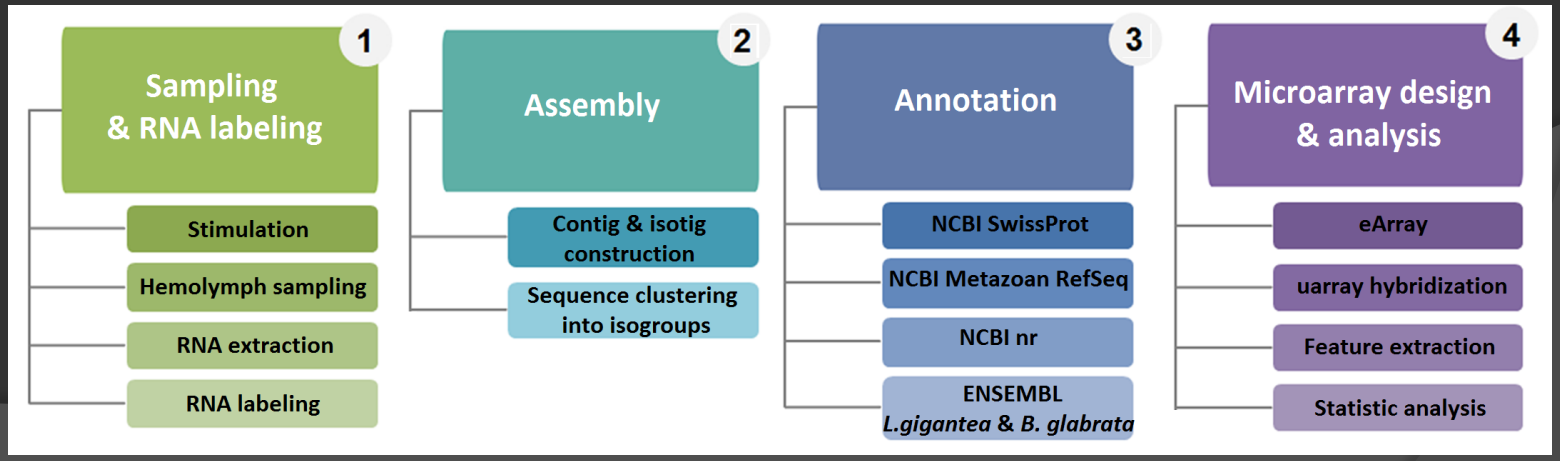


- nº1 China > 90%
- Europe:
  - Italy
  - France
- Spain:
  - ~ 2.000 T
  - ~ 15 M€
  - Galicia ~ 100%

- Diseases → high economical losses
- Limited knowledge of the I.S. of bivalves
- A lot of work to do about genes and proteins

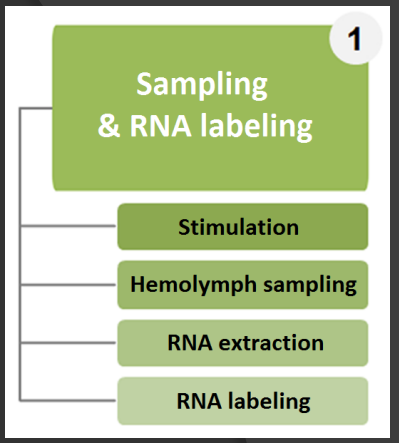
Objectives

- Design a new whole-tissue *R. philippinarum* microarray, including immune-related hemocytes sequences.
- Gene expression of hemocytes against a *V. alginolyticus* (TA15) challenge.
- Transcriptomic profile against a *P. olseni* challenge in a time course.



Vibrio challenge and sampling

- *In vivo* stimmulation

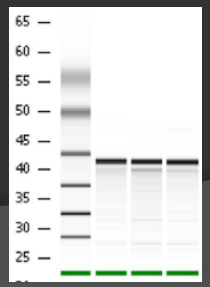


Sampling points:

Vibrio	*Perkinsus*
3h	5d
8h	10d
24h	14d
72h	31d

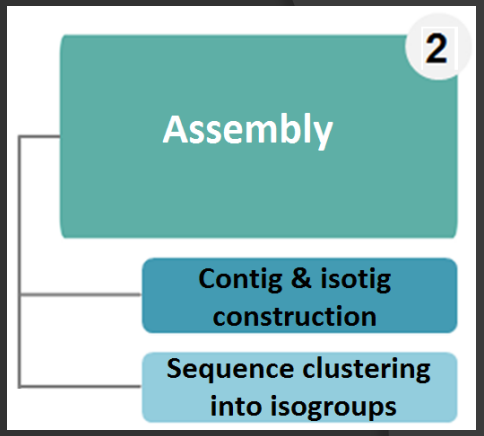
5 biological replicates

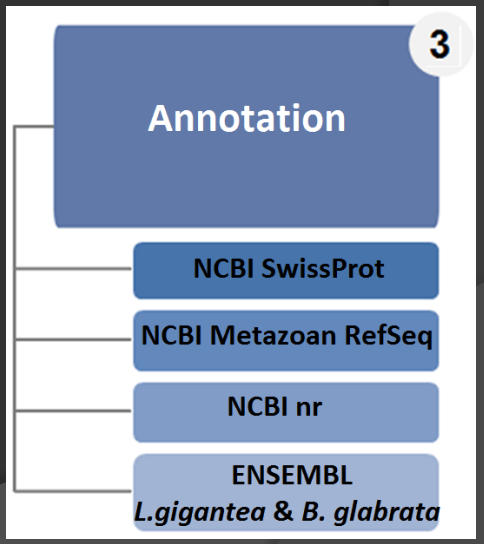
RNA extraction → Bioanalyzer → RNA labeling



Microarray design

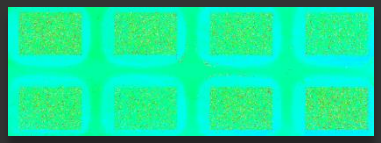
	nº sequences
SEQUENCE ORIGIN	
Sanger (Milan et al., 2011)	5,758
454 tissues (Milan et al., 2011)	457,717
454 hemocytes (Moreira et al., 2012)	975,190
NCBI	2,050
TOTAL	1,440,715
ASSEMBLY	
Not assembled raw sequences:	11,76%
Assembled raw sequences:	88,24%
ANNOTATION	
singletons phred Q > 20	5,914
NCBI, contigs and longest isotig of each isogroup	6,242
TOTAL *R. philippinarum* successfully designed probes	**13,671**

