Electronic supplementary material (ESM) for:

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Specific immune priming in the invasive ctenophore *Mnemiopsis leidyi*
Biology Letters

Content:

S1

Ctenophore handling and treatment

Ctenophores of the species *Mnemiopsis leidyi* were collected in the North Sea (Oostende, Belgium) and transported to the GEOMAR, were they were kept in North Sea water (35psu) under constant conditions in a climate chamber. Ctenophores were separated into plastic beakers (300ml, North Sea water 35psu) were they were kept throughout the experimental procedure. The ctenophores were injected twice with bacterial agents or sterile seawater (InstantOcean, 35psu, sterile filtered 0.22 µm) as control. For the treatment animals were carefully transferred onto a petri dish and injected with 50µl trough the mesoglea directly into their body cavity. The two treatments were conducted in consecutive order with the second injection 84hours after the first treatment. Between the treatments water was exchanged and animals were kept in random array to avoid any container effects.

Bacteria growth conditions

Both bacteria were taken from glycerol stocks transferred into culture medium 101 (5 g Peptone and 3 g meat extract per liter, both Sigma) adjusted for marine bacteria by addition of 3% NaCl and grown at 28°C overnight. Bacteria were transferred to a larger volume and kept in exponential growth for additional 24h until optical density (OD) was measured at 612nm. *L. anguillarum* was grown to an optical density of 0.39 for the first exposure and 0.31 for second injection. *P. citreus* was grown until OD was 0.17 for the first injection and 0.17 for the second treatment. Bacteria cultures were transferred into 1.5 ml eppendorf tubes, heat deactivated at 65°C for 1h, centrifuged at medium speed (2000 rpm), then the bacterial pellet was resuspended in artificial seawater (InstantOcean, 35psu, sterile filtered 0.22 µm).

S2 Target genes:

Target genes were selected based on EST libraries of a single infection experiment (Philipp, Bolte et al. unpublished data). Here ctenophores (*M. leidyi)* were left naive, sham treated or injected with either lipopolysaccharide (LPS) or *Listonella anguillarum* (heat deactivated). RNA extracted six hours after the treatments was pooled from four animals per treatment group and sequenced on Roche 454 FLX. 805 601 reads, with an average length of 373 bp passed the quality control. 676 933 reads could be assembled into 26 165 contigs with an average length of 824 bp, 128 688 reads could not be assembled and remained as singletons. The assembly consists of 171 467 naiv, 104 697 sham, 228 770 Vibrio and 171 999 LPS sequence reads. Differential expression was estimated as the number of reads for each treatment within each contig, corrected by the number of reads in the total assembly. As candidate genes for the qPCR based immune priming experiment we choose genes which were up-regulated in the bacterial treatment compared to naiv/sham treatment and could be annotated to innate immune functions:

- Peroxiredoxins (A12) are a group of anti-oxidant proteins, which protect cells from reactive oxygen species (ROS). Furthermore they play a role in immune modulation [1] and hydrogen peroxide mediated signal transduction [2]. Peroxiredoxin expression can be induced by LPS [3].
- Chitinase (A4) like proteins are involved in inflammatory response in vertebrates [4, 5], they also participate in apoptosis and macrophage activation. For cnidarians a putative double function in chitin degradation and allorecognition has been reported [6].
- Adenosylhomocysteinase (S-adenosylhomocysteine hydrolase, L1N) is an ubiquitous and evolutionarily conserved metabolic enzyme that converts S-adenosylhomocysteine to homocysteine and adenosine [7].
- Lectins (TC1N) bind to surface carbohydrates and play an important role in innate immune activation [8].
- ProPO/Diphenoloxidase (TR2N) is involved in melanisation (phenoloxidase activity) and associated with phagocytosis and encapsulation in invertebrate innate immune defence [9, 10].
- Superoxid Dismutase (SOD, TR3) is involved in oxygen metabolism and regulation of reactive oxygen species (ROS) in inflammation [11].
- Complement factor B (TR4) is part of the alternative pathway of complement activation, directly from pathogen surface (Janeway et al. 2001).

