An immune-enriched oligo-microarray analysis of gene expression in Manila clam (*Venerupis philippinarum*) haemocytes after a *Perkinsus olseni* challenge

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ABSTRACT

Parasites of the genus *Perkinsus* cause high mortality and economic losses in bivalves commonly produced in global aquaculture. Although the immune responses of oysters and clams naturally infected with *P. marinus* or *P. olseni* have been extensively studied, there is not much information on host response at the early stages of infection. In this study, we analysed how *P. olseni* influences the gene expression profiles of haemocytes from the Manila clam (*Venerupis philippinarum*) using temporal experimental infections and an immune-enriched DNA microarray. We identified an early phase of infection that was characterised by no mortality and by the increased expression of genes associated with pathogen recognition, production of nitrogen radicals and antimicrobial activity. Cellular processes such as inhibition of serine proteases and proliferation were also involved in this early response. This phase was followed by an intermediate stage, when the pathogen was most likely multiplying and infecting new areas of the body, and animals began to die. In this stage, many genes related to cell movement were over-expressed. Thirty days after infection metabolic pathway genes were the most affected. Apoptosis appears to be important during pathogenesis. Our results provide novel observations of the broader innate immune response triggered by *P. olseni* at different infection stages.

KEYWORDS: *Venerupis philippinarum*, *Perkinsus*, haemocytes, oligo-microarray, gene ontology, Blast2GO, immune response, apoptosis.

1. Introduction

The Manila clam (*Venerupis philippinarum*) is one of the most extensively cultured bivalves in the world, especially in Asia and Europe. In Europe, 36,000 tons were produced in 2011, and this yield is likely to increase in the near future, either through expanding current production areas or by introduction into new suitable areas (FAO, 2012). The Manila clam was introduced to most Mediterranean countries in the 80's (Breber, 1985; Flassch and Leborgne, 1992) to counter the declining production of the autochthonous Grooved carpet shell clam (*V. decussatus*) due to overfishing, bacterial infections and parasitism (Figueras and Novoa, 2011; Romalde et al., 2014). The most difficult challenge for its production is disease, mainly associated with *Vibrio* spp. (Paillard et al., 2004; Beaz-Hidalgo et al., 2010) and *Perkinsus* spp. (Villalba et al., 2004; Waki et al., 2012).

Perkinsus spp. belong to a family of protistan parasites infecting a wide variety of marine molluscs (clams, oysters, abalones, mussels, scallops, etc) (Villalba et al., 2004). This genus includes seven species, some of which are associated with mass mortalities. For example, *P. marinus* is the causative agent of massive mortalities in native oysters, *Crassostrea virginica*, along the Atlantic coast of the United States and Mexico (Andrews, 1988). *P. olseni* is associated with severe mortality in the abalone and the pearl oyster in Australia (Norton et al., 1993; Goggin and Lester, 1995), and *Perkinsus*-like parasites are associated with mass mortalities of the Manila clam in Korea, Japan and China (Maeno et al., 1999; Liang et al., 2001; Park and Choi, 2001). In Europe, *P. atlanticus/olseni* and *Perkinsus*-like parasites have been reported in different areas of the Mediterranean and Atlantic coasts including Portugal, Spain, France and Italy; these parasites are associated with 50-80% of *V. decussatus* mortality in Algarve (S. Portugal) and 40% of the *V. phillipinarum* annual cumulative mortality in Galicia (NW Spain)

(reviewed in Villalba et al., 2004). *Perkinsus*-associated mortalities are particularly severe when environmental conditions are favourable to the parasite, for example, elevated temperatures and increased salinity (Andrews, 1988).

Perkinsus spp. parasites generally have two phases in their life cycles: an asexual propagation phase in the host tissue called uninucleated trophozoites and a zoosporulation phase in seawater (Bordenave et al., 1995). Although the process is not entirely understood, infection starts with trophozoites colonising connective tissues in the gill, mantle, labial palps, gonads and digestive gland (Park and Choi, 2001; Chintala et al., 2002). The most important histological response to this infection is the intense infiltration of haemocytes in tissues where the parasite is present, including epithelia, connective tissue, muscular fibres and blood spaces. Free and encapsulated parasites propagate by repeated cell divisions and induce the destruction and loss of normal tissue structure, leading to organ dysfunction (Mackin, 1951; La Peyre et al., 1995). Trophozoites are actively engulfed by haemocytes but not eliminated; trophozoites divide within and subsequently rupture haemocytes, resulting in their dissemination throughout the organism (Choi et al., 2002).

The immune response induced by *Perkinsus* spp. includes increased serum agglutinins, changes in lysozyme concentration and the generation of serine protease inhibitors (La Peyre et al., 1995; Faisal et al., 1998; Oliver et al., 1999; Ordás et al., 2000). Host oxygen-dependent killing mechanisms are also suppressed by the pathogen acid phosphatase, which interferes with superoxide anions to help the parasite survive in the host (Hervio et al., 1991; Anderson, 1999).

The effect of *Perkinsus* spp. on the host gene expression profile has been reported in experimentally infected oysters (Tanguy et al., 2004; Wang et al., 2010) and naturally

93 infected clams (Kang et al., 2006; Prado-Alvarez et al., 2009; Leite et al., 2013). A
94 combination of antimicrobial and oxidative stress responses are enhanced in the Eastern
95 oyster (Tanguy et al., 2004; Wang et al., 2010), while metabolic and stress related genes
96 are modulated in the infected carpet shell clam (Leite et al., 2013). Immune-related genes
97 involved in antimicrobial defence, pathogen recognition, anti-oxidant activity and
98 apoptosis are also regulated in both species after *Perkinsus* infection (Wang et al., 2010;
99 Leite et al., 2013). To date, the processes and genes that are differentially expressed in
100 haemocytes at early and late stages of infection, when the pathogen is actively
101 multiplying in tissues and when the infection is well established, are unclear.

102 Oligo-microarrays are a sensitive and reproducible technology for studying complex
103 biological functions because they allow the simultaneous analysis of the expression of
104 thousands of genes. Microarrays have been applied to address different biological issues
105 in cultured bivalves (Xu and Faisal, 2009; Dheilly et al., 2011; Milan et al., 2011). Oligo-
106 microarrays have been used to analyse gene expression in *C. virginica* infected with *P.*
107 *marinus* (Wang et al., 2010) and in *V. decussatus* naturally infected with *P. olseni* (Leite
108 et al., 2013).

109 In this study, we used an immune-enriched DNA microarray (Moreira et al., 2012) to
110 analyse how *P. olseni* influences the gene expression profiles of haemocytes from Manila
111 clams intramuscularly injected with this pathogen; this technique has been previously
112 used to study gene expression in response to *Vibrio alginolyticus* (Moreira et al., 2014).
113 Our results provide novel observations of the innate immune response at different
114 infection stages.

115

116 **2. Materials and methods**

117 *2.1. Animals*

118 Healthy and *Perkinsus*-infected clams (*V. philippinarum*) were obtained from the
119 Carril and Pontevedra shellfish farms, respectively (Galicia, NW Spain). The presence or
120 absence of *P. olseni* was confirmed using the Ray's fluid thioglycollate medium assay
121 (RFTM) (Ray, 1966). Healthy clams were maintained in open-circuit filtered seawater
122 tanks at 15°C with aeration. Naturally infected animals were maintained in the same
123 conditions using closed circuit seawater. All animals were fed daily with a mixture of
124 microalgae containing *Phaeodactylum tricornutum, Isochrysis galbana* and *Rhodomonas*
125 *lens.* Clams were acclimatised to the aquarium conditions for one week before the
126 experiments were conducted. Animal care and challenge experiments were reviewed and
127 approved by the CSIC National Committee on Bioethics.

128

129 *2.2. Experimental infections*

130 *Perkinsus* trophozoites were isolated from naturally infected animals following
131 the protocol established by Ford et al. (2002). The concentration was adjusted to $5x10^4$
132 trophozoites/ml in filtered seawater (FSW). The salinity of the inoculum was 35 ppt.
133 Healthy clams (*P. olseni*-free animals) (n=100) with a weight of 2.25 ± 0.64 g soft tissue
134 were notched in the shell and intramuscularly injected with 100 µl of the trophozoite
135 suspension. Control animals (n=100) were injected with 100 µl of FSW. After infection,
136 clams were maintained in 50-l tanks with aeration. The temperature was increased at
137 1°C/day ratio from 15 to 22 °C to allow the infection to proceed. Clams were fed twice
138 daily with microalgae. Water was renewed once/day for the duration of the experiment.
139 Mortalities were registered daily until 31 days after infection.

