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Abstract: Immunostimulants are key molecules in aquaculture since they heighten defensive responses and protection against pathogens. The present study investigated the treatment of Senegalese sole larvae with a whole-cell crude extract of the microalgae Nannochloropsis gaditana (Nanno) and programming of growth and the immune system. Larvae at hatch were treated with the Nanno extracts for 2 h and thereafter were cultivated for 32 days post-hatch (dph) in parallel with an untreated control group (CN). Dry weight and length at 21 days post-hatch (dph) were higher in post-larvae of the Nanno than CN group. These differences in weight were later confirmed at 32 dph. To evaluate changes in the immune response associated with Nanno-programming treatments, the Nanno and CN post-larvae were supplied with two bioactive compounds yeast beta-glucan (Y) and a microalga extract from the diatom Phaeodactylum tricornutum (MAe). The bioactive treatments were administrated to the treatment groups through the live prey (artemia metanauplii, 200 artemia mL-1) enriched for 30 min with MAe or Y (at 2 mg mL-1 SW) or untreated prey in the case of the negative control (SW). The effect of the treatments was assessed by monitoring gene expression, enzyme activity and mortality over 48h. The post-larvae sole supplied with the bioactive compounds Y and MAe had increased mortality at 48h compared to the SW group. Moreover, mortality was higher in Nanno-programmed than CN post-larvae. Lysozyme and total anti-protease enzymatic activities at 6 and 24h after the start of the trial were significantly higher in the Nanno and MAe supplied post-larvae compared to their corresponding control (CN and SW, respectively). Immune gene transcripts revealed that il1b, cxc10 and mx mRNAs were significantly different between Nanno and CN post-larvae at 6 and 24 h. Moreover, the expression of il1b, tnfa, cxc10, irf3, irf7 and mx was modified by bioactive treatments but with temporal differences. At 48h after bioactive treatments, Y and SW post-larvae were challenged with the lymphocystis disease virus (LCDV). No difference existed in viral copy number between programming or bioactive treatment groups at 3, 6 and 24 h after LCDV challenge although the total number of copies reduced with time. Gene expression profiles in the LCDV-challenged

group indicated that post-larvae triggered a wide defensive response compared to SWC 24h after challenge, which was modulated by programming and bioactive compound treatments. Cluster analysis of expressed genes separated the SW and Y groups indicating long-lasting effects of yeast beta-glucan treatment in larvae. A noteworthy interaction between Nanno-programming and Y-treatment on the regulation of antiviral genes was observed. Overall, the data demonstrate the capacity of microalgal crude extracts to modify sole larval plasticity with long-term effects on larval growth and the immune responses.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Microalgal extracts induce larval programming and modify growth and the immune response to bioactive treatments and LCDV in Senegalese sole post-larvae

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Running title: Microalgal extracts induce trained immunity in sole

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Abstract

Immunostimulants are key molecules in aquaculture since they heighten defensive responses and protection against pathogens. The present study investigated the treatment of Senegalese sole larvae with a whole-cell crude extract of the microalgae *Nannochloropsis gaditana* (Nanno) and programming of growth and the immune system. Larvae at hatch were treated with the Nanno extracts for 2 h and thereafter were cultivated for 32 days post-hatch (dph) in parallel with an untreated control group (CN). Dry weight and length at 21 days post-hatch (dph) were higher in post-larvae of the Nanno than CN group. These differences in weight were later confirmed at 32 dph. To evaluate changes in the immune response associated with Nanno-programming treatments, the Nanno and CN post-larvae were supplied with two bioactive compounds yeast β-glucan (Y) and a microalga extract from the diatom *Phaeodactylum tricornutum* (MAe). The bioactive treatments were administrated to the treatment groups through the live prey (artemia metanauplii, 200 artemia mL^{-1}) enriched for 30 min with MAe or Y (at 2 mg mL^{-1} SW) or untreated prey in the case of the negative control (SW). The effect of the treatments was assessed by monitoring gene expression, enzyme activity and mortality over 48h. The post-larvae sole supplied with the bioactive compounds Y and MAe had increased mortality at 48h compared to the SW group. Moreover, mortality was higher in Nanno-programmed than CN post-larvae. Lysozyme and total anti-protease enzymatic activities at 6 and 24h after the start of the trial were significantly higher in the Nanno and MAe supplied post-larvae compared to their corresponding control (CN and SW, respectively). Immune gene transcripts revealed that *il1b, cxc10* and *mx mRNAs* were significantly different between Nanno and CN post-larvae at 6 and 24 h. Moreover,

the expression of *il1b*, *tnfa*, *cxc10*, *irf3*, *irf7* and *mx* was modified by bioactive treatments but with temporal differences. At 48h after bioactive treatments, Y and SW post-larvae were challenged with the lymphocystis disease virus (LCDV). No difference existed in viral copy number between programming or bioactive treatment groups at 3, 6 and 24 h after LCDV challenge although the total number of copies reduced with time. Gene expression profiles in the LCDV-challenged group indicated that post-larvae triggered a wide defensive response compared to SWC 24h after challenge, which was modulated by programming and bioactive compound treatments. Cluster analysis of expressed genes separated the SW and Y groups indicating long-lasting effects of yeast β-glucan treatment in larvae. A noteworthy interaction between Nanno-programming and Y-treatment on the regulation of antiviral genes was observed. Overall, the data demonstrate the capacity of microalgal crude extracts to modify sole larval plasticity with long-term effects on larval growth and the immune responses.

Keywords: beta-glucans, immune response, LCDV, microalgae, programming, Senegalese sole, virus

1. Introduction

Prebiotics are frequently used as feed supplements to strengthen immune competence in aquaculture. Functional polysaccharides or immunosaccharides, such as β-glucans, can directly stimulate the innate immune system when provided orally or injected and prevent disease outbreaks and mortality [1-3]. The β-glucans are complex polysaccharides present from bacteria to plants, structurally composed of glucose residues linked with β-D-glycosidic bonds. However, the position and distribution of these glycosidic bonds, the degree of branching, the molecular size and solubility characteristics confer specific chemical and bioactive properties to different β-glucans [3-5]. The branched 1,3/1,6 β-glucan variants from yeast and algae are the most frequent β-glucans used as feed ingredients in aquaculture [3, 6]. A new function recently assigned to β-glucans, particularly the soluble β-glucan laminarin, is epigenetic programming of myeloid cells and promotion of innate immune memory in fish [7]. This cell priming by application of stimuli in early larval stages is associated with metabolic programming and an enhanced immune response and survival [7-9]. We recently demonstrated that Senegalese sole (*Solea senegalensis*) larvae can be programmed at hatch by thermal treatments to enhance growth performance in juveniles [10, 11]. However, epigenetic reprograming of early life stages using microalgal extracts to modify growth and the immune response is still poorly explored.

The administration of β-glucans is reported to enhance immune system response and survival rates in several fish species challenged with different pathogens [3]. In Senegalese sole, particulate yeast β-glucans and microalgal extracts enriched in

polysaccharides supplied by injection or oral administration activated an immunomodulatory response [12, 13]. However, trained immunity induced by yeast β-glucans seems to be more specific since in the flatfish *Scophthalmus maximus* only protection against the bacteria *Aeromonas salmonicida* but not against viral haemorrhagic septicaemia virus (VHSV) was observed [14]. The lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease, a highly contagious disease responsible for high economic losses in the aquaculture industry worldwide [15]. Both probiotics and a herbal extract were reported to enhance the innate immune response and resistance to LCDV in *P. olivaceus* [16, 17]. However, no information exists about the capacity of β-glucans to modulate the response to LCDV or if microalgal extracts can program the immune system.

The microalga *Nannochloropsis gaditana* is routinely used in hatcheries for larval rearing due to its nutritional value [18]. This microalgae is a rich source of bioactive molecules including polysaccharides (chrysolaminarin among others), polyunsaturated fatty acids and carotenoid pigments and the extracts have a cytoprotective effect on dermal fibroblasts under oxidative stress *in vitro* due to their potent antioxidant action [19, 20]. In the present study *N. gaditana* whole-cell crude extracts (Nanno) were tested for their capacity to program sole larvae and their long-term effects on growth performance and the immune response in post-larvae were evaluated. Two bioactive treatments (yeast β-glucans and microalgal polysaccharide-enriched extracts from the diatom *Phaeodactylum tricornutum*), and a challenge with LCDV were used to evaluate the antiviral immune response associated with Nanno programming. The results obtained provide data revealing microalgae can modulate fish larval plasticity with benefits for the aquaculture sector.

