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# Turbot (*Scophthalmus maximus*) Nk-lysin induces protection against the pathogenic parasite *Philasterides dicentrarchi* via membrane disruption

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ABSTRACT

P. dicentrarchi is one of the most threatening pathogens for turbot aquaculture. This protozoan ciliate is a causative agent of scuticociliatosis, which is a disease with important economic consequences for the sector. Neither vaccines nor therapeutic treatments are commercially available to combat this infection. Numerous antimicrobial peptides (AMPs) have demonstrated broad-spectrum activity against bacteria, viruses, fungi, parasites and even tumor cells; an example is Nk-lysin (Nkl), which is an AMP belonging to the saposin-like protein (SAPLIP) family with an ability to interact with biological membranes. Following the recent characterization of turbot Nkl, an expression plasmid encoding Nkl was constructed and an anti-Nkl polyclonal antibody was successfully tested. Using these tools, we demonstrated that although infection did not clearly affect nkl mRNA expression, it induced changes at the protein level. Turbot Nkl had the ability to inhibit proliferation of the *P. dicentrarchi* parasite both *in vivo* and *in vitro*. Moreover, a shortened peptide containing the active core of turbot Nkl (Nkl71-100) was synthesized and showed high antiparasitic activity with a direct effect on parasite viability that probably occurred via membrane disruption. Therefore, the *nkl* gene may be a good candidate for genetic breeding selection of fish, and either the encoded peptide or its shortened analog is a promising antiparasitic treatment in aquaculture.

## 1. Introduction

Scuticociliatosis is a parasitic disease caused by histiophagous ciliates of the order *Scuticociliatida*. This disease has been reported as an important emerging problem in aquaculture worldwide in recent decades. Although these ciliates are free-living members of marine ecosystems, they can transform into histiophagous parasites that affect a wide variety of farmed fish, including the olive flounder (*Paralichthys olivaceus*) [1], European sea bass (*Dicentrarchus labrax*) [2], southern bluefin tuna (*Thunnus maccoyii*) [3] and turbot (*Scophthalmus maximus*) [4–7]. These parasites can also affect other animal groups, including mollusks and crustaceans, leading to important commercial losses [8,9].

In Spain, scuticociliatosis outbreaks have been identified in the turbot aquaculture industry during the last two decades, with a concomitant increase in production costs due to mortality and morbidity episodes [2,6,10]. One of the main problems in diagnosing fish scuticociliatosis is identification of the pathogenic species causing the disease. However, studies conducted based on morphological and ultrastructural characteristics have identified *Philasterides dicentrarchi* as the scuticociliate responsible for the outbreaks in turbot farms in Spain [5,6,11].

The natural path of infection is not known, although it probably occurs through lesions in the gills and/or skin [12]. The parasite migrates via the bloodstream and connective tissues and feeds actively on host cells (principally erythrocytes) and tissue components; this severe systemic infection usually leads to death [6]. As the infection progresses, the ciliates increase in number and affect a wide variety of organs and tissues, thereby provoking more severe lesions and inflammatory reactions.

The lethal effects produced by *P. dicentrarchi* in turbot farms and the consequent economic losses have driven the development of

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chemotherapeutic agents to control scuticociliatosis. Synthetic antiprotozoals and natural polyphenols with antiparasitic properties, among other chemicals, have been evaluated *in vitro* [10,13–19]. However, no systemic chemotherapeutic treatments have been shown to be effective against parasites inside the fish [20]. Although several efforts have been undertaken to develop effective vaccines against this parasite [21–28], the ability of the antigen to evade host agglutination [21] and the presence of different isolates that coexist in the same farmed fish [29] have hindered the development of a universal vaccine. As a consequence, a deeper understanding of host immune defenses and the possible mechanisms of eradication of the parasite by fish cells must be acquired.

Several immune-related genes are upregulated after *P. dicentrarchi* stimulation [30], although no functional assays have been conducted with any of these genes. Nevertheless, Piazzon et al. [31] observed that resistance to *P. dicentrarchi* in turbot was more dependent on humoral than on cellular immune responses, which could also be the case for antimicrobial peptides (AMPs). For instance, Nk-lysin (Nkl), which is homologous to human granulysin, has shown direct or indirect effects against pathogenic bacteria [32–35], viruses [32,33,36], tumor cells [37,38] and parasites [39–41]. This protein is structurally related to saposin-like proteins (SAPLIPs), which are a family of lipid-binding proteins that are structurally conserved and possess membrane-perturbing abilities [42,43]. Moreover, Nkl displays amphipathic characteristics due to its 3D structure [44–46], which provides a positive net charge that seems to be required for its interactions with negatively charged biological membranes [46,47]. Nkl is mainly found in the cytolytic granules of cytotoxic T lymphocytes and natural killer cells [44]. Nevertheless, recently we observed that teleost Nkl was not only expressed in innate effector cells belonging to the lymphoid lineage but also in erythrocytes, demonstrating an important role in the autophagic mechanism of these cells [36].

In a previous work, we characterized the turbot *nkl* gene, successfully constructed an expression plasmid encoding Nkl and produced an anti-Nkl polyclonal antibody [36]. Due to previous evidence concerning the antiparasitic activity of porcine and chicken Nkl peptides against protozoan pathogens [39–41], we were interested in analyzing the potential activity of Nkl against *P. dicentrarchi*, which is the main parasite responsible for scuticociliatosis in turbot. Both the expression plasmid encoding the full-length Nkl (pMCV1.4-*nkl*) and a shortened synthetic peptide containing the potential core region of the turbot Nkl (Nkl71-100) were tested using *in vivo* and *in vitro* approaches. Our results suggest that Nkl can affect the viability of the ciliate, thereby reducing the parasite load and increasing the survival of experimentally infected turbot. Moreover, we have demonstrated through scanning electron microscopy (SEM) a direct effect of this molecule on physical destruction of the parasite. These findings provide new information about the immune mechanisms used by teleosts to eliminate this feared pathogen.

## 2. Materials and methods

### 2.1. Fish and ethical approval

Juvenile turbot *Scophthalmus maximus* with an average weight of 2.5 g were obtained from a commercial fish farm (Insuiña S.L., Galicia, Spain). The animals were maintained in 500-l fiberglass tanks with a recirculating saline water system (total salinity of approximately 35 g/l) and a light-dark cycle of 12:12 h at 18 °C. They were fed daily with a commercial dry diet (LARVIVA-BioMar, France). Prior to the experiments, the fish were acclimatized to the laboratory conditions for 2 weeks. Fish care and the challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number ES360570202001/16/FUN01/PAT.05/tipoE/BNG.