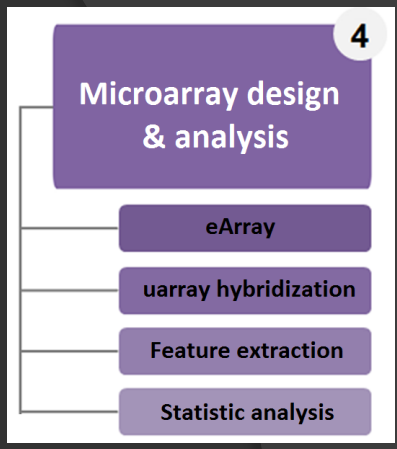




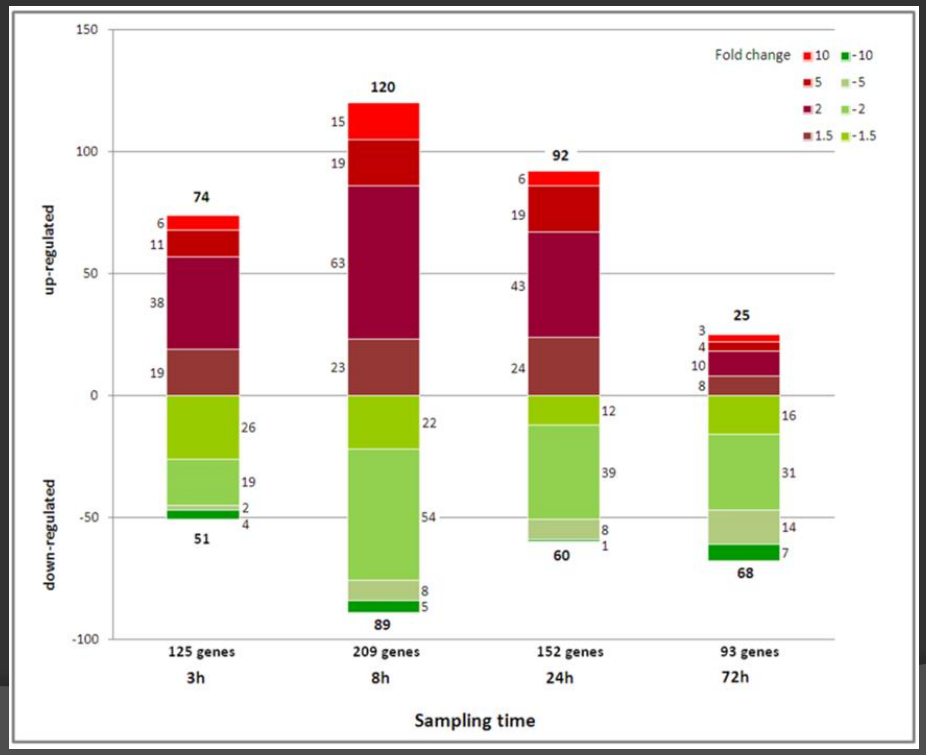
Expression profile

Microarray hybridization → Microarray scanning → Genespring

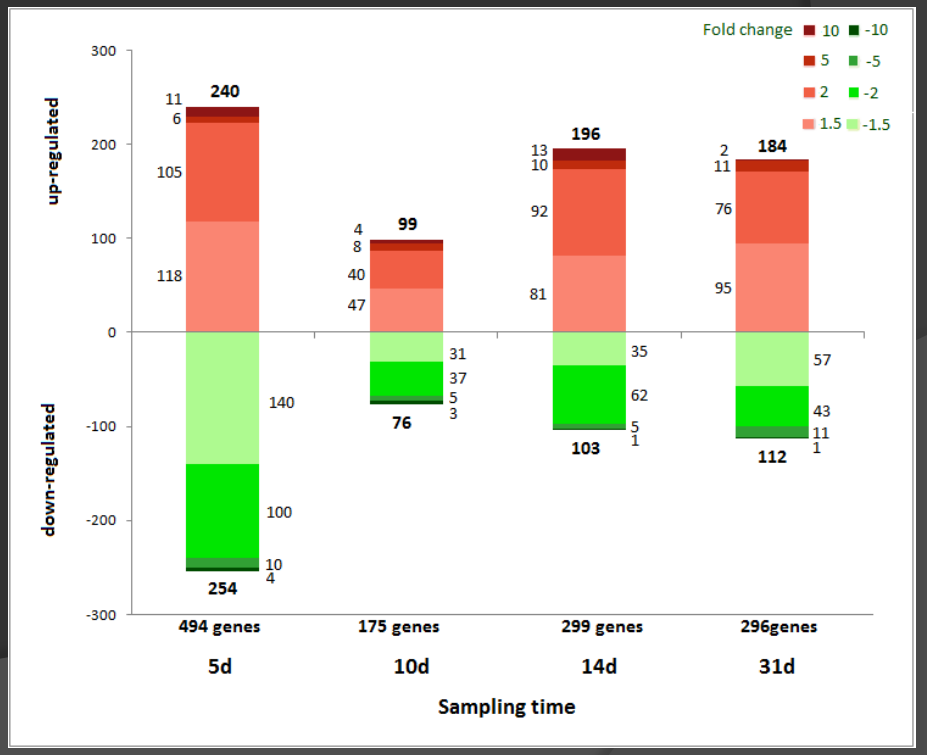




Vibrio alginolyticus



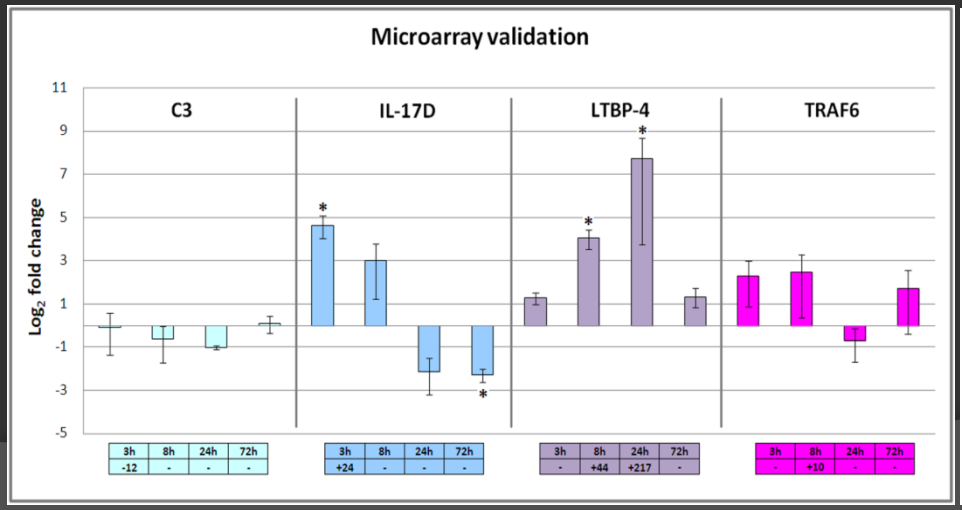
Perkinsus olseni



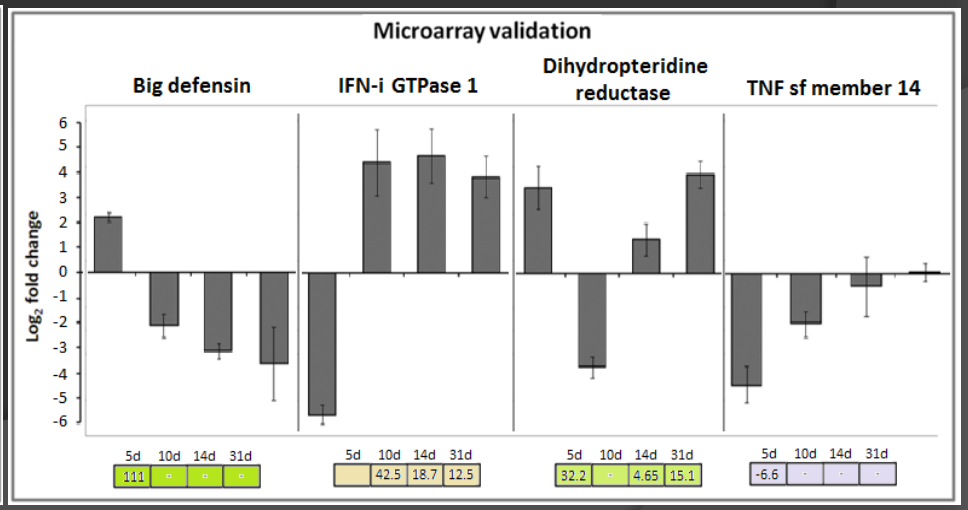
Validation

- Genes selected based on their relevance in the immune system:
  - C3: complement system
  - IL-17D: regulation cytokine production & inhibitory effect on hematopoiesis
  - LTBP-4: related to TGF-β function, cell adhesion and migration
  - TRAF6: activation of NF-κB & AP-1
  - Big defensin: antimicrobial peptide
  - IFN-i GTPase 1: resistance to intracellular pathogens
  - Dihydropteridine reductase: nitric oxide biosynthetic process
  - TNF sf14: activation of NF-κB & proliferation of immune cells