References S2

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2. Wood Z.A., Poole L.B., Karplus P.A. 2003 Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science Signalling* **300**(5619), 650.
3. Bast A., Fischer K., Erttmann S.F., Walther R. 2010 Induction of peroxiredoxin I gene expression by LPS involves the Src/PI3K/JNK signalling pathway. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms* **1799**(5), 402-410.
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5. Lee C.G., Da Silva C.A., Dela Cruz C.S., Ahangari F., Ma B., Kang M.J., He C.H., Takyar S., Elias J.A. 2011 Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annual review of physiology* **73**, 479-501.
6. Mali B., MÃ¶hrlen F., Frohme M., Frank U. 2004 A putative double role of a chitinase in a cnidarian: pattern formation and immunity. *Dev Comp Immunol* **28**(10), 973-981.
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8. Honda S., Kashiwagi M., Miyamoto K., Takei Y., Hirose S. 2000 Multiplicity, structures, and endocrine and exocrine natures of eel fucose-binding lectins. *Journal of Biological Chemistry* **275**(42), 33151-33157.
9. Cerenius L., Söderhäll K. 2004 The prophenoloxidase-activating system in invertebrates. *Immunological reviews* **198**(1), 116-126.
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S3 Q-RT-PCR assay

As reference gene we identified glyceraldehyde 3-phosphate dehydrogenase (GADPH), which has already been used in earlier studies on gene expression in basal metazoans [1]. Expression levels between treatment groups were fairly stable based on the number of reads per treatment group. RNA concentrations were quantified with a photo spectrometer (NanoDrop, Peqlab) and 600ng per sample were used for cDNA synthesis. RNA was transcribed to cDNA using the Quanti Tect Reverses Transcription Kit (Quiagen, Germany) following standard procedure and including a no reverse transcription control (NRTC) for every sample. Quantitative PCR reactions were performed using Fast SYBR Green (Applied Biosystems) on an ABI Step One Plus (Applied Biosystems). The cycling protocol consisted of dissociation at 95°C for 20s followed by 45 cycles of dissociation at 95° for 5s and 20s annealing and elongation at 60°C, this was followed by a melting curve analysis 95°C 15s, 60°C 1min and 15s at 95°+0.5°C Δ T per cycle. For all primers the efficiency was tested with serial dilutions of pooled template cDNA. Standard curves of all included primers showed an efficiency between 90-105% and a R^2 >0.98. For the measurements all individual samples were diluted 1:50 and measured in triplicate on independent plates, including negative controls (NTC) for all samples and primers. Additionally a no reverse transcription control (NRTC) was measured for every template.
The raw qPCR data were checked for consistency. For all included genes amplification of NTCs and NRTCs is at least five CTs lower than template amplification. The threshold was manually set to 0.5 for all reads. Standard deviation for each gene was calculated from the triplicate measurements and only data with an SD <0.5 were considered for further analysis. From the mean CT values -Δ CT were calculated by subtracting CT of each target gene from CT for GADPH measured at the same plate ($-\Delta CT = CT_{GADPH} - CT_{Target}$).

References S3
[1] Duffy D.J., Frank U. 2011 Modulation of COUP-TF expression in a cnidarian by ectopic Wnt signalling and allorecognition. *PLoS One* **6**(4), e19443.

S4 Statistical analysis

a) Two-way MANOVA over all genes b) Two-way ANOVA on single genes c) Planned contrasts

a) Two-way MANOVA over all genes

```
> F.manovatest<-manova
(cbind(dCTA12,dCTA4,dCTL1N,dCTTC1N,dCTTR2N,dCTTR3,dCTTR4N)~T1*T2,
data=IL_MAN)
> summary(F.manovatest)
          Df  Pillai approx F num Df den Df  Pr(>F)
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
```

b) Two-way ANOVAs on single genes

A12 Peroxireoxin

```
anova(lm(dCTA12~T1*T2, data=IL_MAN2))
Analysis of Variance Table

Response: dCTA12
          Df Sum Sq Mean Sq F value  Pr(>F)
T1         2  0.741  0.3706  0.2302 0.79588
T2         2  2.659  1.3295  0.8259 0.44858
T1:T2      4 23.400  5.8499  3.6343 0.01707 *
Residuals 27 43.460  1.6096
```

A4 Chitinase

```
> anova(lm(dCTA4~T1*T2, data=IL_MAN2))
Analysis of Variance Table

Response: dCTA4
          Df Sum Sq Mean Sq F value  Pr(>F)
T1         2 21.186 10.5930  3.5275 0.04539 *
T2         2  9.575  4.7877  1.5943 0.22381
T1:T2      4 27.463  6.8658  2.2863 0.08955 .
Residuals 24 72.072  3.0030
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

L1N Adenosylhomocysteinase