### 2.2. Parasite culture

The ciliates were maintained in our laboratory at 18 °C in complete sterile L15 medium (Leibovitz medium, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 2% penicillin/streptomycin solution (Invitrogen). Once the ciliates became accustomed to the new culture conditions, the antibiotic solution was removed to achieve higher ciliate activity. The virulence of the ciliates was maintained by periodic experimental turbot infections, in which a 50-µl volume of a ciliate suspension ($10^4$ parasites/ml) was administered via intraperitoneal injection every 3 months. When the first mortalities were recorded at 3 or 4 days post-infection, the ciliates were isolated from the peritoneal cavity following previously described indications [12].

### 2.3. Experimental infections

Several experimental infections were conducted *in vivo* with the aim of evaluating and studying different molecular and functional activities of Nkl, which will be described in the following sections. Turbot were intraperitoneally (i.p.) injected with a 50-µl suspension containing $10^4$ parasites/ml for the infection conditions, whereas the same volume of L15 medium + 10% FBS was used for the control animals. At 6, 24 or 72 h post-infection (hpi), the fish were sampled as indicated in the next section.

### 2.4. Evaluation of nkl gene expression after P. dicentrarchi challenge

To evaluate the effect of the ciliate on *nkl* transcription, gene modulation was evaluated by quantitative PCR (qPCR) in the head kidney, whole blood cells and peritoneal exudate cells (PECs) after *in vivo* infection with the parasite for 24 and 72 h. Blood and PECs were obtained as previously described [48]. Tissues were harvested under RNase-free conditions for each individual fish (n = 6), and total RNA was isolated using the Maxwell 16 LEV simplyRNA tissue kit (Promega) in accordance with the manufacturer's instructions. cDNA synthesis was performed with the NZY First-Strand cDNA Synthesis Kit (NZYtech) using 0.2 µg of total RNA. The qPCR reactions were performed using specific primers designed with the Primer 3 software [49], and their efficiencies were previously tested according to the protocol described by Pfaffl [50]. Individual qPCR reactions were conducted in 25-µl reaction volumes using 12.5 µl of SYBR GREEN PCR Master Mix (Applied Biosystems), 10.5 µl of ultrapure water (Sigma-Aldrich), 0.5 µl of each specific primer (10 µM) and 1 µl of cDNA template. All reactions were performed using technical triplicates in the 7300 Real-Time PCR System thermocycler (Applied Biosystems) with an initial denaturation step (95 °C, 10 min), followed by 40 cycles of a denaturation step (95 °C, 15 s) and one hybridization-elongation step (60 °C, 1 min). The relative *nkl* expression was calculated using the Pfaffl method [50]. *nkl* expression was normalized to *eukaryotic translation elongation factor 1 alpha* (*eef1a*) gene expression; this reference gene was not affected by the infection. Fold units were calculated by dividing the normalized expression values in the different samples by the normalized expression values obtained in the controls. The primer sequences for *nkl* were 5′-CATCTGGGAGTTAGTCGAGGAG-3′ and 5′-TTGGATAAAAGAGCAG GTGAGA-3′ and for *eef1a* were 5′-GGAGGCCAGCTCAAAGATGG-3′ and 5′-ACAGTTCCAATACCGCCGATTT-3’.

### 2.5. Flow cytometry

To assess the effect of parasite infection on the Nkl protein abundance and distribution, the blood, PECs and head kidney were sampled from a total of 8 fish per treatment (infected and control) at 24 and 72 hpi for flow cytometry analysis. Head kidney cells were obtained by passing the tissues through a 100-µm nylon mesh. Blood samples were centrifuged at 200 × g for 5 min to separate the plasma from the cells,

and the same procedure was performed with the PECs to recollect the cells. The obtained cells were fixed with 2% paraformaldehyde for 5 min at room temperature (RT) and washed with phosphate-buffered saline (PBS). The cells were resuspended in PBS, and 200 µl of cells/well were distributed in a 96-well flat-bottom plate. The plate was centrifuged at 200 × g for 5 min, and the supernatant was discarded. The cells were blocked and permeabilized by incubation in PBS with 0.1% saponin (Sigma) and 2% bovine serum albumin (BSA) (Sigma) for 1 h at 4 °C. Then, the plates were incubated overnight at 4 °C with the primary antibody (a rabbit anti-Nkl polyclonal antibody [36]) diluted 1:500 in staining buffer (PBS with 0.1% saponin and 1% BSA) or with an antibody negative control solution (pre-bleed serum). The cells were washed and incubated with an Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Molecular Probes-Life Technologies; 1:500) for 1 h at RT. The samples were washed twice and resuspended in PBS. Nkl expression was analyzed using the FACSCalibur flow cytometer (BD Biosciences) in dot plots based on relative size (forward light scatter, FSC) and complexity (side light scatter, SSC) using linear and logarithmic scales. FL1-H histograms were used to compare the fluorescence levels emitted by samples labeled with the anti-Nkl antibody and the pre-immune serum. The percentage of positive fluorescent events and the fluorescence intensity (average) were recorded.

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

The Nkl levels in the fish sera and peritoneal exudates (PEs) were determined by indirect ELISA. Animals were infected with *P. dicentrarchi* as described above. At 24 and 72 hpi, the sera and PEs were collected from a total of 9 turbot per treatment and sampling time point. Sera were obtained after allowing the blood to coagulate for 30 min at RT and removing the clot by centrifugation at 2000 × g for 10 min. The PE was obtained as explained above but was centrifuged at 4000 × g for 10 min to remove fish cells and parasites. A total of 50 µl per well of turbot serum or PE diluted 1:10 or 1:6, respectively, in 20 mM Tris-Cl (pH 4) was dispensed into 96-well flat-bottom high-binding plates (Costar) and incubated overnight at 37 °C. A 100-µl volume per well of an 8% non-fat dry milk solution was used to block the plates at RT for 4 h. Then, the plates were washed three times with distilled water. For detection of the peptide, 50 µl of a solution of the anti-Nkl polyclonal antibody diluted 1:5000 in ELISA buffer (0.5% BSA, 0.01% Tween 20, 0.005% phenol red and 10% PBS in distilled water; pH 7) was added to the wells, incubated for 2 h at RT and then washed three times with distilled water. The same volume and concentration of rabbit pre-bleed serum was used as a negative control. A 50-µl volume per well of goat anti-rabbit antibody labeled with horseradish peroxidase (HRP) (Sigma) diluted 1:500 in ELISA buffer was used as a secondary antibody for detection of the specific Nkl-primary antibody interaction after incubation for 45 min at RT. After three washes, 100 µl of 1 step Ultra TMB-ELISA (Thermo Scientific) was added to each well. The reaction was stopped with 2 N $H_2SO_4$, and the optical density was measured at 450 nm with a spectrophotometer (Labsystems iEMS Reader MF). The rabbit pre-bleed intensity signal was subtracted from the intensity of the signal obtained with the anti-Nkl antibody. These values were directly proportional to the amount of Nkl protein present in each well.

