



## Research

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## Evolutionary biology

# Specific immune priming in the invasive ctenophore *Mnemiopsis leidyi*

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Specific immune priming enables an induced immune response upon repeated pathogen encounter. As a functional analogue to vertebrate immune memory, such adaptive plasticity has been described, for instance, in insects and crustaceans. However, towards the base of the metazoan tree our knowledge about the existence of specific immune priming becomes scattered. Here, we exposed the invasive ctenophore *Mnemiopsis leidyi* repeatedly to two different bacterial epitopes (Gram-positive or -negative) and measured gene expression. Ctenophores experienced either the same bacterial epitope twice (homologous treatments) or different bacterial epitopes (heterologous treatments). Our results demonstrate that immune gene expression depends on earlier bacterial exposure. We detected significantly different expression upon heterologous compared with homologous bacterial treatment at three immune activator and effector genes. This is the first experimental evidence for specific immune priming in Ctenophora and generally in non-bilaterian animals, hereby adding to our growing notion of plasticity in innate immune systems across all animal phyla.

## 1. Introduction

At the base of the metazoan tree four phyla branch off prior to Bilateria: Cnidaria, Porifera, Placozoa and Ctenophora [1]. These simple multi-cellular animals are invaluable to understand comparatively the evolution of key metazoan traits, including development, neurobiology and immune defence. Their large and delicate body surfaces are exposed to a 'soup' of bacteria in the marine environment, prompting the question of how their apparently effective immune defence is ensured.

The immune system has the 'double-edged' task of discriminating and eliminating pathogenic non-self while minimizing damage to self. Specific immune priming permits an induced response upon secondary exposure to the same threat [2,3]. While immunological memory was traditionally considered a hallmark of the vertebrate adaptive immune system [4], there is growing evidence that invertebrate immune responses are also modulated upon repeated infections [5–8]. Such functional analogues to immune memory clearly reach down further in the tree of life [8] but distribution and mechanisms remain to be defined. There are some immune repertoire studies on cnidarians and sponges [9,10], while experimental evidence for immune priming in basal metazoans is lacking. The neighbouring phylum of Ctenophora has been largely ignored in comparative immunology, even though it might represent the most basal metazoans [1,11]. The lobate ctenophore *Mnemiopsis leidyi* gained wide recognition as an invasive species, introduced repeatedly from the Americas into Eurasian Seas [12]. As the first ctenophore with a sequenced genome [13] it may become the 'model' species of this phylum.

To investigate specific immune priming in *M. leidyi*, we measured immune gene expression upon two consecutive bacterial challenges. Ctenophores were

**Table 1.** Experimental treatment combinations. Ctenophores were sequentially injected (T1 and 84 h later T2) with *L. anguillarum* (L), *P. citreus* (P) or sham treated (S) in a fully factorial design. This results in nine treatments, including homologous (ho) and heterologous (ht) bacterial exposures.

	first exposure (T1)		
	<i>L. anguillarum</i>	<i>P. citreus</i>	sham
second exposure (T2)			
<i>L. anguillarum</i>	LL (ho)	PL (ht)	SL
<i>P. citreus</i>	LP (ht)	PP (ho)	SP
sham	LS	PS	SS

exposed twice to heat-killed bacteria in a fully reciprocal design. In heterologous treatments, ctenophores were injected with two different bacteria, and in homologous treatments twice with the same agent. In the absence of specific immune priming, gene expression should solely depend on the second treatment. Alternatively, presence of specific immune priming would be identified if expression depends on the interaction of primary and secondary exposure and differs between homologous and heterologous treatments.

## 2. Material and methods

### (a) Animal collection and experiment

*Mnemiopsis leidyi* were collected in the North Sea (Oostende, Belgium) and acclimatized at GEOMAR in North Sea water (35 psu) for 24 h. Ctenophores were individually kept in beakers (300 ml) throughout the experiment (see the electronic supplementary material, S1). For the immune challenge, two heat-killed bacteria were used: the Gram-negative bacterium *Listonella anguillarum* (DSM no. 11323) and the Gram-positive *Planococcus citreus* (ATCC 14404), dissolved in sterile, artificial seawater. This combination of two abundant marine pathogens has been applied to activate and characterize immunological responses in fishes [14]. Bacteria were grown as outlined in the electronic supplementary material, S1.