Vibrio alginolyticus

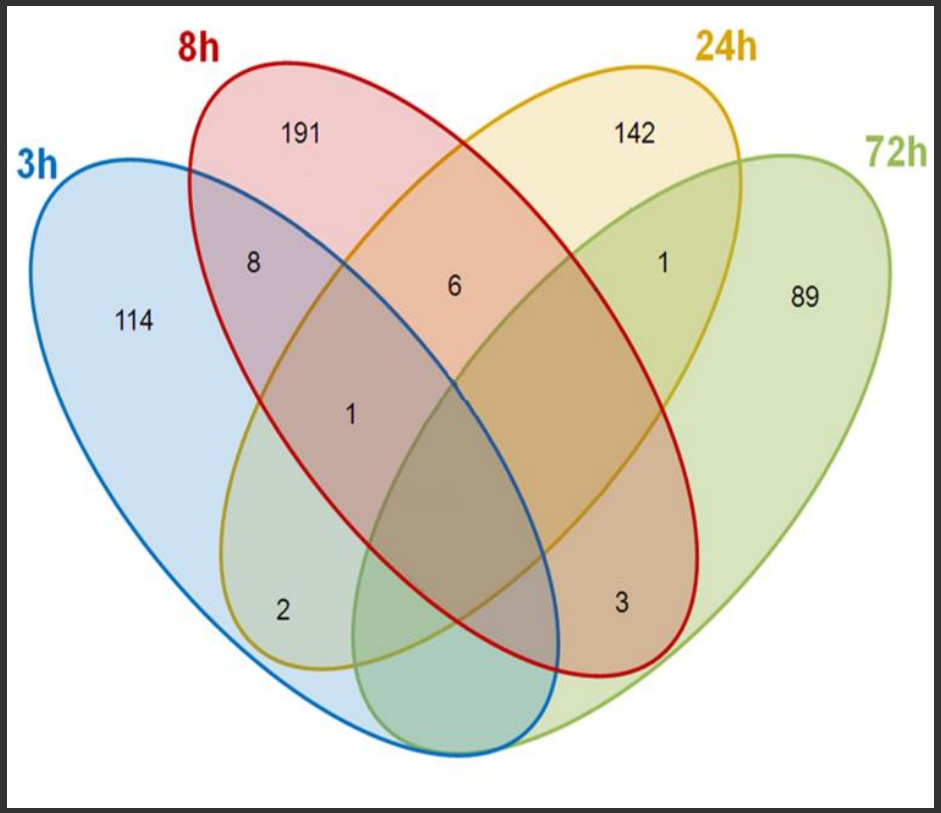


Perkinsus olseni

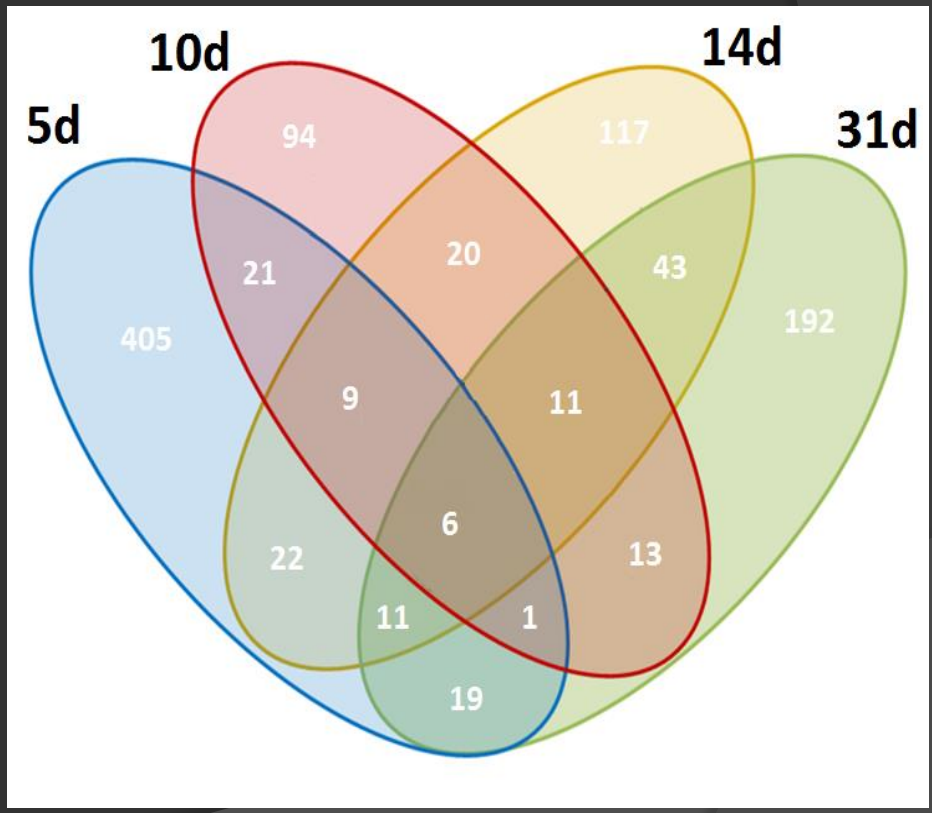


Temporal distribution of d.e.g.

Vibrio alginolyticus

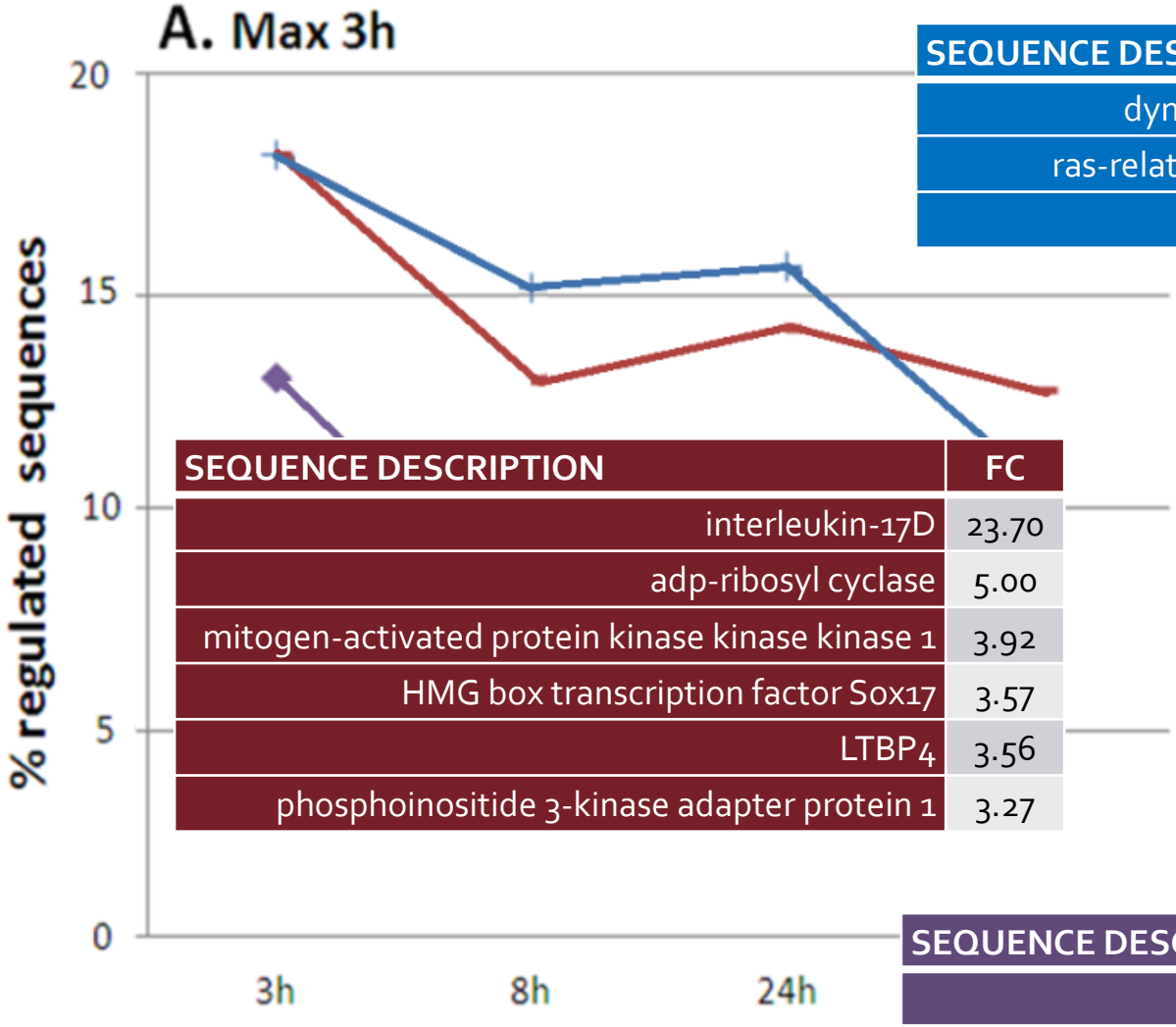


Perkinsus olseni



Timing of the response

Vibrio alginolyticus – 3hpi



SEQUENCE DESCRIPTION	FC
dynein light chain flagellar outer arm	48.91
ras-related c3 botulinum toxin substrate 3	11.05
perlucin	7.94

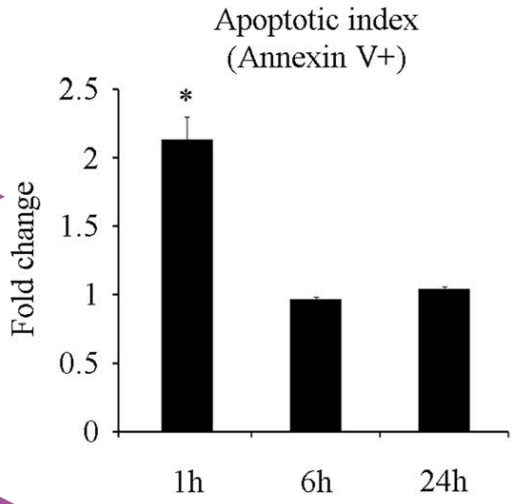
SEQUENCE DESCRIPTION	FC
interleukin-17D	23.70
adp-ribosyl cyclase	5.00
mitogen-activated protein kinase kinase kinase 1	3.92
HMG box transcription factor Sox17	3.57
LTBP4	3.56
phosphoinositide 3-kinase adapter protein 1	3.27