### 2.7. In vivo chemotaxis assay

To test the chemoattractant potential of *P. dicentrarchi*, turbot were i.p. injected with the parasite or culture medium, and PECs were collected from 8 turbot per treatment and sampling time point at 6, 24 and 72 hpi. For this step, the fish were i.p. inoculated with 100 µl of sterile PBS, and the cell suspensions were recovered after a brief abdominal massage. The numbers of leukocytes present in the peritoneum were determined by counting the cells in a Neubauer chamber under an optical microscope.

### 2.8. nkl expression plasmid (pMCV1.4-nkl)

The pMCV1.4-*nkl* expression vector encoding the turbot Nkl mature peptide and the corresponding empty plasmid (pMCV1.4) were produced as previously described [36]. These plasmids were transfected into the human cell line HEK-293 (ATCC CRL-1573) to obtain conditioned supernatants (SNs) with or without Nkl for the *in vitro* and *in vivo* experiments. To accomplish this goal, HEK-293 cell monolayers were grown in 25-$cm^2$ culture flasks at 37 °C in Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% FBS, 100 µg/ml of primocin (InvivoGen), 1× non-essential amino acids (Gibco), and 1 mM sodium pyruvate (Gibco). When the cell cultures reached 70–80% confluence, recombinant or empty plasmids were transfected into cells using the XtremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions. Briefly, plasmids (recombinant or empty) at a final concentration of 6 µg complexed with a final volume of 40 µl of Transfection Reagent (Roche) were incubated for 15 min in 600 µl of Opti-MEM (Gibco) and primocin (InvivoGen) and then added to each flask in 5400 µl of culture medium with 10% FBS. The flasks were incubated at 37 °C for 48 h. SNs from both transfections were collected, passed through a 0.22-µm filter and stored at −80 °C prior to use. Correct protein production was previously confirmed by western blotting [36].

### 2.9. Nkl shortened synthetic peptide (Nkl71-100)

The potential core region of the turbot Nkl was identified based on data previously described for other species in which the active core of Nkl was identified as a small part of the full protein sequence. Several Nkl core regions from other teleosts were aligned using the ClustalW server [51], and the turbot Nkl shortened peptide was selected by maintaining a region centered around two Cys residues separated by nine amino acids and the respective N- and C-terminal regions that maintained the conserved positions of the basic amino acids. The 3D structure was predicted with the PEP-FOLD 2.0 web application at http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD [52]. The synthetic peptide (Nkl71-100) was synthetized by New England Peptide (Gardner, MA, USA) with a purity grade > 95%.

### 2.10. Antiparasitic activity of Nkl and its shortened analog (Nkl71-100)

To analyze the direct effect of Nkl on ciliate viability, several *in vitro* and *in vivo* experiments were conducted to test the effects of both the full recombinant protein and the synthetic peptide Nkl71-100.

For the *in vitro* assays, ciliates in the late exponential or early plateau phase of growth were concentrated by centrifugation at 650 × g for 5 min and then resuspended in PBS. A subsample of the parasite suspension was fixed in 2% paraformaldehyde for 5 min at RT and then washed and resuspended in PBS. After counting in a Neubauer chamber, a 10-µl volume containing a total of $10^4$ ciliates was added to each well of 96-well flat-bottom microtiter plates containing 90 µl of different concentrations of the Nkl71-100 peptide (ranging between 8 and 0.007813 µM) diluted in L15 + 10% FBS + 2% penicillin/streptomycin. Each concentration was assayed in triplicate. To eliminate the potential toxic effect of the synthetic peptide, two additional peptides corresponding to other regions of the turbot Nkl were used as a control. These peptides (RSLEINIDDQEQVC and CLFYPKQEESQTE) were tested together at a concentration of 4 µM each (final concentration 8 µM; Nkl71-100 at 0 µM). The parasites were fixed, and the number of viable ciliates was counted in a Neubauer chamber at 24 h. The viability was determined based on the cell morphology compared with that of the untreated cells.

The viability of the parasites in the presence of SN from transfected HEK-293 cells actively expressing the full-length Nkl protein was also assessed *in vitro*. The same concentration of ciliates was dispensed into