```
> anova(lm(dCTL1N~T1*T2, data=IL_MAN2))
Analysis of Variance Table

Response: dCTL1N
          Df  Sum Sq Mean Sq F value  Pr(>F)
T1         2  0.7765 0.38823  0.8997 0.41998
T2         2  0.4085 0.20427  0.4734 0.62860
T1:T2      4  5.3233 1.33084  3.0841 0.03498 *
Residuals 24 10.3565 0.43152
```

TC1N MACPF/Lectin

```
> anova(lm(dCTTC1N~T1*T2, data=IL_MAN2))
Analysis of Variance Table

Response: dCTTC1N
          Df  Sum Sq Mean Sq F value Pr(>F)
T1         2   5.587  2.7936  0.6180 0.5468
T2         2   7.325  3.6626  0.8102 0.4557
T1:T2      4  10.567  2.6417  0.5844 0.6767
Residuals 26 117.539  4.5207
```

TR2N Propo4

```
> anova(lm(dCTTR2N~T1*T2, data=IL_MAN2))
Analysis of Variance Table

Response: dCTTR2N
          Df Sum Sq Mean Sq F value  Pr(>F)
T1         2  5.170  2.5852  1.0984 0.34784
T2         2  8.439  4.2196  1.7929 0.18574
T1:T2      4 24.644  6.1610  2.6177 0.05718 .
Residuals 27 63.546  2.3536
---
```

TR3 superoxide Dismutase

```
> anova(lm(dCTTR3~T1*T2, data=IL_MAN2))
Analysis of Variance Table

Response: dCTTR3
          Df Sum Sq Mean Sq F value  Pr(>F)
T1         2  1.947  0.9733  0.4172 0.66309
T2         2  2.315  1.1577  0.4962 0.61429
T1:T2      4 21.048  5.2619  2.2553 0.08943 .
Residuals 27 62.996  2.3332
```

TR4N Complement factor B1

```
> anova(lm(dCTTR4N~T1*T2, data=IL_MAN2))
Analysis of Variance Table

Response: dCTTR4N
          Df  Sum Sq Mean Sq F value   Pr(>F)
T1         2  1.5193 0.75965  1.6276 0.217336
T2         2  5.5145 2.75723  5.9077 0.008198 **
T1:T2      4  4.7828 1.19569  2.5619 0.064394 .
Residuals 24 11.2013 0.46672
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

c) Planned Contrasts

Treat: c1) ho vs. ht c2) inf vs. ho+ht

	c1	c2
1_SS	0	0
2_LS	0	0
3_PS	0	0
4_LL	1	-1
5_SL	0	1
6_PL	-1	-1
7_PP	1	-1
8_SP	0	1
9_LP	-1	-1

A12 Peroxiredoxin

```
> contA12<-aov(dCTA12~Treat)
> summary(contA12, split=list(Treat=list("ho vs ht"=1,"inf vs ho+ht"=2)))
                  Df Sum Sq Mean Sq F value Pr(>F)
Treat              8  26.80   3.350   2.081 0.0740 .
Treat: ho vs ht    1   4.14   4.145   2.575 0.1202
Treat: inf vs ho+ht 1   9.62   9.620   5.977 0.0213 *
Residuals         27  43.46   1.610
```

A4 Chitinase

```
> contA4<-aov(dCTA4~Treat)
> summary(contA4, split=list(Treat=list("ho vs ht"=1,"inf vs ho+ht"=2)))
                  Df Sum Sq Mean Sq F value Pr(>F)
Treat              8  58.22   7.278   2.424 0.0446 *
Treat: ho vs ht    1   0.62   0.623   0.208 0.6527
Treat: inf vs ho+ht 1   0.04   0.037   0.012 0.9125
Residuals         24  72.07   3.003
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
3 observations deleted due to missingness
```

L1N Adenosylhomocysteinase

```
> summary(contL1N, split=list(Treat=list("ho vs ht"=1,"inf vs ho+ht"=2)))
                  Df Sum Sq Mean Sq F value  Pr(>F)
Treat              8  6.508   0.814   1.885 0.10977
 Treat: ho vs ht   1  4.858   4.858  11.258 0.00263 **
 Treat: inf vs ho+ht  1  0.325   0.325   0.754 0.39378
Residuals         24 10.357   0.432
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
3 observations deleted due to missingness
```