2. Material and methods

2.1 Microalgae extract and Yeast β-glucan sources

All microalgae in this study were supplied by Fitoplancton Marino S.A (El Puerto de Santa Maria, Spain). The microalgae *N*. *gaditana* was initially grown indoors using autoclaved seawater (salinity 33 psu) enriched with filter-sterilized f/2 nutrients in 50 mL flasks. Filter-sterilized CO_2 was continuously bubbled through the cultures maintained under standard conditions as previously reported [12]. Microalgae were inoculated in outdoor photobioreactors (PBRs) and cultivated under seasonal environmental conditions. After harvesting the microalgae 4 h after sunrise by continuous-flow centrifugation, they were frozen at -20 ºC and freeze-dried. To break the cells, the freeze-dried biomass was hydrated in sea water (SW) at a dilution of 1:4 (mass algae:SW) at 4ºC and broken by high-pressure homogenization (Niro Soavi high-pressure homogenizer) at 1,200 bar using a flow of 9 L h^{-1}. The fragmented microalgae were freeze-dried for preservation until use.

To prepare the whole-cell crude extract (Nanno), the freeze-dried microalgae were suspended in sterile sea water (SW) at a ratio of 1:10 (w/v), respectively. The sterilized SW contained dimethyl sulfoxide (DMSO) at a final concentration of 0.01%. The crude microalgae extract was thoroughly mixed and the suspension was stored at 4ºC for 24h. Before use of the microalgae extracts for sole larval programming (within 30 min of preparation), the crude microalgae extract was centrifuged at 10,000xg for 15 min at 4ºC and the soluble fraction was collected into a clean tube at 4^{o}C.

A previously characterized microalgal polysaccharide-enriched extract (MAe) from the diatom, P. tricornutum [12, 13] and pure yeast β-glucan (Y) (particulate (1,3)-(1,6)- β-glucan (Yestimun®) 91% pure from brewers' yeast, Quimivita, Barcelona, Spain; 42200P-030) were used for bioactive treatments of post-larval Senegalese sole.

2.2 Virus collection and culture conditions

The LCDV genotype VII was recovered from diseased animals (gilthead sea bream specimens) as previously described in [21, 22]. The viral titer was calculated using a $TCID_{50}$ assay and later confirmed by qPCR as previously reported [23, 24].

2.3 Fish trial

All procedures were authorized by the Bioethics and Animal Welfare Committee of IFAPA and given the registration number 26–11–15-374 by the National authorities for the regulation of animal care and experimentation.

For the larval reprogramming trial with Nanno, eggs collected from two independent tanks of wild sole broodstock held by IFAPA Centro El Toruño (El Puerto de Santa Maria, Cadiz, Spain) and CUPIMAR (San Fernando, Spain) were used. The fertilized eggs from each of the broodstock tanks were mixed, viable eggs separated by buoyancy (total 120,000 eggs) and incubated in a 500L-cylindroconical incubator at 20ºC in an open circuit. Recently hatched larvae (~1h after hatching; 0 days post-hatch (dph)) were collected and approximately 80,000 larvae were distributed equally between two buckets containing 4 L of seawater with slight aeration to facilitate larval dispersion. The sea water (SW) temperature was 20ºC, oxygen 6.5 ppm and salinity 37 ppt. To one bucket containing hatched larvae (0 dph) the Nanno crude extract

(Nanno group) was added (0.2 mg dry mass equivalent mL^{-1} SW) and for the control larvae (CN) the Nanno crude extract was omitted. After 2h treatment, the larvae were concentrated using a mesh, rinsed in clean seawater and then both experimental groups were distributed between triplicate 400-L tanks and cultured using standard hatchery protocols until the post-larval stage (after completion of metamorphosis)[10]. The CN and Nanno-exposed groups were sampled at premetamorphosis (7 and 12 dph), metamorphosis (16 dph) and at the end of metamorphosis (21 and 30 dph) to determine their dry weight and length. At each sampling point, individual larvae from each tank (20-25 larvae, each treatment in triplicate) were photographed to determine total length using ImageJ v1.47 software and oven dried (24 h, 60 °C) to estimate mean dry weight. At 32 dph, when post-larvae were sampled to carry out the trial to evaluate the immune response to bioactive treatments, since they were big enough the wet weight of individual larvae (19-24 larvae per tank, each treatment in triplicate) was measured.

To evaluate the immune response of the Nanno and CN post-larvae, a two-part trial was carried out as depicted in Figure 1. In brief, 720 post-larvae at 32 dph from the Nanno and CN groups were randomly distributed between 18 plastic trays (360 cm^2, 80 post-larvae per tray) containing 1L of filtered-sterile seawater (salinity 35 g L^{-1}, 6 ppm oxygen at the beginning of the trial). The trays were placed in a temperature-controlled room to keep the water temperature at 20º C. The post-larvae were kept in these conditions with still water and without an additional oxygen supply for 24h and fed with artemia (~1200 metanauplii/tray; 15 artemia/post-larva). The tank water was totally renewed (~90%) with oxygen oversaturated (300%) filtered seawater every 24 hrs. No mortality was registered at the beginning of the trial.

2.3.1 Treatments with MAe and Y

The aim of the trial was to determine if Nanno programming in hatched larvae affected the immune response triggered by MAe and Y exposure in post-larvae. Both bioactive MAe and Y treatments were administrated to sole post-larvae (32 dph) through the live prey. The experimental groups maintained in triplicate trays were Nanno-MAe and Nanno-Y and CN-MAe and CN-Y. The artemia metanauplii (200 artemia mL^{-1}) were enriched for 30 min with MAe or Y (at 2 mg mL^{-1} SW). The negative control groups were fed artemia metanauplii (200 artemia mL^{-1}) prepared in SW only and consisted of Nanno-SW and CN-SW. After 30 min enrichment, the artemia were filtered through a mesh and suspended in clean seawater and the trial was started by supplying ~1200 metanauplii/tray to the post-larval sole (15 artemia/fish). No artemia were observed in the tanks 1h after feeding and a second dose of Y- or MAe-enriched or control (SW) artemia was supplied to the post-larvae in the triplicate trays/treatment 4h later. Fish sampling was carried out 6 and 24 h after the start of the experiment and used for gene expression and enzyme analysis (n = 6/group, 2 from each of the replicate trays for each of the analysis). The water in the trays remained without circulation throughout the trial but was totally renewed immediately after sampling at 6 and 24h with oxygenated (300% saturated) SW. The sole larvae were kept in the trays for 48h after the start of the treatments and the mortality was recorded at 6, 24 and 48h. For sampling, specimens were euthanized in an overdose (300 ppm) of tricaine methane sulfonate (MS-222) and fixed in RNA-later (Invitrogen) for gene expression analysis (at 6 and 24h) or frozen on dry ice for enzyme analysis (at 6 and 24h). Thereafter, samples were stored at -80 °C until use.

2.3.2 Viral challenge

After evaluating the responses of Nanno and CN sole post-larvae to Y and MAe exposure for 48h, they were challenged with LCDV to evaluate their antiviral response (Fig 1b). Due to the high mortality observed in the Nanno-MAe group it was excluded from the LCDV trial. The Nanno-Y, CN-Y, Nanno-SW and CN-SW post-larvae were pooled by treatment and redistributed between 8 new plastic trays (180 cm^2, 30 fish/tray; 2 trays per treatment) containing oxygenated (300%) filtered seawater. The four experimental groups of post-larvae were immediately challenged with LCDV supplied through the artemia as described in [22] (Fig. 1b). Briefly, artemia metanauplii were enriched with an LCDV suspension containing $5x10^4$ $TCID_{50}$ mL^{-1}, for 30 min, filtered, washed and resuspended in clean seawater. Then 15 infected metanauplii/post-larva (450 artemia/tank) were supplied to each of the 8 trays establishing four post-larvae groups: Nanno-Y-V, CN-Y-V, Nanno-SW-V and CN-SW-V). Matched groups not challenged by viral exposure were fed with non-infected artemia (15 metanauplii/post-larva, 2 trays), and designated Nanno-Y-SWC, CN-Y-SWC, Nanno-SW-SWC and CN-SW-SWC. Approximately 1h after feeding when no artemia were observed in the trays, the seawater was totally renewed with clean sterile oxygenated seawater. Samples (n = 6/treatment; n = 3/tray) of sole post-larvae were collected at 3, 6 and 24 h after LCDV-challenge for quantification of LCDV DNA copies and at 24 h for gene expression analysis. No mortality was registered up to 24 h after LCDV challenge.

2.4 RNA isolation and gene expression analysis

Total RNA for gene expression analysis was extracted from Nanno, CN, Nanno-Y, Nanno-MAe, Nanno-SW, CN-Y, CN-MAe and CN-SW (n = 4 per group) samples collected at 6 and 24h using an Isolate II RNA Mini Kit (Bioline). Individual larvae

were homogenized in a Fast-prep FG120 instrument (Bio101) using Lysing Matrix D (Q-Bio-Gene) for 60 s at speed setting 6. The extracted total RNA was treated twice over 30 minutes with DNase I using the clean-up protocol according to the manufacturer's instructions. The extracted RNA was quantified using a Nanodrop ND-8000 (Thermo Scientific) and the integrity was assessed by agarose gel electrophoresis.