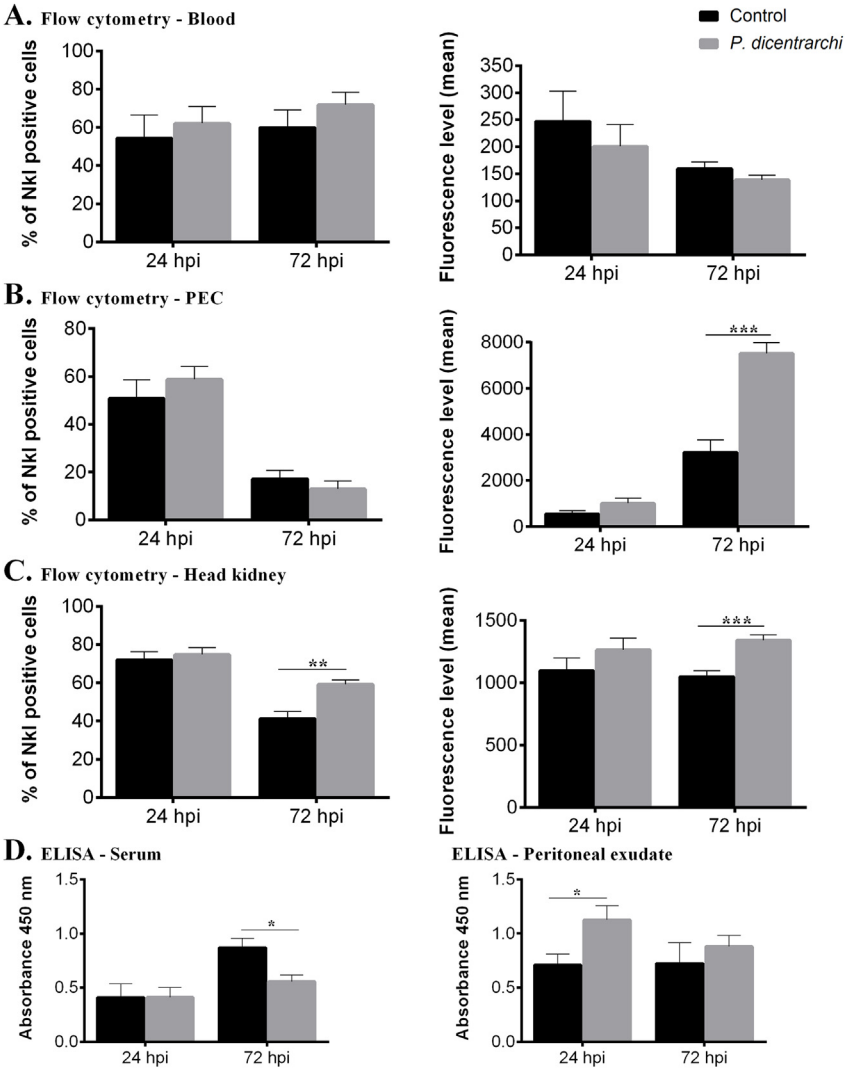


**Fig. 1.** Modulation of the turbot Nkl protein during *Philasterides dicentrarchi* challenge in the blood (**A**), PECs (**B**) and head kidney (**C**) at 24 and 72 hpi as determined by flow cytometry. The percentages of Nkl-positive cells and the mean fluorescence intensity values in Nkl-positive cells are represented. The results are expressed as the mean + SEM (n = 8). (**D**) The Nkl level was measured in turbot sera and peritoneal exudates from control and *P. dicentrarchi*-infected individuals by indirect ELISA at 24 and 72 hpi. The results are represented as the mean values of the absorbance at 450 nm + SEM (n = 9). Significant differences are displayed as *** (0.0001 < p < 0.001), ** (0.001 < p < 0.01) or *(0.01 < p < 0.05).

the plates and incubated with 90 µl of SN from HEK-293 cells transfected with pMCV1.4-*nkl* or empty plasmid and diluted 1:2 in parasite culture medium. Each treatment was assayed in triplicate and compared with the positive growth control wells. For both the short peptide and the supernatant assays, the viability of the parasites after *in vitro* incubation for 24, 48, 72 and 96 h was examined as explained above.

The putative antiparasitic activities of the Nkl71-100 peptide and the full recombinant protein were also analyzed *in vivo*. A 50-µl volume of the synthetic peptide (8 µM) or 50 µl of SN from transfected HEK-293 cells (with pMCV1.4-*nkl* or the empty plasmid) was intraperitoneally injected in the presence or absence of *P. dicentrarchi*. To monitor mortality, a total of 3 biological replicates (10 turbot/replicate) was used for each experimental condition. For the experiment with Nkl71-100, the parasite load in the peritoneal cavity was determined in individuals who were dying at 4 days post-challenge as described for cell counting in section 2.7.

In addition, intramuscular (i.m.) injection of the Nkl expression plasmid was conducted in the caudal peduncle, in which 50 µl of PBS, the control empty plasmid (pMCV1.4) or the expression plasmid (pMCV1.4-*nkl*) was injected (50 ng/µl in PBS), and the turbot were i.p. infected with *P. dicentrarchi* ($10^4$ ciliates/ml) after 48 h. To check the correct replication of the plasmid, five turbot from each treatment were sacrificed, and the muscle (site of injection) was sampled after 2 days. *nkl* transcription was analyzed by qPCR as described above. To monitor mortality, a total of 3 biological replicates (10 turbot/replicate) was used for each experimental condition. After infection, mortalities were recorded daily over a 12-day period.

### 2.11. Scanning electron microscopy (SEM)

To visualize the direct and physical effect of Nkl71-100 on parasite structure, a suspension of ciliates ($10^4$ parasites/ml) in PBS and Nkl71-100 at a final concentration of 8 µM were incubated at 18 °C for 72 h. The ciliates were collected by centrifugation at 1000 × g for 5 min. The cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 at 4 °C for 2 h, pelleted by centrifugation (1000 × g for 5 min), and resuspended again in fixative at 4 °C for 2 h. Finally, the parasites were washed several times with 0.1 M cacodylate buffer. The samples were conserved at 4 °C prior to SEM examination using a Philips XL30 (Electron Microscopy Unit of CACTI, University of Vigo, Spain).

### 2.12. Statistical analysis

Kaplan-Meier survival curves were analyzed with a log-rank (Mantel-Cox) test. For the remaining experiments, the results are represented graphically as the mean + standard error of mean (SEM). Significant differences were obtained using a *t*-test and displayed as *** (0.0001 < p < 0.001), ** (0.001 < p < 0.01) or *(0.01 < p < 0.05).

## 3. Results

### 3.1. P. dicentrarchi does not induce nkl gene expression

To determine the effect of *P. dicentrarchi* on transcription of the turbot *nkl* gene, the gene expression levels in blood, PECs and head kidneys from infected and non-infected turbot were analyzed at 24 and 72 hpi. No significant differences in the *nkl* expression levels were found between the control and parasite-infected samples (Supplementary Figure 1).

### 3.2. P. dicentrarchi infection affects the Nkl protein distribution

The percentage of Nkl-positive cells and their mean fluorescence levels were evaluated in blood, PECs, and head kidney cells from healthy and *P. dicentrarchi*-infected turbot by flow cytometry (Fig. 1A–C). At 24 hpi, no differences between the infected and non-infected (control) turbot were observed for any of the analyzed samples. The percentages of PECs and blood cells positive for Nkl were not affected by the infection, but the mean fluorescence intensity was significantly elevated in the PECs (Fig. 1A and B). However, at 72 hpi, the head kidney showed an increase in the percentage of Nkl-positive cells and in the mean fluorescence value (Fig. 1C).