140

141 *2.3. Sampling protocol*

142 Twenty animals from each experimental group were sampled at 5, 10, 14, and 31
143 days post-infection (pi). Five animals from each group and time were used for
144 histological evaluation. Animals were fixed in Davidson's fixative (Shaw and Battle,
145 1957) for 24 h, embedded in paraffin and stained with haematoxylin and eosin (Merck).
146 Histological sections were examined under light microscopy (Nikon eclipse 80i) for the
147 presence of the parasite and pathological alterations. Haemolymph and gills were
148 extracted from the 15 remaining animals in each group to determine the parasite load.
149 The haemolymph samples were also used for microarray experiments. Haemolymph from
150 five individuals was pooled, and three pools (biological replicates) were taken at each
151 sampling point and condition. After centrifugation of the haemolymph samples at
152 3,000xg for 10 min at 4 °C, the pellets were resuspended in 250 µl of TRIzol reagent
153 (Invitrogen). Total RNA was isolated following the manufacturer's specifications. RNA
154 was treated with DNase I and purified again using an RNeasy Mini kit (Qiagen). The
155 concentration and purity of the RNA were measured using a *NanoDrop ND1000*
156 spectrophotometer (Thermo Scientific), and the RNA integrity was tested on an Agilent
157 2100 Bioanalyser (Agilent Technologies). RNA (1 µg) was reverse transcribed into
158 cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen) according to the
159 supplier's protocol.

160

161 *2.4. Evaluation of the infection levels*

162 The infection levels were evaluated in the gills and haemolymph of the infected
163 animals used for microarray experiments. In gills, the intensity of the infection was
164 estimated by the RFTM assay (Ray, 1966). In haemolymph, *P. olseni* was quantified by

qPCR of its 18S rRNA gene using the Brilliant II SYBR Green qPCR Master Mix protocol (Agilent Technologies) and specific primers (Table 1).

2.5. Microarray experiments

A 8x15K Agilent 60-mer oligo-microarray (Moreira et al., 2014) was used to compare the gene expression profiles of clams with and without *P. olseni* infection. Probe sequences and other details on the microarray platform can be found in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GPL16450. Only high quality RNA samples were used. One hundred ng of RNA from each sample was amplified, labelled with Cy3 using the Low Input Quick Amp labelling kit (Agilent Technologies) and purified with RNeasy mini spin columns (Qiagen). Amplification and dye incorporation rates were verified using a *NanoDrop ND1000* spectrophotometer (Thermo Scientific). Microarray hybridisation was conducted following the protocol previously described by Moreira et al. (2014). Briefly, Cy3-labelled RNA was fragmented and diluted in 2X GE hybridisation buffer (Agilent). Slides were incubated for 17 h at 65 °C in a hybridisation oven (Agilent) and scanned at 5 µm resolution using an Agilent G2565BA DNA microarray scanner. Agilent Feature Extraction software (version 9.5.1) was used for the data extraction and background subtraction following standard procedures. GeneSpring software (Agilent) was used to normalise and analyse the microarray fluorescence data. The raw and normalised fluorescence values were deposited in the GEO database under Accession no. GSE GSE59399 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ezufuoostlujxsd&acc=GSE59399). Significant differences in gene expression between the controls (an unique control sample was generated by pooling RNAs obtained from different animals at the 4 different

189 time points) and the infected samples at each sampling point were analysed by the t-test
190 ($p<0.01$). Genes with <1.5-fold change were not further investigated. A hierarchical
191 cluster of the genes with fold-changes greater than 5 at any stage was implemented in
192 MeV (Saeed et al., 2003),

193

194 *2.6. Gene Ontology (GO) terms and enrichment analysis*

195 After statistical analysis, GO terms (Ashburner et al., 2000) were assigned to the
196 significantly expressed genes (t-test, $p<0.01$) through the time course using Blast2GO
197 software (Conesa et al., 2005). The analysis was performed using default values
198 (annotation cut-off=55, GO weight=5), and the most convenient levels for biological
199 process ontology were selected. Enrichment analyses were conducted using the total
200 microarray information as the "reference set" and the information at each sampling time
201 (5, 10, 14 and 31 days) and changes (up- or down-regulated genes) as the "test sets". A
202 one-tailed Fisher's exact test was performed without removing double IDs and with a
203 false discovery rate (FDR) cut-off value of 0.05.

204

205 *2.7. qPCR microarray validation*

206 To validate microarray results by qPCR, a set of 4 genes covering the fold change
207 (FC) range variation across the different experimental conditions were selected. Specific
208 primers for qPCR were designed using the *Primer3* program (Rozen and Skaletsky, 2000)
209 according to qPCR restrictions (Table 1). The efficiency of each primer pair was analysed
210 with seven serial five-fold dilutions of cDNA, and the slope of the regression line of the
211 quantification cycle versus the relative concentration of cDNA was calculated (Pfaffl,

2001). The same RNA samples were used for microarray and qPCR experiments. qPCR was performed in an Mx3005P machine (Agilent Technologies) using 1 µl of fivefold-diluted cDNA in a 25-µl reaction following the Brilliant II SYBR Green qPCR Master Mix protocol (Agilent Technologies). All reactions were performed as technical triplicates, and an analysis of melting curves was performed in each reaction. The relative expression levels of the genes were normalised to the clam 18S rRNA gene, which was constitutively expressed and not affected by the *P. olseni* challenge, and analysed by the Pfaffl method (Pfaffl, 2001).

2.8. Apoptosis assay

To analyse apoptosis during *P. olseni* infection, a TUNEL assay was conducted in histological sections using an *in situ* cell death detection kit (Roche). Briefly, tissue sections were dewaxed, rehydrated and permeabilised for 30 min with proteinase K (20 µg/ml in 10 mM Tris HCl, pH 7.4). After washing, samples were incubated for 60 min at 37°C with 50 µl TUNEL reaction mixture. Samples were rinsed with PBS and analysed in a drop of PBS under a confocal fluorescent SPE microscope (Leica).

3. Results and discussion

3.1. Experimental infections and mortalities

Manila clams were experimentally infected via intramuscular injection with *P. olseni* that were isolated from naturally infected clams. This inoculation route was selected because injection into the haemolymph sinuses of the adductor muscle leads to a more rapid parasitic infection (Bushek et al., 1997; Chintala et al., 2002). The intramuscular injection of a low concentration of trophozoites (2.25×10^3 trophozoites/g

wet tissue weight) started with a 12-day period with no mortalities, followed by a constant increase in mortality (up to 42%) to the end of the experiment (day 31) (Figure 1A). Similar mortality kinetics were described in *C. virginica* that were intramuscularly infected with *P. marinus* and in *V. philippinarum* infected by bath with *P. olseni* (Chintala et al., 2002; Shimokawa et al., 2010; Waki et al., 2012; Waki and Yoshinaga, 2013).

The percentage of infected animals and the intensity of their infections were evaluated in gills and haemocytes at 5, 10, 14 and 31 days pi. Using the RFTM assay in gills, viable parasites (Ray, 1966) were detected at all sampling points in infected clams; however, the progression of the disease could not be evaluated in most animals because the infection intensities were very low (0.5 on the Ray's scale with less than 10 trophozoites in the analysed gill tissues). Based on this RFTM assay, the number of trophozoites found in the infected clams did not increase during the experimental infection, which agreed with results obtained in *C. virginica* intramuscularly infected with *P. marinus* (Chintala et al., 2002). When the parasite load was quantified by qPCR, there was a significant increase in the parasite 18S gene mRNA from day 5 to day 31 (Figure 1B). The qPCR detection and quantification of *P. olseni* in clams was more sensitive than the RFTM assay, as described for *P. marinus* (Marsh et al., 1995; Robledo et al., 1998). Accordingly, the prevalence of *P. olseni* may have been underestimated by the RFTM assay. Histological examinations revealed the presence of a few trophozoites mainly located in the mantle at day 14 and in the connective tissue at day 31 (Figure 1C and D). *P. olseni* was not detected at day 5 and 10. Our results suggest an early phase of infection (5-10 days) with no mortalities and a middle stage when the pathogen is actively multiplying. After 31 days, new immature trophozoites, produced by binary fission, spread to adjacent tissues and cause severe disease.