To isolate total RNA and DNA from post-larvae at 24 h after LCDV challenge (n=4 per treatment), the TRIsure (Bioline) method was used as described in Carballo, et al. [22]. For post-larvae at 3 and 6 h after LCDV challenge only DNA was extracted (see section 2.6). RNA from the 24 h post-larvae (groups CN_SW_SWC, CN_Y_SWC, Nanno_SW_SWC, Nanno_Y_SWC, CN_SW_V, CN_Y_V, Nanno_SW_V, Nanno_Y_V) was extracted by homogenization in a Fast-prep FG120 instrument (Bio101) using Lysing Matrix D (Q-Bio-Gene) and TRI-Reagent (1 mL) for 60 s at speed setting 6. Chloroform (0.2 ml) was added, incubated for 5 min at room temperature and then samples were centrifuged at 12,000xg, 4ºC for 15 min and the aqueous phase was transferred to a column of Isolate II RNA Mini Kit (Bioline) and treated as describe above.

RNA was reverse-transcribed using an *iScript*™ cDNA Synthesis Kit (Bio-Rad) and qPCR assays were carried out using a CFX96™ Real-Time System (Bio-Rad) in a 10-µl reaction volume containing cDNA generated from 200 ng of original RNA template, 300 nM each of specific forward and reverse primers, and 5 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The primers used in the study have previously been published: *il1b, tnfa, clec, cxc10, irf7* [12], *irf3* [21], *mx* [25] *cd4 and cd8a* [22]. The qPCR amplification cycle was as follows: a first step

of 7 min at 95ºC for enzyme activation, followed by 40 cycles of 30 s at 95ºC and 30s at 60 ºC. Each PCR reaction was carried out in duplicate. Ubiquitin (*ub52*) and β-actin (*actb2*), which did not vary significantly between samples were used as reference genes [26]. Relative mRNA expression in samples was determined using the $2^{-(\Delta\Delta Ct)}$ method. Clustering analysis was carried out using PermutMatrix [27] as previously described in [21, 22].

2.5 Enzymatic assays

The Nanno-Y, Nanno-MAe, Nanno-SW, CN-Y, CN-MAe and CN-SW post-larvae collected at 6 and 24 h (n = 6/group) were weighed and homogenized individually in 500 µL of extraction buffer (1 M Tris-HCl, 0.01 M NaCl, 0.01 M KCl, 0.005 M of $MgCl_2$), following the method described in [28]. Lysozyme and total anti-protease activities were measured using a turbidimetric assay [29] and a spectrophotometric method [30] modified by Hanif et al. [31], respectively. Data obtained from the enzymatic assays were expressed as U per post-larvae for lysozyme specific activity and percent total antiprotease activity per post-larvae calculated relative to the positive control (trypsin), which was designated as 100%. All the measurements were carried out using a microplate reader (BioTek Synergy 4, BioTek Instruments, Inc., USA).

2.6 Quantification of viral DNA copies

Determination of viral DNA copies in LCDV-challenged post-larvae (n = 6) at 3 and 6 h and in the LCDV-enriched and control artemia (n = 6, treatment vehicle) was established by extracting total DNA using an Isolate II Genomic DNA Kit (Bioline). In the case of LCDV-challenged post-larvae at 24h, DNA was isolated from the

organic phase of the TRIsure resulting from the RNA extraction protocol (see section 2.4). In both cases, samples were treated with RNase A (Bioline) following the manufacturer's instructions. DNA was quantified spectrophotometrically using a Nanodrop ND-8000. Absolute quantification of viral DNA copies was carried out according to the protocol specified by Valverde, et al. [24] using a CFX96™ Real-Time System (Bio-Rad) in a 10 µl final volume containing 200 ng of DNA, 300 nM each of the specific forward and reverse primers, and 5 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The amplification protocol used was as follows: 7 min denaturation and enzyme activation at 95 ºC, followed by 40 cycles of 30 s at 95 ºC and 1 min at 59ºC.

2.7 Statistical analysis

Weight and length of sole were compared using a Student t-test. Mortality curves after the administration of bioactive compounds (MAe and Y) were analysed using a Log Rank test. The qPCR data were log-transformed in order to comply with normality and homogeneity of variance. To assess if there were statistical differences in gene expression and enzymatic analysis after bioactive treatments, a General Linear Model (GLM) analysis was used with programming (Nanno or CN), bioactive treatment (Y, MAe or SW), and time (6, 24 or 48 when available) as fixed factors. To identify significant differences after the LCDV challenge, a GLM analysis was carried out using programming treatment, bioactive treatment and LCDV challenge (V and SWC) as fixed factors. When significant interactions between factors were detected, a two-way or one-way ANOVA was carried out followed by an LSD post-hoc test.

For viral quantification, a two-way ANOVA was carried out using programming, bioactive treatment or time as fixed factors. When a significant effect was identified, an LSD post-hoc test was carried out. Statistical analyses were performed using SPSS *v*21 software (IBM) with statistical significance set at $P<0.05$ and data are presented as mean ± standard error of the mean (SEM). Principal component analysis (PCA) was performed in FactoMineR [32].

3. Results

3.1 Long-term effects of larval programming on growth

Treatment of recently hatched larvae with Nanno whole-cell crude extracts for 2h did not cause any mortality. After Nanno treatment larvae were reared until the completion of metamorphosis following a standard rearing protocol in the hatcheries and they had a significantly higher dry weight (1.58±0.21 vs 1.16±0.08 mg) and standard length (0.84±0.01 vs 0.70±0.03 cm) than the CN group at 21 dph ($P<0.05$). At 30 dph differences in dry weight were still observable but high data variability meant that no significant differences were found (Fig. 2). However, the wet weight of 32 dph post-larvae treated with Nanno was significantly higher than the CN group, 13.43±0.19 vs 12.36±0.61, respectively ($P<0.05$).

3.2 Survival associated with larval programming after administration of bioactive treatments in post-larvae

Survival of Nanno and CN sole treated with bioactive treatments (Y or MAe) at 32 dph was monitored over 48h and compared with the negative control (SW) (Fig. 3a). Survival was 100% in all experimental groups (Nanno-Y, Nanno-MAe, Nanno-SW, CN-Y, CN-MAe, CN-SW) 6h after feeding, although a significant increase ($P<0.05$)

in mortality occurred from 24 to 48h associated with bioactive treatments (SW < Y < MAe) and also between the programming groups (CN < Nanno).

Cumulative mortality in the CN-SW and Nanno-SW at 48 h was 0 and 1.0±1.2%, respectively (Fig. 3a). In the group given Y-enriched artemia, a small increase in mortality occurred in the Nanno-Y group at 24 h (2.7±3.2%). The cumulative mortality at 48 h for the CN-Y and Nanno-Y groups was 1.8±2.8% and 16.7±13.4%, respectively (Fig. 3a). The highest mortality rates were observed in larvae supplied with MAe enriched artemia. At 24h, the mortality was 18.6±16.3% for the CN-MAe and 96.6±2.6% for the Nanno-MAe groups and reached 56.7±9.9% and 100%, respectively by 48h.

3.3 Enzymatic activities associated with larval programming and the administration of bioactive treatments in post-larvae

Lysozyme and total anti-protease activities were measured at 6 and 24h using whole post-larvae homogenates. Due to the high mortality in the CN-MAe and Nanno-MAe groups, there was not enough specimens for enzymatic analysis at 24h. Lysozyme but not total antiprotease activities (Fig. 4A and 4B) decreased significantly (P<0.05) with time. With respect to programming, Nanno-treated post-larvae had significantly higher lysozyme (6 h) and antiprotease (24h) activities than the CN group. When bioactive treatments were compared, a significantly higher lysozyme activity in Nanno-MAe and anti-protease activity in CN-MAe exposed post-larvae compared to control groups (Nanno-SW and CN-SW, respectively) at 6 h were found.

3.4 Expression profiles associated with larval programming and the administration of bioactive treatments in post-larvae

To evaluate the long-term effects on the immune response of programming treatment in recently hatched larvae, expression levels of nine genes after administering MAe and Y through the diet to 32 dph Nanno and CN post-larvae were quantified. The genes analysed included a) pro-inflammatory genes, *il1b* and *tnfa*, receptor lectin type-c (*clec)*, chemokine *cxc10*, b) antiviral genes *irf3*, *irf7* and *mx* and c) lymphocyte markers *cd4* and *cd8a* (Fig. 5a). The expression levels of most of the genes analysed (except *il1b*) were significantly modified between sampling time points (6 *vs* 24h). When the programming effect was analysed using GLM, the steady-state gene expression levels of *il1b, cxc10* and *mx* were significantly different between CN and Nanno groups. The Nanno-MAe and Nanno-Y had higher *cxc10* mRNA levels (2.0-fold) than the corresponding CN groups. For *il1b* and *mx*, a significant interaction occurred between time×programming at 24h and *il1b* transcripts were 2.3-fold lower and *mx* mRNAs 1.5-fold higher in Nanno compared to CN. No specific interactions were found between time×programming for the other genes analysed.