When an indirect ELISA was used to determine the Nkl levels in sera from infected and non-infected turbot, no significant differences were observed at 24 hpi, but a lower Nkl protein concentration was detected in the infected fish at 72 h (Fig. 1D). In contrast, a higher Nkl level was observed at 24 hpi in the PE samples from the infected individuals than in those from the control fish, but these differences disappeared after 72 h (Fig. 1D).

### 3.3. P. dicentrarchi induces leukocyte migration to the peritoneum

Because the percentage of Nkl-positive PECs was not affected by *P. dicentrarchi* intraperitoneal challenge, we analyzed leukocyte migration to the peritoneal cavity after infection. At the tested sampling time points (6, 24 and 72 hpi), an increase in the number of leukocytes was observed after infection (Fig. 2); these differences were significant at 24 hpi, indicating strong chemoattractant activity.

### 3.4. Nkl has activity against P. dicentrarchi

After transfecting HEK-293 cells with the Nkl expression plasmid (pMCV1.4-*nkl*) and the corresponding control empty plasmid (pMCV1.4), the conditioned SNs were collected to test their *in vitro* and *in vivo* activities.

For the *in vitro* experiments, no significant differences were observed in parasite growth and viability at 24, 48 and 72 h post-treatment with SN containing Nkl (Fig. 3A). Nevertheless, after 96 h, the number of viable ciliates was significantly lower than that of the parasites treated with SN from HEK-293 cells transfected with the empty plasmid (Fig. 3A).

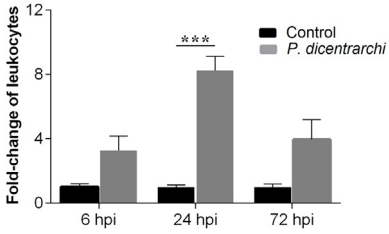


**Fig. 2.** Fold-change of the number of leukocytes in the peritoneal cavity of turbot at 6, 24 and 72 hpi with *P. dicentrarchi* compared to the controls. The results are represented as the mean + SEM (n = 6). Significant differences are displayed as *** (0.0001 < p < 0.001), ** (0.001 < p < 0.01) or *(0.01 < p < 0.05).

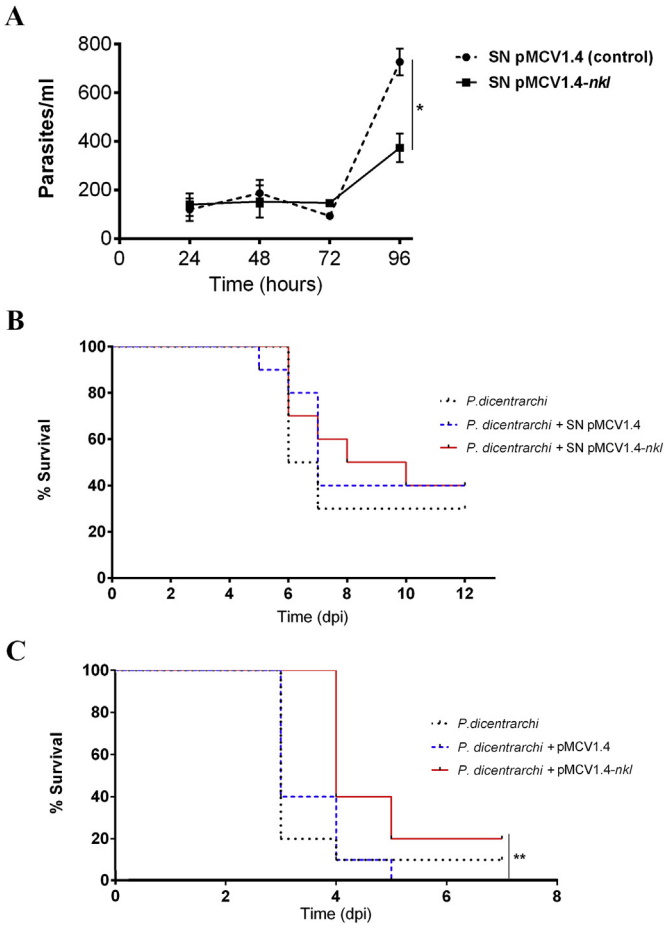


**Fig. 3.** Antiparasitic activity of the turbot Nkl peptide. (**A**) *In vitro* effect of the Nkl-enriched SN or the corresponding controls measured during 4 consecutive days. Data are represented as the mean number of *P. dicentrarchi* counted in the Neubauer chamber ± SEM (n = 3). (**B**) Kaplan-Meier survival graph representing the *in vivo* effect of the Nkl-enriched SN during *P. dicentrarchi* infection (n = 30). Mortality was recorded over a 12-day period. No significant differences were observed between the Nkl-treated fish and the corresponding controls. (**C**) Kaplan-Meier survival graph representing the *in vivo* effect of intramuscular injection of the Nkl expression plasmid (pMCV1.4-*nkl*) compared with the survival of the corresponding controls during *P. dicentrarchi* infection (n = 30). Mortality was recorded over a 7-day period. A slight but significant increase in the survival rate was observed in turbot previously inoculated with pMCV1.4-*nkl*. Significant differences are displayed as *** (0.0001 < p < 0.001), ** (0.001 < p < 0.01) or *(0.01 < p < 0.05).

In contrast, i.p. infection of turbot with the ciliate in the presence of SNs from HEK-293 cells did not produce significant differences in the survival rate, although a certain delay in mortality was observed in the fish that received Nkl (Fig. 3B). Nevertheless, the effect of i.m. injection of the expression plasmid encoding Nkl was further investigated. Correct replication of the *nkl* gene in muscular cells was successfully confirmed at 2 days post-injection, and a fold-change of 100 was obtained compared with expression in the fish inoculated with PBS or empty plasmid (Supplementary Figure 2). In this case, a significant increase in the survival of turbot previously inoculated with the plasmid encoding Nkl was observed (Fig. 3C). At the end of this experiment, the survival rate of the turbot i.m. injected with the empty plasmid was 0%, whereas 20% survival was observed for the pMCV1.4-*nkl* injected fish. No mortality events were observed in the control groups in any of the *in vivo* experiments (non-treated and non-infected fish, data not shown).