3.2. Microarray hybridisation, robustness and validation

A total of 24 microarray experiments were performed. Only robust fluorescence values within the 20-90th percentile were used for the analysis. Results were validated by qPCR, which confirmed the patterns of up-regulated and down-regulated expression of the four selected genes (big defensin, interferon-inducible GTPase 1, quinoid dihydropteridine reductase and the TNF superfamily member 14) (Figure 2). This platform was previously validated through qPCR by Moreira et al. (2014). Our results are similar to those observed in other studies (Pardo et al., 2012; Leite et al., 2013) and support the consistency of our microarray data.

3.3. The general response of haemocytes to P. olseni infection

Haemocytes were selected to analyse the effect of *P. olseni* infection because they are the immune cells in molluscs (Donaghy et al., 2009), and they are targets for trophozoite multiplication and dissemination (Choi et al., 2002). A total of 1264 genes were differentially expressed (DE) in the control and infected groups (Figure 3A). A similar proportion of DE genes were up- and down-regulated (56 and 44 %, respectively) at different timepoints. At five days after infection, there were no mortalities, but the highest number of DE genes were found (39 % of the total modulated genes, 494 out of 1264 genes). The number of DE genes decreased to 13 % (175 out of 1264) at 10 days after infection. When the pathogen possibly started to proliferate at day 14, the number of DE genes increased to 23 % (299 out of 1264) and was maintained until the end of the experiment (Figure 3A). A Venn diagram was constructed to analyse the number of unique and common DE genes at the different sampling times after *P. olseni* infection

285 (Figure 3B). Many DE genes were time-specific (868 out of 1264). The highest
286 percentage of time-specific genes was registered at 5 d after infection (81.98 %; 405 out
287 of 494). At 10 and 14 days after infection, the number of specific genes decreased to 53
288 and 59%, respectively. At the end of the experiment (day 31), the exclusive genes
289 represented 64.8% of the total genes. The unique sets of genes regulated in Manila clam
290 are likely reflections of the dynamic expression of the host genome at different times
291 post-infection, as was previously suggested in *C. virginica* challenged with *P. marinus*
292 (Wang et al., 2010).

293

294 *3.4. Gene expression profile after P. olseni infection*

295 According to the enrichment analysis, the immune response was down-regulated
296 at 5 days after infection. The GO terms included processes such as response to stimulus,
297 signal transduction, cytoskeleton organisation, innate immune response, positive
298 regulation of defence response and type I IFN biosynthesis, which were reduced in the
299 presence of the pathogen (Figure 4). This early parasite-induced inhibition of the immune
300 response has been previously suggested in *C. virginica* infected with *P. marinus* (Hughes
301 et al., 2010), and it involves the reduction of several haemocyte and humoral immune
302 functions including ROS, lysozyme production, cell mobility and hemagglutination
303 (Soudant et al., 2013). The down-modulation of GO terms related to actin filament
304 activity (Figure 4) and the decreased expression of genes such as the TNF superfamily
305 member 14 and the IFN-inducible GTPase 1 gene at day 5 (Table 2, Supp. Figure 1)
306 support this early immune modulation.

307 Despite the initial immune suppression, Manila clams also highly expressed genes
308 involved in pathogen recognition at the beginning of the infection (day 5), including C1q

domain-containing proteins, FRED-related proteins and sialic acid-binding lectins, as previously described in naturally infected clams (Kang et al., 2006; Prado-Alvarez et al., 2009; Adhya et al., 2010). Most of those genes were in the top 25 expressed genes during the infection (Table 2). Lectins are some of the most important *Perkinsus*-inducible proteins; they are involved in recognising the parasite and promoting its opsonisation or encapsulation (Kang et al., 2006; Kim et al., 2006; Tasumi and Vasta, 2007; Adhya et al., 2010; Wang et al., 2010; Leite et al., 2013). A galectin-3 gene was up-modulated 5 days after infection (Table 3). This molecule is crucial for various signalling and molecular recognition processes and is overexpressed in *Perkinsus*-naturally infected clams (Kim et al., 2008) and oysters (Tasumi and Vasta, 2007). Interestingly, the most highly expressed gene at day 5 is fibropellin-3 (FC=276.41) (Table 2). This gene has been described in the sea urchin, and it encodes a glycoprotein containing two epidermal growth factor (EGF) domains at the 5' end. This protein is the principal component of the apical lamina and is involved in cell adhesion (Burke et al., 1998). Fibropellin-3 expressed in the Manila clam haemocytes could aid in the recognition of and adhesion to *P. olseni* in the initial stages of the infection, although this hypothesis should be tested in clams.

Genes encoding antimicrobial peptides were also in the top 25 expressed genes at day 5 (Table 2, Supp. Figure 1). For example, the antimicrobial peptide big defensin was exclusively highly expressed at day 5 (FC=111.23). This gene has been described in clams (Perrigault et al., 2009; Zhao et al., 2010), scallops (Zhao et al., 2007; Pauletto et al., 2014) and oysters (Fleury et al., 2009) and is active against Gram-positive and -negative bacteria and fungi (Rosa et al., 2011). This study is the first to describe the up-regulation of big defensin during *P. olseni* infection. Histones also play a role in innate immune defences due to their antimicrobial properties (Dorrington et al., 2011). Manila clams infected with *P. olseni* showed an up-regulation of several histone genes mainly at

day 5 post-infection (Table 2 and 3, Supp. Figure 1), as described previously in eastern oysters experimentally challenged with *P. marinus* (Dorrington et al., 2011). Our analyses also revealed the involvement of serine proteases inhibitors (SPIs) that counteract serine proteases secreted by *Perkinsus* spp., which are virulence factors responsible for tissue degradation (LaPeyre et al., 1996; Xue et al., 2006). The serine protease inhibitor, cvSI-1, is involved in the *C. virginica* defence against *P. marinus*, possibly by inhibiting parasite proliferation (LaPeyre et al., 2010; Yu et al., 2011). A serine-type peptidase inhibitor (ovomucoid protein) was strongly up-regulated at 5 days after infection (FC=26.55) (Table 2). However, it is possible that SPIs are not expressed exclusively in the initial stages of infection because another SPI similar to cvSI-1 was over-expressed at day 31 (FC=4.44). The late expression of a putative CvSI-1 has been described in oysters 30 days after *P. marinus* challenge (He et al., 2012).

After recognition by the host immune cells, *P. olseni* can be engulfed or encapsulated to prevent the spread of the pathogen. The enrichment analysis revealed the down-regulation of several GO terms related to actin filaments (actin filament bundle organisation and assembly) at day 5 (Figure 4), suggesting that *P. olseni* causes the haemocytes to enter a static state in agreement with the observed inhibition of phagocytosis caused by *P. olseni* in *V. decussatus* and *V. philippinarum* (Ordás et al., 2000; Flye-Sainte-Marie et al., 2009). Encapsulated parasites are frequently observed in histological sections (Montes et al., 1995), and this response involves the active migration of many haemocytes from the haemolymph into the tissues (Lee et al., 2001). At day 14, Manila clams infected with *P. olseni* expressed many genes related to cell movement and migration. Three different beta-tubulins (FC values 42.50, 14.94 and 10.23), a radial spoke head protein 4 homolog gene and a neurocalcin-delta gene were included in the top 25 expressed genes (Table 2), suggesting that haemocytes were most likely recruited to

prevent the spread of the pathogen at this time point. The enrichment analysis and the GO terms confirmed active cell proliferation at the beginning of the infection. GO terms related to proliferation such as the G2/M transition of the mitotic cell cycle, the S phase of the mitotic cell cycle and DNA strand elongation involved in DNA replication, were up-regulated at day 5 (Figures 5 and 6A). These observations agree with previous reports of increased haemocyte densities in tissue and haemolymph after *P. marinus* infections (La Peyre et al., 1993).

The production of free radicals derived from oxygen and nitrogen is associated with phagocytosis. Reactive oxygen and nitrogen components (ROS and NO, respectively), alone or in combination with lysosomal enzymes, efficiently eliminate pathogens (Novas et al., 2004; Villamil et al., 2007). The nitric oxide (NO) biosynthetic process was up-regulated at day 5 (Figure 5). This biological process is mediated by the quinoid dihydropteridine reductase (QDPR). This enzyme is involved in the metabolic pathway of tetrahydrobiopterin (BH4), which is an essential cofactor allowing nitric oxide synthase (NOS) to release nitric oxide (Gu et al., 2013). Manila clams infected with *P. olseni* showed up-regulation of a QDPR-like gene (Table 2, Supp. Figure 1). Although this gene was expressed at all timepoints, its maximum expression occurred at day 5 (FC=32.23). This result is in agreement with the increase in plasma nitrite concentration described in *C. virginica* at 6 and 14 days after infection with *P. marinus* (Villamil et al., 2007).