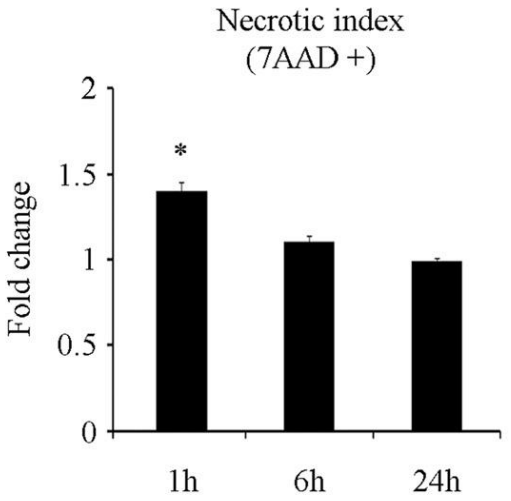
SEQUENCE DESCRIPTION	FC
calmodulin	3.46

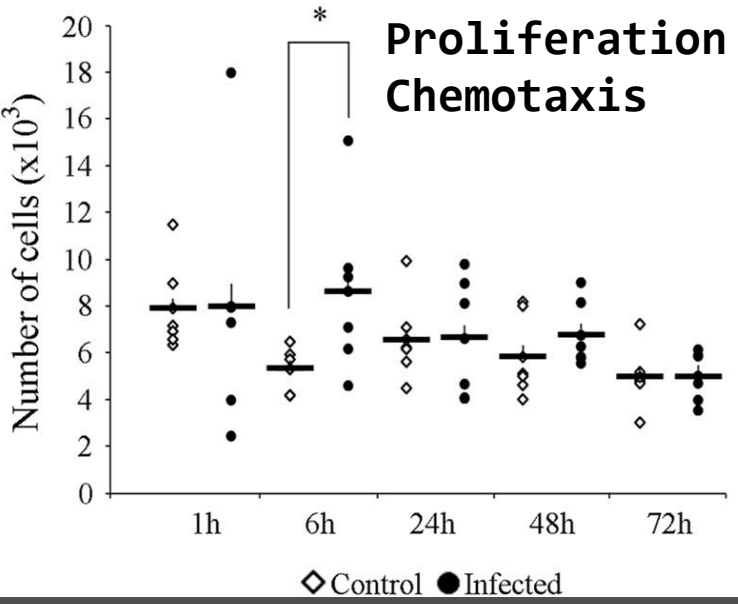
Importance of apoptosis: *Vibrio*

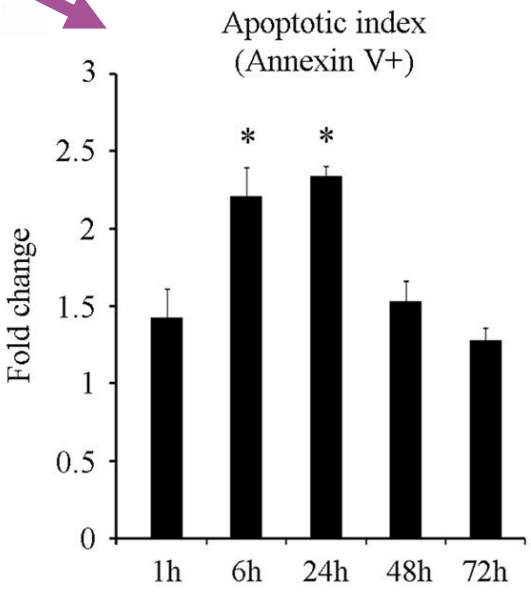
In vitro assay

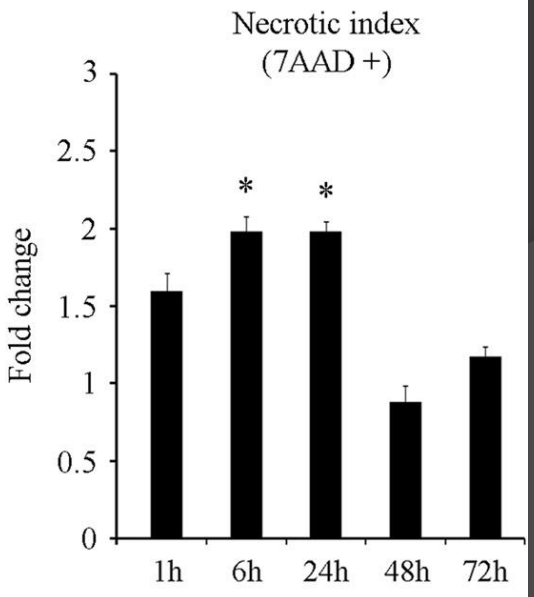
In vivo assay





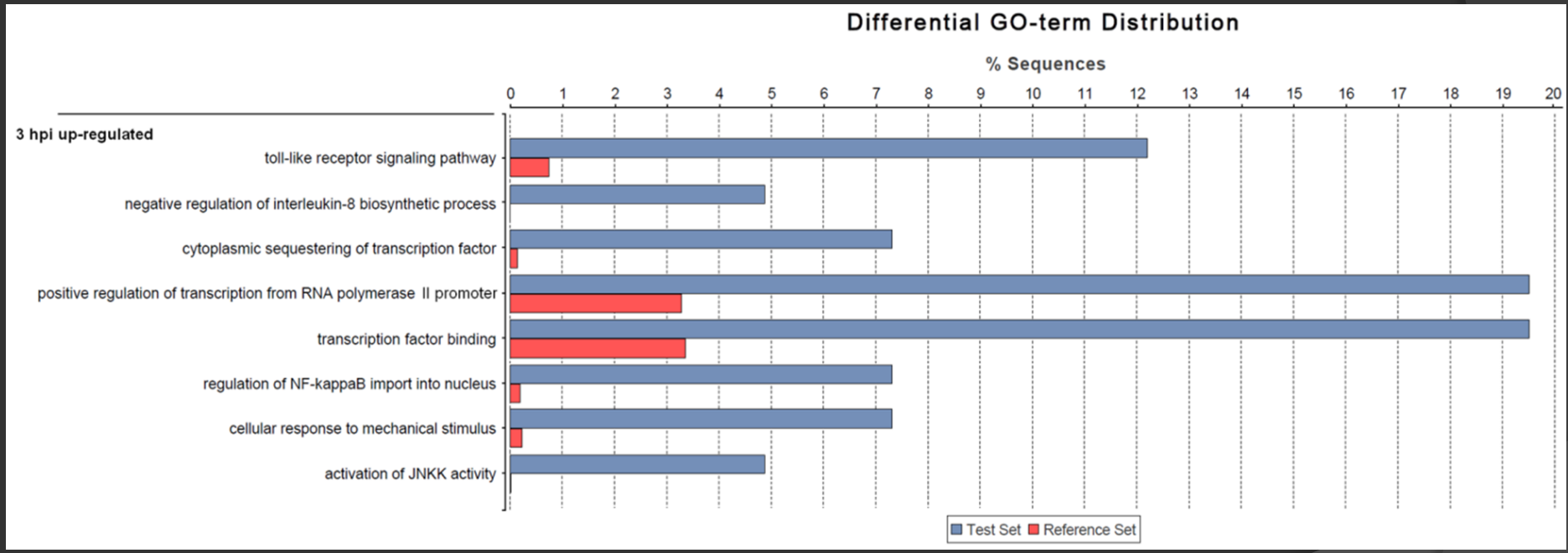






Enrichment analyses:

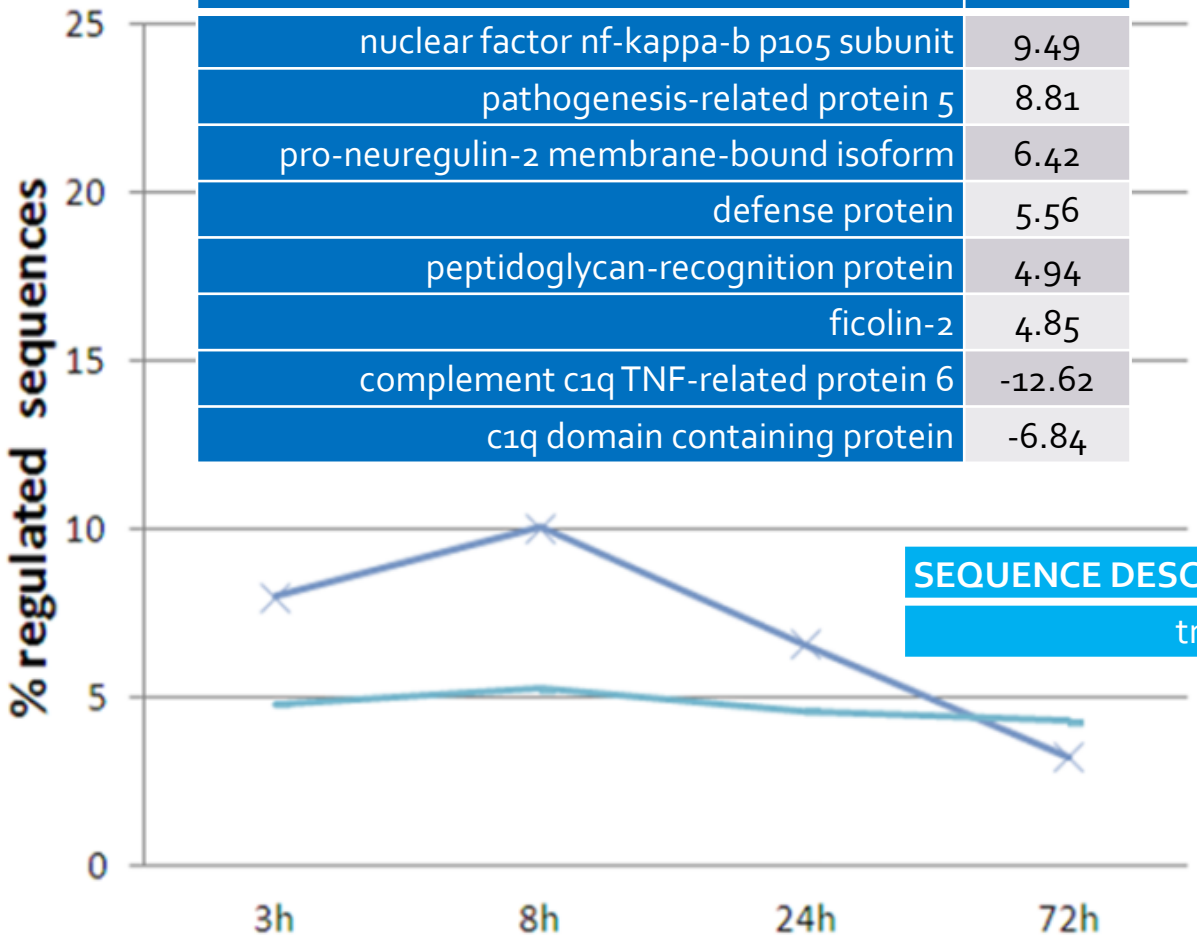
Vibrio alginolyticus – 3hpi up-regulated genes