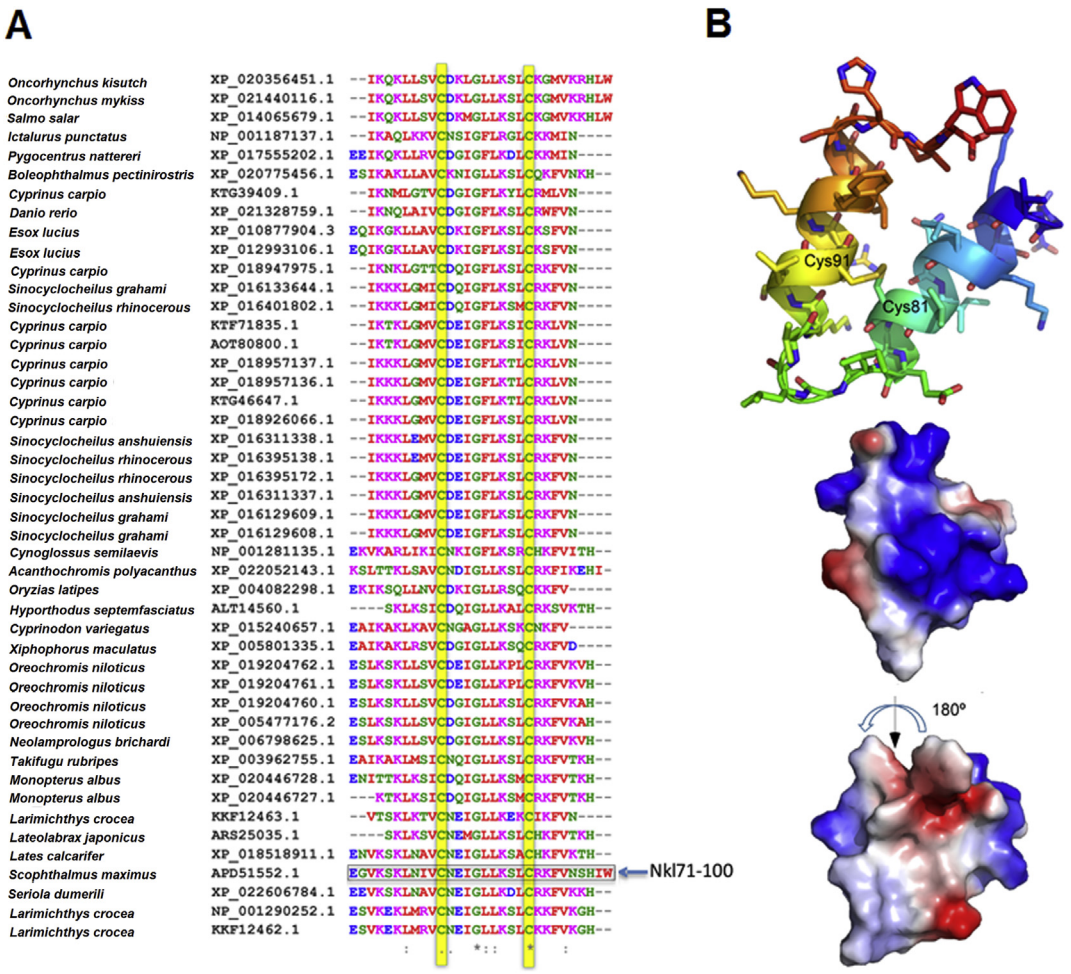


**Fig. 4.** Characteristics of the synthetic shortened peptide Nkl71-100. (**A**) Multiple alignment of vertebrate NK-lysine peptides focused on their predicted active core, including the turbot Nkl. In each sequence, two Cys residues are marked with a yellow box at a distance of 9 amino acids. (**B**) Predicted secondary and tertiary structures of the Nkl71-100 peptide (upper) and electrostatic surface potential showing two faces (middle and lower) of the folded peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### *3.5. Shortened Nkl peptide containing the active core (Nkl71-100)*

The potential core region of the turbot Nkl was predicted by aligning the protein sequence with sequences from other teleosts with a recognized active region (Fig. 4A). The predicted short peptide was composed of 30 amino acids and corresponded to the residues from position 71 to 100, both of which were included (EGVKSKLNIVCNEIGLLKSLCRKFVNSHIW), and had a molecular weight of 3429.10 Da. This peptide presented two well-conserved cysteines (residues 11 and 21), and the predicted 3D-structure revealed the formation of a disulfide bond and an alpha-helical structure (Fig. 4B). The peptide was dissolved in PBS to a final stock concentration of 60 µM for the experiments.

### *3.6. In vitro and in vivo antiparasitic activity of Nkl71-100*

To determine the optimal working dose of the Nkl71-100 synthetic peptide, an *in vitro* dose-response experiment was conducted using 1:2 serial dilutions ranging from 8 to 0.007813 µM. After 24 h of incubation with *P. dicentrarchi*, a significant reduction in parasite survival was observed for the higher doses (8, 4 and 2 µM) (Fig. 5A). A time course *in vitro* experiment was conducted to evaluate parasite growth using the 4- and 8-µM peptide concentrations. Treatment with 4 µM Nkl71-100 significantly reduced the number of ciliates in culture (from 2200 ± 115 parasites/ml to 1067 ± 291 parasites/ml at 96 h post-treatment), and treatment with 8 µM Nkl71-100 almost completely eliminated the parasites (from 2200 ± 115 parasites/ml to 67 ± 67 parasites/ml at 96 h post-treatment) (Fig. 5B).

To examine the *in vivo* antiparasitic potential of Nkl71-100, turbot were simultaneously i.p. injected with 8 µM of the short peptide and a suspension of $10^4$ parasites/ml. The results showed a significant increase (approximately 30%) in the survival of individuals treated with the synthetic peptide compared with that of the non-treated controls (only infected) (Fig. 5C). To corroborate this antiparasitic effect, the parasite load was determined in the peritoneum of the infected turbot. A significant reduction of the parasite load was noted in the individuals treated with the Nkl71-100 peptide compared with the load of the infected controls (Fig. 5D).