Ctenophores were injected through the mesoglea into the body cavity with 50  $\mu$ l of *L. anguillarum* (L), *P. citreus* (P) or with artificial seawater as sham control (S). All animals received a subsequent secondary injection 84 h later with either the same strain (homologous), or a different bacterial strain (heterologous) or sham-exposed in a fully reciprocal set-up (table 1), afterwards they were transferred into fresh water. Six hours after secondary exposure, total RNA was extracted from four individuals per treatment combination (Invitek Spin Tissue RNA Mini, 36 samples). The set-up resulted in nine different treatments, including two homologous (LL and PP) and two heterologous bacterial treatments (LP and PL).

### (b) Quantification of immune gene expression using Q-RT-PCR

Our seven target genes were preselected based on unpublished pooled EST-libraries of *M. leidyi* comprising four treatments (naive, sham, LPS or bacteria exposure; S Bolte\*, EER Philipp\*, L Kraemer, G Hemmrich-Stanisak, J Saphörster, O Roth, TBH Reusch, P Rosenstiel 2010 \*shared first authorship, unpublished data). We identified differentially regulated genes via digital expression profiling that are putatively involved in bacterial sensing (see electronic supplementary material, S3). Primers flanking these target genes were designed using the software PRIMER3 [15] with melting temperatures around 60°C and amplicon length

80–160 bp (table 2; electronic supplementary material, S2). Gene expression was quantified with Q-RT-PCR as outlined in the electronic supplementary material, S3 relative to glyceraldehyde 3-phosphate dehydrogenase (GADPH).

### (c) Data analysis

Prior to (M)ANOVA data normality was tested using a Shapiro–Wilk test (JMP v. 10) and deviations from homogeneity using Levene’s test. A two-way MANOVA across all genes was performed to test the effect of primary (T1) or secondary exposure (T2) or their interaction (priming effect) on gene expression. This was followed by two-way ANOVAs testing which genes contributed to the overall effect (see electronic supplementary material, S4). To unravel whether a priming effect may be specific, we performed planned contrast analyses comparing homologous (LL and PP) versus heterologous (LP and PL) bacterial treatments (see electronic supplementary material, S4). Analyses were performed in R v. 2.15.1 (www.r-project.org).

## 3. Results

Over all tested genes, no main effects of first or second exposure were significant. Rather, the interaction of both exposures significantly influenced gene expression (MANOVA:  $F = 1.71$ ,  $p = 0.04^{**}$ , table 3a; electronic supplementary material, S4), attesting a priming effect. This translated to univariate interactions in six of the seven genes, constituting the overall effect (see electronic supplementary material, S4). Finally, a planned contrast between homologous (ho) and heterologous (ht) bacterial challenges revealed a significantly different expression at four genes (table 3b; electronic supplementary material, S4), supporting specificity of the priming effect.

Relative expression of all genes comparing homologous (ho) or heterologous (ht) bacterial exposure is shown in figure 1 (all groups in the electronic supplementary material, S5). Four genes showed significantly modulated expression: adenosylhomocysteinase (L1N) was higher expressed in the homologous treatment whereas expression of prophenol oxidase (proPO, TR4N), superoxide dismutase (TR3) and complement factor B1 (TR4N) was decreased upon homologous compared with heterologous bacterial exposure.

## 4. Discussion

The expression of immune-related genes in *M. leidyi* was not only determined by the acute bacterial challenge but also depended on previous pathogen exposure. Such a plastic response implies the presence of immune priming in the phylum Ctenophora that comes with a certain degree of specificity regarding the treatment with two distinct bacteria, i.e. a Gram-negative *Vibrio* and a Gram-positive *Planococcus*.