With respect to the specific immune responses associated with the bioactive treatments, the main effect after GLM analysis was observed for *il1b* that had higher mRNA levels in MAe than in Y and SW groups ($P < 0.05$) (Fig. 5). These differences were highest at 24h (average 2.3-fold higher than the SW). For most of the genes, a significant interaction between time×bioactive treatment was detected in GLM, which is indicative of a time course response triggered by these compounds. Compared to the SW control at 6h, *tnfa*, *cxc10*, *irf7* and *mx* mRNAs increased in post-larvae supplied with MAe and only *tnfa* increased in those supplied with Y. After an initial rapid stimulation, *tnfa*, *irf3*, *irf7* and *mx* mRNAs in MAe and Y groups and *cxc10* levels in MAe decreased at 24h and were lower than the SW control.

3.5 Viral loads of post-larvae challenged with LCDV

Two daysafterthe bioactive treatments'sole post-larvae from the Nanno-Y, Nanno-SW, CN-Y and CN-SW groups were challenged with LCDV. The final concentration of the virus used to enrich the artemia via the seawater was $5x10^4$ $TCID_{50}$ mL^{-1} and resulted in $5.3 \pm 3.0x10^5$ *mcp* copies µg metanauplii^{-1}. No virus was amplified by PCR in the post-larvae from the control group (SWC). Viral *mcp* DNA was detected in all post-larvae at 3 and 6 h post-viral challenge (pvc). However, the rates of LCDV positive animals fell at 24 h pvc and ranged from 50 to 83% in the CN_SW and CN_Y groups, respectively. No significant differences in the number of viral DNA copies existed between programming or bioactive treatments (GLM; $P > 0.05$). Significant differences in viral copy number only occurred across time ($P < 0.05$) since they fell from an average of 37 viral *mcp* copies µg DNA^{-1} at 3 h pvc to 27 copies at 24 h pvc (Fig. 6).

3.6 Gene expression profiles of programmed and bioactive exposed post-larvae after LCDV challenge

In order to evaluate the immune response in programmed larvae (Nanno and CN groups) supplied with bioactive treatments (SW and Y groups) and challenged with LCDV the expression patterns of the genes indicated in 3.4 were analysed at 24h after challenge. Clustering analysis of gene expression data clearly separated the LCDV-challenged fish from the SWC group (Fig. 7A). Activation of antiviral genes, proinflammatory cytokines and lymphocyte markers was clear and more intense in post-larvae of the Y group (Fig. 7A). Moreover, a Nanno-SW specific gene cluster in the LCDV-infected group was identified. In non-challenged fish (SWC), two major branches separating SW and Y irrespective of the programming treatment were

identified. These differences were also observed in the PCA analysis that clearly separated the LCDV-challenged from the SWC group (Fig 7B and C) and the SW and Y specimens within them.

A detailed analysis of gene expression profiles (Fig. 7A; Suppl. File 1 and 2) indicated that viral challenge triggered a wider and more intense response of cytokines *il1b* and *tnfa*, chemokine *cxc10*, receptor *clec* and the lymphocyte markers *cd4* and *cd8a* in the Y groups compared to the SW groups. However, an interaction between mRNA levels associated with programming and bioactive treatment in the SWC and V-challenged groups was found. In the non-challenged SWC group, GLM indicated that the steady-state mRNA levels of the three antiviral genes (*irf7*, *irf3* and *mx*) were higher in Nanno-SW than Nanno-Y. Moreover, in the V-challenged group, no differences in the expression patterns between Nanno-Y and CN-Y groups were observed. However, viral challenge activated the expression of antiviral genes higher in the Nanno-SW than CN-SW group without modifying the expression of *il1b* in the Nanno-SW group.

4. Discussion

There is increasing evidence that fish larvae have high genome plasticity as an adaptive mechanism to variable environmental conditions. Previous studies have demonstrated that small changes in the ambient temperature at which eggs and larvae develop can have persistent effects on myogenesis and somatic growth [33], the stress response [34] and sex differentiation [35]. More recently, non-specific immune memory, or trained immunity, has been demonstrated in fish [7, 36, 37]. This early training of immunity has been proposed as a strategy to manipulate the immune

response to improve larval rearing performance with benefits for immunity in adults [38]. Previous data in sole pinpointed the window of time at which thermal sensitive imprinting of sole was most effective, namely, the release of larvae from the egg chorion [10, 11]. The present study demonstrates for the first time that short-term exposure of recently hatched sole larvae to crude extracts of *N*. *gaditana* has long-term effects on both growth and the immune responses. Nanno-programmed post-larvae were bigger at 21 and 32 dph than untreated controls This change in growth performance has previously been associated with changes in muscle cellularity [33, 39] and a shift in energy pathways due to metabolic programming that in turn modifies appetite and the immune response [7, 40]. The data obtained in this study open-up new potential applications of microalgae not explored until now that could benefit the aquaculture industry.

Previous data from our group demonstrated that yeast β-glucans and MAe modulate immune system responses in juvenile sole. Oral administration of yeast β-glucans acted locally in the gut and modulated the immune response and the microbiome, while MAe activated a systemic anti-inflammatory response [13]. In contrast, intraperitoneal injections of MAe provoked a sustained and potent inflammatory response [12]. The higher mortality observed in post-larvae supplied with yeast β-glucans and MAe confirmed their bioactivity and their action on metabolism and the immune response in fish. These effects are supported by the enhanced growth performance of sole post-larvae and their modified response to the immunomodulatory effects of Y and MAe bioactive treatments compared to the SW group.

Lysozyme and antiprotease activities represent two elements of the innate defence against bacteria. An increase in serum lysozyme levels and antiprotease activities in response to bacterial infections or after the administration of immunostimulants has been associated with enhanced disease resistance [41, 42]. Hence, these enzymes are frequently monitored as health markers and are highly modulated by nutrition, stress, infection, pollution [41] and by thermal imprinting in zebrafish [43]. In our study, higher levels of lysozyme and antiprotease activities were detected in Nanno-programmed larvae indicating that our microalgal crude extracts had long-term effects on the enhancement of innate immunity. Moreover, it should be noted that MAe (6h after exposure) increased the activity of these enzymes corroborating the results of previous studies indicating the high potential of this extract as an immunomodulator [12, 13]. The decreased activity of lysozyme observed at 24h may be due to the acute stress associated with the high mortality observed, since stressful situations are known to reduce humoral defences and lysozyme levels [44].

The Nanno programming treatments enhanced the mRNA levels of chemokine *cxc10* and the antiviral (*irf3*, *irf7*, and *mx*) immune transcripts in post-larvae, indicating a heightened response of antiviral genes to the LCDV challenge and a reduction in pro-inflammatory cytokines. The elevated levels of antiviral immune transcripts in fish is a well conserved mechanism to provide rapid protection against lethal virus infections in the aquatic environment [37]. It is well-established that β-glucans, and particularly soluble laminarin, are major inducers of trained immunity in fish and cause increased phagocytosis and expression of inflammatory cytokines and viral resistance [7, 45]. Since a crude Nanno extract was used in this study it was not possible to associate a specific microalgal molecule to the programming of antiviral defences and

proinflammatory cytokines. Nonetheless, the approach used represents a novel and promising approach with potential for the control microbial diseases in fish.

Dietary supply or injection of β-glucans increase the expression and production of pro-inflammatory cytokines and stimulates phagocytic, cytotoxic, and antimicrobial activities although with some differences depending on the β-glucan structure and solubility [3, 6]. In this study, we demonstrated that both MAe and yeast β-glucans modified the steady-state levels of innate immune genes as well as the intensity of the defensive response to LCDV. The administration of both bioactive compounds activated a quick and transient immunomodulatory response that was more intense in MAe treatments. This heightened response observed for MAe supports the more systemic action of this soluble extract than particulate yeast β-glucans as suggested in Carballo, et al. [13] and the strong induction of pro-inflammatory cytokines and other innate immune related genes when injected i.p. in juveniles [12]. It should be noted that the response to Y and MAe exposure or LCDV challenge was dynamic and post-larvae reduced gene expression levels progressively after the treatments. Interestingly, comparison of gene expression levels between SW and Y in the SWC group revealed long-lasting changes at 96h (Fig. 7). The down-regulation of cytokines and antiviral genes after β-glucan exposure is reminiscent of the homeostatic mechanism that balances harmful and defensive inflammatory responses stimulated by pathogens [46, 47].