Timing of the response

Vibrio alginolyticus – 8hpi

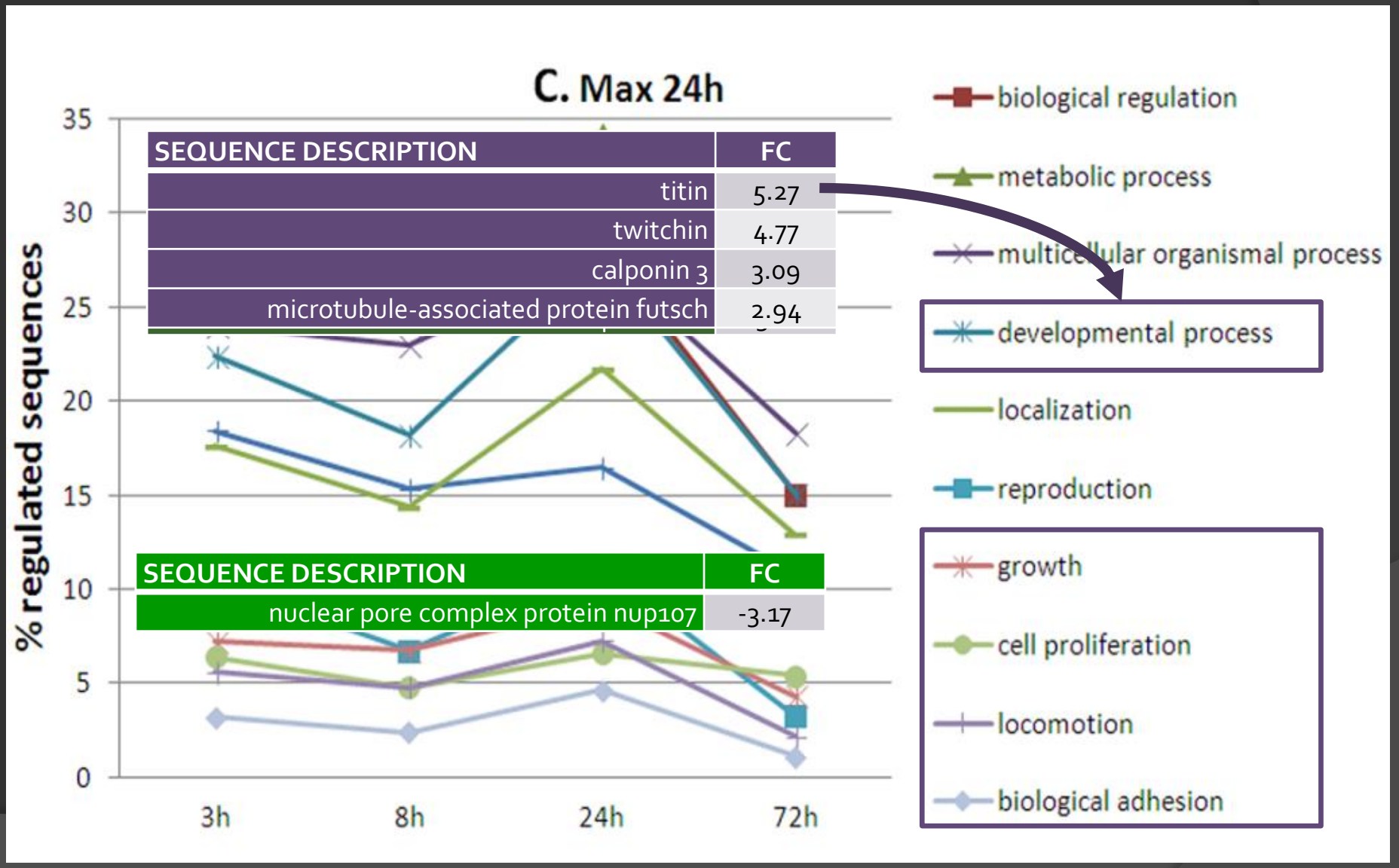
SEQUENCE DESCRIPTION	FC
nuclear factor nf-kappa-b p105 subunit	9.49
pathogenesis-related protein 5	8.81
pro-neuregulin-2 membrane-bound isoform	6.42
defense protein	5.56
peptidoglycan-recognition protein	4.94
ficolin-2	4.85
complement c1q TNF-related protein 6	-12.62
c1q domain containing protein	-6.84