TR2N Propo 4

```
> contTR2N<-aov(dCTTR2N~Treat)
> summary(contTR2N, split=list(Treat=list("ho vs ht"=1,"inf vs ho+ht"=2)))
                  Df Sum Sq Mean Sq F value Pr(>F)
Treat              8  38.25   4.782   2.032 0.0807 .
 Treat: ho vs ht   1  11.84  11.839   5.030 0.0333 *
 Treat: inf vs ho+ht  1   3.38   3.385   1.438 0.2409
Residuals         27  63.55   2.354
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

TR3 Superoxide Dismutase

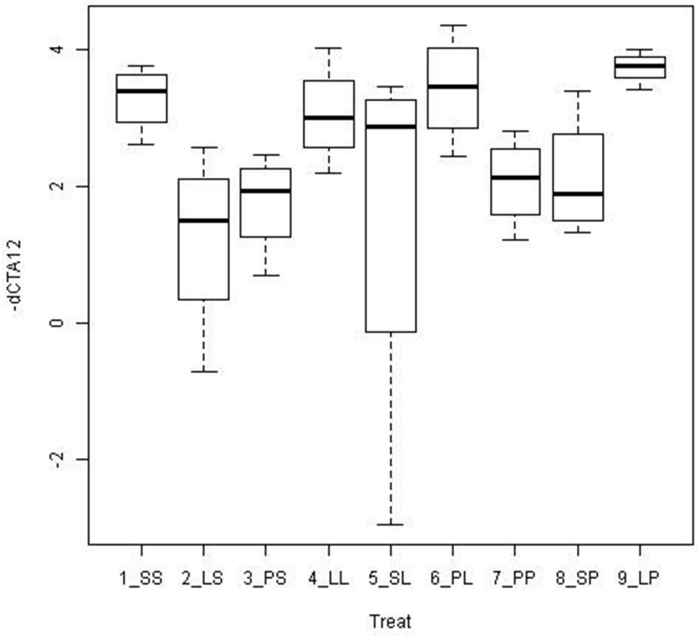
```
> contTR3<-aov(dCTTR3~Treat)
> summary(contTR3, split=list(Treat=list("ho vs ht"=1,"inf vs ho+ht"=2)))
                  Df Sum Sq Mean Sq F value Pr(>F)
Treat              8  25.31   3.164   1.356 0.2598
 Treat: ho vs ht   1  10.61  10.610   4.548 0.0422 *
 Treat: inf vs ho+ht  1   1.31   1.313   0.563 0.4596
Residuals         27  63.00   2.333
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

TR4N Complement factor B1

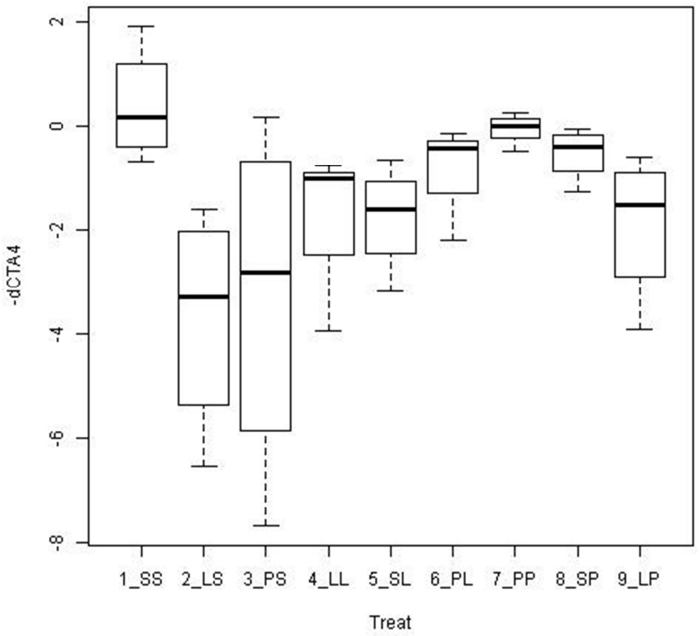
```
> contTR4N<-aov(dCTTR4N~Treat)
> summary(contTR4N, split=list(Treat=list("ho vs ht"=1,"inf vs ho+ht"=2)))
                  Df Sum Sq Mean Sq F value Pr(>F)
Treat              8 11.817   1.477   3.165 0.0136 *
 Treat: ho vs ht   1  3.409   3.409   7.304 0.0124 *
 Treat: inf vs ho+ht  1  1.586   1.586   3.399 0.0776 .
Residuals         24 11.201   0.467
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
3 observations deleted due to missingness
```