### *3.7. Nkl71-100 induces parasite disruption*

The effect of Nkl71-100 on the cell structure of *P. dicentrarchi* was investigated by SEM. A ciliate suspension was incubated for 72 h with the peptide (8 µM), and the structure was compared between the treated and untreated parasites. Fig. 6 shows different images of the control (Fig. 6A and B) and treated parasites (Fig. 6C–E). The images verified that the Nkl71-100 treatment produced swelling of the parasites, retraction of the cilia, and tearing of the surface (Fig. 6C) and was even able to completely destroy the parasites (Fig. 6D). Therefore, the Nkl71-100 peptide clearly damaged the membrane of *P. dicentrarchi*,

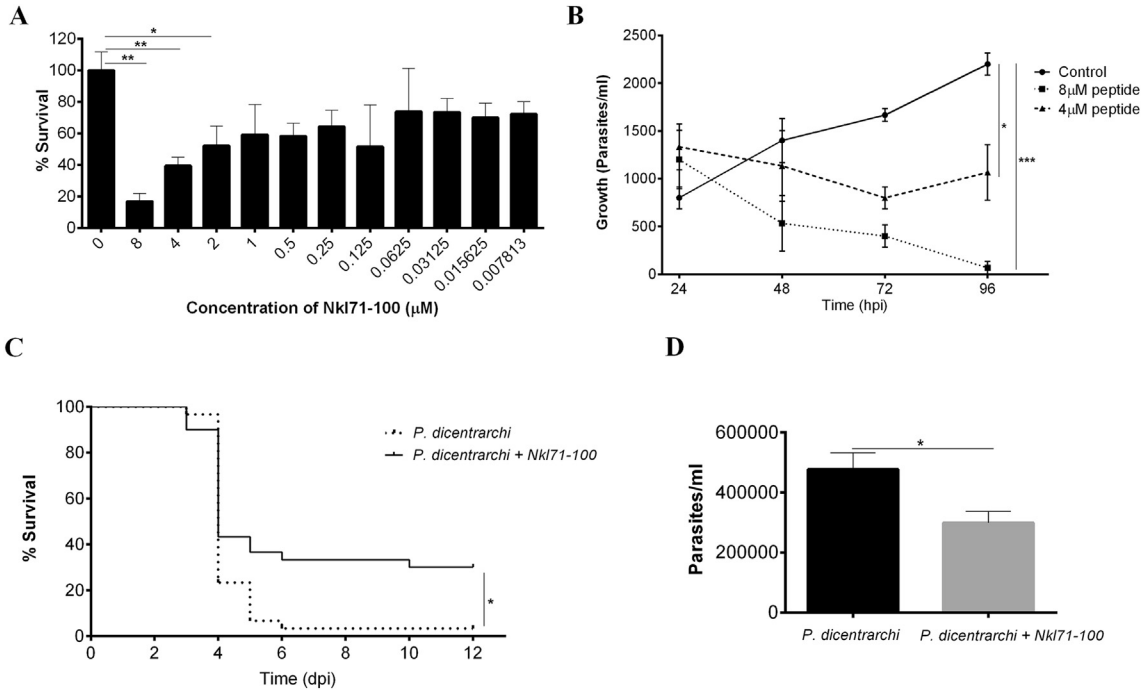


**Fig. 5.** Antiparasitic activity of the Nkl71-100 synthetic peptide. (**A**) *In vitro* dose response of Nkl71-100 against *P. dicentrarchi* over a 24-h period. The data represent the mean percentage of surviving parasites compared with that of the control (untreated parasites) +SEM (n = 3). (**B**) *In vitro* time course of the antiparasitic effect of Nkl71-100 (4 and 8 μM) against *P. dicentrarchi* over a 4-day period. Data represent the mean concentration of parasites + SEM (n = 3). The starting concentration of *P. dicentrarchi* was 1000 parasites/ml. (**C**) Kaplan-Meier survival graph representing the *in vivo* antiparasitic effect of Nkl71-100 (8 μM) during *P. dicentrarchi* infection (n = 30). Mortality was recorded over a 12-day period. (**D**) The parasite load in the peritoneum of turbot infected with *P. dicentrarchi* in the absence or presence of Nkl71-100 at 4 days post-challenge. The results are presented as the mean concentration of parasites + SEM (n = 12 for Nkl71-100–treated fish; n = 20 for untreated fish). Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or *($0.01 < p < 0.05$).

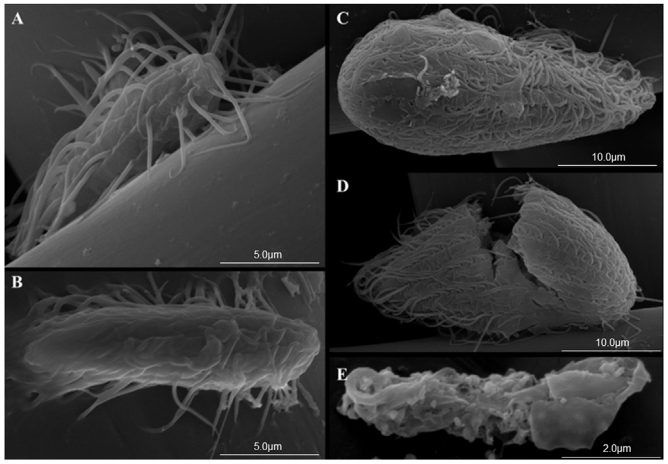


**Fig. 6.** Scanning electron microscopy (SEM) images of *P. dicentrarchi* parasites incubated with parasite culture medium (**A**, **B**) or Nkl71-100 (8 μM) (**C**, **D**, **E**) over a 72-h period.

ultimately leading to cell death and the appearance of empty membranes (ghost cells) (Fig. 6E).

## 4. Discussion

*P. dicentrarchi* is one of the main pathogens affecting turbot culture and usually is associated with high mortality rates and important economic losses. Neither chemotherapeutic treatments nor vaccines have provided successful results against this ciliate to date [53]. To solve this issue, important efforts have been undertaken in recent years to gain a better understanding of host-pathogen interactions and to search for effector molecules and attempt to enhance their action [30,54–56].

Piazzon et al. [31] observed that resistance to *P. dicentrarchi* in turbot was more dependent on humoral than on cellular immune responses, suggesting an important role for complement and nitrogen and oxygen reactive species. The role of humoral factors could be even broader, and other components, such as AMPs, could also be involved.

Although Nkl and its human homolog granulysin have been reported to have antimicrobial, antiviral, antifungal and antitumor properties [44,57–65], few studies have focused on their effects against parasite infections. Granulysin or Nkl has been shown to be effective against intracellular parasites, such as *Trypanosoma cruzi* [39,66], *Toxoplasma gondii, Leishmania major* [66], *Plasmodium falciparum* [40] and *Eimeria* spp [67,68].