SEQUENCE DESCRIPTION	FC
tnf receptor-associated factor 6	9.69

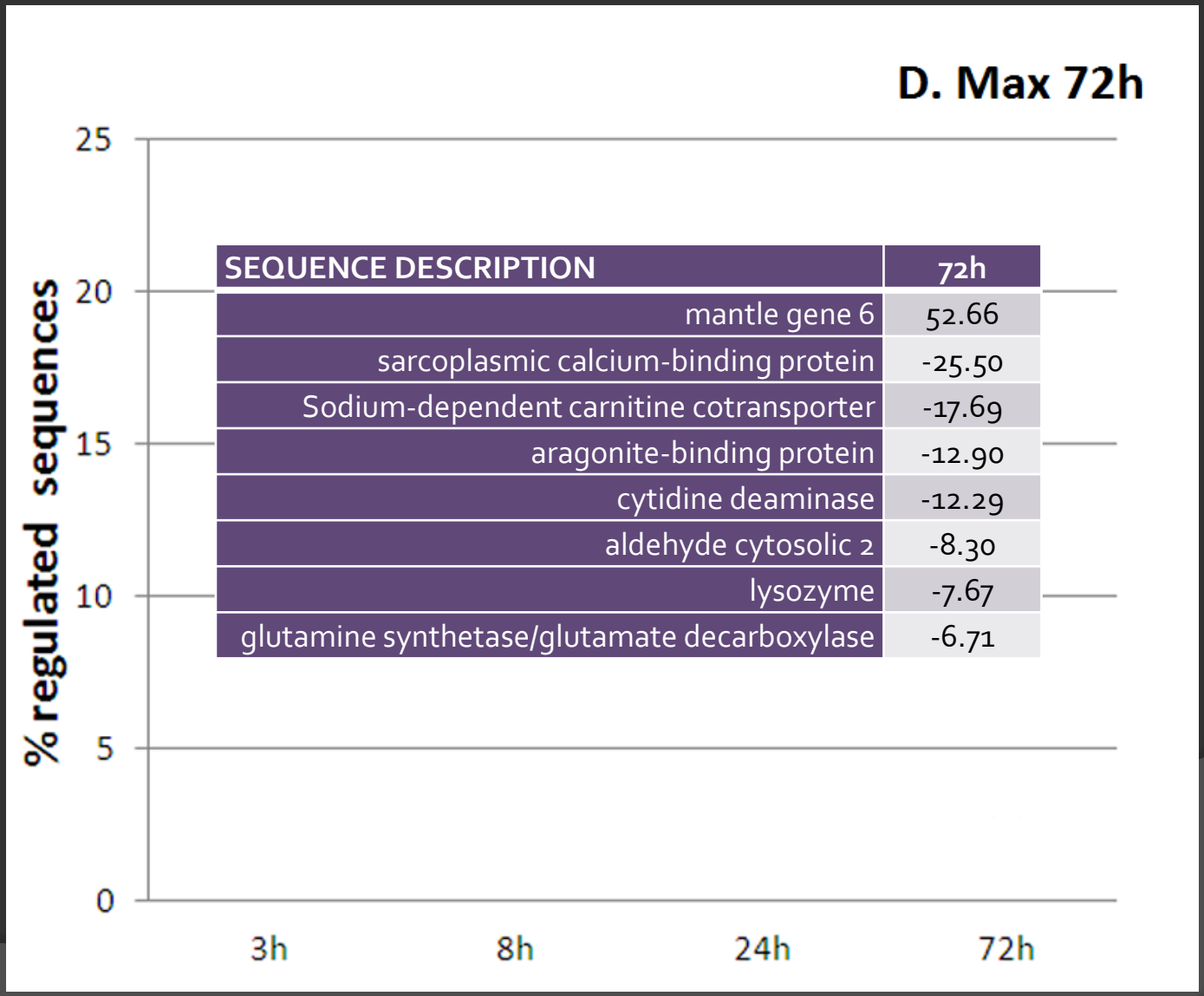
Timing of the response

Vibrio alginolyticus – 24hpi



Timing of the response

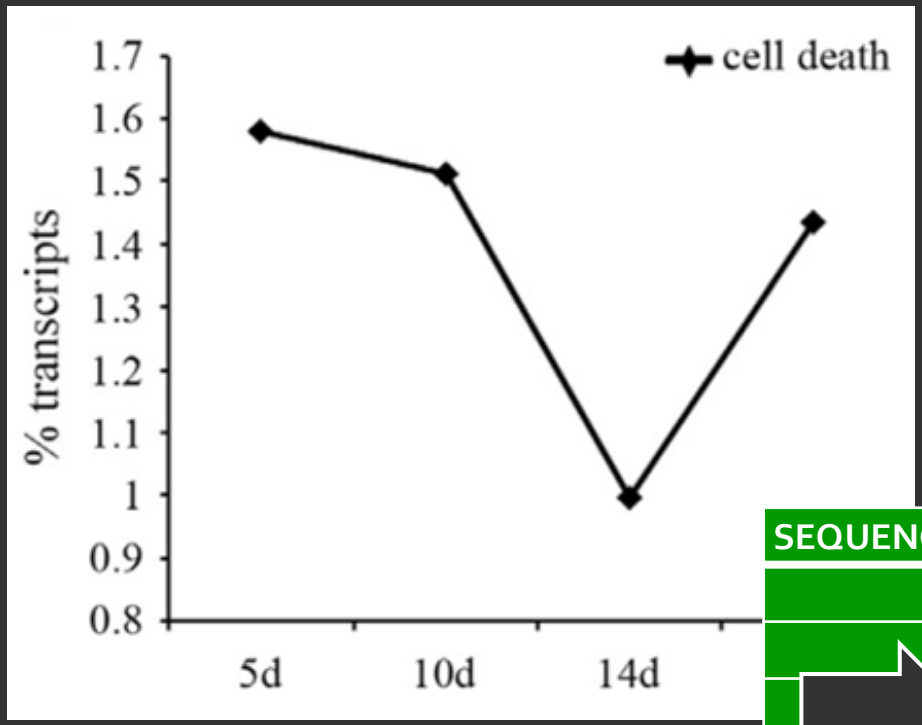
Vibrio alginolyticus – 72hpi



SEQUENCE DESCRIPTION	72h
mantle gene 6	52.66
sarcoplasmic calcium-binding protein	-25.50
Sodium-dependent carnitine cotransporter	-17.69
aragonite-binding protein	-12.90
cytidine deaminase	-12.29
aldehyde cytosolic 2	-8.30
lysozyme	-7.67
glutamine synthetase/glutamate decarboxylase	-6.71

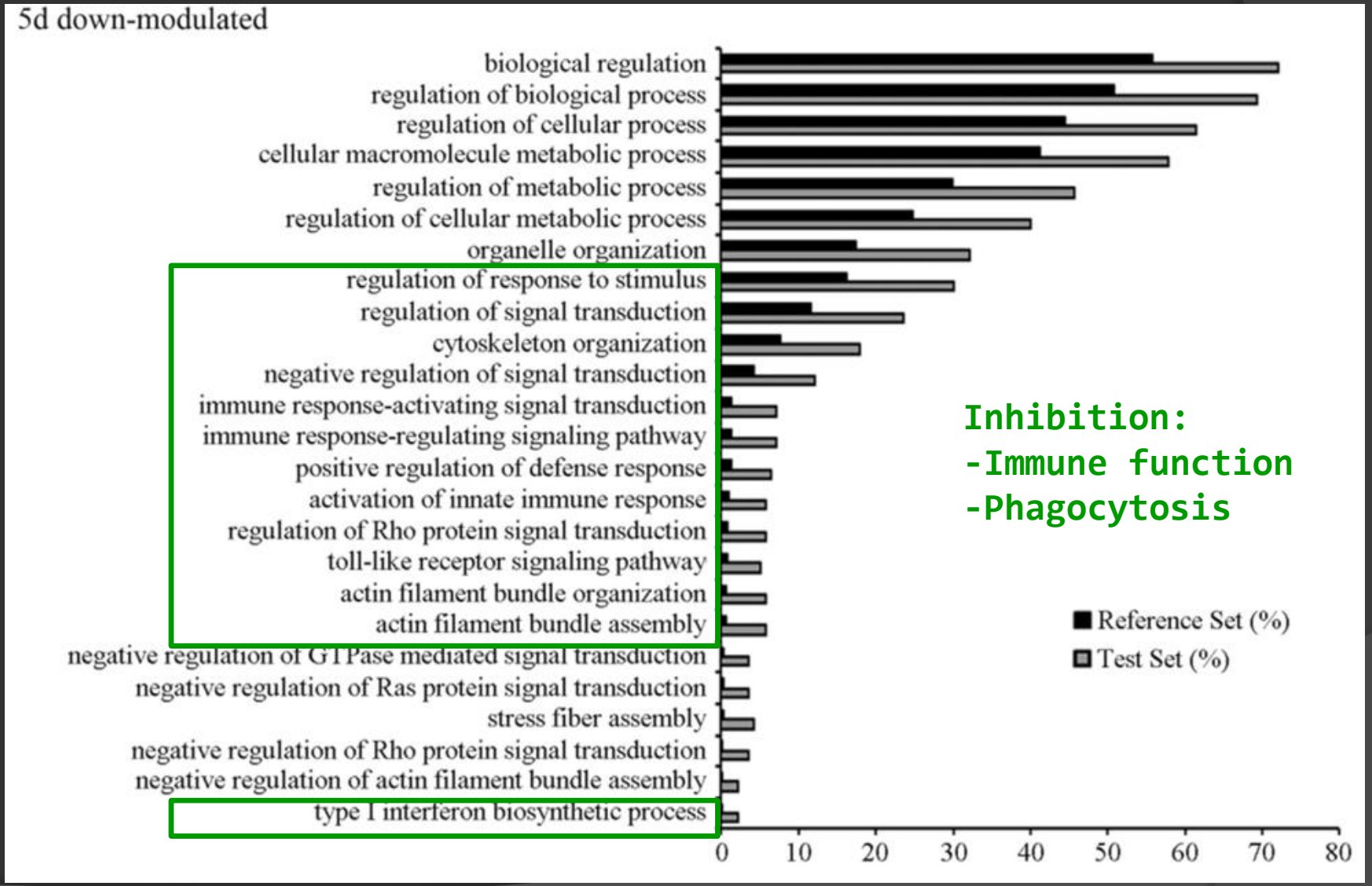
Timing of the response

Perkinsus olseni – 5dpi

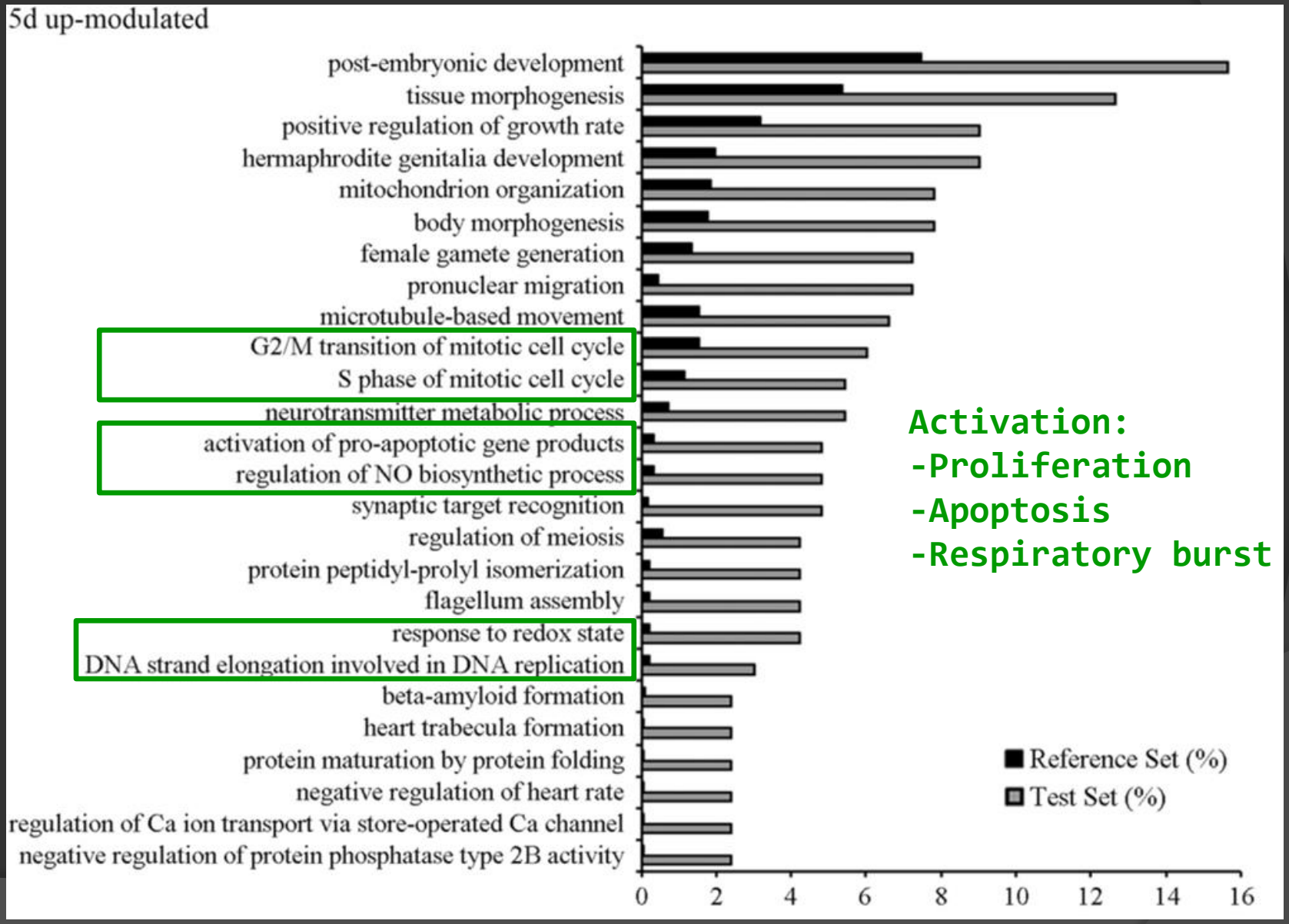


SEQUENCE DESCRIPTION	5d
Fibropellin-3	276.41
Big defensin	111.23
Quinoid dihydropteridine reductase	32.23
Ovomucoid (serine-type peptidase inhibitor)	26.55
C1q tumor necrosis factor-related protein 2	7.52
Histone h2a	3.84
Pathogen-related protein FRED	3.32
Sialic acid binding lectin	-8.71
TNFmsuperfamily member 14	-6.61
Interferon inducible GTPase 1	-4.38