The LCDV challenge triggered a broad defensive response and activated the expression of most of the genes investigated. The quick and systemic antiviral defensive response that involved several antiviral pathways, cellular markers and inflammation-related genes has previously been related to the high resistance of sole

to LCDV [21, 22] and other fish viruses [45, 48]. Reduction in *mcp* copies has been associated with the capacity of the immune response to block viral replication and spreading [22]. Interestingly, the clustering and PCA analysis clearly demonstrated an interaction between Nanno-programming and yeast β-glucan treatments that affected mainly the antiviral genes (*irf7*, *irf3* and *mx)* and pro-inflammatory cytokines *il1b* and *tnfa*. No differences in response to LCDV were found between programming treatments in Y-supplied post-larvae. In contrast, the Nanno-programmed fish of the SW group activated higher expression of antiviral genes and did not modify expression of pro-inflammatory cytokines compared to CN post-larvae. Previous studies have demonstrated that the innate immune system can be trained, through epigenetic modifications, towards a specific response depending on the molecule used to prime the innate immune system [49]. Our results demonstrate that the *Nanno* extracts produced long-term effects on the steady-state level and response of antiviral genes to LCDV. Further research is necessary to identify the molecules in microalgal extracts responsible for the effects identified and how they modified the response of post-larvae sole to β-glucans.

In summary, this study demonstrated that a crude extract of *N*. *gaditana* can be used for larval programming in sole and that the effect lasts into the post-larvae stage. The methodology developed using larvae at hatch was technically simple and can readily be applied in hatcheries and represents a new potential application for microalgae not explored until now. The better growth performance and the enhancement of the immune response after supplying bioactive treatments or challenging with LCDV indicates they have the potential to improve operational methods used in fish hatcheries for management and disease control. The interaction between β-glucans

and Nanno programming after the LCDV challenge suggests that they mediate their actions via different regulatory pathways and this aspect deserves to be further investigated.

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Conflict of Interests

The authors declare they have no conflict of interests.

Authors'contributions

CC: Investigation for gene expression and viral analysis, fish laboratory experiments, gene expression and viral analysis. sampling, data Curation, validation of results, sampling; APM: Investigation for enzymatic activities, Data Curation; CM: Resources. Microalgal MAe extract; LM: Resources. Microalgal extracts. Supervision, Funding acquisition; DMP Conceptualization, Supervision, Funding acquisition, Writing - Review & Editing; MM: Conceptualization, Supervision, Funding acquisition, Writing - Original Draft, Review & Editing.

Captions

Figure 1. A) Experimental design for bioactive treatment. Nanno-programmed (Nanno) and negative control sole post-larvae (CN) were distributed in trays (n=80) and given artemia that was enriched for 30 min with bioactive treatments (Microalgal extract (MAe) or yeast β-glucans (Y)) or seawater (SW) as a control group in triplicate (x3). A second dose was also supplied 4h after the onset of the bioactive trial and the post-larvae were sampled at 6 and 24 h (in blue). After 48 h, post-larvae from SW and Y groups (that also contained the CN and Nanno groups) were distributed in new trays (n=30/tray) in duplicate and challenged with artemia infected with the virus LCDV. Post-larvae fed artemia enriched with clean seawater (SWC) was used as a negative control. Samplings were carried out at 3, 6 and 24 h after the LCDV challenge. B) Codes used in the study to identify the experimental groups

Figure 2. Dry weight (A) and length (B) of programming treatments (CN and Nanno). Asterisks denote the existence of statistically significant differences among treatments ($P < 0.05$).

Figure 3. Cumulative mortality (%) of CN and Nanno-programmed (Nanno) post-larvae administrated the bioactive treatments (MAe in the dashed green line and Y in the orange line) and the negative control (SW). The control groups are indicated with square symbol and Nanno post-larvae with circular symbols. Mortality were recorded for 48h after the onset of the trial.

Figure 4. A) Lysozyme and B) antiprotease enzymatic activities of CN and Nanno post-larvae administrated the bioactive treatments (MAe and Y) and the negative control (SW). Enzyme activity was measured in homogenates of post-larvae sampled at 6 and 24h after the onset of the trial. The mean ± SEM are represented and expressed as U per post-larvae for lysozyme (A) and % inhibition per post-larvae for anti-protease (B) activity. Letters denote significant differences between bioactive treatments at a specific time point for the CN and Nanno (letters added ') groups. Asterisks indicate significant differences between programming treatments at the same time point and the symbol "&" between time points. Significance was set at $P<0.05$.

Figure 5. Relative gene expression levels of CN and Nanno-programmed post-larvae treated with the bioactives, MAe and Y and the negative control (SW). Expression levels were determined at 6 and 24 h after the onset of the trial. The control group (SW) is indicated by blue bars, MAe in green bars and Y in orange bars. Data are expressed as the mean ± SEM, n = 4, the calibrator group is the SW control 6h). Significant differences for programming (Progr), bioactive treatments (Bio T) and time are indicated in the square. The post-hoc result is indicated on the right if significant. Interactions are only shown when significant. When interactions are significant, a two-way analysis was carried out. In this case, letters denote significant differences between bioactive treatments at the same time point (6h in lowercase and 24h in uppercase). Post-hoc analysis is indicated in letters using the colour code indicated above. Asterisks indicate significant differences between programming treatments at the same time point. Significance was set at $P<0.05$.

Figure 6. Quantification of LCDV genomic DNA in infected Senegalese sole post-larvae at 3, 6 and 24h post-challenge (pch). LCDV DNA copies in CN (grey) and Nanno (green) are indicated. Data are expressed as the mean logarithm of viral DNA copies per µg of total DNA ± SEM. Letters denote the significant differences between sampling points (P<0.05).

Figure 7. Hierarchical heat map cluster (A) and Principal components analysis (PCA) (B and C) using the gene expression values at 24 h after a LCDV challenge. A) Data were normalized using log2 of fold-change. Green and red colours indicate low and high expression values, respectively according to the scale shown. Sample codes are as follows: first Letter CN or Nanno indicates if control or Nanno programmed; second letter indicates if post-larvae were administrated MAe, Y or SW; the third letter if post-larvae were challenged with LCDV (V) or the negative control (SWC). The main clusters grouping the LCDV (red) and SWC (green) as well as other subclusters grouping similar samples are indicated. Gene names are shown on the left. B) and C) Principal Component Analysis (PCA) plot based on the complete set of genes analyzed representing the samples (n=4) for programming (B) or bioactive (C) treatments. The confidence ellipses (95%) for LCDV challenge groups (SWC and V) are indicated. Dimension 1 explains 48.6% and dimension 2 the 20.5% of total variation.

Supplementary files

Suppl. file 1 Relative gene expression levels of the control (CN; grey) and Nanno-programmed (Nanno; green) post-larvae administrated Y (solid color) or SW (cross-hatched color). V indicates the infected and SWC the non-infected groups. Expression

levels were determined at 24 h after the challenge. Data are expressed as mean ± SEM, n = 4 from the calibrator group (CN-SW-SWC group). Significant effects of programming (Prog), bioactive treatments (Bio T) and LCDV infection (challg) and post-hoc comparisons are shown. When the interaction was significant, a two-way ANOVA was carried out. In this case, letters indicate significant differences between programming groups for each bioactive treatment. Asterisks denote significant differences between viral treatments for each bioactive treatment. Letters above the colour squares and the hash sign denotes significant differences between groups. Significant differences were set at $P<0.05$

Suppl file 2. The principal component analysis (PCA) biplot of the gene set analysed in samples for programming (A), bioactive compounds (B) and virus challenge (C). The name of the genes is indicated in the arrows. The confidence ellipses (95%) for each experimental group are shown.

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Highlights

Crude extracts of microalga *Nannochloropsis gaditana* modified larval programming for growth performance in sole post-larvae

Yeast β-glucan and microalgal extract activated immune gene responses but modulated by larval programming in sole post-larvae

LCDV DNA copies were not modified by programming or bioactive treatments. .