After the early immune suppression observed at day 5, the expression of some immune genes returned to the levels observed prior to the experimental infection at day 10 and 14. For example, the IFN-inducible GTPase 1 gene is associated with cell-autonomous resistance mechanisms against other apicomplexan intracellular parasites, and it stimulates oxidative, autophagic, membranolytic, and inflammasome-related

antimicrobial activities within the cytosol and pathogen-containing vacuoles (Kim et al., 2012). This gene was down-regulated at the beginning of the infection but highly expressed at days 10 and 14 (FC values 42.54 and 18.75, respectively) (Table 2, Supp. Figure 1).

At the end of the sampling period (31 days post-infection), the enrichment analysis and GO terms (Figure 5 and 6B, respectively) revealed mainly an increase in metabolic processes; changes in metabolism-related genes reflect changes in growth and survival (Leite et al., 2013). Physiological and metabolic changes in the host frequently occur after prolonged exposure to *Perkinsus* parasites (Choi et al., 1989). The GO term analysis at level 4 revealed that lipid, fatty acid, carbohydrate and organic compound metabolism were increased at day 31 (Figure 6B). In particular, lipid metabolism is important for the survival and propagation of *P. marinus* because it cannot synthesise sterols and, thus, must sequester them from its host (Lund et al., 2007). Interestingly, there were also genes related to steroid metabolism (Figure 6B). The 17-beta-hydroxysteroid dehydrogenase 14 gene, involved in steroid metabolism (Table 2) was up-modulated during the final stage of the infection, possible implicating sterols in the survival of *P. olseni* in Manila clams.

Apoptosis as a defence mechanism against *Perkinsus* infection has been described in *C. virginica.* Apoptosis of infected cells without the induction of inflammation could restrict the spread of the pathogen and protect the integrity of nearby tissues (Sunila and LaBlanca, 2003; Hughes et al., 2010). The enrichment analyses showed a significant up-regulation of "activation of pro-apoptotic gene products" at the early stages of the infection (Figure 5), and the classification of the DE genes into different GO categories revealed an increased number of genes related to the apoptotic cell death at those stages (Figure 6C). Apoptosis was down-regulated at the middle stages of infection (between

409 days 10 and 14) when the GO terms “regulation of endopeptidase activity involved in
410 apoptosis” and “cellular disassembly involved in execution phase of apoptosis” were
411 down-regulated (Figure 4) and the lowest number of DE genes related to cell death were
412 observed (Figure 6C). However, apoptosis rebounded at the end of the experiment when
413 the pathogen was potentially re-infecting healthy cells. Apoptosis is also modulated in *C.*
414 *virginica* infected with highly virulent strains of *P. marinus* (Hughes et al., 2010).

415 To corroborate the involvement of apoptosis during *P. olseni* infection, TUNEL
416 assays were conducted in histological preparations (Figure 7). Samples from non-infected
417 animals showed a low number of apoptotic cells, mainly located in the mantle, gills and
418 intestine, which was expected given that apoptosis is a physiological process related to
419 tissue homeostasis and integrity (Kerr et al., 1972). In infected animals, a high number of
420 apoptotic cells were detected at day 5 in the mantle and gills, as previously described in
421 waterborne *P. marinus* infections (Dungan et al., 1996; Allam et al., 2013). Apoptotic
422 cells were mainly detected in connective tissues between muscular fibres surrounding the
423 digestive gland at later sampling points (day 14 and 31); this is the target tissue for
424 pathogen multiplication (OIE, 2013).

425 In animals naturally infected with *P. olseni*, re-infection or co-infections with
426 other pathogens can occur. These events hinder studies of the host response in the initial
427 infection stages. The experimental infections allowed us to describe the early immune
428 modulation induced by *P. olseni* in infected clams, and interestingly, this immune
429 modulation was similar to the reported immune response against a *Perkinsus* spp. natural
430 infection. However, with our approach we identified novel genes related to pathogen
431 recognition (fibropellin-3), the production of nitrogen radicals (quinoid dihydropteridine
432 reductase) and antimicrobial proteins such as big defensin and histones. In conclusion, we
433 have developed a controlled *Perkinsus* infection that models the early bivalve-pathogen

interaction and allows us to analyse parasite-driven gene modulation. Apoptosis was observed in different stages of the infection and confirmed by histological techniques, demonstrating that this is a key process in the response to *Perkinsus* spp. infection.

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700 **Table 1.** qPCR primers for *P. olseni* quantification and *V. philippinarum* microarray

701 validation.

Primer name	Sequence 5' → 3'	Product size (bp)	Ct slope	Acc. nº / Probe name
PerkOlseni 18S F	ATGGCCGTTCTTAGTTGGTG	148	-3.3	HQ339918.1
PerkOlseni 18S R	GCCTCAAGCTTCCTTGTGTT			
Vphil 18S F	CCGAACATCTAAGGGCATCA	169	-3.0	EF426293.1
Vphil 18S R	AGTTGGTGGAGCGATTTGTC			
Vphil BigDef F	TATCGCTGTGTCTGGACCAA	126	-3.1	S_isotig19730_isogroup08197
Vphil BigDef R	TGCAGAAACCTGTACCACCA			
Vphil GTPase1 F	GGAGACGGAGACGGACATT	173	-3.2	P_isotig24006_isogroup12473
Vphil GTPase1 R	GTGCTGCAAGACAAGATTGC			
Vphil PteridinReduct F	AGAGGGAACTGCAGGAATGA	91	-3.4	P_isotig11915_isogroup02100
Vphil PteridinReduct R	AAGGCCACTATTTGGTGCTG			
Vphil TNF14 F	GCCATCAGCACAAGTCAATG	106	-3.4	P_FYX81E202F1YEW
Vphil TNF14 R	AATCAGGTCGGTCAGGATTG			

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Table 2: Top 25 expressed genes along the time course. Specific identifiers have been selected from the immune-enriched DNA microarray (Moreira et al., 2012).

SEQUENCE DESCRIPTION	5 DPI FC	10 DPI FC	14 DPI FC	31 DPI FC
fibropellin-3	276,41			
big defensin	111,23			
quinoid dihydropteridine reductase-like	32,23		4,63	15,5
ovomucoid (serine-type peptidase inhibitor)	26,55			
mechanosensory protein 2	20,81			6,31
60s acidic ribosomal protein p2	12,88	4,31		
neurofilament medium polypeptide	12,70			
vitelline membrane outer layer protein 1 homolog	11,44			
c1q tumor necrosis factor-related protein 2	7,52			
nuclear transcription factor y subunit beta	7,23			
tenascin-N	6,03			4,52
fatty acid hydroxylase domain containing 2	5,88			
probable tocopherol chloroplastic	4,68			
ubiquinol-cytochrome-c reductase complex core protein 1	4,11			
histone h2a	3,84			
pathogen-related protein FRED	3,32			
zinc finger protein	-13,57			
ependymin-related protein	-12,24			
sialic acid binding lectin	-8,71			
lipoxygenase homology domain-containing protein 1	-8,58			
elongation factor 2	-6,77			
tnf superfamily member 14	-6,61			
aldehyde dehydrogenase	-6,45			
26s proteasome non-atpase regulatory subunit 7	-5,95			-1,87
interferon-inducible GTPase 1	-4,38			

SEQUENCE DESCRIPTION	5 DPI FC	10 DPI FC	14 DPI FC	31 DPI FC
interferon-inducible GTPase 1		42,54	18,75	12,53
tena thi-4 family		24,83	10,19	
dolichyl-diphosphooligosaccharide-protein glycosyltransferase		13,18		
40s ribosomal protein s16		9,70		
ras gtpase-activating-like protein iqgap2		7,84		
tctex1 domain-containing protein 1		6,33	20,87	
max dimerization protein 1		5,81		5,40
c1q domain containing protein 1q51		5,21		4,92
sorbitol dehydrogenase		4,67		
v-type proton atpase subunit f 1		4,62		
calcium-binding EGF-like domain		4,52		
histone-binding protein Caf1		4,40		
contactin-tenascin		4,38		
60s acidic ribosomal protein p2	12,88	4,31		
tenascin-r		4,06		
venom protein 164		3,15		
short-chain collagen c4-like		-12,74	-5,16	
BTB/POZ domain-containing protein 6		-11,42	2,63	
40s ribosomal protein s18		-8,02		
nadh dehydrogenase iron-sulfur protein mitochondrial		-7,29		
collagen alpha-1 chain		-6,61	-5,85	
cub and sushi domain-containing protein 3 precursor		-4,99	-2.87	
chemokine-like receptor 1		-4,62	-2,61	-3,76
small heat shock protein	-5,04	-4,03		
78 kda glucose-regulated protein		-3,62		