Enrichment analyses: *Perkinsus*

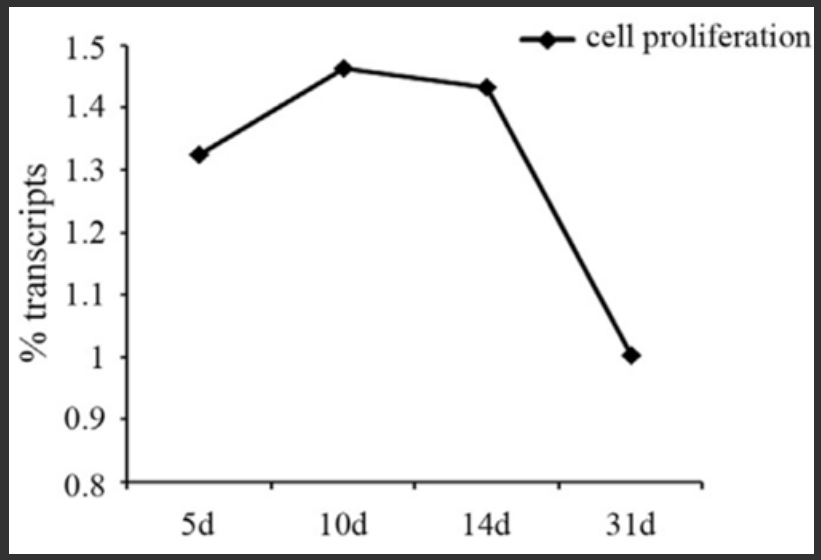


Enrichment analyses: *Perkinsus*

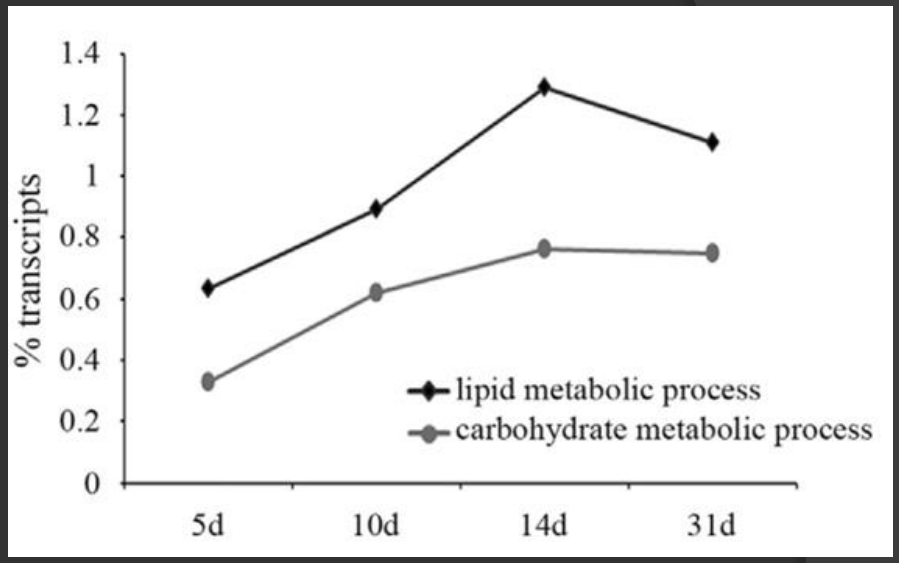


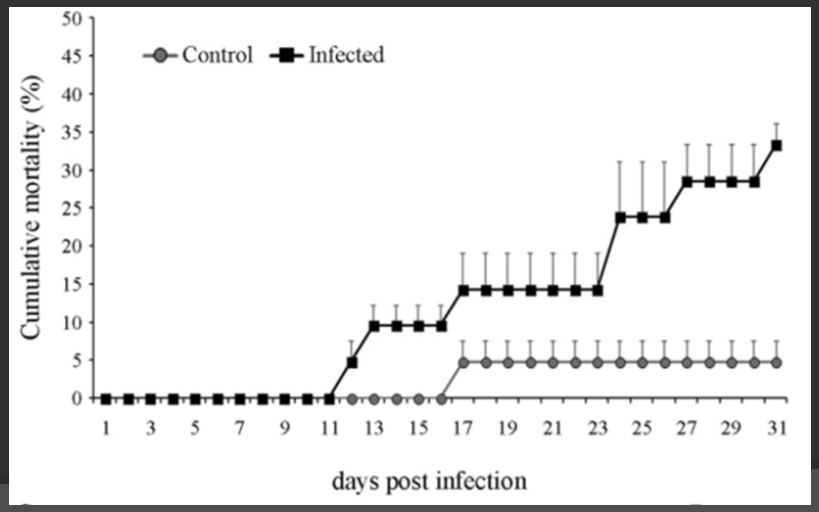
Timing of the response

Perkinsus olseni – 10dpi



14dpi

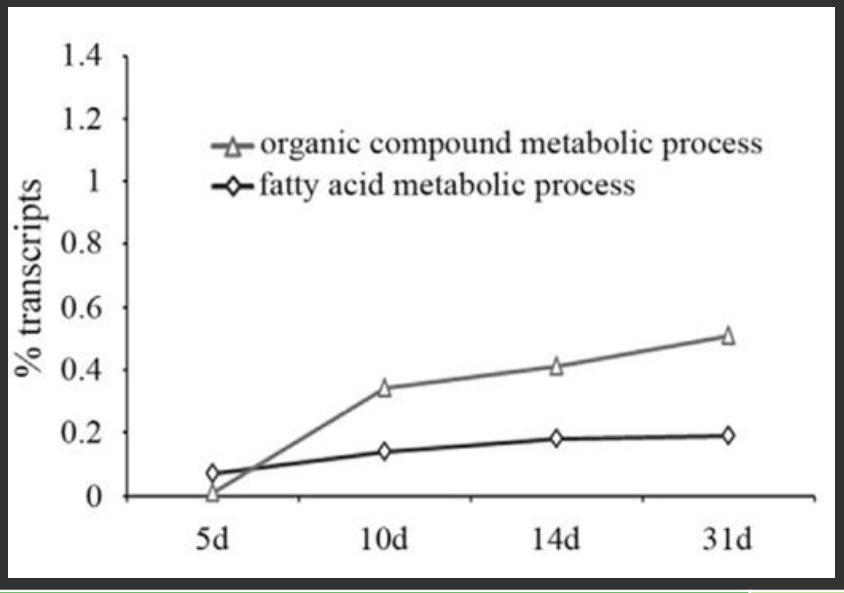


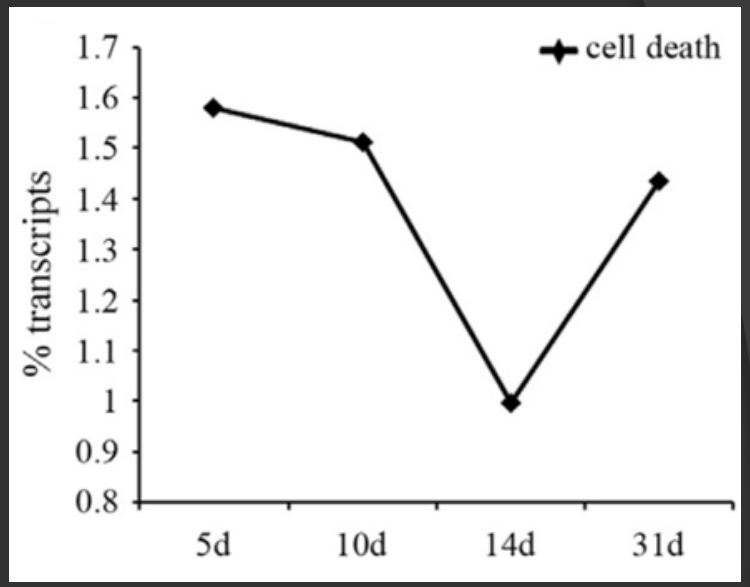


SEQUENCE DESCRIPTION	14d
Beta-tubulin (1)	42.50
Sialic acid binding lectin	21.33
Interferon-inducible GTPase 1	18.75
Beta tubulin (2)	14.94
Beta tubulin (3)	10.23
Glutathione-S transferase	6.55
Neurocalcin delta	5.80
Tyrosinase-like protein	-10.06