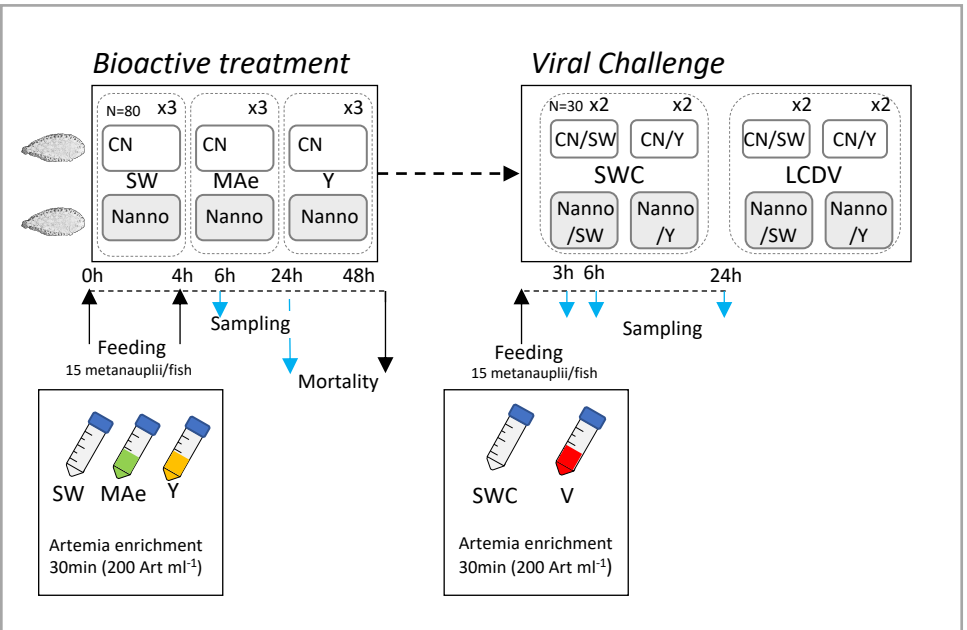
LCDV activated a wide defensive response modulated by yeast β-glucan and Nanno-programming

Credit Author Statement

CC: Investigation for gene expression and viral analysis, fish laboratory experiments, gene expression and viral analysis. sampling, data Curation, validation of results, sampling; APM: Investigation for enzymatic activities, Data Curation; CM; Resources. Microalgal MAe extract; LM: Resources. Microalgal extracts. Supervision, Funding acquisition; DMP Conceptualization, Supervision, Funding acquisition, Writing - Review & Editing; MM: Conceptualization, Supervision, Funding acquisition, Writing - Original Draft, Review & Editing.

Figure1

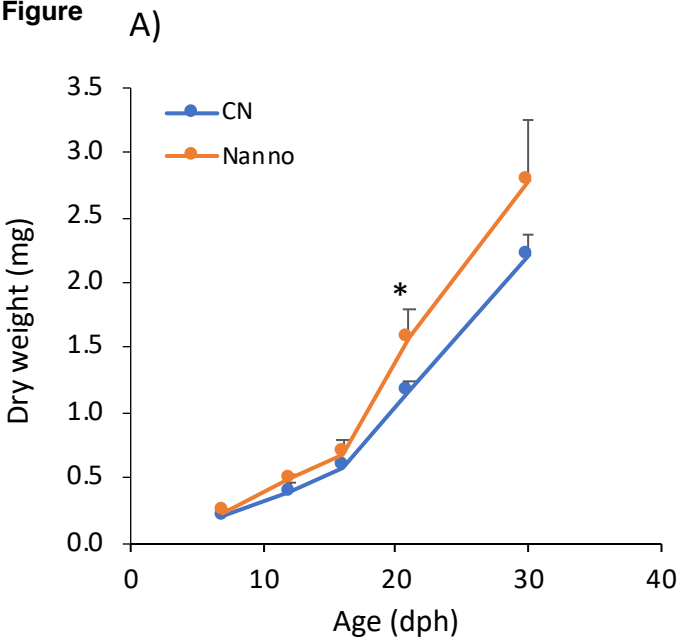
A)



B)

Programming	Larval exposure at 1 dph	
N. gaditana extract	Nanno	
Seawater control	CN	
Biotreatment (BioT)	Post-larvae exposure at 32 dph (programming*BioT)	
yeast β-glucan	Nanno-Y	CN-Y
P. *tricornutum* Microalgal extract	Nanno-MAe	CN-MAe
Seawater control	Nanno-SW	CN-SW
Challenge	Post-larvae viral challenge (programming*BioT*challenge)	
LCDV	Nanno-Y-V Nanno-SW-V	CN-Y-V CN-SW-V
Seawater control	Nanno-Y-SWC Nanno-SW-SWC	CN-Y-SWC CN-SW-SWC

Figure


A)
CN
Nanno
Dry weight (mg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
Age (dph)
*

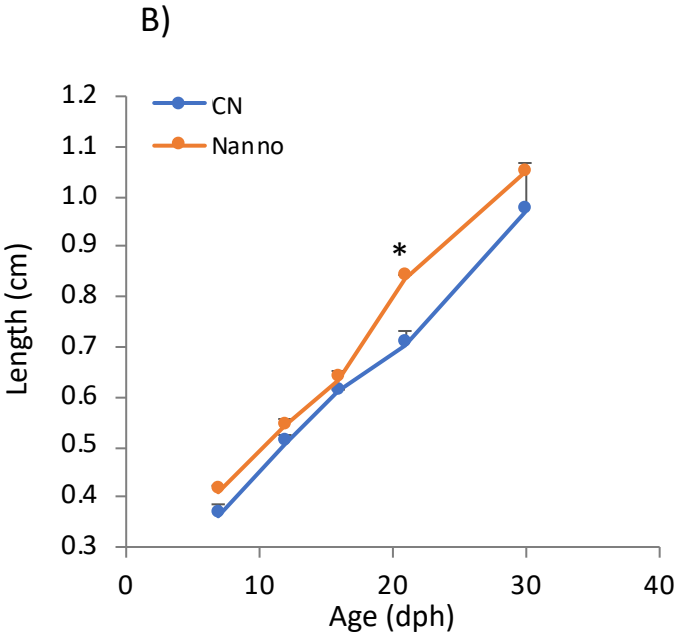

B)
CN
Nanno
Length (cm)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
10
20
30
40
Age (dph)
*

Figure3

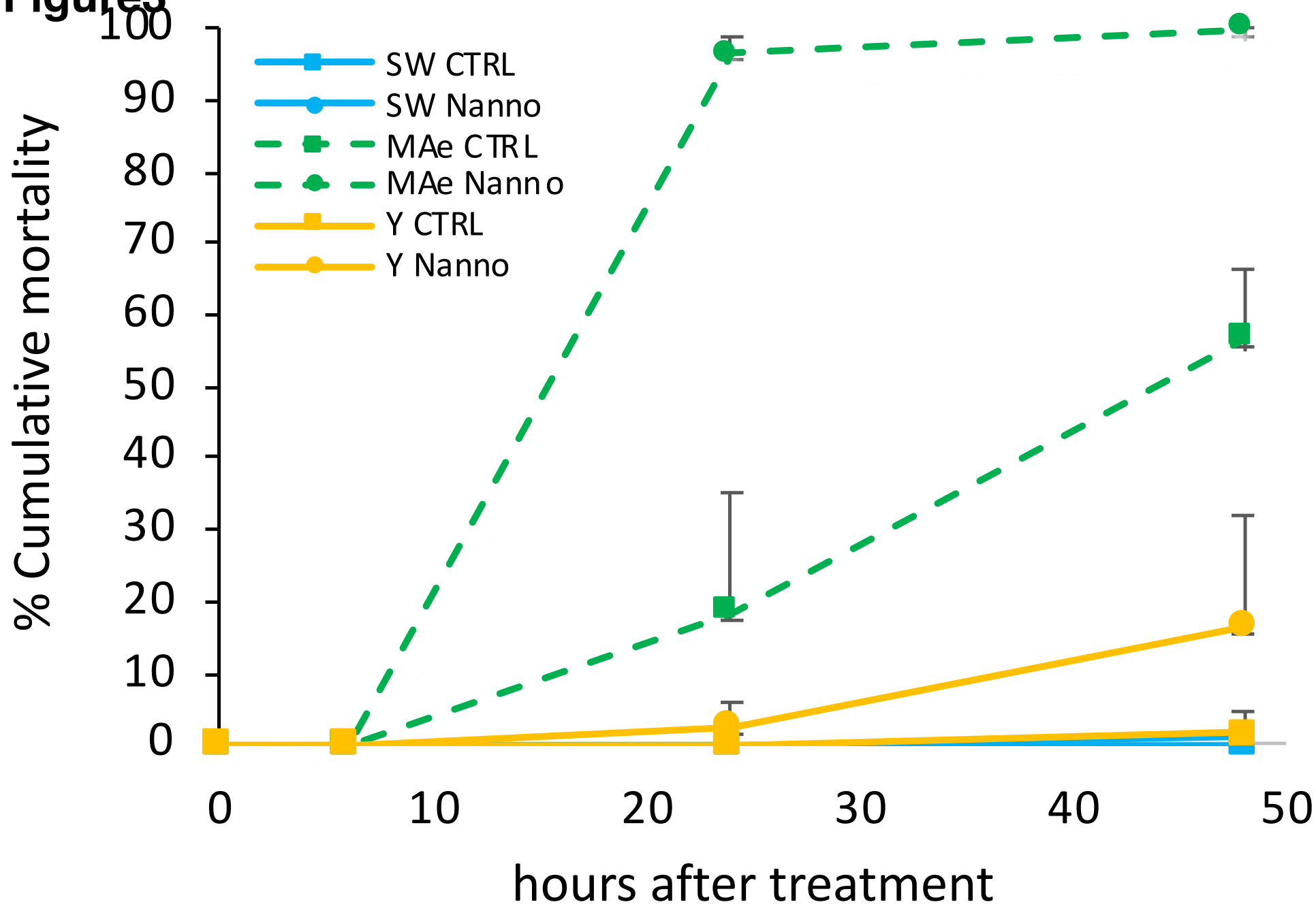
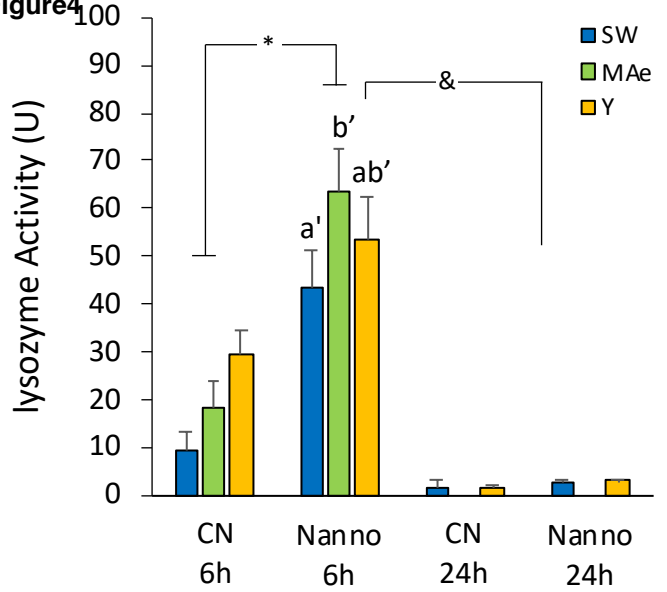

100
90
80
70
60
50
40
30
20
10
0
% Cumulative mortality
SW CTRL
SW Nanno
MAe CTRL
MAe Nanno
Y CTRL
Y Nanno
0
10
20
30
40
50
hours after treatment

Figure4


lysozyme Activity (U)
100
90
80
70
60
50
40
30
20
10
0
SW
MAe
Y
*
&
b'
ab'
a'
CN 6h
Nanno 6h
CN 24h
Nanno 24h

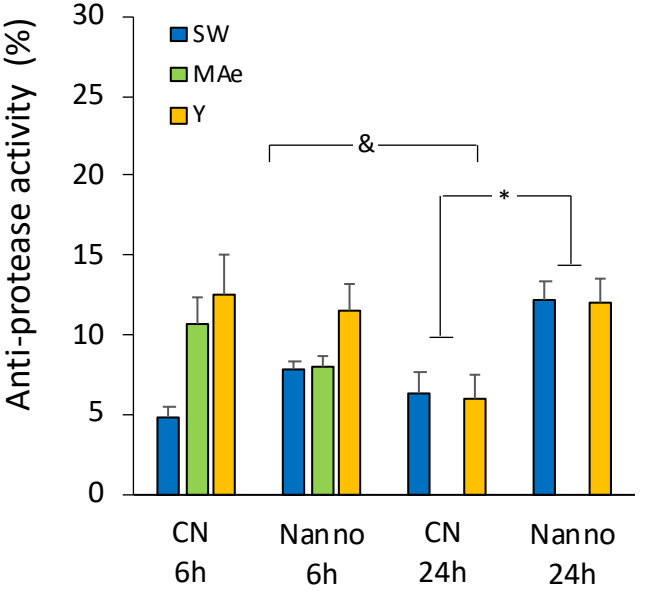
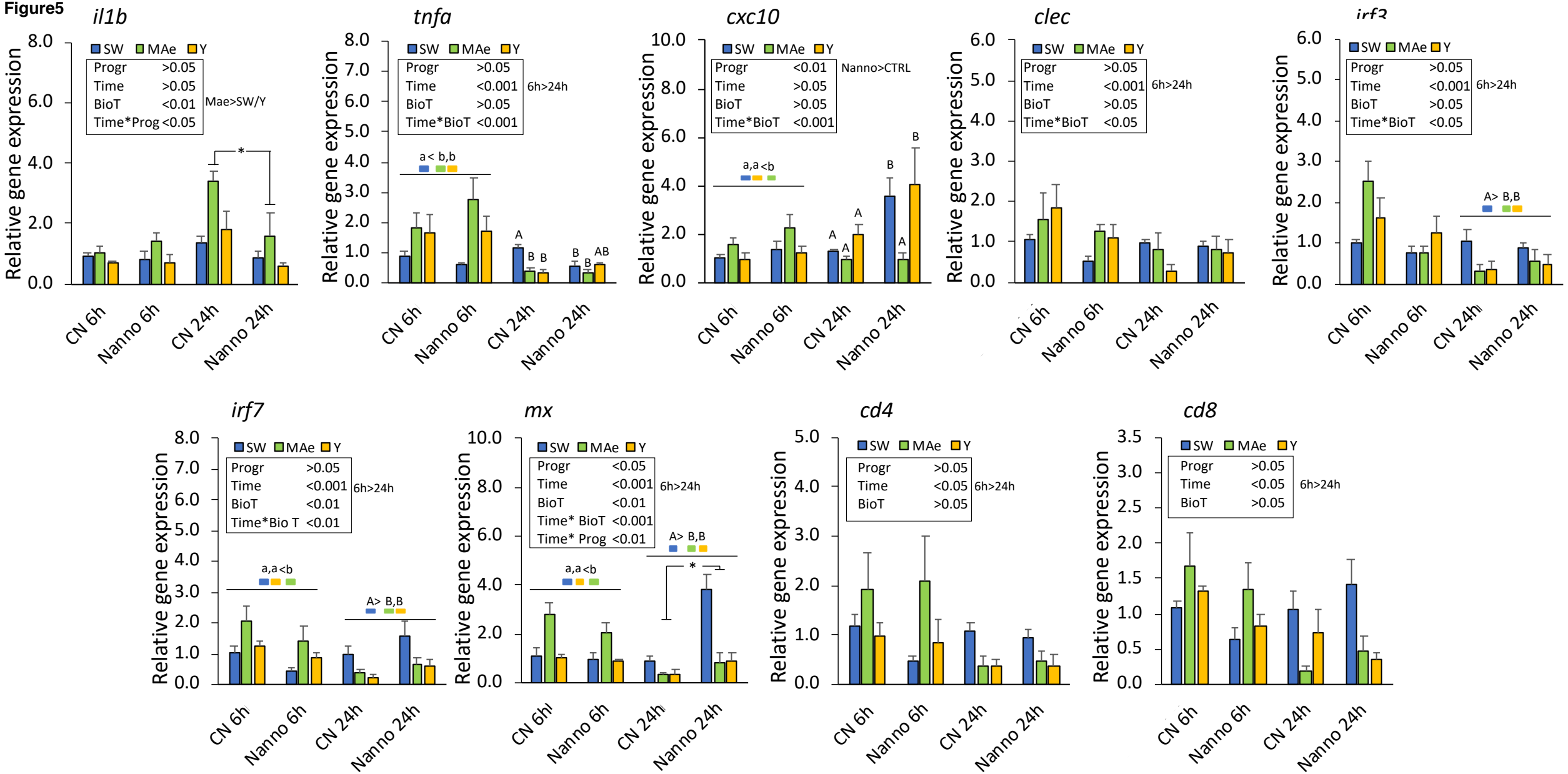

Anti-protease activity (%)
30
25
20
15
10
5
0
SW
MAe
Y
&
*
CN 6h
Nanno 6h
CN 24h
Nanno 24h

Figure5


il1b
SW MAe Y
Progr >0.05
Time >0.05
BioT <0.01
Time*Prog <0.05
Mae>SW/Y
tnfa
Progr >0.05
Time <0.001
BioT >0.05
Time*BioT <0.001
6h>24h
a< b,b
cxc10
Progr <0.01
Time >0.05
BioT >0.05
Time*BioT <0.001
Nanno>CTRL
a,a <b
clec
Progr >0.05
Time <0.001
BioT >0.05
Time*BioT <0.05
6h>24h
irf3
Progr >0.05
Time <0.001
BioT >0.05
Time*BioT <0.05
6h>24h
A> B,B
irf7
Progr >0.05
Time <0.001
BioT <0.01
Time*Bio T <0.01
6h>24h
a,a <b
A> B,B
mx
Progr <0.05
Time <0.001
BioT <0.01
Time* BioT <0.001
Time* Prog <0.01
6h>24h
a,a <b
A> B,B
cd4
Progr >0.05
Time <0.05
BioT >0.05
6h>24h
cd8
Progr >0.05
Time <0.05
BioT >0.05
6h>24h
Relative gene expression
CN 6h
Nanno 6h
CN 24h
Nanno 24h