S 5 Gene expression

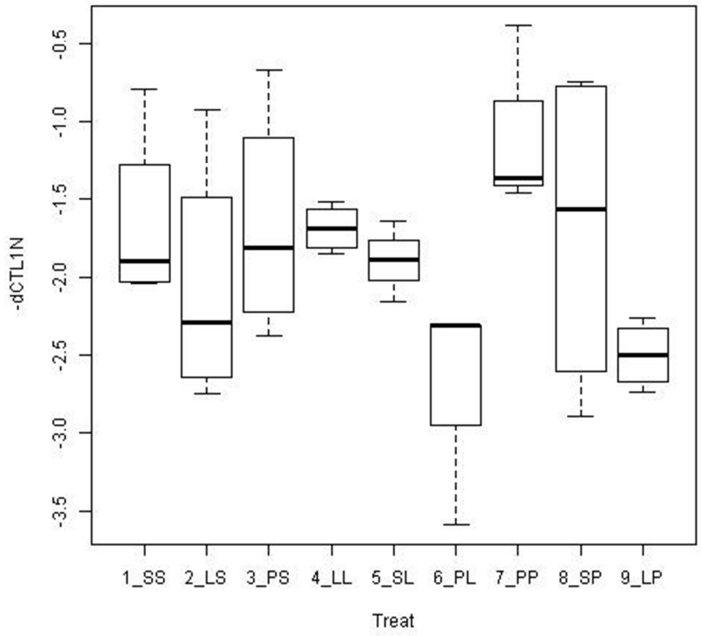
Relative expression of seven target genes (a-g), over all nine treatment combinations first and second treatment with Sham, *Listonella* or *Planococcus* (Treat: 1_SS, 2_LS, 3_PS, 4_LL, 5_SL, 6_PL, 7_PP, 8_SP, 9_LP). Expression is depicted as minus delta CT (-dCT) values relative to GADPH expression levels. Higher values correspond to higher relative expression.