SEQUENCE DESCRIPTION	5 DPI FC	10 DPI FC	14 DPI FC	31 DPI FC
beta-tubulin (P_isotig15436_isogroup03903)			42,50	
sialic acid binding lectin			21,33	
tctex1 domain-containing protein 1		6,33	20,87	
glycoside hydrolase family 18			19,71	
interferon-inducible GTPase 1		42,54	18,75	12,53
beta-tubulin (S_isotig25379_isogroup13846)			14,94	
sodium-dependent glutamate aspartate transporter 3			12,11	
allene oxide synthase-lipoxygenase protein			12,11	
beta-tubulin (P_isotig14697_isogroup03491)			10,23	
tena thi-4 family		24,83	10,19	
radial spoke head protein 4 homolog			8,06	3,83
glutathione s-transferase			6,55	
gtp-binding protein 6			6,46	
elongation factor 2			6,43	
aac-rich mrna			5,96	
neurocalcin-delta			5,80	
60s ribosomal protein l35			5,50	
tyrosinase-like protein			-10,06	-8,60
28s ribosomal protein mitochondrial			-9,92	
collagen alpha-1 chain		-6,61	-5,85	
short-chain collagen c4-like		-12,74	-5,16	
fmrfamide receptor	-3,84	-2,45	-4,45	
partial tbp gene for tata box binding isolate co69			-4,21	
nuclear receptor dax-1		-3,93	-4,15	

SEQUENCE DESCRIPTION	5 DPI FC	10 DPI FC	14 DPI FC	31 DPI FC
quinoid dihydropteridine reductase-like	32,23		4,63	15,15
interferon-inducible GTPase 1		42,54	18,75	12,53
ring finger protein 44				8,09
upf0667 protein c1orf55				7,37
sodium- and chloride-dependent creatine transporter 1				7,25
translation elongation factor 2				6,49
yn10a gene for endo- -beta				6,39
mechanosensory protein 2	20,81			6,31
zinc finger protein 207				5,41
max dimerization protein 1		5,81		5,40
c1q domain containing protein 1q51		5,21		4,92
elongation factor 2 (P_isotig16220_isogroup04687)				4,92
cadherin-related tumor suppressor				4,78
elongation factor 2 (P_isotig17914_isogroup06381)				4,73
c1q tumor necrosis factor-related protein 6				4,47
serine protease inhibitor cvsi-1				4,44
17-beta-hydroxysteroid dehydrogenase 14				4,33
cartilage matrix protein				-11,07
alpha macroglobulin				-9,18
tyrosinase-like protein tyr-3			-10,06	-8,60
blastula protease 10				-7,58
h aca ribonucleoprotein complex subunit 4				-7,50
sorting nexin-2				-7,33
prefoldin subunit 6				-6,83

710

711

712 **Table 3.** Selection of significant expressed genes at different times post infection.
713 Specific identifiers have been selected from the immune-enriched DNA microarray
714 (Moreira et al., 2012).

715

Recognition	FC 5d	FC 10d	FC 14d	FC 31d
complement c1q tumor necrosis factor-related protein 2	7.52			
galectin-3	1.65			
lactose-binding lectin l-2 (c-type lectin)	1.68			
hepatic lectin r2 3	-2.01			
collectin-12	-1.70			
ficolin-2	-1.8	-2.16	-1.9	
sialic acid-binding lectin (P_isotig09382_isogroup01034)	-8.71			
c1q domain containing protein 1q51 (P_GG3IVWD04IPQ8D)		5.2		4.91
sialic acid-binding lectin (P_isotig08863_isogroup00881)			21.33	
c1q domain containing protein 1q15 (P_isotig09706_isogroup01136)			3.23	
c-type lectin domain family 13 member			2.53	
complement component 1 q subcomponent-like 1			-1.78	
complement c1q tumor necrosis factor-related protein 6				4.47
complement c1q tumor necrosis factor-related protein 9b				3.41
sialic acid-binding lectin (N_isotig06599_isogroup00404)				3.00
c1q domain containing protein 1q79 (S_contig03303_isogroup00029)				2.09
complement c1q-like protein 3				2.01
complement c1q-like protein 4				1.92
c1q domain containing protein 1q79 (AS_GIDV13C02HM7P7)				1.58
tandem repeat galectin				-1.69
Histones	**FC 5d**	**FC 10d**	**FC 14d**	**FC 31d**
histone h2a	3.83			
histone h3	3.31			
histone h3-like protein	2.28			
histone-binding protein Caf1		4.39		
histone h1			1.68	
histone h1-like protein 6				-1.62

716

717

718

719 **Figure legends**

720 **Figure 1.** (A) Cumulative mortalities registered in healthy clams intramuscularly infected
721 with 100 µl of a solution containing $5x10^4$ trophozoites/ml. Results represent the mean ±
722 SEM of three independent infections. (B) qPCR quantification of 18S rRNA transcripts
723 from *P. olseni*. Results represent the mean ± SEM of four independent samples. Asterisks
724 indicate a significant difference ($p<0.05$) at day 5. (C) Trophozoites were present in the
725 mantle at day 14. (D) Trophozoites were present in the connective tissue at day 31. Scale
726 bar, 10 µm.

727

728 **Figure 2.** Validation of microarray data by qPCR of the selected genes big defensin,
729 interferon-inducible GTPase 1, dihydropteridine reductase and TNF superfamily member
730 14. Data were log2-transformed to facilitate the illustration of down-regulated genes.
731 Fold change units were calculated by dividing the normalised expression values in
732 infected clams by the normalised expression values in the controls. Each bar represents
733 the mean ± SEM of the four biological replicates. Significant results obtained in
734 microarray experiments are indicated in the boxes below.

735

736 **Figure 3.** (A) Distribution of the number of genes regulated throughout infection. The
737 number of significantly modulated genes is subdivided according to the intensity (fold
738 change) and direction of regulation (up or down). (B) Venn diagram of differentially
739 expressed genes in clam haemocytes at the four sampling times after *P. olseni* infection.

740

741 **Figure 4.** Enrichment analysis. Distribution of GO terms down-regulated between the test
742 set (significantly expressed genes for each sampling point) and the reference set (all of
743 the sequences present in the microarray). Only significant analyses are shown.

744

745 **Figure 5.** Enrichment analysis. Distribution of GO terms up-regulated between the test
746 set (significantly expressed genes for each sampling point) and the reference set (all of
747 the sequences present in the microarray). Only significant analyses are shown.

748

749 **Figure 6.** Percentage of transcripts included in the GO categories associated with cell
750 proliferation (A), metabolic processes (B) and apoptotic cell death (C) at the different
751 sampling points.

752

753 **Figure 7.** Apoptosis in histological samples was evaluated by TUNEL assay. Apoptotic
754 cells are stained green. In infected animals, a high number of apoptotic cells was detected
755 at day 5 in the mantle and gills. Apoptotic cells were mainly detected in connective
756 tissues between muscular fibres and the digestive apparatus at later sampling points (day
757 14 and 31). Scale bar, 50 μm.