In addition to *S. maximus* [36], *nkl* genes have been identified in several fish species, including *Ictalurus punctatus* [34,69], *Paralichthys olivaceus* [70], *Cynoglossus semilaevis* [32], *Danio rerio* [71], *Larimichthys crocea* [72], *Oreochromis niloticus* [73] and *Cyprinus carpio* [35]. Nevertheless, only one publication has reported modulation of *nkl* in fish after a parasitic infection. In that work, Zhou et al. [72] observed that infection of *L. crocea* with the external parasite *Cryptocaryon irritans* decreased *nkl* expression in the head kidney. In the current work, we evaluated modulation of *nkl* expression after *P. dicentrarchi* infection in the blood, PECs and head kidney, but our results did not reveal any significant modulation. Nevertheless, the two infections are not comparable, because *C. irritans* is an external parasite and *P. dicentrarchi* affects the internal tissues of the fish.

This absence of *nkl* gene modulation in turbot may be explained by storage of Nkl together with perforins and granzymes in the cytolytic granules of cytotoxic T lymphocytes and NK cells and their release when action is required [44]. Therefore, variations at the protein level could be detected even in the absence of gene modulation. Indeed, we found significant differences between infected and control turbot at 72 hpi in the number of Nkl-positive cells in the head kidney and in the mean fluorescence intensity in both the head kidney and PECs.

Nkl protein synthesis has been classically ascribed to effector cells belonging to the lymphoid lineage, such as T lymphocytes and natural killer cells [44]. Recently, we demonstrated that teleost erythrocytes also produced Nkl [36]. Red and white cells are produced by hematopoietic stem and progenitor cells, and this process is known to occur in the head kidney of fish [74,75]. These phenomena could explain the increased numbers of Nkl-positive cells in this tissue after parasite challenge. After infection, more cells would be required to control the pathogen, explaining their migration to the site of infection. In experimental i.p. infections, most parasites accumulate in the peritoneal cavity, where effector cells should be required. However, no differences were observed in the numbers of Nkl-positive PEC cells. When the number of leukocytes in the peritoneum was analyzed at different time points post-infection, the number of total leukocytes was significantly higher in the fish infected with *P. dicentrarchi*. Although leukocytes are ingested by the parasite, based on the present results we deduced that the migration of leukocytes to the peritoneum had to be dramatically enhanced to compensate the phagocytic activity of the ciliate. Because the flow cytometry analyses revealed that these migrating leukocytes were not Nkl-positive, we speculated that they corresponded to the myeloid lineage, such as neutrophils and/or macrophages. Nevertheless, we probably did not detect the migration of Nkl-positive cells to the peritoneum because these cells were degranulating and releasing Nkl to combat the parasite, as determined by ELISA in the PEs at 24 hpi. The increase in Nkl in the exudate could reflect the potential direct antiparasitic activity of this peptide.

As a member of the saposin-like protein (SAPLIP) family, Nk-lysin peptides possess lipid-binding activity and therefore can disrupt biological membranes [42]. Both the expression plasmid encoding Nkl and a shorter Nkl peptide encoding the active core (Nkl71-100) were used to analyze this antiparasitic effect against *P. dicentrarchi*. The enriched SN from HEK-293 cells transfected with the expression plasmid reduced the parasite viability after 96 h of *in vitro* incubation compared with the SN from cells transfected with the empty control plasmid. Nevertheless, the *in vivo* results did not show an increase in the survival rate in individuals inoculated with the enriched SN and concomitantly infected with the parasite. The dose of protein present in the SN was probably not sufficient to fight the infection. However, slight but significant protection against *P. dicentrarchi* infection was observed when the pMCV1.4-*nkl* expression plasmid was i.m. injected, suggesting that injection of a constant source of protein production into the fish might produce the dose required to combat and thus be more effective against the infection. Therefore, the full Nkl peptide seems to reduce *P. dicentrarchi* proliferation. Nevertheless, previous studies demonstrated that some Nkl-derived and shorter peptides also possessed biological activity against bacteria, fungi, parasites or tumor cells [39,44,60,67,76,77] in higher vertebrates. Our studies with Nkl71-100 also showed a decrease in the survival percentages of parasites after an *in vitro* incubation and an increase in the survival rate when this peptide was i.p. injected together with the parasite, suggesting that Nkl possessed a core region that was critical for protein activity. Similar results were observed by Jacobs et al. [39], who found that recombinant pig Nkl and the shortened analog NK-2 rapidly permeabilized the plasma membrane of the human pathogenic parasite *Trypanosoma cruzi* and killed the parasites. NK-2 was also tested against the malarial parasite *Plasmodium falciparum*, and a decrease in the parasite viability was observed [40]. In addition to mammalian Nk-lysins, the antiparasitic effects of the full-length chicken Nkl and a shortened synthetic analog were demonstrated against different species of *Eimeria* parasites [41,68].

Nk-lysins have been shown to act via insertion of their α-helices into the target cell membrane, which provokes membrane disruption [43,65]. When the ciliates were incubated with the Nkl71-100 peptide for 72 h, SEM images revealed that this molecule disrupted the cellular membrane and led to degradation of the parasite cells. The mechanism of action of Nkl71-100 could involve an initial disruption of the parasitic membrane, which would produce a loss of intracellular contents leading to the appearance of the observed ghost cells.

Several studies have been conducted in fish to analyze the antibacterial activity of Nk-lysin peptides and shortened analogs [32–35,72]. However, to the best of our knowledge, no experiments have been conducted to analyze the putative effect of a teleost Nkl during parasitic infection. Therefore, the present work represents the first evidence of the antiparasitic activity of an Nkl peptide in fish. This study suggests that the turbot full-length recombinant Nkl protein and especially its derivate peptide Nkl71–100 may represent new candidates for effective protection against *P. dicentrarchi* infection, which is one of the main pathogens in turbot culture. Moreover, a positive correlation between the constitutive *nkl* expression level and resistance against the viral hemorrhagic septicemia virus (VHSV) in turbot was previously reported [36,78]. Due to the resistance against *P. dicentrarchi* provided by the administration of the expression plasmid encoding Nkl, we cannot discard genetic breeding selection of individuals with higher basal *nkl* expression, which may represent an efficient strategy for reducing the natural mortality caused by this parasite in turbot farms.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.08.004.

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