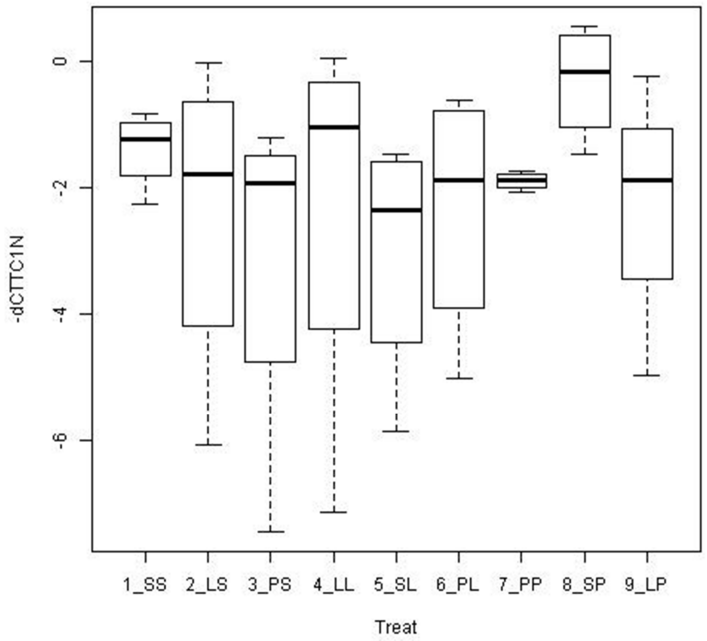
a) A12 Peroxiredoxin



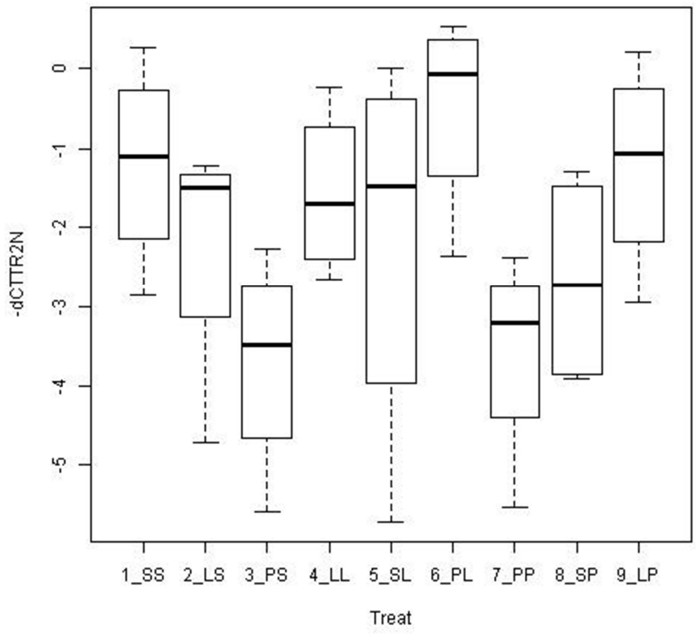
b) A4 Chitinase



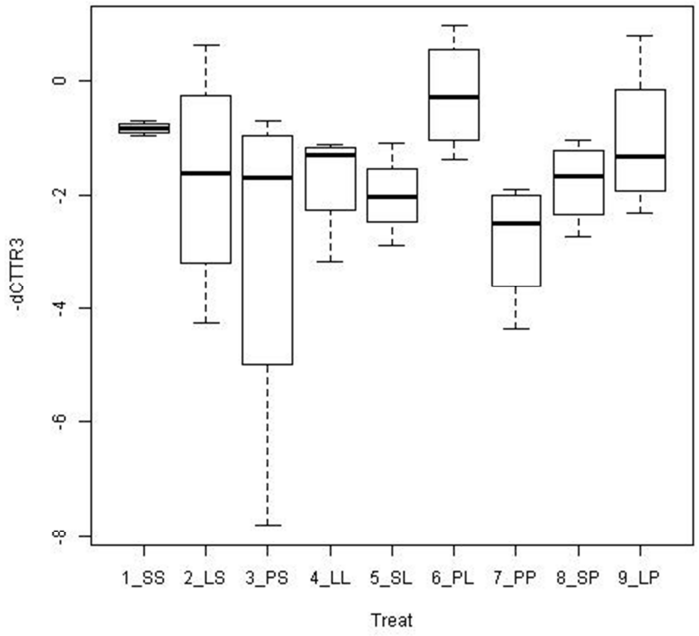
c) L1N Adenosylhomocysteinase



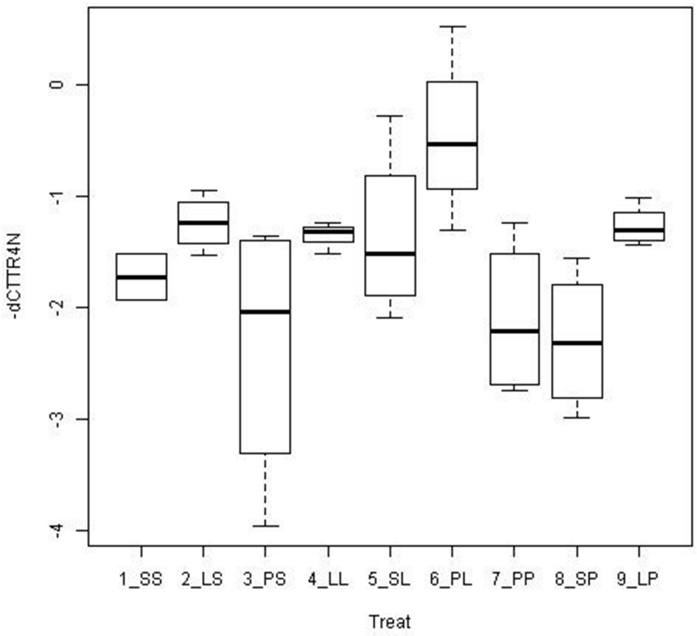
d) TC1N MACPF/Lectin



e) TR2N Propo 4



f) TR3 Superoxide Dismutase



g) TR4N Complement factor B1