Figure

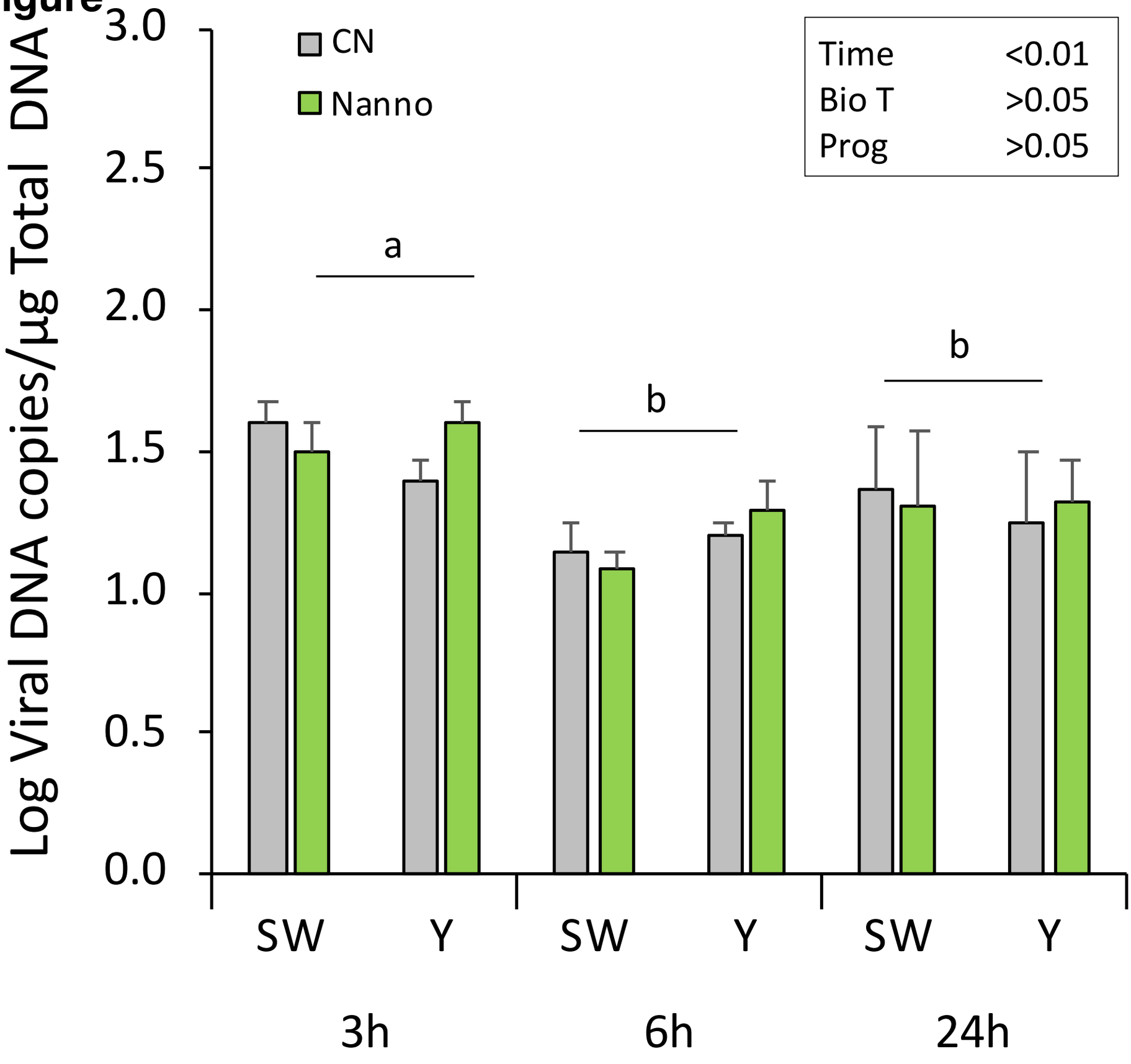
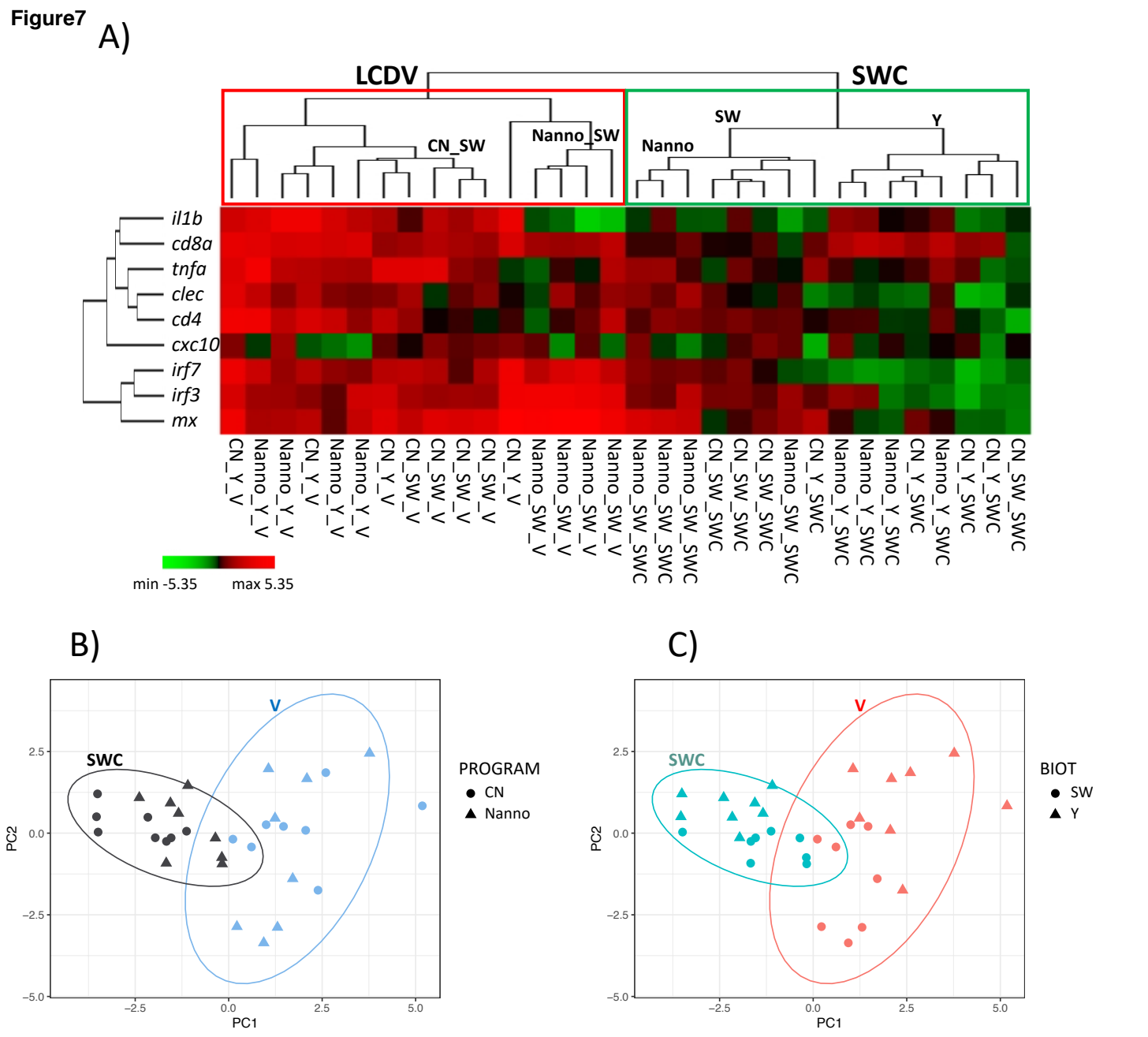

Log Viral DNA copies/µg Total DNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CN
Nanno
Time <0.01
Bio T >0.05
Prog >0.05
a
b
b
SW
Y
SW
Y
SW
Y
3h
6h
24h

Figure7


A)
LCDV
SWC
CN_SW
Nanno_SW
SW
Y
Nanno
il1b
cd8a
tnfa
clec
cd4
cxc10
irf7
irf3
mx
CN_Y_V
Nanno_Y_V
Nanno_Y_V
CN_Y_V
Nanno_Y_V
Nanno_Y_V
CN_Y_V
CN_SW_V
CN_SW_V
CN_SW_V
CN_SW_V
CN_Y_V
Nanno_SW_V
Nanno_SW_V
Nanno_SW_V
Nanno_SW_V
Nanno_SW_SWC
Nanno_SW_SWC
Nanno_SW_SWC
CN_SW_SWC
CN_SW_SWC
CN_SW_SWC
CN_SW_SWC
Nanno_SW_SWC
CN_Y_SWC
Nanno_Y_SWC
Nanno_Y_SWC
CN_Y_SWC
Nanno_Y_SWC
CN_Y_SWC
CN_Y_SWC
CN_SW_SWC
min -5.35
max 5.35
B)
V
SWC
PROGRAM
CN
Nanno
PC2
PC1
C)
V
SWC
BIOT
SW
Y
PC2
PC1

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