758

759 **Supplementary Figure 1.** Heat map showing the evolution of differentially expressed
760 genes with fold changes values higher that 5 at the different sampling points

Figure 1

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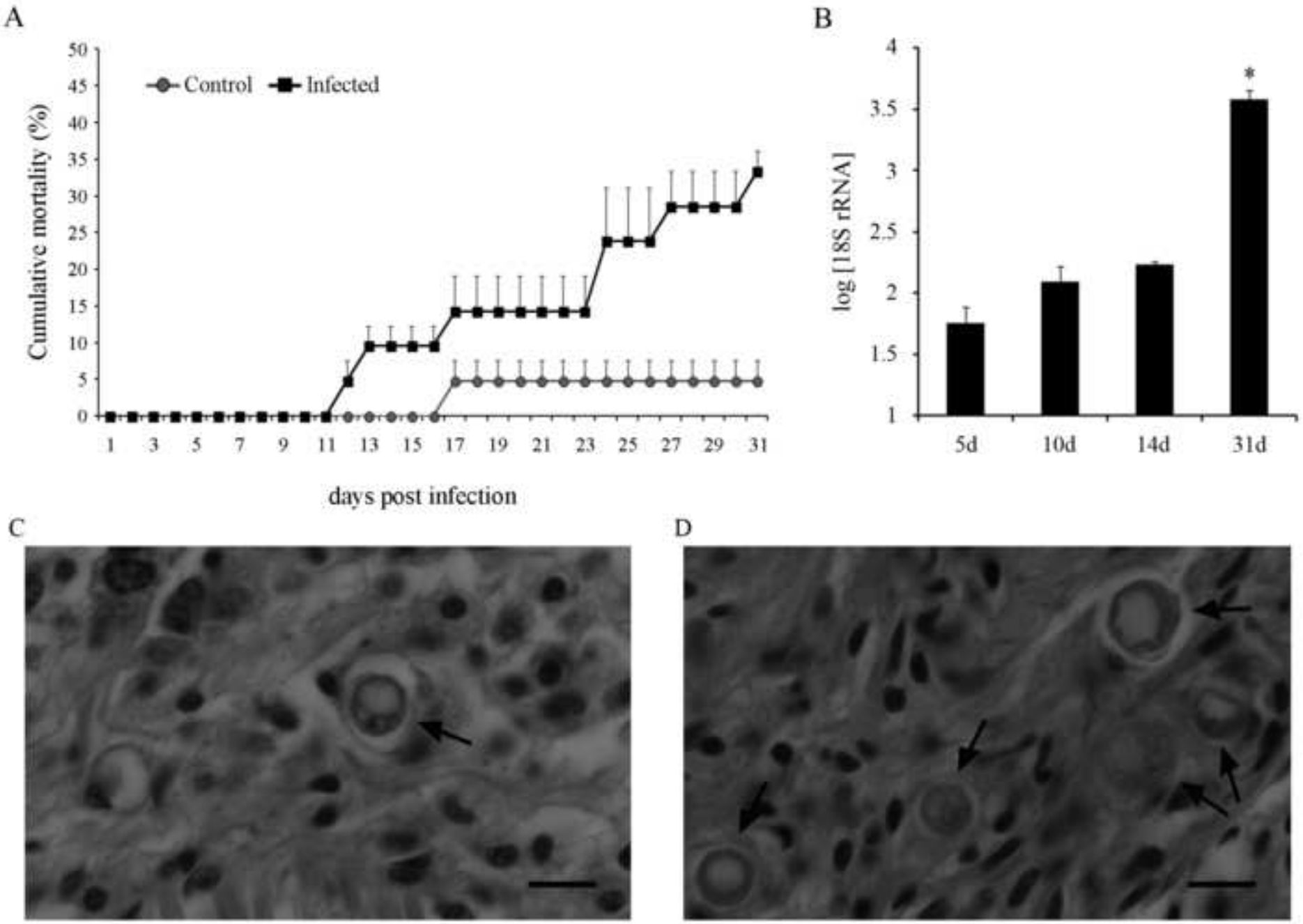

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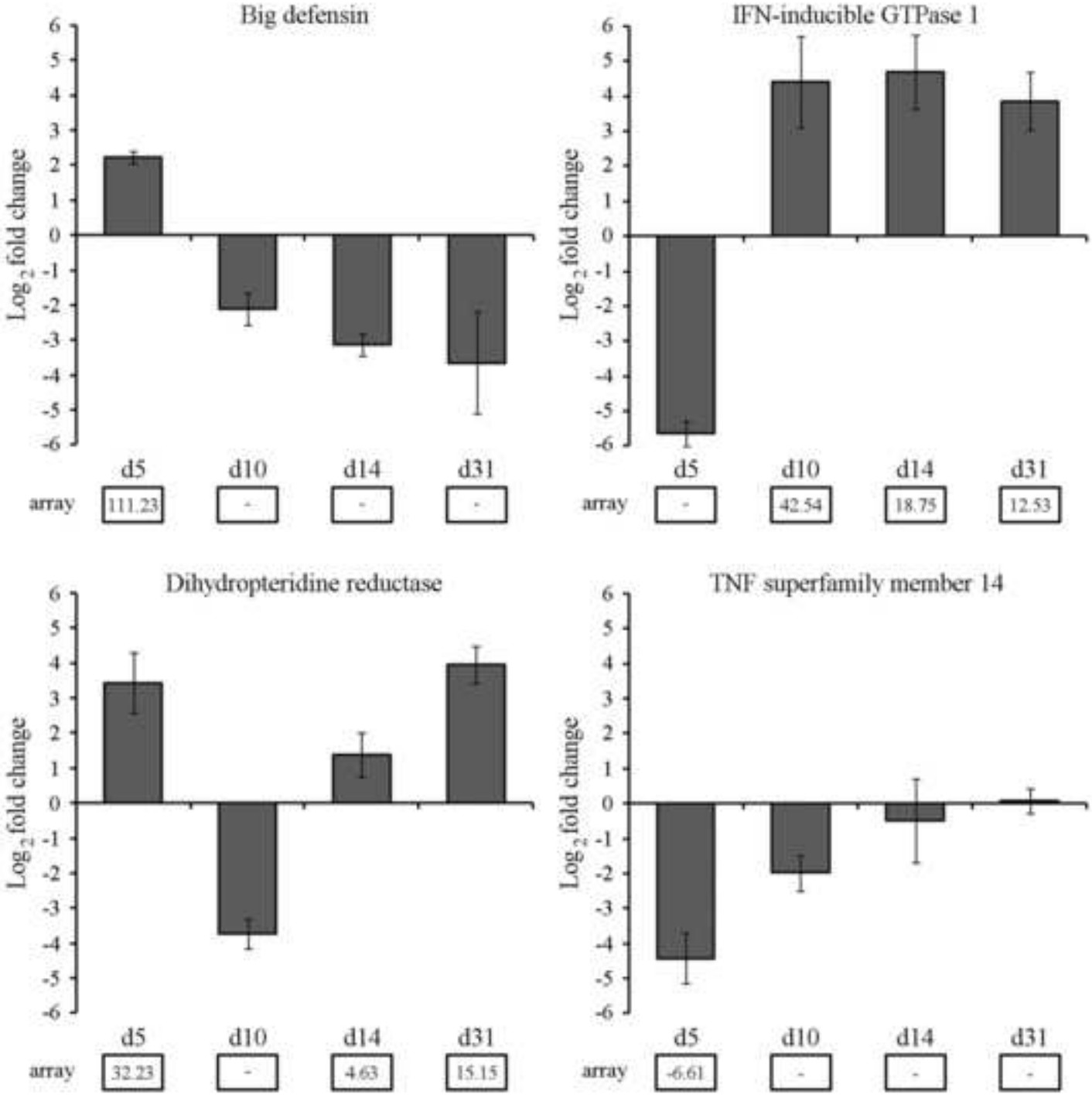

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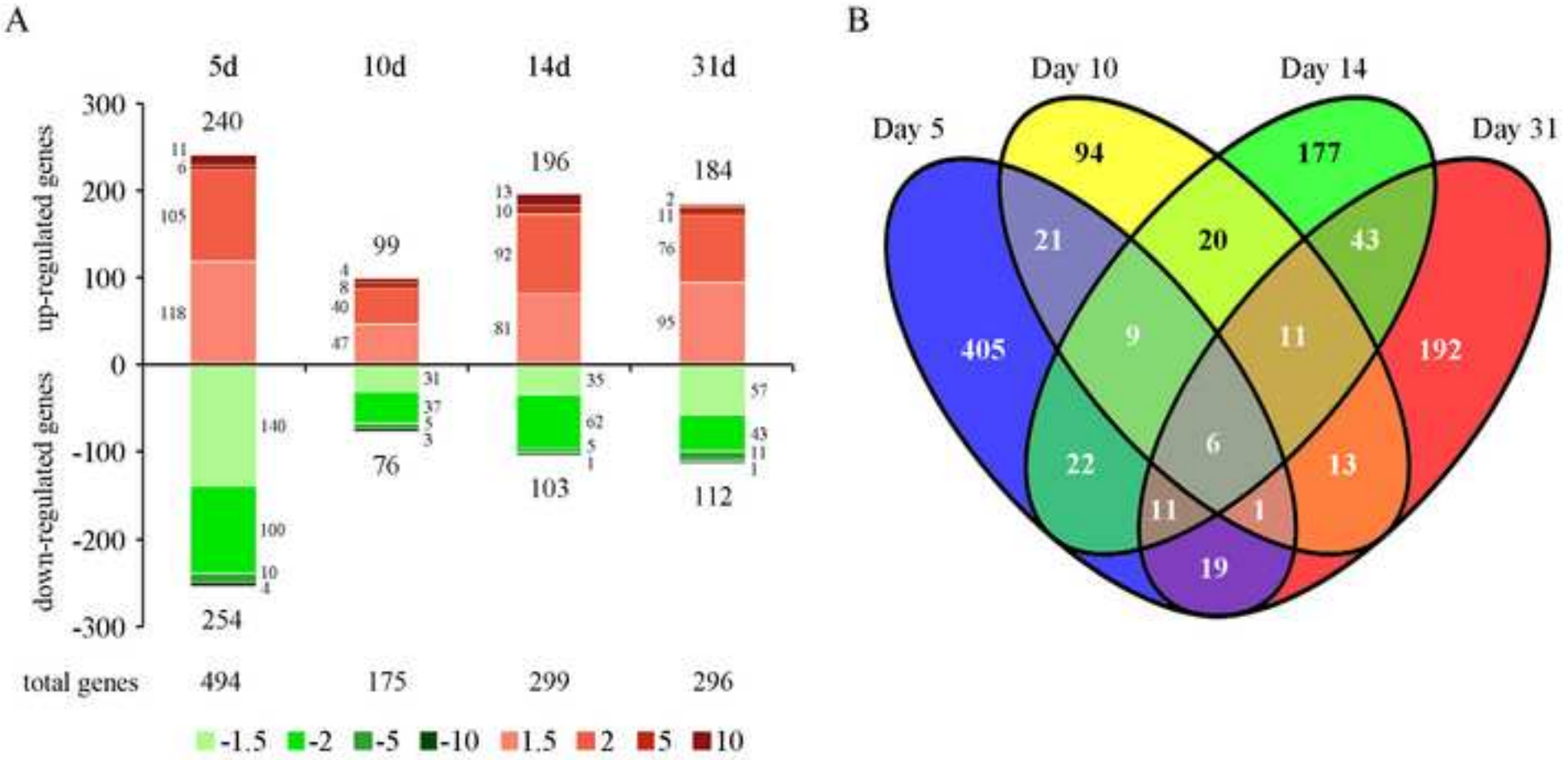

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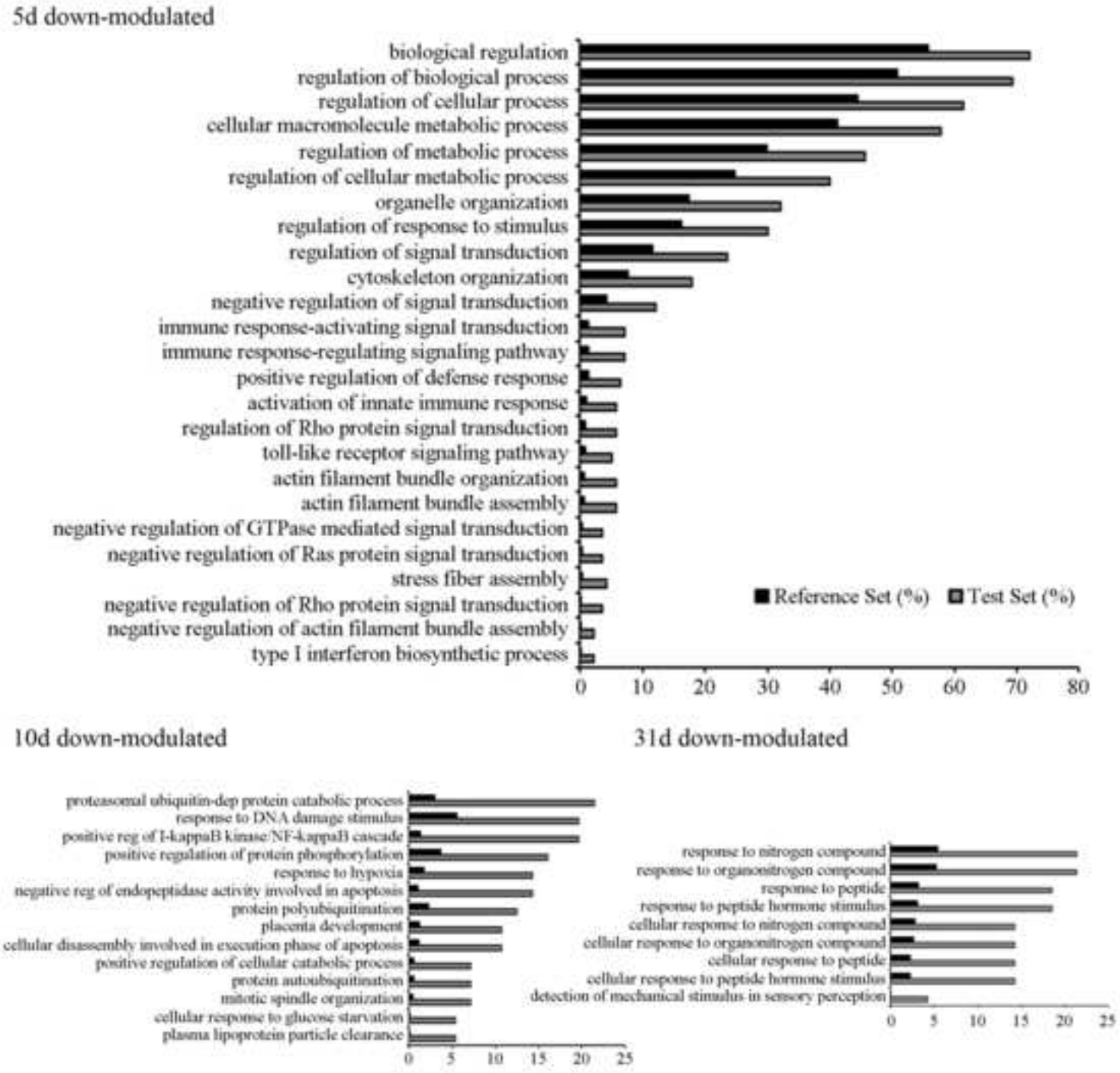

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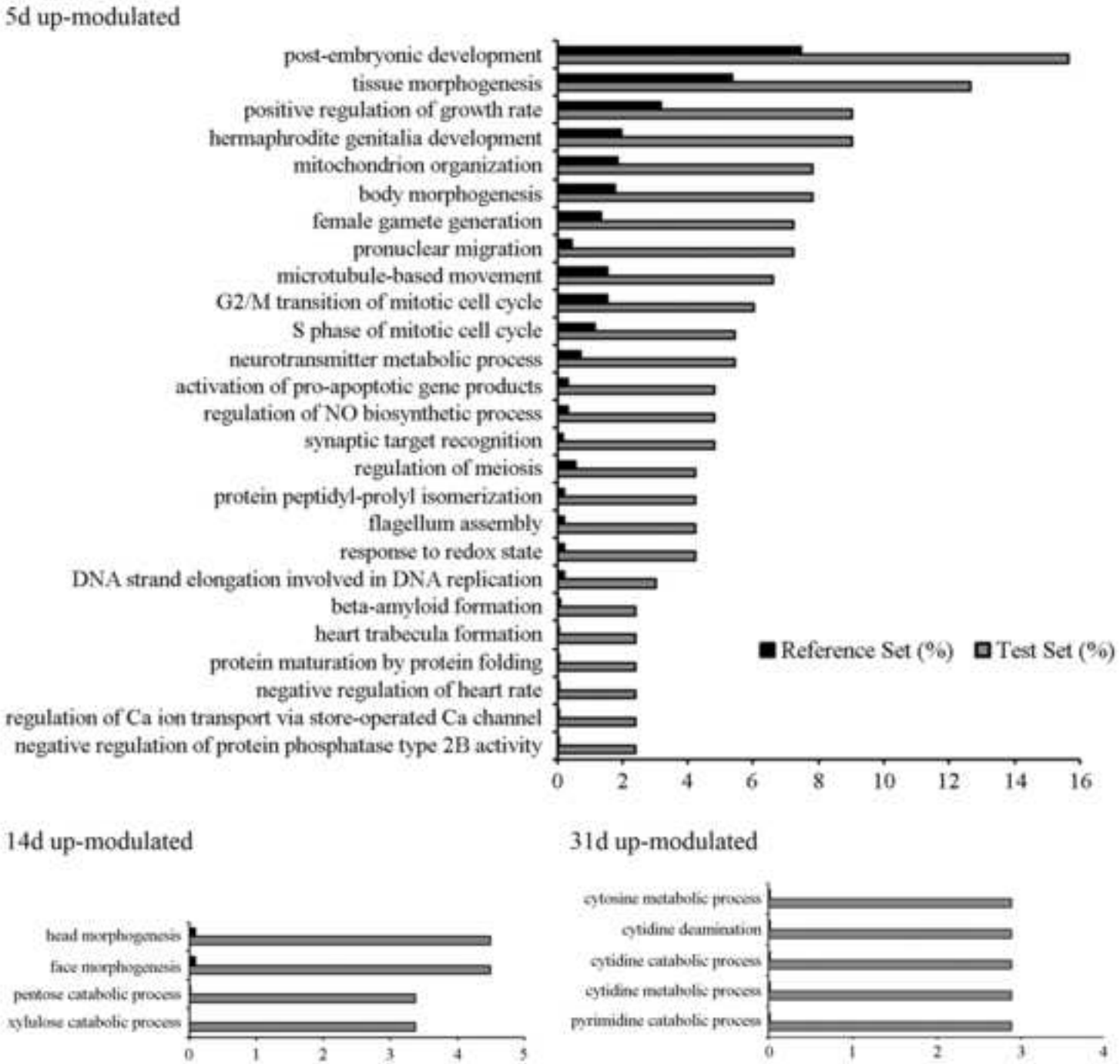

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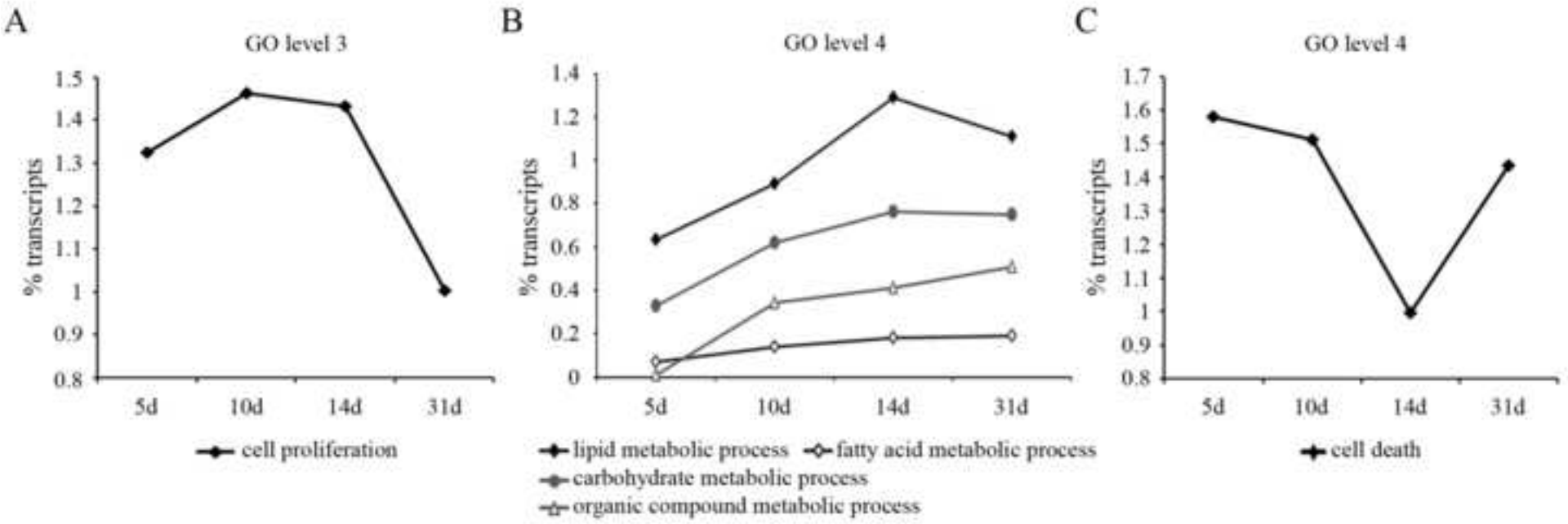

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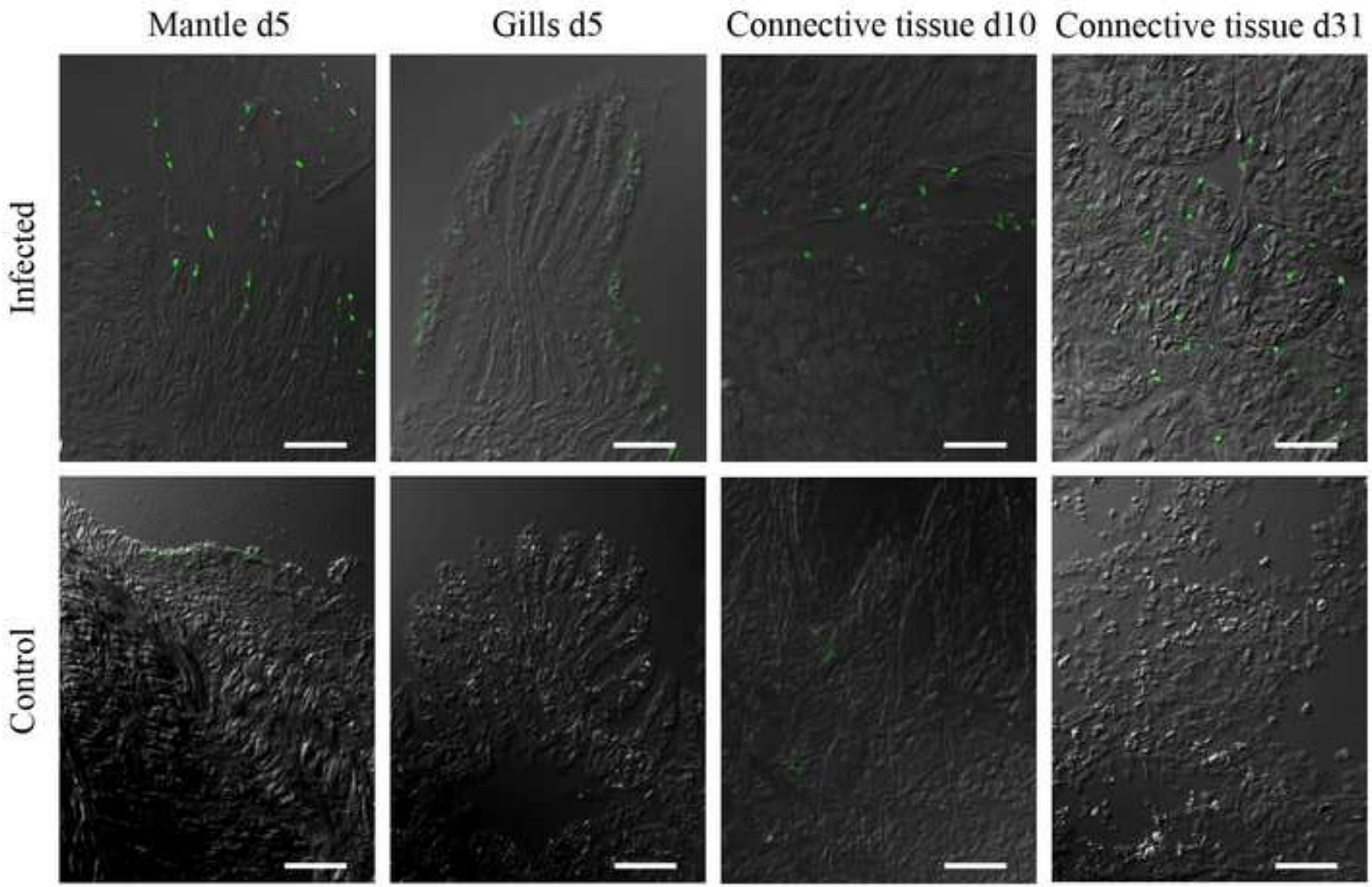

Supplementary Figure 1

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