We described the ctenophore immune response via expression of candidate genes which had been preselected from pooled cDNA libraries of sham and bacteria-challenged individuals. Their putative immune function has not yet been assessed directly in ctenophores and functional interpretation relies on homology to the phylogenetically closest examples (mostly Cnidaria). ProPO/diphenoloxidase expression was reduced upon homologous compared with heterologous exposure. Phenoloxidase activity (melanization) is an important component of innate immunity in invertebrates, mostly studied in arthropods and crustaceans [16] and an important role in

**Table 2.** Genes and primers for quantitative real-time PCR in the ctenophore *M. leidyi*.

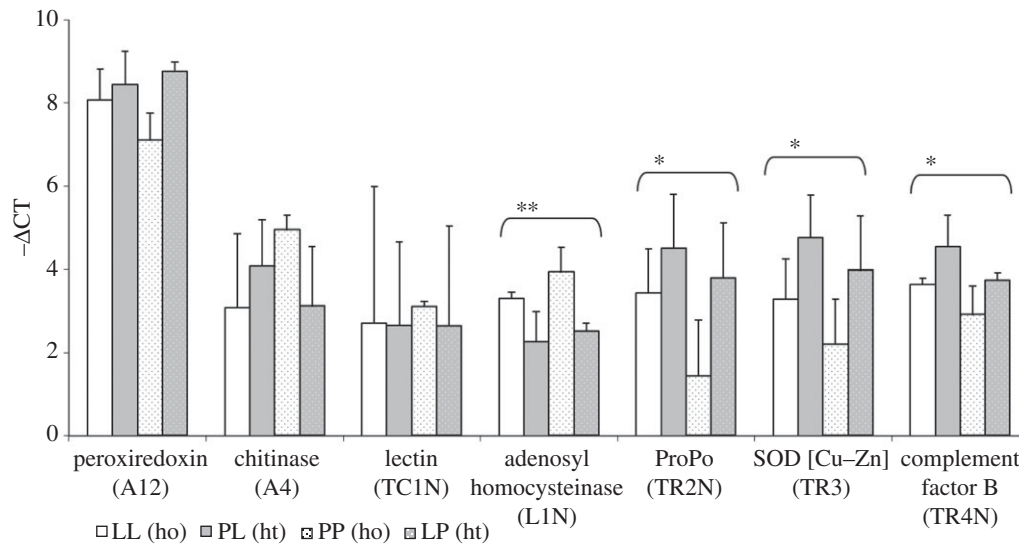
primer	gene annotation	pathway/function	seq. 5'–3'	amp. size (bp)
GADPH	glyceraldehyde 3-phosphate dehydrogenase	glycolysis	AGG GCT GAT GAC TGT TC CCT CTC CCG TCT CTC CAT TT	87
A12	peroxiredoxin	ROS/redox	CCC CAG CCT CAA TAA CTG AA ATG GCC GGT ACC GTA GAT TA	103
A4	chitinase	chitin degradation, put. allorecognition	GTC GGG TCC TTG ACA ACA GT ACT GGG GAA GCA GGA TTT TT	83
TC1N	MACPF 14/lectin	complement	ATT TGC AGA TCG ACC AAA CC CCA AAC ACA CAA CTG GCA AC	121
TR2N	proPOdiphenoloxidase subunit A3	ROS/redox melanization	CTT CCA ATT TGT CAC CAG CA GGA GAG ATA ACC GAC CAG CA	120
TR3	SOD Cu–Zn 7	ROS/redox	AAT CCA CAT GGA GCC ACT TC TGC CCT CTT TGC TCT TGT TT	80
TR4N	complement factor B	complement, alternative pathway	TCG ACC CAT CAC ACC TAA CA CCC ATG ACA ACG TGC ACT AC	93
L1N	adenosylhomocysteinase B	nucleic acid and protein metabolism	GTG GAG ACA CCC AGC GAT AC CTG ACA TCG AGT TGG CAG AA	137

**Table 3.** Statistical analysis of differential gene expression. (a) Two-way MANOVA over all genes testing the effects of first exposure (T1), second exposure (T2) and their interaction (T1 : T2). (b) Planned contrast between homologous (ho) and heterologous (ht) exposure. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ .

	d.f.	Pillai	approx. <i>F</i>	num d.f.	den d.f.	Pr(> <i>F</i> )
(a) two-way MANOVA						
T1	2	0.69615	0.91528	14	24	0.55601
T2	2	1.01764	1.77586	14	24	0.10461
T1 : T2	4	1.85618	1.73166	28	56	0.04044*
residuals	17					
gene	contrast	d.f.	sum sq	mean sq	<i>F</i> -value	Pr(> <i>F</i> )
(b) planned contrasts						
A12 peroxiredoxin	ho versus ht	1	4.14	4.145	2.575	0.1202
A4 chitinase	ho versus ht	1	0.62	0.623	0.208	0.6527
L1N adenosylhomocysteinase	ho versus ht	1	4.86	4.858	11.258	0.00263**
TR2N proPO	ho versus ht	1	11.84	11.839	5.030	0.0333*
TR3 SOD	ho versus ht	1	10.61	10.61	4.548	0.0422*
TR4N complement factor B	ho versus ht	1	3.41	3.409	7.304	0.0124*

cnidarian immune defence of corals, with an upregulation in pigmented tissues as part of an inflammatory response [17]. Superoxide dismutase (Cu–Zn SOD) catalyses the dismutation of superoxide into oxygen and hydrogen peroxide [18], and thus plays an important role in inflammatory processes [19]. SOD is known as a major player in the breakdown of

cnidarian–dinoflagellate symbiosis during coral bleaching [20]. Here, its expression was reduced upon homologous compared with heterologous bacterial challenge. Such reduced inflammatory response after homologous exposure, detected for phenoloxidase and superoxide dismutase, may save resources and reduce self-damage. Complement factor B is



**Figure 1.** Differential gene expression between homologous (ho) and heterologous (ht) pathogen exposure. Ctenophores were injected with *L. anguillarum* (L) or *P. citreus* (P), resulting in homologous (LL and PP) and heterologous (PL and LP) treatments. Expression of seven immune-related genes ( $-\Delta CT \pm$  s.d., all values transferred to positive scale by addition of 5CTs). Four genes showed significantly different expression: adenosylhomocysteinase was upregulated upon homologous treatment; ProPo, SOD and complement factor B showed lower expression after homologous treatment ( $*p \leq 0.05$ ,  $**p \leq 0.01$ , for exact  $p$ -values, see table 3b).

involved in the alternative pathway of complement activation directly from the pathogen surface [21]. This evolutionarily oldest pathway of complement activation [22] is present in Cnidaria but to date unexplored in Porifera and Ctenophora [23]. Here, we observed significantly lower expression in homologous compared with heterologous treatment, indicating that complement activation contributes to specific immune priming of ctenophores. Increased expression of the metabolic enzyme adenosylhomocysteinase in homologous, as opposed to heterologous treatments suggests enhanced metabolic function in these animals.

At the first glance, lower gene expression after homologous compared with heterologous treatment at three immune receptor and effector genes seems puzzling. However, these findings are consistent with evolutionary theory predicting that selection drives species to minimize costs and self-damage of immune defence [2,3]. In line with this, the expression of a general metabolic enzyme adenosylhomocysteinase was increased after homologous compared with heterologous bacterial challenge. According to the concept of immune priming, a specific response to repeated infections would also include upregulation of particular immune pathways matching this encounter (reviewed in [24]). We did not observe such

upregulation of immune effectors after homologous exposure, indicating that either the repeated injections with heat-killed bacteria were not recognized as real threats (infections) or the specifically upregulated genes were not included in our candidate gene set. Future research including transcriptome-wide analysis of gene expression should help to identify pathways specifically upregulated after repeated exposure.

Despite this limitation, our study provides experimental evidence that immune gene expression of *M. leidyi* is induced through pre-exposure. To our knowledge, this is the first observation of immune priming in the phylum Ctenophora and in an invertebrate prior to Bilateria. This study should encourage future research to unravel the significance of this process, its molecular mechanisms and ecological implications. Ultimately, such plasticity will enhance the ecological performance of comb jellyfish and contribute to their success in changing global oceans.

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