Timing of the response

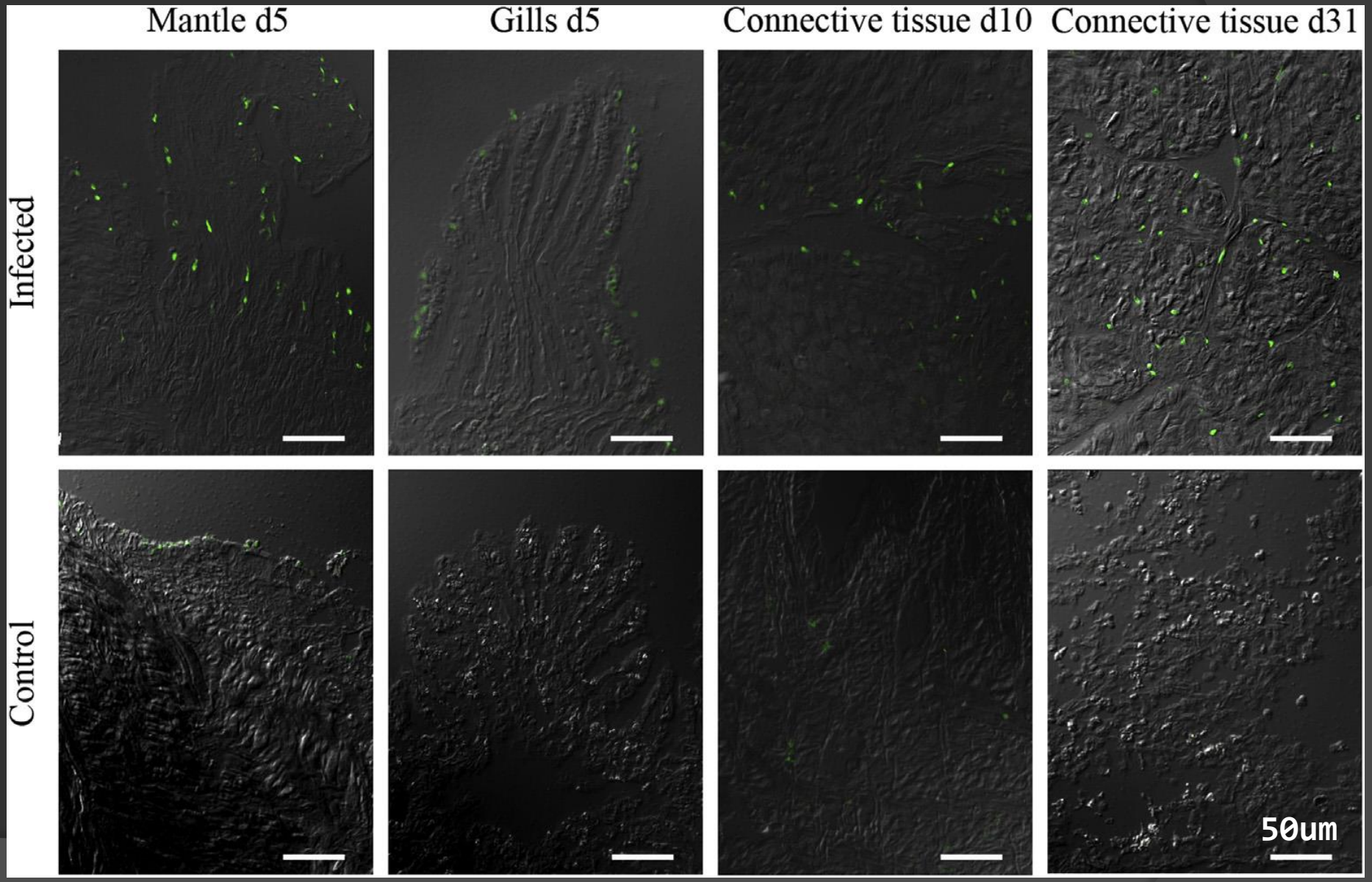
Perkinsus olseni – 31dpi





SEQUENCE DESCRIPTION	31d
Quinoid dihydropteridine reductase	15.15
Interferon-inducible GTPase 1	12.53
C1q domain-containing protein 1q51	4.92
Cadherin-related tumor suppressor	4.78
C1q TNF factor related protein6	4.47
Serine protease inhibitor cvSI-1	4.44
17-Beta-hydroxysteroid dehydrogenase 14	4.39
C1q TNF factor related protein 9b	3.41
Sialic acid binding lection	3.00
C1q domain-containing protein 1q79	2.09

Importance of apoptosis: *Perkinsus*



Conclusions *Vibrio*

- The timing for the response against a *Vibrio* infection was established:
  - Genes related to signaling, transcription and apoptosis were typically expressed as early as 3 h post-infection.
  - Characteristic immune and defense genes appeared at 8 hpi.
  - A high number of processes were activated 24 hpi to overcome the infection, including chemotaxis.
  - 72 h after infection a negative feedback of all of the previously active processes was observed.
- The key point to overcome the infection seemed to be 8 hours after the challenge.
- Importance of a fast response in bivalves and the effectiveness of their innate immune system.

Conclusions *Perkinsus*

- Timing for the response against a *Perkinsus*:
  - 5 dpi: pathogen recognition, NO radicals and AMP. No mortality.
  - 10 dpi: } Chemotaxis. Mortality increases.
  - 14 dps: }
  - 31dps: Metabolism-related genes.

- Apoptosis is a central process to control the infection.

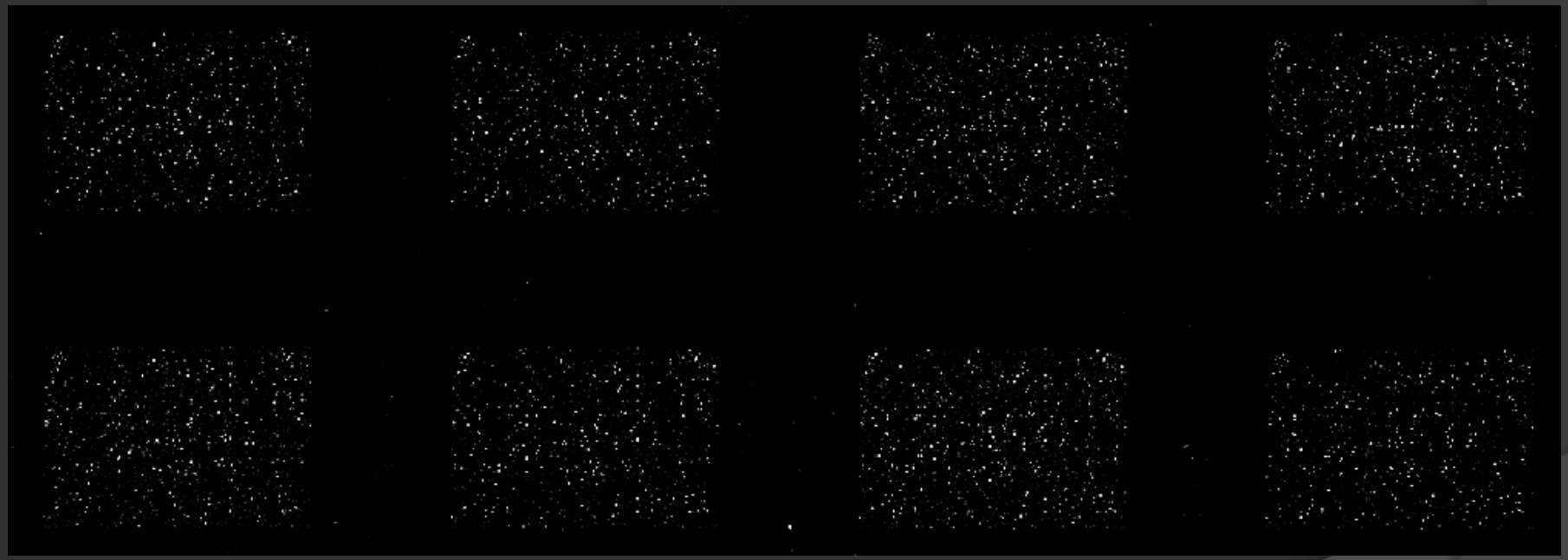
- Identification of novel genes:
  - Pathogen recognition: fibropellin-3
  - Production of nitrogen radicals: quinoid dihydropteridine reductase
  - Antimicrobial proteins: big defensin and histones.

Acknowledgments:

- Ministerio de Ciencia e Innovación

- ReProSeed project

Thank you for your attention!

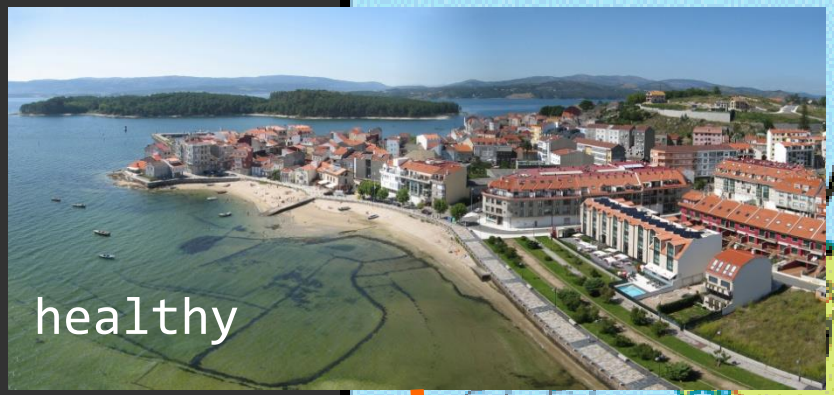


Instituto de Investigaciones Marinas de Vigo

CSIC Consejo Superior de Investigaciones Científicas

International Symposium on Genetics in Aquaculture XIX
SANTIAGO DE COMPOSTELA
2015

R. phil sampling:



Vibrio end point mortality: 44%
Perkinsus mortality and detection: